Original Article

New Metabolites of Phenthoate in Mammalian Systems

Kazuki TOEDA, Toru MIYAMOTO, Shigeru KATO*
and Izuru YAMAMOTO

*Department of Agricultural Chemistry, Tokyo University of Agriculture,
Setagaya-ku, Tokyo 156, Japan*

**NODAI Research Institute, Tokyo University of Agriculture,
Setagaya-ku, Tokyo 156, Japan*

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Twelve transformation products from phenthoate (PAP)[$(\text{CH}_3\text{O})_2\text{P}(\text{S})\text{SCH}(\text{Ph})\text{COOC}_2\text{H}_5$] were identified by using intact mice, rat liver subcellular fractions and peracid. Other than those previously reported, five products were newly found: $(\text{CH}_3\text{O})_2\text{P}(\text{O})\text{SCH}(\text{Ph})\text{COOH}$ by mice and microsome-NADPH system (mfo), $\text{CH}_3\text{S}(\text{O})\text{CH}(\text{Ph})\text{COOH}$ by mice, $\text{HOCH}(\text{Ph})\text{COOC}_2\text{H}_5$ by mice, mfo and cytosol, $(\text{CH}_3\text{S})(\text{CH}_3\text{O})\text{P}(\text{O})\text{SCH}(\text{Ph})\text{COOC}_2\text{H}_5$ (PAP S-isomer) by mfo and $(\text{CH}_3\text{O})_2\text{P}(\text{O})\text{SSCH}(\text{Ph})\text{COOC}_2\text{H}_5$ (phosphinyl disulfide) by peracid oxidation. Based on the identification of the products, the initial modifications seem to occur in five ways on PAP: i) hydrolysis of carboxy ethyl ester; ii) demethylation; iii) formation of phosphorus oxythionate; iv) cleavage of C–S bond; and v) isomerization. Further modifications result in various products. Twenty-three unidentified metabolites were found. PAP S-isomer by mfo and phosphinyl disulfide by peracid oxidation are considered to be produced in mice, but their instability does not allow their finding.

The organophosphorus insecticide phenthoate (dimethyl S- α -ethoxycarbonylbenzyl phosphorodithioate or PAP) has low toxicity to mammals, while highly effective against various pest insects. Its fate in plants, houseflies, mice, air, soils and water, and decomposition by light have been reported.^{1–4)} Also, enantiomers of PAP were prepared and compared in terms of toxicity and efficacy.⁵⁾ This paper reports the metabolic fate of PAP in animals with emphasis on new metabolites by using peracid, rat liver subcellular fractions and intact mice.

MATERIALS AND METHODS

1. ¹⁴C-PAP

¹⁴C-PAP was provided by Sumitomo Chemical Co., Ltd. Specific activity was 5.67 mCi/mmol, over 99% purity.

2. Reference Compounds

Structures and designations are given in Table 1. All the compounds except **1**, **22** and

23 were synthesized in our laboratory which will be reported elsewhere.

3. Thin-layer Chromatography (TLC)

Silica gel chromatoplate (Merck 60F₂₅₄, precoated, 0.2 mm with fluorescence indicator) was used for one or two dimensional separation. Eight solvent systems used and the *R_f* values of the reference compounds are listed in Table 1.

4. Gas Chromatography-Mass Fragmentography (MF) and -Mass Chromatography (MC)

A Shimadzu Auto GCMS-6020 gas chromatograph-mass spectrometer was used for MF and MC. Operating conditions were as follows: column, a silanized 50 cm $\times$ 2.5 mm i.d. glass column packed with 5% OV-1 on Gas chrom Q (60–80 mesh); flow rate of carrier gas, helium, 15 ml/min; ionization source, 260°C; ionizing voltage, 20 eV; accelerating voltage, 3.5 kV. The monitoring ions which were selected for MF and MC of compounds and

Table 1 TLC *R_f* values of PAP and its related compounds.

