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METABOLISM OF DRUGS—LVII

ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF *p*-NITROPHENOXY ACETIC ACID, A METABOLITE OF BUTYL *p*-NITROPHENYL ETHER IN RATS, MICE AND GUINEA PIGS

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Abstract—The metabolism of butyl *p*-nitrophenyl ether *in vivo* in rats, mice, and guinea pigs was investigated and compared with that in rabbits. In the previous paper of this series, it was found that in rabbits this substance was metabolized mainly to (+)-3-hydroxybutyl *p*-nitrophenyl ether through the penultimate hydroxylation on its butyl chain, although the *O*-dealkylation reaction, the common metabolic pathway of alkylaryl ethers, occurred to a lesser extent.

It was shown in the present study that, in addition to the two metabolites described above, rats and mice excreted a considerable amount of a new metabolite, *p*-nitrophenoxy acetic acid, which was produced probably by sucessive ω- and β-oxidation on the butyl group. Rabbits and guinea pigs, however, showed a remarkable species difference in the metabolism, excreting only a little of such carboxylic acid.

THE PENULTIMATE hydroxylation, together with ω-oxidation, is one of the well established metabolic pathways of many drugs possessing a C-alkyl side chain.[1] In the previous paper of this series, it was found that in rabbits this reaction also played an important role in the metabolism of aryl ethers of longer alkyl groups, e.g. butyl or isoamyl, whereas the *O*-dealkylation reaction, the common metabolic reaction of alkylaryl ethers, played only a minor role.[2] On the other hand, in the metabolism of propylaryl ethers a pathway through ω-oxidation was also important as well as the dealkylation and penultimate hydroxylation pathways.[2]

The present investigation was undertaken to determine whether such (ω-1)-or ω-oxidation of *O*-alkyl compounds takes place in mammalian species other than rabbits, using butyl *p*-nitrophenyl ether.

It will be shown in this paper that rats and mice, unlike rabbits and guinea pigs, metabolize a considerable part of the given ether to *p*-nitrophenoxy acetic acid by successive ω- and β-oxidation of the butyl group. As in rabbits, this ether also undergoes dealkylation and penultimate oxidation in the other three species.

METHODS

Materials

Butyl *p*-nitrophenyl ether, m.p. 32°, was synthesized by the method described in the previous paper.[2] *p*-Nitrophenol, m.p. 114°, was obtained from a commercial source and (+)-3-hydroxybutyl *p*-nitrophenyl ether, m.p. 93°, $[\alpha]^{22} + 29°$ (C = 1·0 in $CHCl_3$), from the previous study.[2]

The animals used were rats (Wistar–King), mice (CF no. 1), guinea pigs, and rabbits. Rats of both sexes were used, but only male animals of the other species.

Administration of butyl p*-nitrophenyl ether*

The compound was administered orally in a dose of about 300 mg/kg body wt. as a suspension in an appropriate volume of 10% gum arabic.

Extraction of the metabolites

To the combined 48-hr urines from several animals of each species, which had received butyl *p*-nitrophenyl ether, conc. HCl was added to make a final HCl concentration of 5 per cent. It was then boiled on a water bath for 1 hr to hydrolyze conjugated metabolites.* The hydrolyzed urine was extracted with ether either by shaking mechanically or by continuous extraction for 20 hr. The extract was concentrated to about 100 ml after drying over anhydrous Na_2SO_4, and was shaken 3 times with the same volume of 0·1 N NaOH. The ether layer containing neutral metabolites was washed once with a small volume of water.

The alkaline layer and wash were combined, and the mixture was acidified with dilute HCl and extracted 3 times with the same volume of ether to obtain the acidic fraction.

Paper and thin-layer chromatography

Paper chromatography was carried out by the ascending technique with filter paper (Toyoroshi no. 51A). The solvent system used was *n*-butanol, pyridine, benzene, conc. NH_4OH (40:40:25:6, v/v).

Thin-layer chromatography was carried out by use of silica gel plates, 0·25 mm thick (Silica gel G, Merck) which was activated at 110° for 60 min. The solvent system used was ether, benzene, petroleum ether (2:2:1, v/v). The chromatograms were visualized by an ultraviolet lamp or by spraying with 5% NaOH solution.

Gas chromatography

The instrument used was a Shimadzu model GC-1C equipped with a hydrogen flame ionization detector (dual column and differential flame type). The column was a stainless steel U-shaped tube (2·25 m × 4 mm). The packing was 1·5% SE-30 on Chromosorb W (60–80 mesh), which was pretreated with hexamethyldisilazane. The column temperature was 190°, the injection port temperature 240°, and the detector cell temperature 200°. Nitrogen was used for the carrier gas with a flow rate of 40 ml/min.

RESULTS

Detection of the metabolites

Detection of the metabolites in two fractions, neutral and acidic, of the urine extracts described under Methods was performed by means of paper, thin-layer, and gas chromatography.

The chromatograms of the urine extracts of medicated rats, mice, and guinea pigs revealed essentially the same pattern, indicating existence of only one metabolite in

* It was confirmed that no breakdown of metabolites was observed under this condition by control experiment.

the neutral fraction and two in the acidic fraction. No unchanged material was detected in any of urine samples examined. Sex differences, which were usually observed in the metabolism of rats, were not significant in this case.

