BBA 23329

Effect of deuteration of the *O*-CH_3 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_3 group should substantially decrease the rate of oxidative demethylation. To test this hypothesis, [*Me*-^{2}H]*o*-nitroanisole was prepared and its oxidation was studied in an *in vitro* system.

[*Me*-^{2}H]*o*-Nitroanisole was prepared by the method described by VOGEL[1] except that the reaction was carried out in a sealed steel bomb at 80° for 84 h. [*Me*-^{2}H]Iodomethane (minimum isotopic purity, 99 atom %, Volk Radiochemical Co.) was used as the labeled starting material. The product, [*Me*-^{2}H]*o*-nitroanisole had a b.p. of 146°/19.4 mm as compared with a reported value[2] 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^2H_3]*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); $NADP^+$ (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.	*o-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 × *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[3]. Formaldehyde was assayed colorimetrically after distillation[4]. 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[5] showed an average $K_m{}^{2H}/K_m{}^{H}$ of 0.4 ($9.6 \cdot 10^{-5}$ M/$2.5 \cdot 10^{-4}$ 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–^{2}H bond breaking. It is of interest that we observed no kinetic isotope effect in a somewhat analogous reaction, the oxidation of [C^2H_3]tolbutamide to the hydroxy metabolite of tolbutamide by the microsomal system[6]. On the other hand, the *in vitro* rate of oxidation of [3′-^{2}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[7]. Deuterium kinetic isotope effects were also reported for the oxidation of [α,α-$^2H_2$]tyramine[8] catalyzed by monoamine oxidase and of [C^2H_3]morphine[9], an N-demethylation reaction catalyzed by liver microsomes.

This investigation was supported in part by a U.S. Public Health Service Grant AM 06629 from the National Institute of Arthritis and Metabolic Diseases.

Departments of Biomedical Research and Pharmaceutical Chemistry,
Stanford Research Institute,
Menlo Park, Calif. (U.S.A.)

C. MITOMA
D. M. YASUDA
J. TAGG
M. TANABE

1 A. I. VOGEL, *A Textbook of Practical Organic Chemistry*, 3rd ed., John Wiley and Sons, New York, 1956, p. 670.
2 H. I. HEILBRON AND H. M. BUNBURY, *Dictionary of Organic Compounds*, Vol. 3, Oxford University Press, New York, 1953, p. 632.
3 K. J. NETTER, *Arch. Exptl. Pathol. Pharmakol.*, 238 (1960) 292.
4 M. J. BOYD AND M. A. LOGAN, *J. Biol. Chem.*, 146 (1942) 279.
5 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
6 J. TAGG, D. M. YASUDA, M. TANABE AND C. MITOMA, *Biochem. Pharmacol.*, 16 (1967) 163.
7 J. SOBOREN, D. M. YASUDA, M. TANABE AND C. MITOMA, *Federation Proc.*, 24 (1965) 427.
8 B. BELLEAU, J. BURBA, M. PINDELL AND J. REIFENSTEIN, *Science*, 133 (1961) 102.
9 C. ELISON, H. RAPOPORT, R. LAURSEN AND H. W. ELLIOTT, *Science*, 134 (1961) 1078.

Received December 9th, 1966