Compound	Designation	Solvent system ^{a)}										
		A	B	C	D	E	F	G	G'	H		
(1) (MeO) ₂ P(S)SCH(Ph)COOEt	PAP	0.39	0.56									
(2) MeO(MeS)P(O)SCH(Ph)COOEt	PAP S-isomer	0.05	0.37									
(3) (MeO) ₂ P(O)SCH(Ph)COOEt	PAP oxon	0.04	0.33									
(4) (MeO) ₂ P(S)SCH(Ph)COOH	PAP acid			0.41	0.47	0.34						
(5) (MeO) ₂ P(O)SCH(Ph)COOH	PAP oxon acid			0.34		0.32						
(6) MeO(HO)P(S)SCH(Ph)COOEt	Demethyl PAP			0.38		0.50						
(7) MeO(HO)P(O)SCH(Ph)COOEt	Demethyl PAP oxon			0.20	0.36	0.35						
(8) MeO(HO)P(S)SCH(Ph)COOH	Demethyl PAP acid			0.23	0.23							
(9) MeO(HO)P(O)SCH(Ph)COOH	Demethyl PAP oxon acid			0.04	0.05	0.02						
(10) (MeO) ₂ P(O)SSCH(Ph)COOEt	PAP phosphinyl disulfide	0.35						0.11				
(11) (MeO) ₂ P(O)SCH ₂ CH(Ph)COOEt		0.23						0.08				
(12) (MeO) ₂ P(O)SCH ₂ CH(Ph)COOMe		0.22								0.43		
(13) (MeO) ₂ P(S)SCH(2-MeO-Ph)COOEt										0.46		
(14) (MeO) ₂ P(S)SCH(3-MeO-Ph)COOEt										0.46		
(15) (MeO) ₂ P(S)SCH(4-MeO-Ph)COOEt										0.46		
(16) (MeO) ₂ P(O)SCH(2-MeO-Ph)COOEt							0.53		0.28			
(17) (MeO) ₂ P(O)SCH(3-MeO-Ph)COOEt		0.28					0.61		0.29			
(18) (MeO) ₂ P(O)SCH(4-MeO-Ph)COOEt		0.29					0.58		0.28			
(19) (MeO) ₂ P(O)SCH(2-MeO-Ph)COOMe		0.28					0.48		0.25			
(20) (MeO) ₂ P(O)SCH(3-MeO-Ph)COOMe		0.25					0.53		0.25			
(21) (MeO) ₂ P(O)SCH(4-MeO-Ph)COOMe		0.25					0.50		0.26			
(22) HSCH(Ph)COOEt		0.26										
(23) [-SCH(Ph)COOEt] ₂		0.36										
(24) HSCH(Ph)COOH		0.30	0.58									
(25) [-SCH(Ph)COOH] ₂					0.30							
(26) HOCH(Ph)COOEt	Ethyl mandelate	0.10	0.41									
(27) HOCH(Ph)COOH	Mandelic acid				0.23							
(28) MeSCH(Ph)COOEt		0.35	0.59		0.51		0.32					
(29) MeSCH(Ph)COOH				0.11								
(30) MeS(O)CH(Ph)COOEt					0.14	0.21	0.28					
(31) MeS(O)CH(Ph)COOH		0.09	0.43									
(32) MeS(O ₂)CH(Ph)COOEt		0.36	0.54									
(33) PhCOCOEt					0.19	0.53	0.35					
(34) PhCOCOEt					0.02	0.07						
(35) HO ₂ SCH(Ph)COOH												
(36) MeO ₃ SCH(Ph)COOMe		0.11	0.50									

^{a)} Solvent systems; A: benzene, B: benzene-ethyl acetate (2 : 1 v/v), C: ethyl acetate-methanol-acetic acid (100 : 20 : 4 v/v), D: acetone-butanol-water-ammonia water (7 : 2 : 0.8 : 0.2 v/v), E: acetonitrile-water-ammonia water (8 : 1.8 : 0.2 v/v), F: ether (unsaturated), G: hexane-ether (2 : 1 v/v), G': hexane-ether (2 : 1 v/v, unsaturated), H: hexane-ether (1 : 3 v/v).

Table 2 Monitoring ions and *R_t* of chemicals on MC and MF analysis.

