

Biosynthesis of the Actinomycin Chromophore: Incorporation of 3-Hydroxy-4-Methylanthranilic Acid into Actinomycins by *Streptomyces antibioticus*

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Actinomycin synthesis by washed mycelia of *Streptomyces antibioticus* has been conducted in the presence of 3-hydroxy-4-methylanthranilate-(*carboxyl*-¹⁴C). Incorporation of this compound into actinomycins has been observed, which constitutes further evidence that 3-hydroxy-4-methylanthranilate is an intermediate in actinomycin biosynthesis. The position of the incorporated label has been determined to be within the actinomycin chromophore, and the label appears to be equally distributed between both halves of the chromophore. Incidental to these findings was the observation that the ¹⁴C-labeled actinomycins were subject to rapid reabsorption by the organism with actinomycin V taken up preferentially to actinomycin IV.

Brockmann and Muxfeldt (4) first described the chemical synthesis of actinocin, the actinomycin chromophore, from 3-hydroxy-4-methylanthranilic acid (MHA). On the basis of this and other work, these investigators postulated that MHA was an intermediate in actinomycin biosynthesis. The discovery by Katz and Weissbach (12, 15, 22) of the enzyme phenoxazinone synthetase in *Streptomyces antibioticus*, which catalyzes the oxidative condensation of two molecules of MHA (or MHA-pentapeptide) to form actinocin (or an actinomycin), provided support for this postulate. The latter workers and their collaborators have also furnished other corroborating evidence.

The incorporation of radioactivity from DL-tryptophan-(*benzene ring*-¹⁴C) into actinomycin by washed mycelia of *S. antibioticus* was inhibited by MHA (23, 24). In this experiment the added MHA appeared to trap radioactivity from the tryptophan. If a product-precursor relationship existed, this result was to be expected, since the benzene ring of tryptophan had been established as a precursor of actinocin (20). In addition, the incorporation of ¹⁴C-L-valine into actinomycin was stimulated by MHA (24). Both 3-hydroxyanthranilate and 3-hydroxykynurenine, each of

which could be a precursor of MHA, mimicked the effect of MHA in the latter experiment. However, both of these compounds failed to dilute incorporation of radioactivity from tryptophan-(*benzene ring*-¹⁴C) into actinomycin.

Since firm evidence regarding the precursors of the chromophore was desirable, we decided to subject MHA to direct test as a precursor. For this purpose we synthesized MHA-(*carboxyl*-¹⁴C) chemically and incubated it with *S. antibioticus*.

MATERIALS AND METHODS

Materials. All reagents, unless otherwise specified, were of reagent grade and were obtained from commercial sources. 3-Methoxy-4-methylbenzoic acid and 2-nitro-3-hydroxy-4-methylbenzoic acid were purchased from Aldrich Chemical Co., Milwaukee, Wisc. Actinomycin IV (also named actinomycin D and Dactinomycin) was generously provided by David Hendlin of Merck, Sharp and Dohme Research Laboratories. Prepacked thin-layer plates of Silica Gel G, containing a fluorescent indicator, were obtained from Brinkmann Instruments Inc., Westbury, N.Y.

DL-Tryptophan-[*benzene ring*-¹⁴C (UL)] (3.81 c/mole) was a product of Nuclear-Chicago Corp., Des Plaines, Ill. ¹⁴C-cuprous cyanide (40 c/mole) and ¹⁴C(UL)-L-valine (195 c/mole) and actinomycin IV-(*methyl*-¹⁴C) (16 c/mole) were obtained from New England Nuclear Corporation. Oxalic-1,2-¹⁴C acid (17.3 C/mole) was purchased from Tracerlab, Waltham, Mass.

Organism. *S. antibioticus* (ATCC 14888; Rutgers Institute of Microbiology Culture Collection no. 3720)

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was grown in soya peptone medium; actinomycin synthesis was initiated by incubation of the organism in glutamic acid-galactose medium by the method of Goss and Katz (7). After 72 hr in this medium, the mycelium was harvested by centrifugation, washed with isotonic saline, and incubated at 28 C in a volume of 0.1 M buffer (*see Results*) which was 40% that of the original culture medium.

