## Isotope Effects in Enzymatic N-Demethylation of Tertiary Amines

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The N-demethylation of 1-(N-methyl-N-trideuteriomethylamino)-3-phenylpropane (1) by rodent liver homogenates was studied. The ratio of 1-trideuteriomethylamino-3-phenylpropane (2)/1-methylamino-3-phenylpropane (3) was determined by gc-ms. The ratio of 2/3 in the product of N-demethylation of 1 by liver homogenate from phenobarbital-treated rats was 1.31 and from untreated rats was 1.32, and the differences between the two groups were not statistically significant. The ratio of 2/3 in the product obtained from N-demethylation of 1 by liver homogenates from mice was 1.45. The ratio of 2/3 of greater than unity indicates the presence of a kinetic primary isotope effect in the enzymatic N-demethylation reaction. This effect is exclusive of the secondary isotope effects on the amine nitrogen. The presence of this primary isotope effect indicates that the cleavage of the C-H bond of the N-methyl group is a rate-limiting step in the N-demethylation of tertiary amines by rodent microsomal enzymes.

Since the first in vitro studies on the N-demethylation of p-dimethylaminoazobenzene by liver microsomal fraction,<sup>1</sup> there has been an ever-increasing interest in the nature of this process. This reaction was found to be catalyzed by a member of a group of membrane-bound mixed function monooxygenases, with the cytochrome P-450 hemoproteins as the terminal oxidases. The N-dealkylation reaction is believed to proceed through aminocarbinol intermediates which are produced by direct hydroxylation on the carbon  $\alpha$  to the nitrogen.<sup>2</sup> The observed isotope effect in the enzymatic N-demethylation of trideuteriomethylnormorphine,<sup>3</sup> where the ratio of rate constants  $(k_{\rm H}/k_{\rm D})$  and the ratio of Michaelis constants  $(K_m^{\rm D}/K_m^{\rm H})$  were found to be 1.40 and 1.43, respectively, had been cited to support this mechanism.<sup>2</sup> Since liver microsomes were used in these studies, the measured  $K_{\rm m}$  reflected both the ability of the compound to reach the metabolizing sites within the microsomal membrane and its affinity to the enzyme surface. Inasmuch as trideuteriomethylnormorphine was found to be more basic than morphine, the fraction of the latter compound which will be present in the undissociated form at the pH of the incubation mixture will be greater than that for the deuterated isomer, and since morphine partitioned poorly into the lipid phase from aqueous buffer (log P =-5.0 for heptane, 7.4 buffer<sup>4</sup>) the difference in the amount undissociated present will be reflected on the availability of the substrate to the metabolizing sites within the microsomes. Therefore, the higher  $K_m$  for trideuteriomethylnormorphine could be attributed to secondary isotope effects on the nitrogen basicity.

The isotope effects on the enzymatic O-demethylation of o-nitroanisole have also been studied<sup>5</sup> and the ratio of the rate constants  $(k_{\rm H}/k_{\rm D})$  was found to be approximately 2.0. The ratio of the Michaelis constants for the two substrates  $(K_m^D/K_m^H)$  was found to be approximately 0.4, indicating stronger binding of the deuterated substrate to the O-demethylating enzyme. The above two studies indicate that the secondary isotope effects could have paradoxical effects on the apparent Michaelis constants for substrates of enzymatic N- and O-dealkylation reactions. It can enhance the affinity of substrate to the enzyme surface and hence lower its  $K_m$  as in the enzymatic O-demethylation of o-nitroanisole and it can also increase the  $K_m$  for substrates of enzymatic N-dealkylation by decreasing the availability of the compound to the metabolizing sites. Therefore, a different approach was investigated to examine the primary isotope effects on the rates of enzymatic N-dealkylation of tertiary amines exclusive of the secondary isotope effects on the amine nitrogen.

