Stereoselectivity in Metabolism of the Optical Isomers of Cyanofenphos (*O-p*-Cyanophenyl *O*-Ethyl Phenylphosphonothioate) in Rats and Liver Microsomes

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The racemic, (+)- and (–)-forms of cyanofenphos (*O-p*-cyanophenyl *O*-ethyl phenylphosphonothioate) were rapidly metabolized in the rat by cleavage of P-O-aryl linkage, cleavage of P-O-alkyl linkage and conjugation of *p*-cyanophenol with sulfuric acid. There was a marked difference in the proportion of the major urinary metabolites, *p*-cyanophenol and *p*-cyanophenyl sulfate, with three forms of cyanofenphos.

The three forms of cyanofenphos were metabolized at almost equal rates in rat liver microsomes-NADPH system. (+)-Cyanofenphos underwent oxidation of P=S to P=O and cleavage of P-O-aryl linkage predominantly. In contrast, the (–)-isomer was converted to the corresponding oxon analog by mixed function oxidase, and then the oxon analog was rapidly hydrolyzed to *p*-cyanophenol by microsomal arylesterase-type enzyme. This microsomal enzyme had a remarkable selectivity in hydrolyzing (–)-cyanofenphos oxon versus the (+)-isomer. Stereoselectivity in the metabolism of the cyanofenphos isomers in the rat appears likely to be mainly due to selective hydrolysis of the (–)-oxon analog by the arylesterase-type enzyme.

Toxicity of organophosphorus esters to mammals, insects and plants is greatly affected by chirality at phosphorus.^{1~5)} Although selectivity in toxicity of the enantiomers of asymmetric phosphorus esters to mammals and insects appears to be associated with selective inhibition of acetylcholinesterase,⁶⁾ this selectivity may be due in part to differential metabolism of the enantiomers. However, very little has been reported on the stereoselectivity in metabolism of asymmetric phosphorus esters *in vivo* and *in vitro*. Cox *et al.*⁷⁾ reported that, following administration of racemic cyclophosphamide, a widely used anticancer drug, to patients, the drug recovered from the urine was levorotatory. The enantiomers in racemic S-2571 (*O*-ethyl *O*-2-nitro-5-methylphenyl *N*-isopropyl phosphoramidothioate) were stereoselectively metabolized by rabbit liver mixed function oxidase to give dextrorotatory products of the parent compound, the oxon analog and derivatives hydroxylated at the 5-methyl group.⁸⁾

The insecticide cyanofenphos (*O-p*-cyano-

phenyl *O*-ethyl phenylphosphonothioate) contains an asymmetric phosphorus atom and optical isomers are therefore possible. The present investigation compares metabolism of racemic, (+)- and (–)-cyanofenphos in rats and rat liver microsomal preparations.

MATERIALS AND METHODS

Spectroscopy Nuclear magnetic resonance spectra (NMR) were determined in appropriate deuterated solvents using tetramethylsilane as an internal standard, on a Hitachi R-20B spectrometer at 60 MHz. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane. Electron impact mass spectra (MS) were obtained with a Shimadzu-LKB 9000 mass spectrometer. Samples were analyzed 12 and 70 eV by means of a direct insertion probe. Infrared spectra (IR) were recorded on a Hitachi EPI-G3 spectrometer. Samples were analyzed as a thin film on a NaCl crystal. Specific rotations were measured at 20°C in chloroform solution in 1 cm cells on a Perkin-Elmer 141 spectropolarimeter.

Thin-layer chromatography (tlc). Precoated silica gel 60F-254 chromatoplates (20 × 20 cm, 0.25 mm layer thickness, E. Merck) were used for analytical and pre-

parative purposes. The tlc *R_f* values for a variety of cyanophenphos derivatives and solvent systems used are given in Table I. Resolved compounds were de-

TABLE I. THIN-LAYER CHROMATOGRAPHIC PROPERTIES OF CYANOFENPHOS AND OF VARIOUS METABOLITES

Compound	<i>R_f</i> values with indicated solvent systems		
	A	B	C
Cyanofenphos	0.50	0.93	0.90
Cyanofenphos oxon	0.32	0.86	0.80
<i>p</i> -Cyanophenol	0.15	0.83	0.73
Desethylcyanofenphos	0.05	0.39	0.33
<i>p</i> -Cyanophenyl sulfate	0	0.28	0.46