Incorporation experiments. Labeled compound was added to the incubation mixture to give a concentration of 0.05 $\mu\text{C}/\text{ml}$. Samples of 5 ml each were taken at indicated times and pipetted into 12-ml glass conical centrifuge tubes packed in ice. The supernatant fluid obtained on low-speed centrifugation was extracted with ethyl acetate (13, 16). Both the unextracted supernatant fluid and the neutral (pH 8.0) ethyl acetate extract (containing the actinomycins) were analyzed for radioactivity. Samples were counted in a Nuclear-Chicago liquid scintillation spectrometer with a naphthalene-dioxane scintillation system (1). The neutral ethyl acetate fractions were chromatographed on Silica Gel G thin-layer plates in the ethyl acetate-acetone system (2:1) by the method of Katz, Mauger, and Weissbach (9). The plates were assayed for radioactivity by the method of Nishimura et al. (17, 21) with a Nuclear-Chicago Actigraph III scanner equipped with a thin-layer plate attachment and a digital integrator.

Synthesis of MHA-[carboxyl- ^{14}C]. 3-Methoxy-4-methylbenzoic acid was converted to 2-nitro-3-methoxy-4-methylbenzoic acid by the procedure of Simonsen and Rau (19). Conversion of this compound to 2-nitro-3-methoxy-4-methylidobenzene was accomplished essentially as described by Hanks (8). This compound was treated with ^{14}C -cuprous cyanide to form the labeled nitrile. Hydrolysis of the cyano function and removal of the methyl from the methoxy group was accomplished by treatment in a sealed tube with 6 N HCl at 110 C for 24 hr. This procedure did not result in complete demethylation, but increased yields of the desired product were obtained by repeated acid treatment. 2-Nitro-3-hydroxy-4-methylbenzoic-(*carboxyl- ^{14}C*)-acid was isolated from the reaction mixture by thin-layer chromatography on Silica Gel G plates with a solvent containing chloroform and acetic acid (9:1). The radioactive compound cochromatographed in this system with authentic compound. MHA-(*carboxyl- ^{14}C*) was generated from this material by reduction of the nitro group with sodium dithionite by the method of Brockmann and Muxfeldt (4). The product was purified by chromatography on a column of Dowex-1 chloride by the procedure of Weissbach et al. (24), and was used immediately for the experiment.

Alkaline hydrogen peroxide oxidation of labeled actinomycin: high voltage paper electrophoresis of the product before and after acid hydrolysis. Actinomycin was subjected to oxidation by alkaline hydrogen peroxide by the procedure described by Bullock and Johnson (5), but on a reduced scale. The labeled sample (0.02 to 0.30 μmole) was dissolved in 0.8 ml of methanol and this solution was mixed with 1.0 ml of alkaline hydrogen peroxide (3% H_2O_2 ; 0.75 N NaOH). The reaction mixture was allowed to stand at room temperature for 1 hr, after which time it was acidified

(turned Congo Red test paper blue) by the addition of 1 N HCl. The acidic solution was extracted three times with 2-ml portions of ethyl acetate. The combined ethyl acetate extracts were evaporated under reduced pressure and the residue dissolved in a small volume (ca. 0.3 ml) of methanol. Routinely, half of this solution was subjected to strong acid hydrolysis. For this purpose, dried samples were mixed with 0.5 ml of 6 N HCl in small Pyrex tubes. The tubes were sealed and placed in an oven at 105 C for 12 to 16 hr. The contents of the tubes were then reduced to dryness in vacuo at 30 C. Excess HCl was removed by repeated addition of water and by evaporation. The residues were dissolved in water and spotted on Whatman 3MM strips (2.54 by 75 cm). The strips were moistened with buffer [0.1 M tris(hydroxymethyl)aminomethane, 0.001 M disodium ethylenediaminetetraacetate (EDTA), adjusted to pH 7.4 by the addition of HCl] and subjected to electrophoresis in this buffer at 40 v/cm for 1.5 hr in a Savant "flat-plate" electrophoresis apparatus cooled to 0 C. Electrophoresis of the unhydrolyzed product of the alkaline hydrogen peroxide digest was carried out in the same way. After electrophoresis the paper strips were dried and counted on the Actigraph scanner. Radioactive areas of the strips were eluted with water for further analysis.

