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IN VITRO METABOLIC STUDIES ON PROPYL *p*-NITROPHENYL ETHER*

C. MITOMA, R. L. DEHN AND M. TANABE

Life Sciences Division, Stanford Research Institute, Menlo Park, Calif. 94025 (U.S.A.)

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SUMMARY

1. This study was conducted to examine how the metabolic rates at the three sites on propyl *p*-nitrophenyl ether (PNPE) varied with respect to each other under various experimental conditions. The hepatic microsomal system of the rat and guinea pig showed differences in some respects.

2. The rates of metabolism at the three sites varied independently of each other after pretreatment of the rat or the guinea pig with phenobarbital or 3-methylcholanthrene. Moreover, the major metabolite in these two species was different after enzyme induction, although (ω-2)-alcohol was the major metabolite in both species before induction.

3. Substitution of hydrogen atoms with deuterium atoms at each of the metabolic sites resulted in selective inhibition of metabolism at the particular site. A general trend for a slightly increased formation of other metabolites was observed to compensate for the decreased formation of the major metabolite in both animal systems. Implications of these findings with regard to the number of enzymes involved in the metabolism of PNPE is discussed.

INTRODUCTION

The metabolic fate of propyl *p*-nitrophenyl ether (PNPE) as well as several other alkyl *p*-nitrophenyl ethers has been reported by YOSHIMURA *et al.*[2]. After orally administering PNPE to male rabbits, they isolated from the urine 31 % of the dose as *p*-nitrophenol (oxidation at ω-2), 17 % as 2-hydroxypropyl *p*-nitrophenyl ether (oxidation at ω-1) and 16 % as 3-(*p*-nitrophenoxy)propionic acid (oxidation at ω).

Since the compound is metabolized at three different sites of the molecule, we were interested in investigating whether the rates of metabolism at any one of these sites could be altered independently of those at the other sites. In the course of this study, a difference in the hepatic enzyme system between the rat and the guinea pig was observed.

Abbreviation: PNPE, propyl *p*-nitrophenyl ether.

* A preliminary report of this study was presented before the Fall Meeting of the American Society for Pharmacology and Experimental Therapeutics, 1970[1].

MATERIALS AND METHODS

Incubation conditions and analytical procedures

Male Sprague–Dawley rats weighing 200–250 g were purchased from Simonsen Laboratories, Inc., Gilroy, Calif. Male guinea pigs weighing 300–350 g were obtained from Horton's Laboratory Animals, Inc., Los Gatos, Calif. They were kept in cages using Absorb-Dri (hardwood shavings) as the bedding and were maintained on a stock diet and water *ad libitum*.

To stimulate or induce the hepatic microsomal enzyme systems, the animals were treated twice daily with phenobarbital (38 mg/kg per dose, intraperitoneally) for 3 days or were given a single intraperitoneal injection of 3-methylcholanthrene (50 mg/kg) 2 days before they were sacrificed.

The livers were homogenized in 3 ml of 0.1 M cold potassium phosphate buffer, pH 7.4, for each g of liver. A postmitochondrial supernatant fraction containing the microsomes was prepared by centrifuging the homogenate in a Servall refrigerated centrifuge for 10 min at 10000 $\times$ *g*. The microsome fraction was prepared by centrifuging the 10000 $\times$ *g* supernatant for 30 min at 140000 $\times$ *g*.

