However, it would also appear that hydroxylation of the chlorophenyl ring as well as oxidation of the sulfur atom of T, as seen in the chicken and the rat, is required for rapid elimination of the drug from animals.

ACKNOWLEDGMENT

The authors wish to thank M. Schach von Wittenau for his helpful comments and suggestions; John Stratton for editorial aid; C. L. Holmes for surgical implantation of bile cannula in a dog; Theresa Morse, Carol Rocchetti, Norman Glidden, and Richard Bartolucci for their skilled technical assistance; and Elbridge Luther for preparation of mass spectra.

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Comparison of Cholinesterase Activity, Residue Levels, and Urinary Metabolite Excretion of Rats Exposed to Organophosphorus Pesticides

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Blood cholinesterase activity, urinary levels of phenolic and organophosphorus metabolites, and residues of intact compounds in blood and fat were determined following exposure of rats to organophosphorus pesticides. The eight pesticides studied included representative halogenated compounds (carbophenothion, dichlofenthion, ronnel, and leptophos) and nonhalogenated compounds (parathion, EPN, dimethoate, and dichlorvos). Cholinesterase activity was determined by gas chromatography. Metabolites were extracted, derivatized, subjected to silica gel chromatography, and quantitated by FP–GC and EC–GC. Residues were extracted, cleaned up with silica gel chromatography, and quantitated with EC–GC. Data comparing these various exposure indicators are presented.

A number of methods are available for monitoring exposure to degradable pesticides. These include methodology for determining blood cholinesterase levels, urinary alkyl phosphate metabolites, urinary phenol metabolites, and residues of the intact compounds in blood and in adipose and other tissues. These methods have been used singly to monitor exposure to organophosphorus pesticides. However, it would be valuable to have a comparison of the effectiveness of the four indices of exposure for application to future studies. The work reported in this paper gives a comparison of the four methods, using rats as models, in a feeding study involving eight organophosphorus pesticides.

The pesticides selected for the study are shown in Table I. They include aromatic and aliphatic organophosphate compounds as well as two organophosphonate pesticides. The eight compounds may also be classified by the presence or absence of halogenated moieties. The compounds were selected to provide an opportunity to compare cholinesterase inhibition, tissue residue levels, and urinary excretion of metabolites in rats exposed to different classes of organophosphorus pesticides. Hopefully, it would also give an insight into possible delayed excretion of halogenated organophosphorus pesticides.

Table I. Pesticides Selected

Compound		Halogenated	
Class	Name	Yes	No
Aliphatic organophosphate	Dichlorvos	X	
	Dimethoate		X
Aromatic organophosphate	Carbophenothion	X	
	Dichlofenthion	X	
	Ronnel	X	
	Parathion		X
Organophosphonate	Leptophos	X	
	EPN		X

EXPERIMENTAL SECTION

Male Sprague-Dawley rats, weighing 160–185 g each, were used in the study. So that the rats could be treated as individuals for cholinesterase determinations, they were distinguished by ear notching. They were housed in pairs in stainless steel metabolism cages which were cleaned daily to minimize fecal contamination. Standard laboratory chow and water were furnished ad libitum.

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Urine samples were collected in glass bottles, each sample containing the combined 24-h urine output of two rats. The urine samples were frozen and stored until they were analyzed. Urine was collected for 24 h prior to the beginning of the feeding study to determine background levels of metabolites in the urine.

Blood samples for determination of cholinesterase levels were obtained by shaving off the tip of the tail with a sterile surgical scalpel blade and collecting blood in heparinized microhematocrit tubes. Plasma cholinesterase levels were determined immediately. To establish preexposure cholinesterase levels, blood was taken from each animal just prior to the administration of the first dose.

The pesticides were administered by gavage as solutions of analytical standard quality compounds in peanut oil. Two levels of exposure were used for each compound: approximately 0.1 LD_{50} and approximately 0.01 LD_{50}. Two rats were dosed at each level of each compound and housed together in a metabolism cage. The animals were dosed three times at 24-h intervals, and immediately after dosing a clean bottle was placed under the cage to collect urine. Two hours after each dose, blood was collected for cholinesterase determination. Urine and blood were collected as described for a total of 10 days.

Blood and tissue samples were needed to analyze for residues of intact dichlofenthion, ronnel, carbophenothion, leptophos, and EPN. For this purpose, two extra rats were dosed as previously described at 0.1 LD_{50} for each of these compounds. One animal of each pair was sacrificed by cervical dislocation 6 h after administration of the third dose, and blood and adipose samples were taken. These were frozen until analyzed. The second animal was sacrificed on day 8 of the experiment, and blood and adipose samples were taken for analysis.