Chemicals	Analytical mode	<i>m/z</i> of monitoring ion	<i>R_t</i> (column oven temperature, °C)
PAP (1)	MF	274, 273, 217	2.03 (225)
PAP <i>S</i> -isomer (2)	MF	274, 273, 245, 217	2.76 (225)
PAP oxon (3)	MF	258	1.62 (225)
Ethyl mandelate (26)	MF	180, 107	2.58 (140)
Compound 32	MF	164, 163	1.86 (210)
Compounds 13 , 14 and 15	MF	304, 193	13, 2.92; 14, 3.19; 15, 3.71 (230)
Compounds 16 , 17 and 18	MC	288, 193	16, 2.35; 17, 2.56; 18, 2.52 (230)
Compound 11	MF	208, 187	1.88 (230)
Methyl ester of compound 4	MC	306, 274, 246, 125	7.90 ^{a)}
Methyl ester of compound 5	MF	290, 258, 231	1.91 (215)
Compounds 19 , 20 and 21	MC	288, 177, 151	19, 2.01; 20, 2.20; 21, 2.71 (230)
Compound 12	MF	194, 187	1.65 (230)

^{a)} Temperature programming: 150°C for 1 min, then 150–280°C (8°C/min).

column temperatures were shown in Table 2.

5. Radioactivity Assay

Radioactive compounds were detected on the plates by autoradiography with a SAKURA X-ray film. A radioactive silica gel zone was cut from the plate, kept overnight in a vial with the following scintillator mixture and the radioactivity was counted with an Aloka LSC-903 liquid scintillation counter. The above samples or any organosoluble samples were added with 10 ml of scintillator mixture (DPO 4 g, POPOP 0.1 g, toluene 600 ml and ethyl cellosolve 400 ml). Aquatic samples were mixed with 10 ml of Aquazol-2 (New England Nuclear). Solid samples were burned in an Aloka ASC-112 automatic sample combustion system.

EXPERIMENTAL

1. Peracid Oxidation

To ¹⁴C-PAP (15 mg, 1.4 × 10⁶ dpm) in 5 ml of CH₂Cl₂, a solution of 3-chloroperbenzoic acid (MCPBA, purity 80%, 10.2, 20.4 or 30.6 mg which correspond to equi-, twice or thrice moles to PAP) in 5 ml of CH₂Cl₂ was added dropwise under ice-cooled conditions. After the addition the mixture was stirred for 15 min and kept overnight at –20°C. A portion of the reaction mixture was submitted to two dimensional TLC (systems C and B). A complicated chromatogram (not shown) suggested decomposition of products on TLC. This

Fig. 1 TLC autoradiograms showing peracid oxidation products of ¹⁴C-PAP.

UV irradiation was made after the first development (see text).

might be caused by production of unstable PAP phosphinyl disulfide (**10**) which is expected as a product of peracid oxidation of phosphorodithioate.⁶⁾ Therefore, other approach was tried (Fig. 1). Firstly, the reaction mixture was developed with system A. PAP (*R_f* 0.39) and [–SCH(Ph)COOEt]₂ (**23**) (*R_f* 0.30) were identified. The spots of lower *R_f* values seem to include many products. Secondly, the reaction mixture was added with an excess amount of diazomethane in ether and kept for 3 hr in dark at 4°C. This procedure aims to stabilize phosphinyl disulfide by derivatization and is expected to give the following reaction (a) if PAP phosphinyl

Product	Products found (%)		
	MCPBA/PAP ratio		
	1	2	3
PAP (1)	46.0	12.1	8.0
PAP oxon (3)	31.6	29.6	34.1
PAP phosphinyl disulfide (10)	8.6	18.5	12.8
[–SCH(Ph)COOEt] ₂ (23)	1.7	4.2	<7.3
Others	12.1	35.6	>37.8
Total	100	100	100

Table 4 Metabolism of ^{14}C -PAP by rat liver microsome with or without NADPH under different pH conditions.

