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# Stereochemistry and Mechanism of Reactions Catalyzed by Indolyl-3-alkane α-Hydroxylase*

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**The reaction of tryptamine with indolyl-3-alkane α-hydroxylase is shown to remove stereospecifically the *pro-S* hydrogen at C-2 of the side chain and to give hydroxytryptamine of "*R*" configuration. The reaction therefore proceeds stereospecifically with net inversion of configuration at C-2 of the tryptamine side chain. In the reaction of L-tryptophan methyl ester, the enzyme also catalyzes stereospecific removal of the *pro-S* hydrogen at C-3, but the product 3-hydroxytryptophan methyl ester is racemic at C-3. The unreacted tryptophan methyl ester is shown to incorporate solvent hydrogen into the *pro-S* position at C-3 in an at least partially stereospecific manner, suggesting that the reaction of L-tryptophan methyl ester is reversible. The hydrogens at C-1 of the tryptamine side chain and the α-hydrogen of L-tryptophan methyl ester are shown to be retained in the reactions. The results support the notion that the enzyme catalyzes stereospecific 1,4-dehydrogenation of 3-substituted indoles to the corresponding alkylidene indolenines as the primary reaction, followed by stereospecific or nonstereospecific hydration of these intermediates as a secondary process. Substrate specificity studies with a number of tryptophan analogs are in excellent agreement with such a mechanism.**

Indolyl-3-alkane α-hydroxylase is a novel tryptophan-metabolizing enzyme recently isolated from *Pseudomonas* by Roberts and Rosenfeld (1), and characterized by these authors (1) and by Takai *et al.* (2). This enzyme catalyzes oxidation on the side chain of a variety of 3-substituted indole compounds, including certain tryptophan-containing oligopeptides. Although there is still controversy about the structure of the product(s) formed from *N*-acetyl-L-tryptophanamide (3, 4, 5), the products from tryptamine and L-tryptophan methyl ester have been identified as side chain 2-hydroxytryptamine and 3-hydroxytryptophan methyl ester, respectively (1). In this paper we report results defining the stereochemical course of the reactions of tryptamine and L-tryptophan methyl ester and their mechanistic implications.

## EXPERIMENTAL PROCEDURES

*Materials*—The chemicals used were of reagent grade or of the highest purity commercially available, and they were used without further purification. L-[3-$^{14}$C]tryptophan (~45 mCi/mmol), tritiated water (10 mCi/ml), and [2-$^{14}$C]glycine (40 mCi/mmol) were purchased from Amersham/Searle. [$^{18}$O]Water (95 atom %) was obtained from Merck. Hexamethyldisilazane and trimethylchlorosilane were purchased from Pierce. (2*S*, 3*R*)- and (2*S*, 3*S*)-[3-$^{3}$H]tryptophan were prepared from (2*S*, 3*S*)- and (2*S*, 3*R*)-[3-$^{3}$H]serine, respectively, as previously described (6). DL-[2-$^{2}$H]tryptophan was synthesized by hydrolysis of ethyl 2-formamido-2-carbethoxy-3-indole propionate in $^{2}H_2O$ (7). L-[2-$^{3}$H]tryptophan was synthesized as described previously (8). (2*S*, 3*R*)- and (2*S*, 3*S*)-[3-$^{3}$H]tryptophan methyl ester and L-[2-$^{3}$H]tryptophan methyl ester were obtained from the corresponding tryptophan samples by treatment with $CH_3OH$/HCl and were purified by paper chromatography (System A). Side chain [1,2-$^{2}H_4$]tryptamine was synthesized according to the following procedure. Gramine methiodide (1.1 g), obtained from reaction of methyl iodide with gramine in absolute ethanol at 0°C (9), was converted to indolyl acetonitrile by heating with 0.5 g of NaCN in 10 ml of $^{2}H_2O$ (10). The $CHCl_3$ extract contained 40% gramine and 60% indolyl acetonitrile. NMR spectra indicated that the indolyl acetonitrile formed incorporated 90% of deuterium at the methylene position. The mixture was then dissolved in 20 ml of EtOD, mixed with 20 ml of 2 N NaOD/$^{2}H_2O$, and reduced with 1.5 g of Raney Nickel (Ni/Al, 50:50) (11). The reaction mixture was extracted with benzene. The tryptamine obtained was converted to its HCl salt and recrystallized repeatedly from 95% EtOH/ethyl acetate. Mass spectral analysis indicated that the isotopic purity of the [1,2-$^{2}H_4$]tryptamine obtained was 90%. The various tryptophan analogs used were available from earlier work (12–14).

*Enzymes*—Indolyl-3-alkane α-hydroxylase was purified as described (1) and had a specific activity of 10 units/mg of protein or 40 units/ml. Tyrosine decarboxylase (*Streptococcus faecalis*), tryptophanase (*Escherichia coli*), lactate dehydrogenase (pig heart), catalase (beef liver), and D-amino acid oxidase (hog kidney) were purchased from Sigma, and glutamate/pyruvate transaminase (pig heart) from Boehringer Mannheim.

*Instrumental Methods*—Radioactivity of compounds in solution or their location on radiochromatograms was determined by previously described methods (15). Mass spectra were measured on a Dupont 21-492 spectrometer using electron impact (70 eV) and chemical (isobutane, 0.5 torr) ionization. Nuclear magnetic resonance spectra were recorded on a Varian FT-80 spectrometer. Optical rotations were measured on a Perkin-Elmer 241 polarimeter.

*Chromatography*—Precoated silica gel plates (0.25 mm in thickness) from Brinkmann were employed for thin layer chromatography. Paper chromatography was done using the descending technique on Whatman No. 3 mm paper which had been washed with 1 M citric acid followed by water. The following solvent systems were used: System A, chloroform/95% ethanol/acetic acid/water, 50:34:10:6 ($R_F$ values: tryptophan 0.42, tryptophan methyl ester 0.70); System B, isopropanol/concentrated $NH_4OH$/water, 6:3:1 ($R_F$ values: tryptophan 0.70, serine 0.46).

