3-HYDROXY-4-METHYLKYNURENINE AS AN INTERMEDIATE IN ACTINOMYCIN BIOSYNTHESIS

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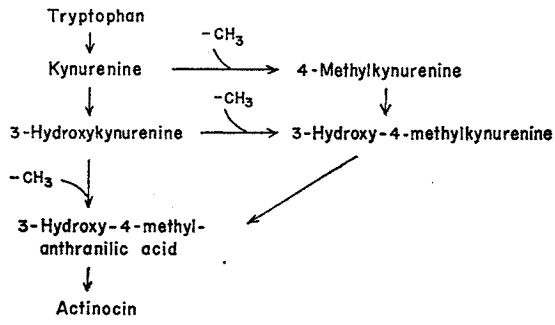
D, L-3-Hydroxy-4-methylkynurenine was prepared from 3-methoxy-4-methylanthranilic acid and found to be a competitive precursor with L-tryptophan in the biosynthesis of actinomycin D by *Streptomyces antibioticus* (ATCC 14888).

The phenoxazinone nucleus of actinomycin D is believed to be formed by the condensation of two units of 3-hydroxy-4-methyl-anthranilic acid or a derivative thereof coming from L-tryptophan[1,2]. The formation of 3-hydroxy-4-methylanthranilic acid probably involves a diversion of the well-known metabolic pathway leading to the formation of 3-hydroxyanthranilic acid from L-tryptophan[2]. However, the mechanism of transmethylation of the aromatic ring has not been examined to the extent of determining which of the alternative compounds mentioned in Fig. 1 is the pentultimate precursor for actinocin formation. We have studied the possibility that 3-hydroxy-4-methylkynurenine might be an intermediate in the formation of actinocin by washed cells of *S. antibioticus* (ATCC 14888).

Fig. 1. Possible metabolic pathways for conversion of L-tryptophan to actinocin



Preparation of 3-Hydroxy-4-methylkynurenine

Two methods were used for the preparation of D, L-3-hydroxy-4-methylkynurenine: As summarized in Fig. 2, 3-methoxy-4-methylbenzoic acid (**I**) was nitrated with concentrated HNO_3 (in acetic anhydride). Treatment of the product (**II**) with thionylchloride gave the acid chloride (**III**) which was then reacted with ethoxymagnesium-malonic ester. After hydrolysis to the ketone (**IV**), the product was brominated (in acetic acid) to give a monobromoketone (**V**). This was condensed with acetaminomalonic ester and sodium hydride (in dimethylformamide) and the product was hydrolyzed and reduced with HI and red phosphorus to give 3-hydroxy-4-methylkynurenine (**VI**).

The second approach (Fig. 3) started with the nitroketone (**IV** of Fig. 2) which upon reaction with diethyloxalate and sodium ethoxide gave the pyruvic ester (**VII**). The oxime (**VIII**) was then hydrolyzed and reduced with HI and red phosphorus (as mentioned in the previous synthesis) to give 3-hydroxy-4-methylkynurenine.

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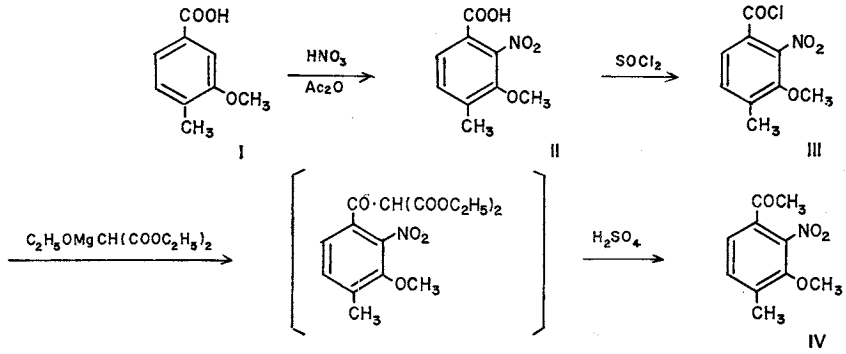
Fig. 2. Plan for synthesis of 3-hydroxy-4-methylkynurenine