Methods of Analysis. *Plasma Cholinesterase.* The method of Cranmer and Peoples (1971) was used for analysis of blood cholinesterase levels. Samples were collected in heparinized microhematocrit tubes, and centrifuged to separate plasma from erythrocytes. A 2-cm section of tube (containing 20 μL of plasma) was transferred to a test tube containing a buffer solution with 3,3-dimethylbutyl acetate as the substrate. This was mixed well to insure complete removal of the plasma from the section of tube. The solution was incubated at 37 °C for 30 min. Formic acid (44% in water) was added to stop the reaction and the reaction product, 2,2-dimethylbutanol (DMB), was extracted with carbon disulfide. The level of DMB, which served as an indication of cholinesterase activity, was determined by gas–liquid chromatography (GLC) using a hydrogen flame ionization detector (FID).

Urinary Alkyl Phosphate and Alkyl Phosphonate Metabolites. The method of Shafik et al. (1973a) was used to determine the level of alkyl phosphates in urine. Acidified urine was extracted with a mixed solvent (1:1 acetonitrile–ether), and the organic extract was derivatized with diazopentane. Silica gel column chromatography was used for cleanup and fractionation of the alkyl phosphates. A gas chromatograph equipped with a flame photometric detector (FPD) in the phosphorus mode was used to quantitate the alkyl phosphates.

The alkyl phosphonate metabolites were determined by the method of Lores et al. (1975). Ion-exchange chromatography was used to isolate the metabolites from urine. The alkyl phosphonates were derivatized with diazopentane and quantified using FPD–GLC.

Urinary Phenol Metabolites. Phenols were analyzed by the method of Shafik et al. (1973b). The urinary conjugates were hydrolyzed with acid in an autoclave, the free phenols were extracted with ether, derivatized with diazoethane, and fractionated with solvents of varying polarity, through a silica gel column. Quantitation was accomplished by electron-capture (EC) GLC.

Residues of Intact Pesticides. Blood and adipose tissue were analyzed for intact residues using modifications of the methods in the Manual of Analytical Methods (1974). After the blood was centrifuged, 1 g of plasma (or as much as could be recovered if less than 1 g) was transferred to a screw-capped culture tube. Two milliliters of 2% Na_2SO_4 and 6 mL of methylene chloride were added. The tube was placed on a Roto-Rack (Fisher Scientific Co.) for 2 h at 50 rpm, then centrifuged to separate the layers. A measured aliquot of the methylene chloride extract was transferred to a graduated centrifuge tube using a disposable Pasteur pipet. A keeper was added (5–10 drops of 1% paraffin oil in isooctane) and the extract was concentrated to an appropriate volume for quantitation by FPD–GLC.

A 500-mg sample of adipose tissue was ground with 2.5 mL of acetonitrile, centrifuged, and the supernatant liquid transferred to a 45-mL graduated centrifuge tube. The residue was reextracted twice with 2.5 mL of acetonitrile, and the extracts were combined in the 45-mL tube. The combined extract was evaporated under nitrogen to 2–3 mL. Then, 25 mL of 2% aqueous Na_2SO_4 was added and the mixture was extracted with 5 mL of methylene chloride, followed by two additional extractions with 2 mL of methylene chloride, for a total of 9 mL. The combined extracts were evaporated to dryness and taken up in 0.5 mL of hexane.

A column containing 1 g of silica gel (deactivated with 0.2 mL water) was prepared and prewashed with hexane. The extract in hexane was transferred to the column, and the tube and column rinsed three times with 0.5-mL portions of hexane. The column was eluted with 8 mL of hexane, the eluates to this point being discarded. The column was then eluted with 15 mL of 60% benzene in hexane and the eluate collected. The eluate was suitably concentrated or diluted for quantitation by FPD–GLC.

RESULTS AND DISCUSSION

Table II lists the compounds investigated in this study, along with LD_{50} values and the dosing levels. Tables III and IV show the amount of alkyl phosphate or phenol metabolites excreted during the course of the study and the percent of the total administered dose which could be accounted for as the metabolites. Figure 1 shows the acetylcholinesterase activity of five control rats dosed only with peanut oil. Figures 2–17 show the pattern of excretion of alkyl phosphates and phenols. In addition, these figures show the effect on acetylcholinesterase activity of exposure to the organophosphorus compounds. (The cholinesterase axis is on the left; each line represents an individual rat. The urinary metabolite axis is on the right; bars show levels of metabolites in pooled urine.)

