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ISOTOPE EFFECTS IN *O*- AND *N*-DEMETHYLATIONS MEDIATED BY RAT LIVER MICROSOMES: AN APPLICATION OF DIRECT INSERTION ELECTRON IMPACT MASS SPECTROMETRY

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SUMMARY

Isotope effects of ~2 have been found for the *O*-demethylation of *p*-nitroanisole, *p*-methoxyacetanilide, and *p*-dimethoxybenzene and the respective trideuteromethyl derivatives, when mediated by rat liver microsomes.

The direct insertion mode of electron impact mass spectrometry (the advantages and limitations of which are discussed) was used together with conventional methods (observation of formaldehyde release, product analysis by spectrophotometry) to determine the isotope effects. Only the mass spectrometry method was applicable for determining the isotope effect associated with the mono-*O*-demethylation of *p*-trideuteromethoxyanisole and an unusually large value (10) was found.

An insignificant isotope effect (≯ 1.05) was found for the mono-*N*-demethylation of 1-(*o*-carbamoylphenyl)-3,3-dimethyltriazene and its di-(trideuteromethyl) analogue. The protium and deuterium forms had closely similar growth-inhibitory activities for the TLX5 lymphoma in mice.

INTRODUCTION

Drugs may be activated or deactivated by metabolism and one or more metabolites may have activity comparable to that of the initial drug[1]. Oxygenases are frequently involved in the initial metabolism of drugs especially in the liver. Although there are several oxygenase systems[2] each apparently activates molecular oxygen and

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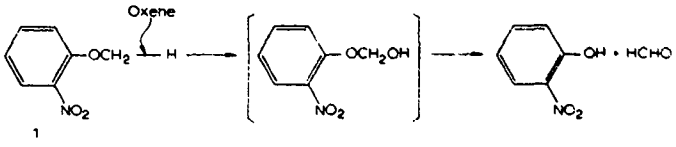
Since this paper was submitted, the use of direct-insertion chemical-ionisation mass spectrometry coupled with stable isotope dilution for the quantitation of quinidine and lidocaine in human plasma has been described (W. A. GARLAND, W. F. TRAJER AND S. D. NELSON, *Biomed. Mass Spectrom.*, 1 (1974) 124–129.

Abbreviations: AR, Analar, DMSO, dimethylsulphoxide; EI, electron impact.

releases "oxene" (the formal equivalent of atomic oxygen)[3] in response to the presence of an appropriate nucleophilic centre in the molecule (drug, carcinogen, or other exogenous chemical) adsorbed by the oxygenase complex. Oxene is isoelectronic with carbenes with which its reactions appear to be analogous[3] in relation to insertion into σ-bonds (especially C–H and N–H to give the corresponding hydroxy derivatives) or addition to π-bonds (especially aromatic C=C bonds to give epoxides). A third type of reaction[4] involves lone pairs of electrons as in the conversion of tertiary amines into *N*-oxides.

If oxene inserts directly into an R–H bond to give R–OH then an isotope effect, with a consequent slower reaction rate, should occur for the corresponding R–D compound. Most of the isotope effects* reported to date involve the conversion C–H(D) → C–OH and a magnitude of ~2, as, for example, in the demethylation of *o*-nitroanisole[5] *(1)*. Recently, however, a value of 7 has been reported for the oxidative metabolism of cotinine[6] *(2)* and we have encountered a value of 10 for the demethylation of *p*-trideuteromethoxyanisole (see below). The maximum theoretical[7] isotope effect is 18.



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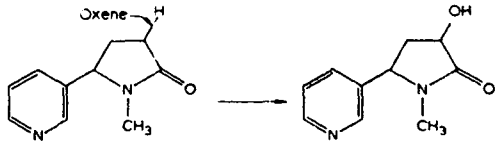

2 (Cotinine)

Significant changes in pharmacological activity are associated with isotope effects of ~2 in, for example, *N*-trideuteronormorphine[8] [the effect on the pharmacological activity of an OCD_3 group in codeine (which is activated by *O*-demethylation[9]) apparently has not been investigated], 3′-deuterobutethal[10], and 3′,3′-dideuteropentobarbital[11]. An isotope effect of 1.9 was observed for the oxidative deamination of (+)-2-deuteroamphetamine[12] (see also VREE *et al.*[13]).

An understanding of the structural features of organic compounds which control the magnitude of isotope effects might allow more rational consideration of the modification of drug activity by deuteration at the site of metabolic attack.

We now report on a series of microsomally mediated *O*- and *N*-demethylation reactions selected in connexion with a preliminary evaluation of the direct insertion mode of EI mass spectrometry for the determination of isotope effects since this mode has potential advantages (see DISCUSSION). G.l.c.-mass spectrometry has been used

* Throughout this paper the isotope effect refers to the hydrogen-deuterium system.

to study isotope effects as, for example, reflected by the urinary excretion of (+)-amphetamine, (+)-*N*-isopropylamphetamine and their deuterated analogues administered to man[13].