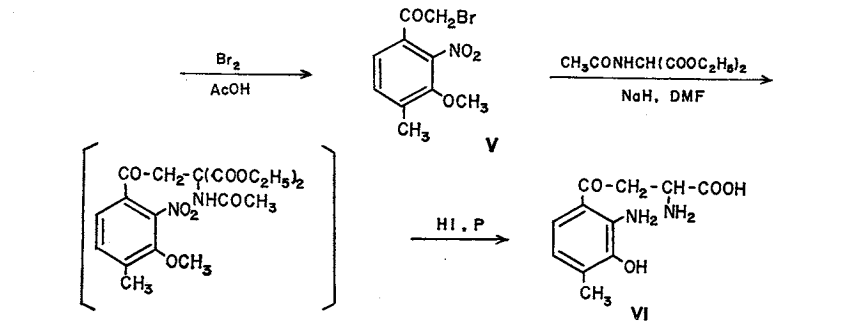
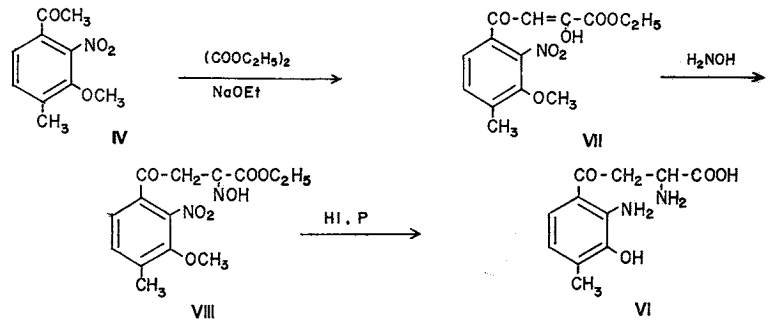


Fig. 3. Alternative method for synthesis of 3-hydroxy-4-methylkynurenine



Experimental

Materials: 3-Methoxy-4-methylbenzoic acid was purchased from Aldrich Chemical Company. Sodium hydride (obtained as a 57 % suspension in oil) and methyllithium (2 M in ethyl ether) were obtained from Alfa-Ventron. The HI (catalog no. A-135) was the product of the Fisher Chemical Company.

2-Nitro-3-methoxy-4-methylbenzoyl chloride (**III**): A solution of 8.0 g of 2-nitro-3-methoxy-4-methylbenzoic acid (prepared by SIMONSEN's method 3) in 80 ml of $CHCl_3$ was boiled with stirring with 9.0 ml of thionylchloride for 1 hour. The excess thionylchloride and $CHCl_3$ were removed by evaporation under reduced pressure and the resultant acid chloride was used immediately in the preparation of 2-nitro-3-methoxy-4-methylacetophenone (**IV**).

2-Nitro-3-methoxy-4-methylacetophenone (**IV**): 0.1 ml of CCl_4 and 1.0 ml of abs. EtOH were

added to 1.0 g of magnesium turnings (maintained under anhydrous conditions). As soon as the reaction had begun, 30 ml of abs. ethylether was added with stirring. This was followed by a mixture of 6.5 g of diethylmalonate, 4.0 ml of abs. EtOH, and 5.0 ml of abs. ethylether. This latter solution was added at such a rate that the rapid boiling was maintained, and when all had been added, the mixture was boiled under reflux for 3 hours until all of the magnesium had dissolved. The acid chloride (**III**) dissolved in 20 ml of abs. ethylether was added to the above gray solution. The reaction mixture was heated throughout the addition of the acid chloride until the dark red solution became too viscous to be stirred. After an additional 15 minutes of heating, the mixture was cooled and shaken with 50 ml of 10 % H_2SO_4 until all of the solids had dissolved. The ethylether phase was separated and the aqueous layer extracted with 25 ml of ethylether. The combined ether extracts were washed with H_2O, dried over $MgSO_4$, and concentrated. A mixture of 12 ml of glacial acetic acid, 1.5 ml of concentrated H_2SO_4, and 8 ml of H_2O was added to the residue and the mixture heated under reflux for 4 hours. The solution was then cooled, made alkaline by addition of sufficient 20 % NaOH and then extracted with ethylether. The ether extract was washed with H_2O, dried over $MgSO_4$, decolorized with charcoal, and concentrated to give 6.5 g of **IV** as a colorless oil. Although this material was pure enough to be used in the next step of the reaction sequence, it could be purified further by chromatography on Florisil with ethylether as eluant.

