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STEREOSPECIFIC DEUTERIUM SUBSTITUTION AT THE α-CARBON POSITION OF DOPAMINE AND ITS EFFECT ON OXIDATIVE DEAMINATION CATALYZED BY MAO-A AND MAO-B FROM DIFFERENT TISSUES

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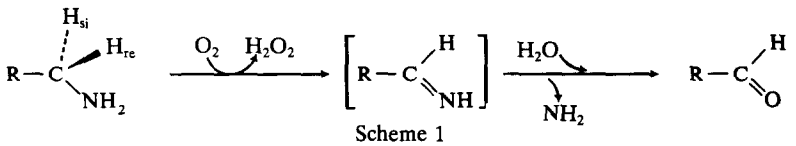
Abstract—Stereospecific replacement of deuterium in the α-carbon side chain position of dopamine (DA) was achieved by decarboxylation of L-3,4-dihydroxyphenylalanine (L-dopa) using hog kidney aromatic aminoacid decarboxylase. The $S[\alpha\text{-}^2H_1]$DA enantiomer was obtained by decarboxylation of L-$[\alpha\text{-}^2H_1]$dopa in H_2O, while the $R[\alpha\text{-}^2H_1]$DA enantiomer was obtained by decarboxylation of unsubstituted L-dopa in ^2H_2O. An inverse solvent isotope effect of L-dopa decarboxylation was observed in ^2H_2O. The deaminated aldehyde products of the four DA analogues, i.e. undeuterated DA, $[\alpha,\alpha\text{-}^2H_2]$DA, $R[\alpha\text{-}^2H_1]$DA and $S[\alpha\text{-}^2H_1]$DA, have been analyzed by the gas chromatography–mass spectrometry (GC–MS) method. It is evident that monoamine oxidase (MAO) catalyzes the stereochemical removal of only *R*-deuterium and that *S*-deuterium was maintained at the α-carbon atom of 3,4-dihydroxyphenylacetaldehyde. The steady-state kinetics of the oxidative deamination of undeuterated, $[\alpha,\alpha\text{-}^2H_2]$, $R[\alpha\text{-}^2H_1]$, and $S[\alpha\text{-}^2H_1]$ dopamine were assessed by determination of the aldehyde products directly by high performance liquid chromatography (HPLC) using electrochemical detection (ECD). MAO-A from rat liver mitochondria (deprenyl-treated) and from human placenta, as well as MAO-B from rat liver (clorgyline-treated) and from human platelet were used in this study. The apparent isotope effects, i.e. $(V/K)_H/(V/K)_D$ ratios of $[\alpha,\alpha\text{-}^2H_2]$DA and $R[\alpha\text{-}^2H_1]$DA, were quite similar (2.34 and 3.13) with respect to both MAO-A and MAO-B. $S[\alpha\text{-}^2H_1]$DA exhibited a slight secondary isotope effect.



Scheme 1

Monoamine oxidase (MAO)† catalyzes the oxidation of monoamines to aldehydes (Scheme 1).

Substitution with deuterium at the α-carbon causes significant isotope effects during enzymatic oxidative deamination [1–3]. The absolute stereochemistry of the abstraction of an H atom from the prochiral methylene group of tyramine adjacent to N has been reported [4]. Abstraction of the Re-H in the deamination of *p*-tyramine by rat liver mitochondrial MAO has also been verified by radioactive tracer methods employing *S*- and R-[α-^{3}H]-*p*-tyramine as substrates [5]. It is not clear, however, why the kinetic isotope effect for *p*-tyramine at saturated concentration levels relative to its α-deuterated analogues is 1.2 [4], which is much lower than that of our own data reported to be 2.3 [2]. In these earlier studies, however, the oxidation of the tyramine was assessed by observing the disappearance of the substrate using Ruheman purple as developed with ninhydrin [1, 4]. We have shown recently that such color development is subject to a profound isotope effect [6]. Richards and Spenser [7] have recently investigated the deuterium isotope effects of several aliphatic amines catalyzed by hog kidney diamine oxidase. In contrast to the MAO catalyzed reaction where the Re-H is abstracted, it is the Si-H that is removed in this latter case. We, therefore, thought it worthwhile to re-examine which α-carbon hydrogen atom was removed from the substrate and to redetermine the magnitude of the deuterium isotope effect during oxidative deamination catalyzed by MAO.

MAO has been categorized into distinct enzyme type MAO-A and type MAO-B on the basis of their sensitivities to inhibition by selective inhibitors and specificity towards different substrates [8–10]. It is also interesting to know whether MAO-A and MAO-

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† Abbreviations: L-dopa, L-3,4-dihydroxyphenylalanine; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAH, 3,4-dihydroxyphenylacetaldehyde; PLP, pyridoxal-5′-phosphate; MAO, monoamine oxidase [EC 1.4.3.4, monoamine:oxygen oxidoreductase (deaminating)]; ADC, aromatic aminoacid decarboxylase (EC 4.1.1.26); HPLC-ECD, high performance liquid chromatography–electrochemical detection; and GC–MS, gas chromatography–mass spectrometry.

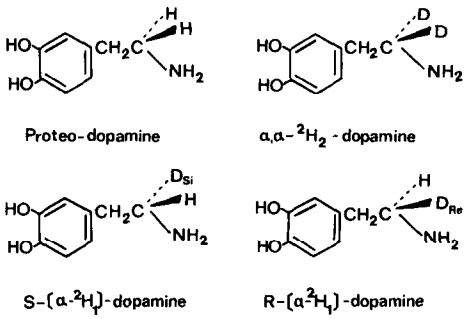


Fig. 1. Structures of dopamine and its α-carbon deuterated analogues.

B may exert any different stereospecific mechanism during deamination of monoamines. In an attempt to investigate further this question, we have measured the deamination of dopamine, a mixed-type substrate, and some of its deuterated analogues and enantiomers; these were proteo-dopamine, [α,α-$^2H_2$]DA, *R*[α-$^2H_1$]DA and *S*[α-$^2H_1$]DA (see Fig. 1 for structures), catalyzed by both MAO-A and MAO-B obtained from several sources.