All compounds produced measurable amounts of alkyl phosphate metabolites at both levels of exposure. Because of the lack of standards, the phosphonate metabolites of EPN were not determined. With the exception of carbophenothion, which produced essentially no *p*-chlorothiophenol, the levels of phenol metabolites were comparable to the alkyl phosphates for all pesticides studied.

The plasma cholinesterase activity presented a less clear-cut picture. In all cases severe inhibition was seen at the high level of exposure. At the lower level, some compounds (dimethoate, parathion, EPN) caused little or no inhibition while others (dichlofenthion, ronnel, car-

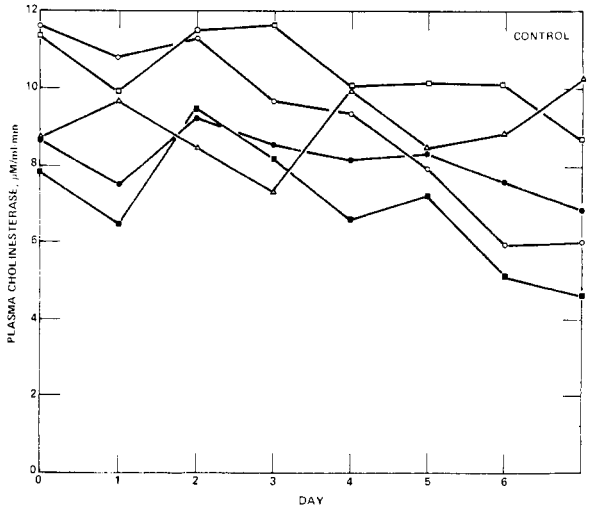

Figure 1. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: control rats (cholinesterase only).

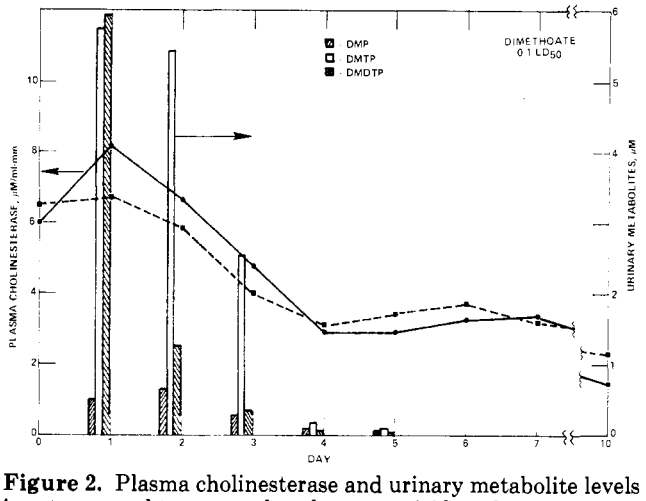

Figure 2. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: dimethoate, 0.1 LD_{50}.

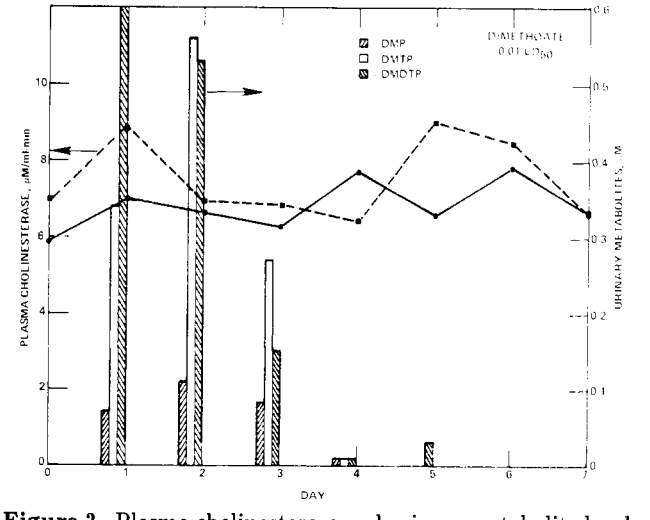

Figure 3. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: dimethoate, 0.01 LD_{50}.

bophenothion, leptophos) caused marked inhibition. Although excretion of urinary metabolites was essentially complete within 48 h of the last dose, the cholinesterase activity did not always recover as rapidly. Ronnel and