A, toluene-acetic acid (7/1, v/v); B, toluene-ethyl acetate-propanol-acetic acid (8/12/5/3); C, ethyl acetate ethanol-water (10/2/1).

tected as follows; quenching of gel fluorescence when viewed under short-wavelength UV light and spraying with 1% (w/v) PdCl₂ in 1 N HCl followed by heating at 110°C for 5 min. Radioactive products were detected by radioautography or radioscanning with a model TRM-1B tlc scanner (Aloka, Tokyo, Japan).

Radioanalysis. The radiocarbon content of organo-soluble fractions from extraction of various materials and gel regions from tlc plates was determined with a Packard model 3385 liquid scintillation spectrometer equipped with an automatic external standard, using 15 ml of a dioxane scintillation fluid (5 g of 2, 5-diphenyloxazole and 100 g of naphthalene in 1 liter of dioxane). Feces were dried over P₂O₅ in a vacuum desiccator and then combusted in a Packard model 306 oxidizer prior to liquid scintillation counting (Isco). Industrial X-ray 150 films (Fuji Photo Film Co., Ltd., Tokyo, Japan) were used for radioautography. Developed tlc plates were placed under film and held at 4°C to 8°C for several days depending on the radioactivity.

Percent distribution of radioactive spots was determined by scraping gel regions from the chromatoplates into scintillation vials and counting them directly with a liquid scintillation spectrometer.

Chemicals. Synthesis of the optical isomers of cyanofenphos was performed in the following. Racemic *O*-ethyl phenylphosphonothioic acid (13 g) was treated with an equivalent of brucine (28 g) in 500 ml methanol. After concentration, the residues were recrystallized from acetone to give the diastereomeric brucine salts in approximately 30% yield. The brucine salts (5 g) in ethyl acetate solution was treated with 20 ml of 1 N HCl and then washed with saturated NaCl solution. After concentration of the ethyl acetate solution, the resolved phosphonothioic acid was obtained;

$[\alpha]_D^{20} - 11.8^\circ$ ($c=4.5$). After recrystallization, the soluble residue was concentrated and then recrystallized from methanol to give the diastereomeric brucine salts in about 30% yield. Treatment of the brucine salts with 1 N HCl gave the other enantiomer of the phosphonothioic acid; $[\alpha]_D^{20} + 12.0^\circ$ ($c=8.3$). Chlorination of (–)-*O*-ethyl phenylphosphonothioic acid (300 mg) with two equivalents of phosphorus pentachloride in 50 ml chloroform was carried out at –10° to 0°C for 1 hr.⁹⁾ After concentration of the reaction mixture, the obtained crude chloride was purified on a silicic acid column (30×3 cm, *i. d.*, 50 g of 100 mesh silicic acid, Mallinckrodt Chemical Works) using benzene. The yield of (–)-*O*-ethyl phenylphosphonochloridothioate was about 60%; $[\alpha]_D^{20} - 72.5^\circ$ ($c=3.4$). (+)-*O*-Ethyl phenylphosphonochloridothioate was obtained from the (+)-phosphonothioic acid in the same manner; $[\alpha]_D^{20} + 71.3^\circ$ ($c=3.2$). An equimolar mixture of the (–)-chloride (500 mg) and *p*-cyanophenol (270 mg) in 20 ml toluene was refluxed for 3 hr in the presence of two equivalents of anhydrous K₂CO₃ (1 g). The reaction mixture was concentrated, and the obtained crude product was purified by silicic acid column chromatography (30×3 cm, *i. d.*) in *n*-hexane-benzene (1/2, v/v). The yield of (+)-cyanofenphos was about 70%; $[\alpha]_D^{20} + 33.5^\circ$ ($c=4.0$). (–)-Cyanofenphos was prepared from the (+)-chloride in the same manner; $[\alpha]_D^{20} - 33.7^\circ$ ($c=3.0$). The structure of both cyanofenphos isomers was confirmed by NMR, IR and MS. Both isomers were identical in these spectra. Also, no impurities were detected by NMR and tlc in *n*-hexane-acetone (5/2, v/v). NMR (CDCl₃), δ 1.35 (3H, t, CH₃), 4.25 (2H, q, CH₂) and 7.00~8.20 ppm (9H, m, aromatic); IR, 2230 (CN), 1600 and 1500 (aromatic ring), 1230 (P–O–aryl) and 1030 cm^{–1} (P–O–alkyl); ms, *m/e* 303 (M), 258 (M–C₂H₅), 226 (M–C₆H₅), 185 (M–OC₆H₄CN), 169, 158, 141, 119, 110 and 77.

