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Dopamine-β-hydroxylase

STEREOCHEMICAL COURSE OF THE REACTION*

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SUMMARY

d-Amphetamine ((S)-2-amino-1-phenylpropane) stereospecifically tritiated at the β-carbon was prepared by hydrogenolysis of the corresponding aziridine in the presence of $^3H_2$ and the configuration of the product was established as (2S)-amino-(1S)-[1-^{3}H]phenylpropane.

Dopamine-β-hydroxylase catalyzed the removal of the pro-R hydrogen atom and the production of 1-norephedrine, (2S,1R)-2-amino-1-hydroxyl-1-phenylpropane, from d-amphetamine. Comparison of the chemical structure of the tritiated substrate with that of the product reveals that the reaction goes with over-all retention of configuration.

The enzyme, dopamine-β-hydroxylase (EC 1.14.17.1) is a copper-containing monooxygenase that catalyzes the hydroxylation of the "β" or benzylic carbon of various phenylethylamines (1–4). *In vivo* it catalyzes the hydroxylation of dopamine to noradrenalin. In an attempt to understand better its chemical mechanism I investigated the stereochemical course of the reaction.

It is possible to conclude whether a substitution reaction goes with over-all retention or inversion of configuration by comparison of the absolute configuration of the starting material with that of the product. Accordingly the absolute configuration of stereospecifically tritiated *d*-amphetamine is compared with that of the product of hydroyxlation catalyzed by dopamine-β-hydroxylase.

EXPERIMENTAL PROCEDURES

Materials

Dopamine-β-hydroxylase—The enzyme was purified from beef adrenals by the procedure of Friedman and Kaufman (1) as modified by Hartman and Udenfriend (5). The active fractions from the first gel filtration step were combined, and dialyzed overnight against a solution 80% saturated with ammonium sulfate. The resulting suspension was stored at −20°. Before use the precipitate was dissolved in 1 to 2 ml of 0.02 M KPO_4, pH 6.4, 0.1 M NaCl, and desalted by passage through a column (1 × 17 cm) of Sephadex G-25 in the same buffer.

Chemicals—Resolution of *d*-norephedrine was done by the method of Kalm (6); $[\alpha]_D$ +14.2° (published, +14.8, 1.2%, MeOH, 26°). The *trans*-β-methylstyrene was purchased from K & K Laboratories, Inc., Plainsville, N. Y., and carrier-free tritium was from New England Nuclear, Boston, Mass. Other chemicals and chromatography materials were of good grade from common suppliers.

Methods

Synthesis of trans-2-Methyl-3-phenylaziridine—Phenylpropanolamine (*dl*-norephedrine), 57 g, was the starting material for the procedure of Brois (7) that had minor modifications (Fig. 2).

Hydrogenolysis I—Small scale hydrogenolysis in the presence of tritium gas was conducted under the conditions that resulted in retention of configuration in the experiments of Sugi and Mitsui (8, 9) (see Fig. 2).

The reaction chamber, which was continuous with a tube of carrier-free $^3H_2$, was charged with approximately 0.05 ml of ethanol containing NaOH (250 nmoles), 2-methyl-3-phenylaziridine (50 μmoles), and 483 μg of finely ground $Pd(OH)_2$ (10). After 2.5 hours at room temperature, the reaction mixture was removed from the reaction chamber, which was then rinsed with 2 volumes of ethanol. To the combined washings and reaction mixture was added *d*-amphetamine (5 mmoles). After filtration of the mixture, the filtrate was made basic with 75 ml of 1 N NaOH and extracted three times with 7 ml of pentane. The combined pentane fractions were extracted with HCl (1 N). The HCl solution was made basic and extracted again with pentane. After removal of the water, most of the pentane was evaporated under vacuum. The remainder was streaked on a plate of silica gel, which contained fluorescent indicator but no binder (500 μm) for preparative thin layer chromatography in System II. After development of the plate, the silica gel bearing the band corresponding to authentic amphetamine was removed and extracted with 1 N HCl. The HCl solution was made basic and extracted with pentane. After the pentane had been dried and removed under vacuum, the remaining material was dissolved in an equal amount (mole per mole) of HCl (0.1 N). Gas chromatography in System I gave a single peak whose retention time corresponded to that of authentic amphetamine. In addition the structure was confirmed by infrared and NMR spectra.

