Paraoxon Hydrolyzing Enzymes in Rat Liver

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H[3]-Paraoxon was shown to be degraded by at least four distinct enzymes in rat liver with different pH optima, reaction constants, and sensitivities to metallic ions, SH-reagents, and inhibitors. These enzymes were present in a soluble and a particulate state in the liver cell. The specific activity was highest in the crude microsomes and lower in the washed mitochondria and the crude soluble fraction at the respective optimum pH's. The crude soluble fraction contained two different enzymes which were partially purified by ammonium sulfate fractionation. The washed mitochondrial, crude microsomal, crude and partially purified soluble enzymes produced diethyl phosphate as the principal metabolite. A partially purified soluble fraction produced O-desethyl paraoxon. The enzymes in microsomes and mitochondria were solubilized by treatment with sodium desoxycholate. The above subcellular fractions also degraded H[3]-parathion, but not necessarily by the enzymes responsible for paraoxon degradation.

Toxic organophosphates and fluorophosphates are enzymatically hydrolyzed by various organisms. These hydrolases reduce the toxicity of the compounds against such organisms (Adie, 1956; Heath, 1961; O'Brien, 1960; Saunders, 1953; Smith, 1955, 1962). Aldridge (1953a) characterized two distinct types of enzymes in mammalian sera in some detail and called them A-esterase and B-esterase. A-esterase hydrolyzes p-nitrophenyl acetate (p-NPA) and paraoxon (Aldridge, 1953b; Augustinsson, 1953; Mounter, 1954). This enzyme is present in the sera and other tissues of mammals (Aldridge, 1953a, b; Wilkinson, 1965) and insects (Metcalf et al., 1956). It is probably identical with the aromatic esterase of Mounter and Whittaker (1953), and the arylesterase of Augustinsson (1953, 1958, 1959, 1961), which is mainly responsible for the hydrolysis of phenyl acetate in human plasma. However, later, Main (1960a, b) obtained a highly purified paraoxon-hydrolyzing enzyme from sheep serum. This enzyme did not hydrolyze phenyl acetate, and therefore, Main named the enzyme paraoxonase to distinguish it from other arylesterases. Erdös and Boggs (1961) also found that in addition to arylesterase, mammalian serum albumin hydrolyzes paraoxon at an appreciable rate; human albumin (fraction V or crystallized) hydrolyzes paraoxon at the rate of 2.4 μmoles per 100 mg. per hour. Although mammalian blood serum may be of importance in the detoxication of paraoxon, rat liver is three or four times more active than rat serum (Aldridge, 1953b). The above studies were concerned with enzymic cleavage of the p-nitrophenyl group; the authors reserve the term paraoxonase for this type of enzyme.

All organophosphates are desalkylated to some extent in mammals, insects, and plants (Bull and Lindquist, 1964, 1966; Dauterman et al., 1959; Knaak and O'Brien, 1960; Krueger et al., 1959; O'Brien et al., 1961; Plapp and Casida, 1958b, 1958c), although usually desalkylation is of minor importance except for cases where a resistance factor of rice stem borer, Chilo supressalis, to parathion was involved (Iyatomi and Saito, 1965; Kojima et al., 1963b); and for some dimethyl organophosphates with low mammalian toxicity, particularly Sumithion (Hollingworth et al., 1967; Miyamoto, 1964). A desalkylating enzyme for dimethyl organophosphates was found in the soluble fraction of rat liver and other tissues, and insect tissues (Fukami and Shishido, 1963, 1966; Hodgson and Casida, 1962; Shishido and Fukami, 1963). There are no reports of the desethylation of paraoxon in vitro. Probably the mammalian liver and insect gut are the primary site of desalkylation. The mammalian liver is the most important source of organophosphate degrading enzymes as shown by the physiological, biochemical, and toxicological studies of organophosphorus insecticides (Heath, 1961; Kojima, 1961; O'Brien, 1960).

This work was undertaken to study systematically the distribution, properties, and identities of paraoxon-degrading enzymes in rat liver.

