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THE DEALKYLATION OF SOME *p*-NITROPHENYLALKYLETHERS AND THEIR α-DEUTERATED ANALOGUES BY RAT LIVER MICROSOMES

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Abstract—The cytochrome P-450 mediated *O*-dealkylation of four *p*-nitrophenylalkylethers and two α-deuterated *p*-nitrophenylalkylethers has been studied in rat liver microsomes. All the compounds showed a typical type I binding spectrum. A strong correlation was found between the log of the binding affinities (log K_s) and the log of the partition coefficients (log *P*). The initial dealkylation rates were in the order *p*-nitrophenylisopropylether > *p*-nitrophenylbutylether > *p*-nitrophenetole (*p*-nitrophenylethylether) > *p*-nitroanisole (*p*-nitrophenylmethylether). The rate of the dealkylation of the deuterated compounds was slower than that of their unlabelled analogues. The velocities decrease in the order *p*-nitrophenetole > *p*-nitroanisole > [α-D_2]*p*-nitrophenetole > [α-D_3]*p*-nitroanisole. The reduced rate of metabolism of the two deuterated analogues does not seem to be due to a decreased rate of cytochrome P-450 reduction or an impairment of substrate binding. Rather the breakage of the carbon–deuterium bond is the rate limiting step in the reaction sequence. A free radical mechanism is proposed to explain this phenomenon.

It is generally accepted that alkylarylethers are cleaved via hemiacetal intermediates into phenols and aldehydes by the mixed function oxidase enzymes of the hepatic endoplasmic reticulum. The oxidation of a wide variety of alkylarylethers has been reported both *in vivo* [1] and *in vitro* [2]. McMahon *et al.* [3] showed that in the case of the *in vitro* dealkylation of a series of alkyl-*p*-nitrophenylethers in rats, the rate of cleavage of longer chain alkyl groups such as butyl and hexyl substituents was considerably less rapid than that for the shorter chain alkyl groups. Mitoma *et al.* [4] have demonstrated that substitution of hydrogen atoms by deuterium atoms in [3-^{2}H]propyl-*p*-nitrophenylether resulted in a selective inhibition of *p*-nitrophenol formation, and a similar effect was also reported for the *O*-demethylation of *o*-nitroanisole and its α-deuterated isomer [5].

Previous spectral observations [6] suggested that the reactive area of cytochrome P-450 is in contact with, or buried within a highly hydrophobic part of the cytochrome protein or of the lipids of the microsomal membrane. This would reflect a possible relationship between binding affinities and partition coefficients of the type I binding compounds.

In the present study, the initial dealkylation rates of *p*-nitroanisole, *p*-nitrophenetole, their deuterated analogues, *p*-nitrophenylisopropylether, and *p*-nitrophenylbutylether were examined. The binding spectra and the K_s values, as well as the partition coefficients, were determined for these compounds.

These studies were designed in an attempt to elucidate the rate limiting step of the dealkylation reaction, whether it be the binding of substrate, reduction of cytochrome P-450, or a hereto unconsidered factor.

MATERIALS AND METHODS

Chemicals. NADP was purchased from P-L Biochemicals Inc. (Agents International Enzymes Ltd.), NADPH was purchased from B.D.H. Chemicals Ltd., Poole, glucose-6-phosphate from International Enzymes Ltd., and glucose-6-phosphate dehydrogenase from Boehringer Corporation. CH_3CD_2I was purchased from Stohler Isotope Chemicals, Switzerland. All other compounds were at least of reagent grade. *p*-Nitroanisole was recrystallized before use. α-Deuterated-*p*-nitroanisole was the generous gift of Dr. F. J. Wolf, Merck, Sharp & Dohme (New Jersey, U.S.A.). *p*-Nitrophenetole was synthesized by the general method of Branch and Jones [7] except that the oily product was twice recrystallized from ethanol, m.p. 55–57°. α-Deutero *p*-nitrophenetole was prepared using the method for *p*-nitrophenetole except that CH_3CD_2I was used as alkylating agent. *p*-Nitrophenylisopropylether and *p*-nitrophenylbutylether were prepared as previously described [8].

Elemental analysis for the *p*-nitrophenylisopropylether was carried out (Found: C = 59·25, H = 6·11, N = 8·15; calculated: C = 59·57, H = 6·08, N = 7·83). The infrared spectrum of all the compounds was used to confirm purity of each compound using a Perkin-Elmer 157G Grating Infrared Spectrophotometer. In addition, the n.m.r. spectrum was measured for *p*-nitrophenetole and its deuterated analogue. (n.m.r. spectrum of *p*-nitrophenetole shows a quadrate at δ 4·13 ppm and triplet at δ 1·46 ppm. The quadrate in the n.m.r. disappeared when deuterated *p*-nitrophenetole was used, and a singlet at δ 1·46 ppm was found).