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Hydrogenolysis II—The conditions are adapted from those described by Sugi and Mitsui (8, 9) to produce inversion of configuration. The procedures were the same as those for "Hydrogenolysis I" with three exceptions. (*a*) The reaction solvent was benzene and (*b*) no NaOH was present. (*c*) At the termination of the reaction the reaction mixture was applied directly to the plate for thin layer chromatography.

Amine-Oxide Elimination Reaction—The methylation, oxidation, and elimination procedures of Cram and McCarty (11) were performed on a somewhat smaller scale. The methylation reaction started with 3.37 mmoles of *d*-amphetamine and 3.0 ml each of formic acid and formaldehyde as well as 0.1 ml of [^{3}H]-amphetamine-HCl solution (0.1 M) in the appropriate experiments. After its isolation and characterization (gas chromatography and infrared spectrum), a measured amount of the product, *N*,*N*-dimethylamphetamine, was counted for radioactivity, and 0.554 mmole of it was incubated 72 hours with 0.333 ml of H_2O_2 (30%). The excess H_2O_2 was destroyed with catalase and the reaction mixture was lyophilized. While connected to vacuum (1 mm Hg) via a Dry Ice trap, the reaction vessel was incubated at 115° for 30 min. Finally the material in the trap was made acid and extracted three times with 1 ml of pentane. The neutral organic product (*trans*-β-methylstyrene) was quantitated by gas chromatography of the combined pentane extracts in System II with authentic *trans*-β-methylstyrene as standard. After removal of most of the pentane, the product was purified by gas chromatography on a column (6.3 mm × 2 m) under the same conditions as in System II with collection of the purified material in a few milliliters of pentane at 0°. A sample from the resulting pentane solution was counted for radioactivity and the *trans*-β-methylstyrene was quantitated by analytical gas chromatography in System II.

Thin Layer Chromatography, System I—The mixture to be analyzed was spotted in one corner of a plate covered with Silica Gel G, that had been activated 60 min at 100°, and the plate was developed in the mixture, MeOH-PhH (10:90 (v/v)). After the solvent had been removed in a fume hood, the plate was reactivated for 30 min at 100°. Solutions of norephedrine and norpseudoephedrine (0.1 μmole) were spotted on a line through the point of application of the unknown sample and parallel to the direction of the first development. With this line as the starting line the plate was developed in the second dimension in acetone. After evaporation of the acetone, the plate was sprayed with ninhydrin solution and heated at 100° for 10 min.

Thin Layer Chromatography, System II—After application of the sample to the plate, it was developed in the mixture of cyclohexane-benzene-diethylamine (75:15:10 (v/v)), described by Fike (12).

Gas Chromatography, System I—Samples to be analyzed were injected into a gas chromatograph (Perkin-Elmer model 154) equipped with a column (3.2 mm × 2 m) at 200° containing Chromosorb W coated with FS-1265 (Perkin-Elmer, Norwalk, Conn.) through which flowed the carrier gas (helium) at 15 ml per min. The various components in the effluent were detected by thermal conductivity.

Gas Chromatography, System II—Analyses were performed as described for System I except that the column contained Carbowax-1500 on Teflon supporting material at 110° and the carrier gas flowed at 50 ml per min.

Gas Chromatography, System III—The procedure was the same as in System II except that the column was operated at 140°.

Radioactivity—Liquid samples were counted in Bray's solution; particulate samples from thin layer chromatograms were suspended in Bray's solution, which also contained 5 g of Thixotropic Gel Powder (Packard Instrument Co.) per 100 ml.

Enzyme Assay—Dopamine-β-hydroxylase was assayed by the method of Creveling *et al.* (3) as modified by Kuzuya and Nagatsu (13). A unit of enzyme is that amount that produces 1.0 nmole of octopamine per min under the conditions of the assay.

RESULTS AND DISCUSSIONS

Stereochemistry of Reaction Product

The action of dopamine-β-hydroxylase upon a substrate with an asymmetrical center at a location other than the reaction center should yield one or more diastereoisomeric products. Since it will catalyze the hydroxylation of amphetamine (4), incubation of the enzyme with the stereoisomer, *d*-amphetamine (*I*, Fig. 1), should yield one or both of the two possible products, norephedrine (*II*) and norpseudoephedrine (*III*) which are separable from each other and from the much larger amount of amphetamine present in the reaction mixture by two-dimensional thin layer chromatography. The results from such a chromatography of the amines present at the end of incubation of *d*-amphetamine with dopamine-β-hydroxylase show that the only ninhydrin-reactive spot from the reaction mixture corresponds to norephedrine. There is no detectable material corresponding to norpseudoephedrine. Neither spot was obtained from the mixture from similar incubations in the absence of either enzyme or substrate. Therefore, the product of the hydroxylation of *d*-amphetamine by dopamine-β-hydroxylase has the "*R*" configuration about the β-carbon (Fig. 1). This is in agreement with the *in vivo* experiments of Carlsson *et al.* (16). It is reassuring that the active isomer of noradrenalin has the same absolute configuration (17).

