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Metabolism of an Acaricide, 4-Methyl-3-(n-propylthio)phenyl 4-Nitrophenyl Ether, in Rat

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^{14}C-Labeled 4-methyl-3-(n-propylthio)phenyl 4-nitrophenyl ether (MPNE) was administered orally to rats at a dosage of 400 mg/kg of body weight. Near-maximum elimination was achieved within 4 days. At the end of 20 days, approximately 56% of radioactivity was found in feces and 27% in the urine. At the same time, a low level of radioactive residue was found mainly in the adipose tissue in the form of the original parent compound. The metabolism of the acaricide is mainly carried out by the mixed-function oxidase. In addition to the parent compound, two major metabolites γ-hydroxy fulfoxide ether and sulfoxide ether were found in feces. One minor metabolite was identified as γ-hydroxy sulfone ether besides a few unknown minor polar metabolites. In urine only γ-hydroxy sulfoxide ether was identified, though there were several other unknown polar metabolites. No conjugated products were found to exist in the feces or urine. While no amino derivative of MPNE was found among in vivo metabolic products, an amino derivative of MPNE sulfoxide was found to form in vitro. This reductive metabolism is stimulated by the presence of NADPH and is apparently coupled with mixed-function oxidase.

MPNE [4-methyl-3-(n-propylthio)phenyl 4-nitrophenyl ether] is a new type of specific acaricide of which chemical structure is quite different from any other acaricide being used today. It has shown promise for control of mites such as *Tetranychus urticae* and *Panonychus citri* (Kato et al., 1975). The most intriguing aspect of this acaricide is that

it is a diphenyl ether compound that was originally intended for a herbicide. Instead, the compound has shown an excellent acaricidal activity without any appreciable herbicidal actions. As a result, many questions may be raised as to its mode of action, metabolism, and biological effects. One of the first questions scientists could ask is the nature of the actual toxic principle to animals, whether it is the starting compound or its metabolites. Another important question is how it is metabolized and whether any of its metabolic products accumulate in biological

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systems as stable intermediates. The investigations reported here were initiated in an effort to provide new informations on the metabolic fate of this new acaricide. We have particularly made attempts to identify the enzymatic processes involved in its metabolism. The results were then compared with those obtained by other workers on herbicidal diphenyl ethers.

MATERIALS AND METHODS

Materials. [^{14}C]MPNE [4-methyl-3-(n-propylthio)-phenyl 4-nitrophenyl ether] was supplied by Nippon Kayaku Co., Ltd., Tokyo, Japan. The specific activity was 4.61 mCi/mmol and MPNE has a purity of greater than 99% as shown by thin-layer chromatography, autoradiography, and liquid scintillation counting. The chemical is labeled with ^{14}C at the methyl group. The analytical reference standard preparation of MPNE and candidate metabolites were also supplied by the same company. All cofactors and biochemicals were obtained from Sigma Chemical Co., St. Louis, MO. SKF-525A was obtained from Smith Kline & French Laboratories, Philadelphia, PA. Silica gel GF 254 was obtained from Scientific Products, Romulus, MI. Analytical TLC precoated plates LK5F was obtained from Whatman, Inc., Clifton, NJ. No-screen X-ray films (NS-5Y) for autoradiography were obtained from Eastman Kodak Co., Rochester, NY. Adult male Sprague-Dawley rats weighing 160–200 g/individual were supplied by Spartan Research Animals, Inc., Haslett, MI.

Treatment of Rats and Collection of Samples. Five adult male rats were given a single oral dose of [^{14}C]-MPNE, specific activity 0.461 mCi/mmol (achieved by diluting the original compound with the analytical reference standard of MPNE), at a dosage rate of 400 mg/kg of body weight. The chemical was dissolved in 0.75 mL of corn oil and administered by using a stomach tube technique followed by a rinse of 0.25 mL of corn oil. The rats were held individually in metabolism cages, and feces and urine were collected separately at regular intervals. Each time a sample of urine was quantitatively measured and aliquots were taken for radioassay. Each sample of feces collected was air-dried, weighed, and ground, and an appropriate quantity was taken for radioassay. Two treated rats were sacrificed 24 h posttreatment by cerebral concussion. Various organs and tissues were dissected out immediately, and representative samples were taken for radioassay. At the end of 20 days posttreatment the remaining rats were sacrificed and the radioactivity in different parts of the body was determined in the same way as described above.

Radioassay. Radioactivity found in organic solvents was determined by mixing the sample with 10 mL of nonaqueous scintillation solution consisting of 5.0 g of PPO, 0.35 g of dimethyl-POPOP and 1.0 L of toluene. Radioactivity in aqueous phase was determined by using aqueous scintillation solution consisting of 5.5 g of PPO, 0.3 g of dimethyl-POPOP, 0.5 L of toluene, and 0.5 L of methyl-Cellosolve. Radioactivity found in fecal material, body organs, and tissues was determined by using a Harvey Instruments' Model OX-200 biological oxidizer. The radioactivity was determined as $^{14}CO_2$ that was trapped in scintillation solution, consisting of 1 part of Carbosorb II and 2 parts of Permafluor V (both from Packard Instrument Co.). These vials were counted in a Tri-Carb Model 3003 liquid scintillation spectrometer, and corrections for radioactive quenching were made by an external standard method.

Thin-Layer Chromatography. For quantitative studies Whatman LK5F precoated plates with a 250-μm

silica gel layer were used for resolving MPNE and its metabolites. The solvent system and R_f values are presented in Table I. The nonradioactive analytical reference standard of the metabolites were detected under UV light, and ^{14}C-labeled metabolites were detected by Berthold TLC scanner, Model LB 2760. For isolation of sufficient quantities of metabolites for further analysis and confirmation, extracts were applied as a band on a 0.5 mm thick laboratory-prepared TLC plates and developed in solvent system B. The ^{14}C bands were detected by autoradiography and removed from the plate and eluted with acetone. Isolated metabolites were further purified by at least another TLC system.

