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MICROSOMAL METABOLISM OF PARATHION*

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Abstract—Studies of the microsomal activation of ^{35}S-labeled parathion showed that a ^{35}S-metabolite was bound onto microsomes probably as the result of desulfuration in the activation reaction. Further work indicated that rabbit liver microsomes also degraded parathion by splitting at the aryl phosphate bond. Both activation and degradation by microsomes required $NADPH_2$ and O_2, and were inhibited by SKF 525-A and insecticide synergists. The system was specific to parathion and did not degrade paraoxon. The degradation was also demonstrated with microsomes from rat liver and cockroach fat body and a model system (EDTA, Fe^{2+}, and ascorbate).

PHOSPHOROTHIOATE insecticides are activated to phosphate analogs by microsomal enzymes which require oxygen and $NADPH_2$ or $NADH_2$ *in vitro*. No report has yet been made on the fate of the sulfur atom detached in this reaction. If the microsomal system does not further metabolize paraoxon (O,O-diethyl *p*-nitrophenyl phosphate) or detached sulfur in the activation *in vitro* of parathion (O,O-diethyl *p*-nitrophenyl phosphorothioate), a sulfur-containing metabolite should be found in an amount equivalent to that of paraoxon. This study was started to find such a metabolite formed by the activation of ^{35}S-labeled parathion.

Previous work with cockroach fat body microsomes showed inactivation of the parathion activation enzymes during the reaction.[1] Binding of a reactive intermediate onto the microsomes was suggested as a possible cause. Therefore, a sulfur-containing metabolite bound onto the fat body microsomes was sought. In later experiments, liver microsomes of male albino rabbits were usually the enzyme source. Fat body microsomes of the American cockroach, *Periplaneta americana* (L.), and liver microsomes of male albino rats were occasionally used. The enzyme studies were supplemented with model experiments (EDTA, Fe^{2+}, and ascorbate) as before.[1]

MATERIALS AND METHODS

Chemicals. ^{35}S-Labeled parathion was purchased from the Radiochemical Centre, Amersham, Bucks., England. Specific activity was originally 50·9 mc/m-mole, although, most experiments were done with specific activities of less than 1 mc/m-mole.The radioactive material was dissolved in absolute ethanol and purified by thin-layer chromatography on silica gel GF_{254} plates, with *n*-hexane:dioxane (2:1) as the solvent. This removed four radioactive impurities. ^{35}S-Parathion on the chromatogram was

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detected under ultraviolet light (most of the sample area was covered with aluminum foil to avoid photochemical reactions). The silica gel section was scraped off, packed in a glass tube with a glass-wool plug, and the parathion was eluted with absolute ethanol. This was chromatographed again with *n*-hexane:ethyl acetate (2:1) as the solvent. Only the parathion spot showed radioactivity. Parathion was then eluted and stored at about −15° as an approximately 10^{-1} M ethanol solution containing 1% Triton X-100.

$NADPH_2$, NADP, $NADH_2$, and NAD were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Potassium O,O-diethyl phosphorothioate was supplied by Dr. E. Clark of American Cyanamid Co., Princeton, N.J. Other chemicals used in this study have been described elsewhere.[1]

Enzyme sources. Liver microsomal pellets were prepared from adult male albino rabbits or rats, by the method of Palade and Siekevitz.[2] Twenty per cent microsomes were made by suspending the pellets in 0·25 M sucrose to make half the volume of the original 10% homogenates. To this suspension was added 1 per cent by volume of 10^{-3} M paraoxon. After standing for 15 min, the mixture was centrifuged at 105,000 *g* for 1 hr to obtain paraoxon-treated microsomes. The treated microsomes were stored frozen (−15°). All experiments were done with the treated microsomes, which degraded less paraoxon than untreated microsomes; e.g. a 10% suspension of treated microsomes degraded 15 per cent of 10^{-6} M paraoxon in 15 min, whereas an untreated control degraded 77 per cent. Preparation of paraoxon-treated fat body microsomes from adult female American cockroaches has been described.[1]

Reaction systems. Parathion was incubated with an enzyme source at room temperature (approximately 25°) without shaking. A standard incubation mixture contained 0·5 ml of 40% liver microsomes in 0·25 M sucrose to make a final concentration of 10%. The following compounds were added to make a total volume of 2 ml (final concentrations indicated): parathion ($2{\cdot}5 \times 10^{-5}$ M), a cofactor $NADPH_2$ or $NADH_2$ (10^{-3} M), nicotinamide (0·01 M), KCl (0·15 M), and trisodium citrate (0·01 M). The pH of the incubation mixture was about 7·1. Trisodium citrate was used instead of phosphate buffer since the latter interfered with analyses of sulfur in the aqueous phase. Since the sensitivity of the method for *p*-nitrophenol was low, the amounts of the reagents were doubled to give a volume of 4 ml for this determination. The reaction was stopped with 0·5 ml of 20% trichloroacetic acid for ^{35}S analyses, with 1 ml of 5% trichloroacetic acid for *p*-nitrophenol determination, and by heating in boiling water for paraoxon determination.

A typical model system was made up of 0·5 ml of each of the following: 10^{-4} M parathion, 0·1 M sodium ascorbate, 10^{-3} M $FeSO_4$, 0·05 M EDTA, and 0·1 M sodium acetate or borax–KH_2PO_4 buffer. The reaction was started by addition of $FeSO_4$, and the mixture was shaken at room temperature for 1 hr.