Identification of oxalate in acid hydrolysates of alkaline hydrogen peroxide digests of ^{14}C -MHA-labeled actinomycin. Two criteria were used to establish the identity of ^{14}C -labeled products of the combined alkaline hydrogen peroxide and acid hydrolysis treatment of actinomycin as oxalate: (i) comparison of the migration of the product with that of authentic ^{14}C -oxalate and (ii) precipitation of the product by calcium ion as calcium oxalate. Aqueous eluates of areas corresponding to oxalate on the paper electrophoresis strips were evaporated to dryness and mixed with 0.30 ml of a 0.007 M solution of sodium oxalate. Precipitation of the oxalate was effected by the addition of 0.70 ml of 0.1 M CaCl_2 . The resulting mixture was heated in a boiling water bath for 2 to 3 min, chilled in ice, and centrifuged. The supernatant fluid was carefully removed by pipette and the precipitate was washed with 1 ml of water. The washing was repeated once and the supernatant solutions combined. The precipitate was dissolved by the addition of 1.0 ml of 0.12 M EDTA (adjusted to pH 9 with sodium hydroxide). Samples of both the supernatant fraction and the dissolved calcium oxalate precipitate were assayed for radioactivity by scintillation counting as described above.

RESULTS

Incorporation of ^{14}C -L-valine into actinomycin as a function of pH. The use of washed mycelia to study the incorporation of ^{14}C -labeled amino acids into actinomycin has been reported by Weissbach and co-workers (24). These workers utilized 0.1 M potassium phosphate buffer at pH 7.3 for their incubations. Preliminary attempts to test the incorporation of MHA into actinomycin resulted in a small incorporation of the compound

into product under these conditions. Analysis of the incubation medium revealed that a large quantity of the labeled substance had become oxidized during the incubation. Since the aerobic oxidation of MHA is facilitated by higher pH, we attempted to find conditions more suitable for MHA by testing the incorporation of ^{14}C -L-valine into actinomycins by washed organisms in 0.1 M buffer at pH values from 5.40 to 7.45. Incorporation of label under these conditions was maximal at 30 min. When potassium phosphate was used as buffer, a linear dependence of incorporation with pH was observed over the range tested. The incorporation of the labeled amino acid into actinomycins at pH 5.40 was 68% of that observed at pH 7.45. The incorporation at pH 6.05 was about 80% of that at pH 7.45; these conditions were chosen for further experiments with MHA.

Incorporation of ^{14}C -MHA into actinomycin. Addition of ^{14}C -MHA to washed mycelia of *S. antibioticus* in 0.1 M potassium phosphate at pH 6.05 resulted in a rapid uptake of ^{14}C from the medium, 70% of the label being taken up in 15 min and 82% of the label taken up in 2 hr. This uptake is similar to that observed with L-amino acid precursors of actinomycin, but it should be noted that this is an apparent uptake by the organism. A significant portion of the medium radioactivity is that which has in fact been taken up by the organism and returned to the medium in the form of actinomycin.

Analysis of the radioactivity in the medium was performed as described above. In a control experiment this procedure resulted in a high value for ethyl acetate-extractable radioactivity (33% of the total), a measure of the extractability of ^{14}C -MHA by this solvent at pH 6.05. Katz and Weissbach (13) have shown that with labeled amino acids, e.g., proline, valine, and threonine, neutral ethyl acetate-extractable radioactivity is a measure of actinomycin synthesis. In the ^{14}C -MHA experiments, therefore, it was necessary to purify the neutral ethyl acetate extract further to ascertain the amount of label in the actinomycins. This was done by chromatography of the extract on Silica Gel G thin-layer plates. A typical chromatogram obtained with ^{14}C -MHA-labeled actinomycins is shown in Fig. 1. The positions of actinomycins IV and V were verified by the location of yellow bands on the thin-layer plates. These bands have been shown to cochromatograph with authentic actinomycins produced by *S. antibioticus* in this system. Except for the relatively large amount of radioactivity located at the origin, this chromatogram is quite similar to those obtained in experiments in which actinomycins were labeled by