The amount of metabolite in the neutral fraction of rats and mice seemed very small, but was identified as 3-hydroxybutyl *p*-nitrophenyl ether by thin-layer and gas chromatography, although the optical activity was not confirmed. It could not be detected by the less sensitive paper chromatographic method. The area on the thin-layer chromatogram corresponding to that of the authentic sample of (+)-3-hydroxybutyl *p*-nitrophenyl ether, which was isolated from the rabbit urine in the previous study,[2] was scraped into a flask and extracted with ethanol (EtOH). The ultraviolet absorption spectrum of this extract had the characteristic maxima of 3-hydroxybutyl *p*-nitrophenyl ether (λ_{max} 228 and 309 mμ). Guinea pigs and rabbits excreted this metabolite more than the other species. As reported previously, it was actually the major metabolite in rabbits.[2]

One of the two metabolites in the acidic fraction was proved to be *p*-nitrophenol by measuring the u.v. absorption spectrum of the extract from a thin-layer chromatogram, as well as by the R_f values of paper and thin-layer chromatography and the retention time of gas chromatography. By preliminary estimation with gas chromatographic analysis, this metabolite was found to be excreted more in rats and mice than in the other species.

Another acidic metabolite had an R_f value of 0·17 by paper chromatography and revealed a yellow color in 0·1 N NaOH. The u.v. absorption spectrum of the EtOH extract from the chromatogram possessed two maxima, around 227 and 306 mμ, characteristic of *p*-nitrophenyl ether,[2] suggesting that the benzene nucleus of this metabolite had remained intact. The peak of this metabolite could not be observed by gas chromatography unless it was methylated with CH_2N_2, indicating that it might be the ω-carboxylic acid. As described later, this metabolite was isolated in crystalline form and identified to be *p*-nitrophenoxy acetic acid. Rats and mice excreted it in considerable amounts, whereas rabbits and guinea pigs excreted only a small amount. In the previous study in rabbits,[2] excretion of this metabolite was not noticed, but it was confirmed by the present careful re-examination.

TABLE 1. R_f VALUES AND RETENTION TIMES (RT) OF THE METABOLITES IN PAPER (PPC), THIN-LAYER (TLC), AND GAS (GC) CHROMATOGRAPHIS*

Compounds	R_f		RT (min)
	PPC	TLC	GC
Butyl *p*-nitrophenyl ether	0·95	0·81	3·0
3-Hydroxybutyl *p*-nitrophenyl ether	0·93	0·25	6·5
p-Nitrophenol	0·40	0·41	2·0
p-Nitrophenoxy acetic acid	0·17	0·0	3·0 (methyl ester)

* The conditions were described under Methods.

R_f values and retention times of the above three metabolites and unchanged compound under conditions described in Methods are shown in Table 1; the metabolites detected in each species are summarized in Table 2. The amount of each metabolite was roughly estimated from the gas chromatogram and expressed as a

relative amount in one species. It must be noted that the amount of the identified metabolites excreted into the 48-hr urine was a part of the dose.

Isolation and identification of the metabolite, p-*nitrophenoxy acetic acid*

After administration of butyl *p*-nitrophenyl ether to 4 female rats in a daily dose of 300 mg/kg for 7 days, extraction of the combined urine from the time of medication

TABLE 2. THE METABOLITES IN VARIOUS ANIMALS

Species	Metabolites*			
	Unchanged	(ω-1)-OH	NP	NPAA
Rats	none	trace	major	major
Mice	none	trace	major	major
Guinea pigs	none	major	minor	minor
Rabbits	none	major	minor	minor

* (ω-1)-OH, 3-hydroxybutyl *p*-nitrophenyl ether; NP, *p*-nitrophenol; NPAA, *p*-nitrophenoxy acetic acid.

to 48 hr after the last dose by the procedure described under Methods gave 0·85 g of the acidic fraction. This brown gum was dissolved in a small volume of benzene and chromatographed through a silica gel column (Wakō gel, 100–200 mesh, Wakō Pure Chem. Ind.). The column was eluted stepwise with benzene, benzene–ether (1:1, v/v), ether and $CHCl_3$. The eluates with benzene and benzene–ether were demonstrated to consist mainly of *p*-nitrophenol and the desired metabolite, respectively, by thin-layer chromatographic examination; both metabolites were isolated in crystalline form.

After decoloration with charcoal, the latter metabolite was recrystallized from acetone–benzene to slightly yellow prisms, m.p. 184–186°. The i.r. absorption spectrum (KBr pellet) of this metabolite, when compared with that of the original butyl *p*-nitrophenyl ether, had an extra doublet at 5·73 and 5·83 μ, possibly owing to a carboxylic acid group, in addition to the peaks due to the aromatic (6·24 μ) and nitro groups (6·59 and 7·42 μ). This, together with the u.v. spectral data, supported the structure of ω-carboxylic acid, and finally the elemental analysis led to the conclusion that it could not be *p*-nitrophenoxy butyric acid, but *p*-nitrophenoxy acetic acid. (Found: N, 7·28. $C_8H_7O_5N$ requires N, 7·11.) The comparison of this metabolite with chemically synthesized *p*-nitrophenoxy acetic acid according to the metnod of Meyer and Duczmal,[3] by m.p., paper chromatography, and infrared absorption spectrum showed their complete identity.