Analyses. For quantitative determination of ^{35}S-parathion and metabolites, samples were digested with Pirie's reagent[3] [a mixture of nitric acid and perchloric acid containing $Cu(NO_3)_2$]. When samples were extracted with an organic solvent, the solvent was evaporated before digestion. The digested material was dissolved in 5 ml of 0·02 N HCl, and $^{35}SO_4$ ions were precipitated with 5 ml of 0·1 M benzidine hydrochloride in 0·4 N HCl after adding 2 ml of 0·01 M K_2SO_4 (nonradioactive). Ten minutes later, 4 ml of acetone was added and the mixture was let stand overnight. The sample was then filtered through Munktell's No. OK paper by using a fritted-

glass filtering apparatus,[4] and the disk of benzidine sulfate (19 mm in diameter) was washed first with 50% and then with 95% acetone. The precipitate on the filter paper was dried over silica gel, mounted on a planchet with glue, and counts were taken by using a Nuclear-Chicago gas flow detector, model D-47, and a Berkeley decimal scaler, model 2105. To convert counts per minute of benzidine sulfate samples at a given time into moles of the ^{35}S-parathion or metabolites, specific activity of ^{35}S-parathion was determined as follows. Two-milliliter aliquots of approximately 2×10^{-5} M ^{35}S-parathion were digested and counted as described above. Precise concentration of the same ^{35}S-parathion solution was determined colorimetrically by a modification of Lausen's method.[5] Duplicate determinations were made for four separate preparations of ^{35}S-parathion solution, and an average value of counts per minute for 2×10^{-8} mole of ^{35}S was obtained. Through this value a line was drawn on a semilog paper with a slope corresponding to the half-life of ^{35}S (87·1 days). This chart gave the specific activity at any given time.

p-Nitrophenol in the incubation mixture was determined spectrophotometrically after extraction. The mixture, acidified with trichloroacetic acid, was extracted twice with 10-ml aliquots of *n*-hexane to remove parathion and paraoxon. Residual *n*-hexane was removed by a stream of N_2, and the solution was extracted with 5 ml of 2-butanone; 3 ml of the butanone phase was transferred to another vial and was shaken with 5 ml of 0·1 N NaOH. Absorbance of the aqueous layer was determined immediately. A value for a blank was obtained with a similarly treated sample without *p*-nitrophenol. A calibration curve was prepared by processing samples of known amounts of authentic *p*-nitrophenol in the standard incubation mixture without parathion or $NADPH_2$.

Ion-exchange chromatography with Dowex 1-X8 anion exchange resin[6] was run for the determination of O,O-diethyl phosphorothioate by using a 2·5 cm (inside diameter) × 29 cm column and the following solvent system: (1) elution gradient (400 ml) pH 2 to pH 1 HCl; (2) elution gradient (400 ml) pH 1 to 1 N HCl; (3) water (115 ml) to bring pH of eluate above 1; (4) elution gradient (400 ml) pH 1 HCl plus methanol (1:3) to 1 N HCl plus methanol (1:3); (5) elution gradient (400 ml) 1 N HCl plus methanol (1:3) to 6 N HCl plus methanol (1:3). Phosphorus was determined by the method of Rockstein and Herron[7] after digestion with perchloric acid.

Infrared spectra were taken for metabolite identification, by using matched microcells and beam condensers, on a Beckman IR-5A infrared spectrophotometer. Paraoxon was assayed manometrically by using cholinesterase from housefly heads.[1]

RESULTS

Binding of ^{35}S onto microsomes

Suspected binding of detached ^{35}S onto microsomes of cockroach fat body was examined. Cockroach microsomes were homogenized in 0·25 M sucrose to make a 40% suspension, and incubated with ^{35}S-parathion in a standard incubation mixture. Protein was precipitated after 30 min with 0·5 ml of 20% trichloroacetic acid, the mixture let stand for 1·5 hr, and then filtered through Whatman No. 42 filter paper. The precipitate on the filter paper disk was washed with 5 ml of 5% trichloroacetic acid, dried over silica gel, and washed with 5 ml benzene. This sample gave a radioactivity of 277 counts/min. After further washings with 5-ml aliquots of benzene (three times), acetone, methanol, and water, an activity of 262 counts/min was

recorded. Protein precipitation with 6 ml acetone instead of trichloroacetic acid gave similar results. Since the radioactivity on the protein precipitate could not be removed by solvents, it was not due to a simple adsorption of ^{35}S-parathion but to a chemical binding of a ^{35}S-containing metabolite. Further experiments revealed the following: (1) protein-bound radioactivity increases with time in a manner similar to that of activation; (2) no binding occurs without $NADPH_2$ or $NADH_2$; (3) binding is inhibited 75 per cent by $2{\cdot}5 \times 10^{-4}$ M sesamex, and 60 per cent by 10^{-4} M WARF antiresistant (N,N-dibutyl-*p*-chlorobenzene-sulfonamide from S. B. Penick & Co., New York 8, N.Y.); (4) binding also occurs with rabbit and rat liver microsomes with the same requirement for pyridine nucleotides. These results were consistent with the assumption that the bound sulfur was derived from the sulfur detached in the activation reaction. It was also possible, however, that bound sulfur was derived from another reaction catalyzed by an enzyme system similar to the activation enzymes.

Parathion activation by rabbit liver microsomes

Rabbit liver was selected as the enzyme source for further studies because of its large size and good activity. Conversion of parathion into paraoxon by rabbit liver microsomes was confirmed by an infrared spectrum of the purified anticholinesterase metabolite from an incubation mixture as described before.[1] One hundred milliliters of an incubation mixture containing 10% rabbit liver microsomes, $2{\cdot}5 \times 10^{-5}$ M ($2{\cdot}5 \times 10^{-6}$ mole) nonradioactive parathion, and 5×10^{-4} M $NADPH_2$ produced $1{\cdot}1 \times 10^{-6}$ mole of anticholinesterase in terms of paraoxon in 1 hr. Sixty-seven per cent was extracted with 100 ml *n*-hexane, and thin-layer chromatography of the metabolite gave a single spot containing a cholinesterase inhibitor, as revealed by manometric assay of methanol eluates from sections of the chromatogram. The R_f value of this spot was the same as that of paraoxon. The infrared spectrum of the eluted material in carbon tetrachloride was identical with that of authentic paraoxon.