NMR ($CDCl_3$): δ 2.38 (s, 3, $-CH_3$); 2.58 (s, 3, $-COCH_3$); 3.83 (s, 3, $-OCH_3$); 7.41 (d, 1 J=7 Hz, C_5-H); 7.10 (d, 1 J=7 Hz, C_6-H).

Analyses: Found, C, 57.06; H, 5.17; N, 6.86%.
Calculated for $C_{10}H_{11}NO_4$, C. 57.41; H, 5.30; N, 6.70%.

2-Bromo (2′-nitro-3′-methoxy-4′-methyl) acetophenone (**V**): A solution of 3.0 g of **IV** dissolved in 15 ml of glacical acetic acid was mixed at room temperature with 2.2 g of bromine dissolved in 15 ml of glacial acetic acid. The solution was warmed to 55~60°C and stirred for 20 minutes by which time the bromine color had completely disappeared. It was then stirred for an additional 10 minutes and poured into ice water. The white crystals were collected by filtration, washed with H_2O, and dried *in vacuo*. A yield of 3.7 g of **V** was obtained (88 % yield) which had a melting point of 82~83°C. Recrystallization from EtOH gave a material which had m.p. 83~84°C in the form of white needles.

NMR ($CDCl_3$): δ 2.41 (s, 3, $-CH_3$); 3.86 (s, 3, $-OCH_3$); 4.40 (s, 2, $-COCH_2$); 7.46 (d, 1, J=7 Hz, C_5-H); 7.61 (d, 1, J=Hz, C_6-H).

Analyses: Found, C, 41.56; H, 3.34; N, 4.93; Br, 27.69%.
Calculated for $C_{10}H_{10}NO_4Br$: C, 41.69; H, 3.49; N, 4.86; Br, 27.74%.

Ethyl acetamino (2-nitro-3-methoxy-4-methylphenacyl) malonate: Diethylacetamidomalonate, 2.4 g, was dissolved in 10 ml of dry dimethylformamide. To this solution was added in small portions with stirring, 0.5 g of a 57 % oil suspension of sodium hydride. The resulting suspension was stirred for an additional 15 minutes until all of the sodium hydride was dissolved. Then, 2.9 g of **V** dissolved in 10 ml of anhydrous dimethylformamide was added and the resulting dark brown solution stirred at room temperature for 16 hours. The dimethylformamide was then removed under reduced pressure at 40~50°C. The residue, a thick brown oil was used immediately for the next step of the sequence.

D, L-3-Hydroxy-4-methylkynurenine (**VI**): The oil from 2.9 g of **V** was refluxed with stirring for 16 hours with 50 ml of 57 % HI and 0.8 g of red phosphorus powder. The reaction mixture was then diluted with 400 ml of H_2O and filtered. The orange filtrate was further diluted with H_2O and passed through a Dowex 50×12 resin column (200~400 mesh; H^+ form; 5×30 cm column). The column containing the absorbed reaction mixture was washed with 500 ml of distilled H_2O and then with 2,000 ml of 2.4 N HCl. The 3-hydroxy-4-methyl-kynurenine (**VI**) was eluted with 4,000 ml of 5N HCl. The 5N HCl was removed by evaporation under reduced pressure. EtOH was added to the residue and the solution evaporated under reduced pressure thrice. Ethylether was then added and removed under reduced pressure. The cream colored powder was quickly collected by filtra-

tion, washed with ethylether, and dried *in vacuo* to give 1.1 g of D, L-3-hydroxy-4-methylkynurenine dihydrochloride.