Enzymatic N-demethylation of compounds of type 1 should provide a mixture of the isotope isomers 2 and 3. The ratio of 2/3 in the product would reflect the magnitude of the primary isotope effects in this reaction. The

$$\begin{array}{c} \text{RN} \xrightarrow{\text{CH}_3} \longrightarrow \text{RNHCH}_3 + \text{RNHCD}_3 \\ \xrightarrow{\text{CD}_3} 2 3 \end{array}$$

secondary isotope effects on the nitrogen in compound 1 will influence the overall rate of N-dealkylation of the substrate but will have no effect on the ratio of 2/3. In the present paper we report the primary isotope effects on the enzymatic N-demethylation of 1-(N-methyl-N-trideuteriomethylamino)-3-phenylpropane (1a).

### **Results and Discussion**

Compound 8 was obtained via the synthesis represented in Scheme I. In this synthesis 1-amino-3-phenylpropane (4) was treated with benzaldehyde with concomitant removal of water to provide the methine base 5. The latter was allowed to react with trideuteriomethyl iodide (99.5% D) to produce 6 which on hydrolysis provided 7.6 Treatment of 7 with formic acid and formaldehyde provided 8. The lowresolution mass spectrum of 8 at an electron beam energy of 70 eV in the ion source showed a molecular ion at m/e166. There were peaks, however, at m/e 165, 164, and 163 with intensities of 9.2, 5.0, and 14.4% relative to that of the molecular ion, respectively. It was of interest, therefore, to ensure that these peaks are primarily due to fragments of the molecular ion and not due solely to impurities of the hydrogen isotopes of 8. When the mass spectrum of 8 was determined at an electron beam energy of 8.9 and 20 eV, the ratio of peak intensities at m/e 163/166 and 165/166 progressively decreased as the electron beam energy decreased. This indicated that peaks at m/e 163 and 165 represent, at least in part, fragments of the molecular ion. The ratio m/e 164/166, however, changed in an unexplainable fashion since it increased as the electron' beam energy was decreased.

The enzymatic N-demethylation of 8 would result in the formation of a mixture of 9 and 10 (Scheme II). The quantitative separation of these products (9 and 10) from the

#### Scheme I



Scheme II



unchanged substrate 8 was achieved by treatment of the mixture with acetic anhydride to produce the acetamides 11 and 12, followed by extraction of 8 from the nonbasic 11 and 12 with dilute aqueous acid. A method was developed for the determination of the relative amounts of 11 and 12 in the product using glc-ms.

An authentic sample of 10 was treated with acetic anhydride and the product, after work-up, was examined by glc-ms. The mass spectrum of the peak which eluted off the chromatographic column with the same retention time as authentic 12 was examined. It showed peaks at m/e 191 (molecular ion), 87, and 44 (base peak). When a sample of 9 was treated similarly it showed a peak which eluted off the chromatographic column with essentially the same retention time as that for 12. The mass spectrum for this chromatographic peak showed major peaks at m/e 194 (M<sup>+</sup>), 90, and 47 (base peak) as well as a peak at m/e 191 with an intensity of less than 2% of that at m/e 194 (M<sup>+</sup>). It was evident that these three major peaks in the mass spectra of both 11 and 12 represented fragments of the molecule which contained the -NCD<sub>3</sub> and -NCH<sub>3</sub> moieties respectively intact and that any of these could be used for the estimation of the ratio of 11 to 12 in a mixture. It was felt, however, that the use of the base peaks at m/e 44 and 47, for 12 and 11, respectively, would not be appropriate since the peak at m/e 44 could be contaminated with the peak due to  $CO_2$  impurities. Furthermore, it was felt that since the parent peaks for these compounds were of good intensity (15% of base peak), it would be best to use the ratio of the intensities of the parent peaks (i.e., 194/191) to estimate the relative amounts of 11 and 12 present in the product of the enzymatic N-demethylation of 8.