MATERIALS AND METHODS

Chemicals. H[3]-Paraoxon was synthesized in this laboratory. An unsuccessful attempt first was made to synthesize p-nitrophenyl phosphorodichloridate by the methods of Tolkmith (1958a, b) and Hilton and O'Brien (1965), who synthesized the thiono analog using a 1-to-1 mixture of anhydrous magnesium chloride and copper chloride or pyridine as a catalyst. However, sodium

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chloride (NaCl) was an effective catalyst for *p*-nitrophenyl phosphorodichloridate. A solution of 13.9 grams (0.1 mole) of *p*-nitrophenol and 92.0 grams (0.6 mole) of phosphorus oxychloride (POCl₃) was refluxed while stirring in the presence of 0.5 gram of (4.2 moles) NaCl for 5 hours at 115° C. After the reaction, unreacted POCl₃ was removed immediately by distillation. The liquid remaining after the removal of the POCl₃ was then distilled under a pressure of less than 1 mm. The product was a viscous, almost colorless, liquid boiling at 125° C. per 0.02 mm. which could be crystallized at dry ice temperatures. The yield of crude *p*-nitrophenyl phosphorodichloridate (m.p. 41–2° C.) was 16.5 grams (64%). Recrystallization from *n*-hexane (60–70°) raised the melting point to 42–3° C.

p-Nitrophenyl phosphorodichloridate was relatively stable to heat below 200° C. and in nonpolar organic solvents such as *n*-hexane, petroleum ether, cyclohexane, benzene, and chloroform, but was unstable in acetone. In general, it was much less stable than its thiono analog and tended to decompose rapidly in air at room temperature. Therefore, it was necessary to seal it into a glass ampoule immediately after distillation.

H³-Paraoxon was synthesized by reacting H³-ethanol and *p*-nitrophenyl phosphorodichloridate. An ampoule containing H³-ethanol, 25 mc. (713 mc. per mmole), was immersed to a depth of 15 cm. in a cooling bath of dry ice and trichloroethylene. Then the upper part of the ampoule was opened, and the inside was quickly rinsed down with 1 ml. of anhydrous benzene, and the required amounts of unlabeled absolute ethanol (0.3 ml. of total ethanol, 5.0 mmoles), 1 ml. of anhydrous benzene, 3 ml. of freshly distilled pyridine (2.5 mmoles), and 960 mg. (3.75 mmoles) of *p*-nitrophenyl phosphorodichloridate were added. The insides of the ampoule were again rinsed with 1 ml. of anhydrous benzene. The ampoule was resealed and shaken for 79 hours at room temperature. After the reaction, the ampoule was opened after cooling in a dry ice–trichloroethylene bath. The reaction mixture was allowed to warm up to room temperature and was transferred to a small separatory funnel with 20 ml. of benzene. The benzene solution was washed three times with 10-ml. portions of 5% sodium carbonate until only a very pale yellow aqueous phase was obtained and washed five times with water. The benzene layer was filtered through anhydrous sodium sulfate, and the benzene evaporated in a stream of dry air.

The yield of H³-paraoxon was 250 mg. (36%). It was purified further according to the method of Aldridge (1953b) to remove hydrolysis products before the preparation of H³-paraoxon stock solution. The specific activity of H³-paraoxon was 3.8 mc. per mmole after preparation. The radiochemical purity was greater than 99.5%, as determined by paper chromatography (Metcalf and March, 1953) and silica gel G chromatoplate (Snyder, 1964).

The H³-parathion (4.5 mc. per mmole) was material previously synthesized in this laboratory (Hilton and O'Brien, 1965).

Cofactors. These were obtained from Sigma Chemical Co.: Oxidized glutathione Grade III (GSSG), reduced glutathione crystalline (GSH), nicotinamide (NIC), nicotinamide adenine dinucleotide (NAD), reduced nicotin-amide adenine dinucleotide (NADH₂), nicotinamide adenine dinucleotide phosphate (NADP), and reduced nicotinamide adenine dinucleotide phosphate (NADPH₂).