Product	% of applied radiocarbon					
	pH 7.5		pH 7.0		pH 6.5	
	+	-	+	-	+	-
PAP (1)	1.5	0.8	2.6	0.5	6.1	1.3
$[-\text{SCH}(\text{Ph})\text{COOEt}]_2$ (23)	0.3	ND	0.5	ND	0.7	ND
PAP oxon (3)	0.3	ND	2.0	ND	12.0	0.1
PAP acid (4)	67.6	93.0	63.2	94.4	67.4	93.0
M-1 (Ethyl mandelate)	1.4	0.3	2.1	0.3	2.2	0.9
M-2 (PAP <i>S</i> -isomer)	0.9	ND	0.5	ND	0.4	ND
M-3	0.3	0.1	0.6	ND	0.4	0.6
M-4	1.9	2.8	2.9	2.0	1.3	2.5
M-5	3.4	ND	3.0	ND	1.0	ND
M-6	0.5	1.3	0.5	0.4	0.3	0.3
M-7 (PAP oxon acid)	12.2	0.1	12.3	0.1	2.8	0.1
Origin	1.5	0.1	0.8	0.1	0.6	0.1
Aqueous fraction	8.2	1.5	9.0	2.2	4.8	1.1
Total	100	100	100	100	100	100

^{a)} +: with NADPH, -: without NADPH.

Fig. 3 TLC autoradiograms of metabolites of ^{14}C -PAP by rat liver microsome.

acid (**4**), $[-\text{SCH}(\text{Ph})\text{COOEt}]_2$ (**23**), M-1 through M-7 appeared on the chromatograms. Quantitation of these metabolites is shown in Table 4. With or without NADPH, PAP acid (**4**) was the major metabolite. Without NADPH, PAP acid (**4**) produced more than 93% at any pH examined. However, in the presence of NADPH, it reduced to 63–67%, and many minor metabolites appeared or increased in amount except M-4 and M-6. Also, oxon forma-

tion at pH 6.5 and M-7 formation at pH 7.5 and 7.0 became remarkable. Radiocarbon in the aqueous layer also increased with NADPH. M-1 was identified as ethyl mandelate (**26**) by MC as discussed later. Hydroxylation on the phenyl ring did not seem to occur because methylation of the extract did not give any corresponding spots to compounds **13** through **21**. M-2 produced in the presence of NADPH seems to be PAP *S*-isomer (**2**) by *R_f* value. Confirmation was made by MF as discussed later. M-7 was identified as PAP oxon acid (**5**) by MC as discussed later.

2.1.2 Cytosol

After incubation with cytosol at 37°C for 60 min, the mixture was adjusted to pH 1 with 1 N HCl and extracted thrice each time with 20 ml of CH_2Cl_2 . With cytosol fraction in the presence or absence of GSH, metabolism proceeded extensively. Identification of the metabolites in the extract was conducted by TLC as shown in Fig. 4. PAP (**1**), PAP acid (**4**), demethyl PAP (**6**), ethyl mandelate (**26**), S-1 through S-6 appeared on the chromatograms. Quantitation of these metabolites is shown in Table 5. With or without GSH, PAP acid (**4**) was the major metabolite. However, it decreased with GSH, while aqueous fraction,

Table 5 Metabolism of ^{14}C -PAP by rat liver cytosol with or without GSH under different pH conditions.

Product	% of applied radiocarbon					
	pH 7.5		pH 7.0		pH 6.5	
	+ ^{a)}	-	+	-	+	-
PAP (1)	4.5	1.7	2.0	7.1	7.8	9.7
Ethyl mandelate (26)	0.8	0.9	0.8	0.6	1.3	1.0
PAP acid (4)	60.3	74.1	55.6	80.4	48.6	79.4
Demethyl PAP (6)	4.3	2.3	2.8	1.5	7.5	1.1
S-1	0.7	0.4	0.5	0.3	0.8	0.3
S-2	0.8	0.5	0.7	0.3	0.5	0.3
S-3	5.4	0.8	4.8	0.9	2.2	0.7
S-4	0.4	0.6	0.5	0.6	0.4	0.4
S-5	0.2	0.2	0.2	ND	0.4	ND
S-6	0.1	0.2	0.2	ND	0.1	ND
Origin	0.1	0.2	0.1	0.2	0.1	0.2
Aqueous fraction	22.4	18.1	31.8	8.1	30.3	6.9
Total	100	100	100	100	100	100

^{a)} + : with GSH, - : without GSH. Fig. 4 TLC autoradiograms of metabolites of ^{14}C -PAP by rat liver cytosol.

demethyl PAP (6) and several minor metabolites, particularly S-3, increased or appeared. This trend was more remarkable, in general, at lower pHs. This result suggests that with GSH, particularly at lower pHs, demethylation and conjugation by GSH S-transferase-GSH system proceed as well as hydrolysis of ethoxycarbonyl moiety by esterase system. Formation of ethyl mandelate (26) was not affected by GSH. For confirmation, ethyl mandelate (26), after the addition of unlabeled

compound, was hydrolyzed to mandelic acid (27) with aqueous 1% NaOH at 40°C; PAP acid (4) and demethyl PAP (6) were treated with diazomethane to give the corresponding methylated derivatives.