Solutions containing mixtures of varying known concentrations of 9 and 10 were prepared and each of these was subjected to the same extraction procedure which was used for the work-up of the incubation mixtures for the enzymatic N-demethylation of 8. The nonbasic fraction, which contained the mixture of 11 and 12, was subjected to glcms analysis. The mass spectrum of the peak which eluted off the glc column with the same retention time as 11 and 12 was scanned, repetitively, between m/e 175 and 225. Five scans were normally obtained for each peak and one additional scan immediately prior to the peak to ensure that the eluate was free of impurities which produce peaks in the mass spectrum at m/e 191 or 194. The average of the ratios of the peak intensities at m/e 194/191 was calculated for each solution and the correlation between these values and the ratios of the concentration of 9 to 10 in the solutions was determined by the method of least squares. Table I represents the results of these studies and shows that there is good linear correlation between the ratios of peak intensities at m/e 194/191 and the ratios of concentrations of 9 to 10. It was therefore concluded that the relative amounts of 9 and 10 in the product of enzymatic N-demethylation of 8 could be determined using the aforementioned procedure.

**Table I.** Correlation between the Ratio of 9/10 and the Ratio of Peak Intensities at m/e 194/191

Ratio of concn of 9/10	Ratio <sup><i>a</i></sup> of peak intensities of $m/c$ 194/191 (±SE)	
0.57 0.95 1.14 1.52	$\begin{array}{c} 0.56 \ (0.04) \\ 0.94 \ (0.04) \\ 1.25 \ (0.05) \\ 1.60 \ (0.02) \end{array}$	

<sup>a</sup>Each value is the mean for five measurements. Regression analysis gave a correlation coefficient (r) = 0.996.

If the secondary amines 9 and 10 were the terminal metabolites in the enzymatic N-demethylation of 8, the relative concentrations of these two products would reflect the relative rates of removal of the NCD<sub>3</sub> and NCH<sub>3</sub>. However, if 9 and 10 were substrates for the microsomal enzymes, the relative concentrations of these compounds would reflect both the relative rates of removal of NCD<sub>3</sub> and NCH<sub>3</sub> and the relative rates of metabolism of 9 and 10 by microsomal enzymes. The latter process would encompass both primary and secondary isotope effects. Therefore, it was essential to establish that 9 and 10 are not substrates for the microsomal enzymes or that if they are substrates to these enzymes there will be no significant difference in the rate of metabolism of 9 and 10 due to isotope effects. This was established by the incubation of a mixture of 9 and 10 of known composition  $([CD_3]/[CH_3] = 0.640)$  with microsomal enzymes under the same conditions as used for the study of N-demethylation of 8. The composition of the mixture at the end of the incubation period was found to be identical with that before incubation  $([CD_3]/[CH_3] = 0.640)$  indicating that 9 and 10 were not substrates for the microsomal enzymes or even if they were substrates there was no measurable difference in the rates of metabolism of the two compounds. Furthermore, we used saturating concentrations of the substrate 8 in the N-demethylation studies. This was done to ensure that the concentration of the substrate 8 was much greater than the concentration of the products 9 and 10 and, hence, would minimize the potential interference of the latter as substrates to the microsomal enzymes.

Compound 8 was used as a substrate for the microsomal enzymes obtained from livers of untreated rats, rats pretreated with phenobarbital (75 mg/kg ip daily for 5 days), and mice. The 9000g supernatant from the liver homogenate was used as the source of microsomal enzymes. The incubation was carried out in the presence of saturating concentration of the substrate for 60 min. The reaction mixture was deproteinized by HClO<sub>4</sub> and the basic substrate and products were extracted in benzene. The benzene extracts were treated with acetic anhydride to form the acetamides 11 and 12 from the secondary amines 9 and 10, respectively, produced by the enzymatic demethylation of the substrate 8. The nonbasic fraction was subjected to the gc-ms assay previously described and the ratios of the intensity of the peak at m/e 194 to that at m/e 191 were determined. The ratios of 9 to 10 in the products of the enzymatic demethylation of 8 were then calculated from the linear equation obtained from Table I. The results obtained in a typical experiment are presented in Table II. The ratio of 9/10 in the product of N-demethylation of 8 by liver homogenate from phenobarbital-treated rats was  $1.39 (\pm 0.03)$ and from the untreated rats was  $1.32 (\pm 0.07)$  and the differences between the two groups were not statistically significant. The liver homogenate from the phenobarbital