*Conversion of Tryptophan to Tryptamine by Tyrosine Decarboxylase*—Tyrosine decarboxylase (16, 17) possesses only poor tryptophan decarboxylase activity (18, 19), which is, however, sufficient for our purpose, namely to prepare tryptamine stereospecifically labeled at any one of the four side chain hydrogens from tryptophan. A typical reaction mixture contained in 0.5 ml of 50 mM potassium phosphate buffer (pH 5.5): stereospecifically labeled L-tryptophan, 0.1 mg; pyridoxal phosphate, 0.1 mg; tyrosine decarboxylase, 5 IU. After incubation at 37°C for 8 h, NaOH was added to bring the solution to pH >10, followed by extraction with benzene (three 2-ml

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portions). The benzene extract was evaporated to dryness, dissolved in 2 ml of 0.1 N NaOH, and extracted with benzene again. The benzene extract was then evaporated to dryness and dissolved in 1 ml of 10% HCl and extracted with benzene. The aqueous layer contained tryptamine-HCl, which was identified by UV and NMR spectral comparison with authentic samples. The yield varied from 3 to 10%. In some cases the sample was further purified by recrystallization from ethanol + ethyl acetate. In the decarboxylation of [α-$^2$H]tryptophan, the DL mixture was used directly, since we have shown that D-tryptophan is neither a substrate nor an inhibitor of tyrosine decarboxylase by a control experiment in which two parallel incubations, one containing 1 mg of L-tryptophan and $4.5 \times 10^5$ dpm L-[3-$^{14}$C]tryptophan, and the other containing 1 mg of L-tryptophan, 1 mg of D-tryptophan, and $4.5 \times 10^5$ dpm L-[3-$^{14}$C]tryptophan, gave the same amounts of tryptamine (0.21 and 0.26 μmol, respectively) with the same specific activity ($1.02 \times 10^5$ dpm $^{14}$C/μmol and $0.95 \times 10^5$ dpm $^{14}$C/μmol, respectively).

*Indolyl-3-alkane α-Hydroxylase Reactions*—The hydrochloride salt of the substrate, tryptamine or tryptophan methyl ester, was dissolved in 50 to 100 μl of $H_2O$ and then incubated with an appropriate amount of indolyl-3-alkane α-hydroxylase (approximately 0.1 to 0.31 units/μmol of substrate) at 37°C for 20 min. Longer incubation very often resulted in the formation of by-products. The reaction mixture was then dialyzed twice at 0–4°C against 1 ml of $H_2O$. The dialyzate was lyophilized, and the $H_2O$ distilled was counted for tritium activity, when desired. A proton NMR spectrum of the sample was then taken to determine the purity of the product and the extent of conversion. For the reaction of tryptamine, >90% conversion could be achieved (no impurity was detectable by NMR), and the 2-hydroxytryptamine thus obtained was used for the measurement of optical rotations. In the case of L-tryptophan methyl ester, the best conversion obtained without by-product formation was 70%. In the radioisotope experiments, an aliquot of the sample was lyophilized to remove $^2H_2O$ (the 100% $^2H_2O$ used contained a low amount of tritium activity), and then the $^3H/^{14}C$ ratio was determined. In the cases in which the reaction was carried in $^2H_2O$, the enzyme was dialyzed against 10 mM potassium phosphate buffer in $^2H_2O$ (pD 6.6) before incubation, and $^2H_2O$ was used in the work-up of the product.

To isolate the unreacted tryptophan methyl ester, the reaction mixture after the above treatments was mixed with 1 mg of nonlabeled compound, dissolved in 5 N NaOH, and heated on a steam bath for 3 h. The reaction mixture was then neutralized with HCl, and tryptophan was isolated by paper chromatography (System A).

In the reaction of tryptamine, the unreacted material was isolated by repeated recrystallization with a large excess of nonlabeled compound from 95% ethanol/ethyl acetate.

*Incorporation of Solvent Tritium into the β Position of Tryptophan Methyl Ester*—L-tryptophan methyl ester (0.5 mg) was dissolved in 0.1 ml of tritiated water (~10 mCi) and incubated with 10 μl of indolyl-3-alkane α-hydroxylase at room temperature for 30 min. Then an additional 3 mg of the substrate was added, and the reaction mixture was further incubated for 20 min. After repetitive lyophilization, the residue was found to retain 0.65 μCi of tritium activity. The sample was then hydrolyzed with 5 N NaOH, neutralized with HCl, and chromatographed in System B. The tryptophan isolated (6 μmol based on UV) contained 0.5 μCi of tritium. Half of this sample was mixed with 5 mg of L-tryptophan and L-[3-$^{14}$C]tryptophan to give a $^3H/^{14}C$ ratio of 3.47, dissolved in 25 ml of 0.2 M potassium phosphate buffer in $^2H_2O$ (pH 8.0) containing 7 mg of glutathione, 0.2 mM pyridoxal phosphate, 0.5 mg of bovine serum albumin, 10 μmol of NADH, 5 IU of lactate dehydrogenase, and 0.4 IU of tryptophanase. The reaction mixture was incubated at 37°C for 20 h, and the reaction was followed by measuring the decrease in the absorbance at 340 nm. The reaction was stopped by boiling for 2 min, and the lactate was isolated (60% yield, $^3H/^{14}C$, 3.76), oxidized to acetate ($^3H/^{14}C$, 3.66), and analyzed for the configuration of the methyl group according to the procedures described previously (8).

*β-Hydroxytryptophan Methyl Ester/[$^{18}$O]Water Exchange*—β-Hydroxytryptophan methyl ester (0.5 mg, containing 40% tryptophan methyl ester) was dissolved in 0.1 ml of [$^{18}$O]water (95 atom %) and incubated at room temperature for 1 h. After lyophilization, 0.2 mg of the residue was dissolved in 30 μl of dry pyridine, and 10 μl of hexamethyldisilazane and 5 μl of trimethylchlorosilane were added. The reaction mixture was kept for 30 min at room temperature under a nitrogen atmosphere and then evaporated to dryness for mass spectral analysis (chemical ionization, isobutane). The $M + 1$ ion showed no detectable enrichment in the isotope satellites.