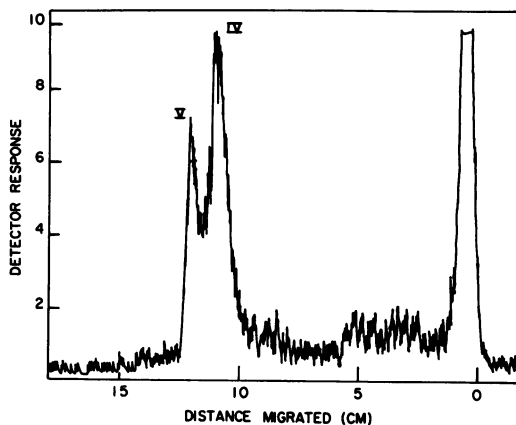


FIG. 1. Thin-layer chromatography of ^{14}C -MHA-labeled actinomycin. The actinomycin fraction was derived from a 60-min incubation of *S. antibioticus* with ^{14}C -MHA and chromatographed on a Silica Gel G plate. The plate was scanned in the Actigraph III at a scan rate of 30 cm/hr with a slit width of 3 mm. Full scale deflection represents 500 counts/min.

radioactive amino acids. In addition, the results obtained in this chromatographic system closely parallel those seen in the circular paper chromatographic system with the solvent system of 10% sodium *o*-cresotinate:n-dibutyl ether:symmetrical tetrachloroethane at a ratio of 4:3:1 (11). Although in the present study actinomycin mixtures were not crystallized prior to chromatography, there appears to be little doubt that the two major components correspond to actinomycins V and IV.

The peak at the origin of the chromatograms did not correspond to a yellow band, indicating that it was probably not associated with an actinomycin. Moreover, it disappeared from the medium with time, being almost absent at 2 hr.

The time course of the incorporation of ^{14}C -MHA into actinomycins (taken as the sum of actinomycins IV and V on the plates) is summarized in Table 1. Exact time points were difficult to obtain, particularly during the early part of the experiment. This was due to the rapid uptake of ^{14}C -MHA by the organism, apparently even during centrifugation in the cold. Incorporation of ^{14}C -MHA appeared to reach a peak at about 15 min after addition of the labeled compound and then declined. Addition of L-tryptophan at a concentration of 0.1 mM resulted in a 20% decrease in incorporation at the peak, whereas a 10-fold molar excess of unlabeled MHA resulted in a 50% decrease in incorporation at the peak and, surprisingly, almost no label in actinomycins by 2-hr. To explore this apparently anomalous

TABLE 1. Incorporation of ^{14}C -MHA into actinomycins^a

Addition to incubation	^{14}C in actinomycins (dpm/5 ml) at				
	Approx 2 min	15 min	30 min	60 min	120 min
None.....	10,700	22,900	22,200	11,500	7,200
L-Tryptophan.....	6,700	18,800	15,100	8,900	6,600
MHA.....	2,000	12,400	6,100	500	400

^a ^{14}C -MHA ($2.5\ \mu\text{C}$) was added to 50-ml incubation mixtures of *S. antibioticus* in 0.1 M potassium phosphate buffer at pH 6.05. Incorporation into actinomycin was determined by chromatography of the neutral ethyl acetate extract. Where indicated, L-tryptophan ($5\ \mu\text{moles}$) or unlabeled MHA ($0.6\ \mu\text{mole}$) was added.

lous result, an incorporation experiment was conducted in which the ratio of ^{12}C -L-valine to ^{14}C -L-valine was increased from 0 to 220. The results presented in Fig. 2 indicate that there is indeed a linear relationship between this ratio and the relative dilution of incorporation of ^{14}C -L-valine into actinomycin. However, the nature of the relationship is such that a 10-fold dilution of ^{14}C -L-valine is reflected in less than a 2-fold dilution of label in actinomycin, a situation which is completely analogous to that observed with ^{14}C -MHA. These results are best explained by increased uptake of amino acid or MHA at higher concentrations. Incorporation values above those expected from the dilution of labeled compound by the unlabeled species actually indicate an increase in

synthesis of actinomycins. For example, without dilution, approximately 1.5 nmoles of L-valine was incorporated. Upon the addition of increasing amounts of unlabeled amino acid, this value rose linearly until it reached a maximum value of 9.6 nmoles incorporated, at approximately a 1 to 100 dilution of the ^{14}C -L-valine.