Substrates. A stock solution (10m*M*) was prepared by dissolving 27.5 mg. of purified H³-paraoxon in 2 ml. of absolute ethanol and adding to 8 ml. of Sörensen's 0.067*M* sodium phosphate buffer, pH 7.2, containing 2% (w./v.) Triton X-100 (Rohm and Haas Co.). A buffered substrate solution (0.1m*M*) was prepared by diluting 1 ml. of the stock solution in 100 ml. of the buffered Triton solution and shaking vigorously. The latter solution was diluted 30-fold on addition to the tissue sample (see below) giving final concentrations of 3.3 μM paraoxon, 0.007% ethanol, and 0.067% Triton.

A buffered H³-parathion substrate solution (0.1m*M*) was prepared by adding 5 ml. of a stock acetone solution to 15 ml. of the buffered Triton solution. Both stock and substrate solutions were stored in the refrigerator.

Enzyme Preparation. Female albino rats (200 to 250 grams, Holtzman Co., Madison, Wis.) were killed by decapitation. The livers were removed as quickly as possible and transferred to a glass beaker in an ice bucket. After weighing, the livers were homogenized in the appropriate volume of a suitable ice cold buffer with an Omni-Mixer for 2 minutes to make the desired concentrations (w./v.). The crude homogenate was centrifuged (Lourdes centrifuge) at 800 × G for 10 minutes to sediment the nuclear fraction (undisrupted cells, nuclei, etc.). This fraction was washed three times by resuspending it in buffer, followed by centrifugation at 800 × G for 10 minutes. The first supernatant was then centrifuged at 15,000 × G (Lourdes centrifuge) for 10 minutes. The second sediment (mitochondrial fraction) was washed three times. The crude microsomal fraction was obtained by centrifuging the remaining supernatant at 127,000 × G for 30 minutes at about 5° C. by using a Type SW 50 L rotor in a Spinco Model L-2 ultracentrifuge. The final supernatant was referred to as the crude soluble fraction. The microsomal pellet was not washed. All three particulate fractions were resuspended in the buffer and made up to the original volume of the suspension before centrifugation. All enzyme concentrations described are those of the suspensions before addition to the incubation mixtures.

Ammonium Sulfate Fractionation. A 20% liver homogenate in 0.1*M* barbital buffer, pH 8.8, was centrifuged in a Servall centrifuge for 10 minutes at 800 × G (to reduce the volume), then at 30 minutes at 127,000 × G in an L-2 centrifuge. Solid ammonium sulfate was added to the supernatant with stirring in an ice bath to make solutions which were 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100% saturated [amount calculated by the table of Osborne (Akabori, 1955)]. The pH of each solution was adjusted to about 8.8 with 5*N* ammonium hydroxide and acetic acid. The solutions were left overnight at 5° C. The 10 to 60% saturated solutions were centrifuged at 3000 × G for 10 minutes, the 70% saturated solution at 6780 × G for 10 minutes, and the 80 to 100% saturated solutions at 27,000 × G for 10 minutes. The precipitates were suspended in the same buffer and made up to the same volume as the solutions before the addition of the solid ammonium sulfate. The ammonium sulfate preparations

were then dialyzed for 2 to 2.5 days against the same buffer. During dialysis, the buffer was changed eight times to keep a constant pH. The prolonged dialysis proved necessary to remove all ammonium sulfate.

Solubilization of Enzymes. The crude microsomal and washed mitochrondrial enzymes were treated as follows: Five milliliters of 25mM sodium desoxycholate in Sörensen's 0.067M sodium phosphate buffer, pH 7.0 or 7.7, were slowly added to the 20 ml. of 25% crude microsomal or washed mitochondrial fractions, respectively, then centrifuged at 15,000 × G for 10 minutes for mitochondria, and 127,000 × G for 30 minutes for microsomes at 5° C. The precipitate was made up to a volume of 25 ml. with the buffer. These preparations were designated as treated soluble and insoluble fractions. Controls were prepared by diluting 25% crude microsomal and washed mitochondrial fractions, respectively, to 20% enzyme concentration with the buffer, omitting the sodium desoxycholate.