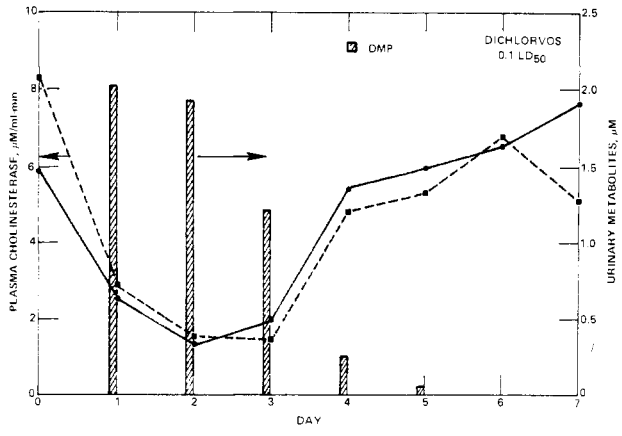

Figure 4. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: dichlorvos, 0.1 LD_{50}.

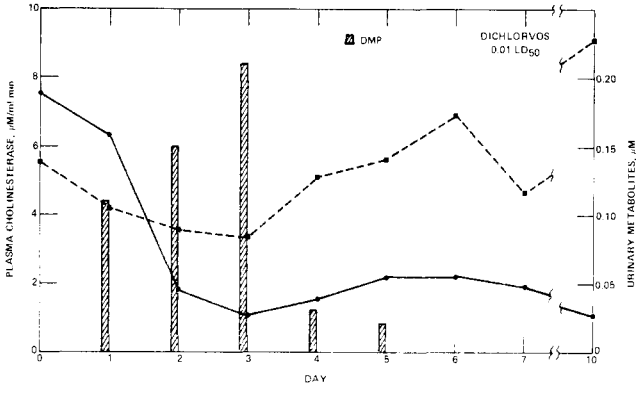

Figure 5. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: dichlorvos, 0.01 LD_{50}.

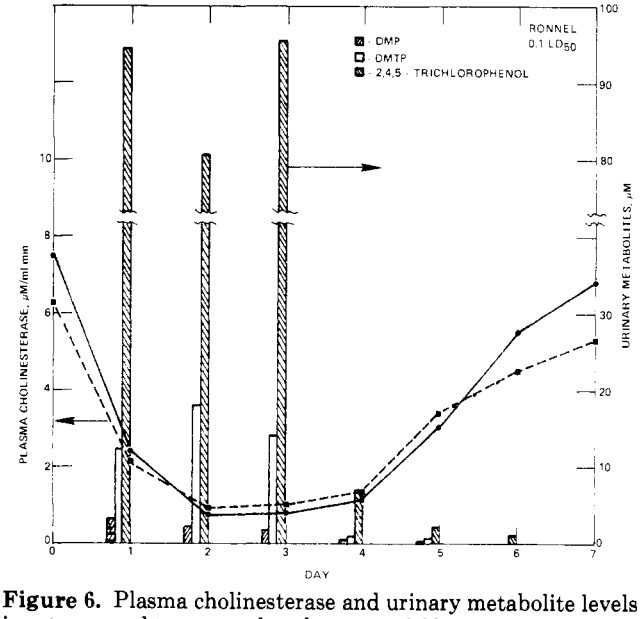

Figure 6. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: ronnel, 0.1 LD_{50}.

carbophenothion at both the high and low levels, and dichlofenthion and leptophos at the high level, caused some delay in recovery. Cholinesterase activity in rats administered 0.1 LD_{50} dimethoate did not recover during the period of the investigation.

Table V shows the levels of intact residues found in blood and adipose tissue of rats exposed to ronnel, carbophenothion, dichlofenthion, EPN, and leptophos. The

Table II. Levels of Exposure

Compound		LD_{50}, mg/kg	Mg fed $(rat)^{-1}$ $(day)^{-1}$	
Common name	Chemical name		0.1 LD_{50}	0.01 LD_{50}
Dimethoate	*O,O*-Dimethyl-*S*-(*N*-methylcarbamoylmethyl) phosphorodithioate	327	6.54	0.654
Dichlorvos	*O,O*-Dimethyl-*O*-(2,2-dichlorovinyl) phosphate	97	1.94	0.194
Ronnel	*O,O*-Dimethyl-*O*-(2,4,5-trichlorophenyl) phosphorothioate	1865	37.3	3.73
Dichlofenthion	*O,O*-Diethyl-*O*-(2,4-dichlorophenyl) phosphorothioate	270	5.4	0.54
Carbophenothion	*O,O*-Diethyl-*S*-[(*p*-chlorophenylthio)methyl] phosphorodithioate	53	1.06	0.106
Parathion	*O,O*-Diethyl-*O*-(*p*-nitrophenyl) phosphorothioate	16	0.32	0.032
EPN	*O*-Ethyl-*O*-(*p*-nitrophenyl) phenylphosphonothioate	36	0.72	0.072
Leptophos	*O*-(4-Bromo-2,5-dichlorophenyl)-*O*-methyl phenylphosphonothioate	91	1.82	0.182

