Studies of the Enzymic Mechanism of the Metabolism of Diethyl 4-Nitrophenyl Phosphorothionate (Parathion) by Rat Liver Microsomes

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1. The metabolism of parathion by rat liver microsomes is affected by various enzyme inhibitors in a manner quite typical of the 'mixed-function oxidase' enzyme systems. 2. With many of these inhibitors (*p*-chloromercuribenzoate, Cu^{2+} , 8-hydroxyquinoline) the conversion of parathion into diethyl hydrogen phosphorothionate is less inhibited than conversion into diethyl 4-nitrophenyl phosphate (paraoxon). 3. Compounds containing reduced sulphur stimulate the overall metabolism of parathion. However, the conversion of parathion into diethyl hydrogen phosphorothionate is stimulated more than its conversion into paraoxon. 4. The metabolism of parathion to diethyl hydrogen phosphorothionate is also stimulated by EDTA, Ca^{2+} and Ba^{2+} , but these stimulatory effects are not additive. 5. The electron acceptors FAD, riboflavine, menadione and methylene blue exhibit a concentration-dependent differential inhibition of the metabolism of parathion to diethyl hydrogen phosphorothionate and to paraoxon. 6. The concentration of parathion required for the half-maximal rate of production of diethyl hydrogen phosphorothionate is significantly different from the concentration required for half-maximal rate of production of paraoxon. 7. The results are discussed in terms of either two separate enzyme systems metabolizing parathion to diethyl hydrogen phosphorothionate and to paraoxon or two different binding sites for parathion, which share a common electron-transport pathway.

In a previous paper (Neal, 1967) the metabolism *in vitro* of the cholinergic phosphorothionate diethyl 4-nitrophenyl phosphorothionate (parathion) by an enzyme system or systems located in microsomal particulate fractions isolated from various animal species and from various tissues was described. The present paper reports an investigation of the effects of various enzyme inhibitors and activators on the enzymes or enzyme systems that metabolize parathion.

There have been numerous reports providing evidence for the existence of more than one 'mixed-function oxidase' enzyme system in liver microsomes (Kampffmeyer & Kiese, 1964; Creaven, Parke & Williams, 1965; Conney, Gillette, Inscoc, Trams & Posner, 1959; Gaudette & Brodie, 1959; Vardanis, 1966; Gram & Fouts, 1966). In the studies described below, an attempt was made to determine whether more than one 'mixed-function oxidase' enzyme system was involved in the initial reactions leading to the metabolism of parathion and whether separate enzyme systems were responsible for the formation of diethyl hydrogen phos-

phorothionate and of diethyl 4-nitrophenyl phosphate (paraoxon) from parathion.

The data presented support the view that the initial metabolic reactions that parathion undergoes do involve two separate 'mixed-function oxidase' enzyme systems, one that metabolizes parathion to diethyl hydrogen phosphorothionate and *p*-nitrophenol and another that metabolizes parathion to paraoxon.

METHODS

The procedures for isolating and measuring the amounts of diethyl hydrogen phosphorothionate, diethyl hydrogen phosphate and paraoxon formed during the incubation were previously described (Neal, 1967). The incubation procedures used were modified.

Except where noted, the incubation system was as follows: phosphate buffer (pH 8.0), 100 μmoles ; NADP, 1.3 μmoles ; glucose 6-phosphate, 5.6 μmoles ; glucose 6-phosphate dehydrogenase, 0.5 unit; parathion, 0.35 μmole (added as a 13.6 mM solution in ethanol); microsomes equivalent to 100 mg. of liver. Distilled water was added to make a final vol. of 2.0 ml. The mixture was incubated with shaking at 37° in 25 ml. Erlenmeyer flasks open to the air.

Except where noted in the text, the incubation period was 1 hr. Inhibitors that were insoluble in water were added to the incubation medium dissolved in a minimum amount of ethanol and an equal amount of ethanol was added to the control flasks.

There is a rather large variation in the ratio paraoxon: diethyl hydrogen phosphorothionate with different microsomal preparations. This ratio also varies in a predictable manner with the age of the microsomes. Thus in isolated microsomes stored at -20° the ratio decreases steadily for about 8–12 days and then increases to the original value after about 28 days. The total activity of the microsomes toward parathion decreases approx. 10–20% during this 28-day period. Because of these variations, the comparisons of the activities under various conditions of incubation were always made in simultaneous experiments.