Quantitative analyses of the incubation mixture

^{35}S-Parathion was incubated with rabbit liver microsomes for 1 hr, and the mixture was analyzed for *n*-hexane-soluble, aqueous, and protein-bound radioactivity, and for paraoxon (Table 1). Radioactivity was determined after digesting the sample with

TABLE 1. ANALYSIS OF INCUBATION MIXTURE

Sample	^{35}S (10^{-6} M)		
	Total	*n*-Hexane-extractable*	Aqueous
Whole incubation mixture	8·26	3·70	4·02
Washed precipitate	1·49		
Acetone washings of precipitate	1·74	1·69	0·01
Filtrate	4·25	1·28	2·85
Controls (whole incubation mixture)			
0-time	8·32	7·72	0·06
No $NADH_2$	8·46	7·76	0·03
Paraoxon formed: $1{\cdot}85 \times 10^{-6}$ M			

* Some radioactivity was lost during evaporation of *n*-hexane; cf. Fig. 5.
^{35}S-Parathion was incubated with 10% rabbit liver microsomes and 10^{-3} M $NADH_2$ for 1 hr. The incubation mixture was analyzed in the scheme shown in Fig. 3 for *n*-hexane-soluble, aqueous, and protein-bound radioactivity. Paraoxon was determined simultaneously.

Pirie's reagent. At least 49 per cent ($4{\cdot}02 \times 10^{-6}$ M) of the parathion had been metabolized to aqueous ^{35}S-metabolites, whereas paraoxon formed and bound sulfur were 22 and 18 per cent respectively. Paraoxon degradation by the treated microsomes was too small to account for the difference between the amounts of the aqueous metabolite and paraoxon (Table 2). This meant that at least one metabolite other than paraoxon

TABLE 2. DEGRADATION OF PARAOXON BY PARAOXON-TREATED MICROSOMES

Paraoxon added	Cofactor	% Paraoxon remaining					
		0 min	15 min	30 min	60 min	90 min	120 min
10^{-6} M	10^{-3} M $NADPH_2$	85	76	71	64	48	
	10^{-3} M $NADH_2$	86	80	74	64	41	
	None	85	76	66	56	49	
$2{\cdot}3 \times 10^{-5}$ M	None	101	91		102		85
$9{\cdot}4 \times 10^{-6}$ M		96	87		89		85
$2{\cdot}2 \times 10^{-6}$ M		90	85		82		69
$8{\cdot}8 \times 10^{-7}$ M		73	66		57		41

Ten per cent rabbit liver microsomes were incubated with paraoxon. Aliquots of the incubation mixture were heated at intervals and paraoxon concentration determined.

was produced in the presence of $NADH_2$. Other experiments showed that chloroform extracted more radioactivity than *n*-hexane, indicating that chloroform extracted some ^{35}S-metabolite(s) as well as ^{35}S-parathion. When ^{35}S-parathion was removed by extracting with *n*-hexane, chloroform extracted radioactivity only from strongly

TABLE 3. EXTRACTION OF ACIDIC METABOLITE FROM INCUBATION MIXTURE

Second solvent	pH	^{35}S (10^{-6} M)			
				2nd Solvent	
		Parathion added	*n*-Hexane extracts*	1st extract	2nd extract
Chloroform	<1	22·75	11·98	1·60	1·07
	3·5		12·64	0·19†	
Ethyl acetate	<1	19·50	7·41	3·09	0·46
	3·5		7·60	0·36†	

* Some radioactivity was lost during evaporation of *n*-hexane; cf. Fig. 5.

† First and second extracts combined.

^{35}S-Parathion was incubated with 10% rabbit liver microsomes and 10^{-3} M $NADH_2$ for 1 hr, and reaction was stopped with trichloroacetic acid with a resulting pH of less than 1. Na_2HPO_4 was used to raise pH to 3·5. The mixture was extracted twice with 5-ml aliquots of *n*-hexane, and then with a second solvent.

acidic media (Table 3). This indicated that the radioactive material extracted with chloroform was strongly acidic. Since chloroform did not extract the acidic metabolite(s) very efficiently, other solvents were examined. Ethyl acetate was a better solvent, and benzene and ether were less efficient. Production of the acidic metabolite(s) was also shown with rat liver microsomes, fat body microsomes of the American cockroach, and the EDTA–Fe^{2+}-ascorbate model system (Table 4).

TABLE 4. PRODUCTION OF PARAOXON AND ACIDIC METABOLITE

Reaction system	Incubation time (hr)	Parathion added (10^{-6} M)	Metabolite (10^{-6} M)	
			Paraoxon	Acidic metabolite
Rat liver microsomes	2	27·2	5·48	4·28
Cockroach microsomes	2	41·5	2·58	2·55
Model system	1	39·0	4·34	12·7

Ten per cent rat liver microsomes were incubated with 10^{-3} M $NADH_2$, and 15% cockroach fat body microsomes were incubated with 10^{-3} M $NADPH_2$. Borax–KH_2PO_4 buffer, pH 6·3, was used for the model system. See Fig. 3 for operational definition of acidic metabolite.

Yellow metabolite

Parathion was incubated with rabbit liver microsomes for 1 hr, and the mixture was centrifuged at 105,000 *g* for 1 hr. Extraction of the supernatant with *n*-hexane left a yellow aqueous layer which turned colorless on acidification to pH 4·3 with HCl. The yellow solution showed the same spectrum as *p*-nitrophenol in the range of 360~500 mμ when a blank incubation mixture without parathion was used for reference. The production of yellow color required $NADPH_2$ or $NADH_2$. These results indicated that *p*-nitrophenol was produced in the order of 10^{-5} M from parathion by an enzyme system similar to that effecting activation.