Preparation of the optical isomers of *O*-*p*-cyanophenyl *O*-ethyl phenylphosphonate (cyanofenphos oxon) was carried out in the following. *m*-Chloroperoxybenzoic acid (172 mg) was added to a stirring solution of (+)-cyanofenphos (303 mg) in 5 ml dichloromethane over 30-min period at 0°C. The resulting solution was concentrated to about 20% of the original volume and then cooled to partially precipitate *m*-chlorobenzoic acid. The dichloromethane-soluble material was purified by preparative tlc first in toluene-acetic acid (7/1, v/v) and then in benzene-ethyl acetate (2/1, v/v). The yield of (+)-cyanofenphos oxon was about 90%; $[\alpha]_D^{20} + 45.3^\circ$ ($c=7.5$). Preparation of (–)-cyanofenphos oxon from (–)-cyanofenphos was performed as above; $[\alpha]_D^{20} - 46.7^\circ$ ($c=7.5$). Both cyanofenphos oxon isomers showed identical NMR, IR and MS. NMR (CDCl₃), δ 1.30 (3H, t, CH₃), 4.25 (2H, q, CH₂) and 6.70~8.00 ppm (9H, m, aromatic); IR, 2230 (CN), 1600 and 1490 (aromatic ring), 1260 (P=O), 1220 (P–O–aryl) and 1030 cm^{–1} (P–O–alkyl); MS, *m/e*

286 (M), 257 (M-C₂H₅), 209 (M-C₆H₅), 168 (M-OC₆H₄CN), 169, 141, 119 and 77.

Desethylcyanofenphos (*O-p*-cyanophenyl phenylphosphonothioic acid) was prepared in the following. A mixture of 300 mg cyanofenphos and 160 mg potassium *N,N*-dimethyldithiocarbamate in 10 ml methanol was refluxed for 10 hr. The reaction mixture was concentrated, purified by preparative tlc in toluene-ethyl acetate-acetic acid (5/7/1, v/v/v) and then by passing through Sephadex LH20 column using methanol. NMR (CD₃OD), δ 6.80~8.00 ppm (9H, m, aromatic); IR, 3440 (OH), 2230 (CN), 1600 and 1490 (aromatic ring), 1240 (P=O) and 1230 cm⁻¹ (P-O-aryl).

p-Cyanophenyl sulfate was prepared according to Whitehead *et al.*¹⁰⁾ Tetraethyl pyrophosphate (TEPP) was prepared according to the method of Toy.¹¹⁾ Other chemicals used were; *p*-cyanophenol from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin; and brucine from Wako Pure Chemical Industries, Osaka, Japan; reduced nicotinamide adenine dinucleotide phosphate (NADPH) from Oriental Yeast Co., Ltd., Tokyo, Japan.

Labeled compounds. The individual racemic, (+)- and (-)-forms of each ¹⁴C-labeled cyanofenphos and ¹⁴C-labeled cyanofenphos oxon were prepared by the same procedures as with unlabeled compounds. ¹⁴C-*p*-Cyanophenol labeled at the CN group was prepared by Hazue and Makari of this institute,¹²⁾ with specific activity of 8.57 mCi/mmole. Each preparation was purified by preparative tlc in toluene-acetic acid (7/1, v/v) and benzene for the cyanofenphos isomers, and toluene-acetic acid (7/1) and benzene-ethyl acetate (2/1) for the cyanofenphos oxon isomers. The radiochemical purity of ¹⁴C-preparations was determined

by tlc in toluene-acetic acid (7/1), radioautography and lsc. Six radiolabeled chemicals used are presented in Table. II.