The disappearance of actinomycins in these relatively short-term experiments was surprising. One possibility that occurred to us was that the actinomycins actually underwent degradation. Since the degradation of actinomycin by the producing species had not been previously described, the authenticity of this observation was confirmed by direct experiment. To this end, $0.25\ \mu\text{C}$ of actinomycin IV-(methyl- ^{14}C) was incubated with washed organisms under conditions that were completely analogous to those employed in the incorporation experiments. Under these conditions, the decline of neutral ethyl acetate-extractable material was linear for 30 min, by which time 37% of the added actinomycin had been degraded. Uptake of label from the medium into the cells was also observed, but this reaction was somewhat slower with 32% of the added label taken up in 2 hr.

To gain further insight into the meaning of these results, the formation of actinomycins IV and V were studied separately with MHA as the labeled compound (Table 2). Incorporation of MHA into total actinomycin showed the same pattern as that presented earlier. However, the kinetics of incorporation of MHA into actinomycin IV differed markedly from that observed for actinomycin V. For the first 30 min of the incubation, actinomycin V was the predominant labeled species present, after which time labeled actinomycin IV was present in larger quantities. Actinomycin V showed the rapid increase and decline characteristic of the total actinomycin fraction, whereas actinomycin IV appeared to be a more stable species with a pattern similar to those previously reported (14, 24). In preliminary experiments to investigate this phenomenon, it

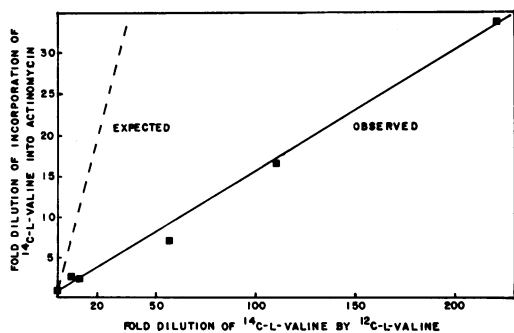


FIG. 2. The effect of addition of ^{12}C -L-valine on ^{14}C -L-valine incorporation into actinomycins. ^{14}C -L-valine ($2.5\ \mu\text{C}$) were added to 50-ml incubation mixtures of washed cells of *S. antibioticus* in 0.1 M potassium phosphate buffer at pH 6.05. Additions of 0 to 2500 nmoles of ^{12}C -L-valine were made to each of six incubations to make dilutions of the ^{14}C -L-valine from 0- to 220-fold. After 30 min of incubation, the radioactivity incorporated into actinomycin was determined. The fold dilution of incorporation of ^{14}C -L-valine into actinomycin was calculated as the ratio of radioactivity incorporated into actinomycin at no dilution to radioactivity incorporated at the indicated dilution. The line labeled "expected" would be obtained if the added ^{12}C -L-valine did not stimulate the incorporation of total L-valine into actinomycin.

TABLE 2. Incorporation of ^{14}C -MHA into separated actinomycins^a

Actinomycin	^{14}C in actinomycins (dpm/5 ml) at				
	Approx 2 min	15 min	30 min	60 min	120 min
IV	1,500	5,400	4,600	4,000	3,200
V	2,200	9,000	6,600	3,200	2,100

^a ^{14}C -MHA (2.5 μC) was added to 50-ml incubations of *S. antibioticus* in 0.1 M potassium phosphate buffer at pH 6.05. Incorporation into actinomycins IV and V was determined by thin-layer chromatography of the neutral ethyl acetate extracts of samples taken at the times indicated.