*Stereochemistry of Tryptophan Decarboxylation by Tyrosine Decarboxylase*—L-[2-$^3$H]tryptophan (0.1 mg, 1 μCi) was decarboxylated by incubation with tyrosine decarboxylase as described above to give a 4% yield of tryptamine. The latter was diluted with 1 mg of nonlabeled tryptamine·HCl and incubated with 5 ml of an oxidation mixture (153 mg of $K_2Cr_2O_7$, 24 ml of $H_2SO_4$, made up to 100 ml with $H_2O$) in a steam bath for 15 min. After adjustment to pH ~3 with NaOH, the reaction mixture was passed onto a Dowex 50W $H^+$ column, which was then washed thoroughly with $H_2O$ and eluted with 1 N $NH_4OH$. The effluent, after evaporating to dryness, contained about 10% of the original radioactivity of tryptamine. It was mixed with [2-$^{14}$C]glycine and recrystallized twice with excess of nonlabeled glycine from 95% EtOH/ethyl acetate to give [2-$^3H_1$, 2-$^{14}$C]glycine with a $^3H/^{14}C$ ratio of 1.02. The mother liquid had approximately the same $^3H/^{14}C$ ratio. Two milligrams of this sample was incubated with 30 IU of glutamate-pyruvate transaminase (dialyzed against $^2H_2O$ buffer for 3 h) in 0.5 ml of 0.1 M potassium phosphate buffer in $^2H_2O$, pD 7.9, containing 0.1 mM pyridoxal phosphate at 37°C. The α-hydrogen exchange of glycine was followed by observing the decrease in their NMR signal. After 14 h the exchange was complete, the singlet peak of the two α-hydrogens had changed to a triplet signal ($J = 2.4$ Hz due to H-$^2$H coupling) of one hydrogen, and the incubation was continued for 10 h. The reaction mixture was lyophilized and then dialyzed against water to remove enzyme. The [2-$^2$H, 2-$^3$H]-glycine isolated from the dialysis water showed a $^3H/^{14}C$ ratio of 0.86. In a separate experiment, the tryptamine obtained from L-[2-$^3$H]tryptophan was mixed with side chain [2-$^{14}$C]tryptamine to give a $^3H/^{14}C$ ratio of 2.04. The glycine obtained from oxidation was recrystallized with an excess of nonlabeled glycine, incubated with D-amino acid oxidase (5 IU) in 0.5 M potassium phosphate buffer, pH 7.8, in the presence of oxygen gas and excess catalase. The resulting glyoxylate was diluted with 5 mg of nonlabeled glyoxylate and converted to its 2,4-dinitrophenylhydrazine derivative. The isolated product showed a $^3H/^{14}C$ ratio of 0.22.

## RESULTS

*Stereospecific Removal of Side Chain C-2 Hydrogen from Tryptamine*—In order to determine which of the two side chain C-2 hydrogens is lost in the reaction, we prepared side chain (2*R*)- and (2*S*)-[2-$^3$H]tryptamine from (2*S*, 3*R*)- and (2*S*, 3*S*)-[3-$^3$H]tryptophan, respectively, by decarboxylation with tyrosine decarboxylase. The two tryptamine samples were mixed with side chain [2-$^{14}$C]tryptamine to give a $^3H/^{14}C$ ratio of approximately 2, and then incubated with indolyl-3-alkane α-hydroxylase. The product was not purified, because of its instability, but was dialyzed into water followed by lyophilization. The extent of reaction was determined by NMR spectroscopy, and the samples were then counted to determine their $^3H/^{14}C$ ratios. As shown in Columns 1 and 2 of Table I, the product from 2*R*-[2-$^3$H]tryptamine retained 98% of the tritium, whereas that from 2*S*-[2-$^3$H]tryptamine retained only 59% of the tritium activity. Since the product was not purified to remove unreacted tryptamine, the 59% tritium in the product mixture from 2*S*-[2-$^3$H]tryptamine could come from the residual [$^3$H]tryptamine, which reacted much slower than

TABLE I

*Stereochemical data for the removal of hydrogen in the reactions catalyzed by indolyl-3-alkane α-hydroxylase*[a]

| | Side chain [2-$^3$H]tryptamine | | | | [3-$^3$H]tryptophan methyl ester | | | |
|---|---|---|---|---|---|---|---|---|
| | 2*S* | 2*R* | 2*S* | 2*R* | 3*S* | 3*R* | 3*S* | 3*R* |
| $^3H/^{14}C$, substrate | 1.96 | 2.13 | 1.96 | 2.13 | 3.23 | 3.52 | 1.94 | 2.19 |
| % conversion[b] | >90 | >90 | 75 | 45 | 70 | 60 | 90[d] | 70 |
| $^3H/^{14}C$ crude product | 1.17 | 2.08 | 1.40 | 2.15 | 2.32 | 3.49 | 0.83 | 2.07 |
| % $^3$H retention[c] | 59 | 98 | 71 | 100 | 72 | 99 | 43 | 95 |
| $^3H/^{14}C$, recovered substrate | | | 3.49 | 2.29 | | | 1.24 | 1.93 |

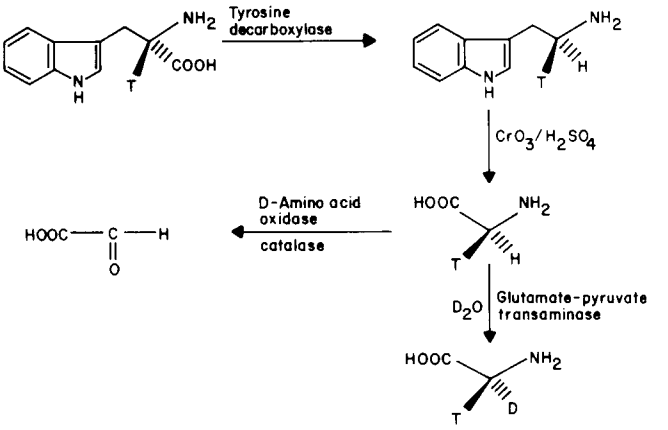
[a] No decrease in the $^3H/^{14}C$ ratios was observed in the control experiments in which the enzyme was omitted.
[b] Analyzed by peak area in the NMR spectra, subject to ±5% error.
[c] Most tritium activity lost was found in the distillate.
[d] In this incubation some unidentified by-products formed.