In 10 ml of 60 % EtOH was dissolved 0.5 g of the dihydrochloride and the solution adjusted to pH 4.0 by dropwise addition of saturatee sodium acetate solution. The D, L-3-hydroxy-4- methylkynurenine (**VI**) preciptated as cream colored crystals. The solution was cooled, filtered, and the crystalline material washed with a small amount of water. The crystals were then dried to give 0.3 g of pure **VI** with a melting point 240~241°C (dec.). The crystals in solution gave a brown color test with $FeCl_3$ reagent and a purple ninhydrin test.

NMR (D_2O): δ 2.33 (s, 3, $-CH_3$); 3.91 (d, 2, $-CH_2$-); 4.50 (t, 1, $-CH_2$-); 7.41 (d, 1, J=8 Hz, C_5-H); 7.61 (d, 1, J=8 Hz, C_6-H).

u.v. spectra: max. at pH 7 (H_2O): 365 nm (ϵ 3,403); 272 nm (ϵ 8,576); 228 nm (ϵ 17,834). In 6N HCl: 310 nm (ϵ 33,09); 263 nm (ϵ 11,514); 215 nm (ϵ 12,176).

Analyses: Found, C, 55.64; H, 5.89; N, 11.42%.
Calculated for $C_{11}H_{14}N_2O_4$, C, 55.46; H, 5.92; N, 11.76%.

Ethyl (2-nitro-3-methoxy-4-methylbenzoyl) pyruvate (**VII**): To a solution of 0.2 g sodium in 10 ml of abs. EtOH was added a mixture of 1.5 g of **IV**, 2.0 ml of diethyloxalate, and 5.0 ml of abs. EtOH. This final solution first turned purple and then dark brown in color. It was kept for 16 hours at 0°C and then 15 ml of 2N H_2SO_4 and 10 ml of H_2O were added. The solution was then extracted 3 times with 25 ml portions of ethylether. The combined ether extracts were dried (Na_2SO_4) and the solvent evaporated under reduced pressure. The resulting syrup crystallized. These crystals (**VII**) were collected by filtration, washed with cold EtOH and dried (1.1 g, 40 % yield). A sample was recrystallized from EtOH for analytical purposes and had m.p. 80~81°C.

NMR ($CDCl_3$): δ 2.40 (s, 3, $-CH_3$); 3.95 (s, 3, $-OCH_3$); 6.83 (s, 1, $-CH=$); 7.33 and 7.47 (d, 1, J=10 Hz) (C_5-H and C_6-H); 1.43 (t, 3, $-OCH_2CH_3$) (2, q, $-OCH_2CH_3$).

Analyses: Found, C, 54.35; H, 4.83; N, 4.51%.
Calculated for $C_{14}H_{15}NO_7$, C, 54.37; H, 4.89; N, 4.53%.

Ethyl (2-nitro-3-methoxy-4-methylbenzoyl) pyruvate oxime (**VIII**): To a solution of 0.7 g of pyruvic ester (**VII**) in 20 ml of warm EtOH was added a solution of 0.5 g hydroxylamine acetate in 20 ml of hot EtOH. The mixture was kept at room temperature for 16 hours and then evaporated to dryness under reduced pressure. The colorless syrup was dissolved in a small amount of 9:1(v/v) mixture of beozene-EtOH and applied to a Florisil column. The oxime was eluted with more of the benzene-EtOH mixture. The eluate fractions were combined and evaporated under reduced pressure to yield 0.6 g of pure **VIII** as a colorless syrup.

NMR ($CDCl_3$): δ 1.3(t, 3, $-OCH_2CH_3$); 4.30 (q, 2, $-OCH_2CH_3$); 2.35 (s, 3, $-CH_3$); 3.80 (s, 3, $-OCH_3$); 3.50 (br s, 2, $-CH_2$-); 7.30 (br s, 2, aromatic).