To assay PNPE metabolism, 1 ml of the 10000 $\times$ *g* supernatant fraction (25–35 mg protein) was mixed with 1.7 ml of solution containing Tris buffer, pH 8.0 (200 μmoles), glucose 6-phosphate (40 μmoles), $MgCl_2$ (25 μmoles), $NADP^+$ (0.25 μmole), NADH (5 μmoles) and finally 20 μmoles of PNPE in 0.1 ml of 50 % glycerol–dimethyl sulfoxide (1:2, v/v). The incubation was carried out in a Dubnoff metabolic shaker for 30 min at 37° in air. The reaction was stopped by acidifying with 1 ml of 3 M HCl. The reaction mixture was extracted twice with 10 ml of ether. The combined ether extract was evaporated and the residue was dissolved in 0.5 ml of methanol. An aliquot of the methanol solution was spotted on a silica gel F-254 plate (Brinkmann) and the plate was developed in a benzene–ether (9:1, v/v) solvent system. The carboxylic acid, alcohol, *p*-nitrophenol and PNPE gave R_F values of 0, 0.17, 0.28 and 0.68, respectively. Each area of the thin-layer plate containing these compounds was scraped into a scintillation vial. The silica gel in the vial was soaked in 0.5 ml of methanol to elute the compounds. The vial was counted in a Nuclear-Chicago Mark I liquid scintillation counter after adding 10 ml of toluene scintillation fluid containing 0.02 % *p*-bis-[2-(4-methyl-5-phenyloxazoyl)]-benzene and 0.4 % 2,5-diphenyloxazole. The above described procedure was shown to almost quantitatively account for the most polar metabolite, carboxylic acid, after it was spotted on a thin-layer plate. The percent of counts in each metabolite area was calculated and was multiplied by 20 μmoles to obtain the values for each metabolite. Where indicated, the incubation was assayed only for *p*-nitrophenol formation. The colorimetric procedure used was similar to that used for the assay of *o*-nitrophenol[3].

Preparation of ^{14}C-labeled, ^2H-substituted propyl p-nitrophenyl ether: [3-^2H,^{14}C]*PNPP*

To a 50-ml two-neck round bottom flask equipped with dry ice condenser and magnetic stirrer was added 1.04 g (8.25 mmoles) of [3-$^2H_3$]propyl bromide (Merck, Sharpe and Dohme, Canada, 98 % isotopic purity), 1.12 g (8.0 mmoles) *p*-nitro-[^{14}C]phenol in 25 ml hexamethylphosphoramide and finally 1.12 g (8.1 mmoles) anhydrous K_2CO_3. The reaction mixture was stirred at 75°. After the first 8 h the dry ice condenser was not recharged. After 20 h reaction time, the mixture was cooled,

poured into a mixture of 80 ml ice water and 20 ml 1 M NaOH, and was extracted 4 times with 50 ml benzene. The combined benzene extracts were washed twice with 20 ml 1 M NaOH, 4 times with 20 ml distilled water, and dried over anhydrous Na_2SO_4. Removal of the benzene at reduced pressure yielded 1.25 g of yellow oil which was chromatographed on 12.5 g of neutral Alumina (Woelm activity Grade I). The second 50 ml ether fraction contained 0.56 g of [3-$^2H_3$,^{14}C]PNPP. [^{14}C]Phenol was purchased from Amersham/Searle Corp. *p*-Nitro[^{14}C]phenol was prepared by the method of GORTATOWSKI *et al.*[4].

Analytical data. λ_{max} in ethanol 309 nm (ε = 12000); literature[2]: λ_{max} in ethanol 309 nm (ε = 11500). NMR: δ 1.90 (^{3}H-2); 4.05 (^{3}H-2); 6.95 (^{2}H-2); 8.20 (^{2}H-2). Examination of the residual methyl resonances at δ 1.30 indicated the product contained less than 2 % hydrogen and hence was minimally 98 % deuterated.

Similarly, [2-$^2H_2$,^{14}C]PNPE and [1-$^2H_2$,^{14}C]PNPE were prepared starting with *p*-nitro[^{14}C]phenol and [2-$^2H_2$]propyl bromide and [1-$^2H_2$]propyl bromide, respectively. The specific activity of all of these doubly labeled PNPE was approx. 0.01 μC/μmole.

Preparation of 2-hydroxypropyl p-nitrophenyl ether

p-Nitrophenol, 1.0 g (7.2 mmoles) was placed in a 45-ml stainless steel high pressure reaction vessel and dissolved in 15 ml hexamethylphosphoramide followed by 0.7 ml (11.0 mmoles) propylene oxide. The reaction vessel was immediately sealed and the reaction was heated to 140° with stirring for 16 h. The reaction was cooled and poured into 75 ml benzene. The benzene was washed three times with 25 ml 1 M NaOH, 6 times with 10 ml distilled water, dried over Na_2SO_4 and evaporated under reduced pressure to yield 1.34 g of dark yellow oil.