After the incubation mixture was extracted with *n*-hexane to remove parathion and paraoxon, the yellow compound was extracted with chloroform from acidic media. Chloroform was evaporated and the residue dissolved in 0·1 N NaOH. The spectrum was the same as that of *p*-nitrophenol in the 340~500 mμ range, with a peak at 400 mμ. The yellow material had the same R_f values as *p*-nitrophenol on thin-layer chromatograms on silica gel GF_{254} with the following solvent systems; chloroform:isobutanol (10:1, $R_f = 0{\cdot}46$; 5:1, $R_f = 0{\cdot}71$); *n*-hexane:dioxane (1:1, $R_f = 0{\cdot}41$; 2:3, $R_f = 0{\cdot}60$). Also, co-chromatography of the material with *p*-nitrophenol gave a single spot with the above systems. After the compound extracted with chloroform was purified by thin-layer chromatography and dissolved in water, the absorbance at 400 mμ was determined at various pH values (Fig. 1). The pK of the compound (7·06) matched that of *p*-nitrophenol determined similarly. An infrared spectrum of the yellow compound eluted from a chromatogram showed that the compound was *p*-nitrophenol (Fig. 2). Although extra peaks were seen on the spectrum of the yellow metabolite at 3000, 1725, and around 900 cm^{-1}, they are probably due to contamination; positions and shapes of major peaks were identical with those of *p*-nitrophenol. Therefore, the yellow compound was identified as *p*-nitrophenol.

Incubation of paraoxon (10^{-5} M) with microsomes and $NADPH_2$ or $NADH_2$ for 2 hr did not produce *p*-nitrophenol. Paraoxon degradation by treated microsomes, which was not affected by addition of $NADPH_2$ or $NADH_2$ (Table 2), was too small to account for the *p*-nitrophenol produced in the parathion incubation mixture. Therefore, *p*-nitrophenol was not produced secondarily from paraoxon but was derived directly from parathion metabolism. The yellow metabolite was also produced by the model system.

Identification of the acidic metabolite

A balance sheet of the microsomal metabolism of parathion was made by analyzing a ^{35}S-parathion incubation mixture (Fig. 3). The acidic metabolite and bound sulfur accounted for most of the ^{35}S of parathion metabolized. Therefore, *p*-nitrophenol was produced in connection with one of these two ^{35}S-metabolites. The production

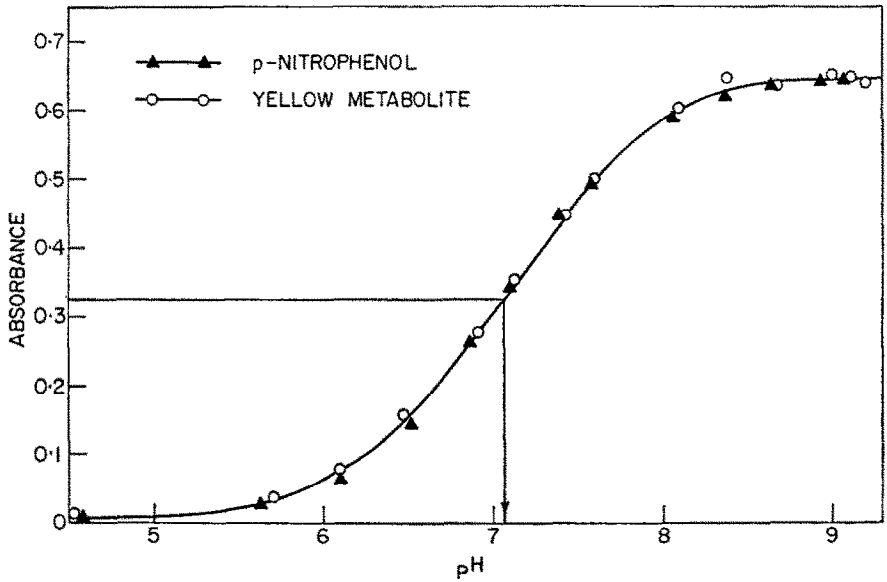


FIG. 1. Determination of p*K* values for *p*-nitrophenol and yellow metabolite. One hundred milliliters of a mixture containing 2×10^{-4} M nonradioactive parathion, 10% rabbit liver microsomes, and 10^{-3} M $NADPH_2$ were incubated for 1 hr, acidified with 25 ml of 0·4 N HCl, and centrifuged at 105,000 *g* for 1 hr. Supernatant was washed with *n*-hexane and yellow metabolite was extracted with chloroform. After evaporation of the solvent, the metabolite was dissolved in 8 ml water. Two milliliters of a borax–KH_2PO_4 buffer was mixed with 0·5 ml of the metabolite solution, and absorbance at 400 mμ and pH were determined by using a Beckman DU spectrophotometer and a Beckman GS pH meter respectively.

of *p*-nitrophenol meant that parathion was split at the aryl phosphate bond; therefore the phosphate moiety had to give rise to another metabolite. It was likely that the latter was the acidic ^{35}S-metabolite described above. The acidic metabolite was extracted with chloroform (ethyl acetate was not suitable for purification because acetic acid from its hydrolysis interfered), and chromatographed by thin-layer chromatography (cellulose G; isopropanol:2 N ammonia, 8:2). The chromatogram was scanned with an end-window Geiger counter through a 5-mm slit made in aluminum foil. The radioactive spot was positive to a P=S test[8] and a phosphorus test,[9] and the R_f value (~0·8) was about the same as that of potassium O,O-diethyl phosphorothioate. However, R_f values were not constant, which may have been due to salt contamination. Therefore, the extracted metabolite was dissolved in about 5 ml water after evaporation of chloroform, and chromatographed on a Dowex 1-X8 column and compared with authentic potassium O,O-diethyl phosphorothioate When the metabolite was co-chromatographed with the standard compound, the radioactive metabolite was eluted simultaneously with the standard compound as