treated rats N-demethylated 7.0-9.0% of the substrate while the homogenates from the untreated rats resulted in only 1.5-2.0% N-demethylation. Changing the incubation time of the substrate from 60 to 15 min did not result in any change in the ratio of 9/10 in the product. The ratio of 9/10 in the product obtained from N-demethylation of 8 by liver homogenate from mice was 1.45 (±0.16).

#### Conclusions

The ratio of 9/10 of greater than unity in the product of enzymatic N-demethylation of 8 indicates the presence of a kinetic primary isotope effect in this process. This effect is exclusive of the secondary isotope effects of deuterium on the amine nitrogen. The ratio of 9/10 in the product is equal to the  $k_{\rm H}/k_{\rm D}$  for the cleavage of the N-methyl group. The low ratio of  $k_{\rm H}/k_{\rm D}$  (1.4) is not inconsistent with a primary isotope effect since ratios of  $k_{\rm H}/k_{\rm D}$  of only slightly greater than unity have been observed in numerous studies, and Westheimer has provided a theory which explains these smaller deuterium isotope effects.<sup>7</sup> The presence of this primary isotope effect on the cleavage of the N-methyl group indicates that the cleavage of the C-H bond of the N-methyl group is a rate-limiting step in the enzymatic Ndemethylation reaction. This is consistent with the mechanism proposed by McMahon which involves direct hydroxylation on the carbon  $\alpha$  to the nitrogen to form an  $\alpha$ -carbinolamine intermediate.<sup>2</sup>

#### **Experimental Section**

Elemental analysis was performed by M-H-W Laboratories, Garden City, Mich. The ir spectra were obtained with a Perkin-Elmer 237B or Beckman IR-9 spectrophotometer. The nmr spectra were taken on a Varian A-60D in CDCl<sub>3</sub> with TMS as internal standard. Mass spectra analyses were performed on AEI MS-30. Gc-ms analyses were performed on a LKB 9000 instrument equipped with a 0.625  $\times$  180 cm glass column packed with 3% OV-1 on Chromosorb W (80-100 mesh). The rate of flow of carrier gas  $(N_2)$  was 30 ml/min and the temperature of the column oven was 150°. A 1- $\mu$ l portion of the sample to be analyzed was injected in the gas chromatograph and the mass spectrum of the eluted peak was scanned repetitively between m/e 175–225 at an electron beam energy of 70 eV in the ion source. Five scans were obtained for each peak and one additional scan immediately prior to the peak. The ratios of peak height at m/e 194 to that of m/e 191 were calculated. Regression analysis was used to determine the line of best fit for the correlation between the ratios of peak intensity  $(m/e \ 194/$ 191) and the ratio of concentration of 9/10. Calculations were performed on a Hewlett-Packard 9100A calculator.

1-(N-Methyl-N-trideuteriomethylamino)-3-phenylpropane (8). A solution of benzaldehyde (7.42 g, 0.07 mol) and 1-amino-3phenylpropane (9.46 g, 0.07 mol) in benzene (100 ml) was placed in a flask connected to a Dean-Stark distillation receiver. The mixture was heated under reflux until the distillation of water was complete (6 hr). The solution was concentrated under reduced pressure: the viscous residue was transferred into a stainless steel pressure bottle and treated with 10 g of CD<sub>3</sub>I (99.5% D, Stohler Isotope Chemicals, Inc.). The bottle was immediately closed and the mixture was heated in a steam bath for 16 hr. The tube was cooled and its contents were transferred into a beaker, treated with  $H_2O$  (150 ml), and boiled for 1 hr. The cooled reaction mixture was extracted with ether and basified with 10% NaOH and the separated base was extracted with ether. The ether extract was dried (MgSO<sub>4</sub>) and the ether was removed under reduced pressure. The residue was distilled and the major fraction [bp 83-85° (0.3 mm)] was identified as compound 7 (11.9 g, 80%) from its nmr and mass spectrum and its purity was established by glc.