The compounds chlorpromazine and SKF-525A (2-diethylaminoethyl 2,2-diphenylvalerate-HCl) were supplied by Smith, Kline and French Laboratories, Philadelphia, Pa., U.S.A.

In some experiments the metabolism of parathion was determined by measuring the quantity of *p*-nitrophenol released as described by Neal & DuBois (1965). In the statistical analysis of the data the Student's *t* distribution was used as a test of the null hypothesis by using a level of significance of *P* equal to or less than 0.05. The standard deviations were calculated by using $\sqrt{n-1}$.

RESULTS

Attempts were made to solubilize and fractionate the component enzymes of the microsomes responsible for the metabolism of parathion, but they all resulted in partial or total disappearance of the enzymic activity. Among the techniques used were solubilization with deoxycholate, Tween 80 (polyoxyethylene sorbitan; Atlas Chemical Industries, Inc., Wilmington, Del., U.S.A.) and Triton X-100 (alkylphenoxypolyethoxyethanol; Rohm and Haas, Philadelphia, Pa., U.S.A.), and fractionation by ammonium sulphate precipitation and Sephadex (Pharmacia Fine Chemicals Inc., New York, N.Y., U.S.A.) filtration.

The metabolism of parathion by rat liver microsomes *in vitro* may be represented as shown in Scheme 1 (Neal, 1967).

Reactions (1) and (2) require NADPH and oxygen for activity. Reaction (3) is catalysed by an esterase located in microsomes that is similar to or identical with the enzyme detected in various tissues by Aldridge (1953), and that requires only Ca^{2+} for optimum activity.

In preliminary experiments both parathion and paraoxon were incubated with microsomes in the presence of various inhibitors, to determine whether the inhibition of the release of *p*-nitrophenol from parathion was caused by inhibition of the metabolism of parathion to diethyl hydrogen phosphorothionate and *p*-nitrophenol or of the hydrolysis of paraoxon by the microsomal esterase. Metabolism of parathion was inhibited by heavy-

metal ions (Cu^{2+} , Hg^{2+} and Zn^{2+}), by the thiol reagent PCMB,* by the metal-binding agents 8-hydroxyquinoline and bathophenanthroline (4,7-diphenyl-1,10-phenanthroline) and by SKF-525A, the inhibitor of the microsomal drug-metabolizing enzymes. The compounds $\alpha\alpha'$ -bipyridyl, sodium arsenite, sodium cyanide and iodine did not inhibit the metabolism of parathion in this system.

The effect of the various inhibitors of parathion degradation on the amounts of the various metabolites of parathion other than *p*-nitrophenol appearing after 1 hr. incubation was then investigated, and the ratio paraoxon:diethyl hydrogen phosphorothionate was determined (Table 1). The sum of paraoxon and diethyl hydrogen phosphate was taken as a measure of the total metabolism of parathion to paraoxon.

The metal-binding agent bathophenanthroline strongly inhibited the metabolism of parathion to both diethyl hydrogen phosphorothionate and paraoxon. The chelating agent 8-hydroxyquinoline also inhibited the metabolism of parathion to the same products. However, at a concentration of 3.0 mM this compound inhibited the metabolism to paraoxon more than that to diethyl hydrogen phosphorothionate, as is shown by the decrease in the ratio of these two metabolites in comparison with the control. At 1.0 mM concentration 8-hydroxyquinoline was still inhibitory, but the ratio paraoxon:diethyl hydrogen phosphorothionate was not significantly different from that in the control incubation. The thiol reagent PCMB inhibited the metabolism of parathion to paraoxon more strongly than that to diethyl hydrogen phosphorothionate. At 0.01 mM, PCMB actually stimulated the metabolism of parathion to diethyl hydrogen phosphorothionate but inhibited the metabolism to paraoxon. SKF-525A was inhibitory in this system *in vitro*, inhibiting the formation of diethyl hydrogen phosphorothionate and paraoxon about equally.

The effect of different concentrations of Cu^{2+} on the metabolism of parathion is shown in Table 2. At the highest concentration of Cu^{2+} used, 0.05 mM, the metabolism of parathion to diethyl hydrogen phosphorothionate was inhibited to a greater extent than that to paraoxon. However, at the lower Cu^{2+} concentrations (5 and 1 μM) the formation of diethyl hydrogen phosphorothionate was stimulated. The conversion of parathion into paraoxon was inhibited at all concentrations of Cu^{2+} .