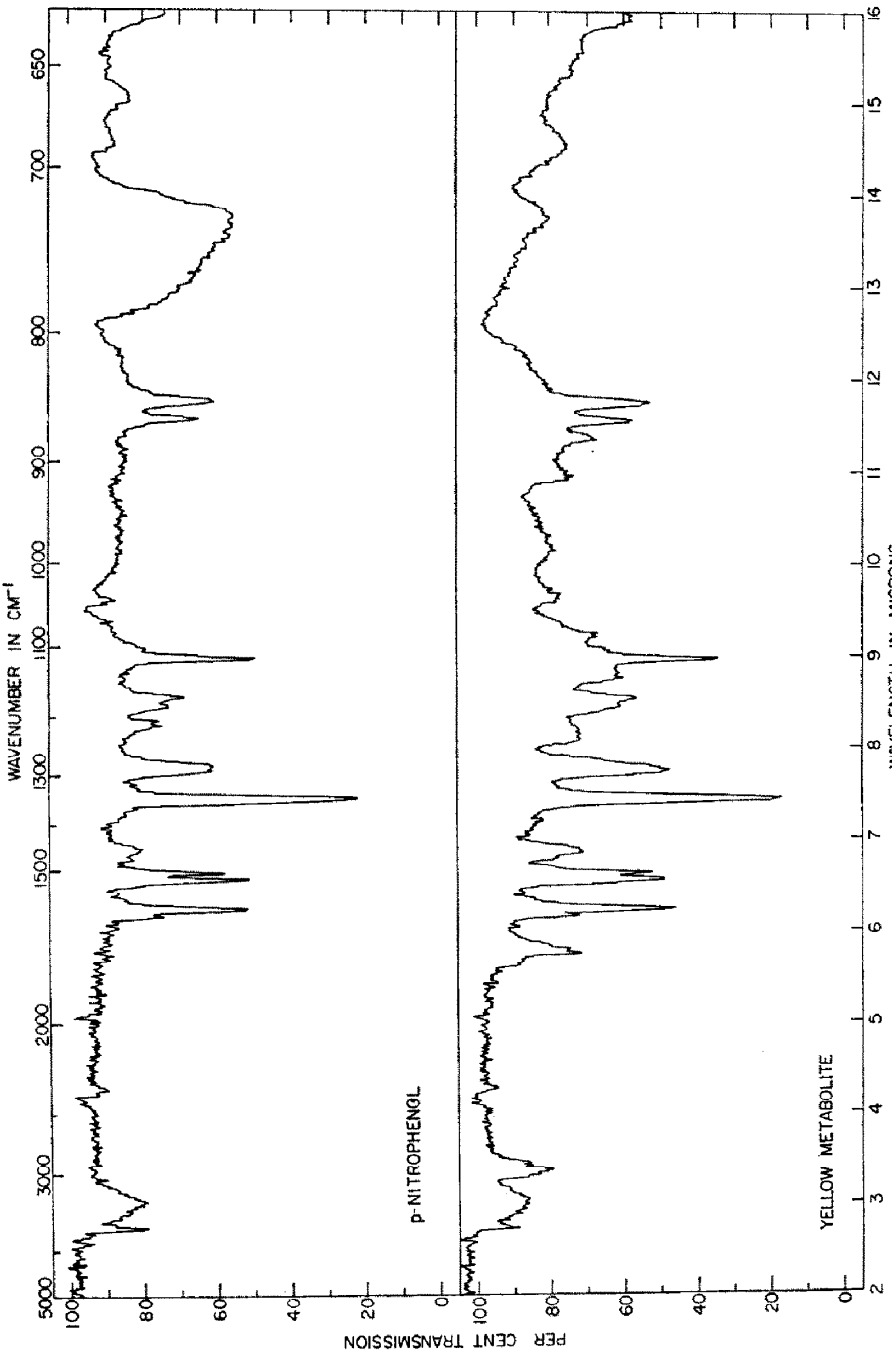


FIG. 2. Infrared spectra of *p*-nitrophenol and yellow metabolite. The metabolite was extracted as described for Fig. 1 and isolated by thin-layer chromatography; silica gel GF_{254} was used with *n*-hexane:dioxane (1:1) as the solvent. An infrared spectrum of the metabolite in $CHCl_3$ was taken by using matched 0·05 mm cells.