MATERIALS AND METHODS

Materials. Wistar male rats (200 g) were used; hog kidney was supplied by Intercontinental Packers Ltd., Saskatoon; human placenta was obtained from the Department of Obstetrics and Gynecology, University of Saskatchewan; DL-3,4-dihydroxyphenylalanine[2-$^2H_1$]HBr (98 atom% D) and [α,α-$^2H_2$]DA were obtained from Merck Sharp & Dohme Isotope, Montreal, Quebec, and deuterium oxide (99.8 atom% ^2H) from the Aldrich Chemical Co., Milwaukee, WI. Deprenyl (phenylisopropylmethylpropinylamine HCl) was a gift from Prof. J. Knoll (Budapest, Hungary) and clorgyline [*N*(2,4-dichlorophenoxy-*n*-propyl)-*N*-methylpropargylamine HCl] from May & Baker Ltd. (Dagenham, U.K.). All other chemicals were of analytical grade.

Preparation of aromatic aminoacid decarboxylase. A partially purified aromatic-L-aminoacid decarboxylase (ADC) was prepared from hog kidney. The tissues were homogenized in 0.01 M phosphate buffer (pH 7.4) containing pargyline (1×10^{-4} M) and centrifuged at 14,000 *g* for 20 min. Ammonium sulfate was added to the supernatant fraction to produce 30% saturation; it was centrifuged again and more ammonium sulfate was added to the supernatant until 50% saturation was reached; the resultant precipitate was isolated by centrifugation. The protein was dissolved in 0.02 M phosphate buffer (pH 7.5) and applied to a column (5 × 80 cm) of Sephadex G-200 equilibrated with 0.02 M phosphate buffer (pH 6.8). The column was developed with the same buffer at a flow rate of 54 ml/hr. Fractions containing ADC were pooled, and the enzyme was precipitated by 55% saturation with ammonium sulfate followed by centrifugation. The pellet was dissolved in 0.01 M phosphate buffer (pH 7.4) and kept frozen at −20°; it was stable for at least 1 month. A part of the enzyme was also prepared in ^2H_2O. The pellet was resuspended in 55% saturation of ammonium sulfate, which was prepared in ^2H_2O, and centrifuged, and the enzyme was redissolved in phosphate buffer in ^2H_2O.

Preparation of rat liver mitochondrial MAO-A and MAO-B and human placenta MAO-A. Freshly dissected rat livers, as well as frozen human placenta, were rinsed with chilled saline, cut into small pieces, and homogenized immediately in ice-cold 0.32 M sucrose in 0.01 M phosphate buffer (pH 7.5). The mitochondrial fractions were obtained by differential centrifugation as previously described [2]. The mitochondrial membrane fragments were prepared by lysing the mitochondria in chilled distilled water and then centrifuging at 105,000 *g* for 30 min. These membrane preparations were further washed twice by resuspension in chilled distilled water and centrifugation. The pellet was resuspended in distilled water and further homogenated by ultrasonic disruption at 75 W (PEP) for 20 sec using a needle probe tip (Braun-sonic 1510).

Rat liver MAO-A and MAO-B were obtained by treatments using selective MAO inhibitors. The mitochondrial membrane enzyme preparations were incubated with either the MAO-B inhibitor deprenyl (1×10^{-6} M) or the MAO-A inhibitor clorgyline (5×10^{-7} M) at room temperature for 1 hr.

Preparation of human platelet MAO-B. Platelets were obtained from freshly collected blood with sodium citrate as anticoagulant following differential centrifugation and washing as previously described [11]. The platelet suspensions were homogenized by ultrasonic disruption before analysis of enzyme activities.

Radioenzymatic method for assay of aminoacid decarboxylase. The aromatic-L-aminoacid activity towards L-dopa was determined by using a radioenzymatic method and separation of the ^{14}C-labeled product formed through an ion exchange column (Amberlite CG 50) as previously described [12]. The incubation mixture for the assays contained pyridoxal-5-phosphate (2.5×10^{-5} M), pargyline (1×10^{-5} M) and substrate (5×10^{-4} M) in a total volume of 200 μl in 0.1 M phosphate buffer (pH 7.2.).

Assay of MAO activity. A very sensitive HPLC procedure has been developed for the assay of MAO activity (Yu *et al.*, manuscript submitted for publication) based on a direct measurement of the aldehyde-produced proteo-dopamine along with [α,α-$^2H_2$]dopamine, and the *R*[α-$^2H_1$]- and *S*[α-$^2H_2$]dopamine enantiomers were incubated with MAO-A or MAO-B (10–20 μg protein) in a total volume of 200 μl of 0.05 M phosphate buffer (pH 7.5) for 30 min. The enzyme reaction was stopped by adding 800 μl of 0.1 N perchloric acid containing 2.5×10^{-4} M EDTA and 1×10^{-4} M sodium metabisulfite ($Na_2O_5S_2$). After centrifugation, 20 μl was subjected to HPLC analysis.

HPLC analysis. Chromatographic separations were performed as previously described [13] at ambient room temperatures on a 250 × 4.6 mm ID Ultrasphere I.P. analytical column packed with C-18, spherical 5 μM particles (Beckman, Toronto, Ont.). An SSI precolumn filter (0.5 μm filter elements) (Terochem, Rexdale, Ont.) and a 30 × 4.6 mm ID Brownlee MPLC RP-18 SPHERI-5 guard column

(Technical Marketing Associates, Calgary, Alberta) were installed between the Waters WISP 710B automated sample injector and the analytical column (a 20-μl aliquot of each prepared sample was injected onto the column). The mobile phase consisting of 75 mM monobasic sodium phosphate, 1 mM sodium octyl sulfate, 500 μM EDTA, 12.5% acetonitrile with the final pH adjusted to 2.75 with phosphoric acid was pumped through the column at 1.0 ml/min using a Waters M45 solvent delivery system (Millipore, Mississauga, Ont.). Prior to the addition of acetonitrile, the buffer was filtered through a 0.22 μm millipore filter. Degassing of the mobile phase was accomplished by either vacuum or helium sparging. The detector consisted of a BAS model TL-5A thin-layer amperometric electrode (Mandel Scientific, Rockwood, Ont.) which was controlled at 0.7 V vs a Ag/AgCl reference electrode. Signals for the detector were integrated by peak height using a Shimadzu C-R3A recording data processor (Tekscience, Oakville, Ont.). Deuterium substitution at the α-carbon position of dopamine was found to have no effect on the sensitivity of the electrochemical detector.