The effect of various compounds containing reduced sulphur on the metabolism of parathion by rat liver microsomes was examined (Table 3). Mercaptoethanol, dithiothreitol (*threo*-2,3-dihydroxy-1,4-dithiobutane) and 1-phenylthiourea markedly enhanced the production of both diethyl

* Abbreviation: PCMB, *p*-chloromercuribenzoate.

Table 1. *Effect of various enzyme inhibitors on the metabolism of [³²P]parathion*

The incubation procedures are described in the Methods section. The values for paraoxon represent the sum of the paraoxon and the diethyl hydrogen phosphate formed during the incubation. The concentration of [³²P]parathion in each incubation flask was 0.18mM. The values for each experiment are the means $\pm$ S.D. of the means of three separate determinations, carried out simultaneously with microsomes isolated from the same pooled sample of male rat livers. The *P* values, in parentheses, indicate the significance of differences between mean results of incubations in the presence and absence of inhibitors. N.S., not significant.

Expt. no.	Inhibitor	Metabolite formed (mμmoles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
		Diethyl hydrogen phosphorothionate	Paraoxon	
1	None	194 $\pm$ 4	466 $\pm$ 14	2.53 $\pm$ 0.09
	Bathophenanthroline (1.0mM)	39 $\pm$ 6 (0.005)	101 $\pm$ 18 (0.005)	2.64 $\pm$ 0.15 (N.S.)
2	None	243 $\pm$ 10	651 $\pm$ 12	2.69 $\pm$ 0.16
	8-Hydroxyquinoline (3.0mM)	160 $\pm$ 14 (0.005)	278 $\pm$ 13 (0.005)	1.76 $\pm$ 0.15 (0.010)
	8-Hydroxyquinoline (1.0mM)	178 $\pm$ 5 (0.005)	422 $\pm$ 5 (0.005)	2.37 $\pm$ 0.04 (N.S.)
	PCMB (0.05mM)	175 $\pm$ 9 (0.005)	374 $\pm$ 20 (0.005)	2.15 $\pm$ 0.16 (0.050)
	PCMB (0.01mM)	277 $\pm$ 4 (0.025)	491 $\pm$ 12 (0.005)	1.77 $\pm$ 0.07 (0.010)
	SKF-525A (3.0mM)	141 $\pm$ 9 (0.005)	326 $\pm$ 22 (0.005)	2.33 $\pm$ 0.23 (N.S.)

Table 2. *Effect of Cu²⁺ on the metabolism of [³²P]parathion*

The experimental conditions and statistical analyses were as in Table 1. The values for each experiment are the means $\pm$ S.D. of three separate determinations at each concentration of Cu²⁺, carried out simultaneously. The *P* values are in parentheses. N.S., not significant.

Concn. of CuSO ₄ (mM)	Metabolite formed (mμmoles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
	Diethyl hydrogen phosphorothionate	Paraoxon	
None	141 $\pm$ 5	403 $\pm$ 4	2.87 $\pm$ 0.11
0.050	74 $\pm$ 2 (0.005)	304 $\pm$ 12 (0.005)	4.07 $\pm$ 0.10 (0.005)
0.025	135 $\pm$ 2 (N.S.)	314 $\pm$ 12 (0.005)	2.34 $\pm$ 0.12 (0.025)
0.010	134 $\pm$ 12 (N.S.)	338 $\pm$ 15 (0.010)	2.54 $\pm$ 0.31 (N.S.)
0.005	159 $\pm$ 6 (0.050)	343 $\pm$ 3 (0.005)	2.16 $\pm$ 0.09 (0.005)
0.001	194 $\pm$ 8 (0.005)	329 $\pm$ 5 (0.005)	1.71 $\pm$ 0.09 (0.005)

Table 3. *Effect of compounds containing reduced sulphur on the metabolism of [³²P]parathion*

The experimental conditions and statistical analyses were as in Table 1. The values for each experiment are the means $\pm$ s.d. of the means of three separate determinations, carried out simultaneously. The *P* values are in parentheses.

