ENZYMATIC CONVERSION OF 5-TRITIOTRYPTOPHAN TO 4-TRITIO-5-HYDROXYTRYPTOPHAN

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The hydroxylation of tryptophan to 5-hydroxytryptophan (5HTP) is the first step in the biosynthesis of 5-hydroxytryptamine (serotonin) (16). This reaction has been observed in whole cells of Chromobacterium violaceum (10), in cell-free extracts of brain (3,12) and mast cells (9). However, the lack of a sensitive and convenient assay has hindered studies on the purification and characterization of the hydroxylating enzyme. Experiments were initiated using, as substrate, tryptophan labelled with tritium in the 5-position, with the hope of measuring the rate of hydroxylation by the release of tritium into water according to the following reaction sequence:

$$\text{Tryptophan-5-}{}^{3}\text{H} + 1/2\ O_2 + H_2O \xrightarrow[\text{Hydroxylase}]{\text{Tryptophan}} 5\text{-OH-tryptophan} + {}^{3}H^{+}$$

$${}^{3}H^{+} + H_2O \rightarrow H^{+} + {}^{3}HOH$$

Similar exchange reactions have been used successfully for assaying tyrosine hydroxylase (11), tyrosinase (13), and collagen

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proline hydroxylase (7). However, when attempts were made to devise such an assay for tryptophan-5-hydroxylase using an enzyme system from neoplastic mast cells, it was observed that the rate of hydroxylation of tryptophan, as measured by a colorimetric procedure, occurred at 4-20 times the rate of tritium release into water. The present studies afford an explanation for the unexpected low tritium release during hydroxylation of tryptophan. The data show that hydroxylation is accompanied by a migration of the tritium from the 5-position of the substrate to the 4-position of the product with almost complete retention. A comparable shift of tritium and deuterium from the 4-position to the 3-position during the enzymatic hydroxylation of phenylalanine was recently reported from this laboratory by Guroff, et al (4,5).

Preparation of DL-tryptophan-5-^{3}H (^{2}H) -- This was prepared by reduction of 5-bromo-DL-tryptophan with tritium gas (New England Nuclear Corporation). Preparative purification of ^{3}H-tryptophan was carried out in a Beckman-Spinco Model 120 Amino Acid Analyzer, using a 0.95 x 52 cm column of PA-28 resin and elution with 0.2 M citrate buffer pH 4.2 (6). Only the fractions corresponding to authentic tryptophan were used in these experiments.

The position of the tritium was ascertained in several ways. Bromination of the tritiated tryptophan with 2-3 equivalents of N-bromosuccinimide in acetate buffer pH 5.0, which displaces hydrogen at the 2 and 5 positions (8) caused a loss of 87% of the activity. Independent studies on the conversion of 5-^{3}H-tryptophan to actinomycin by whole cells of Streptomyces antibioticus indicated that

80 to 90% of the radioactivity in the tritiated tryptophan was either in the 4 or 5 positions (18). Finally, deuterated tryptophan, prepared in an identical manner, was assayed by nuclear magnetic resonance, and shown to contain at least 90% of the deuterium in the 5-position.

Preparation of 5-hydroxy-DL-tryptophan-4-^{3}H (4-^{3}H-5HTP) -- 5-Hydroxy-DL-tryptophan (4.4 mg, 20 μmoles) was dissolved in a mixture of 300 μl of tritiated water (30 mc) and 200 μl of dimethylformamide containing 20 μl of triethylamine. The solution was heated in a sealed tube under nitrogen for 3 hrs at 65°. After dilution, lyophilization and preparative paper electrophoresis (pH 6.5, acetic acid, pyridine buffer), the zone corresponding to the 5-hydroxytryptophan was extracted into water and lyophilized.

Tryptophan-5-hydroxylase preparation -- Mouse neoplastic mast cells (9,14) were suspended in an equal volume of 0.05 Tris buffer pH 6.8 containing mercaptoethanol (0.01 M) and ferrous ammonium sulfate (0.25×10^{-3} M). Cells were broken by passage through a French pressure cell and the soluble fraction was obtained by centrifugation at 100,000 x g for one hour. Solid ammonium sulfate (enzyme grade) was added and the proteins which precipitated between 0-60% saturation were collected and dissolved in the same buffer solution as above.

Isolation of ^{3}H-5-hydroxytryptophan -- Following incubation, 1.5 ml of 10% $ZnSO_4$ and 1.5 ml of 1 N NaOH were added to 5 ml of the reaction mixture and the precipitate was removed by centrifugation. The supernatant fluid was adjusted to pH 3.5 with acetic acid and

passed through a short Dowex-50 pH 3.2 column. The resin was washed and the amino acids eluted with 0.35 M citrate buffer pH 5.2. The eluate was lyophilized and the inorganic salts precipitated with ethanol-acetone-water (1:10:1) at 0°. After evaporation the amino acids in the desalted extract were separated on a 0.9 x 25 cm column of PA-35 resin using the Spinco-Beckman Model 120C Amino Acid Analyzer. Citrate buffer pH 5.2, 0.35 M, was the eluting fluid (6). 5-Hydroxyindoles and tryptophan were measured fluorometrically (16,2).