2.2 Phosphinyl disulfide

Phosphinyl disulfide type compounds are generally unstable and requires derivatization with diazomethane for detection. As the formation of PAP phosphinyl disulfide (10) was confirmed by peracid oxidation, an attempt was made to find this compound from CH_2Cl_2 extract obtained on microsome-NADPH metabolism by the method which was successful for detecting the phosphinyl disulfide from S-(4-chlorophenyl) diethyl phosphorodithioate.⁷⁾ However, the corresponding compounds 11 and 11' were not detected. Probably PAP phosphinyl disulfide (10) is too unstable to be present in such incubation mixture.

3. Analysis of PAP Metabolites from Unlabeled PAP by MF and MC

Many metabolites were identified from ^{14}C -PAP by cochromatography with the authentic compounds. However, more rigid confirmation will be required for some of the metabolites. For example, M-2 was assigned as PAP S-

Fig. 5 Analysis by MF and MC of metabolites of PAP.

isomer, but such isomerization occurs usually by heat or light, and formation under biological conditions has only been reported recently by us for other compounds.⁷⁾ M-7 was methylated with diazomethane, and the methylated product and its photoproducts gave a little lower *R_f* values than PAP oxon (3) and its photoproducts on TLC (system B). Therefore, M-7 seems to be either PAP oxon acid (5) or PAP oxon hydroxylated on the phenyl moiety. In other aspects, other compounds having carboxylic acid or carboethoxy group are expected to be present as PAP metabolites. Identification of these metabolites was tried by MF and MC by the following procedures.

A microsomal incubation mixture consisted of PAP (2 μ M) in 2 ml of 40% aqueous ethanol, 10 ml of 20% homogenate of microsome in 0.25 M sucrose, NADPH (100 μ M) in 1.0 ml of 0.25 M sucrose, and 26 ml of 0.25 M sucrose in 1/15 M phosphate buffer (pH 7.5) (total 40 ml). Modification was made by subtracting NADPH or NADPH plus microsome. After incubation at 37°C for 15 min, the reaction was stopped by the addition of 5 ml of CH₂Cl₂. Two sets of incubation were carried out and combined for the following treatment (see Fig. 5). The combined incubation mixture (A)

Table 6 Metabolites of PAP by rat liver microsome with or without NADPH.

Product	% of products		
	+	—	c
PAP oxon (3)	0.13	ND	ND
PAP S-isomer (2)	3.90	1.90	ND

a) +: with NADPH, —: without NADPH, c: without NADPH and microsome.

was extracted thrice each time with 160 ml of CH₂Cl₂. The extract (B) was dried over sodium sulfate, filtered through milipore filter (0.5 μ m) and concentrated under reduced pressure and below 30°C. A portion was subjected to TLC with system B and separated into nine zones according to *R_f* values. By MF and/or MC, PAP from zone 7, PAP S-isomer (2) and ethyl mandelate (26) from zone 5, and PAP oxon (3) from zone 4 were identified. Particular attention was paid for identification of PAP S-isomer. As Table 6 indicates, PAP S-isomer (2) as well as PAP oxon (3) did not produce when both NADPH and microsome were absent. The result indicates that isomerization of PAP to its thiol isomer (2) occurred with

mixed function oxidase system. Formation of ethyl mandelate (**26**) was also followed by MF. After incubation with microsomal system for 15 min at pH 6.5 with or without NADPH, 0.15 or 0.04% was found, respectively. Therefore, formation of ethyl mandelate seems to be dependent on mixed function oxidase system. M-1 metabolite in Fig. 3, produced by microsomal system, corresponded to ethyl mandelate (**26**) in *R_f* values and $\text{MeS(O}_2\text{)CH(Ph)COOEt}$ (**32**). However, the latter was not detected by MC, therefore M-1 is ethyl mandelate.

