Biochemical Pharmacology, Vol. 30, No. 22, pp. 3089–3094, 1981.
Printed in Great Britain.

0006-2952/81/223089-06 $02.00/0

DEUTERIUM ISOTOPE EFFECTS ON THE ENZYMATIC OXIDATIVE DEAMINATION OF TRACE AMINES

PETER H. YU,* SHANE BARCLAY, BRUCE DAVIS and ALAN A. BOULTON
Psychiatric Research Division, University Hospital, Saskatoon, Saskatchewan S7N 0X0, Canada

(*Received* 15 *January* 1981; *accepted* 6 *April* 1981)

Abstract—The steady-state kinetics of the oxidative deamination of some trace amines [*p*-tyramine (*p*-TA), *m*-tyramine (*m*-TA) and β-phenylethylamine (PE)] and the same trace amines containing deuterium in their side-chain (i.e. αα-d_2 and ββ-d_2) have been assessed using rat liver mitochondrial monoamine oxidase (MAO) incubated at 37° in 0.05 M phosphate buffer (pH 7.5). In these *in vitro* reactions, a considerable reduction in deamination occurred when the deuterium substitution was in the α position. In addition, the isotope effect was found to be related to hydroxyl substitution on the phenyl ring. The apparent $(V/K)_H/(V/K)_D$ ratios were 4.44, 4.24 and 2.06 for *p*-TA, *m*-TA and PE respectively. We have confirmed that the cleavage of the C—H bond at the α position is involved in the rate-limiting step of the enzymatic deamination. In the case where the deuterium substitution was in the ββ position, a slight enhancement of deamination occurred with the $(V/K)_H/(V/K)_D$ ratio becoming 0.89, 0.86 and 0.95, respectively, for *p*-TA, *m*-TA and PE. The selective inhibition of the deamination of the αα-deuterated amines by the specific MAO inhibitors clorgyline (type A) and deprenyl (type B) was not different from that of the corresponding non-deuterated trace amines. The isotope effect was found to be somewhat greater at lower temperatures. Using mixed substrates (i.e. trace amine + corresponding deutrated trace amine), the deuterated amines were observed to exhibit a weak inhibitory effect due simply to competition for the same site on the enzyme.

Trace amines such as *p*-tyramine (*p*-TA), *m*-tyramine (*m*-TA) and β-phenylethylamine (PE), are constituents of peripheral tissues, the central nervous system and body fluids [1]. They are synthesized endogenously, are pharmacologically [2] and behaviorally active [3], and can have profound effects on neuronal activity [4, 5]. They appear to be excreted in abnormal amounts by persons suffering with various psychiatric and neurological disorders [6]. They have been proposed to act as synaptic activators (neuromodulators) [1, 3, 6, 7]. They are inactivated by enzymatic oxidative deamination through monoamine oxidase [amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4, MAO] which deaminates to the aldehyde which is then converted either to the acid with aldehyde oxidase or to an alcohol with alcohol dehydrogenase [8]. TA labelled with deuterium causes a marked increase in its sympathomimetic effects [9]. The MAO reaction involves the cleavage of an α-hydrogen from the side-chain carbon atom; substitution of this α-hydrogen with deuterium results in a profound reduction in the amount of TA deaminated (i.e. an isotope effect) [10]. In a study by Belleau *et al.* [10], the oxidation of TA and αα-d_2-TA was determined based on the rate of color development with ninhydrin. Recently, we have observed that there was a profound isotope effect in the ninhydrin reaction with αα-deuterated monoamines (P.H. Yu, unpublished observation). We have, therefore, reinvestigated this study adopting a sensitive fluorometric method that measured the amount of hydrogen peroxide produced [11, 12]. In addition, we have also compared the effects of different deuterium substitutions of *p*-TA, *m*-TA and PE on the mechanism of proton transfer in the deamination process by monoamine oxidase.

