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

(Received for publication, December 12, 1978, and in revised form, March 8, 1979)

Ming-Daw Tsai,‡ Heinz G. Floss,‡ Henry J. Rosenfeld,§ and Joseph Roberts§

From the ‡Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907, and §The Sloan-Kettering Institute for Cancer Research, New York, New York 10021

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 alkylidine indolines 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 esters, 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-[3-14C]Tryptophan (45 mCi/mol), tritiated water (10 mCi/ml), and [2-14C]glycine (40 mCi/mmol) were purchased from Amersham/Searle. [10O]Water (98 atom %) was obtained from MERCK. Hexamethyldisilazane and trimethyloctasilazane were purchased from Pierce. (2S, 3R)- and (2S, 3S)-[3-14H]Tryptophan were prepared from (2S, 3S)- and (2S, 3R)-[3-14H]serine, respectively, as previously described (6). DL-[2-14H]tryptophan was synthesized by hydrolysis of ethyl 2-formamido-2-carbethoxy-3-indole propionate in H2O (7). 1-2-14Htryptophan was synthesized as described previously (8, 15, 16, and 21). Z-[2-14H]tryptophan methyl ester and m-[2-14H]tryptophan methyl ester were obtained from the corresponding tryptophan samples by treatment with CH3OH/HCl and were purified by paper chromatography (System A). Side chain [1,2-14H]tryptophan 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 H2O (10). The CHCl3 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 30 ml of EtOH, mixed with 20 ml of 2 N NaOD/H2O, 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 [1,2-14H]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), tryptophan decarboxylase (16, 17), 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 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/acidic acid/water, 50:34:10:6 (RF values: tryptophan 0.70, serine 0.46). Tyrosine decarboxylase activity (18, 19), which is, however, sufficient for our purposes, namely to prepare tryptamine stereospecifically labeled at C-2, and 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
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 and acetic acid. The reaction mixture 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 and acetic acid.

**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 H2O and then incubated with an appropriate amount of indolyl-3-alkane α-hydroxylase (approximately 0.1 to 0.3 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 H2O. The dialyate was lyophilized, and the H2O distilled was counted for tritium activity by liquid scintillation. A portion of this mixture 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 was obtained by incubating 1 μg of L-tryptophan and 4.5 × 10^6 dpm L-[1-14C]tryptophan, the other containing 1 μg of L-tryptophan, 1 μg of L-tryptophan, and 4.5 × 10^6 dpm L-[l-3H]tryptophan, gave the same amounts of tryptamine (0.21 and 0.26 μmol, respectively) with the same specific activity (1.02 × 10^6 dpm/14C μmol and 0.95 × 10^6 dpm/14C μmol, respectively).

**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 (2R) and (2S) [2-3H]tryptamine from (2S, 3R) and (2S, 3S)-[3-14C]tryptophan, respectively, by decarboxylation with tyrosine decarboxylase. The two tryptamine samples were mixed with side chain (2S)-[2-3H]tryptamine to give a 1H/14C ratio of 2.04. The glycine obtained from oxidation was recrystallized with an excess of nonlabeled glycine, incubated with d-amino acid oxidase (5 U) 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 3,4-dihydroxyphenylalanine derivative. The isolated product showed a 1H/14C ratio of 0.22.

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

<table>
<thead>
<tr>
<th>Side chain [2-3H]tryptamine</th>
<th>[3-14C]tryptophan methyl ester</th>
</tr>
</thead>
<tbody>
<tr>
<td>2S</td>
<td>2R</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>H/14C, substrate</td>
<td>&gt;90</td>
</tr>
<tr>
<td>H/14C, crude product</td>
<td>1.17</td>
</tr>
<tr>
<td>% retention</td>
<td>93</td>
</tr>
</tbody>
</table>