Tissue preparation. Hepatic microsomes were obtained from male Wister albino rats (140–180 g) pretreated with sodium phenobarbitone for 3 days (100 mg/kg body wt, i.p. once per day) and killed by cervical fracture 24 hr after the last injection. Livers were immediately removed and immersed in ice-cold 1·15% potassium chloride solution followed by homogenization in 0·25 M sucrose. Washed microsomal fractions were then obtained by differential centrifugation [9]. The final microsomal suspension was diluted with 0·07 M phosphate buffer pH 7·8 to contain 5 mg/ml of protein. The protein content of the microsomal suspension was determined by the method of Lowry *et*

al. [10], and cytochrome P-450 content using the method of Ullrich [11].

Determination of dealkylation rate. The incubation mixture contained glucose-6-phosphate (2·65 mM), glucose-6-phosphate dehydrogenase (2 units), nicotinamide (0·4 mM), *p*-nitrophenylalkylether (0–50 mM), microsomal suspension (0·2 ml) and 0·07 M phosphate buffer pH 7·8 to produce a final volume of 2 ml in the cuvette. The reaction was started by the addition of NADP (0·2 mM) and the initial rate of *p*-nitrophenol formation was measured spectrophotometrically in the dual beam mode with wavelength setting at 420 nm, using 490 nm as a reference on a Perkin–Elmer 356 spectrophotometer.

NADPH–cytochrome P-450 reductase. A Perkin–Elmer 356 spectrophotometer in the dual wavelength collimated beam mode was used. The sample and reference beams were set at 450 and 490 nm, respectively. The initial rate of anaerobic reduction of cytochrome P-450 by NADPH was determined in the presence of carbon monoxide by measuring the increase in absorbance at 450 nm with 490 nm as a reference as described by Gigon *et al.* [12], except that NADPH was used rather than a NADPH-generating system.

Apparent spectral changes. The difference spectra produced by the compounds studied were determined as described by Schenkman *et al.* [13] using a Perkin–Elmer 356 Spectrophotometer in the split beam mode. The spectral dissociation constant (K_s) was determined by using double reciprocal Lineweaver–Burk plot of the absorption differences of spectral changes against concentration of the drug [13]. The absorbance (ΔA) was measured between 420 nm trough and the baseline only, because the intrinsic light absorption of the *p*-nitrophenylethers at lower wavelengths prevented measurement of the peak.

Partition coefficients. The apparent partition coefficient of each compound was determined between *n*-octanol and 0·1 M phosphate buffer pH 7·4. After 2 hr agitation on a rotary shaker, the concentrations of the compound in both phases were assayed spectrophotometrically. The wavelengths chosen were those of the absorption maxima in respective solvents. The values obtained were found to be in good agreement with the theoretical values derived by the method of Leo *et al.* [14], except for the deuterated compounds where no theoretical values are available.

RESULTS

Spectral interaction of *p*-nitrophenyl alkyl ethers with hepatic microsomes indicate a type 1 spectral change. A decrease in the K_s values was found with the increase in the number of carbon atoms in the straight aliphatic chain of the ethers studied, but there was no significant change in their A_{max} values (Table 1). *p*-Nitroanisole and *p*-nitrophenetole showed similar K_s values to their deuterated analogues, and similarly, there was no change in the A_{max} values.

The lipophilicity is unchanged by deuteration (Figs. 1 and 2) and a good correlation is observed between the lipophilicity of the straight chain alkyl-*p*-nitrophenylethers (including the deuterated compounds) and their K_s values (Fig. 1). Equation (1) was found to hold for the log K_s vs log P (log partition coefficient) plot with five of the alkyl-*p*-nitrophenylethers, but not