Stereochemistry of Substrate

Synthesis of Stereospecifically Tritiated Amphetamine—In a series of papers Sugi and Mitsui (8, 9) described the hydrogenolysis of optically active 2-methyl-2-phenylaziridine to give 2-phenyl-1-propylamine with either retention or inversion of configuration, dependent upon the reaction conditions. Similarly the hydrogenolysis of an isomeric aziridine, *trans*-2-methyl-3-phenylaziridine (*IV*, Fig. 2), in the presence of tritium gas should go with either retention or inversion of configuration to yield *d*-amphetamine (*I*) labeled with tritium at either the pro-*S* or

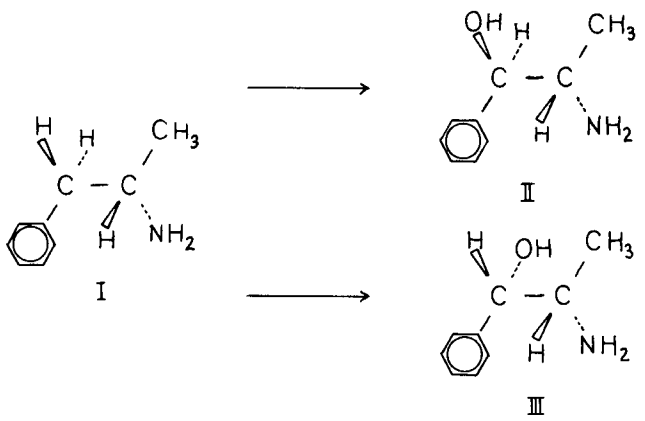

FIG. 1. Possible products of the enzymatic hydroxylation of *d*-amphetamine. The absolute configuration of *d*-amphetamine (*I*) is from Leithe (14), whereas that of *l*-norephedrine (*II*) is from Fodor *et al.* (15). "*III*" is norpseudoephedrine.

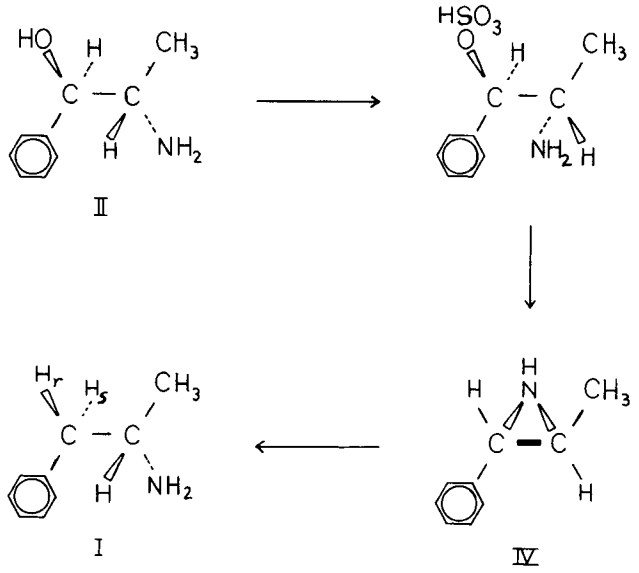

FIG. 2. Synthetic pathway for *d*-amphetamine tritiated at the β-carbon. The pro-*R* hydrogen is designated "*r*" whereas the pro-*S* is "*s*." "*IV*" is *trans*-2-methyl-3-phenylaziridine. Detailed reaction conditions are described under "Methods."

the pro-*R* hydrogen, respectively. The 2-methyl-3-phenylaziridine, synthesized from *dl*-norephedrine, was hydrogenolyzed under conditions to produce retention of configuration, Hydrogenolysis I.

The *dl*-norephedrine, starting material for this synthetic sequence, should yield *dl*-amphetamine as the final product. However, since the hydrogenolysis product was diluted 150-fold with *d*-amphetamine, the purified product consists of less than 1.0% of *l*-amphetamine, which, however, contains half of the total radioactivity. The possible implications of the *l*-amphetamine for the results of subsequent experiments will be discussed below.