Another portion of CH_2Cl_2 extract (B) was treated with diazomethane and divided into two fractions (C and D). Fraction C was searched by MF for detection of PAP or PAP oxon hydroxylated on the phenyl moiety (as the methylated products), but such products were not detected. Fraction D was used for search of PAP phosphinyl disulfide (**10**). TLC separation with system B into zones of *R_f* ca. 0.3 and ca. 0.7, followed by irradiation of zone of *R_f* 0.7 with ultraviolet light and extraction of both zones with ethyl acetate, is expected to give a derivative of PAP phosphinyl disulfide (**10**) as described in sections 1 and 2.2. However, there was no indication of the presence by MF.

The aqueous layer (E) which contains acidic metabolites was acidified to pH 1 with 1 N HCl and extracted thrice each time with 160 ml of ethyl acetate. The extract was dried over sodium sulfate, treated with diazomethane and concentrated for MC analysis. Methyl analogs of PAP and PAP oxon, $(\text{MeO})_2\text{P(S)SCH(Ph)COOMe}$ and $(\text{MeO})_2\text{P(O)SCH(Ph)COOMe}$, were identified. The latter was identified as the methylated product of M-7. There is a possibility that M-7 is PAP oxon acid (**5**) or demethyl PAP oxon acid (**9**). To examine the possibility, M-7 was treated with deuteriodiazomethane and submitted to MC to monitor the incorporation of deuterium into $[(\text{MeO})_2\text{P(O)SCH}]^+$ and M^+ . The deuterium was incorporated into M^+ , but not into $[(\text{MeO})_2\text{P(O)SCH}]^+$. Therefore, M-7 was confirmed as PAP oxon acid (**5**). By MC compound **12**, $(\text{MeO})_2\text{P(O)SCH}_2\text{SCH(Ph)COOMe}$, was not detected, thus further confirming the absence of PAP phosphinyl disulfide formation.

4. *In Vivo* Metabolism of ^{14}C -PAP in Mice

Three doses of ^{14}C -PAP, 25.0, 43.8 and 70.0 mg/kg (each 10 μCi), were given to mouse which correspond to 1/14, 1/8 and 1/5 of LD_{50} values, respectively. ^{14}C -PAP was prepared as emulsifiable concentrate (PAP-solpol-xylene, 11:3:6 w/w) and then diluted with water to 80 μl /mouse before administering orally to 5 male mice of ddN strain (5 weeks old, body weight 20–22 g), which were starved overnight before the administration of PAP.

Each mouse was confined in a metabolic cage. Urine, feces and expired gas were trapped at definite intervals. The urine was diluted with water, filtered through glass fiber and measured. Feces were homogenized with acetonitrile, filtered and the acetonitrile layer was measured. Expired gas was trapped in methyl cellosolve-ethanolamine (2:1 w/w) mixture and the radioactivity was measured.

For study of radiocarbon level in blood, 4.5×10^6 dpm/mouse (43.8 mg/kg) of ^{14}C -PAP was administered and at every definite interval five mice were anesthetized and the as much as possible was taken by syringe from the heart (1–2 ml/mouse). The blood was then diluted with heparin solution to 3 ml and the radioactivity was measured.

The radioactivity in blood increased rapidly, reaching a maximum within 30 min, then gradually decreased and was only detectable after 24 hr (Fig. 6). Excretion of radiocarbon was mainly into urine. A small portion was found in feces, but not in expired gas. Effect of dose was not significant as shown in Fig. 7. Within 6 hr about the half was excreted into urine, and within 24 hr about 95% was excreted into urine. Feces represented only 1% or less.

Fig. 6 Radioactivity level in blood after administration of ^{14}C -PAP to male mice.

Fig. 7 Cumulative excretion of radiocarbon after oral administration of ^{14}C -PAP to male mice (Dose 25–70 mg/kg).

●: urine, ○: feces.

Complete excretion of radiocarbon was made after 8 days. The results indicate that the absorption of PAP is rapid, metabolism is extensive (as indicated later by nature of radiocarbon) and excretion is rapid and complete.