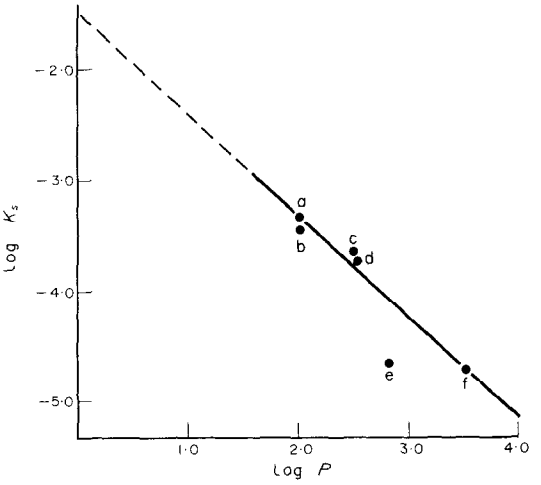

Fig. 1. The relationship between log K_s and log P of *p*-nitrophenylalkylethers. The binding spectra were determined by varying the amount of the drug added to the sample cuvette, while an equal quantity (in μl) of the solvent (ethanol) was added to the reference cuvette in each case. The suspension contained 2 mg/ml protein from phenobarbitone pretreated rats in 0·1 M phosphate buffer pH 7·4. The partition coefficients were determined between *n*-octanol (saturated with phosphate buffer) and 0·1 M phosphate buffer (saturated with octanol) in duplicate. Ten ml of a 5–20 mM substrate solution in *n*-octanol were mixed with an equal quantity of phosphate buffer. The partition coefficient was expressed as the ratio between concentrations of the drug in the organic and aqueous layers (as determined spectrophotometrically). Key: a = p-nitroanisole; b = [α-D_3]*p*-nitroanisole; c = *p*-nitrophenetole; d = [α-D_2]*p*-nitrophenetole; e = *p*-nitrophenylisopropylether; f = *p*-nitrophenylbutylether.

Table 1. Spectrally apparent interactions of various *p*-nitrophenylalkylethers

Compound	$K_s \times 10^{-5}$ M*	A_{max}* ($A \times 10^{-3}$/2 mg)
p-Nitroanisole	42·0 ± 2·0	8 ± 1·0
[α-D_3]*p*-Nitroanisole	39·0 ± 3·0†	8 ± 1·0†
p-Nitrophenetole	20·0 ± 1·0‡	6 ± 1·0†
[α-D_2]*p*-Nitrophenetole	17·0 ± 1·0†	6 ± 0·5†
p-Nitrophenylisopropylether	2·2 ± 0·2§	6 ± 1·0†
p-Nitrophenylbutylether	1·6 ± 0·15§	11 ± 1·0†

Data were derived from Lineweaver–Burk plots, using a microsomal protein concentration of 2 mg/ml.

* Values are mean ± S.E.M. of 3 determinations.

Student's *t*-test was applied using *p*-nitroanisole as a reference, except for [α-D_2]*p*-nitrophenetole, where *p*-nitrophenetole was used as a reference; † non-significant, ‡ $P < 0{\cdot}01$, § $P < 0{\cdot}001$.

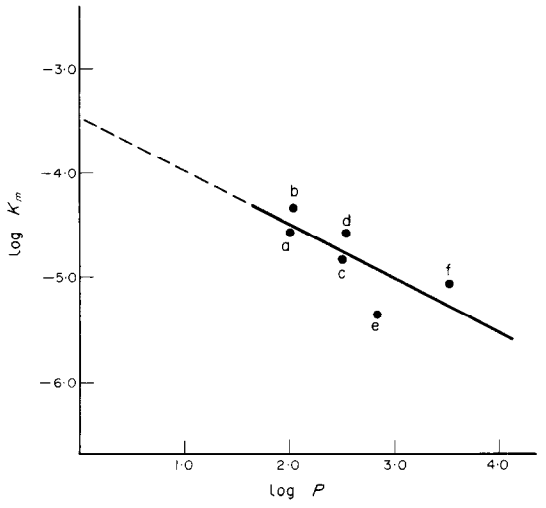

Fig. 2. The relationship between log K_m and log P for the *p*-nitrophenylalkylethers. The initial dealkylation rate was determined as described in the text, using 0·5 mg/ml microsomal protein in 0·07 M phosphate buffer pH 7·8. K_m was determined by a double reciprocal plot of the rate against the substrate concentration. Partition coefficients were determined as in Fig. 1. Key: a = *p*-nitroanisole; *b* = [α-D_3]*p*-nitroanisole; c = *p*-nitrophenetole; d = [α-D_2]*p*-nitrophenetole; e = *p*-nitrophenylisopropylether; f = *p*-nitrophenylbutylether.

with the branched chain compound *p*-nitrophenylisopropylether.

$$\log K_s = -0{\cdot}91124 \log P + 1{\cdot}48663 \quad (1)$$
$$n = 5 \quad r = 0{\cdot}994 \quad \text{standard deviation} = 0{\cdot}0235$$

Equation (2) takes into account all six compounds tested and therefore can be used to describe the effect of *p*-nitrophenylisopropylether on the correlation coefficient.