Table III. Urinary Excretion of Alkyl Phosphate Metabolites of Organophosphorus Pesticides

Compound	Total μM fed 2 rats/3 days	Urinary metabolites[a]	Total μM excreted	% of dose accounted for
Dimethoate	171.0	DMP, DMTP, DMDTP	23.0	13.5
	17.1	DMP, DMTP, DMDTP	2.77	16.2
Dichlorvos	52.7	DMP	5.37	10.2
	5.27	DMP	0.522	9.9
Ronnel	696	DMP, DMTP	52.1	7.5
	69.6	DMP, DMTP	9.81	14.1
Dichlofenthion	102.8	DEP, DETP	60.1	58.5
	10.3	DEP, DETP	5.75	55.9
Carbophenothion	18.6	DEP, DETP	17.4	93.8
	1.86	DEP, DETP	0.751	40.4
Parathion	6.57	DEP, DETP	2.71	41.2
	0.657	DEP, DETP	0.260	39.6
Leptophos	21.4	MPTPn, MPPn, PPn	6.45	30.1
	2.14	MPTPn, MPPn, PPn	1.58	73.8

[a] DMP, *O,O*-dimethylphosphoric acid; DMTP, *O,O*-dimethylphosphorothioic acid; DMDTP, *O,O*-dimethylphosphorodithioic acid; DEP, *O,O*-diethylphosphoric acid; DETP, *O,O*-diethylphosphorothioic acid; MPTPn, *O*-methylphenylphosphonothioic acid; MPPn, *O*-methylphenylphosphonic acid; PPn, phenylphosphonic acid.

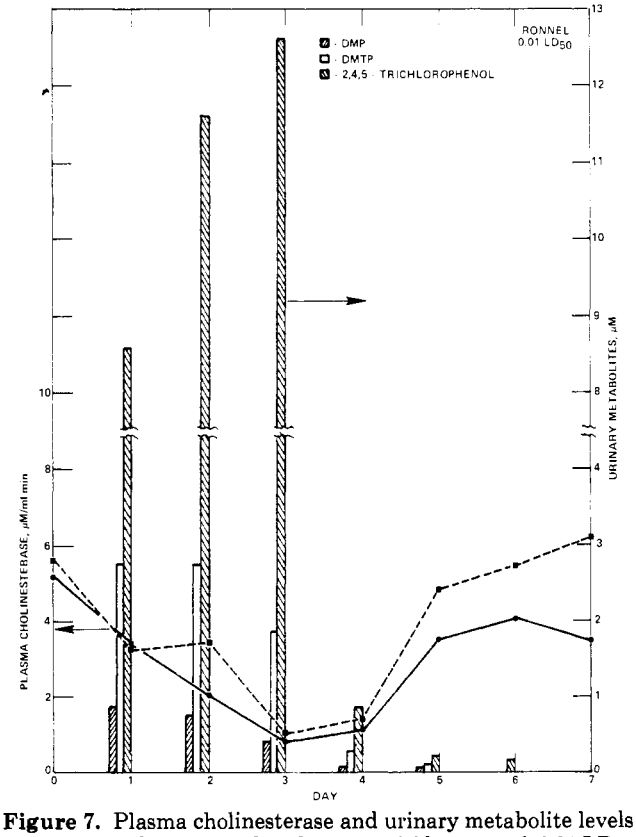

Figure 7. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: ronnel, 0.01 LD_{50}.

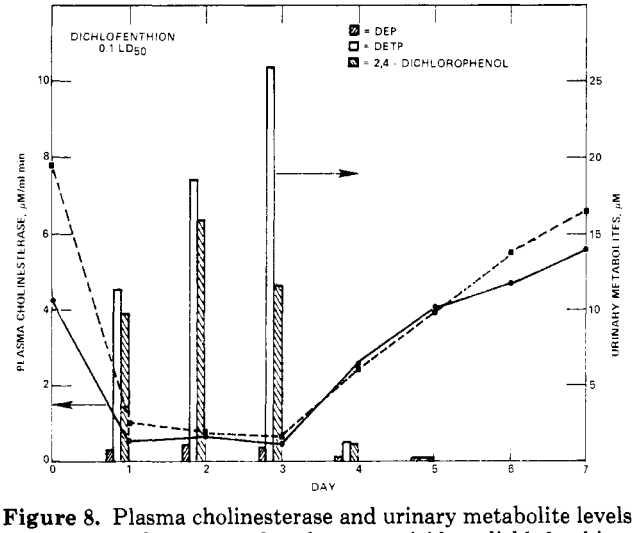

Figure 8. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: dichlofenthion, 0.1 LD_{50}.

residue levels of these compounds in blood were not remarkable. Shortly after exposure, some of the intact pesticide was found in the blood of animals that had been fed ronnel, dichlofenthion, and leptophos (the compounds fed at the highest levels). No residues were found in blood taken from animals 5 days after the last exposure.