Synthesis of [α-$^2H_1$]*dopamine enantiomers.* The two dopamine enantiomers were synthesized using preparative enzymatic procedures as follows:

$$[\alpha\text{-}^2H_1]\text{L-dopa} \xrightarrow[\text{PLP}]{\text{ADC in } H_2O} S[\alpha\text{-}^2H_2]\text{dopamine}$$

$$\text{L-dopa} \xrightarrow[\text{PLP}]{\text{ADC in } {}^2H_2O} R[\alpha\text{-}^2H_1]\text{dopamine}$$

L-Dopa (110 mg) was incubated with hog kidney ADC in 0.05 M phosphate buffer at 37° in a total volume of 100 ml; the ADC was added three times at 30-min intervals. After 2 hr the enzyme reaction was terminated by adding perchloric acid to make the solution 0.1 N. The supernatant fraction, obtained after centrifugation at 27,000 *g* for 30 min, was applied to an Amberlite CG 50 column (2.5 × 12 cm) which had been equilibrated with 0.1 M phosphate buffer (pH 6.5). The column was first eluted with distilled water and then by 0.5 N HCl at a flow rate of 134 ml/hr. Dopamine fractions, which were eluted by the HCl and totally separated from the unreacted L-dopa, were pooled and lyophilized to dryness. The fractions were dissolved in H_2O, and the pH was adjusted to 8.5 with $NaHCO_3$ and subjected to adsorption on an alumina column (2.5 × 5 cm) which had been equilibrated with 0.01 Tris–HCl buffer (pH 8.6). Dopamine was eluted with 0.5 N HCl; the column was then washed with 200 ml H_2O. The dopamine eluate was lyophilized to dryness. The samples were finally separated by a preparative cellulose thin-layer chromatographic procedure in the solvent system *n*-butanol–acetic acid–H_2O (4:1:5, by vol.). The chemical purity of the synthesized DA enantiomers was determined by HPLC–ECD using pure proteo-DA as reference substance. Purities of 70 and 83%, respectively, for R[α-$^2H_1$]DA and S[α-$^2H_1$]DA were obtained.

The isotopic purity was measured by a high resolution mass-spectrometric procedure using dansyl derivatives of the amines as previously described [14]. The isotopic purities of R[α-$^2H_1$]DA and S[α-$^2H_1$]DA were 93 and 98.5% respectively.

GC–MS procedure for the determination of 3,4-*dihydroxyphenylacetaldehyde.* The deaminated products, 3,4-dihydroxyphenylacetaldehydes, were eluted from a small Amberlite CG 50 ion exchange column (0.5 × 6 cm), which had been equilibrated with 0.05 M phosphate buffer (pH 6.5), to remove

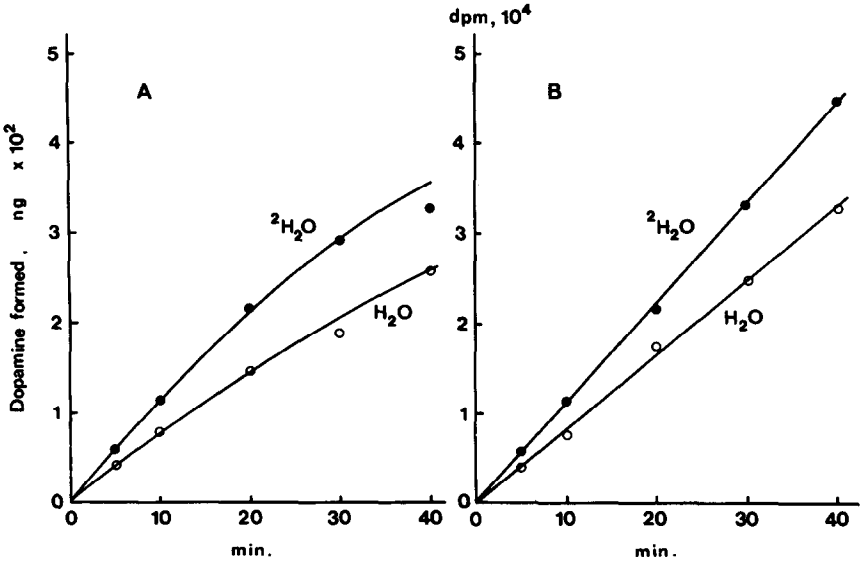


Fig. 2. Effect of deuterium oxide on the enzymatic decarboxylation of L-dopa. The initial velocities of decarboxylation of L-dopa in H_2O (○——○) and in ^2H_2O (●——●) as assayed by (A) a high-performance liquid chromatographic procedure and (B) a radioenzymatic method using [^{14}C]R-dopa as tracer. The labeled DA product was separated by ion exchange chromatography (Amberlite CG 50). Hog kidney aromatic aminoacid decarboxylase was used as described in Materials and Methods.