Metabolism in vivo. Male Sprague-Dawley rats (*ca.* 200 g) were orally treated by stomach tube with each of ¹⁴C-cyanofenphos isomers in 10% Tween 80 at the rate of 4 mg/kg. After administration, the treated rats were each held in metabolism cages. The urine and feces were collected for up to 3 days and monitored for radiocarbon. The collected urine (0~2 days) were lyophilized to dryness and the residues were dissolved in methanol-water (4/1, v/v). The concentrated solution was subjected to tlc in solvent systems A, B and C. The individual metabolites were identified by tlc cochromatography with authentic standards from synthesis.

Metabolism in vitro. Liver enzymes were prepared from male Sprague-Dawley rats (*ca.* 200 g). A 20% (w/v) homogenate of rat livers in 1.15% KCl was centrifuged at 9,000 × *g* for 20 min to obtain the 9,000 × *g* supernatant fraction which was in turn centrifuged at 105,000 × *g* for 60 min to sediment microsomes. The microsomal pellet was washed by resuspension and recentrifugation in the same solution. Incubation mixtures in 5 ml of 0.05 M Tris-HCl buffer, pH 7.5, contained; the microsomes equivalent to 400 mg fresh liver weight; 0 or 5.5 μmole NADPH; 0 or 2.5 μmole EDTA; 0 or 2.5 μmole CaCl₂; 0.5 μmole substrate added last in 50 μl acetone. When TEPP (2.5 μmole) was used to inhibit microsomal esterase, it was added in 50 μl acetone to the bottom of the flask, the acetone was evaporated at 25°C, the microsome preparation and buffer were added. Incubations were carried out in air with shaking at 37°C for 1 hr in 50-ml Erlenmeyer flasks. After incubation, each reaction mixture was adjusted to pH 1~2 by addition of 1 N HCl and 1 g of (NH₄)₂SO₄ was added. The mixtures were extracted three times with chloroform. The combined extracts were concentrated and analyzed by tlc in solvent systems A and B. Metabolites from tlc detected in less than 0.1% amounts are recorded as zero. All experiments were repeated two or more times for confirmation of the results presented.

Toxicity. Insecticidal activity was determined on a susceptible strain of housefly (*Musca domestica*, WHO) and rice stem borer larvae (*Chilo suppressalis*) according to the procedure reported previously.⁹⁾ Each test chemical was dissolved in acetone and 0.4 μl of the solution was topically applied to 3- to 4-day old male houseflies and 20-day old (4th instar) larvae of rice stem borers. Two replications of 30 insects each were used at each dose level. Mortalities were determined 24 hr for houseflies and 48 hr for rice stem borers after treatment.

TABLE II.

Compound	mCi/mmole	Radiochemical purity (%)	[α] _D ²⁰ (CHCl ₃)
¹⁴ C-(±)-Cyanofenphos	8.57	>99	0
¹⁴ C-(+)-Cyanofenphos	7.12	>99	+31.0° (<i>c</i> =8.2)
¹⁴ C-(-)-Cyanofenphos	7.12	>99	-31.8° (<i>c</i> =8.2)
¹⁴ C-(±)-Cyanofenphos oxon	9.10	94.9	0
¹⁴ C-(+)-Cyanofenphos oxon	7.52	83.1	+42.3° (<i>c</i> =1.0)
¹⁴ C-(-)-Cyanofenphos oxon	7.52	86.3	-43.3° (<i>c</i> =1.0)

Toxicity was also determined on male dd strain mice weighing 18~20 g. Each chemical was dissolved in 10% Tween 80 and 0.4 ml of the solution was intraperitoneally (ip) injected to mice. Mortality was evaluated 24 hr after treatment.