For identification of ^{14}C -PAP metabolites urine from one day after the administration of 43.8 mg/kg was filtered through glass fiber, diluted to 25 ml with water (count), acidified to pH 1 with 1 N HCl, and extracted thrice each time with 100 ml of ethyl acetate (count). The aqueous layer (count) was neutralized with 1 N NaOH and its portion (10 ml, 1 μCi) was concentrated under reduced pressure to a half, added with 0.5 ml of 0.2 M acetate buffer (pH 5.0) and 10 mg (10,000 units) of β -glucuronidase or 3 mg (3.48 units) of aryl sulfatase preparation. After incubation for 6 hr with glucuronidase or 20 hr with sulfatase, the mixture was acidified to pH 1 and extracted with ethyl acetate. The above two ethyl acetate extracts were used for the identification of metabolites by TLC and by the methods described above. Autoradiograms of the extract from urine are shown in Fig. 8. PAP and PAP oxon (3) were not found. Seven metabolites, PAP acid (4), PAP oxon acid (5), demethyl PAP (6), demethyl PAP acid (8), demethyl PAP oxon acid (9), MeSCH(Ph)COOH (29), and MeS(O₂)CH(Ph)COOH (31), were identified. However, eight metabolites remain unidentified. Intentional search for PAP S-isomer (2) and PAP phosphinyl disulfide (10) failed. Also, treatment with β -glucuronidase and aryl sulfatase preparations did not give any solubilization in ethyl acetate

Fig. 8 TLC autoradiograms of metabolites of ^{14}C -PAP in urine.

Table 7 Relative amounts of PAP metabolites excreted into urine during 24 hr after oral administration of 43.8 mg/kg of ^{14}C -PAP.

Metabolite	% of administered radiocarbon
MeSCH(Ph)COOH (29)	8.78
PAP acid (4)	34.03
Demethyl PAP (6)	3.50
PAP oxon acid (5)	2.75
Demethyl PAP acid (8)	1.74
Demethyl PAP oxon acid (9)	5.05
MeS(O ₂)CH(Ph)COOH (31)	10.20
U-1	1.34
U-2	0.37
U-3	1.60
U-4	3.72
U-5	0.09
U-6	0.14
U-7	0.15
Aqueous fraction	20.98
Total	94.44

of radiocarbon from aqueous fraction. Quantitation of these metabolites is shown in Table 7. Feces collected until 6 days represented 1.1% of applied radioactivity, from which unchanged PAP (0.52%) and ethyl mandelate (26) (0.04%) as well as 6 unidentified metabolites were found (Fig. 9). PAP metabolites in mice have been

Fig. 9 TLC autoradiograms of metabolites of ^{14}C -PAP in feces.

investigated by Takade *et al.*²⁾ Using ^{32}P - and ^{14}C -PAP, they found demethyl PAP oxon (7), mandelic acid (27), $[-\text{SCH}(\text{Ph})\text{COOEt}]_2$ (23), $[-\text{SCH}(\text{Ph})\text{COOH}]_2$ (25) as well as $(\text{MeO})_2\text{P}(\text{S})\text{-SH}$ and $(\text{MeO})_2\text{P}(\text{S})\text{OH}$ other than those found by us. They also found trace amounts of PAP and PAP oxon in feces. On the other hand, we newly found PAP oxon acid (5) and $\text{Me-S}(\text{O})\text{CH}(\text{Ph})\text{COOH}$ (31).

Based on the identification of the transformation products of PAP under various conditions including mice, metabolic reactivity of PAP is considered as shown in Fig. 10. Initial modifications seem to occur in five ways on the PAP molecule: i) hydrolysis of carboxy ethyl ester (1→4); ii) demethylation (1→6); iii) formation of phosphorus oxythionate (1→X); iv) cleavage of C-S bond (1→26); v) isomerization (1→2). Modification i) is catalyzed by carboxy-esterase of microsome or cytosol origin. Modification ii) proceeds by GSH S-transferase-GSH system.