The **VIII**, 0.6 g, was treated with 25 ml of HI and 0.4 g of red phosphorus as described above to give 0.42 of **VI** (60 % yield).

Biochemical Studies

Experimental

Methods.

S. antibioticus (ATCC 14888) was maintained on yeast extract-peptone-glucose agar slants and transferred at monthly intervals. Cells for biochemical investigations were obtained by growing the culture in a medium containing (g/liter); monosodium glutamate, 2.3 g; K_2HPO_4, 1 g; $MgSO_4$, 0.025 g; $ZnSO_4 \cdot 7H_2O$, 0.025 g; $FeSO_4 \cdot 7H_2O$, 0.025 g; $CaCl_2 \cdot 2H_2O$, 0.025 g; D-galactose, 10 g; distilled water q.s. 1 liter. This chemically defined medium, 1,000 ml, were placed in a 2-liter cotton-plugged Erlenmeyer flask and autoclaved at 121°C for 20 minutes. After cooling the flasks were inoculated with approximately 5 ml of a washed cell suspension of a 48-hour old culture of *S. antibioticus* which had been grown on an NZ-Amine^R-glucose type medium in shaken flasks at 30°C. After 65 hours growth in the chemically defined medium (flasks placed on a rotary shaker at 30°C;

280 rpm, 1-inch displacement), the cells were collected by filtration, and washed with 1 liter of physiological saline solution. After re-collection by centrifugution, the washed cells were re-suspended in 0.1 M pH 6.8 phosphate buffer and diluted to give 5 g wet cells/100 ml.

In the biosynthetic experiments 5 ml of incubation mixture containing 4.5 ml of cell suspension and appropriate solutions of amino acids was placed in 25 ml Erlenmeyer flasks and these, in turn, placed in a water bath shaker (30°C, 160 rpm 1-inch displacement). The amino acid solutions usually contained enough L-threonine, L-proline, sarcosine, glycine, L-methionine, L-valine, L-tryptophan (or D, L-tryptophan-^{14}C labelled in the benzene ring) to give 500 μ moles of each per flask. (When the radioactive tryptophan was used 200 mμ moles were added, and in some experiments 200 mμ moles of L-valine-^{14}C(U) were included). In the case of double labelled studies, 200 mμ moles each of L-valine-^{14}C and L-methionine-C^{3}H$_3$ were added as the tracers.

At the end of the 60-minute incubation period the contents of the flasks were filtered through glass wool to remove the mycelium, and the actinomycin was extracted from the filtrate using ethylacetate. Half of the ethylacetate extract (*c.* 2.5 ml) was concentrated *in vacuo* and the residue dissolved in a small amount of methanol. This solution was placed on a sheet of Eastman 6061 silica gel thin-layer plate and the chromatogram developed (along with appropriate standards) with a solution containing *n*-butanol-ethanol-water (2:1:1). The actinomycin area of the chromatogram was collected in a small pipette (by suction) and the actinomycin eluted with methanol. The methanolic solution was placed in vial and evaporated to dryness. Ten ml of a naphthalene-based scintillation solution were then added, and the radioactivity present measured in a Packard Tri-Carb liquid scintillation counting system (model 2002). (The efficiency for the counter when counting ^{14}C was about 74 % and for ^{3}H about 28 %).

Materials.

All of the non-radioactive aminoacids were obtained from Sigma Chemical Company. The L-valine-^{14}C(U) (280 mCi/mmole) and the D, L-tryptophan-^{14}C (benzene ring labelled) (45 mCi/mmole) were obtained from Amersham/Searle. The L-methionine-C^{3}H$_3$ (1 mCi/0.037 mg) was purchased from New England Nuclear Corporation.