Configuration of Stereospecifically Tritiated Amphetamine—Cram and McCarty (11) demonstrated that the amine-oxide elimination reaction culminates in a *cis* elimination and hypothesized the existence of a cyclic transition state. The analogous elimination from *d*-amphetamine (*I*) could take two possible courses (Fig. 3). In *Scheme a* elimination of *N*,*N*-dimethylhydroxylamine containing the pro-*R* hydrogen yields *cis*-β-methylstyrene (*VI*) as the product, whereas in *Scheme b* elimination of the pro-*S* hydrogen yields *trans*-β-methylstyrene (*VII*). *Scheme b* seems somewhat favored, since in *Scheme a* the methyl and the phenyl groups have an eclipsed relationship in the transition state. When *d*-amphetamine was reacted under the conditions described by Cram and McCarty (11), the product, obtained in a yield of 80 to 100%, was about 90% pure by gas chromatography in System II. The infrared and NMR spectra were identical with those of authentic *trans*-β-methylstyrene and distinguishable from those of the *cis* isomer. Therefore, the reaction goes almost exclusively as depicted in *Scheme b* of Fig. 3, and the pro-*S* hydrogen atom is removed in the course of the amine-oxide elimination reaction.

Reaction of the tritiated amphetamine, whose synthesis was described above, under the conditions of the amine-oxide elimination reaction resulted in the loss of almost all of the radioactivity from dimethylamphetamine (*V*) (Table I, Experiment 1). Therefore, the tritium incorporated into *d*-amphetamine by the synthetic reactions in the previous section must confer the "*S*" configuration on the β-carbon.

Similar reasoning leads to the prediction that the hydrogen

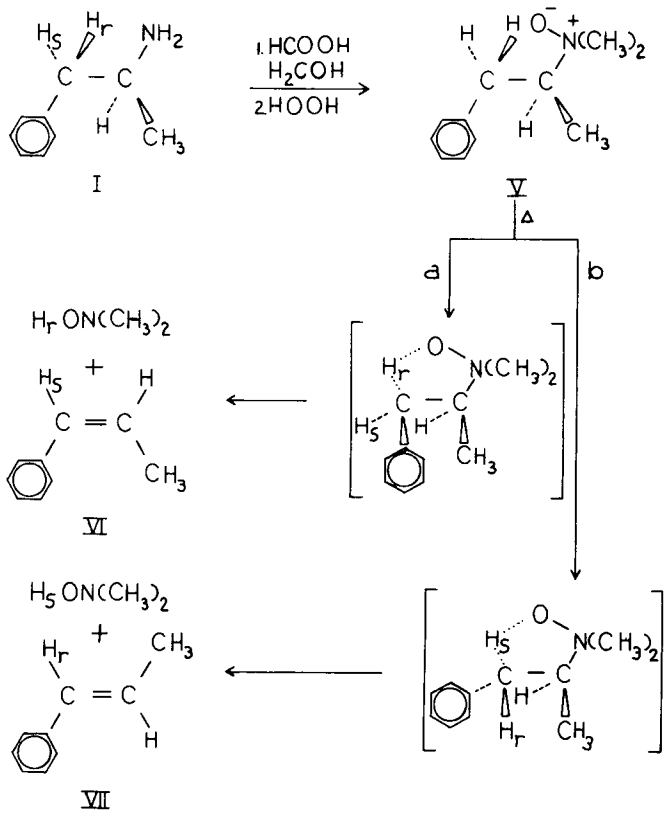

FIG. 3. Possible products of the amine-oxide elimination from *d*-amphetamine. *Structures V*, *VI*, and *VII* are *N*,*N*-dimethylamphetamine-*N*-oxide, *cis*-β-methylstyrene, and *trans*-β-methylstyrene, respectively. The symbol "△" indicates the application of heat (115°).

TABLE I

Stereospecific removal of tritium from β-carbon of amphetamine by amine-oxide elimination

The reaction as well as the purification and quantitation of product and radioactivity were done as described under "Methods."