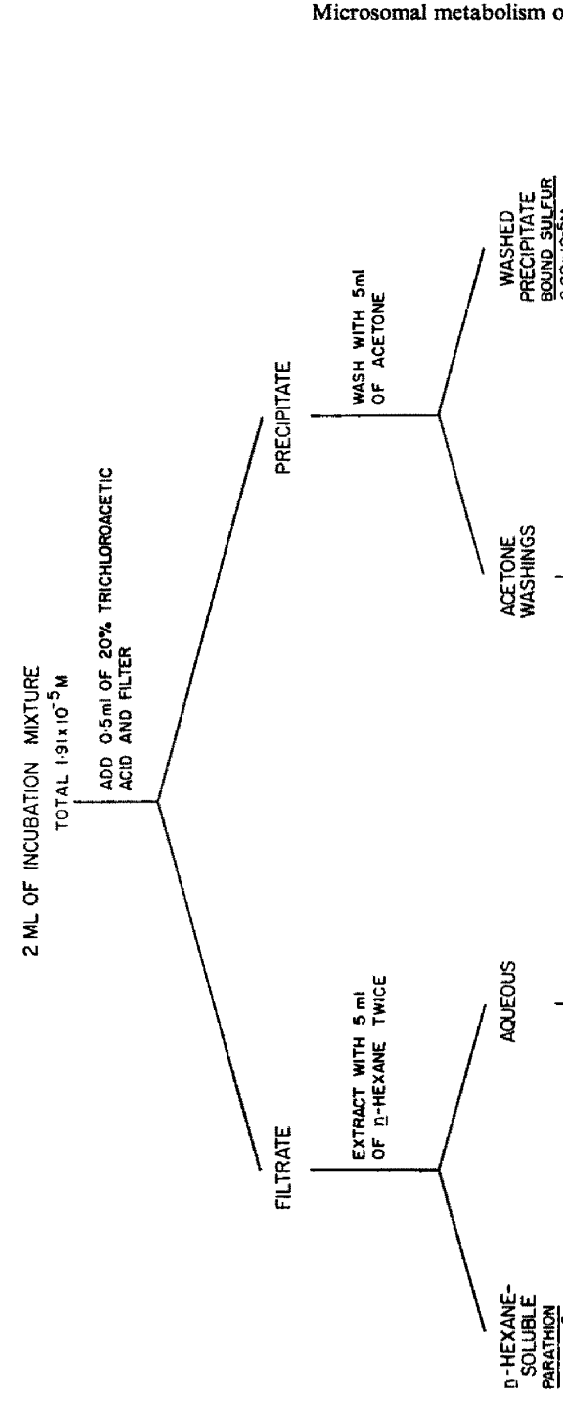


FIG. 3. Fractionation of microsomal incubation mixture containing ^{35}S-parathion and metabolites. ^{35}S-Parathion was incubated with 10% rabbit liver microsomes and 5×10^{-4} M $NADPH_2$ for 2 hr, and ^{35}S in various fractions was quantitatively determined. Concentrations given are the average values of triplicate analyses and refer to original concentration in the incubation mixture. Assays of samples incubated simultaneously showed production of $1{\cdot}07 \times 10^{-5}$ M *p*-nitrophenol and $0{\cdot}53 \times 10^{-5}$ M paraoxon.

measured by phosphorus content. Furthermore, the elution volume was the same as that for the metabolite chromatographed alone (Fig. 4). Yield of the metabolite through the chromatographic column was 100 per cent as calculated by radioactivity and eluate volumes in the sample tubes. These results indicated that the acidic metabolite was O,O-diethyl phosphorothioic acid.

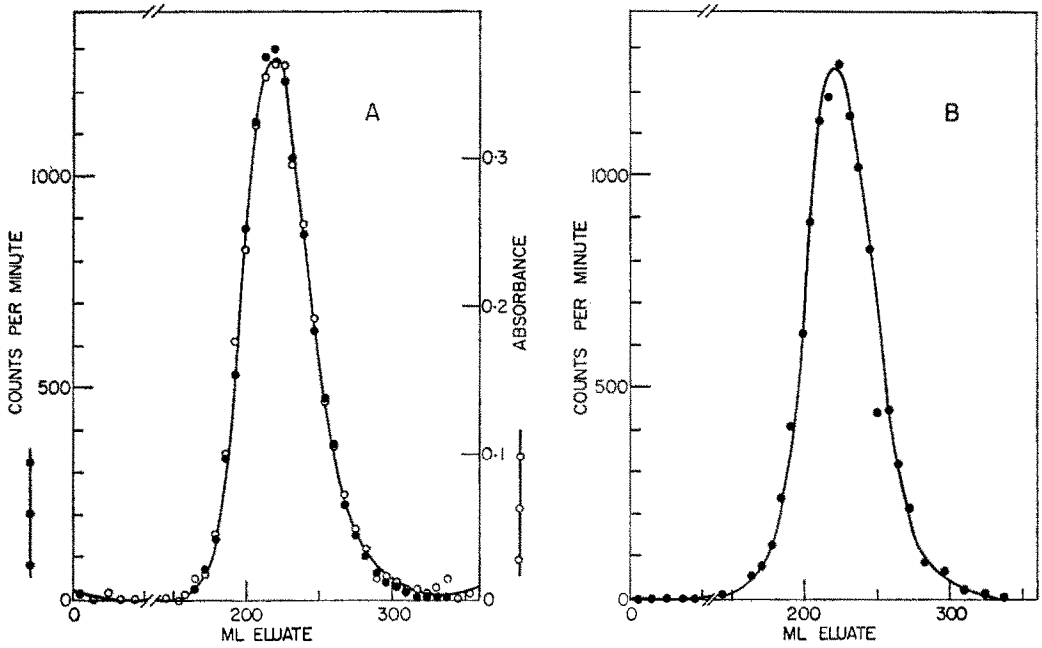


FIG. 4. Ion-exchange chromatography of acidic metabolite. Approximately $2{\cdot}5 \times 10^{-5}$ M ^{35}S-parathion was incubated with 10% rabbit liver microsomes and 5×10^{-4} M $NADPH_2$ for 2 hr (total volume 120 ml). The mixture was acidified to pH 4·3 with 1 N HCl and centrifuged at 78,000 *g* for 1 hr. The supernatant, which contained all the acidic metabolite, was washed with *n*-hexane and then with chloroform, and acidified with 20 ml of 6 N HCl. The acidic metabolite was extracted with chloroform and the solvent evaporated. The metabolite was dissolved in water and chromatographed on a Dowex 1-X8 column with the solvent system described in Materials and Methods. Graphs show only a part of chromatograms, where all radioactivity was recovered, and volumes of the eluate refer to those of elution gradient, pH 1 HCl plus methanol (1:3) to 1 N HCl plus methanol (1:3). Graph A: Co-chromatography of $4{\cdot}34 \times 10^{-7}$ mole of the metabolite and $1{\cdot}60 \times 10^{-5}$ mole of potassium O,O-diethyl phosphorothioate. Graph B: Chromatography of acidic metabolite alone.

