Dopamine-β-hydroxylase

STEREOCHEMICAL COURSE OF THE REACTION*

KENNETH B. TAYLOR

From the Department of Biochemistry, The University of Alabama in Birmingham, Birmingham, Alabama 35294

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 3H2 and the configuration of the product was established as (2S)-amino-(1S)-[1-3H]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 hydroxylation 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 KPO4, pH 0.4, 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); [α]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-β-Methyl-3-phenylaziridine—Phenylpropylamine (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 3H2, was charged with approximately 0.05 ml of ethanol containing NaOH (250 nmoles), 2-methyl-3-phenylaziridine (50 nmoles), 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. The combined washings and reaction mixture was added d-amphetamine (500 nmoles). 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.
**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 thin layer chromatography.

**Amine-Oxide Elimination Reaction**—The methylation, oxidation, and elimination procedures of Cram and McCormy (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 [3H]-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 H2O2 (30%). The excess H2O2 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°C 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 X 2 m) under the same conditions as in System II with collection of the purified material in a few milliliters of pentane at 0°C. 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 110°C, 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°C. Solutions of norpseudoephedrine and norpseudoephedrine (0.1 mmoles) 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 the direction of the first development. The procedures were the same as those for "Hydrogenolysis I" except that the column was operated at 140°C. The neutral organic product (trans-β,β-methylstyrene) was quantitated by gas chromatography in System II.

**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 X 2 m) at 200°C 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 Carbopack X-1500 on Teflon supporting material at 110°C 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°C.

**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 Thioctic Gel Powder (Packard Instrument Co.) per 100 ml.

**Enzyme Assay**—Dopamine-β-hydroxylase was assayed by the method of Creveling et al. (8) as modified by Kuzuya and Nagatsu (13). A unit of enzyme is that amount that produces 1.0 m mole 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 stereosomer, d-amphetamine (l), should yield one or both of the two possible products, norpseudoephedrine (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 norpseudoephedrine. 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).

**Synthesis of Stereo-specifically 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-propyamine with either retention or inversion of configuration, dependent upon the reaction conditions. Similarly the hydrogenolysis of an isomeric aziridine, trans-2-methyl-2-phenyl-1-propyamine (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 pro-R configuration. 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).

**Fig. 1.** Possible products of the enzymatic hydroxylation of d-amphetamine. The absolute configuration of d-amphetamine (I) is from Leib et al. (14), whereas that of l-norpseudoephedrine (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 d-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-dimethylhydrosylamine 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 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-[3H]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. 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 "Δ" indicates the application of heat (115°).

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Amount</th>
<th>Yield</th>
<th>Radioactivity</th>
<th>Specific activity</th>
<th>Yield of radioactivity %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>10^4 dpm</td>
<td>10^-4 dpm/mole</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethylamphetamine</td>
<td>0.551</td>
<td>100</td>
<td>4.80</td>
<td>8.87</td>
<td>100.0</td>
</tr>
<tr>
<td>β-Methylstyrene</td>
<td>0.508</td>
<td>0.210</td>
<td>0.36</td>
<td>100.0</td>
<td></td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dimethylamphetamine</td>
<td>0.531</td>
<td>84.7</td>
<td>0.4444</td>
<td>0.835</td>
<td>100.0</td>
</tr>
<tr>
<td>β-Methylstyrene</td>
<td>0.449</td>
<td>0.152</td>
<td>0.339</td>
<td>40.7</td>
<td></td>
</tr>
</tbody>
</table>
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-ß-hydroxylase. After various times of incubation, samples from the incubation mixture were added to a solution of norephedrine (10 μmoles) and trichloroacetic acid. 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 45°C. The residue was taken up in MeOH (3 X 0.01 ml) and applied to a plate (Silica Gel GF, 250 μm, activated 1 hour at 105°C) 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.

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. The 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.
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 hydroxypyruvate 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 stereoischemical 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.

REFERENCES