Addition	Metabolite formed (m μ moles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
	Diethyl hydrogen phosphorothionate	Paraoxon	
None	257 $\pm$ 11	604 $\pm$ 5	2.36 $\pm$ 0.12
Mercaptoethanol (1.0mM)	440 $\pm$ 5 (0.005)	732 $\pm$ 11 (0.005)	1.67 $\pm$ 0.04 (0.005)
Dithiothreitol (1.0mM)	553 $\pm$ 3 (0.005)	820 $\pm$ 12 (0.005)	1.48 $\pm$ 0.02 (0.005)
1-Phenylthiourea (1.0mM)	458 $\pm$ 21 (0.005)	682 $\pm$ 13 (0.005)	1.49 $\pm$ 0.04 (0.005)
Na ₂ S (1.0mM)	323 $\pm$ 4 (0.005)	485 $\pm$ 13 (0.005)	1.50 $\pm$ 0.05 (0.005)

hydrogen phosphorothionate and paraoxon, the greatest effect being on the production of the thionate. This differential stimulation is reflected in the marked decrease in the paraoxon:diethyl hydrogen phosphorothionate ratio. Sodium sulphide also stimulated phosphorothionate formation but with this compound the production of paraoxon was inhibited. GSH, propane-1,2-dithiol and propane-1,3-dithiol had only a slight stimulatory effect.

If the compounds containing reduced sulphur stimulate parathion metabolism by preventing the oxidation of an essential thiol group, the stimulatory effect of these compounds would be expected to be less on shorter incubation. This prediction was tested with dithiothreitol. The results are shown in Table 4. After 10min. incubation in the presence of dithiothreitol there was a significant difference in the production of diethyl hydrogen phosphorothionate but not in that of paraoxon. After 20min. and 30min. the effect of dithiothreitol became more pronounced and the production of both metabolites was significantly stimulated.

The effect of NAD⁺ and NADH on the metabolism of parathion in the presence of NADPH was examined at 1:1 and at 1:5 NADPH:NAD⁺ and NADPH:NADH molar ratios. NAD⁺ and NADH exhibited neither inhibition nor stimulation.

Addition of Mg²⁺ or Mn²⁺ to the reaction mixture had no consistently significant effect on the metabolism of parathion, but Ba²⁺ and, to a smaller extent, Ca²⁺ significantly stimulated the formation of diethyl hydrogen phosphorothionate (Table 5). Phosphorothionate production was also stimulated by EDTA. The stimulatory effects of EDTA and Ba²⁺ as well as EDTA and Ca²⁺ are not additive, however (Table 5).

The effects of various electron acceptors on the metabolism of parathion are shown in Table 6. Riboflavin (0.2mM) and FAD (0.1mM) inhibited the conversion of parathion into the phosphorothionate to a greater extent than the conversion

into paraoxon. At concentrations of 10 μ M and greater, menadione and methylene blue strongly inhibited the conversion of parathion into both metabolites. However, menadione at 5 μ M and methylene blue at 1 μ M stimulated the production of diethyl hydrogen phosphorothionate but continued to inhibit the metabolism of parathion to paraoxon.

The effects of various potential alternative substrates on the microsomal metabolism of parathion are shown in Table 7. α -Naphthol and α -naphthyl phosphate are both strong inhibitors of the metabolism of parathion; however, the ratios of the metabolites formed in the presence of these inhibitors is not significantly different from the ratio in the control incubation. The compounds α -naphthyl acetate, β -naphthyl acetate and α -naphthyl butyrate also inhibited the metabolism of parathion to about the same degree as did α -naphthol and α -naphthyl phosphate. Naphthalene itself was less inhibitory than its derivatives and appeared to inhibit the formation of diethyl hydrogen phosphorothionate more than the formation of paraoxon. Chlorpromazine was a strong inhibitor of parathion metabolism and seemed to inhibit paraoxon formation preferentially. Codeine, *p*-nitrophenyl acetate and hexobarbital had no significant effect on the metabolism of parathion to the phosphorothionate but did inhibit the conversion into paraoxon to a slight degree. Both *p*-nitrophenyl acetate and codeine significantly altered the ratio paraoxon:diethyl hydrogen phosphorothionate, whereas hexobarbital did not. Aniline and zoxazolamine (2-amino-5-chlorobenzoxazole) at high concentrations inhibited metabolism of parathion to both metabolites but had no significant effect on the ratio.

The effects of preheating the enzyme mixture to 55° for 30sec. and for 60sec. are shown in Table 8. Heating at 55° for 60sec. had no significant effect on the production of diethyl hydrogen phosphorothionate. In contrast, the metabolism to paraoxon

Table 4. *Effect of different incubation times on the stimulation of the metabolism of [³²P]parathion by dithiothreitol*

The incubation procedures are as described in the Methods section, except for the time of incubation. The experimental conditions and statistical analyses were as in Table 1. The values given are the means $\pm$ s.d. of three separate determinations, carried out simultaneously. The *P* values are in parentheses. N.S., not significant.