Tritium release and 5HTP formation from 5-^{3}H-tryptophan -- Incubation of the mast-cell extract with 5-^{3}H-tryptophan yielded appreciable amounts of 5HTP, as measured colorimetrically, but there was little, although variable, release of tritium into water. Typical experiments are shown in Table I. Under the conditions used only

Table I

Comparison of 5-Hydroxytryptophan and Tritium Water Formed from 5-Tritiotryptophan

Experiment	5-Hydroxytryptophan	Tritium water
	millimicromoles	
1	150	8
2	120	11
3	140	33

The incubation mixture (total volume 5 ml) contained the following components per ml: 0.5 ml of enzyme preparation (5-10 mg of protein), 300 μmoles of Tris-acetate buffer pH 6.8, 250 μmoles of mercaptoethanol, 0.5 μmole of p-bromo-m-hydroxybenzyloxyamine (NSD-1055) (11) and 0.25 μmole of ferrous ammonium sulfate. After a 5-min preincubation, 0.25 μmole of 2-amino-6,7-dimethyl-4-hydroxy-5,6,7,8-tetrahydropteridine (Aldrich) and 0.25 μmole of 5-^{3}H-tryptophan (0.5-2.5 μC/μmole) were added to start the reaction. The tryptophan solution was prepared by dissolving a trace quantity of lyophilized 5-^{3}H-DL-tryptophan with nonradioactive L-tryptophan. Incubations were carried out

at 37° for 30 min using a metabolic shaker. At the end of the incubation, 1 ml of the reaction mixture was removed, and 1 ml of 20% TCA was added to precipitate proteins. The 5-hydroxytryptophan in the TCA filtrate was measured colorimetrically by the nitroso-naphthol procedure (17) after two extractions with washed ether to remove mercaptoethanol. Tritium water was measured by transferring 0.2 ml of the reaction mixture to a tube and distilled in vacuo. Radioactivity was measured in a Packard Scintillation Spectrometer

5-20% of the theoretical amounts of tritium were released into water. Since the position of the label in the tryptophan had been verified, these results could only have been due to a marked isotope effect or to retention of the tritium in the product, 5HTP. In order to select between these two alternatives, experiments were performed in which μmole quantities of product were formed enzymatically and separated from the substrate by ion-exchange chromatography on the Amino Acid Analyzer. As seen in Fig 1, the 5HTP, which was completely separated from tryptophan, contained appreciable radioactivity. No radioactive 5HTP peak was observed when enzyme or cofactors were omitted. The findings suggested that during the conversion of 5-^{3}H-tryptophan to 5HTP, the tritium in the 5-position migrated to another position in the product without release into water. It can also be seen in Fig 1 that the specific activity of the enzymatically formed 5HTP was almost the same as that of the tryptophan, indicating that there was >85% retention of the tritium originally in the 5-position of the substrate 5-^{3}H-tryptophan. It was subsequently found that when the reaction mixtures were exposed to 10% trichloroacetic acid for 4 hrs at room temperature, the 5HTP had lost most of its radioactivity and the amounts of tritium water obtained on distillation were almost equivalent (75-95%) to the 5HTP determined colorimetrically.

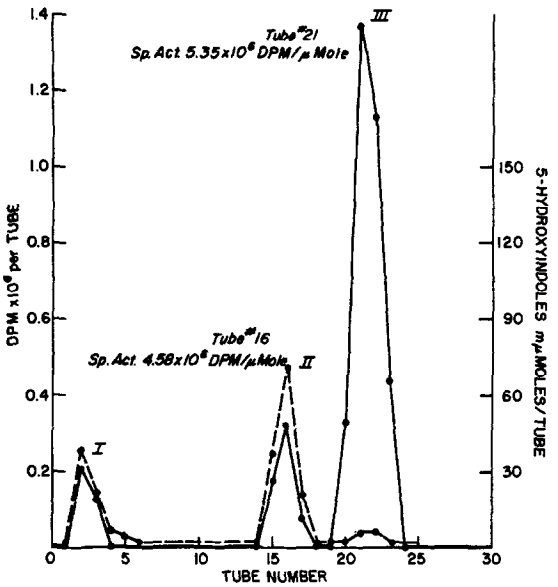

Fig. 1 - Separation of tryptophan and 5-hydroxytryptophan on the Amino Acid Analyzer. A typical reaction mixture was purified as described in the Methods section and chromatographed on a Beckman Amino Acid Analyzer, Model 120. Each tube contained 3.2 ml. Aliquots were removed for radioactive measurement and 5-hydroxyindole determination. Peak II is 5-hydroxytryptophan, peak III is tryptophan. The radioactive components in peak I have not been identified but represent degradation products obtained from the substrate, 5-^{3}H-tryptophan, during the purification procedure; they are also present in control incubations.