RESULTS

Metabolism of racemic, (+)- and (-)-cyanofenphos in rats

When administered orally to rats at 4 mg/kg, the radiocarbon from the racemic, (+)- and (-)-forms of cyanofenphos was almost completely recovered in the excreta after 3 days, most being eliminated in the urine during the first 24 hr. The three forms of cyanofenphos gave nearly the same recoveries, as shown in Table III. The ^{14}C -labeled metabolites excreted within 2 days after administration of the three forms of cyanofenphos were detected mainly in the urine (96.7 to 98.6% of the administered radiocarbon). Table IV shows

were identified as *p*-cyanophenol and *p*-cyanophenyl sulfate. Although the sum of both metabolites was almost equal with the three forms of cyanofenphos, the ratio of *p*-cyanophenol and *p*-cyanophenyl sulfate varied with the three forms. The ratio was approximately 1:1, 1:3 and 1:2 for (+)-, (-)- and racemic cyanofenphos, respectively, indicating that differential metabolism occurred in the rat. Desethylcyanofenphos was found as a minor metabolite in almost equal amounts with the cyanofenphos isomers. No parent compounds and oxon-analogs were detected in the urine.

Metabolism of racemic, (+)- and (-)-cyanofenphos in rat liver microsomes-NADPH system

The three forms of cyanofenphos were stable on incubation with either microsomes or NADPH, and more than 97% of the initial radiocarbon was recovered as the unchanged

TABLE III. RADIOCARBON IN THE URINE AND FECES AT ONE AND THREE DAYS AFTER ORAL ADMINISTRATION OF ^{14}C -LABELED ISOMERS OF CYANOFENPHOS AT 4 mg/kg

Compound	% of administered radiocarbon					
	1 day			3 days		
	Urine	Feces	Total	Urine	Feces	Total
(+)-Cyanofenphos	94.7	1.5	96.2	97.0	2.0	99.0
(-)-Cyanofenphos	96.4	1.5	97.9	98.1	1.9	100.0
(±)-Cyanofenphos	97.5	1.8	99.3	98.7	1.9	100.6

TABLE IV. IDENTIFIED METABOLITES IN THE URINE OF RATS TWO DAYS AFTER ORAL ADMINISTRATION OF ^{14}C -LABELED ISOMERS OF CYANOFENPHOS

Metabolite	% of administered radiocarbon		
	(+)	(-)	(±)
<i>p</i> -Cyanophenol	54.1	23.8	32.1
<i>p</i> -Cyanophenyl sulfate	41.3	72.5	65.3
Desethylcyanofenphos	1.0	0.6	0.7
Others	0.4	0.9	0.5
Total	96.8	97.8	98.6

the proportions of the urinary metabolites of racemic, (+)- and (-)-cyanofenphos tentatively identified by tlc cochromatography in solvent systems A, B and C. The major metabolites

compounds. But, when microsomes and NADPH were present, each of the cyanofenphos isomers was metabolized to the extent of 58.0~59.2%, as shown in Table V. Three metabolites were identified by their tlc characteristics in three different solvent systems, listed in Table I. Cyanofenphos oxon and *p*-cyanophenol were detected as major metabolites. Desethylcyanofenphos occurred to a minor extent. The sum of both major metabolites, cyanofenphos oxon and *p*-cyanophenol, was nearly the same with three forms of cyanofenphos, accounting for 51.0~53% of the initial radiocarbon. However, the ratio of cyanofenphos oxon relative to *p*-cyanophenol was quite different with the cyanofenphos

TABLE V. METABOLISM OF THE RACEMIC, (+)- AND (–)-FORMS OF CYANOFENPHOS BY RAT LIVER MICROSOMES-NADPH SYSTEM IN THE PRESENCE AND ABSENCE OF EDTA, CaCl_2 AND TEPP

More than 97% of the initial radiocarbon was recovered as unchanged compounds from the incubation mixtures without microsomes or NADPH.