Incubation time (min.)	Addition	Metabolite formed (μ moles/g. of liver)	
		Diethyl hydrogen phosphorothionate	Paraoxon
10	None	52 $\pm$ 2	115 $\pm$ 7
10	Dithiothreitol (1.0mm)	72 $\pm$ 1 (0.005)	111 $\pm$ 10 (N.S.)
20	None	95 $\pm$ 4	176 $\pm$ 1
20	Dithiothreitol (1.0mm)	131 $\pm$ 1 (0.005)	206 $\pm$ 9 (0.025)
30	None	132 $\pm$ 2	226 $\pm$ 8
30	Dithiothreitol (1.0mm)	212 $\pm$ 13 (0.005)	324 $\pm$ 23 (0.005)

Table 5. *Effect of Ca²⁺, Ba²⁺ and EDTA on the metabolism of [³²P]parathion*

The experimental conditions and statistical analyses were as in Table 1. The values for each experiment are the means $\pm$ s.d. of three separate determinations for each substance, carried out simultaneously. The *P* values are in parentheses. N.S., not significant.

Additions	Metabolite formed (μ moles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
	Diethyl hydrogen phosphorothionate	Paraoxon	
None	129 $\pm$ 2	270 $\pm$ 3	2.10 $\pm$ 0.04
CaCl ₂ (2mm)	150 $\pm$ 5 (0.010)	285 $\pm$ 12 (N.S.)	1.90 $\pm$ 0.10 (N.S.)
BaCl ₂ (2mm)	180 $\pm$ 7 (0.005)	279 $\pm$ 4 (N.S.)	1.55 $\pm$ 0.04 (0.005)
CaCl ₂ (2mm) + EDTA (3mm)	173 $\pm$ 3 (0.005)	294 $\pm$ 9 (0.050)	1.70 $\pm$ 0.07 (0.005)
BaCl ₂ (2mm) + EDTA (3mm)	183 $\pm$ 2 (0.005)	288 $\pm$ 8 (0.050)	1.58 $\pm$ 0.03 (0.005)
EDTA (3mm)	190 $\pm$ 5 (0.005)	273 $\pm$ 4 (N.S.)	1.43 $\pm$ 0.05 (0.005)

Table 6. *Effect of various electron acceptors on the metabolism of [³²P]parathion*

The experimental conditions and statistical analyses were as in Table 1. The values given for each experiment are the means $\pm$ s.d. of three separate determinations for each electron acceptor, carried out simultaneously. The *P* values are in parentheses. N.S., not significant.

Addition	Metabolite formed (μ moles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
	Diethyl hydrogen phosphorothionate	Paraoxon	
None	220 $\pm$ 5	584 $\pm$ 16	2.65 $\pm$ 0.07
FAD (0.1mm)	160 $\pm$ 5 (0.005)	487 $\pm$ 22 (0.025)	3.06 $\pm$ 0.18 (0.050)
Riboflavin (0.2mm)	109 $\pm$ 8 (0.005)	367 $\pm$ 37 (0.005)	3.37 $\pm$ 0.18 (0.010)
Riboflavin (0.1mm)	132 $\pm$ 4 (0.005)	388 $\pm$ 18 (0.005)	2.97 $\pm$ 0.16 (N.S.)
Menadione (10 μ M)	189 $\pm$ 5 (0.010)	397 $\pm$ 10 (0.005)	2.10 $\pm$ 0.13 (0.025)
Menadione (8 μ M)	236 $\pm$ 6 (N.S.)	472 $\pm$ 7 (0.005)	2.00 $\pm$ 0.03 (0.005)
Menadione (5 μ M)	262 $\pm$ 19 (0.050)	445 $\pm$ 25 (0.005)	1.70 $\pm$ 0.05 (0.005)
Methylene blue (5 μ M)	198 $\pm$ 3 (0.010)	385 $\pm$ 11 (0.005)	1.94 $\pm$ 0.07 (0.005)
Methylene blue (1 μ M)	258 $\pm$ 5 (0.005)	445 $\pm$ 8 (0.005)	1.73 $\pm$ 0.06 (0.005)

was significantly inhibited after 30sec. heating at this temperature. When the enzyme mixture was heated for 120sec. at 55°, all metabolic activity towards parathion was lost.