MATERIALS AND METHODS

Materials. Male Wistar rats (150–200 g) were used. *para*-[1-^{14}C]Tyramine, β-[ethyl-^{14}C]phenylethylamine, 5-[2-^{14}C]hydroxytryptamine (5-HT) and Omnifluor were purchased from the New England Nuclear Corp. (Boston, MA); horse radish peroxidase, homovanillic acid and *p*-TA were from the Sigma Chemical Co. (St. Louis, MO); $LiAlD_4$ was from Fluka AG. (Basel, Switzerland). Deprenyl (phenylisopropylmethylpropynylamine hydrochloride) was a gift from Prof. J. Knoll, Semmelweis University (Budapest, Hungary) and clorgyline (2,4-dichlorophenoxypropyl-*N*-methylpropynylamine hydrochloride) from May & Baker, Ltd. (Dagenham, U.K.). All other chemicals were of analytical grade.

Preparation of m- and p-tyramine-α, α-d_2. *p*-Hydroxybenzaldehyde was converted to *p*-benzyloxybenzaldehyde and then reduced with sodium borohydride. The resulting alcohol was treated with thionyl chloride in chloroform and pyridine to give *p*-benzyloxybenzyl chloride, which was then converted to the corresponding cyanide by gentle refluxing with potassium cyanide in aqueous ethanol. This was then reduced with lithium aluminum deuteride and debenzylated by hydrogenolysis over palladium

* Author to whom all correspondence should be addressed.

on charcoal to give the desired product, *p*-tyramine-α, α-d_2 (*p*-TA-$\alpha\alpha$-d_2), which was isolated and purified by recrystallization of the hydrochloride from ethanol–ether. The melting point was 276–278°.

m-Tyramine-α, α-d_2 (*m*-TA-$\alpha\alpha$-d_2) was prepared in the same way but starting from *m*-hydroxybenzaldehyde. The melting point of this hydrochloride was 139.5–140.5°.

Preparation of phenylethylamine-α, α-d_2. Phenylacetonitrile was reduced with lithium aluminum deuteride. Phenylethylamine-α,α-d_2 (PE-$\alpha\alpha$-d_2) was then isolated and purified by recrystallization of the hydrochloride from ethanol–ether. The melting point was 210–212° (with sublimation beginning around 150°).

Preparation of m- *and* p-tyramine-β, β-d_2. *p*-Hxdroxybenzoic acid was esterified (with ethanol) and then the phenolic group benzylated. The ester was reduced with lithium aluminum deuteride to give the alcohol which was then converted into the chloride with thionyl chloride. The cyanide was prepared by refluxing the chloride with potassium cyanide in deuterium oxide–ethanol–OD. Reduction with lithium aluminum hydride and hydrogenolysis over palladium on charcoal yielded *p*-tyramine-β, β-d_2 (*p*-TA-$\beta\beta$-d_2) which was isolated and purified as the hydrochloride, m.p. 276–278°.

m-Tyramine-β, β-d_2 (*m*-TA-$\beta\beta$-d_2) hydrochloride was prepared similarly, starting from *m*-hydroxybenzoic acid. Its melting point was 141.5–142.5°.

Preparation of phenylethylamine-β, β-d_2. Ethylbenzoate was reduced with lithium aluminun deuteride, the resulting alcohol was converted to the chloride with thionyl chloride, which was then treated with potassium cyanide in deuterium oxide–ethanol–OD to give benzyl cyanide-α,α-d_2. Reduction with lithium aluminum hydride produced phenylethylamine-β,β-d_2 (PE-$\beta\beta$-d_2) which was isolated and purified by recrystallization of the hydrochloride from ethanol–ether.

Chemical and isotopical purity of the amine isomers. The chemical purity of all the above amines was determined by melting point and by nitrosonaphthol fluorometry [13]. They were all greater than 98 percent.