Gas–Liquid Chromatography. For further confirmation of the nature of metabolites, a Varian Aerograph series 2400 gas chromatograph equipped with alkali flame ionization detector was used. The column packings used were 3% SE-30, 3% OV-101, and 3% PF-1, all on Gas-Chrom Q, 80–100 mesh. The SE-30 was packed in a glass column 6 ft long and with a 2-mm i.d., whereas the other two were packed in metal columns 6 ft long and with a 2-mm i.d. Operating conditions were as follows: injector temperature 270 °C, detector temperature 270 °C, nitrogen carrier gas flow 30 mL/min, air flow 235 mL/min, and hydrogen gas flow 48 mL/min. Column temperature and retention time are presented in Table I.

Extraction and Analysis of Metabolites (Figure 2). The combined air-dried and ground feces was transferred to an Erlenmeyer flask and acetone (20 mL/g) was added. The flask was shaken on a Burrel wrist action shaker for 30 min. The extract was collected through decantation. The extraction was repeated twice, and all three extracts were filtered, combined, and concentrated on a rotary evaporator to an oily residue. Hexane was added to dissolve the hexane-soluble metabolites, leaving the hexane-insoluble materials in the flask which were later dissolved in acetone for radioassay. The hexane-soluble fraction was subjected to partitioning 3 times with acetonitrile (1:1 v/v). The acetone fraction (i.e., hexane insoluble) was again evaporated to dryness and subjected to partitioning between distilled water and ethyl acetate. The remaining dry feces (after acetone extraction) was extracted again 3 times with methanol (20 mL/g). All three methanol extracts were combined, concentrated to an oily residue, and subjected to partitioning between distilled water and ethyl acetate. Radioactivity was determined for each fraction in every extraction step. The remaining dry feces (after methanol extraction) was weighed and small samples were taken for radioassay by using the biological oxidizer. The acetonitrile fraction, ethyl acetate fraction from acetone extract, and ethyl acetate fraction from methanol extract were further analyzed by TLC and an autoradiogram was developed. For quantitation of the radioactive metabolites, silica gel on the plate corresponding to the dark spots on the autoradiogram was scraped and counted. Further purification and identification of metabolites were carried out by both TLC and GLC.

The combined urine samples were saturated with NaCl and the pH was adjusted to 3.0 with HCl (Figure 3). This solution was extracted 3 times with ethyl acetate (3.0 mL/mL of urine) in a separatory funnel. All three extracts were combined and evaporated to a few milliliters for radioassay. The remainder was concentrated to an oily residue. Hexane was added first to obtain the hexane-soluble metabolites followed by ethyl acetate to dissolve the remaining hexane-insoluble material. The hexane-soluble fraction was subjected to partitioning 3 times with acetonitrile (1:1 v/v). The ethyl acetate fraction was

Table I. Structures, TLC R_f Values and GLC Retention Time of MPNE and Its Metabolites[b]

Designation and Trivial Name	Structure	R_f Values in Solvent System					GLC Retention Time (min)							
							3% SE 30			3% OV 101			3% QF 1	
		A	B	C	D	E	210°	230°	250°	180°	210°	250°	210°	230°
I. Sulfide Ether (MPNE)	(structure)	0.77	0.76	0.66	0.87	0.68	6.9	3.1	–	–	6.5	–	5.2	–
II. Sulfoxide Ether	(structure)	0.67	0.65	0.46	0.72	0.60	1.4	–	–	3.2	1.4	–	–	1.4
III. Sulfone Ether	(structure)	0.72	0.73	0.54	0.76	0.66	–	6.8	–	–	15.4	–	–	19.1
IV. γ-Hydroxy Sulfide Ether	(structure)	0.66	0.66	0.46	0.62	0.52	–	–	–	–	–	–	–	–
V. γ-Hydroxy Sulfoxide Ether	(structure)	0.28	0.26	0.10	0.38	0.15	1.5	–	–	–	1.4	–	1.3	–
VI. γ-Hydroxy Sulfone Ether	(structure)	0.47	0.51	0.23	0.43	0.30	–	–	7.5 [a]	–	–	6.9 [a]	–	–
VII. β-Hydroxy Sulfide Ether	(structure)	0.69	0.71	0.52	0.67	0.60	–	–	–	–	–	–	–	–
VIII. β-Hydroxy Sulfoxide Ether	(structure)	0.38	0.38	0.20	0.49	0.23	1.4	–	–	–	1.4	–	1.3	–
IX. β-Hydroxy Sulfone Ether	(structure)	0.58	0.63	0.37	0.56	0.48	–	5.3	–	–	–	–	–	14.9
X. Amino Sulfoxide Ether	(structure)	0.41	0.38	0.23	0.43	0.26	–	–	–	2.2	–	–	–	–

[a] This is the Me₃Si derivative. [b] Solvent systems: A, hexane–chloroform–acetonitrile (3:2:2); B, hexane–chloroform–acetonitrile (12:8:11); C, hexane–ether–acetone (2:1:1); D, hexane–chloroform–methanol (4:5:1); E, chloroform–acetone (9:1).

Table II. Distribution of Radioactive Chemicals in Different Body Organs and Tissues 24 h and 21 Days Posttreatment

types of organ or tissue	concentration of radioactive chemicals,[a] ppm	
	24 h posttreatment	21 days posttreatment
brain	0.4069 (0.3513–0.4624)	0.089 (0.0083–0.0294)
lung	2.5511 (2.4412–2.6610)	
heart	1.1541 (0.9268–1.3813)	
liver	6.1790 (5.0158–7.3421)	0.0572 (0.0420–0.0724)
kidney	5.3467 (4.4296–6.0437)	0.0579 (0.0306–0.0852)
spleen	0.6208 (0.5394–0.7021)	0.1077 (0.0360–0.1794)
G.I. tract	6.1516 (4.7459–7.5552)	0.0132 (0.0039–0.0024)
adipose tissue	5.8497 (3.0938–8.6055)	0.0832 (0.0817–0.0847)
muscle	0.5963 (0.5485–0.6440)	0.0133 (0.0104–0.0161)
blood	7.7010	

[a] Each figure is mean of two rats, except for the blood sample.

concentrated to an oily residue and subjected to partitioning between distilled water and ethyl acetate. The amount of radioactivity was determined for each fraction obtained in every extraction step. The last ethyl acetate fraction was analyzed by TLC and an autoradiogram was prepared, and the metabolites were analyzed by the same procedure.