Effect of Tryptophan Metabolites on the Incorporation of Tryptophan-^{14}C into Actinomycin

If 3-hydroxy-4-methylkynurenine is an intermediate in the biosynthesis of actinocin the addition of an excess of this compound to the washed cell suspension supplemented with labelled tryptophan would be expected to dilute the amount of radioactivity in the actinomycin. When 3-hydroxy-4-methyl-kynurenine was added to the cell suspension, a significant reduction in the radioactivity was noted as shown in Table 1. However, methylhydroxyanthranilic acid was even more effective and hydroxyanthranilic acid, hydroxykynurenine, and kynurenine were also active. If the concentrations of the competitive 3-hydroxy-4-methylkynurenine and methylanthranilic acid were increased there was some increase in effectiveness in dilution of radioactivity of the actinomycin, while the effectiveness of the hydroxyanthranilic acid as an antagonist was not significantly concentration dependent.

Effect of Tryptophan Metabolites on the Incorporation of L-Valine-^{14}C into Actinomycin

In order to determine whether the addition of the presumed D-tryptophan metabolites to the washed cell suspensions might affect total actinomycin synthesis the effect of these substances on incorporation of L-valine-^{14}C was examined. As summarized in Table 2 we noted that in several instances the incorporation was increased by the addition of the tryptophan metabolites.

Table 1. Effect of tryptophan metabolites on the incorporation of D, L-tryptophan-^{14}C into actinomycin D

Added metabolite	cpm noted at indicated concentration of added metabolite*	
	2 μmoles	400 mμ moles
None	1,300	989
Kynurenine	570	745
3-Hydroxykynurenine	835	1,118
3-Hydroxy-4-methylkynurenine	460	457
3-Hydroxyanthranilic acid	762	736
3-Hydroxy-4-methylanthranilic acid	350	184

* Reaction mixture contained: L-tryptophan-^{14}C (benzene ring labelled); L-threonine; L-proline; sarcosine; glycine; L-valine; and L-methionine.

Table 2. Effect of tryptophan metabolites on the incorporation of L-valine-^{14}C into actinomycin D

Added metabolite	Radioactivity of actinomycin (cpm)
Kynurenine	995
3-Hydroxykynurenine	2,005
3-Hydroxy-4-methylkynurenine	4,030
3-Hydroxyanthranilic acid	4,332
3-Hydroxy-4-methylanthranilic acid	5,105
Anthranilic acid	2,505

Reaction mixture contained: L-tryptophan; L-threonine; L-proline; sarcosine; L-valine-^{14}C(U); glycine; L-methionine.

Effects of Tryptophan Metabolites on the Incorporation of L-Valine-^{14}C and L-Methionine-C^{3}H$_3$ into Actinomycin

Since each mole of actinomycin has 6 methyl groups derived from L-methionine (2 in the sarcosine, 2 in the N-methyl-L-valine, and 2 in the actinocin), it seemed possible that if non-radioactive 3-hydroxy-4-methylkynurenine was directly incorporated into the actinomycin, the specific activity of actinomycin synthesized by the washed cell suspensions in the presence of L-methionine-C^{3}H$_3$ might be lower. An experiment designed to test this hypothesis is summarized in Table 3. While we noted that the addition of the 3-hydroxy-4-methylanthranilic acid did affect the specific activity of the actinomycin formed (as shown by the decrease in ratio of ^{3}H to ^{14}C in the actinomycin formed in the presence of L-valine-^{14}C and L-methionine-C^{3}H$_3$), hydroxyanthranilic acid was also effective.

Table 3. Effect of tryptophan metabolites on the incorporation of L-valine-^{14}C and L-methionine-C^{3}H$_3$ into actinomycin D

Added metabolite	Radioactivity of actinomycin D		
	^{14}C	^{3}H	H/C
L-Tryptophan (control)	4,630 dpm	27,000 dpm	5.8
3-Hydroxy-4-methylkynurenine	6,550	26,400	4.0
3-Hydroxyanthranilic acid	5,260	25,300	4.8
3-Hydroxy-4-methylanthranilic acid	5,800	24,360	4.2

Reaction mixture contained: L-tryptophan; L-proline; L-valine-^{14}C(U); L-methionine-C^{3}H$_3$; L-threonine; sarcosine; glycine.