The extent of deuteration was calculated by measuring the mass-spectrometric integrated ion current signals for the dansyl derivatives of the unsubstituted and the deuterium amines with an A.E.I. MS-902S high resolution mass spectrometer [14]. It was found that the d_2 purity was quite high in all cases (>95 percent) except *p*-TA-$\beta\beta$-d_2 where it was about 89 percent.

Assay of MAO activity. A sensitive fluorometric method, based on the formation of an intense fluorescence from homovanillic acid and the hydrogen peroxide released during the oxidation of the amine substrates, was adopted as previously described [11, 12].

In the study ot the effects of the deuterated analogues on the oxidation of the non-deuterated amines, a previously described radiochemical method was used [15]. Carbon-14 labelled *p*-TA, PE and serotonin (5-HT) were used as substrates.

Protein was determined by the method of Lowry *et al.* [16] using crystalline serum albumin as a standard.

Preparation of mitochondrial MAO. Freshly dissected rat livers 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). Mitochondria were obtained by differential centrifugation as previously described [15]. Mitochondrial membrane fragments were prepared by lysing the mitochondria in chilled distilled water followed by centrifugation at 105,000 *g* for 30 min. The membrane preparations were then washed by suspension in chilled distilled water and recentrifuged.

Kinetics. Reciprocal velocities of the enzyme

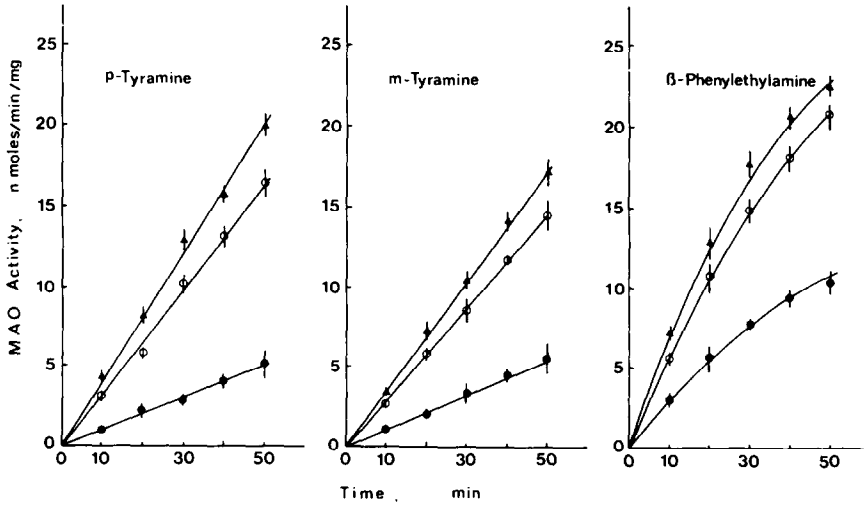


Fig. 1. Initial velocities of the deamination of different deuterated analogues of *p*-TA, *m*-TA and PE by rat liver mitochondrial MAO. Enzyme activity [nmoles·(mg protein)$^{-1}$·min^{-1}] was measured fluorometrically at 37° with $\alpha\alpha$-d_2 (●—●), $\beta\beta$-d_2 (▲—▲), and non-deuterated (○—○) amines as substrate. The substrate concentrations used were 2×10^{-4} M for the tyramine and 25×10^{-5} M for PE.