The nature of water-soluble metabolites in the aqueous fraction of methanol extract from feces, in urine after ethyl acetate extraction, and in the aqueous phase of the hexane-insoluble fraction of urine was studied by enzymatic hydrolysis as follows: 0.5-mL extracts were incubated with either 7000 units of β-glucuronidase type B-3, 150 units of sulfatase type VIII in 3.5 mL of 0.2 M acetate buffer at pH 5.0, or 3.5 mL of dilute HCl (or 3.5 mL of buffer only for control). The reaction mixtures were incubated at 37 °C for 24 h. The reaction was terminated by adding 4.0 mL of ether and directly extracting the products. Extraction was repeated twice and all three extracts were combined for radioassay.

Preparation of Liver Homogenate. In this study three types of liver homogenate preparations were used, viz., 20000g crude supernatant, 100000g microsomal fraction, and 100000g supernatant. The method for preparation of these subcellular fractions has been described by Conaway et al. (1977).

Incubation of [¹⁴C]MPNE with Liver Homogenate. In a typical experiment 0.05 μmol (0.2305 μCi) of [¹⁴C]-MPNE in 10 μL of methyl-Cellosolve was incubated with 0.5 mL of either crude supernatant, 100000g microsomal fraction, or 100000g supernatant plus various combinations of cofactors in a 25-mL Erlenmeyer flask. The protein concentration in crude supernatant was equal to that in 200 mg of liver tissue, whereas in both 100000g microsomal and supernatant fractions the protein concentration was adjusted to approximately 8 mg/mL as determined by the method of Lowry et al. (1951). The required cofactors such as GSH, NADPH, UDPGA, ATP, APS, and MgCl₂ were dissolved in the standard buffer (0.15 M sodium phosphate, pH 7.4, and 0.05 M sucrose) at desired concentrations. By the addition of standard buffer, the incubation mixture was finally made up to 2.0 mL. In some cases inhibitors dissolved in standard buffer were added to the system at the same time as the addition of the substrate. The reaction mixtures were incubated aerobically at 37 °C in a Lab-line shaking water bath for 2 h. At the end of the incubation period 3.0 mL of ether was added to stop the reaction, and the system was immediately shaken and transferred to a screw-cap culture tube and extracted. Extraction was repeated twice and all three ether extracts were combined for analysis. Control was prepared by incubating [¹⁴C]MPNE with 0.5 mL of liver homogenate and 1.5 mL of standard buffer without cofactor or inhibitor. The radioactivities in both the ether and aqueous

phase were determined. The combined ether phase was evaporated to a small volume under a stream of nitrogen gas and analyzed by TLC with a mobile phase which consists of hexane–chloroform–acetonitrile (3:2:2). Autoradiography was prepared and those spots having radioactivity were quantitated. Further identification was carried out by TLC as well as GLC. The aqueous phase accumulated from several control and NADPH-fortified incubation mixtures was concentrated to approximately 2.0 mL by using a Virtis freeze-dryer. It was divided into four parts and further studied by using hydrolytic enzymes as described in the previous section.

Reductive Metabolism of Sulfoxide Ether in Vitro. In this study both 100000g microsomal fraction and supernatant were used. For the 100000g microsomal suspension and supernatant the protein concentration was adjusted to approximately 8 and 32 mg/mL, respectively. In a typical experiment, 1.0 μmol of sulfoxide ether dissolved in 20 μL of methyl-Cellosolve was incubated with 0.5 mL of either 100000g microsomal fraction or supernatant and cofactors such as NADH, NADPH, and FAD. Finally, the volume of the mixtures were adjusted to 2.0 mL with 0.2 M sodium phosphate buffer, pH 6.6. Incubation was carried out in a Hungate tube under anaerobic condition, which was achieved by successive evacuation and nitrogen flushing 3 times. The tubes were incubated in a Lab-line shaking water bath for 2 h at 37 °C in the dark. At the end of the incubation period, 3.0 mL of ether was added to stop the reaction and was extracted directly. Extraction was repeated twice and all three extracts were combined for analysis by GLC.

RESULTS

Excretion of [¹⁴C]MPNE and Its Metabolites. Orally administered [¹⁴C]MPNE was rapidly absorbed and efficiently voided from the body (Figure 1). For the first 12 h posttreatment, the majority of the radioactivity was excreted in urine and a near-maximum elimination was reached within 4 days. At the end of the 20th day, approximately 83% of the administered dose was found in the excreta. The feces provided the major route of elimination, accounting for about 56% of the dose, and 27% was voided in the urine.

¹⁴C-Labeled Residue in the Tissue. At 24 h posttreatment the maximum total residue present in the body of rat was $5.3 \times 10^{-4}\%$ of the administered dose and decreased to only $0.06 \times 10^{-4}\%$ at the end of the 20th day. The tissue distribution of residual radiocarbon in tissues and organs is shown in Table II. At 24 h posttreatment the highest radiocarbon levels were found in the blood followed by the liver, G.I. tract, adipose tissue, kidney, lung, heart, spleen, and muscle. After 21 days a large part of the radiocarbon was eliminated from the tissue except in the adipose tissue, where most apolar pesticides are

Table III.   Relative Abundance (in Percent) of Metabolic Products in Feces Extracts As Analyzed by TLC in Solvent System B