Enzyme Assay. Buffered substrate, 0.1 ml., was pipetted into each 10-ml. plastic tube (100 × 15 mm.). Then 0.6 ml. of buffer, 0.3 ml. of buffered cofactors, activators, inhibitors, or buffer alone (in the case of controls), and, finally, 2.0 ml. of enzyme fraction, were added. In all experiments, one tube was used for a reagent blank (buffer only) without the substrate and enzyme fraction. The second tube was used to correct for nonenzymic hydrolysis without the enzyme fraction. The final concentration of substrate was 3.33 μM.

The tubes were shaken at 37.5° C. for 1 hour (unless otherwise indicated) on a water bath shaker. After the incubation, the reaction was stopped by the addition of 1.0 ml. of 5% trichloroacetic acid (TCA) to each tube; this lowered the pH of the sample to approximately 2 (when pH 7.2 sodium phosphate buffer was used), and afterwards the pH was made to 7 with 1.25 ml. of 0.25N KOH. The unmetabolized substrate was extracted by shaking in the tube with 4 ml. of toluene and centrifuging the tube at 3000 r.p.m. for 10 minutes to give toluene and water extracts and protein residue.

Radioactivity was counted in a Packard Tri-Carb Series 314 E liquid scintillation spectrometer. One milliliter samples of the toluene extract were counted in 10 ml. of toluene counting solution (Hayes, 1963). Similarly, 1-ml. samples of the aqueous phase were counted with 10 ml. of dioxane counting solution (Bray, 1960). The protein residue was washed once with 3 ml. each of toluene and distilled water and centrifuged at 3000 r.p.m. for 10 minutes, and toluene and water were discarded. Samples of whole protein were counted in 10 ml. of dioxane counting solution. Internal standards were employed to compensate for quenching and to determine efficiency in the samples (Kinnory *et al.*, 1958).

Protein Determination. One milliliter of distilled water was added to a 1-ml. portion from each fraction. Protein was precipitated by adding 1 ml. of 28% (w./v.) TCA. After centrifuging, the precipitate was washed once with 5 ml. of 5% (w./v.) TCA and dissolved in 0.1N NaOH. Protein concentration was then determined in a Beckman DU spectrophotometer at 280 mμ, using a blank of 0.1N NaOH. Crystallized human albumin (Nutritional Biochemicals Corp.) was used as a standard. Duplicate

protein determinations were run on each sample.

Metabolite Analysis. The metabolites of the water-soluble extract were analyzed by ion exchange and paper chromatography according to the method of Plapp and Casida (1958a). However, good results were not obtained by ion exchange because of water-quenching of the small amounts of radioactivity.

For paper chromatography, the reaction mixtures were extracted either with 3 ml. of chloroform immediately after incubation or frozen and stored at 0° C. for one to two days and then extracted. After extraction, the chloroform phase was discarded, and the aqueous phase was evaporated under reduced pressure in a rotary evaporator at 53–4° C. These concentrates were applied to 15 strips of Whatman No. 1 paper (2 × 27 cm.), and the chromatograms were developed by the ascending technique to a height of 20 cm. at 23–5° C. Two developing solvent systems were used: a 75 to 25 mixture of 2-propanol–ammonium hydroxide (Plapp and Casida, 1958a), and a 40:9:1 mixture of acetonitrile–water–ammonium hydroxide (Hacskaylo and Bull, 1963). After development, the strips were dried and cut into 21 (1 cm.) pieces, which were counted in 10 ml. of toluene counting solution.

Nonlabeled pure diethyl phosphate and phosphoric acid were cochromatographed as reference compounds for the identification of metabolites and detected by the colorimetric method of Hanes and Isherwood (1949), using ultraviolet light as the reducing agent.