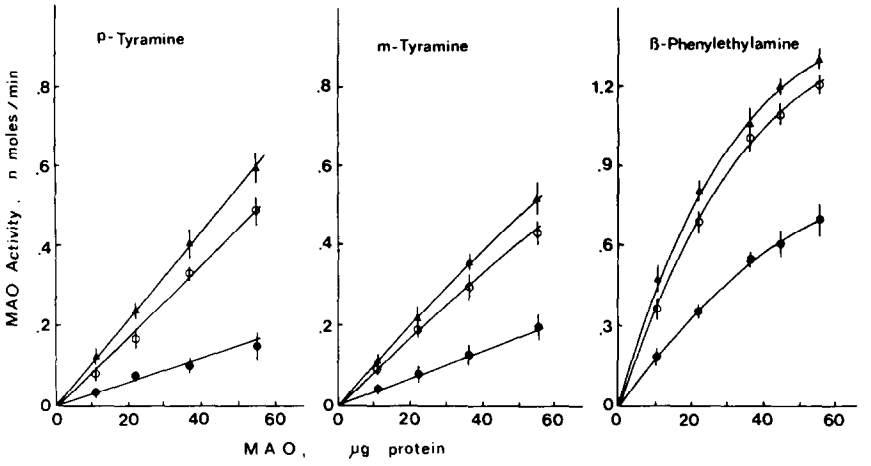


Fig. 2. Deamination of deuterium substituted and unsubstituted *p*-TA, *m*-TA and PE with increasing concentration of MAO. The crude rat liver mitochondrial membrane fragments (0–55 µg/assay) were incubated with $\alpha\alpha$-d_2 (●—●), $\beta\beta$-d_2 (▲—▲), and nonsubstituted (○—○) amines. The substrate concentrations used were the same as those described in the legend of Fig. 1.

reactions were plotted graphically against reciprocals of the substrate concentration. In all cases a reasonable linear relationship was obtained. These data were then fitted to the following equation:

$$v = \frac{VA}{K_m + A}$$

(where v = observed velocity, V = maximum velocity, A = substrate concentration and K_m = Michaelis–Menten constant) using a least squares method according to Wilkinson [17].

RESULTS

MAO activity towards *p*-TA, *m*-TA and PE as well as towards their corresponding $\alpha\alpha$-d_2 and $\beta\beta$-d_2 analogues was assayed. As can be seen from Fig. 1, the oxidation of the $\alpha\alpha$-d_2-deuterated amines was much reduced in comparison with the non-deuterated amines. The magnitude of the $\alpha\alpha$-d_2 isotope effect varied with the amine. As can also be seen from Fig. 1, the isotope effect of the $\alpha\alpha$-d_2 amines was in the order *p*-TA > *m*-TA > PE. In contrast the $\beta\beta$-d_2 analogues were deaminated by MAO somewhat better than the non-deuterated amines.

The deuterium isotope effect was also demonstrated by incubating the amines with increasing amounts of enzyme (Fig. 2). Similar results were found, namely, that the isotope effects were greater with respect to *p*-TA and *m*-TA than for PE.

As can be seen from the Lineweaver–Burk plots in Fig. 3, the replacement of hydrogen by deuterium changes 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 decrease in V_{max} and an increase in K_m were observed with respect to all three of the $\alpha\alpha$-d_2 amine analogues. Substitution in the β position did not affect the K_m values but it did increase the V_{max} values slightly. The isotope effects of the $\alpha\alpha$-d_2 substitution, expressed by V_H/V_D and $(V/K)_H/(V/K)_D$, are shown in Table 2.

The results plotted in Fig. 4 summarize the deuterium isotope effects at different temperatures. As can be seen, the effect decreased somewhat with increasing temperature.

The ability of rat liver MAO in the presence of the specific type A inhibitor, clorgyline, and the type B inhibitor, deprenyl, to deaminate $\alpha\alpha$-d_2 and non-substituted *p*-TA was compared. As can be seen

Table 1. Comparison of the apparent V_{max} and Michaelis–Menten constants for MAO in the enzymatic deamination of *p*-TA, *m*-TA and Pe labelled in the α and β positions with deuterium*

	p-TA		*m*-TA		PE	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Non-deuterated	33.3 ± 1.5	1.1 ± 0.1	30.0 ± 1.8	1.1 ± 0.1	78.9 ± 3.8	0.078 ± 0.005
Deuterium label						
$\alpha\alpha$-d_2	13.7 ± 1.7†	2.0 ± 0.1†	13.5 ± 1.2†	2.1 ± 0.2†	44.7 ± 2.1†	0.091 ± 0.006
$\beta\beta$-d_2	37.5 ± 1.0‡	1.1 ± 0.2	34.9 ± 1.2‡	1.2 ± 0.1	82.3 ± 4.1	0.08 ± 0.005

* V_{max} [maximal velocities, nmoles·(mg protein)$^{-1}$·min^{-1}] and k_m (Michaelis–Menten constant, 1×10^{-4} M) values were obtained by Lineweaver–Burk double-reciprocal plots. Experimental data were fitted to the equation described in Materials and Methods based on least squares analysis assuming equal variance for the velocities [17].