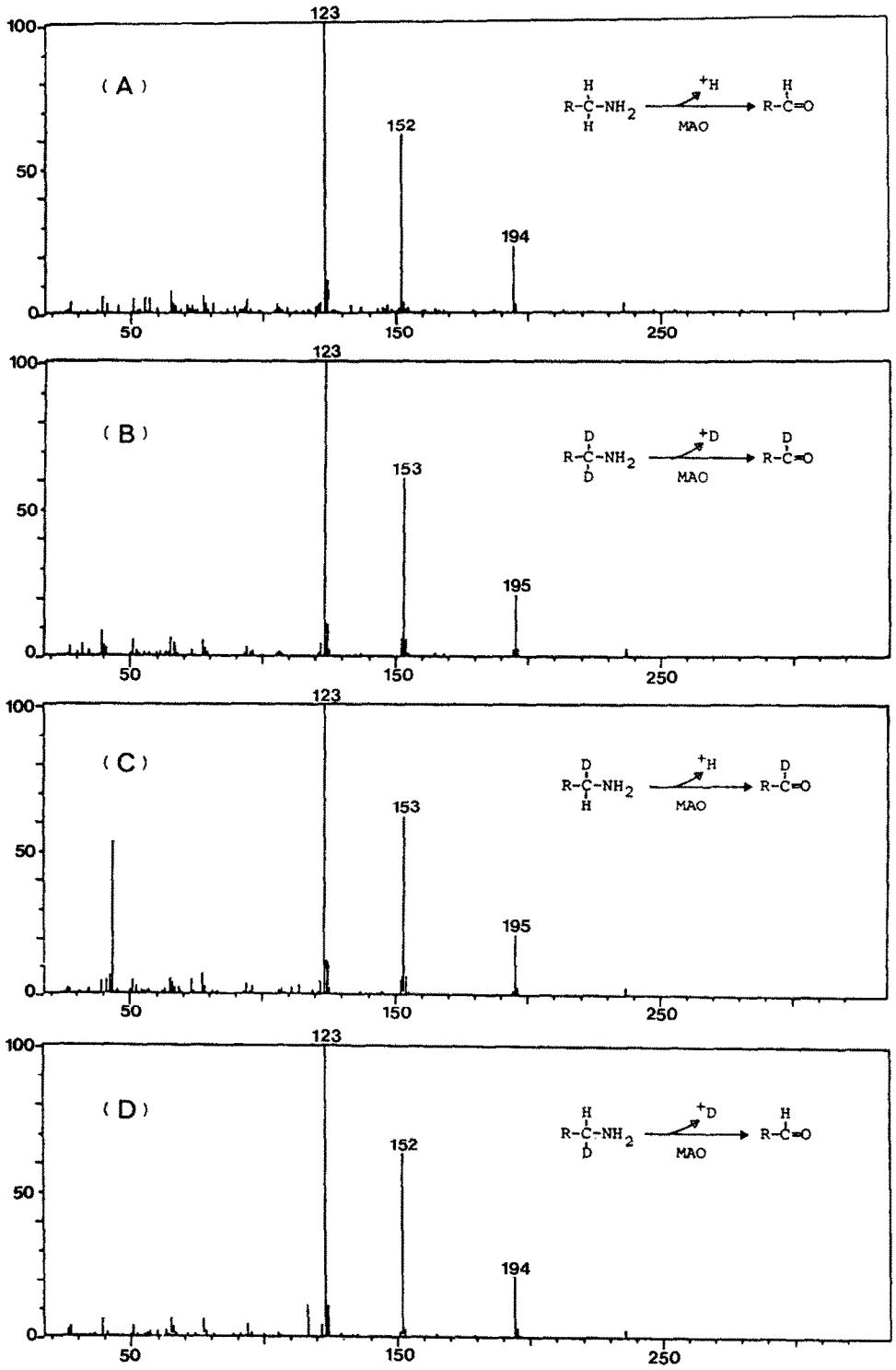


Fig. 3. Mass spectra of the dopamine deaminated product, 3,4-dihydroxyphenylacetaldehyde, after derivatization with acetic anhydride. The aldehyde products were from deamination of (A) proteo-DA, (B) [α,α-$^2H_2$]DA, (C) S[α-$^2H_1$]DA, and (D) R[α-$^2H_1$]DA. See Materials and Methods for details.

unreacted DA substrates. The separation of DA from the aldehyde products was >99% as checked by HPLC. The aldehydes were acetylated under mild alkaline conditions. To the aldehyde eluates (2 ml), 0.4 ml of distilled acetic anhydride and approximately 250 mg $NaHCO_3$ were gradually added 50 mg at a time until evolution of gas ceased. The tubes were then shaken and allowed to react for 1 hr. The acetylated aldehydes were extracted into ethyl acetate (2 × 2 ml). The extract was reduced to 100 μl under a stream of nitrogen, and 3-μl aliquots were injected into the GC–MS.

Mass spectra and quantitation by selected ion monitoring (SIM) were obtained on a VG 70-70 double-focussing mass spectrometer interfaced to an HP-5700 gas chromatograph. The GC conditions were as follows: column 2 m × 2 mm i.d. glass packed with 3% OV-101 on Gas Chrom Q; the carrier was helium at 30 ml/min with a temperature program of 140°, 2 min, 10°/min to 240°. The mass spectrometric conditions were: ion source temperature 200°, interface 240° and resolution of 1000 for spectra and 5000 for SIM analysis. The elemental compositions of the molecular ion (m/z 236) of the undeuterated and m/z 237 of the deuterated compounds were verified to be $C_{12}H_{12}O_5$ and $C_{12}H_{11}O_5{}^2H_1$, i.e. m/z 236.0685 and m/z 237.0747 using m/z 218.9856 of perfluorotri-*n*-butylamine as the reference ion. These masses were observed to be within 20 ppm of the calculated mass values during SIM.

RESULTS

Solvent isotope effect on enzymatic decarboxylation of L-*dopa.* We synthesized $R[\alpha\text{-}^2H_1]$DA by enzymatic decarboxylation of L-dopa in deuterium oxide. Many enzymic properties, including catalytic rates, can be profoundly affected by deuterium oxide (i.e. solvent isotope effect) [15]. It was important to know, therefore, whether/how ^2H_2O affects L-dopa decarboxylation and thus the production of $R[\alpha\text{-}^2H_1]$ DA. In the above described preparation, all solutions such as buffer, substrate, cofactors and enzyme were prepared in 99.8% ^2H_2O. The decarboxylation of L-dopa was not inhibited in D_2O when compared with the rate in H_2O. In fact, an activation (about 25%) was observed. As can be seen from Fig. 2A, the initial rate of decarboxylation of L-dopa in ^2H_2O was markedly higher than the rate in H_2O. This enhanced decarboxylation by reaction in ^2H_2O was also demonstrated by a radioisotopic method (Fig. 2B). Kinetic analysis indicates that the activation was noncompetitive, i.e. the increase of V_{max} and K_m values remained unchanged in ^2H_2O (results not shown).