RESULTS AND DISCUSSION

Subcellular Distribution of the Degrading Systems. The activity of H³-paraoxon-degrading enzymes in rat liver was almost proportional to the enzyme concentrations at pH 7.2 (Figure 1). Centrifugal studies on rat liver homogenates showed that paraoxon-degrading enzymes were present in rat liver in both soluble and particulate fractions (Figure 1, Table I). In considering the activity of subcellular fractions, the pH is of major importance. The relative total activities in soluble, microsomal, and mitochondrial fractions were 71:34:7 at pH 8.8 and 22:49:8 at pH 7.7. At pH 7, the relative activities were similar to those at pH 7.7. Although the lowest total activity is
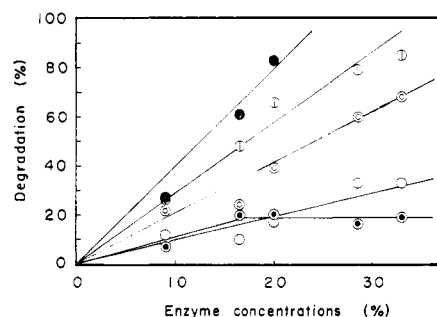
Figure 1. Relationship between enzyme concentration and degradation of H³-paraoxon by rat liver fractions

Reaction mixture in 0.067M sodium phosphate buffer, pH 7.2, incubated at 37.5° C. for 1 hour. Substrate was 3.3 μM in a final concentration. ● Original homogenate; ⊛ Washed nuclear fraction; ○ Washed mitochondria; ⊚ Crude microsomes; ⊕ Crude soluble fraction

therefore in the mitochondria, if one examines specific activity and employs the optimum pH for each fraction, the approximate order of activities is microsomes (3.5)> mitrochondria (1.8) = soluble (1.7 to 1.9) (Table VI).

Enzymes of rat liver degrade sarin, dichlorvos, methyl paraoxon, methyl parathion, and methyl sumithion. The order of our specific activity for paraoxon differs from that of sarin, which is found in the soluble fraction, microsomes, mithochondria, and nuclei, roughly in the proportions 65:20:10:5, with some variations according to the animal species and the tissues (Adie and Tuba, 1958). Microsomes have no dichlorvos-degrading activity (Hodgson and Casida, 1962). The enzymic degradation of methyl paraoxon, methyl parathion, and methyl sumithion by microsomes and mitochondria observed by Fukami and Shishido (1963) and Shishido and Fukami (1963) may be due to different enzymes from those reported here, since they used EDTA (5mM) to prevent coagulation of the preparations tested. EDTA at a concentration of 1mM inhibits about 56 to 57% of the activity of paraoxon-degrading enzymes in microsomes and mitochondria (Table IV).

Optimum pH. The effect of pH on the activity of H³-paraoxon-degrading enzymes in the rat liver was determined by using a combination of 0.067M sodium phosphate, 0.1M glycine-NaCl-NaOH buffers, and 0.1M sodium barbital–HCl buffer (Figure 2). Spontaneous H³-paraoxon hydrolysis became rapid at about pH 10.5. The optimum pH of the various paraoxon-degrading enzymes occurred at about 7.0 for the washed mitochondrial fraction, 7.7 for the crude microsomal fraction, and 8.8 for the crude soluble fraction, although activity was observed throughout the range of pH 6.0 to 11.0.

The results suggest that paraoxon-degrading enzymes of rat liver are not identical with paraoxon hydrolyzing A-esterase of rabbit serum, which has an optimum pH of 7.5 (Aldridge, 1951, 1953b). The soluble fraction of rat liver has enzymes hydrolyzing sarin (Adie and Tuba, 1958), diazinon (Suwanai and Shishido, 1965), the vinyl phosphate of dichlorvos (Hodgson and Casida, 1962), and also desmethylating enzymes which are capable of catalyzing the desalkylation of dichlorvos (Hodgson and Casida, 1962) and methyl parathion (Fukami and Shishido, 1963). Optimal pH values showed that soluble paraoxon-degrading enzymes were not identical with that for diazinon, which has an optimum pH of 5.8 (Suwanai and Shishido, 1965), but were about the same as the enzyme which

desalkylates methyl parathion, pH 8.8 (Fukami and Shishido, 1963). The pH optima of microsomal and mitochondrial paraoxonases were also different from the corresponding diazoxon hydrolyzing enzymes, which both had an optimum pH of 8.5 (Suwanai and Shishido, 1965).