† Values are significantly different from the control, $P < 0.001$.

‡ Values are significantly different from the control, $P < 0.05$.

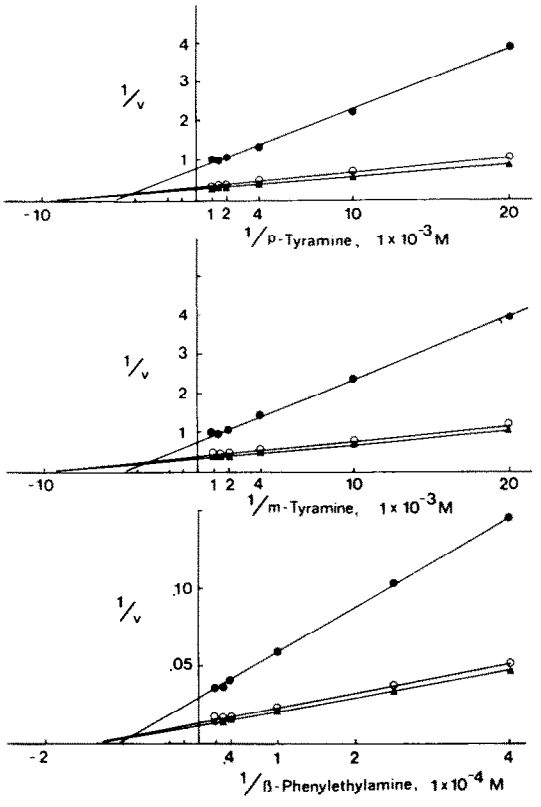


Fig. 3. Lineweaver–Burk plots for the deamination of different deuterated isomers of *p*-TA, *m*-TA, and PE by rat liver mitochondrial MAO. MAO (19–35 μg protein/assay) was incubated with $\alpha\alpha$-d_2 (●—●), $\beta\beta$-d_2(▲—▲), and the non-deuterated amines (○—○) [v = velocity, nmoles (mg protein)$^{-1}\cdot hr^{-1}$; S = substrate concentrations, from 0 to 1×10^{-3} M for the tyramine and 0 to 1×10^{-4} M for PE].

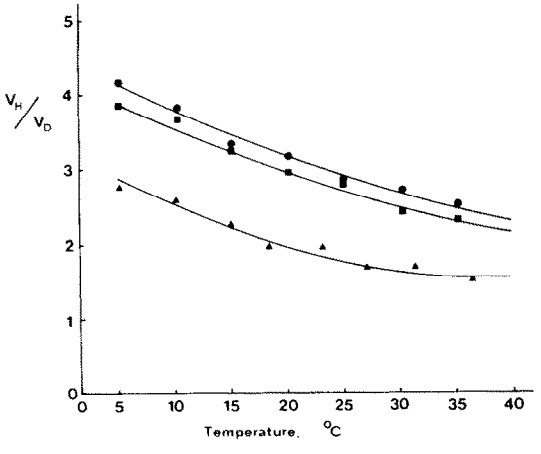


Fig. 4. Variation of the deuterium isotope effect with temperature. Assays were carried out as described in the legend to Fig. 1. The enzyme was incubated at different temperatures in the range of 5 to 40°. The ratio between the non-deuterated and $\alpha\alpha$-deuterated *p*-TA (●—●), *m*-TA (■—■), and PE (▲—▲) is shown.