Identification of Actinomycin formed in Presence of Tryptophan Metabolites

Since there was a possibility that 3-hydroxy-4-methylkynurenine or 3-hydroxykynurenine might be directly incorporated into the phenoxazinone portion of actinomycin without conversion to 3-hydroxy-4-methylanthranilic acid, the actinomycins formed when these L-tryptophan-related materials were added to the washed cell suspensions were isolated on LH-20 Sephadex^{R} chromato-

graphic columns. The absorption spectra of the purified actinomycin formed by the washed cell suspensions in the presence of 3-hydroxy-4-methylkynurenine and in the presence of 3-hydroxykynurenine were identical with that obtained from pure actinomycin D, and the elution patterns from the LH-20 columns were identical with those found with actinomycin D.

Enzymatic Conversion of 3-Hydroxy-4-methylkynurenine to 3-Hydroxy-4-methylanthranilic Acid

Enzymatic conversion of 3-hydroxy-4-methylkynurenine to 3-hydroxy-4-methylanthranilic acid was noted with both washed cells and cell-free systems. In both cases the 3-hydroxy-4-methylanthranilic acid was detected on the chromatographic plates (used for separation of the ethylacetate extracts of the cell suspensions mentioned above) by fluorescence. Boiled (inactivated) cells did not carry out the conversion.

The cell-free enzyme preparation was obtained by crushing the washed *S. antibioticus* cells in a French press. (The cells were suspended in a pH 7.2 0.01M Tris-HCl buffer containing 20 % (v/v) glycerol and 1 mM dithiothreitol). The broken cells were centrifuged at 5,000 rpm for 15 minutes and the cell-free supernatant solution used for the enzyme studies. The reaction mixture contained 0.5 μmole of 3-hydroxy-4-methylkynurenine, 50 μg of pyridoxal phosphate and 0.5 ml of the crude enzyme, with a total volume of 0.7 ml. After 2-hour incubation at 30°C the reaction was stopped by addition of 0.05 ml of 2 N HCl. The 3-hydroxy-4-methylanthranilic acid was extracted with ethylacetate. After washing the extract with water, the ethylacetate was evaporated to dryness and the residue (after dissolving in a minimum of methanol) was spotted on the tlc plate along with authentic 3-hydroxyanthranilic acid (obtained from Sigma Chemical Company). The chromatogram was developed using *n*-butanol-ethanol-water (2:1:1) system. Further study of the effect of increased incubation time on the formation of the hydroxymethylanthranilic acid showed that the rate decreased after 60-minute incubation. No significant conversion of 3-hydroxy-4-methylkynurenine to 3-hydroxy-4-methylanthranilic acid was obtained with a boiled enzyme preparation.

Discussion

Our studies show that addition of 3-hydroxy-4-methylkynurenine to actinomycin-forming cells of *S. antibioticus* suspended in radioactive tryptophan containing buffer resulted in reduction in the amount of radioactive tryptophan converted to actinomycin D while at the same time the amount of actinomycin synthesized was increased. These results suggest that the 3-hydroxy-4-methylkynurenine is an intermediate in the conversion of L-tryptophan to the actinocin portion of the molecule. We also noted an increase in actinomycin biosynthesis when 3-hydroxykynurenine and 3-hydroxyanthranilic acid were each added to the cell suspensions, and deduce from these observations that perhaps actinomycin synthesis may be limited by the rate of L-tryptophan conversion to the hydroxymethylanthranilic acid.

Since no 3-hydroxy-4-methylkynurenine could be isolated from actinomycin-producing cells, the essential role of this compound as an intermediate in conversion of L-tryptophan to actinocin is not proved. On the other hand, the present studies do suggest that enzymes present in the actinomycin-forming *S. antibioticus* can convert the 3-hydroxy-4-methylanthranilic acid to actinomycin, and hopefully these enzymes are present for that purpose.

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