was found that solvent extraction of mycelium harvested after incubation with ^{14}C -MHA with acetone or with chloroform-methanol by the method of Folch et al. (6) released considerable amounts of actinomycin. Analysis of this material by thin-layer chromatography showed that the major labeled component, as well as the major unlabeled component, as judged by the intensity of the yellow band on the plate, was actinomycin V. It is conceivable that the virtually complete disappearance by 60 min of the ^{14}C -MHA-labeled actinomycin in the experiment in which a 10-fold excess of unlabeled MHA was added (Table 1) was due to a preferential synthesis and reabsorption of actinomycin V by the organism under the particular conditions of the experiment. Further work bearing on this point is in progress.

Location of labeled carbons in actinomycin labeled with ^{14}C -MHA. It was important to deter-

mine the location of label incorporated into actinomycins, since the possibility existed that ^{14}C -MHA was first metabolized by the organism and the labeled metabolites incorporated by indirect and more or less random means into the antibiotic. If, on the other hand, the ^{14}C -MHA were incorporated directly into actinomycins, the label should have been located specifically in the carboxyl groups of the actinomycin chromophore. Actinomycin was degraded in such a way as to permit selection between the two alternatives. This was accomplished by the alkaline peroxide degradation of actinomycin by a modified method of Bullock and Jonsson (5); the scheme for this reaction is shown in Fig. 3. Actinomycin (compound I), when treated with alkaline hydrogen peroxide, is degraded into two major fractions and unidentified components. The A peptide of actinomycin is recovered after this treatment in compound II which is 7-methylbenzoxazalone-4-carboxyl pentapeptide A, whereas peptide B is recovered as compound III which is oxalyl pentapeptide B. Residues A and B may be the same amino acid as in actinomycin IV where A = B = L-proline (2, 3, 5, 14), or A and B may be different amino acids as in actinomycin V where one of these residues is L-proline and the other is 4-keto-L-proline.

When actinomycin IV labeled by ^{14}C -MHA and isolated by thin-layer chromatography was treated with alkaline hydrogen peroxide and the reaction mixture was analyzed by paper electrophoresis and scanning of the paper for radioactivity, the pattern shown in Fig. 4 (upper half) was obtained. As expected, two major components were ob-

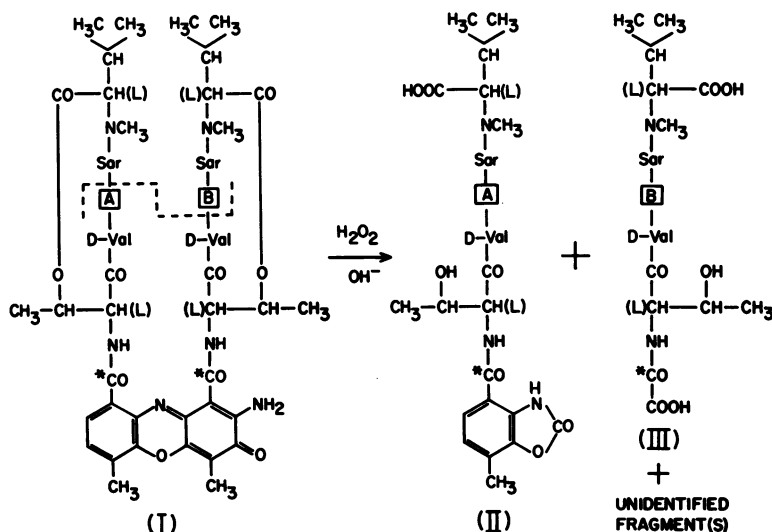


FIG. 3. Alkaline hydrogen peroxide digestion of actinomycin. Symbols: Sar, sarcosine; D-Val, D-valine; asterisks, suspected positions of ^{14}C in actinomycin labeled by MHA (carboxyl- ^{14}C).