The residues in adipose tissue presented a somewhat different picture. As expected, residues were found in samples taken shortly after the last exposure. The levels

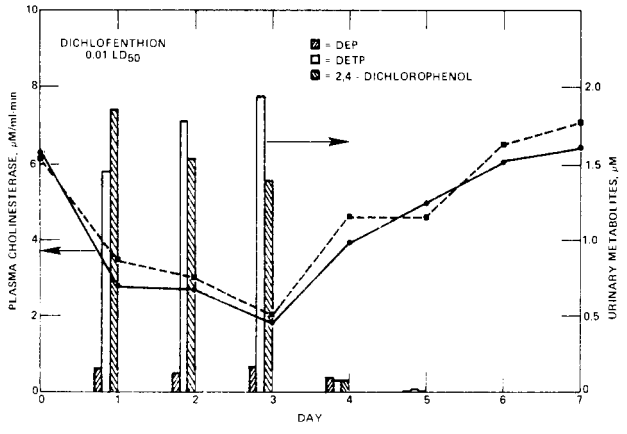

Figure 9. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: dichlofenthion, 0.01 LD_{50}.

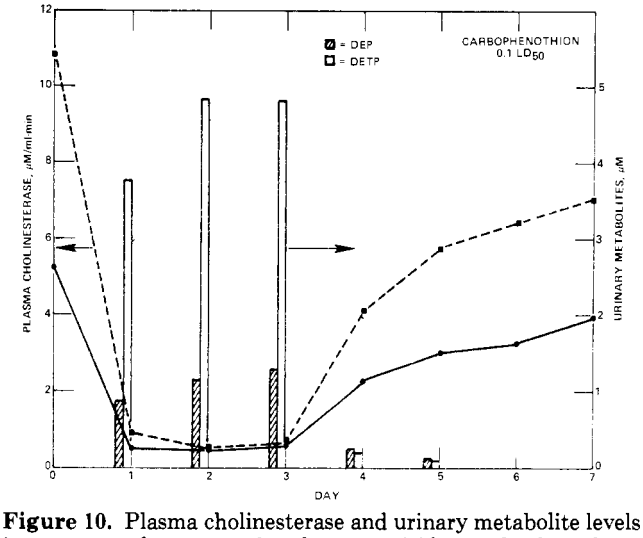

Figure 10. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: carbophenothion, 0.1 LD_{50}.

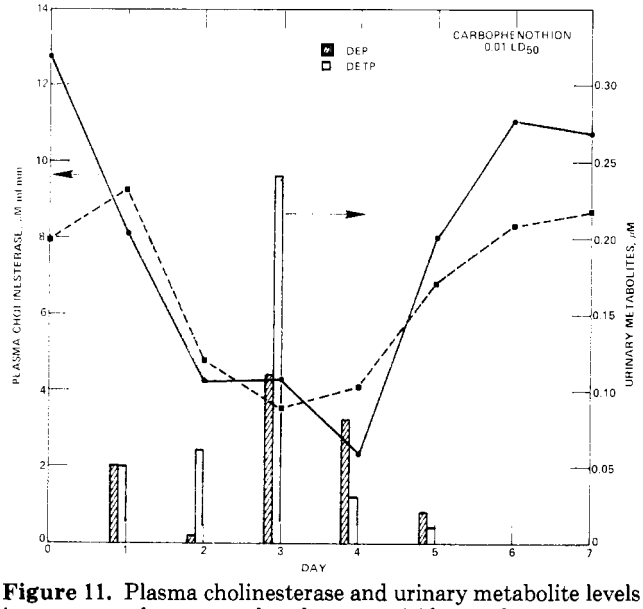

Figure 11. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: carbophenothion, 0.01 LD_{50}.

found were roughly proportional to the level of exposure although the rate of decrease in the residue levels varied widely. Ronnel dropped sharply; the level on day 8 was only 0.1% of the level on the day 3. Carbophenothion and