$$\log K_s = -0{\cdot}99592 \log P + 1{\cdot}35732 \quad (2)$$
$$n = 6 \quad r = 0{\cdot}929$$

The V_{max} and K_m values for the dealkylation of alkyl-*p*-nitrophenylethers are presented in Table 2. The initial velocity of the dealkylation of *p*-nitrophenetole (3 μM) was twice that of *p*-nitroanisole, whilst the increases over *p*-nitroanisole for the dealkylation of *p*-nitrophenylisopropylether and *p*-nitrophenylbutylether were 3 and 2·5 times, respectively. An increase in V_{max} was found with the increase in the number of the carbon atoms of the *p*-nitrophenylalkylethers, but interestingly, the highest V_{max} value and the fastest dealkylation rate were found with the branched chain compound *p*-nitrophenylisopropylether. K_m values decrease with the increase of the number of carbon atoms of the ethers studied, with the exception of *p*-nitrophenylisopropylether which showed a smaller K_m value than the *p*-nitrophenylbutylether.

The initial dealkylation velocity of deuterated *p*-nitroanisole (3 μM) and deuterated *p*-nitrophenetole (3 μM) were found to be slower than their non-deuterated analogues. The V_{max} results for the dealkylation of the unlabelled compounds were found to be 2–3 times greater than those of their deuterated analogues. The K_m values of the deuterated compounds were greater than those for the corresponding unlabelled compounds.

The K_m values for dealkylation were generally one order of magnitude lower than the corresponding K_s values. It is also to be noted that the A_{max} values for the six ethers studied are similar, whereas V_{max} values are not (Tables 1 and 2).

Poor correlation was noticed between log K_m and log P (Fig. 2). The slope of this relationship differs from that for log K_s vs log P.

Equation (3) was found to hold for the compounds studied:

$$\log K_m = -0{\cdot}513 \log P + 3{\cdot}449 \quad (3)$$
$$n = 6 \quad r = 0{\cdot}788$$

All the compounds studied showed similar stimulation of NADPH cytochrome P-450 reductase when employed in a concentration what was approximately the K_m concentration of dealkylation. Typically, an increase of about 19 per cent was found for each substate against controls (13·25 units for the control microsomes to 15·8 units with substrate).

No significant differences between the stimulation produced by various substrates were observed.

DISCUSSION

All the various alkyl-*p*-nitrophenylethers studied interact with the hepatic microsomes to produce a type I spectral change. The K_s values calculated for the interaction of the straight chain compounds give an excellent correlation with their partition coefficients. The slope of the plot of log K_s vs log P is in good agreement with that for the binding of aliphatic carbamates, aromatic hydrocarbons and cyclohexane derivatives to cytochrome P-450 [15]. *p*-Nitrophenylisopropylether did not fit the relationship between log K_s

Table 2. Dealkylation rates of various *p*-nitrophenylalkylethers

Compound	$K_m \times 10^{-6}$ M‡	V_{max}* ($A \times 10^{-3}$/0·5 mg/min)
p-Nitroanisole	30·0 ± 4·0	16 ± 2
[α-D_3]*p*-Nitroanisole	46·0 ± 5·0†	6 ± 1§
p-Nitrophenetole	15·0 ± 2·0†	33 ± 3§
[α-D_2]*p*-Nitrophenetole	26·0 ± 3·0†	11 ± 2‡
p-Nitrophenylisopropylether	5·0 ± 0·7‡	50 ± 4‡
p-Nitrophenylbutylether	8·3 ± 1·3‡	43 ± 6§

Data were derived from Lineweaver–Burk plots, using phenobarbitone pretreated rat liver microsomes.

* Values are mean ± S.E.M.

A Student's *t*-test was applied using *p*-nitroanisole as a reference, except [α-D_2]*p*-nitrophenetole, where *p*-nitrophenetole was used as a reference. † $P < 0{\cdot}05$, ‡ $P < 0{\cdot}01$, § $P < 0{\cdot}02$.

and log P observed for the straight chain compounds, presumably indicating the contribution of a steric factor to binding. A similar observation has been made for the binding of branched chain aliphatic carbamates. No significant differences either in the K_s values or in the partition coefficients could be detected between the deuterated compounds and their unlabelled analogues.

The rate of dealkylation of the ethers studied increased as the number of carbon atoms in the straight chain increased. The highest rate, however, was found with the branched-chain p-nitrophenylisopropylether. Although it ranks in the expected position of the log P-scale (Figs. 1 and 2), it shows a lower K_s than would seem appropriate for its straight-chain analogues. The same holds true for the K_m plot. This unusual behaviour might explain the enhanced reaction rate. Presumably, the enhanced dealkylation rate for the straight chain compounds with increasing chain length is largely a reflection of the higher substrate binding to cytochrome P-450 with increasing chain length.