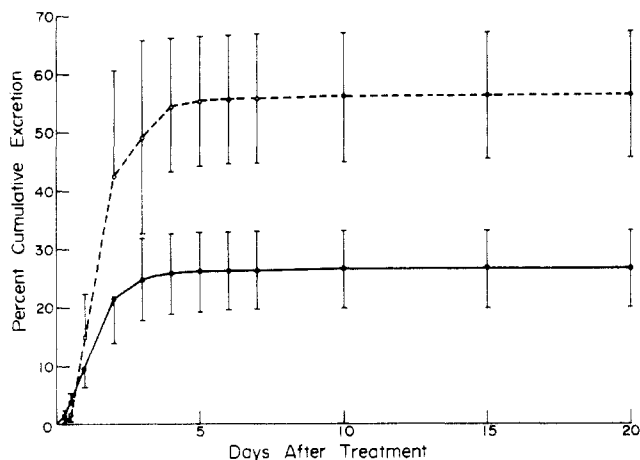| chemicals | MPNE | acetone phase | | methanol phase, ethyl acetate fraction |
		acetonitrile fraction	ethyl acetate fraction	
unknown	0	2.56	18.14	50.11
unknown	0	0.46	9.85	6.44
γ-hydroxy sulfoxide ether	0	1.09	11.17	31.79
β-hydroxy sulfoxide ether	0	0.53	7.41	4.97
γ-hydroxy sulfone ether	0	0.69	25.76	3.96
sulfoxide ether	0.70	23.81	22.26	1.55
sulfide ether (MPNE)	99.30	70.86	5.46	1.19

Figure 1. Cumulative excretion of radioactivity in feces (O) and urine (●) of rats (average of three rats).

stored. Only radioactive compound found in the adipose tissue was MPNE, which is the most apolar compound compared to the metabolites. It is noteworthy that radiocarbon levels in spleen, liver, and kidney was also high.

Extraction and Characteristics of ¹⁴C-Labeled Residues. The general scheme of extraction and partition of radiocarbon residues in the feces and urine are shown in Figures 2 and 3. Over 55% of the radiocarbon in feces was readily extracted by organic solvents; 22% was present in the acetone phase, and the remaining 33% was in methanol. Another 40% was nonextractable. In urine approximately 56% of the radiocarbon was present as

Table IV.   Relative Abundance (in Percent) of Metabolic Products in Urine Extract As Analyzed by TLC in Solvent System B

TLC fraction no.	MPNE	ethyl acetate fraction
unknown	0	36.09
unknown	0	23.88
unknown	0	18.01
γ-hydroxy sulfoxide ether	0	7.78
unknown	0	9.00
unknown[a]	0.58	5.18
sulfide ether (MPNE)	99.48	0.05

[a] There is no definite band in this fraction; it is just a dark shadow due to streaking.

solvent-extractable metabolites, and the remaining 41% was found in the aqueous phase. From the 56% solvent-extractable metabolites, practically none was hexane soluble, whereas over 91% of the radiocarbon was hexane insoluble.

Analysis of Metabolites. The nature of the metabolites in both ethyl acetate fractions from methanol and acetone extracts and the acetonitrile fraction of feces plus ethyl acetate fractions of urine were studied on TLC with a mobile phase, hexane–chloroform–acetonitrile (12:8:11), and compared to [¹⁴C]MPNE and nonradioactive analytical reference standards of the metabolites. Autoradiography of those plates were prepared, and the amount of radioactivity in silica gel corresponding to the darkened spots on the autoradiograms was radioassayed. The results are summarized in Tables III and IV. Further cleanup

Figure 2. Extraction scheme for the analysis of in vivo metabolites of MPNE in the feces. (*) These fractions were further analyzed by TLC. (**) This fraction was analyzed by deconjugation enzymes and dilute acid. The figures below each phase represent the percent radioactivity present in that phase as calculated from the initial amount. The solvent-extractable metabolites equal 55.27%; the solvent-inextractable metabolites equal 40.47%; the total equals 95.74%.

4-Methyl-3-(n-propylthio)phenyl 4-Nitrophenyl Ether

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```
                                    U r i n e
                                     100%
                                        |
                                        |  - Saturate with NaCl
                                        |  - Add HCL - pH 3.0
                                        |  - Ethyl acetate - 3 ml/ml (3X)
                        _____|_____
                       |                                    |
               Aqueous phase **                     Ethyl acetate phase
                   41.24%                                 55.68%
                                                             |
                                                             |  - Na2SO4 anhydrous
                                                             |  - Evaporate
                                                        Dry residue
                                                             |
                                                             |  - n-Hexane
                                            _____|_____
                                           |                                   |
                                   Hexane soluble                      Hexane insoluble
                                    metabolites                          metabolites
                                      0.07%                                   |
                                        |                                     |  - Ethyl acetate
                                        |  -Acetonitrile            Ethyl acetate phase
                                        |   partition (3X)                 50.92%
                    _____|_____                        |
                   |                                 |                       |  - Evaporate
             Hexane phase             Acetonitrile phase              Dry residue
                0.02%                      0.05%                             |
                                                                             |  - Ethyl acetate
                                                                             |  - Distilled
                                                                             |    water
                                                              _____|_____
                                                             |                               |
                                                     Aqueous phase **           Ethyl acetate *
                                                        14.25%                       phase
                                                                                    33.34%
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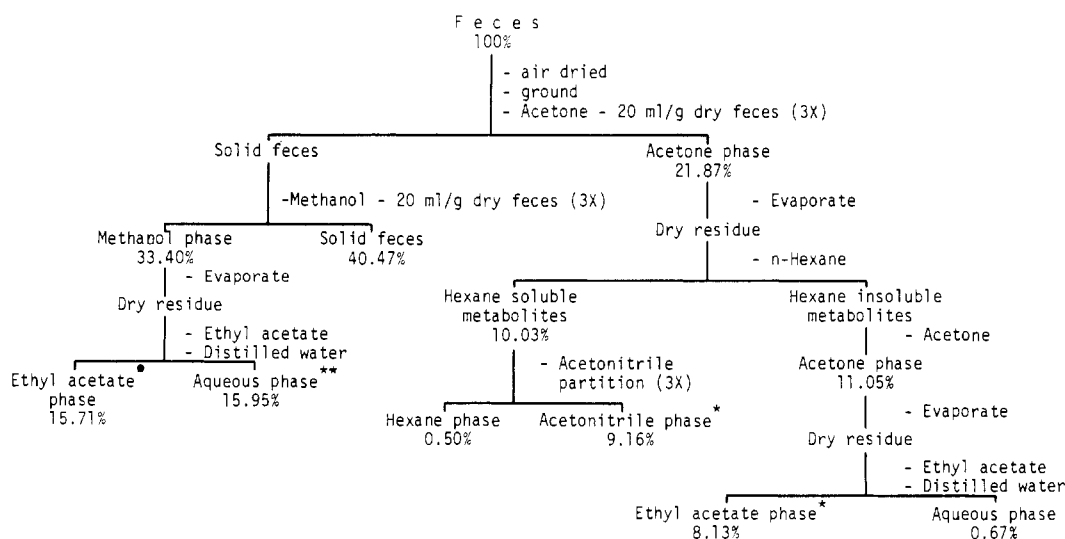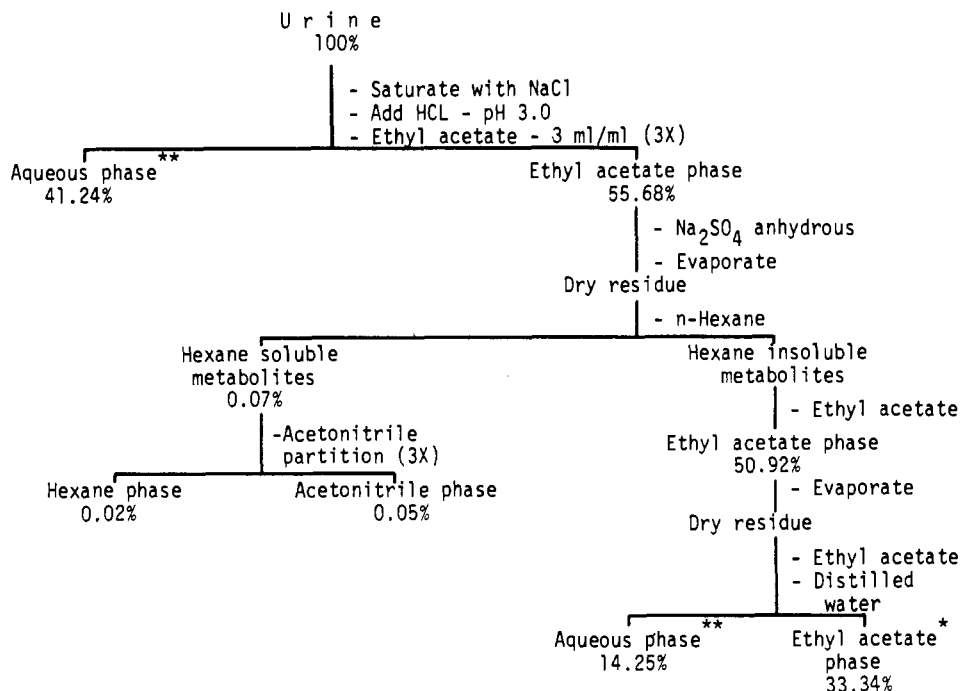**Figure 3.** Extraction scheme for the analysis of in vivo metabolites of MPNE in the urine. (*) This fraction was analyzed by TLC. (**) These fractions were analyzed by deconjugation enzymes and dilute acid. The figures below each phase represent the percent radioactivity present in that phase as calculated from the initial amount.