Time Course of Enzymic Reaction. The rate of enzymic degradation of H³-paraoxon was determined using the optimal pH 7.0, 7.7, and 8.8 buffers for the fractions tested (Figure 3). Under the conditions of the experiment, there appeared to be at least two first-order reactions. Reaction constants were obtainable only for the slower reaction and were 0.0016 min.⁻¹ for the 20% washed
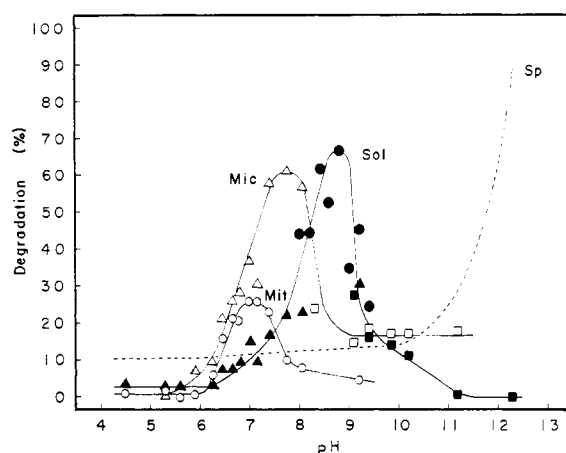
Figure 2. Effect of pH on the H³-paraoxon-degrading enzymes in rat liver fractions

Reaction mixture incubated at 37.5° C. for 1 hour. Substrate was 3.3 μM in a final concentration. Sp. Spontaneous hydrolysis; Mit. 33% washed mitochondria (O); Mic. 20% crude microsomes (Δ and □); Sol. 20% Crude soluble fraction ($\blacktriangle$, $\bullet$, and $\blacksquare$): O $\triangle$ $\blacktriangle$ 0.067M sodium phosphate buffer; □ $\blacksquare$ 0.1M glycine–NaCl–NaOH buffer; $\bullet$ 0.1M sodium barbital–HCl buffer; liver data corrected for spontaneous hydrolysis

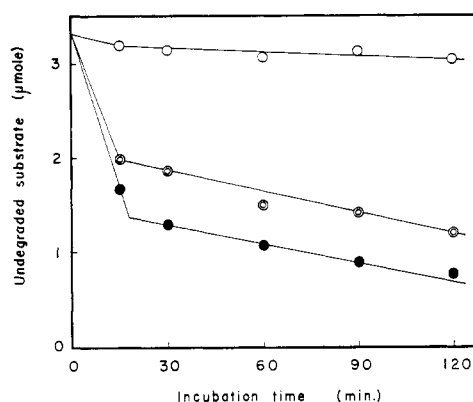**Figure 3. Rate of enzymic degradation of H³-paraoxon by rat liver fractions**

Reaction mixture incubated at 37.5° C. O 20% Washed mitochondria, pH 7.0; ⊚ 20% crude microsomes, pH 7.7; $\bullet$ 20% crude soluble fraction, pH 8.8

Table I. Activity of H³-Paraoxon-Degrading Enzymes in Rat Liver Fractions[a]

	Degradation, %, ± Standard Error		
pH Tested	20% crude soluble fraction	20% crude microsomal fraction	20% washed mitochondrial fraction
8.8	71 ± 2 (8)	34 ± 9 (3)	7 ± 0 (2)
7.7	22 ± 3 (3)	49 ± 4 (13)	8 ± 0.7 (2)
7.0	16 ± 1 (2)	37 ± 3 (5)	10 ± 0.4 (4)

[a] Reaction mixture incubated at 37.5° C. for 1 hour. Figures in parentheses indicate number of experiments.

mitochondrial fraction, 0.0074 min.$^{-1}$ for the 20% crude microsomal fraction, and 0.0067 min.$^{-1}$ for the 20% crude soluble fraction, respectively. In comparison, Aldridge (1953b, c) obtained a first-order constant for nonenzymic paraoxon hydrolysis at 37° C. with phosphate buffer, pH 7.6 of 3.9 to 4.9 × 10^{-5} min.$^{-1}$, corresponding to a half-life under his experimental condition of 9.7 to 11.2 days.