[$^{14}$C]tryptamine due to a kinetic isotope effect. In order to verify this assumption, the reactions were repeated to less than 100% completion, and the unreacted tryptamines were isolated by recrystallization with addition of excess nonlabeled tryptamine. As shown in Columns 3 and 4 of Table I, the tryptamine recovered from the reaction of 2*S*-[2-$^{3}$H]tryptamine shows an increased $^{3}$H/$^{14}$C ratio whereas that from 2*R*-[2-$^{3}$H]tryptamine does not, indicating that there is a kinetic isotope effect in the removal of the *pro-S* hydrogen at C-2. Although the magnitude of the tritium isotope effect cannot be easily calculated from these data, a comparison of the initial rates of oxidation of [1,2-$^{2}H_4$]tryptamine and tryptamine, as determined by polarographic measurement of oxygen consumption, indicates a $k_H/k_{^2H}$ of 1.81. Thus the results suggest that the *pro-S* hydrogen is stereospecifically removed in this reaction.

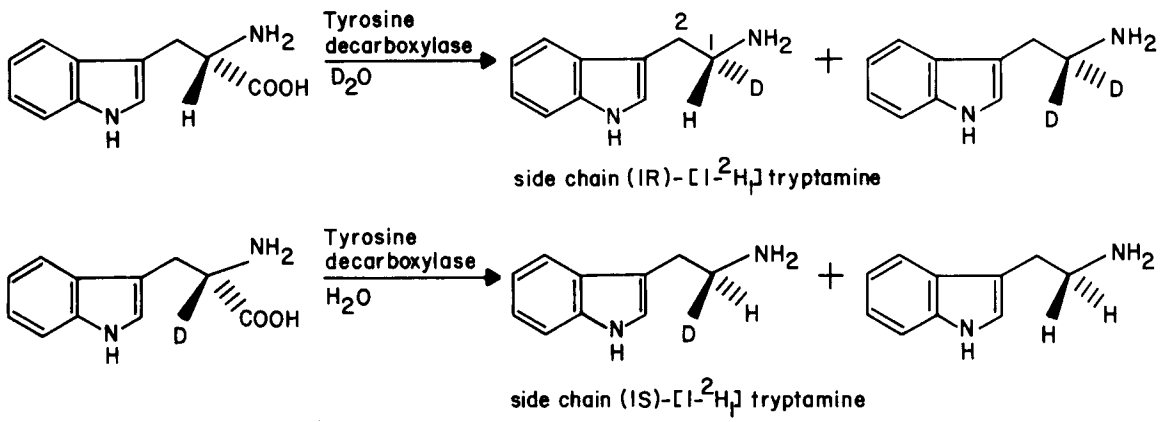
*Absolute Configuration of Hydroxytryptamine*—Although it has previously been suggested that the reaction product from tryptamine may be racemic (3), we have found that it is actually optically active and have determined its absolute configuration at C-2 of the side chain. The molecular rotations of the hydroxytryptamine observed in 5 mM aqueous solution at 25°C are: −41.0° (589 nm), −44.5° (578 nm), −49.8° (546 nm), −83.2° (436 nm), and −136.4° (375 nm). When the hydroxylase reaction was carried out in $^{2}H_2O$, no deuterium incorporation into the product hydroxytryptamine was observed, suggesting that the side chain C-1 hydrogens are retained in the reaction, thus allowing us to determine the absolute configuration at side chain C-2 by stereospecific deuteration at C-1. The two stereospecifically labeled species, side chain (1*R*)-[1-$^{2}$H]tryptamine and side chain (1*S*)-[1-$^{2}$H]tryptamine, were prepared from tryptophan with tryosine decarboxylase according to Scheme I. The stereochemistry of tyrosine decarboxylase from the same organism (*S. faecalis*) has previously been studied with tyrosine as substrate; the reaction proceeds in a retention mode (16). Although it seems reasonable to assume the same stereochemistry for the decarboxylation of tryptophan, we have independently verified the configuration of the resulting tryptamines. The tritium-labeled tryptamine obtained from the decarboxylation of L-[2-$^{3}$H]tryptophan was oxidized with $CrO_3/H_2SO_4$. Glycine was isolated by ion exchange chromatography followed by recrystallization with [2-$^{14}$C]glycine and excess of nonlabeled glycine. After complete equilibration of one of the two α-hydrogens with solvent deuterium by incubation with glutamate-pyruvate transaminase, which is known to exchange stereospecifically the *pro-2R* hydrogen of glycine (20, 21), the resulting [2-$^{2}$H, 2-$^{3}$H, 2-$^{14}$C]glycine retained 86% of the tritium activity. On the other hand, when the [2-$^{3}H_1$, 2-$^{14}$C]glycine sample was incubated with D-amino acid oxidase in the presence of excess catalase, known to remove the *pro-2S* hydrogen of glycine (20, 22); the resulting glyoxylate (isolated as the 2,4-dinitrophenylhydrazone) retained only 11% of the tritium activity. These results indicate that the decarboxylation of L-tryptophan by tyrosine decarboxylase does indeed proceed in a retention mode, as shown in Scheme II.