Table V. Effect of Various Cofactors and Inhibitors[a] upon the Production of Water-Soluble Metabolites in Vitro Using 20000g Crude Supernatant

treatment	% water-soluble metabolites formed	pmol of water-soluble metabolites formed/mg of protein in 2 h[b]
control	2.34 ± 0.33 (3)	120.93 ± 19.20 (3)
control + SKF-525A	1.04 ± 0.21 (4)[c]	52.35 ± 10.49 (4)[c]
control + GSH	1.13 ± 0.32 (4)	54.40 ± 14.52 (4)
control + GSH + NEM	0.85 ± 0.36 (4)	41.82 ± 18.06 (4)
control + GSH + DTT	0.98 ± 0.34 (4)	48.42 ± 17.78 (4)
control + GSH + PCMB	1.60 ± 0.58 (4)	79.30 ± 29.69 (4)
control + NADPH	9.96 ± 2.97 (4)[d]	493.89 ± 156.21 (4)[d]
control + NADPH + SKF-525A	1.14 ± 0.49 (4)[c]	65.03 ± 23.99 (4)[c]
control + UDPGA	1.34 ± 0.30 (4)	69.15 ± 17.38 (4)
control + UDPGA + NADPH	16.96 ± 2.48 (4)[d]	873.53 ± 163.82 (4)[d]
control + APS + ATP + Mg^{2+}	1.14 ± 0.47 (3)	61.17 ± 43.62 (3)
control + APS + ATP + Mg^{2+} + NADPH	17.94 ± 6.19 (4)[d]	944.02 ± 357.12 (4)[d]

[a] Concentrations of GSH, NEM, DTT, and PCMB were 4 mM, NADPH, UDPGA, ATP, and APS were 2 mM, MgCl$_2$ was 10 mM, and SKF-525A was 0.05 mM (2 × substrate concentration). Abbreviations: GSH for glutathione reduced, NEM for N-ethylmaleimide, DTT for dithiothreitol, and PCMB for p-(chloromercuri)benzoate. [b] Those above data are mean ± SE of two individual experiments with two replications each except treatments control + SKF-525A and control + APS + ATP + Mg^{2+}. [c] AKF-525A inhibition over either control or NADPH-fortified system is significant at $P \leqslant 0.05$. [d] NADPH stimulation over either control, UDPGA-fortified, or APS + ATP + Mg^{2+} fortified system is significant at $P \leqslant 0.05$.

and confirmation works were done on TLC and GLC, respectively. In addition to MPNE, two major metabolites γ-hydroxy sulfoxide ether and sulfoxide ether were found in feces. One minor metabolite was positively identified to be γ-hydroxy sulfone ether. In addition, there were a few unknown minor polar metabolites. In urine only γ-hydroxy sulfoxide ether was identified, though there were several unknown polar metabolites. No metabolites were released upon enzymatic or acid hydrolysis attempts on the aqueous phases of both feces and urine. It is quite possible, however, that the water-soluble metabolites were in other conjugated forms than glucuronide and sulfate.