Effects of Cofactors. The effects of various cofactors on the activity of H^3-paraoxon-degrading enzymes in rat liver fractions are shown in Table II. The activities of the fractions were not greatly affected by the addition of any cofactors tested, except that glutathione inhibited washed mitochondrial enzyme by 43% at 10mM. In other experiments, however, the activity of a partially purified soluble fraction (S 40) was stimulated by reduced glutathione and some other cofactors (Kojima and O'Brien, 1967). A similar phenomenon was observed by Fukami and Shishido (1963, 1966) during the enzymic degradation of methyl parathion by the soluble fraction of rat liver.

Effects of Metallic Ions and Inhibitors. An attempt was made to differentiate the enzymes degrading paraoxon in the rat liver fractions by determining the effects of a number of commonly used metallic ions (Table III) and inhibitors (Table IV) on the enzyme activity. In this broad-scale survey, a single concentration (1mM) was employed in every case. The enzymes of these fractions differ greatly in their responses to many agents. Nevertheless, they have certain features in common. All are activated by 1mM Ca^{2+}, and little affected by Na^{+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, Ag^{+}, Pb^{2+}, Hg^{+}, Mn^{2+}, Bi^{2+}, and Cu^{2+}. Heavy metallic cations such as Hg^{2+}, Cd^{2+}, and Co^{2+} were strong inhibitors of all these enzymes. Zn^{2+} was a

potent inhibitor of the soluble enzyme, but had a weak inhibitory effect on microsomal and mitochondrial enzymes. By contrast, Cu^{2+} and Ni^{2+}, and to a lesser extent Fe^{2+}, Fe^{3+}, and Al^{3+}, inhibited the microsomal and mitochondrial enzymes but had little effect on the soluble enzyme.

The paraoxon-degrading enzymes were also sensitive to some SH-reagents, metal complexing agents, carbamates, and other compounds (Table IV). There was considerable variation in response. For example, iodoacetate and p-chloromercuribenzoate inhibited the activity of soluble enzyme. Iodoacetate had a very small effect on the microsomal and mitochondrial enzymes, and p-chloromercuribenzoate inhibited mitochondrial enzyme but did not inhibit microsomal enzyme. p-Iodosobenzoate had no effect on any fractions.

Among the metal-chelating agents tested, tetrasodium ethylendiaminetetraacetate strongly inhibited mitochondrial and microsomal enzymes, but did not inhibit soluble enzyme. 8-Hydroxyquinoline inhibited soluble enzyme moderately and microsomal enzyme weakly. It had no effect on the mitochondrial enzyme. 2,2'-Dipyridyl was not an inhibitor for any fractions. The inhibition by tetrasodium ethylendiaminetetraacetate may be due to removal of Ca^{2+}.

Rat liver enzymes showed different sensitivities to eserine sulfate. This compound inhibited the mitochondrial enzyme strongly, microsomal enzyme moderately, and soluble enzyme weakly. Both sodium diethyldithiocarbamate and o-chlorophenyl N-methylcarbamate inactivated weakly the soluble or mitochondrial enzymes only.

N-Bromosuccinimide was a strong inhibitor of mito-

Table II. Effect of Various Cofactors on Activity of H^3-Paraoxon-Degrading Enzymes in Rat Liver Fractions[a]

| Enzyme Source | Cofactors | Final Concentration, mM | Recovery of Radioactivity, % | Radioactivity, % | | | Degradation, % | Effect[b] |
				Toluene extract	Water extract	Protein residue		
20% crude soluble fraction, pH 8.8	Nil	...	96	18	79	3	79.0	
	GSSG	10	97	16	81	3	81.3	+3
	GSH	10	94	20	77	3	76.6	−3
	NIC	10	94	19	79	2	78.3	−1
	NAD	0.1	88	18	79	3	79.1	0
	NADH$_2$	0.1	82	18	80	2	79.3	+1
	NADP	0.1	92	17	81	2	80.4	+2
	NADPH$_2$	0.1	84	18	79	3	79.2	0
20% crude microsomal fraction, pH 7.7	Nil	...	67	32	63	5	63.0	
	GSSG	10	70	28	67	5	67.9	+8
	GSH	10	70	33	62	5	61.7	−2
	NIC	10	64	34	60	6	61.0	−3
	NAD	0.1	72	28	66	6	67.4	+7
	NADH$_2$	0.1	71	34	60	6	60.6	−4
	NADP	0.1	71	29	65	6	66.6	+5
	NADPH$_2$	0.1	71	39	56	5	54.8	−3
33% washed mitochondrial fraction, pH 7.0	Nil	...	86	70	26	4	18.4	
	GSSG	10	86	77	20	3	10.5	−43
	GSH	10	87	73	23	4	15.3	−17
	NIC	10	81	73	24	3	14.9	−19
	NAD	0.1	81	72	24	4	16.4	−11
	NADH$_2$	0.1	84	72	24	4	16.9	−8
	NADP	0.1	84	72	25	3	16.5	−10
	NADPH$_2$	0.1	86	73	22	5	15.1	−18