It was observed that decarboxylation of L-tryptophan was accompanied by partial exchange of α-hydrogen of tryptophan with solvent. This was confirmed by the $^{3}$H/$^{14}$C decrease from 2.60 in the starting material to 1.54 in the product when the reaction was carried out with L-[2-$^{3}$H, 3-$^{14}$C]tryptophan. Whether this exchange of the α-hydrogen is caused by contaminating enzymes in the commercial tyrosine decarboxylase preparation or by tyrosine decarboxylase itself has not been further investigated. Mass spectral analysis indicated that the solvent exchange of L-[2-$^{2}$H]tryptophan in $H_2O$ is 40% under the reaction conditions.

The decarboxylation of L-tryptophan by tyrosine decarboxylase in $^{2}H_2O$ thus gave (1*R*)-[1-$^{2}$H]tryptamine, in addition to some [1-$^{2}H_2$]tryptamine. The H-1 and H-2 coupling patterns of the 2-OH-tryptamine obtained from this tryptamine sample are shown in Fig. 1*B*. The H-2 signal is composed of a doublet and a singlet. The singlet corresponds to the species with two deuterium atoms at C-1, and is not of interest to our analysis. The doublet corresponds to the species with a deuterium at the *pro-R* position of C-1, and the coupling constant (8.5 Hz) is larger than the apparent coupling constant in the nonlabeled compound (6.5 Hz, triplet). Analogously, decarboxylation of L-[2-$^{2}$H]tryptophan by tyrosine decarboxylase in $H_2O$ gave (1*S*)-[1-$^{2}$H]tryptamine, in addition to some nonlabeled tryptamine. As shown in Fig. 1*C*, the H-2 signal of the 2-OH-tryptamine obtained from this tryptamine sample is composed




SCHEME II




SCHEME I

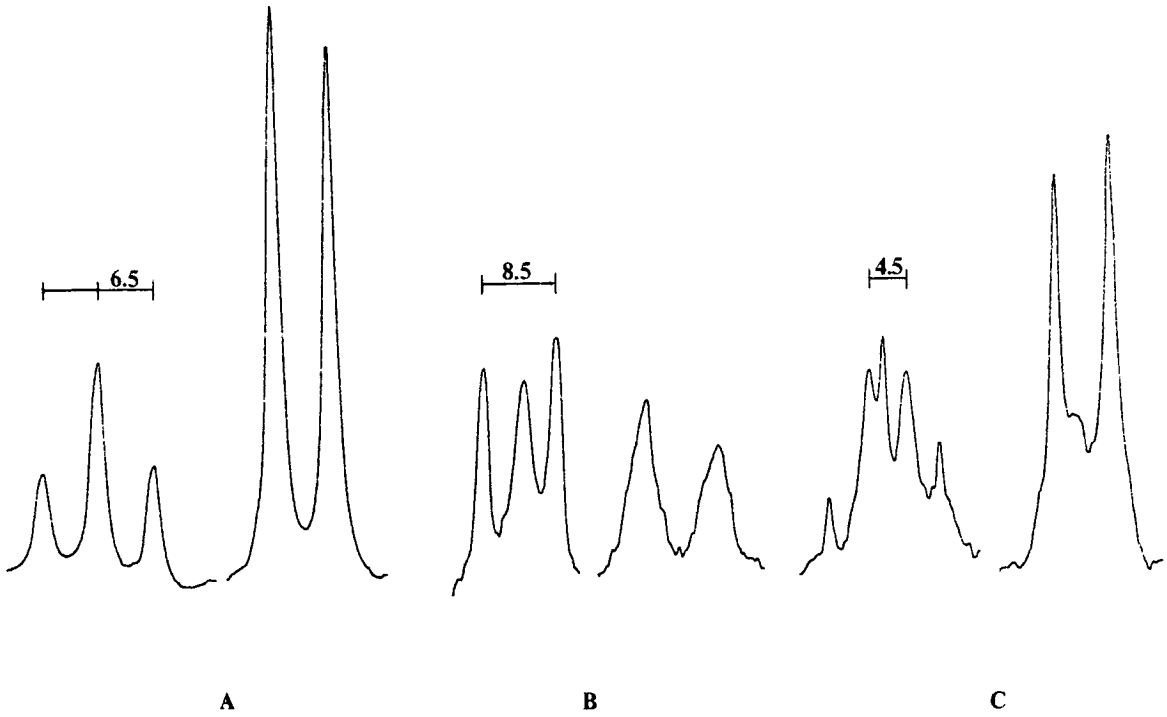
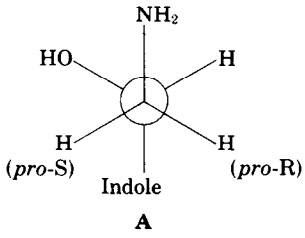
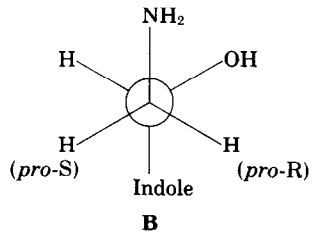


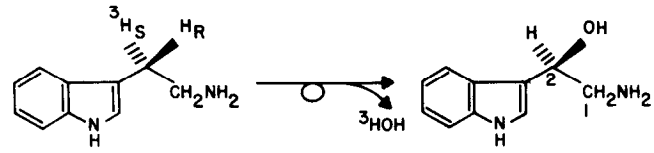

FIG. 1. The $^1H$ NMR coupling patterns of side chain H-1 (right hand side, $\delta$ 3.49 ppm) and side chain H-2 (left hand side, $\delta$ 5.35 ppm) of 2-hydroxytryptamine. *A*, obtained from nonlabeled tryptamine; *B*, from (1*R*)-[1-$^2H$]tryptamine plus [1-$^2H_2$]tryptamine; *C*, from (1*S*)-[1-$^2H$]-tryptamine plus nonlabeled tryptamine. The spectra were taken in $^2H_2O$ solution at room temperature. The coupling constants are expressed in Hz.