The total crude reaction mixture was almost exclusively 2-hydroxypropyl *p*-nitropropyl ether since 2-*p*-nitrophenoxy-1-propanol (the other possible condensation product) can be distinguished by a small separation of shifts of the methyl doublet in the NMR. Recrystallization of the sample from benzene gave 210 mg light yellow solid; m.p. 69–71°, literature[5] 71°. Recrystallization of the mother liquor by addition of light petroleum to separate out oily drops which solidified upon standing at —5° yielded an additional 460 mg. The total yield was 60 %.

Preparation of ^{14}C-labeled 3-(p-nitrophenoxy)propionic acid

To 100 mg solid NaOH (2.5 mmoles) in 10 ml of water was added 310 mg (2.5 mmoles) *p*-nitro[^{14}C]phenol. The mixture was warmed on a steam cone for 10 min then 180 mg of β-propionolactone (2.5 mmoles) was added. The reaction mixture was heated for an additional 35 min then cooled. After acidification to pH 4 with conc. HCl, the aqueous mixture was extracted 3 times with 25 ml portions of benzene. The combined benzene extracts were extracted 3 times with 20-ml portions of fresh 2 % $NaHCO_3$. The combined bicarbonate extracts were washed twice with 10-ml portions of benzene, acidified with conc. HCl to pH 4 and extracted 3 times with 20-ml portions of benzene. The final benzene extracts were washed with 10 ml water, dried with Na_2SO_4 and evaporated under reduced pressure to yield 85 mg. The specific activity of the product was approx. 0.01 μC/μmole.

RESULTS

Preliminary studies were carried out with the enzyme preparation from the rat to approximate optimal incubation conditions for the overall metabolism of PNPE, based on the colorimetric determination of *p*-nitrophenol formed. As shown in Fig. 1, the reaction proceeded linearly for 45 min, therefore we chose 30 min as the standard incubation time. The incubation was routinely carried out with 20 μmoles of PNPE which was sufficient to saturate the enzyme system (Fig. 1).

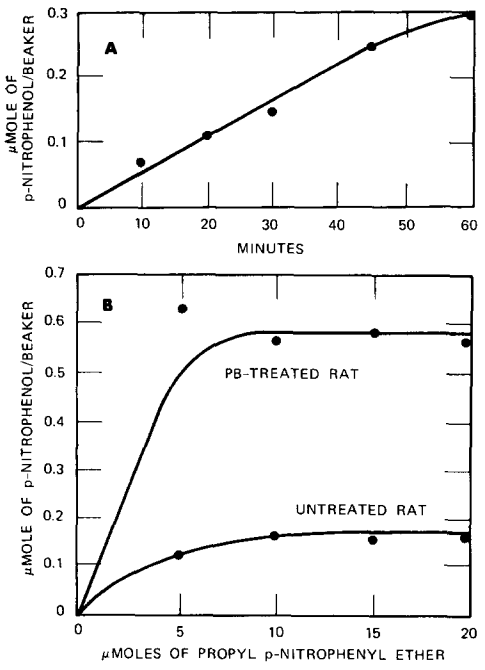

Fig. 1. Formation of *p*-nitrophenol by the rat liver enzyme system as a function of time and substrate concentration. The incubation conditions are described under METHODS AND MATERIALS. PB = phenobarbital.

A typical incubation mixture containing 1 ml of the rat liver supernatant fraction catalyzed the formation of 0.23 μmole of *p*-nitrophenol, 0.37 μmole of alcohol and 0.10 μmole of ω-carboxylic acid per beaker. The corresponding values obtained with the guinea pig liver preparation were 0.14, 0.45 and 0.20, respectively. Thus, the alcohol was the major metabolite in the two species when the enzyme system of the uninduced animals was used.