Metabolite	% of initial radiocarbon											
	Micro. + NADPH			Micro. + NADPH + EDTA			Micro. + NADPH + CaCl_2			Micro. + NADPH + TEPP		
	(+)	(–)	(±)	(+)	(–)	(±)	(+)	(–)	(±)	(+)	(–)	(±)
Cyanofenphos	40.8	42.0	40.8	34.7	29.0	27.3	40.5	38.4	37.2	40.5	37.9	36.5
Cyanofenphos oxon	27.0	10.9	19.5	31.9	49.5	44.0	25.3	5.4	16.3	23.4	11.3	21.1
<i>p</i> -Cyanophenol	26.0	40.1	32.9	26.5	15.2	23.0	27.5	50.2	40.1	29.6	44.0	35.9
Desethylcyanofenphos	0.5	0.6	0.8	1.5	1.2	1.8	0.6	0.6	0.8	0.7	0.8	0.8
Others	0.8	0.8	0.8	0.3	0.3	0.4	0.7	0.8	0.5	0.9	0.9	0.9
Total	94.1	93.4	93.8	94.9	95.2	96.5	94.6	95.4	95.9	95.1	94.9	95.2

isomers. The ratio was approximately 1:1, 1:4 and 1:1.5 for (+)-, (–)- and racemic cyanofenphos, respectively.

The addition of 0.5 mM EDTA to the microsomes-NADPH system had two effects; the metabolism was slightly increased to the extent of 65.3~72.7% of the applied, and the amount of cyanofenphos oxon relative to *p*-cyanophenol was increased with the (–)- and racemic compounds, but not with the (+)-isomer. When metabolism was run in the presence of 0.5 mM CaCl_2 , the amount of *p*-cyanophenol relative to cyanofenphos oxon was markedly increased with (–)- and racemic cyanofenphos. But the metabolism of the (+)-isomer was not affected by addition of CaCl_2 . The addition of 0.5 mM TEPP to the microsomes-NADPH system had almost no effect on the metabolism of the three forms of cyanofenphos. The results were similar to those of the microsomes-NADPH system alone.

These data suggest that two enzyme systems function in the microsomes-NADPH system used. One enzyme system is mixed function oxidase which was not affected by addition of CaCl_2 and TEPP. This enzyme system catalyzed oxidation of the cyanofenphos isomers to the corresponding oxon analog and cleavage of P-O-aryl and P-O-alkyl linkages. The other microsomal enzyme was inhibited by EDTA and enhanced by CaCl_2 , but not affected by

TEPP. This enzyme functioned in formation of *p*-cyanophenol when (–)- and racemic cyanofenphos were incubated with the microsomes-NADPH system.

Hydrolysis of racemic, (+)- and (–)-cyanofenphos oxon by rat liver microsomes

There was a marked difference in the rate of hydrolysis of three forms of cyanofenphos oxon in rat liver microsomes, as shown in Table VI. (–)-Cyanofenphos oxon was rapidly hydrolyzed to *p*-cyanophenol in the microsomes, but the (+)-isomer was resistant to hydrolysis by the microsomes. The racemic compound showed the value intermediate between two optical isomers. More than 99% of the applied radiocarbon was recovered as unchanged compounds from the incubation mixtures without microsomes.

The addition of 0.5 mM EDTA to the microsomes markedly inhibited the hydrolysis of (–)- and racemic cyanofenphos oxon. TEPP had almost no effect on hydrolysis by the microsomes. On incubation with the microsomes-NADPH system, (–)- and racemic cyanofenphos oxon were also hydrolyzed to *p*-cyanophenol, and the results were similar to those of the microsomes alone, except for formation of a polar metabolite which was assumed to be desethylcyanofenphos oxon based on its tlc characteristics.

These data suggest that a liver enzyme hy-

TABLE VI. HYDROLYSIS OF THE RACEMIC, (+)- AND (–)-FORMS OF CYANOFENPHOS OXON BY RAT LIVER MICROSOMES IN THE PRESENCE AND ABSENCE OF EDTA, TEPP AND NADPH

More than 99% of the initial radiocarbon was recovered as unchanged compounds from the incubation mixtures without microsomes.