Modification iii) occurs by mixed function oxidase system. However, mechanism of formation of ethyl mandelate (26) is not certain. Seemingly, hydrolysis occurs simply. However, there was a positive effect of NADPH when microsome was used, and cytosol also catalyzed the reaction without GSH. When benzyl position is hydroxylated, the product seems to be unstable and hydrolyzed to PhCOCOEt (33). But compound 33 and its corresponding acid (34) were not found under any conditions examined. Oxidation at thiol sulfur followed by hydrolysis to ethyl mandelate (26) is only assumed at this point. Ethyl mandelate may

Fig. 10 Metabolic reactivity of PAP.

a: MCPBA, b: microsome, c: microsome-NAD-PH, d: cytosol, e: cytosol-GSH, f: mice, f*: mice by Takade *et al.*, (): minor production.

also be produced from intermediate **X** through compound **22**. Such replacement of benzylic SH with OH group was observed for $\text{ClC}_6\text{H}_4\text{-CH}_2\text{SH}$ in rat.⁸⁾ Isomerization of PAP to its thiol isomer (**2**) did occur under *in vitro* microsomal conditions. Such thiono-thiol isomerization had not been recognized under biological conditions, but our recent work⁷⁾ indicated that this isomerization was rather common. However, further modifications will not allow its finding under *in vivo* conditions. Many secondary reactions follow the initial modifications, resulting in various products illustrated in Fig. 10.

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REFERENCES

- 1) M. Hirose, I. Miyata, T. Saito & M. Hayashi: *Botyu Kagaku* **36**, 43 (1971)
- 2) D. Y. Takade, T. Allsup, A. Khasawinah, T. S. Kao & T. R. Fukuto: *Pestic. Biochem. Physiol.* **6**, 367 (1976)
- 3) D. Y. Takade, M. S. Seo, T. S. Kao & T. R. Fukuto: *Arch. Environ. Contam. Toxicol.* **5**, 63 (1976)
- 4) N. Mikami, H. Ohkawa, & J. Miyamoto: *J.*

Pesticide Sci. **2**, 279 (1977)

- 5) H. Ohkawa, N. Mikami, K. Kasamatsu & J. Miyamoto: *J. Agric. Food Chem.* **40**, 1857 (1976)
- 6) T. Miyamoto & I. Yamamoto: *Agric. Biol. Chem.* **44**, 2581 (1980)
- 7) T. Miyamoto, K. Toeda & I. Yamamoto: *J. Pesticide Sci.* **8**, 613 (1983)
- 8) K. Ishikawa, I. Okuda & S. Kuwatsuka: *Agric. Biol. Chem.* **37**, 165 (1973)

要 約

哺乳動物系における phenthoate の新代謝物

戸枝一喜, 宮本 徹, 加藤 茂, 山本 出

Phenthoate (PAP) $[(\text{CH}_3\text{O})_2\text{P}(\text{S})\text{SCH}(\text{Ph})\text{COOC}_2\text{H}_5]$ からの 12 種の変化生成物をマウス, ラット肝画分, 過酸により同定した. すでに報告されている化合物のほかに, 5 種の生成物が新規に見いだされた: $(\text{CH}_3\text{O})_2\text{P}(\text{O})\text{-SCH}(\text{Ph})\text{COOH}$ [マウス, ミクロゾーム-NADPH 系 (mfo)], $\text{CH}_3\text{S}(\text{O})\text{CH}(\text{Ph})\text{COOH}$ [マウス], $\text{HOCH}(\text{Ph})\text{-COOC}_2\text{H}_5$ [マウス, mfo, サイトソル], $(\text{CH}_3\text{S})(\text{CH}_3\text{O})\text{-P}(\text{O})\text{SCH}(\text{Ph})\text{COOC}_2\text{H}_5$ (PAP S-isomer)[mfo], $(\text{CH}_3\text{O})_2\text{-P}(\text{O})\text{SSCH}(\text{Ph})\text{COOC}_2\text{H}_5$ (phosphinyl disulfide) [過酸酸化]. 代謝物同定に基づき PAP の代謝経路を推定したところ, PAP は次の 5 通りの変化をまず受け, さらに 2 次的に種々の生成物を与えると思われる: i) カルボキシエチルエステルの加水分解, ii) 脱メチル化, iii) phosphorus oxythionate の形成, iv) C-S 結合の開裂, v) 異性化. mfo による PAP S-isomer, 過酸酸化による phosphinyl disulfide はマウス中においても生成したものと考えられるが, 不安定なため見いだされないであろう.