The concentration of parathion required for a

half-maximal rate of production of both metabolites was examined to see if there was any significant difference in the substrate requirement. Three separate experiments were run simultaneously and the *K_m* values determined mathematically, the

Table 7. *Effect of various alternative substrates for the microsomal 'mixed-function oxidases' on the metabolism of [³²P]parathion*

The experimental conditions and statistical analyses were as in Table 1. The values for each experiment are the means $\pm$ s.d. of three separate determinations with each alternative substrate, carried out simultaneously. The *P* values are in parentheses. N.S., not significant.

Expt. no.	Additions	Metabolite formed (m μ moles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
		Diethyl hydrogen phosphorothionate	Paroaxon	
1	None	192 $\pm$ 5	562 $\pm$ 34	2.92 $\pm$ 0.12
	α -Naphthol (0.5 mm)	133 $\pm$ 6 (0.005)	360 $\pm$ 15 (0.005)	2.71 $\pm$ 0.16 (N.S.)
	α -Naphthyl phosphate (0.5 mm)	114 $\pm$ 4 (0.005)	316 $\pm$ 3 (0.005)	2.78 $\pm$ 0.09 (N.S.)
	<i>p</i> -Nitrophenyl acetate (1.0 mm)	226 $\pm$ 19 (N.S.)	483 $\pm$ 11 (0.050)	2.16 $\pm$ 0.13 (0.010)
	Chlorpromazine (0.5 mm)	124 $\pm$ 8 (0.005)	260 $\pm$ 11 (0.005)	2.11 $\pm$ 0.10 (0.010)
2	None	207 $\pm$ 5	462 $\pm$ 6	2.23 $\pm$ 0.03
	Naphthalene (3.0 mm)	105 $\pm$ 4 (0.005)	272 $\pm$ 6 (0.005)	2.59 $\pm$ 0.06 (0.010)
	Naphthalene (1.0 mm)	174 $\pm$ 8 (0.025)	408 $\pm$ 13 (0.025)	2.34 $\pm$ 0.02 (0.025)
3	None	230 $\pm$ 8	545 $\pm$ 9	2.38 $\pm$ 0.06
	Hexobarbital (3.0 mm)	218 $\pm$ 2 (N.S.)	484 $\pm$ 17 (0.025)	2.23 $\pm$ 0.08 (N.S.)
	Aniline (2.0 mm)	188 $\pm$ 4 (0.010)	422 $\pm$ 26 (0.010)	2.25 $\pm$ 0.11 (N.S.)
	Zoxazolamine (3.0 mm)	194 $\pm$ 5 (0.025)	452 $\pm$ 13 (0.005)	2.34 $\pm$ 0.07 (N.S.)
	Codeine (1.0 mm)	241 $\pm$ 4 (N.S.)	509 $\pm$ 2 (0.025)	2.12 $\pm$ 0.03 (0.025)

Table 8. *Effect of preheating the enzyme mixture on the metabolism of [³²P]parathion*

Before incubation, two 5 ml. samples of the enzyme solution containing microsomes equivalent to 100 mg. of liver/ml. of solution were cooled to 2° and then heated to 55°, one sample for 30 sec. and the other for 60 sec. Then 1 ml. portions of the enzyme solutions were incubated with substrate and cofactors, as described in the Methods section. The values given are the means $\pm$ s.d. of three separate determinations with each enzyme preparation, carried out simultaneously. The experimental conditions and statistical analyses were as in Table 1. The *P* values are in parentheses. N.S., not significant.

Treatment	Metabolite formed (m μ moles/hr./g. of liver)		Ratio paraoxon: diethyl hydrogen phosphorothionate
	Diethyl hydrogen phosphorothionate	Paroaxon	
Unheated	140 $\pm$ 6	293 $\pm$ 7	2.11 $\pm$ 0.12
Heated for 30 sec. at 55°	142 $\pm$ 7 (N.S.)	262 $\pm$ 1 (0.010)	1.86 $\pm$ 0.09 (N.S.)
Heated for 60 sec. at 55°	132 $\pm$ 3 (N.S.)	216 $\pm$ 6 (0.005)	1.64 $\pm$ 0.07 (0.025)

location of the 'best straight line' being calculated by the method of least squares. The results ($5.33 \pm 0.25 \times 10^{-5}$ M for diethyl hydrogen phosphorothionate and $3.37 \pm 0.06 \times 10^{-5}$ M for paraoxon) show there is a significant ($P < 0.005$) difference in the K_m values for the formation of these two metabolites.