Table 2. Deuterium isotope effects on the enzymatic deamination of $\alpha\alpha$-d_2 labelled *p*-TA, *m*-TA and PE*

Substrates	V_H/V_D	$(V/K)_H/(V/K)_D$
p-Tyramine	2.43	4.42
m-Tyramine	2.22	4.20
β-Phenylethylamine	1-76	2.05

* The kinetic data were obtained for substrates of protonated (H) and $\alpha\alpha$-d_2 substituted (D) amines from Table 1.

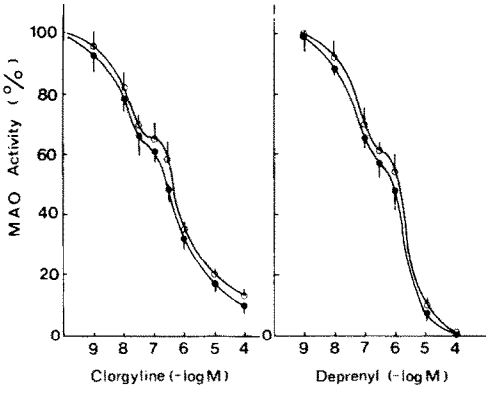


Fig. 5. Effect of clorgyline and deprenyl on the deamination of *p*-TA and *p*-TA-$\alpha\alpha$-d_2 by rat liver mitochondrial MAO. Both *p*-TA-$\alpha\alpha$-d_2 (●—●) and *p*-TA (○—○) at 2×10^{-4} M were incubated with the rat liver mitochondrial membrane fragments (19–35 μg protein/assay) in the presence of the drugs at different concentrations.

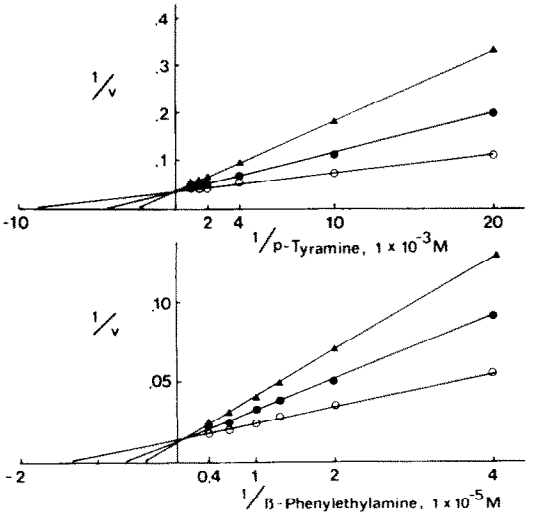


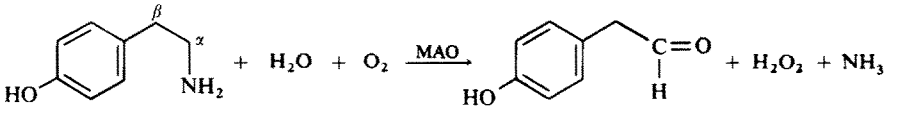
Fig. 6. Inhibition by *p*-TA-$\alpha\alpha$-d_2 and PE-$\alpha\alpha$-d_2 on the deamination of *p*-TA[^{14}C] and PE[^{14}C] in mixed substrate reactions. Lineweaver–Burk plots of the reciprocal of the initial velocity of *p*-TA[^{14}C] oxidation against the reciprocal of substrate concentration were obtained from incubation in the absence (○—○) and presence of $\alpha\alpha$-d_2 (●—●) or non-deuterated amine (▲—▲). The K_m^D concentrations of these amines were used, namely 2.0×10^{-4} M for *p*-TA and 9.1×10^{-5} M for PE.

from Fig. 5, typical biphasic inhibitory response curves were obtained in both cases as the concentrations of deprenyl and clorgyline increased. No significant difference could be found, however, between the oxidation of $\alpha\alpha$-d_2 and the non-labelled amine. The effects (results not shown) of these two inhibitors on the deamination of $\alpha\alpha$-d_2 and non-deuterated *m*-TA and PE were found to be virtually identical.