The isotope effect involved in the demethylation of *o*-nitroanisole by rabbit liver microsomes[5] was determined by UV absorption spectroscopy and by monitoring the release of formaldehyde; oxene insertion into a C–H bond of the OMe group *(1)* affords an unstable hemiacetal of formaldehyde which decomposes into formaldehyde and *o*-nitrophenol. The former method can be used only when there is a significant difference in the UV spectra of the starting material and product and both methods require that the metabolism of the compound and its deuterated analogue be carried out separately.

Mass spectrometry not only allows the metabolism of microgram amounts of a drug and its deuterated analogue (separately or in admixture) to be studied but is also potentially applicable to a wide variety of metabolic reactions. The application of mass spectrometry in the determination of isotope effects is limited to compounds which give molecular ions or appropriate fragment ions of reasonable intensity. With the advent of field ionization[14], chemical ionization[15], and field desorption[16] mass spectrometry molecular ions can be obtained for a much wider range of compounds than is possible by the use of the EI method and the scope of the technique for determining isotope effects has been greatly increased.

MATERIALS AND METHODS

Preparation of rat liver microsomes

Microsomes were isolated from the livers of male Wistar rats (6 weeks old, fed *ad libitum*) for which the drinking water contained 0.1% phenobarbitone for at least three days prior to killing. The microsomes were prepared routinely by the calcium aggregation method[17]. The washed microsomal fraction was suspended in 0.1 *M* Tris–HCl buffer (pH 7.5) so that microsomes isolated from 1 g of liver were contained in a final volume of 1 ml. Such preparations were used immediately and found to be as active in their demethylating ability as a microsomal fraction prepared by more conventional means[18]. This observation has been confirmed by CINTI *et al.*[19] and BAKER *et al.*[20] for rat and mouse liver microsomes, respectively.

Analytical methods

Formaldehyde was determined by the procedure of NASH[21] and *p*-nitrophenol by the method of MCMAHON *et al.*[22].

Mass spectrometric analysis of samples was carried out by the direct insertion technique using an AEI MS-12 spectrometer, an ionizing voltage of 70 eV, a trap current of 100 μA, and an ion-source temperature of 60–100°. For the determination of isotope ratios the chloroform solutions containing the products of metabolism and/or starting compounds with or without added reference standard (see RESULTS) were concentrated onto the direct insertion probe and each mass spectrum was scanned over a limited mass range which contained the peaks of the appropriate

protium- and deuterium-containing molecular ions. Concordant ratios for each pair of peak heights (*i.e.* a variation of not greater than ± 1% from the mean value obtained from 5 scans) were obtained at a scan speed of 34 sec/decade in mass with a resolving power of 1000 providing that the total ion current monitor reading was first allowed to reach a steady value (*cf.* ref. 23).

Synthesis of deuteromethyl ethers

Light petroleum refers to the fraction b.p. 40–60°. Acetone was AR grade and was dried over potassium carbonate before use. Melting points were determined on a Kofler block and are corrected. The homogeneity of products was routinely monitored by t.l.c. using Kieselgel (7731, Merck) and conventional detection with sulphuric acid or Kieselgel GF_{254} (7730, Merck) and detection with UV light at 254 nm (Hanovia Chromatolite); R_F values for individual compounds were similar for both adsorbents.

p-Trideuteromethoxyacetanilide. Sodium hydride (240 mg) was added to a cooled (0°) solution of *p*-acetamidophenol (1.51 g) in *N,N*-dimethylformamide (10 ml) and the mixture was stirred for 20 min before the addition of trideuteromethyl iodide (2.84 g). After further stirring and cooling for 30 min the solution was allowed to attain room temperature and then left for 24 h. Methanol was added and the mixture was concentrated to dryness to give the title compound (256 mg, 16%), m.p. 127–129° (from ethanol–light petroleum). Mass spectral data: *m/e* 168 ($M^{+\cdot}$, 70%) 126 ($[M-CH_2CO]^{+\cdot}$, 68%), and 108 ($[126-CD_3]^+$, 100%).

p-Methoxyacetanilide, prepared by a similar procedure had m.p. 127–128°. Mass spectral data: *m/e* 165 ($M^{+\cdot}$, 80%), 123 ($[M-CH_2CO]^{+\cdot}$, 80%), and 108 ($[123-CH_3]^+$, 100%).

p-Trideuteromethoxynitrobenzene. A mixture of *p*-nitrophenol (500 mg), silver oxide (2 g), *N,N*-dimethylformamide (4 ml), and trideuteromethyl iodide (0.75 ml) was stirred with the exclusion of light, for 1 h at 0° and then for 24 h at room temperature. The mixture was filtered and concentrated to a small volume. Chloroform was added and the solution was washed twice with saturated aqueous sodium bicarbonate, dried ($MgSO_4$), and concentrated to give the title compound m.p. 52–53° (from ethanol–light petroleum). Mass spectral data: *m/e* 156 ($M^{+\cdot}$, 100%), 126 ($[M-NO]^{+\cdot}$, 18%), and 92 ($[126-CD_3O]^+$, 40%).