Compound	Amount	Yield	Radioactivity	Specific activity	Yield of radioactivity
	mmole	%	10^{-6} *dpm*	10^{-9} *dpm/mole*	*% original specific activity*
Experiment 1					
Dimethylamphetamine	0.541		4.80	8.87	100.0
β-Methylstyrene	0.598	100+	0.216	0.36	4.06
Experiment 2					
Dimethylamphetamine	0.531		0.4444	0.835	100.0
β-Methylstyrene	0.449	84.7	0.152	0.339	40.7

atom of the pro-*R* configuration on the β-carbon of the other isomer of amphetamine, *l*-amphetamine, would be lost in the amine-oxide elimination reaction. However, since the hydrogenolysis of the aziridine derived from *d*-norephedrine in the presence of tritium gas yields *l*-[^{3}H]amphetamine in which the tritium confers the "*R*" configuration on the β-carbon, tritium should be lost in the amine-oxide elimination on the latter compound also. This prediction is substantiated by the fact that the specific activity of the methylstyrene in Table I, Experiment

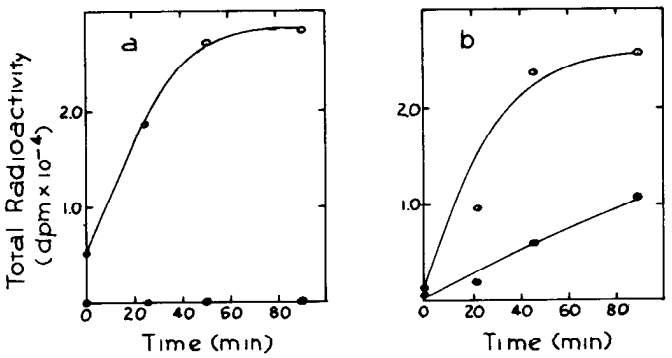

FIG. 4. The fate of tritium from tritiated amphetamine during incubation with dopamine-β-hydroxylase. The *closed circles* (●——●) indicate the radioactivity in water whereas the *open circles* (○——○) indicate that in norephedrine. *a*, stereospecifically tritiated amphetamine (20 μmoles, 10.8 μCi) and enzyme (154 units) were incubated together with 600 μmoles, of KPO_4 (pH 5.5), potassium ascorbate (20 μmoles), and potassium fumarate (20 μmoles) in a total volume of 2 ml. After various times of incubation (37°) a sample of the reaction mixture was removed and added to a solution of norephedrine (10 μmoles) and trichloroacetic acid (600 μmoles). The resulting mixture was applied to a column (0.5 × 3.5 cm) of Dowex 50W (200 to 400 mesh) and the column was washed with 4.0 ml of H_2O. One milliliter of the effluent collected since the application of the sample to the column was counted for the radioactivity released to water. After the column had been washed with additional water (6.0 ml), the amines were eluted with 4.0 ml of 0.5 M NaOH. The basic effluent was extracted three times with 1.0 ml of diethyl ether and the combined extracts taken to dryness under a stream of air at 43°. The residue was taken up in MeOH (3 × 0.01 ml) and applied to a plate (Silica Gel GF, 250 μm, activated 1 hour at 100°) for thin layer chromatography in System II. After development, the silica gel bearing the ultraviolet-absorbing spots that corresponded to norephedrine were removed and counted for radioactivity. *b*, [^{3}H]amphetamine labeled at either hydrogen atom attached to the β-carbon (20.0 μmoles, 2.52 μCi) was substrate for the experiment that was otherwise the same as that reported in Fig. 4*a*.

1, fell to a value less than 50% of that of the dimethylamphetamine.

Although the yield of β-methylstyrene in the above experiment and others similar to it was 80 to 100%, the yield of radioactivity was only 25 to 50%. Therefore, tritiated amphetamine was synthesized under conditions that gave inversion of configuration in the experiments of Sugi and Mitsui (9) (Hydrogenolysis II). Subjection of this amphetamine to the amine-oxide elimination experiment described above produced the results in Table I, Experiment 2. Although the latter results indicate that the *d*-[^{3}H]amphetamine produced by Hydrogenolysis II is a mixture of stereoisomers rather than the expected single stereoisomer, they demonstrate that the results in Table I, Experiment 1, cannot be explained convincingly on any basis other than configuration.