The effects of *p*-TA-$\alpha\alpha$-d_2 and PE-$\alpha\alpha$-d_2 on the oxidation of ^{14}C-labelled *p*-TA and ^{14}C-labelled PE were investigated. The K_m concentrations of these deuterated isomers were used (i.e. 2×10^{-4} M for *p*-TA-$\alpha\alpha$-d_2 and 9×10^{-6} M for PE-$\alpha\alpha$-d_2). Lineweaver–Burk plots (Fig. 6) reveal that the $\alpha\alpha$-d_2 analogues exhibit some competitive inhibitory effects on the corresponding non-deuterated amines. The $\alpha\alpha$-d_2 amines, however, were found to be weak competitive substrates, since their inhibitory effects were even less than those induced by the non-deuterated amines at the same concentration.

DISCUSSION

During oxidative deamination of monoamines by MAO the C—H bond at the α position is broken.



We have confirmed that replacement of the α hydrogens by deuterium in the amine substrates used in these experiments yielded remarkable decreases in their catabolism by MAO [10, 18]. The magnitude of the deuterium isotope effect with respect to deamination of *p*-TA, was found to be different from the previous observation [18]. For example, the V_H/V_D ratio was obtained in our study was 2.34, while 1.2 was reported earlier [18]. The discrepancy seems to be due to the application of different methods for MAO analysis. Belleau *et al.* have measured the disappearance of *p*-TA with ninhydrin. There was a profound isotope effect in the ninhydrin reaction with $\alpha\alpha$-d_2 monoamines (i.e. $K_H/K_D > 4.0$) (P. H. Yu, unpublished observation). This observation was not surprising because in this color reaction cleavage of the C—H bond at the α position of the monoamines was involved [19]. It would be very complicated to use the ninhydrin method for measuring the isotope effect of MAO reaction.

The $(V/K_m)_H/(V/K_m)_D$ ratios obtained in this study were 4.43, 4.20 and 2.05 for *p*-TA, *m*-TA and PE respectively. From this it would appear that the C—H bond is broken in the transition state and that cleavage of the C—H bond is involved in the rate-determining step of the enzymatic deamination reaction. It is not yet known whether the C—N bond is broken simultaneously or whether it is connected in some interdependent way with the C—H cleavage.

Substitution in the phenyl ring (i.e. an hydroxyl group in the meta or para position) of the amine substrate appears to affect the transient state and increase the isotope effect. Evidently $\alpha\alpha$-d_2 substitution in trace amines renders them poor substrates for MAO. Because of this we investigated the effect of $\alpha\alpha$-d_2 substituted amines on the deamination of non-deuterated *p*-TA and PE. In the presence of two substrates, A_H (non-deuterated amine) and A_D (deuterated amine), the effect of A_D on the deamination of A_H at different concentrations by MAO can be expressed as:

$$v_H = \frac{dP_H}{dt} = \frac{V_H A_H}{K_H(1 + A_D/K_D) + A_H} \quad (1)$$

where K_D and K_H are the apparent Michaelis constant with respect to A_D and A_H, respectively and dP_H/dt is the incremental formation of product with time. The velocities (V_H) obtained from the mixed-substrate experiments (as shown in Fig. 6) agree quite well with the theoretically calculated values derived from equation 1. These observations indicate that *p*-TA-$\alpha\alpha$-d_2 behaves as a weak MAO inhibitor merely due to competition for the same site as *p*-TA on the MAO. Similarly, PE-$\alpha\alpha$-d_2 competes with PE for the same site of MAO. Obviously the deuterated amines possess relatively low binding affinities for MAO compared to that of the non-deuterated analogs.