p-Methoxynitrobenzene (*p*-nitroanisole), prepared by a similar procedure, had m.p. 52–54°. Mass spectral data: *m/e* 153 ($M^{+\cdot}$, 100%), 123 ($[M-NO]^{+\cdot}$, 30%), and 92 ($[123-CH_3O]^+$, 40%).

p-(Trideuteromethoxy)anisole. A mixture of *p*-methoxyphenol (250 mg), trideuteromethyl iodide (0.2 ml), potassium carbonate (0.3 g), and acetone (3 ml) was stirred and boiled under reflux for 4.75 h. T.l.c. (ether–light petroleum, 1 : 1) then showed a strong spot due to the title compound (R_F 0.65) and a weaker spot due to starting material (R_F 0.45). After the addition of more trideuteromethyl iodide (0.1 ml) the mixture was boiled under reflux for 3 h, then cooled, and concentrated to dryness. A solution of the residue in benzene was twice extracted with *M* potassium hydroxide, washed thrice with water, filtered, and concentrated. T.l.c. of the residue

showed only the component having R_F 0.65. The residue was recrystallized from aqueous ethanol to give *p*-trideuteromethoxyanisole (108 mg), m.p. 55–56°. A second crop (45 mg) was obtained from the filtrate. Mass spectral data: *m/e* 141 ($M^{+\cdot}$, 100%), 126 ($[M-CH_3]^+$, 50%), and 123 ($[M-CD_3]^+$, 50%); there was virtually no peak at *m/e* 138.

By using a similar procedure *p*-dimethoxybenzene, m.p. 55–56°, was obtained.

p-Di-(trideuteromethoxy)benzene. A mixture of hydroquinone (0.2 g), trideuteromethyl iodide (0.4 ml), potassium carbonate (0.25 g), and acetone (3 ml) was stirred and boiled under reflux for 6 h. The title compound, isolated using the above procedure, had m.p. 55–56°. Mass spectral data: *m/e* 144 ($M^{+\cdot}$, base peak); there were virtually no peaks at *m/e* 141 and 138.

p-Trideuteromethoxyphenol. A mixture of hydroquinone (1 g), trideuteromethyl iodide (0.2 ml), potassium carbonate (0.3 g), and acetone (3 ml) was stirred and boiled under reflux for 5 h. The residue left on evaporation of the solvent was partitioned between water (20 ml) and benzene (20 ml). The benzene layer was washed with water (5 ml) and the combined aqueous solutions were extracted with ethyl acetate (25 ml). The extract was washed with two small volumes of water, then added to the benzene solution and the mixture was concentrated. A solution obtained by treatment of the residue with ether (2 ml) and light petroleum (6 ml) was added to a column (height 23 cm) of Kieselgel (40 g, Merck, 7734, packed in ether–light petroleum, 1 : 4) followed by a second solution obtained by retreatment of the residue with ether (2 ml) and light petroleum (6 ml). The column was eluted with ethyl ether–light petroleum (1 : 4) and the fractionation was monitored by t.l.c. Combination and concentration of the appropriate (18–24) 10 ml fractions gave the title compound (180 mg) m.p. 54.5–55.5° (from benzene–light petroleum). Mass spectral data: *m/e* 127 ($M^{+\cdot}$, 100%), and 109 ($[M-CD_3]^+$, 90%); there was virtually no peak at *m/e* 124.

p-Methoxyphenol has m.p. 55–56°. Mass spectral data: *m/e* 124 ($M^{+\cdot}$, 100%) and 109 ($[M-CH_3]^+$, 90%).

1-(o-Carboxyphenyl)-3,3-di-(trideuteromethyl)triazene

o-Carboxybenzenediazonium tetrafluoroborate[24] (2.69 g) was added in small portions of finely ground solid, to a well stirred solution of di-(trideuteromethyl)-ammonium chloride (1 g) and triethylamine (5.5 ml) in distilled water (10 ml) and ethanol (5 ml) maintained at 0°. The mixture was stirred for 1 h, then acidified with acetic acid, and the resulting solid was extracted with ethyl acetate. The dried (Na_2SO_4) extract was concentrated and the residue was recrystallized from ethyl acetate after treatment with charcoal to give the title compound (1.25 g) as colourless needles, m.p. 127°. (Found: C, 54.23; N, 21.00%. $C_9H_5D_6N_3O$, requires C, 54.27; N, 21.10%.)

1-(o-Carbamoylphenyl)-3,3-di-(trideuteromethyl)triazene

A solution of but-2-yl chloroformate (1.3 ml) in tetrahydrofuran (25 ml) was added dropwise to a well stirred solution of 1-(*o*-carboxyphenyl)-3,3-di-(trideutero-

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