Determination of deuterium loss at α-carbon after oxidative deamination. The oxidative deamination of proteo-DA, $[\alpha,\alpha^2H_2]$DA, $R[\alpha\text{-}^2H_1]$DA and $S[\alpha\text{-}^2H_1]$DA was carried out by incubating rat liver mitochondrial MAO with the above substrates under identical incubation conditions. To permit an analysis of the aldehyde products by GC–MS, it was essential to derivatize the phenolic groups by acetylation under mild alkaline conditions. Such treatment does not cause exchange of the aldehyde hydrogen. The spectra of the four acetylated products are shown in Fig. 3. The molecular ion was 236 ($C_{12}H_{12}O_5$) for proteo-DA and $R[\alpha\text{-}^2H_1]$DA and 237 ($C_{12}H_{11}O_5{}^2H_1$), for $[\alpha,\alpha\text{-}^2H_2]$DA and $S[\alpha^2H_1]$DA. The elemental compositions of these ions were confirmed by high resolution mass analysis; other ions in the spectra, m/z 194 (or 195) and 152 (153), were consistent with the loss of one or two acetyl groups C_2H_2O (42) from the proteo and deutereo aldehydes, whereas m/z 123 was due to further cleavage of the alkyl chain and was consistent with loss of the aldehyde moiety from both the proteo and deuterated compounds. This shows unambiguously that it is the R hydrogen that is cleaved during oxidation of the primary amines by MAO.

Determination of MAO activity towards DA; measurement of proteo and deuterated 3,4-*dihy-*

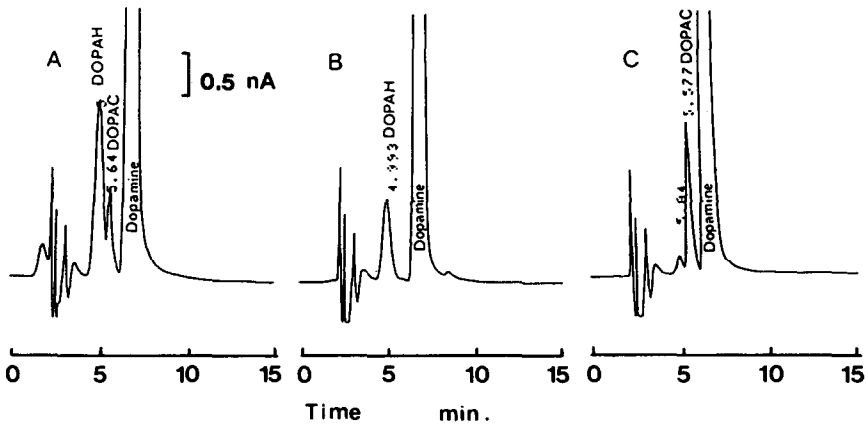


Fig. 4. High-performance liquid chromatographic traces of the dopamine deaminated products catalyzed by (A) crude rat liver homogenate, (B) washed rat liver mitochondrial membrane fragments, and (C) mitochondrial membrane fragments to which was also added yeast acetaldehyde dehydrogenase and β-nicotinamide adenine dinucleotide (NAD^+). The retention times with respect to 3,4-dihydroxyphenylacetaldehyde (DOPAH), DOPAC and dopamine are 5.0, 5.7 and 6.6 min respectively.

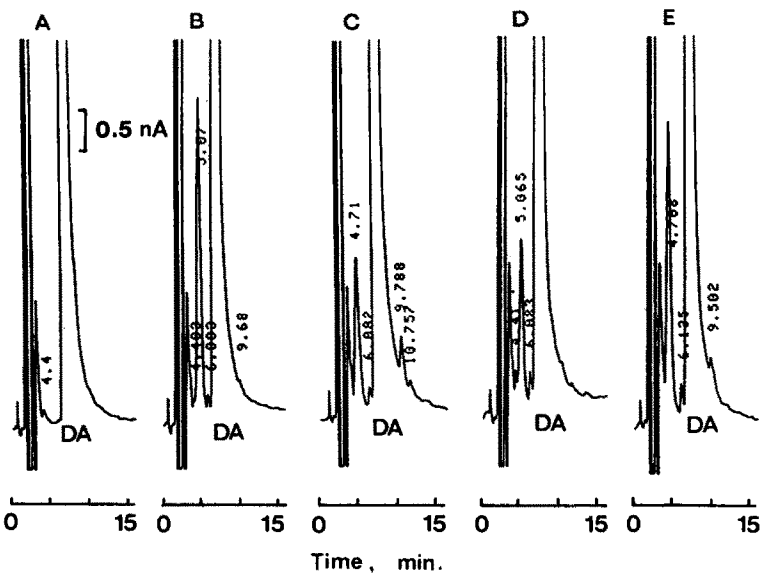


Fig. 5. High-performance liquid chromatographic traces of 3,4-dihydroxyphenylacetaldehydes after deamination of (A) blank (zero time), (B) proteodopamine, (C) [α,α-$^2H_2$]DA, (D) R[α-$^2H_1$]DA and (E) S[α-$^2H_1$]DA, catalyzed by rat liver mitochondrial MAO. Conditions are identical to those described in the legend of Fig. 4.

droxyphenylacetaldehyde by HPLC–ECD. When the washed mitochondrial membrane fragments were used for the determination of MAO actvity towards dopamine, 3,4-dihydroxyphenylacetaldehyde (DOPAH) was found to be the only product formed by HPLC (see Fig. 4B). As can be seen in Fig. 4, 3,4-dihdroxyphenylacetic acid (DOPAC) was formed when either the crude homogenate was used or when yeast aldehyde dehydrogenase was included in the reaction mixture. In the case of the crude homogenate both products, DOPAH and DOPAC, could be identified. The aldehyde intermediate is quite stable and did not deteriorate during at least 1 week in 0.1 N perchloric acid and in the dark in the refrigerator (4°).