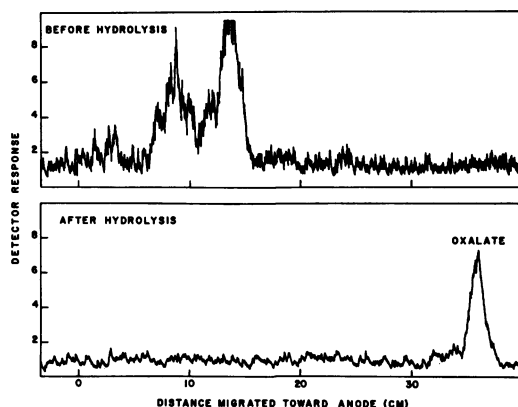


FIG. 4. Paper electrophoresis of alkaline hydrogen peroxide digest of ^{14}C -MHA-labeled actinomycin IV, before and after acid hydrolysis. ^{14}C -MHA-labeled actinomycin IV (0.024 μmole , 7,120 counts/min) was subjected to alkaline H_2O_2 treatment and half of this digest hydrolyzed in 6 N HCl. Both fractions (unhydrolyzed and hydrolyzed) were subjected to paper electrophoresis at pH 7.4 and the electropherograms scanned. The scan rate was 60 cm/hr with the slit width at 12 mm. Full scale deflection in the scans represents 300 counts/min.

tained corresponding to compounds II and III in approximately equal amounts. Strong acid hydrolysis of the unfractionated alkaline peroxide reaction products gave an electrophoresis pattern shown in the lower half of Fig. 4. Oxalate was the major product recovered from this hydrolysis. The absence of radioactivity near the origin of the electrophoresis strip is significant since neutral amino acids from the pentapeptide chains of actinomycin would migrate to this position under these conditions. Isolation of each of the com-

pounds produced by alkaline peroxide treatment of actinomycin IV was accomplished by eluting each of the two major peaks of radioactivity seen in Fig. 4 (upper half). Acid hydrolysis performed in these materials showed that only the leading peak gave rise to oxalate. This allowed it to be identified as compound III; the other peak represents compound II. That compound II is 7-methylbenzoxazalone-4-carboxyl pentapeptide has been verified by two procedures. First, when this material was eluted from the paper and then examined, it had spectral properties which were the same as those described for it by Bullock and Johnson (5). Secondly, the pentapeptide nature of this compound was ascertained by the detection of all of the expected amino acids in acid hydrolysates of the material. However, attempts to obtain free 7-methylbenzoxazalone-4-carboxylic acid by acid hydrolysis were not successful. In fact, this substance appeared to be destroyed upon acid hydrolysis, as assayed by the loss of its characteristic spectrum. Precise localization of the label was therefore not possible. However, since electrophoretic analysis of the hydrolysates clearly indicated that none of the radioactivity was present in the amino acid residues, the conclusion can be made that the label must have been in the 7-methylbenzoxazalone-4-carboxylic acid residue.

Alkaline hydrogen peroxide degradations were performed on actinomycin IV and actinomycin V labeled with ^{14}C -MHA and with DL-tryptophan-[benzene ring- ^{14}C (UL)]. The results of these studies are presented in Table 3. If ^{14}C -MHA was equally distributed between both halves of the molecule, 50% of the radioactivity in actinomycin should be recoverable as labeled

TABLE 3. Alkaline hydrogen peroxide digestion of actinomycins IV and V labeled with ^{14}C -MHA and with DL-tryptophan-[benzene ring- ^{14}C (U)]^a

Description	Actinomycin IV				Actinomycin V			
	^{14}C -MHA		^{14}C -tryptophan		^{14}C -MHA		^{14}C -tryptophan	
	counts/min	%	counts/min	%	counts/min	%	counts/min	%
Amount at start	7,030	(100)	105,000	(100)	8,370	(100)	61,400	(100)
Recovered from alkaline peroxide digest	6,480	92	50,200	48	7,200	86	41,000	67
Recovered from acid hydrolysis	2,600	37	34,100	32	3,160	38	24,700	39
Recovered as oxalate	1,160	23	4,430	4.2	1,140	14	3,460	5.7