Product	% of initial radiocarbon											
	Microsome			Microsome +EDTA			Microsome +TEPP			Microsome +NADPH		
	(+)	(–)	(±)	(+)	(–)	(±)	(+)	(–)	(±)	(+)	(–)	(±)
Cyanofenphos oxon	82.7	8.9	49.5	98.1	87.2	89.4	85.4	10.8	50.4	76.7	11.2	40.9
<i>p</i> -Cyanophenol	16.3	89.8	49.6	0.9	11.6	8.1	13.4	88.3	48.2	12.3	70.7	39.6
Others	0	0	0	0	0	0	0	0	0	9.6	16.8	15.7
Total	99.0	98.7	99.1	99.0	98.8	97.5	98.9	99.1	98.6	98.6	98.7	96.2

drolyzing cyanofenphos oxon to *p*-cyanophenol was present in the microsomes. This enzyme showed a remarkable selectivity in hydrolyzing (–)-cyanofenphos oxon versus the (+)-isomer. In the racemic compound, the (–)-enantiomer appears to be selectively cleaved by the microsomes. The enzyme activity was specifically inhibited by EDTA, but not affected by TEPP.

Toxicity to insects and mice

The optical isomers of cyanofenphos exhibited a marked selectivity in toxicity to houseflies and rice stem borer larvae, as showed in

TABLE VII. TOXICITY OF RACEMIC, (+)- AND (–)-CYANOFENPHOS TO HOUSEFLIES (TOPICAL), RICE STEM BORER LARVAE (TOPICAL) AND MICE (IP)

Compound	LD ₅₀		
	μg/g		mg/kg
	Housefly	Rice stem borer	Mouse
(+)-Cyanofenphos	1.32	2.92	32
(–)-Cyanofenphos	3.61	> 73	35
(±)-Cyanofenphos	1.67	7.30	34

Table VII. (+)-Cyanofenphos was 2.7-fold more toxic to houseflies than the (–)-isomer. The toxicity of the racemic compound was close to that of the (+)-isomer. Also, the (+)-isomer was 2.5-fold more toxic to rice stem bores than the racemic compound. The (–)-isomer was almost inactive even at 73 μg/g.

On the other hand, when administered intraperitoneally, the three forms of cyanofenphos were almost equally toxic to mice.

DISCUSSION

Orally administered ¹⁴C-cyanofenphos rapidly metabolized in mice, and the radiocarbon was almost completely eliminated from the body within a few days. The identified metabolites included desethylcyanofenphos, *p*-cyanophenol and *p*-cyanophenyl sulfate.¹³⁾ In the present study, it was also demonstrated that racemic, (+)- and (–)-cyanofenphos were rapidly metabolized in living rats by P-O-aryl cleavage, by P-O-alkyl cleavage and by conjugation of *p*-cyanophenol with sulfuric acid. There was a marked difference in the proportion of major urinary metabolites, *p*-cyanophenol and *p*-cyanophenyl sulfate, with three forms of cyanofenphos. These data suggest that stereoselective metabolism occurred in the rat.

Figure 1 gives proposed metabolic pathways

FIG. 1. Proposed Metabolic Pathways for Cyanofenphos in the Rat.

for cyanofenphos in the rat and rat liver microsomal preparations generalized to include the three forms of cyanofenphos. The microsomes-NADPH system catalyzed oxidation of the cyanofenphos isomers to the corresponding oxon analogs and oxidative cleavage of P-O-aryl linkage predominantly. Also, desethylation occurred to a minor extent in the enzyme system with the three forms of cyanofenphos. *p*-Cyanophenol was further conjugated to *p*-cyanophenyl sulfate in the rat. Possibly (+)-cyanofenphos undergoes oxidation of P=S to P=O and oxidative cleavage of P-O-aryl linkage at almost equal rates in the microsomes-NADPH system. (–)-Cyanofenphos is similarly oxidized to the oxon analog, which is then rapidly hydrolyzed to *p*-cyanophenol in the microsome preparation. The latter route was established by incubating the cyanofenphos oxon isomers with rat liver microsomes in the presence or absence of EDTA, TEPP and NADPH. The microsomal enzyme hydrolyzing (–)-cyanofenphos oxon was inhibited by EDTA, but not affected by TEPP and NADPH. These properties are very similar to those of diazoxon or paraoxon hydrolyzing enzymes.^{14,16)} Thus, the cyanofenphos oxon-hydrolyzing enzyme is thought to be an arylesterase-type enzyme. The microsomal arylesterase-type enzyme rapidly cleaved (–)-cyanofenphos oxon, but hardly the (+)-isomer. With respect to *p*-cyanophenol formation, an arylesterase-type enzyme appears to be important for (–)-cyanofenphos oxon whereas oxidative cleavage of P-O-aryl linkage by the microsomes-NADPH system is likely to be important for (+)-cyanofenphos. Moreover, the cyanofenphos oxon isomers may undergo oxidative reactions mediated by the mixed function oxidase when the arylesterase-type enzyme attack is minimal.