**A**




**B**




SCHEME III

of a triplet, corresponding to the nonlabeled compound, and a doublet, with a coupling constant (4.5 Hz) smaller than that in the nonlabeled compound. Thus the two hydrogens at side chain C-1 of the hydroxytryptamine are intrinsically different. They appeared equivalent in the NMR spectrum of the nonlabeled compound only because they happen to have the same chemical shift. The major conformations of (2*R*)-2-hydroxytryptamine and (2*S*)-2-hydroxytryptamine are shown as **A** and **B**, respectively, based on the theory that the conformer with the two largest groups *anti* to each other has the lowest energy (23). Intramolecular hydrogen bonding between OH and $NH_2$, if of any significance in aqueous solution, will also contribute to the stability of conformers **A** and **B**. Since the vicinal coupling constant is larger (8.5 Hz) when C-1 is deuterated at the *pro-R* position, whereas it is smaller (4.5 Hz) when C-1 is deuterated at the *pro-S* position, it can be concluded, based on the Karplus relationship, that the reaction product from tryptamine has "*R*" configuration at side chain C-2, as shown in **A**.

The results described so far indicate that the hydroxylation of tryptamine catalyzed by indolyl-3-alkane α-hydroxylase proceeds with net inversion of configuration at C-2 of the side chain, as shown in Scheme III.

*Stereospecific Removal of C-3 Hydrogen from Tryptophan Methyl Ester*—As shown in Columns 5 and 6 of Table I, when 3*S*-[3-$^3H$]tryptophan methyl ester was incubated with the enzyme and the reaction proceeded to 70% completion (as determined by NMR), the mixture of substrate and product after lyophilization to remove solvent retained only 72% of the tritium activity, whereas tritium was quantitatively retained in the parallel reaction of the 3*R* isomer. As discussed in the case of tryptamine, a kinetic isotope effect could be involved in the removal of the *pro-S* hydrogen. In a separate set of reactions, the reaction mixtures were hydrolyzed with base and the tryptophan was isolated with addition of nonlabeled compound and purified by paper chromatography. The results in Columns 7 and 8 of Table I indicate that the tryptophan recovered from the reaction of the 3*S*-isomer has a $^3H/^{14}C$ ratio (1.24) smaller than that of the starting material (1.94), which is consistent with the finding to be discussed in the following section that tryptophan incorporates solvent hydrogen into the *pro-S* position at C-3. This loss of tritium to solvent obscures any tritium enrichment the unreacted tryptophan may have encountered due to a kinetic isotope effect. For the reaction of the 3*R* isomer, the starting material, the reaction mixture after lyophilization, and the recovered tryptophan all have approximately the same $^3H/^{14}C$ ratios. These results suggest that the *pro-S* hydrogen at C-3 of the side chain is stereospecifically removed in the reaction of tryptophan methyl ester.

*Stereospecific Incorporation of Solvent Hydrogen at C-3 of Tryptophan Methyl Ester*—When the reaction of L-tryptophan methyl ester was carried out in tritiated water followed by alkaline hydrolysis, the recovered tryptophan was found to

contain substantial amounts of tritium incorporated from the solvent. This tritiated tryptophan was mixed with L-[3-$^{14}$C]-tryptophan ($^{3}$H/$^{14}$C, 3.47), incubated with tryptophanase in $^{2}H_2O$ to give pyruvate, which was trapped *in situ* as lactate by coupling the reaction to the lactate dehydrogenase systems (8). The lactate obtained ($^{3}$H/$^{14}$C, 3.76) was oxidized to acetate ($^{3}$H/$^{14}$C, 3.66) with $CrO_3/H_2SO_4$, and the chirality of the methyl group in the acetate was determined by the method of Cornforth *et al.* (24) and Arigoni and co-workers (25). In this analysis procedure, which involves conversion to malate with malate synthetase followed by reaction with fumarase, optically pure *R*-[2-$^{2}$H, 2-$^{3}$H]acetate is expected to give malate showing 79.7% tritium retention in the fumarase reaction, whereas the malate from pure *S* acetate should show 20.3% tritium retention upon incubation with fumarase (26). In our results the malate obtained above ($^{3}$H/$^{14}$C, 3.00) showed 61% tritium retention in the fumarase reaction ($^{3}$H/$^{14}$C, 1.82 after fumarase reaction), indicating that the methyl group of acetate had "*R*" configuration with approximately 40% optical purity. Based on the known stereochemistry of the tryptophanase reaction (8), these results indicate that solvent hydrogen is incorporated into the *pro-S* position at C-3 of tryptophan methyl ester, as shown in Scheme IV.

*Configuration of 3-Hydroxytryptophan Methyl Ester*—Rosenfeld *et al.* (3) have recently reported that the NMR spectrum of the reaction product from L-tryptophan methyl ester showed it to be a mixture of two closely related compounds, since there are two methyl signals and two sets of pairs of doublets for the side chain H-2 and H-3 protons. In order to prove unambiguously that these two sets of signals represent diastereomers rather than conformational isomers due to restricted rotation, we have carried out a temperature variation study. Conformational isomers would be expected to vary in their relative proportions with temperature, with the coupling constants being less sensitive to temperature inasmuch as the two sets of signals are still well separated. On the other hand, the relative proportions of the configurational isomers should be independent of temperature, whereas their coupling constants should shift towards the direction of an averaged value with increasing temperature (27), since at higher temperature each isomer will assume a more random conformer distribution. The result of this study is illustrated by the two signals for the H-3 proton as shown in Fig. 2. Upon increasing the temperature from 5 to 50°C, the relative proportions of the two isomers remain constant, whereas the coupling constants between H-2 and H-3 increase from 4.3 to 4.9 Hz for one isomer and decrease from 5.8 to 5.1 Hz for the other isomer. The 3-hydroxytryptophan methyl ester obtained from the reaction of L-tryptophan methyl ester with indolyl-3-alkane α-hydroxylase is therefore a mixture of two diastereomers, which must be epimeric at one or both of the two chiral centers, C-2 and C-3. When the reaction was carried out in $^{2}H_2O$, no deuterium incorporation into the product could be detected by NMR. Repetition of the reaction with L-[2-$^{3}$H, 3-$^{14}$C]tryptophan methyl ester ($^{3}$H/$^{14}$C, 2.74) gave product with a $^{3}$H/$^{14}$C ratio of 2.72. These results show that H-2 of the amino acid side chain is completely retained and strongly suggest that the chiral center of C-2 remains intact during the reaction. The two products from the enzymatic oxidation are epimeric at C-3. In order to determine whether racemization at C-3 occurs nonenzymatically via a reversible dehydration-hydration process, we incubated 3-hydroxytryptophan methyl ester with $H_2{}^{18}O$ and analyzed the product by mass spectrometry. No incorporation of $^{18}$O was observed, excluding such a nonenzymatic racemization process.