Compound 7 (10.0 g, 0.066 mol) was treated with formaldehyde (20 ml of 37% solution) and formic acid (25 ml of 97%). The mixture was heated under reflux for 12 hr and then treated with concentrated HCl (15 ml). The mixture was concentrated under reduced pressure; the residue was dissolved in water, basified with 10% NaOH, and extracted with ether. The ether extract was dried (MgSO<sub>4</sub>) and concentrated under reduced pressure to provide the crude title compound. The residue was distilled and the major fraction [bp 48° (0.1 mm)] was identified as compound 8 (8.5 g, **Table II.** Isotope Composition of the Products of in Vitro

 N-Demethylation of 8 by Rodent Liver Homogenate

	%	
	metabolized	Ratio of 9/10
Phenobarbital-		
treated rats		
Rat 1	9.4	1.43
Rat 2	6.8	1.38
Rat 3	7.1	1.37
$Mean(\pm SE)$		$1.39 (\pm 0.03)^{\circ}$
Untreated rats		
Rat 1	2.0	1.36
Rat 2	1.4	1.25
Rat 3	1.4	1.37
$Mean(\pm SE)$		$1.32 (\pm 0.07)^{\circ}$
Mice		
Experiment 1 <sup>a</sup>	ь	1.34
Experiment 2		1.57

<sup>a</sup>Livers of five mice were pooled and the homogenate was used in each experiment. <sup>b</sup>Per cent metabolism was not determined. <sup>c</sup>The difference between the two groups was not statistically significant (p < 0.05).

78%) by nmr and mass spectrum and its purity was established by glc. Anal.  $(C_{11}H_{14}D_3N)$  C, N, H + D.

**Correlation Curve.** Four solutions each containing a mixture of 9 and 10 at a different ratio, a solution which contained only 9, and a solution which contained only 10 in benzene were prepared. A 5.0-ml aliquot of each solution was treated with acetic anhydride (2 ml) and heated under reflux for 3 hr. The mixture was cooled, diluted with 50 ml of benzene, extracted with 10% NaOH (2 × 10 ml), brine (2 × 15 ml), 0.1 N hydrochloric acid (3 × 20 ml), and brine (15 ml), and dried (MgSO<sub>4</sub>). The solvent was distilled *in vacuo* and the residue was dissolved in 1 ml of ether and the solution was used for the glc-ms analysis as described in the introduction to the Experimental Section.

In Vitro Studies. Adult male Sprague-Dawley rats (180-250 g) and adult male Swiss-Webster mice (20-25 g) were used in these studies. Phenobarbital-treated rats received phenobarbital-sodium ip (in water) at a dose of 75 mg/kg per day for 5 days. Animals were sacrificed by decapitation 24 hr after the last injection of phenobarbital. The livers were immediately removed and washed with ice-cold 1.15% KCl. Rat livers were weighed individually, and livers from five mice were pooled and weighed. The livers were homogenized in 3 vol of ice-cold 1.15% KCl and centrifuged at 9000g  $(0-4^{\circ})$  for 30 min, and the supernatant was used as the source of metabolizing enzymes and NADPH-generating system. The incubation mixture contained in 5 ml of 0.1 M Tris buffer (pH 7.4); 1 ml of the supernatant fraction (250 mg of liver), nicotinamide (20  $\mu$ mol), MgCl<sub>2</sub>·6H<sub>2</sub>O (10  $\mu$ mol), NADP (2.25  $\mu$ mol), glucose 6phosphate (20  $\mu$ mol), and substrate (15  $\mu$ mol). Incubations were carried out in O2 at 37° in an Eberbach water bath shaker for 60 min and the reactions were stopped by the addition of 0.5 ml of 0.6 N perchloric acid. The mixture was centrifuged and a 4-ml aliquot of the supernatant was extracted as described under Extraction Procedure

**Extraction Procedure.** The supernatant was shaken with an equal volume of benzene and the latter was discarded. The aqueous phase was adjusted to pH > 12 and extracted with benzene. The benzene extract was concentrated to a volume of approximately 5 ml and treated with acetic anhydride in exactly the same way as described under Correlation Curve.