It was reported that oxidation of P=S to P=O by microsomal mixed function oxidase is stereospecific and proceeds with retention of configuration at phosphorus.^{8,16)} Also, oxidation of the cyanofenphos isomers to the corresponding oxon analogs appears likely to follow the same stereochemical processes as

those of fonofos and S-2571, since both the oxon analog derived from (–)-cyanofenphos and (–)-cyanofenphos oxon showed similar substrate specificity for microsomal arylesterase-type enzyme.

Faster hydrolysis of (–)-cyanofenphos oxon relative to the (+)-isomer by an arylesterase-type enzyme resulted in formation of a larger amount of *p*-cyanophenol, which is then conjugated to *p*-cyanophenyl sulfate in the rat. In fact, a larger amount of *p*-cyanophenyl sulfate was obtained with (–)-cyanofenphos relative to the (+)-isomer in the rat. Therefore, stereoselectivity in metabolism of the cyanofenphos isomers appears likely to be mainly due to selective hydrolysis of (–)-cyanofenphos oxon by an arylesterase-type enzyme.

Although the optical isomers of cyanofenphos exhibited differences in toxicity to houseflies and rice stem borers, these were almost equally toxic to mice. Since each of the cyanofenphos isomers appears to be very rapidly metabolized in mice, a difference in *in vivo* metabolism of the cyanofenphos isomers may not be reflected in the presented LD₅₀ values.

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REFERENCES

- 1) G. Hilgetag and G. Lehmann, *J. Pract. Chem.*, **280**, 224 (1959).
- 2) T. R. Fukuto and R. L. Metcalf, *J. Econ. Entomol.*, **52**, 739 (1959).
- 3) D. A. Wustner and T. R. Fukuto, *J. Agr. Food Chem.*, **21**, 756 (1973).
- 4) T. W. Holmsen, *Weed Science*, **17**, 187 (1969).
- 5) H. Ohkawa, N. Mikami, A. Mine and J. Miyamoto, *Agric. Biol. Chem.*, **39**, 2265 (1975).
- 6) H. Ohkawa, N. Mikami, K. Kasamatsu and J. Miyamoto, *ibid.*, **40**, 1857 (1976).
- 7) P. J. Cox, P. B. Farmer, M. Jarman, M. Jones, W. J. Stec and R. Kinas, *Biochem. Pharmacol.*, **25**, 993 (1976).
- 8) H. Ohkawa, N. Mikami and J. Miyamoto, *Agric. Biol. Chem.*, **40**, 2125 (1976).
- 9) J. Michalski and M. Mikolajczyk, *Tetrahedron*, **22**, 3055 (1966).
- 10) J. E. Whitehead, A. R. Morrison and L. Young,

- Biochem. J.*, **51**, 585 (1952).
- 11) A. D. F. Toy, *J. Amer. Chem. Soc.*, **70**, 186 (1948).
 - 12) M. Hazue and Y. Makari, unpublished data.
 - 13) S. Kato and I. Yamamoto, *J. Pesticide Sci.*, submitted.
 - 14) T. Shishido and J. Fukami, *Pest. Biochem. Physiol.*, **2**, 39 (1972).
 - 15) P. A. Cammer and R. M. Hollingworth, *Biochem. Pharmacol.*, **25**, 1799 (1976).
 - 16) P. W. Lee, R. Allahyari and T. R. Fukuto, *ibid.*, **25**, 2671 (1976).
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