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### Effect of deuteration of the O-CH<sub>3</sub> group on the enzymic demethylation of *o*-nitroanisole

O-Demethylation is one of the several oxidative reactions catalyzed by the liver microsomal system in the detoxication of drugs. This enzymic reaction involves the breaking of a C-O and a C-H bond, resulting in the formation of formaldehyde. If breaking of the C-H bond is rate limiting in this reaction, complete deuteration of the CH<sub>3</sub> group should substantially decrease the rate of oxidative demethylation. To test this hypothesis, [*Me*-<sup>2</sup>H]*o*-nitroanisole was prepared and its oxidation was studied in an *in vitro* system.

[*Me*-<sup>2</sup>H]*o*-Nitroanisole was prepared by the method described by VOGEL<sup>1</sup> except that the reaction was carried out in a sealed steel bomb at 80° for 84 h. [*Me*-<sup>2</sup>H]Iodomethane (minimum isotopic purity, 99 atom %, Volk Radiochemical Co.) was used as the labeled starting material. The product, [*Me*-<sup>2</sup>H]*o*-nitroanisole had a b.p. of 146°/19.4 mm as compared with a reported value<sup>2</sup> of 150.5-151°/19 mm and a deuterium content\* of 42.20 atom %, calc. 42.86 atom %.

Liver microsomes were prepared from a rabbit that was pretreated with phenobarbital (50 mg/kg, once daily) for three days. The liver was homogenized in three

TABLE I

RELATIVE RATES OF DEMETHYLATION OF UNLABELED AND [*Me*-C<sup>2</sup>H<sub>3</sub>]*o*-NITROANISOLE

The incubation mixture consisted of *o*-nitroanisole (10 μmoles); Tris buffer, pH 8.0 (500 μmoles); nicotinamide (15 μmoles); glucose 6-phosphate (20 μmoles); NADP<sup>+</sup> (0.25 μmole); NADH (5 μmoles); glucose-6-phosphate dehydrogenase (100 units) or 0.5 ml of postmicrosomal supernatant fraction and 0.3 ml of microsomes in a final volume of 3.5 ml. Incubation was carried out for 15 min at 37° in a Dubnoff shaker. Fresh *o*-nitroanisole solutions (10 μmoles/ml) were prepared from stock solutions for each experiment. Figures in parentheses refer to the number of incubations conducted in each experiment. The results are expressed as the average ± S.D.

Expt. No.	<i>o</i> -Nitrophenol formed (μmoles)		$k_H/k_{2H}$ ratio (A/B)
	Control Expt. (A)	Labeled Expt. (B)	
1	0.90 ± 0.05 (5)	0.53 ± 0.03 (4)	1.70
2	0.72 ± 0.04 (6)	0.37 ± 0.03 (6)	1.95
3	0.99 ± 0.07 (5)	0.61 ± 0.01 (6)	1.62
4A	0.84 ± 0.01 (3)	0.36 ± 0.09 (4)	2.34
5	0.89 ± 0.06 (5)	0.32 ± 0.09 (5)	2.78
6A	0.73 ± 0.07 (3)	0.49 ± 0.03 (3)	1.46
			Average 1.98
	Formaldehyde formed (μmoles)		
	Control Expt. (A)	Labeled Expt. (B)	
4B	0.71 ± 0.03 (4)	0.26 ± 0.02 (5)	2.73
6B	0.60 ± 0.00 (3)	0.31 ± 0.04 (3)	1.93
			Average 2.33

\* The deuterium combustion analysis was performed by Mr. JOSEF NEMETH of Urbana, Ill.

volumes (v/w) of 1.15 % KCl and was centrifuged at  $10000 \times g$  for 10 min. The supernatant fraction was centrifuged in a Spinco ultracentrifuge at a maximum speed using a No. 30 head for 30 min to sediment the microsomes. The postmicrosomal supernatant fraction was kept as a source of glucose-6-phosphate dehydrogenase. The microsomal fraction was washed once by recentrifuging in 1.15 % KCl and was suspended in one volume of KCl for every g of the initial liver weight.

*o*-Nitrophenol was assayed spectrophotometrically<sup>3</sup>. Formaldehyde was assayed colorimetrically after distillation<sup>4</sup>. Hexamethylenetetramine was used as a standard for formaldehyde.

As shown in Table I, substitution of deuterium for hydrogen in the methyl group resulted in approx. 50 % reduction in the rate of O-demethylation as evidenced by the ratio of the velocity constants,  $k_H/k^2_H$ , of almost 2 regardless of which product was measured.

Determination of the Michaelis constants by the LINEWEAVER-BURK plot<sup>5</sup> showed an average  $K_m^{2H}/K_m^H$  of 0.4 ( $9.6 \cdot 10^{-5} \text{ M}/2.5 \cdot 10^{-4} \text{ M}$ ) in three separate determinations indicating, if any, a stronger binding of the deuterated *o*-nitroanisole to the O-demethylating enzyme. Thus the difference in the rate of metabolism between unlabeled and deuterated *o*-nitroanisole must be due to differences in the rates of C-H and C-<sup>2</sup>H bond breaking. It is of interest that we observed no kinetic isotope effect in a somewhat analogous reaction, the oxidation of [<sup>2</sup>H<sub>3</sub>]tolbutamide to the hydroxy metabolite of tolbutamide by the microsomal system<sup>6</sup>. On the other hand, the *in vitro* rate of oxidation of [3'-<sup>2</sup>H]5-butyl-5-ethylbarbituric acid (Neonal) to 5-ethyl-5-(3'-hydroxybutyl)-barbituric acid was slower than the unlabeled Neonal and the deuterated Neonal accordingly prolonged the sleeping time of mice<sup>7</sup>. Deuterium kinetic isotope effects were also reported for the oxidation of [ $\alpha,\alpha$ -<sup>2</sup>H<sub>2</sub>]tyramine<sup>8</sup> catalyzed by monoamine oxidase and of [C<sup>2</sup>H<sub>3</sub>]morphine<sup>9</sup>, an N-demethylation reaction catalyzed by liver microsomes.

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