a No decrease in the H/14C 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.
[14C]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 2S-[2-^3H]tryptamine shows an increased 3H/14C ratio whereas that from 2R-[2-^3H]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-^3H]tryptamine and tryptamine, as determined by polarographic measurement of oxygen consumption, indicates a kH/kS 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 hydroxylation reaction was carried out in H2O, 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 (1R)-[1-2H]tryptamine and side chain (1S)-[1-2H]tryptamine, were prepared from tryptophan with tryosine decarboxylase according to Scheme I. The stereochromy of tyrosine decarboxylase from the same organism (S. faecalis) has previously been studied with tyrosine as substrate; the absolute configuration at side chain C-2 by stereospecific labeling with [2-14H]tryptamine and [2-3H]tryptamine, were prepared from tryptophan with tryosine decarboxylase as determined by polarographic measurement of oxygen consumption, indicates a kH/kS 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 hydroxylation reaction was carried out in H2O, 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 (1R)-[1-2H]tryptamine and side chain (1S)-[1-2H]tryptamine, were prepared from tryptophan with tryosine decarboxylase according to Scheme I. The stereochromy of tyrosine decarboxylase from the same organism (S. faecalis) has previously been studied with tyrosine as substrate; the absolute configuration at side chain C-2 by stereospecific labeling with [2-14H]tryptamine and [2-3H]tryptamine, were prepared from tryptophan with tryosine decarboxylase as determined by polarographic measurement of oxygen consumption, indicates a kH/kS 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 hydroxylation reaction was carried out in H2O, 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 (1R)-[1-2H]tryptamine and side chain (1S)-[1-2H]tryptamine, were prepared from tryptophan with tryosine decarboxylase according to Scheme I. The stereochromy of tyrosine decarboxylase from the same organism (S. faecalis) has previously been studied with tyrosine as substrate; the absolute configuration at side chain C-2 by stereospecific labeling with [2-14H]tryptamine and [2-3H]tryptamine, were prepared from tryptophan with tryosine decarboxylase as determined by polarographic measurement of oxygen consumption, indicates a kH/kS 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 hydroxylation reaction was carried out in H2O, 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 (1R)-[1-2H]tryptamine and side chain (1S)-[1-2H]tryptamine, were prepared from tryptophan with tryosine decarboxylase according to Scheme I. The stereochromy of tyrosine decarboxylase from the same organism (S. faecalis) has previously been studied with tyrosine as substrate; the absolute configuration at side chain C-2 by stereospecific labeling with [2-14H]tryptamine and [2-3H]tryptamine, were prepared from tryptophan with tryosine decarboxylase as determined by polarographic measurement of oxygen consumption, indicates a kH/kS of 1.81. Thus the results suggest that the pro-S hydrogen is stereospecifically removed in this reaction.
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 (2R)-2-hydroxytryptamine and (2S)-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 NH₂, 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 3S-[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 3R 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 3S-isomer has a 3H/14C 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 3R isomer, the starting material, the reaction mixture after lyophilization, and the recovered tryptophan all have approximately the same 3H/14C 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-[3-14C]-tryptophan (3H/14C, 3.47) incubated with tryptophanase in 2H2O to give pyruvate, which was trapped in situ as lactate by coupling the reaction to the lactate dehydrogenase systems (8). The lactate obtained (3H/14C, 3.76) was oxidized to acetate (3H/14C, 3.66) with CrO3/ H2SO4, 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 by reaction with fumarase, optically pure R-[2-3H, 2-3H]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 (3H/14C, 3.00) showed 61% tritium retention in the fumarase reaction, indicating that the methyl group of acetate had “K” 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. This is consistent with the view that the side chain configuration can be well defined (8). 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 2H2O, no deuterium incorporation into the product could be detected by NMR. Repetition of the reaction with L-[2-3H, 3-14C]tryptophan methyl ester (3H/14C, 2.74) gave product with a 3H/14C 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 occurs nonenzymatically via a reversible dehydration-hydration process, we incubated 3-hydroxytryptophan methyl ester with H218O and analyzed the product by mass spectrometry. No incorporation of 18O 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 Analogs. The results for the reaction product of L-tryptophan methyl ester at 5 (A), 25 (B), and 50°C (C). The spectra were run in 2H2O using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an external reference (0 ppm). The coupling constants are expressed in Hz.

**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 2H2O using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as an external reference (0 ppm). The coupling constants are expressed in Hz.
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 side chain by one or two carbon atoms is entirely compatible with the reaction. Not surprisingly, extension of the tryptophan analogs, which were selected to probe mechanistic aspects of the reaction. In the 2s 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-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 nonenzymatic, 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-alkyldene indolene 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-alkyldene 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-alkyldene indolene 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 non-activated 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 stereochemical studies.

### Table II

**Relative rates of O₂ consumption by tryptophan analogs**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-tryptophan</td>
<td>100</td>
</tr>
<tr>
<td>D,L homotryptophan</td>
<td>70</td>
</tr>
<tr>
<td>D,L-bis-homotryptophan</td>
<td>50</td>
</tr>
<tr>
<td>D,L-thiotryptophan</td>
<td>0</td>
</tr>
<tr>
<td>D,L-L-1-methyltryptophan</td>
<td>0</td>
</tr>
<tr>
<td>β-methyltryptophan, A isomer</td>
<td>50</td>
</tr>
<tr>
<td>(2R, 3S) + (2S, 3R)</td>
<td></td>
</tr>
<tr>
<td>β-methyltryptophan, D isomer A (2R, 3R) + (2S, 3S)</td>
<td></td>
</tr>
</tbody>
</table>

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).
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-2R 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-2S 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).

Acknowledgments—We thank Dr. K. A. Watanabe, Sloan-Kettering Institute, for discussions, and Ms. Janet Weaver, Purdue University, for carrying out the chirality analysis of acetate.

REFERENCES