DISCUSSION

Dealkylation has been shown to be one of the important metabolic pathways of alkylaryl ethers in the animal body and to proceed through the α-hydroxylated intermediate having the unstable semiacetal structure shown below.[4–6]

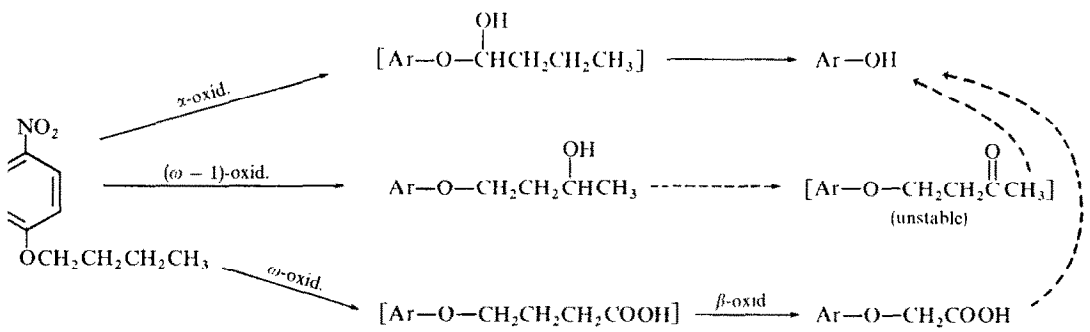
$$\mathrm{Ar{-}O{-}CH_2R} \xrightarrow[\text{liver microsomes}]{\mathrm{NADPH_2,\ O_2}} \mathrm{[Ar{-}O{-}\underset{\displaystyle OH}{\underset{|}{C}}HR]} \longrightarrow \mathrm{Ar{-}OH + R{-}CHO}$$

In our previous paper, however, it was found that, although this was true in aryl ethers of shorter alkyl groups (methyl or ethyl), ω- or (ω-1)-oxidation played a more important role in those having longer alkyl groups in rabbits.[2] Such ω- and (ω-1)-oxidation have been established as the common metabolic pathway of various C-alkyl compounds, but there has been no evidence concerning such metabolism of O-alkyl compounds, except that in rabbits.[2]

The present investigation further proved that these oxidations of *O*-alkyl groups occurred not only in rabbits but also in rats, mice, and guinea pigs, which suggests their general occurrence in various animals.

The importance of ω- and (ω-1)-oxidations varied considerably from species to species. Rabbits and guinea pigs metabolized butyl *p*-nitrophenyl ether mainly by (ω-1)-hydroxylation, whereas rats and mice metabolized it more through α- and ω-oxidations; the latter oxidation was followed by β-oxidation.

The demonstrated metabolic pathways of butyl *p*-nitrophenyl ether described above are illustrated below.



Among the three urinary metabolites in rats and mice, the (ω-1)-hydroxyderivative was excreted in much smaller amounts and *p*-nitrophenol in much larger amounts than in rabbits and guinea pigs, and this suggested the possibility that *p*-nitrophenol might be produced not only by α-hydroxylation but also through a (ω-1)-hydroxy-metabolite. In fact, by a study *in vitro* in rabbit liver, the (ω-1)-hydroxymetabolite was found to be easily dehydrogenated by a soluble enzyme to the corresponding ketone, which was in turn cleaved nonenzymatically to *p*-nitrophenol and an unidentified substance.[7]* *p*-Nitrophenol produced in rabbits by this pathway seemed to be more than that produced by the α-hydroxylation pathway.* If the soluble enzyme which dehydrogenates the (ω-1)-hydroxymetabolite to the corresponding ketone is involved more in rats and mice than in rabbits and guinea pigs, less excretion of (ω-1)-hydroxymetabolite and more of *p*-nitrophenol in the urine of the former animals could be explained, but the authors have no experimental evidence at hand to support this hypothesis. We have also shown that *p*-nitrophenoxy acetic acid produces *p*-nitrophenol by alkaline hydrolysis, although the rate was rather slow at room temperature. A minor part of *p*-nitrophenol isolated from the urine might originate in this chemical hydrolysis. McMahon *et al.*[6] reported that *p*-nitrophenol was not produced by the incubation of *p*-nitrophenoxy acetic acid with a microsomal preparation of rat liver, but only a little was excreted into the urine of the animal given this ether.

* H. Yoshimura, S. Îda, H. Tsuji, M. Mori and H. Tsukamoto, unpublished data.

Total recovery of the identified metabolites from the 48-hr urine was quite low (about a half or less than half of the dose), and preliminary examination of the faeces showed only small quantities of metabolites or of the original compound. These findings lead us to suggest that there may be some other metabolic pathways or rather strong tissue binding of the metabolites or the original compound. In order to understand this metabolism more fully, further study will be necessary.

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