Stereochemistry of Enzymatic Extraction of Hydrogen—The stereospecifically tritiated amphetamine whose configuration was determined by the results in Table I, Experiment 1, was incubated with dopamine-β-hydroxylase. After various times of incubation, samples from the incubation mixture were added to a solution of trichloroacetic acid and norephedrine. The amines in the resulting solution were separated from the water by passage through a column of Dowex 50, and the product of the reaction, norephedrine, was separated from the substrate, amphetamine, by thin layer chromatography. The norephedrine as well as the water were counted for radioactivity (Fig. 4*a*). The amount of radioactivity retained in the product (*open circles*) increased during the reaction whereas that released to water (*closed circles*) remained the same as in a similar tube without enzyme. Therefore, the pro-*R* hydrogen on the β-carbon of amphetamine is removed in the enzymatic hydroxylation. Examination of the configuration of both the substrate and the product of the reaction (Fig. 1), leads to the conclusion that the hydroxylation catalyzed by dopamine-β-hydroxylase goes with over-all retention of configuration.

The radioactivity present at zero time in Fig. 4*a* is not considered significant, since in similar experiments with the same substrate, which yielded similar curves, the radioactivity in norephedrine at zero time was 5.0% that present later in the reaction.

An experiment similar to the one described in Fig. 4*a* was conducted with the mixture of stereoisomers of tritiated *d*-amphetamine, whose composition was established by the results in Table I, Experiment 2. The results (Fig. 4*b*) show that tritium is both released to water and retained in the product.

As indicated earlier the stereospecifically tritiated amphetamine contained less than 1.0% of *l*-amphetamine, which had about half of the total radioactivity. This small amount of *l*-amphetamine takes on added importance in view of the fact that only about 0.1% of the total amphetamine is converted to products in Fig. 4 (23.6 nmoles in Fig. 4*a* and 12.8 nmoles in Fig. 4*b*). However, *l*-amphetamine is not a substrate for dopamine-β-hydroxylase even when the concentration is comparable to that of the *d*-amphetamine in the experiments above. When *d*-amphetamine and *l*-amphetamine, at 10 mM were incubated separately with the enzyme in the spectrophotometric assay of Goldstein and Contrera (4), the A_{400} in the former case increased to 0.214 at the end of 11 min whereas it showed no change in the latter. Even less reaction would be expected, when the total concentration of *l*-amphetamine is 0.005 times that of *d*-amphetamine. In addition stereospecifically tritiated *l*-amphetamine was synthesized by hydrogenolysis ("Hydrogenolysis I") of the aziridine prepared from optically pure *d*-norephedrine and the position of the label was ascertained by the amine-oxide elimination as described above. During incubation of this *l*-[^{3}H]amphetamine with dopamine-β-hydroxylase as described in Fig. 4, tritium appeared neither in the water nor in the norephedrine spot from thin layer chromatography. Therefore, the 0.5% of *l*-amphetamine in the tritiated substrate should have no effect upon the experimental results in Fig. 4.

It is not clear why the reaction stops when less than one per cent of the substrate has reacted. However, this phenomenon is also observed with other substrates under the same incubation conditions. The answer to this question might also explain the disparity in the yield of product in the two experiments of Fig. 4.

It can be seen from Fig. 4 that although the 2 hydrogen atoms on the β-carbon of amphetamine are labeled almost equally with tritium (Table I, Experiment 2), it is released to water considerably more slowly than it appears in norephedrine. This discrepancy might be explained by a primary hydrogen isotope effect. However, additional experiments are necessary in order to define more precisely the conditions under which this effect is demonstrable.

The results presented here are in agreement with the results from other enzymatic hydroxylation reactions. Several hydroxylations of steroid compounds go with over-all retention of configuration (18–21) in both microsomes and fungi, as does the hydroxylation of fatty acids and esters by *Torulopsis* (22, 23) and the hydroxylation of ethylbenzene by liver microsomes (24). The stereochemical course of two enzymatic hydroxylations at a

saturated α-carbon to an amino carbon has been determined and both were found to go with over-all retention of configuration. These are the hydroxylation to form hemanthamine in daffodils by Battersby *et al.* (25) and the hydroxylation to form hydroxyproline in chick embryo by Fujita *et al.* (26).

A hydrogen isotope effect was reported in several of the studies above (22–24) but such an effect was not seen in one of them (26). Although a hydrogen isotope effect is suggested by the results reported here, further work is obviously necessary in order to define more precisely the conditions under which it is observed.

It is difficult to draw mechanistic conclusions from the results presented here as it is with the results from the previous stereochemical studies of hydroxylations. Although it is tempting to think of the initial removal of hydrogen from the substrate followed by the insertion of an oxygen species, it is impossible to eliminate other mechanisms without additional experiments.

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