Figure 5 indicates typical HPLC traces from the MAO reaction with dopamine, [α,α-$^2H_2$]DA and the R[α-$^2H_1$]DA and S[α-$^2H_1$]DA enantiomers as substrates. A considerable deuterium isotope effect was observed in the case of the oxidative deamination of [α,α-$^2H_2$]DA and R[α-$^2H_1$]DA in comparison to those of proteo-DA and S[α-$^2H_1$]DA.

We have also noticed that the proteo aldehyde derived from oxidation of proteo-DA and R[α-$^2H_1$]DA exhibited a retention time (5.07 ± 0.002 min, N = 8, mean ± S.D.) which was slightly but significantly ($P < 0.001$) different from that of the peak (4.71 ± 0.002 min) of monodeutero DOPAH derived from the oxidation of [α,α-$^2H_2$] and S[α-$^2H_1$]DA.

Kinetic deuterium isotope effects on the oxidative deamination of dopamine by rat liver MAO-A and MAO-B. Rat liver MAO-A and MAO-B were obtained by treatment of a fresh mitochondrial fragment preparation with the selective inhibitors deprenyl (MAO-B-I) and clorgyline (MAO-A-I) respectively. A highly selective inhibition was obtained, as indicated previously by the MAO-I titration response curve [16].

As can be seen from Fig. 6, the initial rate of formation of 3,4-dihydroxyphenylacetaldehyde from each of the four DA substrates was linear for at least 30 min. Substitution at the α-carbon position by either two deuteriums or by a single deuterium in the Re position reduced the rate of oxidation catalyzed by both rat liver MAO-A and MAO-B. This isotope effect is quite stereospecific, since only a slight reduction in the reaction rate was observed with respect to the S[α-$^2H_1$)DA enantiomer oxidation.

Figure 7 presents the comparative Lineweaver–Burk plot analyses of oxidative deamination of the above four DA analogues. The replacement of the Re hydrogen by deuterium at the α-carbon position changed both the Michaelis–Menten constants (K_m) and the V_{max} values. The apparent K_m and V_{max} values are summarized in Table 1. A significant decrease of V_{max} ($P < 0.001$) and increase of K_m ($P < 0.01$) were observed with respect to MAO-A activity towards [α,α-$^2H_2$]DA and R[α-$^2H_1$]DA. Interestingly, MAO-B exhibited a reduction of V_{max} towards [α,α-$^2H_2$]DA and R[α-$^2H_1$]DA, but the K_m values were not changed significantly. A slight reduction of V_{max} was observed with respect to deamination of S[α-$^2H_1$]DA with respect to rat liver MAOs.

The isotope effects of the deuterated DA enantiomers in relation to MAO-A and MAO-B expressed as the ratio of the rat constants V_H/V_D and $(V/K)_H/(V/K)_D$ are shown in Table 2.

Deuterium isotope effects on dopamine deamination catalyzed by human placenta MAO-A and platelet MAO-B. Mitochondrial MAOs obtained from human placenta (MAO-A) and human platelet

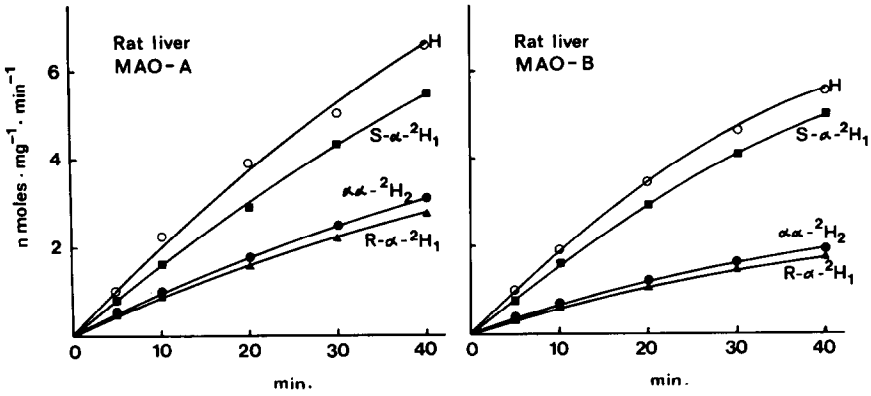


Fig. 6. Initial velocities of the deamination of different dopamine analogues by rat liver mitochondrial MAO-A and MAO-B. Enzyme activity [nmoles · (mg protein)$^{-1}$ · min^{-1}] was measured by direct analysis of the 3,4-dihdroxyphenylacetaldehyde by an HPLC method. Proteo-DA (○——○), [α,α-$^2H_2$]DA (●——●), R[α-$^2H_1$]DA (▲——▲) and S[α-$^2H_1$]DA (■——■), 5×10^{-4} M, were incubated with washed rat liver mitochondrial membrane fragments (15–20 μg protein) at 37°.

(MAO-B) were also used to examine whether stereospecific deuterium substitution of dopamine may exhibit distinguishable isotope effects. As summarized in Tables 1 and 2, the magnitudes of the effects were only slightly different one from the other and are quite similar to those obtained for rat liver MAO-A and MAO-B. Thus, R-deuterium substitution at the α-carbon is involved in bond cleavage and it exhibits an appreciable deuterium isotope effect.

DISCUSSION

It has been established that appropriate enantiomers of monoamines or diamines can be prepared by enzymatic decarboxylation of the corresponding aminoacid [5, 7, 18–20]. R-Enantiomers, for example, can be obtained by decarboxylation of unlabeled aminoacids in deuterium oxide, whereas the S-enantiomers are obtained by decarboxylating α-$^2H_1$-substituted amino acids in H_2O. Using this principle, we synthesized the R- and S-enantiomers of dopamine using hog kidney aminoacid decarboxylase. A solvent deuterium isotope effect, which normally inhibits chemical reaction rates, was not observed in our study during the decarboxylation of L-dopa; on the contrary, a slight increase in the rate of decarboxylation of L-dopa in ^2H_2O occurred. Although one of the hydrogen (or deuterium) atoms of the water was being transferred to the α-carbon of the L-dopa, it was not the rate-limiting step. It has