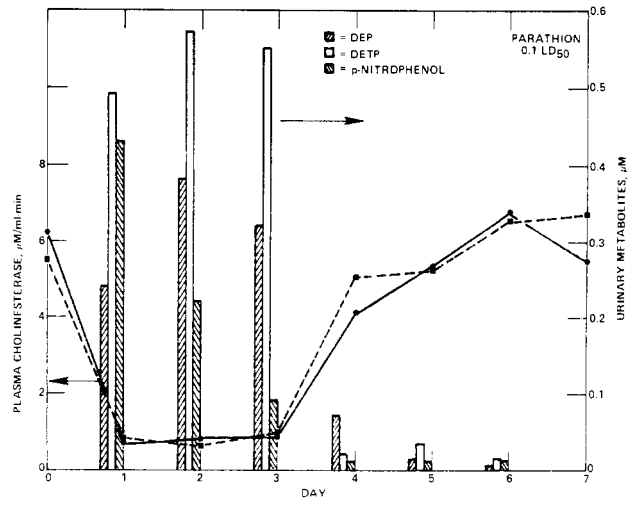

Figure 12. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: parathion, 0.1 LD_{50}.

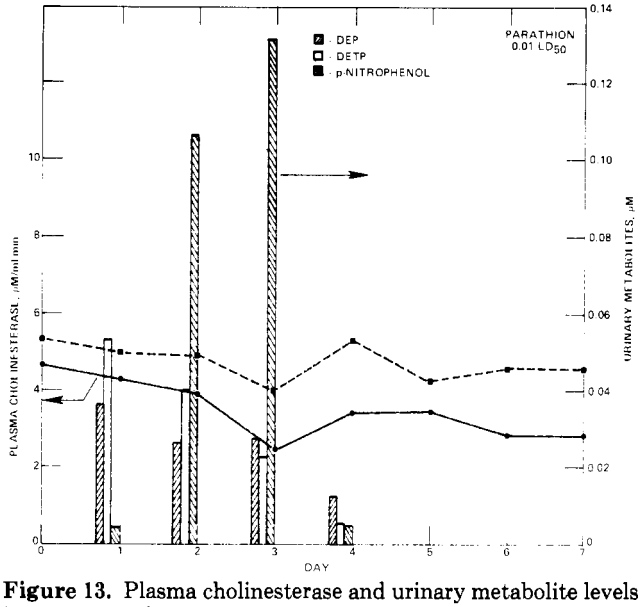

Figure 13. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: parathion, 0.01 LD_{50}.

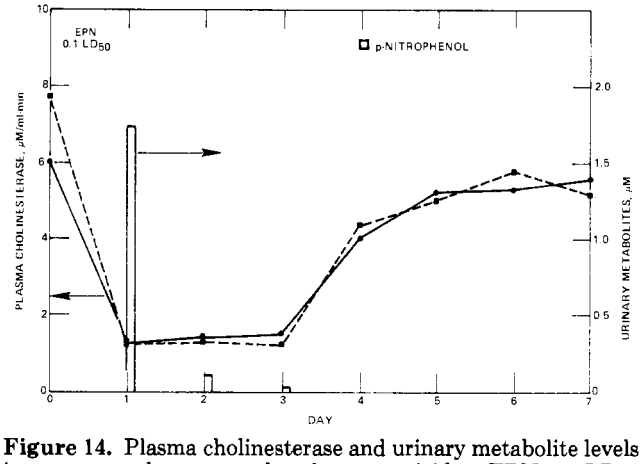

Figure 14. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: EPN, 0.1 LD_{50}.

dichlofenthion both showed a drop of 90% or more between day 3 and day 8. By contrast, the residues of leptophos on day 8 were still 38% of the levels on day 3, indicating a much slower rate of decline. This situation,

Table IV. Urinary Excretion of Phenol Metabolites of Organophosphorus Pesticides

Compound	Total μM fed 2 rats/3 days	Urinary metabolite	Total μM excreted	% of dose
Ronnel	696	2,4,5-Trichlorophenol	284	40.8
	69.6	2,4,5-Trichlorophenol	32.6	46.9
Dichlofenthion	103	2,4-Dichlorophenol	38.6	37.5
	10.3	2,4-Dichlorophenol	4.94	48.0
Carbophenothion	18.6	*p*-Chlorothiophenol	0.139	0.75
	1.86	*p*-Chlorothiophenol	ND	0
Parathion	6.57	*p*-Nitrophenol	0.775	11.8
	0.657	*p*-Nitrophenol	0.245	37.3
EPN	13.4	*p*-Nitrophenol	1.88	14.0
	1.34	*p*-Nitrophenol	0.346	25.8
Leptophos	21.4	4-Bromo-2,5-dichlorophenol	2.71	12.6
	2.14		0.251	11.7