In Vitro Studies. The results of in vitro incubation studies with crude supernatant are summarized in Table V. NADPH, a known cofactor to stimulate the mixed-function oxidase, significantly and consistently increased the conversion of [^{14}C]MPNE into water-soluble metabolites. SKF-525A, a known inhibitor of the mixed-function oxidase, significantly inhibited the production of water-

soluble metabolites. These data support the notion that these oxidative enzymes are important in the metabolic process of MPNE. The addition of GSH did not increase the production of water-soluble metabolites. The presence of inhibitors such as NEM, DTT, and PCMB did not result in the decrease of the production of water-soluble metabolites. The result indicates that the metabolism of MPNE is mainly carried out by mixed-function oxidase and not by glutathione S-transferase. As for conjugation potentials of these metabolites, it is known that UDPGA is required for the formation of glucuronide conjugate, and ATP, APS, and Mg^{2+} are required for the formation of sulfate conjugate. In both cases the presence of NADPH is a prerequisite to significantly increase the production of primary metabolites. For verification of the localization of these metabolic enzymes, experiments were conducted by using both the 100000g microsome and supernatant. The results are summarized in Table VI. In these experiments only the NADPH-UDPGA combination sig-

Table VI. Effect of Various Cofactors and Inhibitors[a] upon the Production of Water-Soluble Metabolites in Vitro Using Both 100000*g* Microsome and Supernatant

treatment	% water-soluble metabolites formed from added MPNE[b]	pmol of water-soluble metabolites formed/ mg of protein in 2 h
microsome		
control	11.26 ± 1.54 (4)	1279.31 ± 175.03 (4)
control + SKF-525A	3.91 ± 0.36 (4)[c]	444.76 ± 40.97 (4)[c]
control + NADPH	29.48 ± 0.91 (4)[d]	3348.91 ± 102.60 (4)[d]
control + NADPH + SKF-525A	17.71 ± 1.41 (4)[c]	2012.58 ± 160.35 (4)[c]
control + UDPGA	8.94 ± 1.19 (4)	1015.52 ± 135.44 (4)
control + UDPGA + NADPH	40.44 ± 3.57 (4)[d]	4593.48 ± 405.72 (4)[d]
control + UDPGA + NADPH + SKF-525A	19.33 ± 0.47 (4)[c]	2196.13 ± 53.61 (4)[c]
supernatant		
control	1.18 ± 0.31 (4)	140.01 ± 37.28 (4)
control + GSH	3.09 ± 0.91 (4)	367.02 ± 107.59 (4)
control + GSH + NEM	2.96 ± 0.57 (4)	351.98 ± 67.88 (4)
control + GSH + DTT	2.12 ± 0.32 (4)	252.19 ± 37.85 (4)
control + GSH + PCMB	3.00 ± 0.63 (4)	357.30 ± 74.69 (4)

[a] Concentrations of GSH, NEM, DTT, and PCMB were 4 mM; NADPH and UDPGA were 2 mM, and SKF-525A was 0.05 mM. [b] Those above data are mean ± SE of two individual experiments with two replications each. [c] SKF-525A inhibition over either control or NADPH-fortified system is significant at $P \leq 0.05$. [d] NADPH stimulation over either control or UDPGA-fortified system is significant at $P \leq 0.05$.

Table VII. Relative Abundance (in Percent) of Ether-Extractable Metabolites of [14C]MPNE in Vitro As Analyzed by TLC in Solvent System A

chemicals	MPNE	crude supernatant only	crude supernatant + GSH	crude supernatant + NADPH	crude supernatant + NADPH + SKF-525A
unknown	0	0	0	11.47	1.06
γ-hydroxy sulfoxide ether	0	0	0	61.48	3.94
β-hydroxy sulfoxide ether	0	0	0	4.62	6.60
γ-hydroxy sulfone ether	0	0	0	3.12	0.89
β-hydroxy sulfone ether	0	0.42	0.19	17.50	3.67
sulfoxide ether	0.37	11.61	6.39	0.40	40.78
sulfone ether	0	0	0	0.38	13.37
sulfide ether (MPNE)	99.63	87.97	93.42	1.03	29.70

Table VIII. Relative Abundance (in Percent) of the Ether-Extractable Metabolites of [14C]MPNE in Vitro As Analyzed by TLC in Solvent System A

chemicals[a]	MPNE	crude supernatant without NADPH[b]	crude supernatant with NADPH at incubation time (min)						
			5	10	15	30	60	120	180
unknown	0	0.20	0.77	1.95	2.65	5.72	7.76	9.33	9.11
unknown	0	0.07	0.53	0.92	1.17	1.59	2.17	1.71	1.19
γ-hydroxy sulfoxide ether (II)	0	0.30	0.88	2.88	6.12	20.38	40.86	58.35	63.88
β-hydroxy sulfoxide ether (IX)	0	0.17	6.34	9.12	11.42	12.77	8.80	5.16	3.94
γ-hydroxy sulfone ether (V)	0	0.30	0.80	1.56	3.15	4.02	3.72	3.97	5.30
β-hydroxy sulfone ether (VIII)	0	4.60	6.75	14.12	22.62	35.08	30.37	17.91	13.15
sulfoxide ether (II)	1.93	19.22	45.52	29.90	26.09	10.93	1.90	2.92	2.06
sulfone ether (III)	0	34.07	12.05	16.35	17.15	7.92	1.53	0.65	0.34
MPNE (I)	98.07	41.08	26.37	23.27	9.64	1.60	2.89	0.68	1.02

[a] Compound number corresponds to that shown in Figure 5. [b] This system was incubated for 120 min.

nificantly increased the production of water-soluble metabolites.