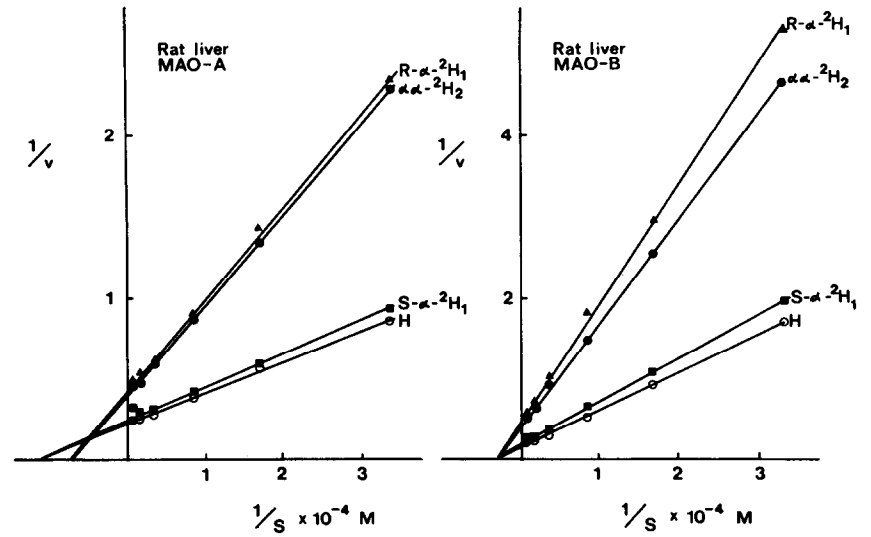


Fig. 7. Lineweaver–Burk plots for the deamination of different DA analogues by rat liver mitochondrial MAO-A and MAO-B. The assay conditions are similar to those described in the legend to Fig. 1, except that substrate concentrations were varied [V = velocity, nmoles · (mg protein)$^{-1}$ · min^{-1}; S = substrate concentration with respect to undeuterated DA (○——○), [α,α-$^2H_2$]DA (●——●), R[α-$^2H_1$]DA (▲——▲) and S[α-$^2H_1$]DA (■——■)].

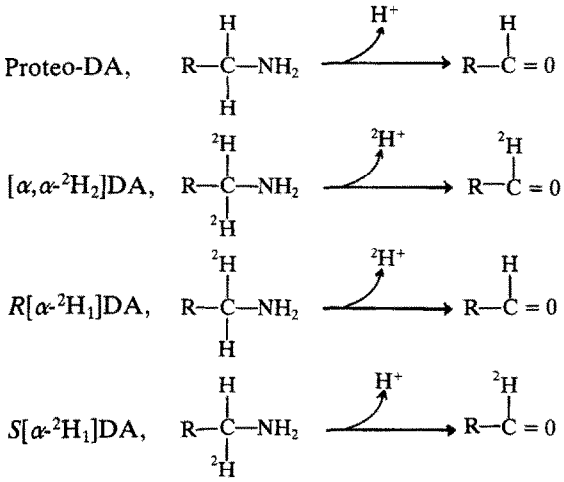
Table 1 Comparison of the apparent V_{max} and Michaelis–Menten constants (K_m) for MAO-A and MAO-B in the oxidative deamination of proteo DA and its deuterated enantiomers

	DA		[α-α-$^2H_2$]DA		R[α-$^2H_1$]DA		S[α-$^2H_1$]DA	
	V_{max}	K_m	V_{max}	K	V_{max}	K_m	V_{max}	K_m
MAO-A								
Rat liver	4.65 ± 0.17	0.96 ± 0.11	2.61 ± 0.06*	1.62 ± 0.11†	2.46 ± 0.02*	1.32 ± 0.03†	4.20 ± 0.04‡	0.89 ± 0.03
Human placenta	8.65 ± 0.24	0.97 ± 0.08	5.13 ± 0.08*	1.39 ± 0.03†	5.05 ± 0.20*	1.71 ± 0.17†	8.17 ± 0.43	1.32 ± 0.16
MAO-B								
Rat liver	6.36 ± 0.15	2.80 ± 0.15	2.70 ± 0.25*	3.17 ± 0.71	2.05 ± 0.06*	2.50 ± 0.17	5.07 ± 0.16*	2.72 ± 0.18
Human platelet	0.76 ± 0.06	2.42 ± 0.51	0.30 ± 0.01*	2.59 ± 0.19	0.29 ± 0.02*	2.53 ± 0.04	0.77 ± 0.05	3.37 ± 0.41

V_{max} (maximal velocities, nmoles/mg protein/min) and K_m (Michaelis–Menten constant, 1×10^{-4} M) values were obtained from the equations described by Wilkinson [17] using a Commodore PET 4016 computer.
*–‡ Significances were obtained by comparison of deuterated DA with proteo-DA as substrate (Student t-test): *$P < 0.001$; †$P < 0.01$; and ‡$P < 0.05$.

been shown that heavy water stabilizes an association of monomers in some enzymes by forming polymers [21]; this appears to exert a protective effect by maintaining the molecular tertiary structure for enzymatic function [22]. It is possible, therefore, that ^2H_2O may stabilize the association of the hog kidney decarboxylase which is known to be composed of several subunits [23]. It is also possible that deuterium oxide may affect the pK_a of the amino acid residues of the enzymes controlling the catalysis [15]. Inverse isotope solvent effects have also been observed in aldehyde reduction and oxidation [24]. The mechanisms of these inverse isotope effects, however, have not yet been established.

Analysis of the aldehydes produced from different DA enantiomers by GC–MS showed quite clearly (see Fig. 3) that cleavage of hydrogen at α-carbon during MAO reactions follows the scheme given below.



This direct analysis of the aldehydes is in agreement with earlier findings that it was the *R*-hydrogen of the α-carbon of tyramine, another mixed-type MAO substrate, that was involved in bond cleavage during its oxidative deamination [4, 5]. Diamine oxidase in contrast catalyzes deamination of amines such as cadaverine, putrescine and agmatine by removing the *S*-hydrogen [7].