Evidence for the formation of 4-^{3}H-5-hydroxytryptophan -- Daly and Witkop (1) have shown that the hydrogen in the 4-position of 5HTP is labile and readily exchanges in acidic solution with the medium. The acid lability of the enzymatically formed product suggested that it might be 4-^{3}H-5HTP. Therefore, the radioactive 5HTP formed from 5-^{3}H-tryptophan, which was isolated after chromatography on Dowex-50, was compared with synthetic 4-^{3}H-5HTP with regard to its ability to lose tritium under a variety of conditions. As seen in Table II both

Table II

Comparison of the Exchange of Tritium from Enzymatically Formed ^{3}H-5HTP with Synthetic 4-^{3}H-5HTP Under a Variety of Conditions

Conditions	Time	Tritium Released - Percent	
		Synthetic 4-^{3}H-5HTP	Enzymatically formed ^{3}H-5HTP
	hrs		
10% trichloroacetic acid	1	100	100
0.1 N H_2SO_4	10	83	78
pH 1.2 HCl-KCl	10	72	72
pH 2.5-9.0	4	<10	<10
0.8 N NH_4OH	1	66	66
0.1 N NaOH	10	68	60

A similar procedure as described in Table I was used for measuring the amount of tritium exchanged from 4-^{3}H-5HTP at 50° in solutions at various pH values.

synthetic 4-^{3}H-5HTP and the enzymatic product completely lost their tritium on incubation at 50° for 60 min in 10% TCA. Furthermore, under other conditions the rates of tritium release were the same from both compounds. Between pH 2.5-9.0 little tritium loss occurred with either compound; above pH 9.0 both compounds lost tritium at comparable rates.

These studies and prior observations on the unique lability of the hydrogen at C-4 of 5HTP indicate that the primary product formed through the action of the mast cell tryptophan-5-hydroxylase on 5-^{3}H-tryptophan is 4-^{3}H-5-hydroxytryptophan. Such a migration of deuterium and tritium, with retention, during substitution by a hydroxyl group was first observed in this laboratory during the enzymatic hydroxylation of phenylalanine to tyrosine (4,5). These and other observations in our laboratories suggest that the mechanism

of enzymatic hydroxylation of an aromatic compound involves migration of the substituent which is displaced to an adjacent position on the ring. For the enzymatic hydroxylation of tryptophan to 5HTP alternative mechanisms may be envisaged as shown in Fig 2.

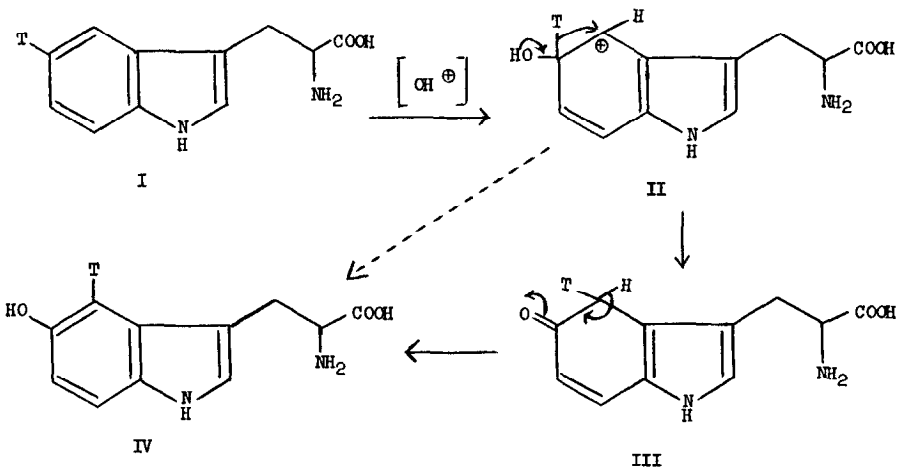

Fig. 2

The virtually complete retention of tritium in 5HTP (IV) necessitates either a stereospecific removal of the hydrogen from III or an extremely large isotope effect in the conversion of III to IV. It is equally possible that the enzyme catalyzes a concerted reaction where III is not an intermediate. Experiments are in progress to test these alternate hypotheses.

Migration of a substituent during enzymatic hydroxylation of an aromatic compound may involve hydrogen, deuterium, tritium or other groups. In the latter category would be the displacement of the pyruvic acid sidechain during the enzymatic hydroxylation of p-hydroxyphenylpyruvate to homogentisic acid (15) and the conversion of 4-chlorophenylalanine to 3-chloro-4-hydroxyphenylalanine by phenylala-

nine hydroxylase.[2] Parallel studies are being undertaken in these laboratories on the chemical substitution of various aromatic and heterocyclic ring systems to establish the general nature of this migration during aromatic substitution.

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