*Substrate Specificity towards Tryptophan Analogs*—Table II shows initial rates of oxidation of a number of tryptophan

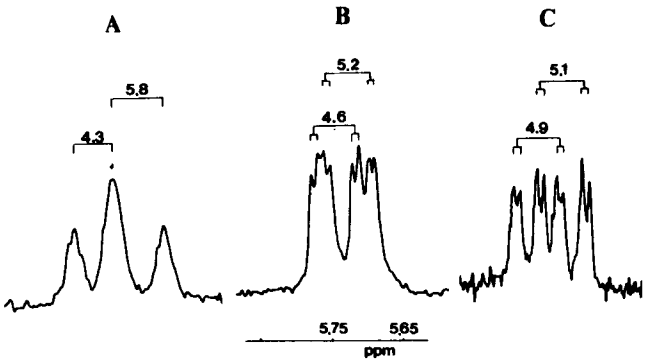
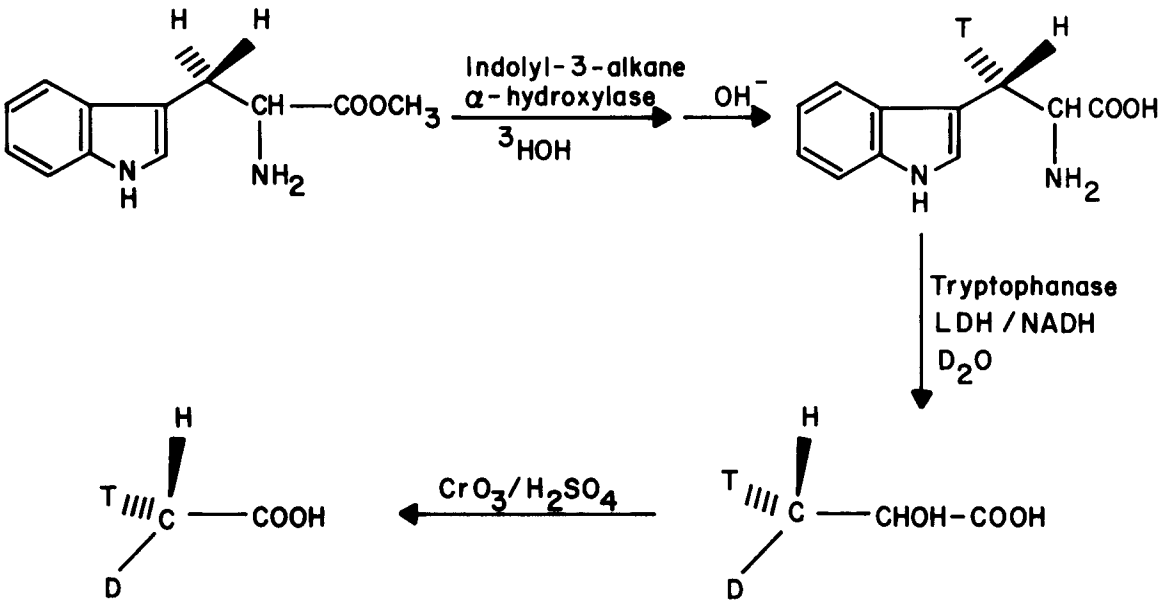



FIG. 2. Coupling patterns of the NMR signals for the β-proton of the reaction product of L-tryptophan methyl ester at 5 (*A*), 25 (*B*), and 50°C (*C*). The spectra were run in $^{2}H_2O$ using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as external reference (0 ppm). The coupling constants are expressed in Hz.




SCHEME IV

TABLE II

*Relative rates of $O_2$ consumption by tryptophan analogs*[a]

| Substrate | Rate |
|---|---|
| | % |
| L-tryptophan | 100 |
| D,L-homotryptophan | 70 |
| D,L-bis-homotryptophan | 50 |
| D,L-thiotryptophan | 0 |
| D,L-*N*-1-methyltryptophan | 0 |
| β-methyltryptophan, A isomer (2*R*, 3*S*) + (2*S*, 3*R*) | 50 |
| β-methyltryptophan, B isomer (2*R*, 3*R*) + (2*S*, 3*S*) | 0 |

[a] All compounds (1 mM in 0.1 M sodium acetate buffer, pH 5.5) were assayed polarographically, using a YSI model 53 oxygen meter, at 37°C in a 1-ml cell with approximately 0.1 unit of enzyme, as described earlier (1).

analogs, which were selected to probe mechanistic aspects of the reaction. Not surprisingly, extension of the tryptophan side chain by one or two carbon atoms is entirely compatible with the reaction. The results with thiotryptophan and *N*-1-methyltryptophan indicate that the enzyme reaction is completely dependent on the presence of an unsubstituted indolic nitrogen in the substrate. The stereochemical conclusions drawn from the experiments with the tritiated substrates are supported by the finding that β-methyltryptophan isomer A, but not isomer B, is a substrate for the enzyme. In isomer A, a racemic mixture of (2*R*, 3*S*) and (2*S*, 3*R*)-β-methyltryptophan, the enzymatically active 2*S* enantiomer carries the methyl group in the position occupied by the *pro*-3*R* hydrogen of tryptophan, leaving a hydrogen in the position involved in the reaction. In the 2*S* enantiomer of isomer B, the hydrogen normally removed by the enzyme has been replaced by the methyl group.