DISCUSSION

It has been shown previously that the microsomal 'mixed-function oxidase' enzymes are inhibited by heavy-metal ions (Gillette, Brodie & LaDu, 1957; Booth & Boyland, 1958), by PCMB (Mitoma, Posner, Reitz & Udenfriend, 1956; Booth &

Boyland, 1958; Gillette & Kamm, 1960; Liebman, 1965), by electron acceptors (Gillette *et al.* 1957) and by SKF-525A (Axelrod, Reichenthal & Brodie, 1954). Thus the metabolism of parathion is affected by these various inhibitors in a way that is generally typical of the 'mixed-function oxidases'. However, with some of these inhibitors there is a differential inhibition of the formation of diethyl hydrogen phosphorothionate and of paraoxon, thus indicating that parathion is metabolized by two separate enzyme systems. That a single compound may be acted upon by different microsomal 'mixed-function oxidases' is not in itself unusual. Chlorpromazine can be demethylated (Beckett, Beaven & Robinson, 1963; Robinson, 1966), converted into its sulfoxide

(Gillette & Kamm, 1960) and undergo ring hydroxylation (Robinson, 1966), presumably by the action of different 'mixed-function oxidases'. Also, the aromatic compound biphenyl may be hydroxylated at different sites, presumably by different enzymes (Creaven *et al.* 1965). The unique feature of the metabolism of parathion is that in both reactions the site of attack by oxygen appears to be the phosphorus atom. On the one hand sulphur would be the leaving group, giving rise to paraoxon, and on the other *p*-nitrophenol would be the leaving group, giving rise to diethyl hydrogen phosphorothionate. In the reaction leading to the formation of the thionate, the electrophilic phosphorus atom is considered to be a more favourable point of attack than the carbon atom in the *p*-nitrophenol ring.

The inhibitory effects of the metal-binding reagents bathophenanthroline and 8-hydroxyquinoline and the thiol reagent PCMB are probably due, at least in part, to inactivation of cytochrome *P*-450 (Mason, North & Vanneste, 1965). The stimulatory effect of lower concentrations of PCMB on the formation of diethyl hydrogen phosphorothionate is more difficult to explain. It may be that PCMB is inactivating a naturally occurring inhibitor such as a thiol-containing protein. NADPH-cytochrome *c* reductase, an enzyme present in the microsomal electron-transport chain that activates oxygen for the 'mixed-function oxidase' reactions, is stimulated by PCMB when in an impure state but is inhibited in the purified state, suggesting that some natural inhibitor has been removed from the purified enzyme (Phillips & Langdon, 1962). Stimulation of NADPH-cytochrome *c* reductase by low concentrations of PCMB has also been reported by Kamin, Masters, Gibson & Williams (1965), who showed that addition of 2 moles of PCMB/mole of flavine in the enzyme can activate the enzyme even though thiol groups do not appear to be involved in the catalyses. As with parathion metabolism (Table 1), higher concentrations of PCMB were inhibitory.

Cu^{2+} is a strong inhibitor of parathion metabolism. At low concentrations, however, Cu^{2+} also stimulates the conversion into phosphorothionate. The above discussion on the action of PCMB might therefore also apply to Cu^{2+} .

The inhibition of the metabolism of parathion by the various electron acceptors is probably due to a diversion of reducing equivalents from the electron transport pathway of the 'mixed-function oxidases' to these compounds that transfer electrons to, perhaps, oxygen. Menadione oxidizes the reduced FAD in NADPH-cytochrome *c* reductase (Kamin *et al.* 1965) and stimulates NADPH oxidation in microsomes as shown by Nishibayashi,

Omura & Sato (1966), whose data indicated that liver microsomes contain a NADPH oxidase requiring menadione as a cofactor. It is possible that the stimulatory effects of small concentrations of menadione on phosphorothionate production (Table 6) may be due to stimulation of a 'mixed-function oxidase' requiring menadione as a cofactor. However, as Nishibayashi *et al.* (1966) point out, no naphthaquinones have yet been detected in microsomes.

The differential inhibition and stimulation of parathion metabolism by menadione seems to contradict the existence of any common electron-transfer pathway for the two metabolites, since this compound acts as an electron acceptor at some point between the flavoprotein NADPH-cytochrome *c* reductase and the so-called non-haem iron protein as shown in Scheme 2 (Omura, Sato, Cooper, Rosenthal & Estabrook, 1965).

There is additional evidence against a common metabolic pathway for the metabolism of parathion to paraoxon and to diethyl hydrogen phosphorothionate, since the formation of phosphorothionate is inhibited more than the formation of paraoxon in the presence of FAD (0.1 mM) and riboflavine (0.2 mM), whereas the opposite is true in the presence of menadione (10 μM) and methylene blue (5 μM).