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- Notes

# Antiparasitic Nitroimidazoles. 9. Synthesis of Some 2-(4-Dialkylaminomethylstyryl)and 2-(4-Amidinostyryl)-5-nitro-1-vinylimidazoles

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A series of 2-styryl-5-nitro-1-vinylimidazoles carrying alkylaminomethyl or amidino functions in the 4 position of the styryl ring was prepared and evaluated for antitrypanosomal activity in mice infected with Trypanosoma rho-desiense. The alkylaminomethyl compounds were found inactive against Trypanosoma cruzi infections in mice. One compound, 2-(4-methylamidinostyryl)-5-nitro-1-vinylimidazole hydrochloride, showed antitrypanosomal activity comparable to the standard drugs suramin, pentamidine, diminazene, and melarsoprol when tested ip against T. rho-desiense infected mice and also showed some activity when tested ip against T. cruzi infected mice.

In paper  $3^1$  we described the antitrypanosomal activity of 1a and various amides derived from it. These amides can be regarded as intermediates, the further elaboration of which may yield compounds with enhanced biological activity. Firstly, reduction of the amides would give rise to 4aminoalkylmethylstyrylnitroimidazoles 1b in which the polar carboxyl group of 1a has been replaced by a strongly basic group, thus changing the character of the compounds. Secondly, the conversion of the amides to amidines 1c would give tautomeric compounds which might be regarded as basic analogs of the acidic carboxyl group in 1a.

The preparation of the amidines has the added attraction that the compounds may be considered as nitroimidazole analogs of 4,4'-diamidinostilbene (stilbamidine), a compound found to be active against a number of protozoal infections.<sup>2</sup> Although simple monoamidines are generally inactive,<sup>3</sup> Sexton<sup>4</sup> has pointed out that in the diamidine series it is possible to replace one amidine residue with another type of polar function and retain antiprotozoal activity. In our case, we would regard the nitro group as the second polar function.



**Chemistry.** The presence of a nitro group and an olefinic function in our starting compounds prevented the use of conventional reducing agents such as lithium aluminium hydride or diborane for the preparation of the alkylaminomethyl compounds.

However, Borch<sup>5</sup> has recently described a procedure for

Scheme I



the reduction of amides, *via* the imino ethers, using sodium borohydride (Scheme I) and we applied this method successfully to a number of tertiary amides. We were able to prepare only one example of a secondary amine 2 (Table I) because the intermediate imino ethers were either too unstable to isolate or did not form readily.

The amidines (Table II) were prepared via the imino ether derived from the primary amide<sup>1</sup> 1d using modifications of the methods described by Barber and Slack<sup>6</sup> or Dox.<sup>7</sup> (Scheme II). The amidoxime 15 was prepared from the nitrile 1e.<sup>1</sup>

#### Scheme II



**Biological Results.** All the compounds in Table III were tested ip or po against infections of *Trypanosoma rho*desiense and *Trypanosoma cruzi* in mice according to procedures described by Hawking.<sup>8</sup> Apart from the secondary amine 2 none of the compounds approached the activity of **1a** against *T. rhodesiense* and all were inactive against *T. cruzi* at doses of up to 200 mg/kg  $\times$  5 ip. It thus appears that replacement of the carboxyl group of **1a** by a basic function reduces antitrypanosomal activity.

Compounds 10-15 (Table IV) were tested sc and po