Characterization of in Vitro Metabolites. The ether extracts were analyzed by TLC using a mixture of hexane–chloroform–acetonitrile (3:2:2) as a mobile phase, and autoradiograms were prepared. Quantitation of the radioactive metabolites was carried out, and the results are summarized in Table VII. Approximately 99% of the added parent compound was degraded. The addition of the inhibitor to the system reduced the degradation of the parent compound to 70% without producing any significant amount of polar metabolites. When the inhibitor was present, the metabolism proceeded only until the formation of sulfoxide and sulfone ether. The overall rate of metabolism can be judged from the rate of production of water-soluble metabolites. The result of such an experiment is summarized in Figure 4. Without NADPH the amount of ether-extractable metabolites decreased from 89% to approximately 79%. Within 3 h of incubation time, the amount of ether-extractable metabolites decreased to 56% in the presence of NADPH. The content of the ether extracts were analyzed by TLC and treated in the same way as described before. The relative abundance of metabolites are summarized in Table VIII. From these results a better understanding of the metabolic sequence has been obtained. During the oxidation process, sulfoxide ether must be formed as the first metabolite which was further oxidized into sulfone ether and eventually to more polar metabolites such as alcohols. The less polar compounds such as MPNE, sulfoxide ether, and sulfone ether began to disappear after 30 min of incubation, whereas polar metabolites (R_f 0.28) started to appear
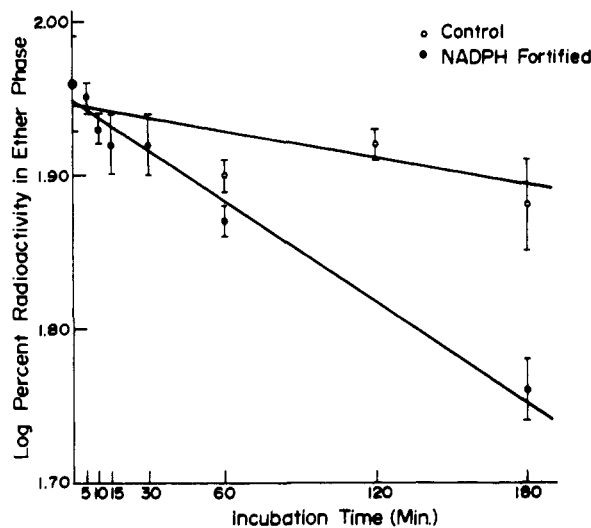
Figure 4. Percentages of ether-extractable radiocarbon after in vitro incubation with the rat liver microsomal preparation.

Table IX. Metabolism of Sulfoxide Ether in Rat Liver Microsomes and Supernatant under Anaerobic Conditions[a]

treatment	amino sulfoxide ether formed, nmol/mg of protein in 2 h	
	microsome	supernatant
no cofactor	3.36 ± 1.35	3.02 ± 0.29
boiled + NADPH + FAD	2.96 ± 0.13	2.92 ± 1.04
+NADPH	20.33 ± 3.12	18.45 ± 1.55
+NADH	5.48 ± 0.33	12.62 ± 2.38
+FAD	3.67 ± 0.17	7.89 ± 0.46
+NADPH + FAD	52.49 ± 0.39	12.53 ± 1.04
+NAD + FAD	25.31 ± 1.84	8.02 ± 0.44

[a] All data are mean ± SE of three replications. Concentrations of NADH and NADPH were 2.5 mM, FAD was 0.25 mM, and amount of sulfoxide ether initially added was 1 µmol.

within 15 min. The major metabolic products of sulfoxide and sulfone were γ-hydroxy sulfoxide ether and β-hydroxy sulfone ether, sulfoxide ether, and sulfone ether. In addition, five unidentified minor polar metabolites were recognized. Their total amount was approximately 10%.

Treatments of the aqueous phase with acid or deconjugation enzymes did not increase the amount of ether-extractable metabolites. It might be due to the increase of forms other than those two types of conjugates. This result also indicates the absence of phenolic (i.e., ring hydroxylated) metabolites.

Reductive Metabolism of Sulfoxide Ether in Vitro.
Reductive metabolism of sulfoxide ether in rat liver microsome and 100000g supernatant was studied under anaerobic condition, and the results are summarized in Table IX. GLC analysis of the ether extracts showed that only NADPH enhances the reductive metabolism activity of sulfoxide ether to form amino sulfoxide ether in the microsome. FAD, when added to a microsomal mixture containing NADPH, showed a strong stimulation ability. When FAD was added to a microsomal mixture containing NADH, stimulation of the reductive metabolism was not as strong compared to FAD plus NADPH. In 100000g supernatant all three cofactors stimulate the reductive metabolism of sulfoxide ether, but only NADPH showed the strongest stimulation. FAD, when added to 100000g supernatant mixture containing either NADPH or NADH, did not show the stimulating ability. Heat inactivation of the enzymes destroyed its activity, which indicated that the enzymes responsible for the reductive metabolism of
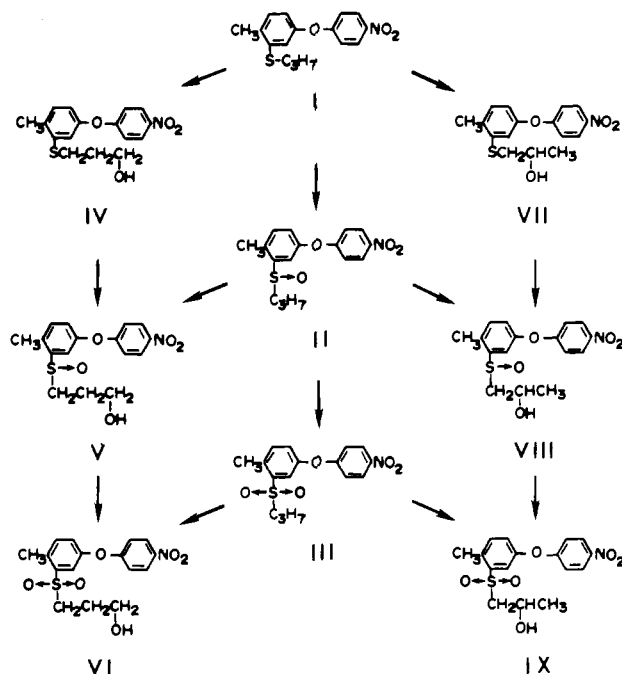

Figure 5. Proposed major metabolic pathway of MPNE in vivo in the rat.

sulfoxide ether is thermolabile.