Tritiated monoamines (i.e. the usual commercially available ones) are frequently used in many studies of metabolism and enzymology [20, 21]. Because the mass of tritium is higher than that of deuterium, the zero point energy of a C—T bond is lower than that of the C—D bond. The magnitude of the isotope effect in $\alpha\alpha$-T_2 substitution on deamination, therefore, is potentially greater than the effects seen here with deuterium [22].

$$\log k_H/k_T = 1.44 \log k_H/k_D$$

The specific radioactivities of these amines, however, are usually obtained by dilution with corresponding nonlabelled amines. If the two analogues are metabolically different, however, the results obtained will be misleading. We have recently observed, for example (P. H. Yu, unpublished observations), that *p*-TA-$\beta\beta$-d_2 is significantly less well β-hydroxylated than *p*-TA. When deuterium- or tritium-labelled isotopes are used as internal standards or markers for tracing metabolic or enzymatic reactions, therefore, it is essential to take into account whether or not they behave as the protonated substrate or whether there is an isotope effect.

It is worth considering whether or not there might be examples and applications in chemotherapy for the use of stable isotope-labelled precursors or drugs which after ingestion could be enzymatically converted into active principles (dopa is an example), so that they might either persist longer or else become physiologically more effective.

Acknowledgements—We thank Dr. S. L. Wu, Department of Chemistry, University of Saskatchewan, for helpful dis-

cussions, Ms. J. Fraser and Mrs. D. Cordeiro for their excellent technical assistance, and the Medical Research Council of Canada and Saskatchewan Health for their continuing financial support.

REFERENCES

1. A. A. Boulton, *Int. Rev. Biochem.* **26**, 179 (1979).
2. A. V. Juorio, *Br. J. Pharmac.* **66**, 377 (1979).
3. A. Faurbye, *Compreh. Psychiat.* **9**, 155 (1968).
4. R. S. G. Jones and A. A. Boulton, *Can. J. Physiol. Pharmac.* **58**, 222 (1980).
5. R. S. G. Jones, *J. Neurosci. Res.* **6**, 49 (1981).
6. A. A. Boulton, in *Trace Amines and the Brain* (Eds. E. Usdin and M. Sandler), p. 21. Marcel Dekker, New York (1976).
7. A. A. Boulton, in *Neurochemistry and Clinical Neurology* (Ed. A. Lajtha), p. 291. Alan R. Liss, New York (1980).
8. L. C. Leeper, H. Weissbach and S. Udenfriend, *Archs Biochem. Biophys.* **77**, 417 (1958).
9. B. Belleau, J. Burba, M. Pindell and J. Reiffenstein, *Science* **133**, 102 (1960).
10. B. Belleau. M. Fang, J. Burba and J. Moran, *J. Am. chem. Soc.* **82**, 5752 (1960).
11. S. H. Snyder and E. D. Hendley, *J. Pharmac. exp. Ther.* **163**, 386 (1968).
12. P. H. Yu and A. A. Boulton, *J. Neurochem.* **35**, 255 (1980).
13. T. P. Waalkes and S. Udenfriend, *J. Lab. clin. Med.* **50**, 733 (1957).
14. D. A. Durden, B. A. Davis and A. A. Boulton, *Biomed. Mass Spectrom.* **1**, 83 (1974).
15. P. H. Yu, in *Monoamine Oxidase: Structure, Function and Altered Functions* (Eds. R. W. Von Korff, T. P. Singer and D. Murphy), p. 233. Academic Press, New York (1979).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
18. B. Belleau and J. Moran, *Ann. N.Y. Acad. Sci.* **107**, 471 (1963).
19. D. J. McCaldin, *Chem. Rev.* **60**, 39 (1960).
20. G. A. Lyles and J. W. Greenawalt, *Biochem. Pharmac.* **27**, 923 (1978).
21. C. J. Fowler and B. A. Callingham, *Molec. Pharmac.* **16**. 546 (1979).
22. C. G. Swain, E. C. Stivers, J. F. Renwer, Jr. and L. J. Schaad, *J. Am. chem. Soc.* **80**, 5885 (1958).