Nature of the microsomal enzymes system for parathion metabolism

Reaction conditions *in vitro* were quite similar for the production of acidic metabolite, *p*-nitrophenol, paraoxon, and bound sulfur. (1) $NADPH_2$ was the best cofactor and $NADH_2$ was also effective; NAD had a lower, but definite, activity and NADP was ineffective (Table 5). The reason for the activity of NAD was not found. (2) Anaerobic incubation in the Thunberg tube suppressed the production of the metabolites by 73 to 90 per cent of the control values, indicating a requirement for O_2. (3) Formation of the metabolites was inhibited by SKF 525-A, WARF antiresistant,

and several pyrethrum synergists (Table 6) just as in the case of the parathion activation reaction by cockroach microsomes.[1] (4) Optimum pH for the microsomal metabolism was about 7 with $NADPH_2$ as the cofactor. Parathion metabolism by the model system was little affected by pH values between 4·9 and 7·7.

TABLE 5. COFACTOR REQUIREMENT FOR PARATHION METABOLISM BY RABBIT LIVER MICROSOMES

Cofactor	Metabolite produced (10^{-6} M)			
	Acidic metabolite	*p*-Nitrophenol	Bound sulfur	Paraoxon
$NADPH_2$	8·9	11·7	9·8	7·0
$NADH_2$	5·0	5·0	3·2	4·8
NADP	0·1	0·1	0·2	0·1
NAD	1·5	2·7	2·2	1·5
None	0·0	0·4	0·2	0·2

Cofactors were tested at a final concentration of 10^{-3} M. Substrate and microsome concentrations and incubation time were, respectively: $2{\cdot}36 \times 10^{-5}$ M, 10% and 60 min for acidic metabolite and paraoxon determinations; 5×10^{-5} M, 5% and 30 min for *p*-nitrophenol determination; and $2{\cdot}32 \times 10^{-5}$ M, 5% and 30 min for bound sulfur determination. Since the determinations of all metabolites were not done with the same microsomes, figures should be compared only between the cofactors.

TABLE 6. PER CENT INHIBITION OF PARATHION METABOLISM

Inhibitor	Inhibitor concentration (M)	Metabolite			
		Acidic metabolite	*p*-Nitrophenol	Bound sulfur	Paraoxon
WARF antiresistant	4×10^{-4}	48	45	45	40
	1×10^{-4}	16	12	16	14
SKF 525-A	4×10^{-4}	64	53	62	55
	1×10^{-4}	26	34	37	29
MGK 264	4×10^{-4}	56	66	67	54
	1×10^{-4}	28	15	29	11
Piperonyl butoxide	4×10^{-4}	85	76	64	87
	1×10^{-4}	35	15	48	34
Sesamex	4×10^{-4}	30		35	36
	1×10^{-4}	15	6	16	10
Propyl isome	4×10^{-4}	61	68	56	60
	1×10^{-4}	22	15	30	31
Sulfoxide	4×10^{-4}	74	44	57	76
	1×10^{-4}	33	21	36	35

Ten per cent rabbit liver microsomes were incubated in the standard incubation mixture with approximately $2{\cdot}5 \times 10^{-5}$ M parathion, 5×10^{-4} M $NADPH_2$, and an inhibitor for 1 hr. Metabolites produced were compared with those produced in the absence of an inhibitor.

Time course of the reaction was examined for the four metabolites. When about 2×10^{-5} M parathion was incubated with 10^{-3} M $NADPH_2$ and 10% rabbit liver microsomes, most of the parathion was metabolized in 1 hr, as indicated by the radioactivity in *n*-hexane extracts. The increase of acidic metabolite was roughly parallel to that of *p*-nitrophenol, and binding of sulfur proceeded in a manner similar

to paraoxon production. This indicated the occurrence of two reactions, one producing acidic metabolite and *p*-nitrophenol, and the other, paraoxon and bound sulfur. The ratio of acidic metabolite to paraoxon ranged from 1:1 to 2:1. Figure 5 illustrates an example.

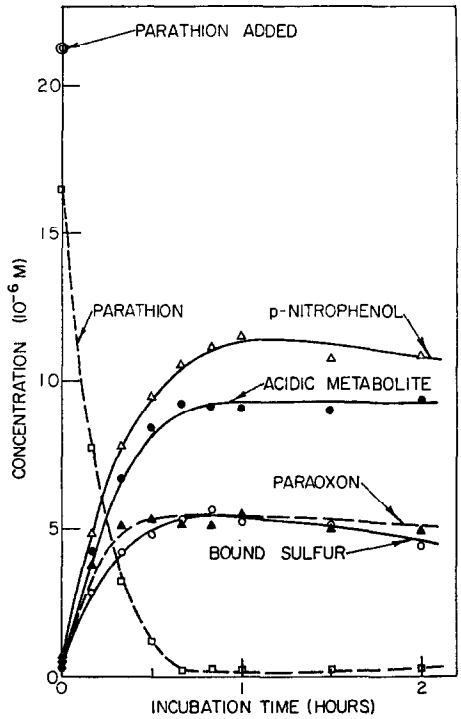
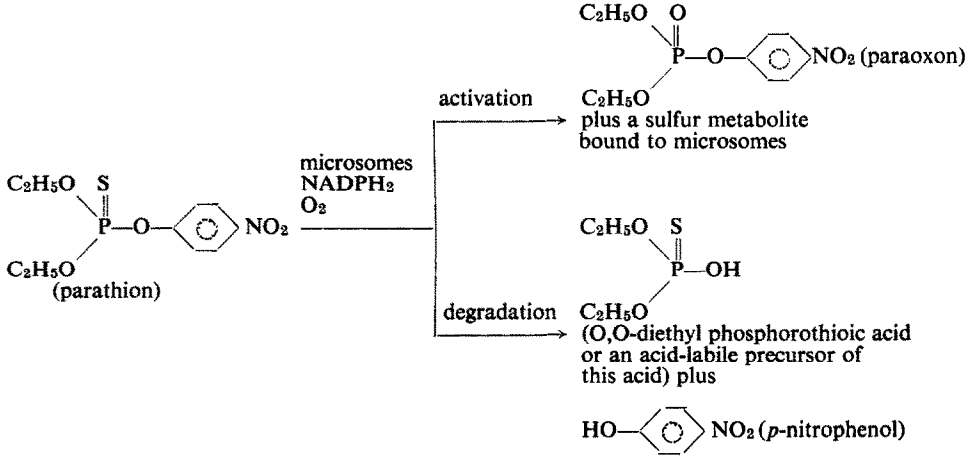