Subsequent studies revealed interesting species differences with regard to the metabolism of PNPE. The results obtained on the metabolic pattern after pretreating the rat with phenobarbital and 3-methylcholanthrene are summarized in Table I. Whereas alcohol was the major metabolite with the untreated rat liver system, phenol became the major metabolite after enzyme induction. Phenobarbital stimulated metabolism at all three sites, particularly alcohol and phenol formation. 3-Methylcholanthrene, on the other hand, stimulated only phenol formation. This stimulation

TABLE I

EFFECT OF INDUCERS ON THE METABOLISM OF PROPYL *p*-NITROPHENYL ETHER BY THE RAT AND GUINEA PIG HEPATIC ENZYME SYSTEMS

There were four rats or two guinea pigs in each group.

Animal	*Inducer*	*Product*	*μmoles/g 10000 × g protein*		*Ratio a/b*	*Significance*
			(*a*) *Control*	(*b*) *Exptl.*		
Rat	Phenobarbital	Carboxylic acid	3.0 ± 1.5	4.7 ± 2.9	1.6	N.S.*
		Alcohol	10.1 ± 2.7	28.9 ± 9.1	2.9	$P<0.01$
		Phenol	8.3 ± 1.9	46.2 ± 10.8	5.6	$P<0.001$
	3-Methyl-cholanthrene	Carboxylic acid	5.3 ± 2.4	4.3 ± 2.2	0.8	N.S.
		Alcohol	16.6 ± 3.9	14.1 ± 2.3	0.9	N.S.
		Phenol	9.8 ± 3.9	110.9 ± 19.7	11.3	$P<0.001$
Guinea pig	Phenobarbital	Carboxylic acid	7.9	14.3	1.8	—
		Alcohol	17.9	81.8	4.6	—
		Phenol	5.5	12.1	2.2	—
	3-Methyl-cholanthrene	Carboxylic acid	7.9	5.6	0.7	—
		Alcohol	17.9	28.4	1.6	—
		Phenol	5.5	3.7	0.7	—

* Not significant.

TABLE II

EFFECT OF DEUTERIUM SUBSTITUTION ON THE METABOLISM OF PROPYL *p*-NITROPHENYL ETHER BY THE RAT AND GUINEA PIG HEPATIC ENZYME SYSTEMS

Figures in parentheses refer to the number of liver preparations from separate animals used in each group.

Animal	*Position of deuterium*	*Product*	*μmoles/g 10000 × g protein*		*Ratio a/b*	*Significance*
			(*a*) *Control*	(*b*) *Exptl.*		
Rat	ω (2)	ω-COOH	8.3	0.5	0.06	
		Alcohol	14.6	12.8	0.88	
		Phenol	22.5	19.7	0.87	
	ω-1(3)	ω-COOH	12.3 ± 3.3	7.7 ± 2.9	0.62	N.S.
		Alcohol	26.4 ± 2.9	5.7 ± 0.6	0.22	$P<0.001$
		Phenol	47.5 ± 4.9	56.3 ± 5.3	1.2	N.S.
	ω-2(3)	ω-COOH	5.4 ± 2.7	6.4 ± 2.9	1.2	N.S.
		Alcohol	27.6 ± 8.3	37.3 ± 14.0	1.4	N.S.
		Phenol	47.4 ± 10.4	11.1 ± 3.3	0.24	$P<0.001$
Guinea pig	ω (3)	ω-COOH	11.5 ± 2.8	3.6 ± 0.6	0.31	$P<0.01$
		Alcohol	38.6 ± 7.3	42.6 ± 1.3	1.1	N.S.
		Phenol	8.2 ± 2.2	11.0 ± 1.3	1.3	N.S.
	ω-1(3)	ω-COOH	16.2 ± 2.5	24.0 ± 1.2	1.5	$P<0.01$
		Alcohol	47.2 ± 2.0	32.7 ± 10.8	0.7	N.S.
		Phenol	10.6 ± 0.6	15.4 ± 5.2	1.5	N.S.
	ω-2(3)	ω-COOH	14.0 ± 6.3	8.0 ± 2.6	0.57	N.S.
		Alcohol	60.2 ± 20.2	57.7 ± 13.4	0.96	N.S.
		Phenol	10.4 ± 3.1	1.6 ± 0.3	0.15	$P<0.001$

was considerably more than that observed with phenobarbital. In similar studies with guinea pigs, the oxidation at the (ω-1)-position to form alcohol was the most stimulated instead of the (ω-2)-position that results in phenol formation, as was the case for the rat. In the guinea pig the stimulation of alcohol formation by 3-methylcholanthrene was not as great as that seen with phenobarbital.