The greater stimulation of phosphorothionate formation as compared with paraoxon formation in the presence of compounds containing reduced sulphur is noteworthy. The stimulatory effect of dithiothreitol with increasing time of incubation suggests that these compounds are probably acting to maintain certain essential thiol groups in a reduced state. The greater relative stimulation of phosphorothionate formation could indicate that a thiol group is more involved in the binding of parathion to the surface of the enzyme when the product is diethyl hydrogen phosphorothionate. It could also mean that a thiol group is functioning directly as a redox couple in the microsomal electron transport, and that the system transferring reducing equivalents for the reaction leading to diethyl hydrogen phosphorothionate formation is more susceptible to oxidation than the system that forms paraoxon. It does not appear that the thiol groups that are protected by the addition of compounds containing reduced sulphur are the same as those that react with PCMB and Cu^{2+} , since with these reagents it is the metabolism to paraoxon that is more strongly inhibited. An exception to this is that Cu^{2+} at 0.05 mM concentration (Table 2) appears to inhibit the formation of phosphorothionate more strongly than that of paraoxon. However, since Cu^{2+} may also bind to amino groups and carboxyl groups, its effect cannot be attributed solely to interaction with a thiol group. PCMB

Scheme 2.

may also be reacting with a group other than a thiol.

The enzyme system that converts parathion into paraoxon appears to be more easily inactivated by physical means than the system forming diethyl hydrogen phosphorothionate. Thus the paraoxon-forming system is more easily inactivated by heating (Table 8).

The stimulatory effect of EDTA may be through binding of adventitious metal ions such as Cu^{2+} or Fe^{2+} , which could be inhibitory as such or act in conjunction with oxygen to oxidize essential thiol groups. Stimulation by Ba^{2+} is more difficult to understand. Since the stimulatory effects of Ba^{2+} and EDTA are not additive, they may be acting by a similar mechanism, which could be, as proposed for low concentrations of PCMB and Cu^{2+} , the inactivation of a natural inhibitor that requires a bivalent cation such as Ca^{2+} for activity.

The K_m values for the formation of both diethyl hydrogen phosphorothionate and paraoxon are quite low and are in the range reported for the metabolism of steroids by liver microsomes (Kuntzman, Lawrence & Conney, 1965). The fact that the K_m values for parathion for the formation of these two metabolites are significantly different further supports the view that two separate enzyme systems are involved in the metabolism of parathion.

Finally, with certain of the alternative substrates for the microsomal 'mixed-function oxidases' there is a differential inhibition of the formation of the two metabolites, which also suggests that two separate enzyme systems are involved in the metabolism of parathion. *p*-Nitrophenyl acetate is a substrate for the esterase that metabolizes paraoxon to diethyl hydrogen phosphate and *p*-nitrophenol. The inhibitory effect of *p*-nitrophenyl acetate on the metabolism of parathion to paraoxon might be due to the mass-action effect of paraoxon accumulating because of the inhibition of its hydrolysis by the esterase. However, the addition of non-radioactive paraoxon ($0.2 \mu\text{mole}$) to the medium at the start of an incubation inhibits the formation of both paraoxon and diethyl hydrogen phosphorothionate to about the same degree (15%). Thus the mechanism of inhibition by *p*-nitrophenyl acetate may be other than by the inhibition of the hydrolysis of paraoxon.

An alternative explanation for the stimulation of diethyl hydrogen phosphorothionate formation at low concentrations of enzyme inhibitors such as

Cu^{2+} and menadione (Tables 2 and 6) is suggested by the observation that the degree of inhibition of the formation of paraoxon in comparison with the control appears to be about the same as the degree of stimulation of diethyl hydrogen phosphorothionate formation. The same effect is seen with low concentrations of PCMB (Table 1) and methylene blue (Table 6). These results suggest that a portion of the metabolic pathway leading to phosphorothionate and to paraoxon may be in common and that the stimulation of the formation of phosphorothionate with an accompanying decrease in paraoxon formation could be a result of a shift of reducing equivalents from the reaction leading to activation of oxygen, which in turn reacts with parathion to form paraoxon, to the reaction in which oxygen reacts with parathion to yield diethyl hydrogen phosphorothionate. Further studies will be required to show whether there are two entirely separate enzyme systems or whether a mechanism of different binding sites sharing a common electron-transport system is involved.

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