^a Actinomycin IV (0.024 μmole) and actinomycin V (0.038 μmole) labeled with ^{14}C -MHA and actinomycin IV (0.30 μmole) and actinomycin V (0.19 μmole) labeled with DL-tryptophan-[benzene ring- ^{14}C (UL)] were subjected to alkaline hydrogen peroxide digestion. Oxalate was determined in 6 N HCl hydrolysates of digests either by paper electrophoretic analysis at pH 7.4 or after precipitation with calcium ion.

oxalate. The lower-than-expected yields obtained with actinomycins IV and V suggested either that ^{14}C -MHA was incorporated into compound II preferentially, or that oxalate was being lost during the hydrolysis. The latter hypothesis was tested by subjecting 1.0 μmole of oxalic acid containing 0.1 μC of authentic oxalic- $1,2\text{-}^{14}\text{C}$ acid in the presence of 1 mg of actinomycin IV to hydrolysis with 6 N HCl at 105 C for 15 hr. Analysis of the hydrolysate showed that 54.5% of the oxalate was recoverable. Applying this value to the oxalate recovery data, the theoretical recovery of oxalate from an actinomycin randomly labeled by ^{14}C -MHA would be 26.8%, which is in good agreement with the value obtained for actinomycin IV. The comparatively low yield of oxalate from actinomycin V has not yet been satisfactorily explained. A similar calculation for the theoretical yield of oxalate from actinomycin randomly labeled with DL-tryptophan-[benzene ring- ^{14}C (UL)] would be 4.5%, which is in excellent agreement with the values obtained for both actinomycins IV and V.

DISCUSSION

Mechanisms for actinomycin biosynthesis have been proposed utilizing MHA or MHA-peptides as intermediates on the basis of the known chemistry of MHA (2, 3). In these propositions MHA is hypothesized as either first condensing to form actinocin and then combining with peptide chains, or by combining with the peptide chains first and then condensing to form the complete actinomycin (24). The demonstration that exogenous MHA can be incorporated into actinomycin is a further indication that this compound is an intermediate in actinomycin biosynthesis. The question as to whether actinocin is an intermediate in this process is not yet answered, but preliminary results indicate that exogenous actinocin is not incorporated into actinomycin (*unpublished data*). Another question relates to the role of the unidentified product of ^{14}C -MHA metabolism, which remained at the origin in the thin-layer chromatography system. The slow disappearance of this material, compared with the rapid labeling and attainment of peak labeling of the actinomycin fraction, help to argue against its participation in antibiotic synthesis. Nevertheless, since this unknown material appears to undergo relatively rapid formation, its identification may be important in ascertaining alternate routes of MHA metabolism in the organism.

The rapid decline of labeled actinomycin represents an interesting aspect of these results. Metabolism of actinomycin has been reported to be

accomplished by a species of *Achromobacter* (10) and also by an *Actinoplanes* species which contains an enzyme that hydrolyzes the lactone bonds of the pentapeptide chains (18). However, degradation of actinomycin by the producing species has not been reported. Yoshida et al. (25) observed that actinomycin could inhibit the growth of the producing species when added to growing organisms. Older organisms which were producing the antibiotic were insensitive to exogenous actinomycin. The authors speculate that producing cells may develop a permeability barrier to the antibiotic they are excreting. In our experiments, antibiotic has been shown to be degraded by washed mycelia of *S. antibioticus*. The conversion of actinomycin IV- ^{14}C to compounds nonextractable with ethyl acetate is rapid, and is consistent with observations made on actinomycins synthesized from radioactive precursors such as ^{14}C -L-valine and ^{14}C -MHA. In addition, a slower reaction in which radioactivity was removed from the medium was also observed. This observation may be associated with the finding that considerable quantities of actinomycin can be released from the organism by organic solvent extraction, suggesting either that the newly synthesized antibiotic is stored intracellularly or that it may be reabsorbed by the organism from the medium. When the separated actinomycins are compared, it is significant that actinomycin V seems to disappear selectively. The finding that the actinomycin in the cells is primarily actinomycin V lends additional support to the hypothesis that this actinomycin may be selectively taken up into the cells from the medium. Further investigation of this phenomenon may yield new information concerning the interrelationship of the actinomycins produced by *S. antibioticus*.

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