The elimination of the *R*-hydrogen from the α-carbon of dopamine has been further substantiated by our results of the separation of deuterated and proteo-3,4-dihydroxyphenylacetaldehydes using HPLC incorporating a reverse phase column (Ultrasphere I.P.). The retention times with respect to deuterated products derived from [α,α-$^2H_2$]DA and S[α-$^2H_1$]DA were identical and markedly different from the retention times with respect to the aldehydes obtained from proteo-DA and R[α-$^2H_1$]DA. This effect is possibly due to a change in polarity by deuterium substitution at the aldehyde group, thus creating a different hydrophobic interaction in the chromatographic system.

Using the highly sensitive and reliable HPLC method for the assay of MAO activity, we addressed the magnitude of the deuterium isotope effects in DA oxidative deamination. It was not surprising to find that R[α-$^2H_1$]DA exhibited an isotope effect as significant as that found with [α,α-$^2H_2$]DA, since the

Table 2. Deuterium isotope effects on the enzymatic deamination of [α,α-$^2H_2$], R[α-$^2H_1$] and S[α-$^2H_1$] dopamines

	V_H/V_D			$(V/K)_H/(V/K)_D$		
	[α,α-$^2H_2$]	R[α-$^2H_1$]	S[α-$^2H_1$]	[α,α-$^2H_2$]	R[α-$^2H_1$]	S[α-$^2H_1$]
MAO-A						
Rat liver	1.78 ± 0.08	1.89 ± 0.07	1.11 ± 0.04	3.01 ± 0.37	2.60 ± 0.32	1.03 ± 0.13
Human placenta	1.69 ± 0.05	1.71 ± 0.08	1.06 ± 0.06	2.42 ± 0.22	3.02 ± 0.43	1.44 ± 0.24
MAO-B						
Rat liver	2.36 ± 0.23	3.10 ± 0.12	1.25 ± 0.05	2.67 ± 0.67	2.77 ± 0.26	1.22 ± 0.11
Human platelet	2.53 ± 0.22	2.62 ± 0.27	0.99 ± 0.08	2.71 ± 0.65	2.74 ± 0.65	1.37 ± 0.36

The kinetic data for different MAO analogues were obtained from Table 1. Standard errors of the ratios were calculated as the mean square error of the individual errors following Wilkinson [17], assuming that the values were statistically independent.

absolute stereochemical cleavage of the R-deuterium-carbon bond was proved to be involved in both cases. The V_H/V_D ratios obtained for DA in this study (ranging from 1.69 to 3.10) were higher than those obtained in an earlier study (i.e. V_H/V_D = 1.2 with respect to R[α-$^2H_1$]tyramine [1] and 1.6 for [α,α-$^2H_2$]DA [3]). In these earlier studies, the MAO activities were determined by measuring the disappearance of substrates usually by a colorimetric procedure. But in this situation the change in substrate concentration could be very small and, hence, less accurate than that of the measurement of product concentration. In enzyme kinetic studies it is also less satisfactory to assess activity by measuring the depletion of substrate since the substrate may be converted by other nonspecific chemical reactions.

In these earlier studies, rat liver MAOs, which contain both MAO-A and MAO-B [25], were used. When different types of MAO were tested in the present study, i.e. MAO-A from rat liver MAO pretreated with clorgyline or from human placenta [26], as well as MAO-B from rat liver MAO pretreated with clorgyline or from human platelets [27], a distinguisable isotope effect with respect to MAO-A and MAO-B was observed. In the deamination of DA catabolized by MAO-A, V_{max} decreased and K_m increased with respect to [α,α-$^2H_2$]DA and [α-$^2H_1$] DA oxidation (see Tables 1 and 2). In the case of MAO-B catalyzed deamination, although V_H/V_D ratios (2.36–3.10) were markedly higher than that of MAO-A (1.69–1.89), the K_m values were not changed significantly when the deamination of [α,α-$^2H_2$]DA and R[α-$^2H_1$]DA was compared with that of proteo-DA. This difference in isotope effects has suggested that MAO-A and MAO-B may exert different mechanisms in the oxidative deamination of monoamines. In fact, it has been shown that rate-limiting steps are dependent on the nature of the substrate [28]. The kinetic mechanism followed by bovine liver MAO, for example, may involve either a ternary or binary complex (ping-pong) mechanism.

We have also observed a relatively small reduction of reaction rate with respect to deamination of S[α-$^2H_1$]DA using rat liver MAO (Table 2). It could be due to a secondary isotope effect since S-α-deuterium was not removed during deamination. We cannot, however, exclude the possibility that other factors may also be involved.

It can be concluded that absolute stereochemistry is involved in the elimination of R-hydrogen during dopamine oxidative deamination catalyzed by both MAO-A and MAO-B from different sources. Dopamine or p-tyramine are typical mixed-type substrates. It is expected to be established whether such a stereochemical relationship can also be applied to the deamination of typical MAO-A substrate (serotonin) or MAO-B substrate (β-phenylethylamine) (work in progress).

L-Dopa has been applied effectively in the treatment of Parkinsonism [29]. The disease is associated with deficiency of DA in the brain; biochemical manipulations that temporarily replenish DA stores could improve the extrapyrimidal motor symptoms [30]. L-Dopa, the precursor of dopamine, was used in the treatment of Parkinsonism because DA itself does not pass the blood–brain barrier [31]. The large clinical dose of L-dopa required for effective treatment causes considerable unwanted side effects [32]. The profound isotope effect on oxidative deamination after α-deuterium substitution [1–4] has been demonstrated *in vivo*, i.e. [α,α-$^2H_2$]β-phenylethylamine was found to be resistant to oxidation after intraperitoneal administration [33] and caused prolonged behavioral effect [34]. It is, therefore, worthwhile to consider whether deuterium substitution of L-dopa could increase the half-life of DA formed and subsequently improve its pharmacological properties. Unfortunately, our study has shown that α-$^2H_1$ substituted L-dopa would be decarboxylated to form S[α-$^2H_1$]DA. This DA enantiomer only exhibits small secondary deuterium isotope effects during deamination. Therefore, it is unlikely that "replenished" DA after administration of [α-$^2H_1$]L-dopa should be more resistant to oxidation in comparison to the undeuterated L-dopa.

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