Table V. Residues of Intact Organophosphorus Pesticides in Blood and Adipose Tissue

Compound	Blood		Adipose		Ratio: day 8/day 3
	Day 3, ppm	Day 8, ppm	Day 3, ppm	Day 8, ppm	
Ronnel	0.36	ND	370	0.40	0.0011
Dichlofenthion	0.01	ND	3.7	0.29	0.08
Carbophenothion	ND	ND	0.42	0.04	0.10
EPN	ND	ND	0.06	ND	
Leptophos	0.04	ND	4.8	1.8	0.38

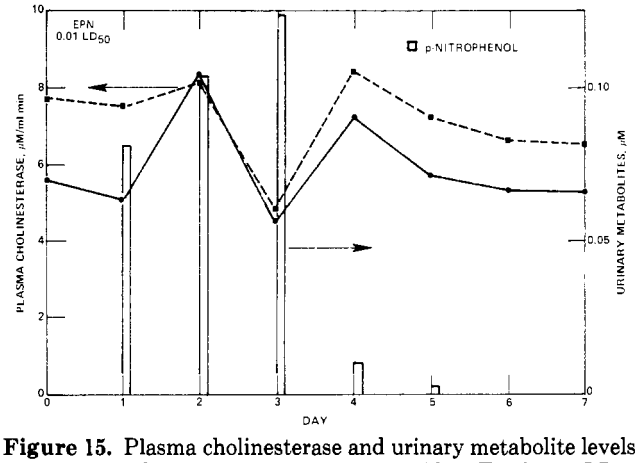

Figure 15. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: EPN, 0.01 LD_{50}.

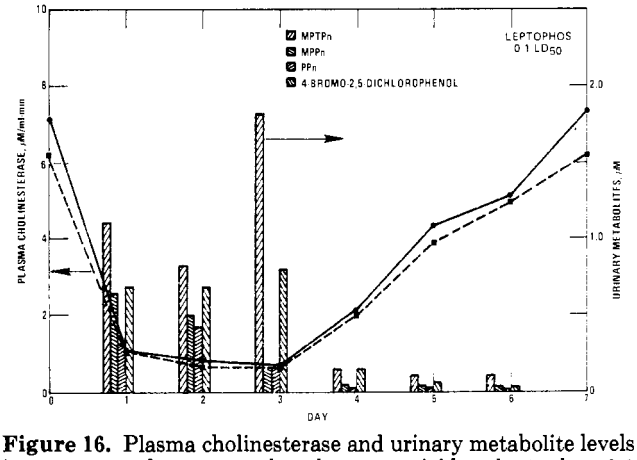

Figure 16. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: leptophos, 0.1 LD_{50}.

which raises the interesting possibility of "storage" of leptophos in adipose tissue, certainly warrants further consideration.

In general, the various analytical methods for monitoring exposure to organophosphorus pesticides which were examined in this study were quite complimentary. Using these techniques, the analytical chemist can obtain a better picture of the indices of exposure and the relationship of these indices to each other.

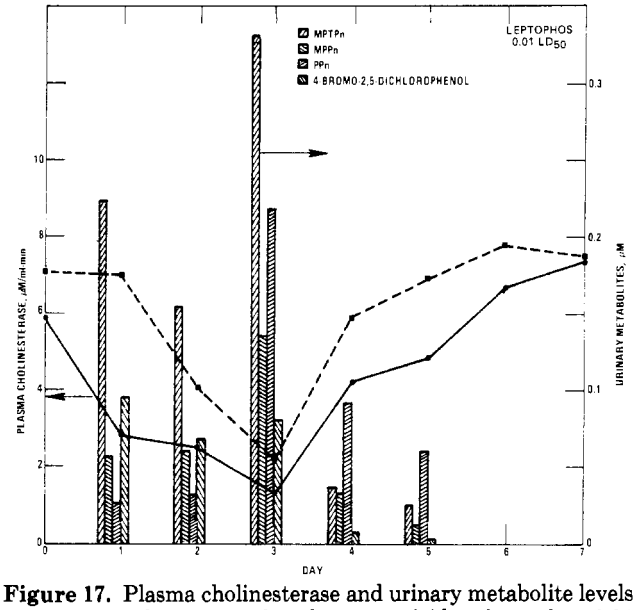

Figure 17. Plasma cholinesterase and urinary metabolite levels in rats exposed to organophosphorus pesticides: leptophos, 0.01 LD_{50}.

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