DISCUSSION

A proposed metabolic pathway of MPNE in rats is shown in Figure 5. The sulfoxidation reaction and hydroxylation at the γ-position of the n-propyl side chain are the major reactions. To understand the significance of such a metabolic pattern, one must look into various metabolic reactions that have been reported to occur on diphenyl ether compounds. Hydrolysis of diclofop-methyl to diclofop was the initial degradation reaction, and aryl hydroxylation and phenol conjugation were predominant in the detoxication process of diclofop-methyl in wheat. No ether bond cleavage was observed (Shimabukuro et al., 1979). In sheep 11% of the total administered [14C]-nitrofen was excreted in feces. Conjugates of amino-nitrofen, 5-hydroxy ether, and a small amount of phenol were found in urine. Ether bond cleavage was a minor pathway (Hunt et al., 1977). The study on dairy cows showed that no nitrofen residue was found in milk, urine, and feces when they were fed 5 ppm of nitrofen for 4 days. The major metabolite found was aminonitrofen which was thought to be formed by nitro reduction in the rumen fluid, and no conjugates were found after hydrolysis (Gutenmann and Lisk, 1967). It was found that in rape, redroot pigweed, and green foxtail, only a small amount of metabolite might be formed by ether bond cleavage of nitrofen (Hawton and Stobbe, 1971). In all those studies it is clear that ether bond cleavage is not a major degradation reaction among diphenyl ethers, indicating that diphenyl ethers are biologically stable compounds (Williams, 1959). Fluorodifen seems to be an exception, since it is rapily cleaved at this position. It was found that hydrolysis of the diphenyl ether linkage of fluorodifen to be the major pathway of degradation resulting in the p-nitrophenyl moiety and formation of conjugates with β-D-glucoside in soybean and corn plants (Geissbuehler et al., 1972). The major metabolite of fluorodifen found in rat urine was [2-nitro-4-(trifluoromethyl)phenyl]mercapturic acid, which accounted for 41% of the dose administered. This means that in rat the mercapturic acid pathway is very important in its detoxication process (Lamoureux and Davison, 1975).

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The principal metabolite of unsubstituted diphenyl ethers fed to rabbits was 4-hydroxydiphenyl ether which was excreted mainly as its glucuronide. In this case, there is no ether bond cleavage (Williams, 1959). However, the limited reduction of the nitro substituents and rapid cleavage of the ether linkage to form the corresponding phenol derivatives were found to be the major reactions of fluorodifen degradation in soybean plants (Rogers, 1971). It was found that glutathione transferase catalyzed the most important reaction in the metabolism of fluorodifen in peanuts. The enzyme catalyzes the cleavage of fluorodifen and forms conjugates (Shimabukuro et al., 1973). The reason why no ether bond cleavage takes place in the case of MPNE could be that the ortho substitution in this case is not strongly electron withdrawing.

Another characteristic of MPNE metabolism is that there were no detectable glucuronide or sulfate conjugates found among in vivo metabolic products. By contrast, in vitro studies indicated that some of the metabolites are capable of forming conjugates as judged by the increase in water-soluble metabolites as a result of incubation with UDPGA or APS (plus ATP and $MgCl_2$) in the presence of NADPH. Two reasons could be postulated for this discrepancy. First, the in vitro studies may not necessarily represent the processes that take place in vivo. Second, there is a chance that the conjugates which were formed in the liver system were enzymatically cleaved during the extraction process in the intestine or in the blood so that no conjugates were recognized either in urine or feces.

The third characteristic of MPNE is that the addition of reduced glutathione, a known cofactor for the formation of glutathione conjugates, did not increase the formation of water-soluble metabolites. It means that MPNE and its metabolites were not preferred substrate for the enzyme glutathione transferase, in contrast to the case with fluorodifen. MPNE is also unique in that it is not ring hydroxylated despite the fact that it is metabolized by the mixed-function oxidase system. In the case of diclofop-methyl ring hydroxylation was the major degradation reaction. From in vitro studies γ-hydroxy sulfoxide ether and β-hydroxy sulfone ether were recognized as major metabolites, whereas β-hydroxy sulfoxide ether, γ-hydroxy sulfone ether, sulfoxide ether, and sulfone ether were minor ones. In feces the major metabolites detected other than MPNE were γ-hydroxy sulfoxide ether and sulfoxide ether plus γ-hydroxy sulfone ether as a minor one, while in urine γ-hydroxy sulfoxide ether was the only one that was recognized. It is probable that in the case of MPNE there are a number of readily oxidizable sites in its side chain as shown above, and as such, ring hydroxylation does not proceed.

Upon analysis of body fat for its residue, it was found that the parent compound was the only compound stored in the fat tissue. This is understandable because MPNE is more apolar than any of its metabolites.

Liver has been known primarily as an oxidative organ, reductive metabolism is considered as minor reaction, and yet, in many cases reductive reactions could play an important role in the degradation of pesticides and toxic chemicals. Good examples are the cases with DDT and toxaphene, where reductive dechlorination is the essential and the initial rate-limiting reaction (Esaac and Matsumura, 1979, 1980). A few nitro compounds are reduced to primary amines by a liver enzyme system that can use either NADH or NADPH as its electron donor (Fouts and Brodie, 1957). Nitro reductase is known to be active under anaerobic conditions and is virtually inactive in air. Reduction of *p*-nitrobenzoate to *p*-aminobenzoate is catalyzed by an enzyme localized in liver microsome, and the process might be mediated by one of the cytochromes, either cytochrome b_5 or cytochrome P-450 (Gillette et al., 1968).

In the case of MPNE, it is rather surprising that amino-MPNE was not found among in vivo degradation products, as in the case of nitrophen where the amino derivative is the most frequently encountered metabolic products in many biological systems.

Reductive metabolism reactions, however, take place under anaerobic condition in an in vitro experiment. It is possible that the amino moiety of amino sulfoxide ether might have been formed in the liver but oxidized back to the nitro moiety during the excretion process, which makes it undetectable in vivo. The system responsible for the reduction of the nitro moiety of MPNE to primary amine is NADPH stimulated. In addition, in the microsomal system FAD strongly stimulated the reductive metabolism when NADPH was present and, to a lesser extent, when NADH was present. Some reductive metabolism activity does occur in the supernatant fraction to a lesser extent as compared to the microsomal system. An interesting observation is that neither the sulfoxide nor sulfone moiety is being reduced under the condition that reduced the nitro group. It is not possible to conclude that the latter group is generally more susceptible to reducing reactions than the former group because of the lack of examples in the literature, but the phenomenon is interesting enough to warrant a future investigation.

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