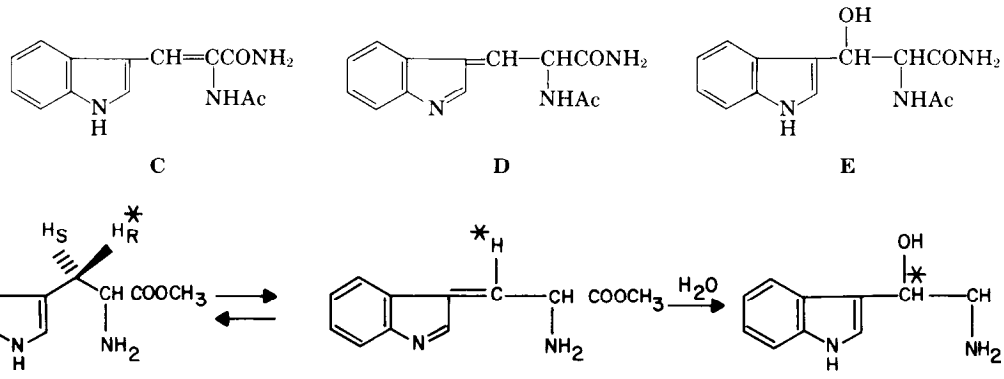
## DISCUSSION

Hayaishi and coworkers (4) have reported that the reaction of *N*-acetyltryptophanamide with indolyl-3-alkane α-hydroxylase gave *N*-acetyl-α,β-didehydrotryptophanamide (**C**) as the major product. They suggested that the reactions of other tryptophan-containing peptides also proceed through α,β-unsaturated intermediates. Rosenfeld and co-workers (3, 28) subsequently isolated **D** (in mixture with its oxazoline derivative), and suggested that the primary product is **D** and that **C** is an artifact formed due to nonenzymic, acid-catalyzed conversion of the primary product. However, Hayaishi and coworkers (5) have recently reported that **C** is the major product (80%) at pH 7.0 but a minor product at pH below 5.5, and that the major product at pH 5.5 is *N*-acetyl-β-hydroxytryptophanamide (**E**), which is dehydrated to **C** at low pH but not at pH 7.0. Furthermore, enzymatic formation of dehydrotryptophan in several peptides has been reported (4, 29). Since our results indicate that the side chain C-1 hydrogen of tryptamine and the α-hydrogen of L-tryptophan methyl ester remain intact in the reaction, the formation of hydroxytryptamine and hydroxytryptophan methyl ester cannot proceed through side chain α,β-didehydro intermediates analogous to **C** unless one makes the unlikely assumption that there is complete recycling of the α-hydrogen. Our results are, however, not incompatible with Hayaishi's finding that the enzyme function is pH-dependent, with **C** as the major product at neutral pH and **E** as the major product at acidic pH for the reaction of *N*-acetyltryptophanamide. Although the pH of our reactions has not been strictly controlled, it is approximately 4 to 5. We have also noticed that longer incubation always results in the formation of by-products, which, however, were not characterized, since this was not of interest for our stereochemical studies.

Our current results strongly support the proposal of Rosenfeld *et al.* (3) that the 3-alkylidene indolenine derivative is an intermediate in the hydroxylation reaction, based on the following arguments: (*a*) removal of one of the two heterotopic methylene hydrogens is always stereospecific; (*b*) the side chain C-1 hydrogens of tryptamine and the side chain H-2 of tryptophan methyl ester are not exchanged in the reactions; and (*c*) the reaction of tryptophan methyl ester is at least partly reversible, as evidenced by incorporation of solvent hydrogen into the unreacted substrate. Since the reverse reaction is at least partially stereospecific, and since the product is racemic at C-3, the reaction must be stepwise, involving first a reversible, stereospecific step and then an irreversible hydroxylation or hydration step, as shown in Scheme V.

The finding that the hydroxytryptamine formed from tryptamine is optically active indicates that in this case the hydration of the intermediate must occur in the chiral environment of the enzyme. It seems most likely that in this latter case, addition of water is faster than release of the 3-alkylidene intermediate from the enzyme, whereas with those substrates which undergo nonstereospecific hydroxylation the release of the intermediate from the enzyme may be the faster process. This would support the suggestion of Rosenfeld *et al.* (3) that addition of water to the 3-alkylidene indolenine intermediate is a spontaneous, nonenzymatic process. However, the alternative possibility that the process is enzyme-mediated in all cases, but proceeds stereospecifically with some compounds and nonstereospecifically with others, cannot be ruled out, although it is somewhat difficult to visualize.

The stereochemical mode of operation of indolyl-3-alkane α-hydroxylase is unusual, as enzymatic hydroxylations of nonactivated secondary carbons, with rare exceptions (30), proceed in a retention mode (31). This is true also for the superficially similar reaction catalyzed by dopamine-β-hydroxylase (32). However, the unusual stereochemistry of indolyl-3-alkane α-hydroxylase is merely a reflection of the




SCHEME V

unusual nature of this enzyme and its reaction mechanism. Most of the hydroxylases examined for their reaction stereochemistry are P-450 enzymes which incorporate molecular oxygen into the product. The latter is true also for dopamine-β-hydroxylase, a copper-containing enzyme which requires ascorbic acid as cofactor, and which catalyzes stereospecific removal of the *pro*-2*R* hydrogen from dopamine to give noradrenaline with net retention of configuration (32). On the other hand, indolyl-3-alkane α-hydroxylase contains iron, with heme as prosthetic group (1), and catalyzes stereospecific removal of the side chain *pro*-2*S* hydrogen atom from tryptamine to give hydroxytryptamine with net inversion of configuration and with water as the source of the oxygen atom incorporated into the product (3).

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