In Table II, the effect on the metabolic rate of labeling PNPE with ^2H at each of the carbon sites on the propyl side-chain is shown. The enzyme preparation used was obtained from animals pretreated with phenobarbital. The primary effect of deuteration was to decrease the rate of oxidation at the carbon site labeled with deuterium. The oxidation rate at the other carbon sites was not appreciably affected. A trend for an increase in the oxidation at the ω- and (ω-1)-positions was observed when the formation of the major metabolite, *p*-nitrophenol, was inhibited by deuteration at the (ω-2)-position in the rat enzyme system. Similar results were obtained when the enzyme system from phenobarbital-treated guinea pigs was used. In this case, the oxidation rate at the ω-position was significantly stimulated when the formation of the major metabolite, (ω-1)-alcohol, was partially inhibited by deuteration at the (ω-1)-site.

DISCUSSION

Species difference between the rat and guinea pig was observed in the metabolism of PNPE. Although the metabolic pattern of PNPE by the hepatic enzyme system of the untreated rat and guinea pig was similar, the inducers altered the metabolic pattern in the two species differently. The metabolic rate primarily stimulated by phenobarbital and singularly affected by 3-methylcholanthrene was that of (ω-2)-oxidation in the rat, whereas it was that of (ω-1)-oxidation in the guinea pig. A possible implication of this difference in the response to inducers is that if one of the metabolites were to be an active or a toxic metabolite, the species difference in the sensitivity to a drug in some instances will become evident only if the animals are in the induced state so far as the hepatic enzyme system is concerned and will not be apparent in the uninduced state.

As was reported previously, substitution of hydrogen atoms by deuterium atoms at the metabolic site decreased the rates of drug metabolism, providing that the breaking of the carbon–hydrogen bond is rate-limiting in the oxidative process[6,7]. In the present studies, it was observed that the rate of oxidation at each of the carbon sites was selectively decreased when it was labeled with deuterium atoms.

The deuterium experiment was conducted to hopefully obtain an indirect evidence for the participation of more than one enzyme in the metabolism of PNPE. Our reasoning was that if PNPE is being metabolized at all three sites by separate enzymes, then blocking the metabolism at one site, particularly at the oxidation site for the formation of the major metabolite, should not alter the rate of oxidation at the other sites, since these reactions are presumably proceeding maximally. If, however, one enzyme is responsible for the formation of all three metabolites, then such a blockage should be compensated by the increased formation of the other metabolites. This, of course, assumes that deuteration of PNPE does not appreciably change its K_m value.

A general trend was observed for the increased formation of other metabolites

when the formation of the major metabolite was decreased as a result of deuteration. Thus, 75 % inhibition of phenol formation in the rat enzyme system resulted in 40 % increase in alcohol formation. A partial inhibition of alcohol formation in the guinea pig enzyme system resulted in 50 % increase in the formation of both the carboxylic acid and phenol. However, the observed increase could not be statistically supported except for one case in the guinea pig system.

Although the data are not conclusive, the results from the deuterium experiment tend to favor the one-enzyme hypothesis. In contrast, the results from the induction studies in the two species examined indicate that the metabolism of PNPE at the three sites may be catalyzed independently by separate enzymes.

An alternative pathway by which *p*-nitrophenol can be formed is through ω-oxidation of PNPE followed by β-oxidation of the resulting *p*-nitrophenoxy propionic acid[8]. This pathway is probably of little significance since the amount of *p*-nitrophenol formed from [3-$^2H_3$]PNPE by the rat and guinea pig microsomal systems was not reduced even though the formation of the acid was considerably reduced.

It is of particular interest that KUNTZMAN and co-workers[9,10] have presented elegant data that strongly suggest the participation of separate enzyme components for the 6β-, 7α- and 16α-hydroxylation of testosterone. However, their findings are not universally accepted[11]. The ultimate proof for the number of enzyme components involved in the metabolism of a particular substrate must await purification of the enzymes in question.

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