Our findings are in apparent conflict with those of McMahon *et al.* [3], who reported a decreased dealkylation rate with increasing size of the alkoxy group after a 30-min incubation. However, since in our experiments only the initial kinetics of dealkylation were used to determine the kinetic constants, McMahon's results cannot be compared directly with our own.

Obviously we are not measuring all the metabolites [4], but if the formation of other metabolites has an influence on the results via competition for an active intermediate, it would tend to reduce rather than enhance α-hydroxylation and therefore result in an underevaluation of dealkylation rate for the dealkylation of the longer chain compounds.

The microsomal oxidative demethylation of amines has been suggested to occur by a free radical mechanism via the displacement of an α-hydrogen atom [16]. A free radical involvement also appears to be likely for the dealkylation of the p-nitrophenylethers (see diagram). The stability of free radical intermediates will generally increase where several resonance possibilities exist and should lead to enhance carbon–hydrogen bond breakage. Assuming that a single mechanism is concerned in the dealkylation of the p-nitrophenylalkylethers studied, p-nitrophenylisopropylether might be expected to yield a more stable radical (structure b, see diagram) compared with the straight chain compounds and this may explain why it is dealkylated more rapidly.

Since binding to cytochrome P-450, steric hindrance, and effects on cytochrome P-450 reductase activity appear not to be the determinants of the slower rate of dealkylation of the deuterated compounds compared with their hydrogen-substituted analogues, it is probably that the rate-limiting step for dealkylation is the displacement of the hydrogen or deuterium atom from the α-carbon atom. (Stage 1, see diagram). This view is supported by the observation that the energy required to break a carbon–deuterium bond via a free radical mechanism is greater than that needed to cleave a carbon–hydrogen bond; this is because a deuterium–carbon bond has a lower ground state energy [17]. It is interesting to note that the regulation of mixed function oxidation activity by the reduction of cytochrome P-450 has also been questioned by Archakov *et al.* [18], who found that the rate of oxidation of aniline and aminoantipyrine derivatives is not dependent either on the rates of reduction reactions of cytochrome P-450, or on the binding of the substrate to the cytochrome.

The influence of deuterium on the dealkylation rates has important implications for the assay of oxidation enzymes based on tritium or deuterium displacement. The rate of metabolism of such substituted compounds may be considerably less than that of the hydrogen-containing substrate to which it is intended to extrapolate.

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REFERENCES

1. R. T. Williams, *Detoxication Mechanisms*, p. 324. John Wiley, New York (1959).
2. J. Axelrod, *Biochem. J.* **63,** 634 (1956).
3. R. E. McMahon, H. W. Culp, J. Mills and F. J. Marshall, *J. med. Chem.* **6,** 343 (1963).
4. C. Mitoma, R. L. Dehn and M. Tanabe, *Biochem. biophys. Acta.* **237,** 21 (1971).
5. C. Mitoma, D. M. Yasuda, T. Tagg and M. Tanabe, *Biochem. biophys. Acta* **136,** 566 (1967).
6. Y. Imai and R. Sato, *J. Biochem.* **62,** 239 (1967).
7. S. J. Branch and B. Jones, *J. Chem. Soc.* 2921 (1955).
8. C. F. H. Allen and J. W. Gate, *Organic Synth., Coll. Vol.* **3,** 140 (1955).
9. H. Remmer, J. B. Schenkman, R. W. Estabrook, H. Sasame, J. R. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Molec. Pharmac.* **2,** 187 (1966).
10. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
11. V. Ullrich, *Hoppe-Seyler's Z. physiol. Chem.* **350,** 357 (1969).
12. P. L. Gigon, T. E. Gram and J. R. Gillette, *Molec. Pharmac.* **5,** 109 (1969).
13. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3,** 113 (1967).
14. A. Leo, C. Hansch and D. Elkins, *Accounts Chem. Res.* **71,** 525 (1971).
15. K. A. S. Al-Gailany, J. B. Houston and J. W. Bridges, in press.
16. C. Hansch and R. Kerley, *J. med. Chem.* **13,** 957 (1970).
17. J. March, in *Advanced Organic Chemistry*, p. 153 McGraw-Hill, New York (1968).
18. A. I. Archakov, I. I. Karuzina, V. N. Tveritinov and I. S. Kokareva, *Biochem. Pharmac.* **23,** 1053 (1974).