FIG. 5. Time course of parathion metabolism by rabbit liver microsomes. ^{35}S-Parathion was incubated with 10% rabbit liver microsomes and 10^{-3} M $NADPH_2$. Parathion was recovered as *n*-hexane extract of the whole incubation mixture; concentrations for parathion are probably lower than actual values because of evaporation losses. Metabolites were assayed as described in Materials and Methods (after fractionation as in Fig. 3 where ^{35}S was determined).

DISCUSSION

Analyses of ^{35}S in the incubation mixture showed that bound sulfur and acidic metabolite constitute most of the ^{35}S-metabolites produced by rabbit liver microsomes. The amount of residual radioactivity found after removal of parathion and the two ^{35}S-metabolites was too small to account for the sulfur metabolite from the activation reaction. The acidic metabolite is probably produced in connection with *p*-nitrophenol by an enzymatic splitting of the aryl phosphate bond of parathion. The results, therefore, indicate that bound sulfur is the sulfur detached in the conversion of parathion to paraoxon. This conclusion is based on the assumption that all or most of the detached sulfur was bound to microsomes. It is possible, however, that only part of the detached sulfur was represented by bound sulfur. It is also possible that a reactive intermediate of parathion metabolism is bound without any relation to paraoxon formation. Nevertheless, the above conclusion seems to be the most likely possibility.

Since all four metabolites were produced from parathion by the microsomes–$NADPH_2$–O_2 system, the following scheme may be suggested for parathion metabolism by microsomal enzymes:



If this scheme is a complete picture of the reactions *in vitro*, the amounts of paraoxon and acidic metabolite should be equal to those of bound sulfur and *p*-nitrophenol, respectively, and the sum of paraoxon and acidic metabolite should account for the parathion metabolized. Our results, however, deviate from this idealized situation (Fig. 5). This may be partly due to imperfections in the assay procedures. It is also likely that part of the deviation was due to further degradation of the metabolites. Although microsomes were pretreated with paraoxon, some paraoxon was still degraded in the system, indicating the presence of phosphatase. The slight decrease of paraoxon in the time-course plot (Fig. 5) might be due to such enzymes. Acidic metabolite, bound sulfur, and *p*-nitrophenol might also be degraded further. A small amount of the residual radioactivity might be due to such secondary metabolites. A third reaction of parathion metabolism might also contribute to the residual activity.

The scheme suggested, however, would represent the major pathways of microsomal metabolism of parathion. Under *in vitro* conditions, degradation was at least as extensive as activation. Whether the two reactions are catalyzed by the same enzyme system or each is catalyzed by a separate enzyme system is not clear. If the former is the case, the two pairs of metabolites would arise from decomposition of an intermediate formed enzymatically. However, variation of the ratio of acidic metabolite to paraoxon might be an indication of separate enzyme systems.

Since the model system produced paraoxon,[1, 10] acidic metabolite, and yellow metabolite, the reaction scheme for the microsomal system would probably apply to the model except that detached sulfur is not bound. Knaak *et al.*[10] reported that parathion was hydrolyzed more than activated by the model system. Since the ratio of hydrolysis to activation was fairly constant, they suggested that the system formed an activated intermediate of parathion which rapidly decomposed to form paraoxon or hydrolysis products. Our results with the model system indicate that the reported hydrolysis is at the aryl phosphate bond. However, the suggested mechanism of the

reaction must be reconsidered. The ratio of degradation to activation seems much greater in the model than in the microsomal system.

Although binding of a sulfur metabolite was originally suspected as a cause of inactivation of parathion activation enzymes of cockroach fat body, our discovery of such binding does not necessarily prove that it is the case. Binding occurs with rabbit and rat liver microsomes as well, but inactivation in the liver microsomes has not been examined.

Paraoxon degradation was not affected by reduced pyridine nucleotides, indicating that the microsomes–$NADPH_2$–O_2 system does not metabolize paraoxon but is specific to parathion. The system also metabolizes methyl parathion and sumithion, as evidenced by formation of phenolic products (unpublished results). Although it is difficult to predict phenomena *in vivo* from evidence *in vitro*, the microsomal system might be a direct detoxication mechanism of phosphorothioate insecticides *in vivo*. O,O-Dialkyl phosphorothioates have been reported as major metabolites of several organophosphorus insecticides *in vivo*.[11–15] Although it has been assumed that O,O-dialkyl phosphorothioates are produced by phosphatase action and hydrolytic degradation has been demonstrated *in vitro*,[16, 17] microsomal degradation might also play an important role in the "hydrolytic" degradation *in vivo*. Studies of significance *in vivo* of microsomal enzymes in the phosphorothioate detoxication are in progress.

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