[a] Reaction mixture incubated at 37.5° C. for 1 hour.
[b] Per cent inhibition (−) or activation (+).

Table III. Effect of Various Metallic Cations on Activity of H³-Paraoxon-Degrading Enzymes in Rat Liver Fractions[a]

Cations[c]	20% Crude Soluble Fraction,[b] pH 8.8				20% Crude Microsomal Fraction, pH 7.7			33% Washed Mitochondrial Fraction, pH 7.0		
	Recovery of radioactivity, %	Degradation, %, ± standard error	Coefficient of variability	Effect[d]	Recovery of radioactivity, %	Degradation, %	Effect[d]	Recovery of radioactivity, %	Degradation, %	Effect[d]
Ca^{2+}	87	79 ± 3	3.8	+11	77	64	+16	96	31	+11
Na^-	84	70 ± 2	2.9	−1	80	57	+4	98	25	−11
Mg^{2+}	84	75 ± 7	9.3	+6	81	51	−7	95	22	−21
Sr^{2-}	90	71 ± 4	5.6	0	83	54	−2	95	22	−21
Ba^{2+}	89	70 ± 3	4.2	−1	81	54	−2	95	26	−7
Hg^{2+}	88	40 ± 25	62.5	−44	84	11	−80	100	6	−79
Cd^{2+}	87	28 ± 20	71.5	−61	86	20	−64	96	10	−64
Co^{2-}	83	53 ± 6	11.3	−25	92	47	−16	94	21	−25
Cu^{2+}	88	66 ± 2	3.0	−7	87	16	−71	95	8	−71
Ni^{2-}	83	66 ± 4	6.1	−7	88	14	−78	98	10	−64
Fe^{3+}	85	68 ± 1	1.5	−4	84	39	−29	96	18	−36
Fe^{2-}	87	69 ± 3	4.3	−3	81	42	−24	98	22	−21
Al^{3-}	92	66 ± 1	1.5	−7	81	46	−16	97	22	−21
Ag^-	86	67 ± 3	4.5	−6	82	59	+7	95	28	0
Pb^{2-}	86	70 ± 2	2.9	−1	77	53	−4	95	26	−7
Hg^+	87	65 ± 2	3.1	−8	83	55	0	94	24	−14
Mn^{2-}	87	76 ± 5	6.6	+7	81	53	−4	95	24	−14
Bi^{2-}	88	68 ± 2	2.9	−4	81	51	−7	96	24	−14
Cu^+	87	67 ± 1	1.5	−6	78	50	−9	98	26	−7
Zn^{2+}	91	32 ± 18	56.3	−55	83	54	−2	95	24	−14
Control	91	71 ± 1			84	55		101	28	

[a] Reaction mixture incubated at 37.5° C. for 1 hour.
[b] Contained paraoxonase and desethylating enzymes.
[c] Final concentration 1mM.
[d] Per cent inhibition (−) or activation (+).

Table IV. Effect of Some SH-Reagents and Inhibitors on Activity of H³-Paroxon-Degrading Enzymes in Rat Liver Fractions[a]

Compound[c]	20% Crude Soluble Fraction,[b] pH 8.8				20% Crude Microsomal Fraction, pH 7.7				33% Washed Mitochondrial Fraction, pH 7.0			
	R[d]	D[e]	V[f]	Effect[g]	R	D	V	Effect[g]	R	D	V	Effect[g]
SH-reagents												
Iodoacetate	76	50 ± 5	12.8	−31	82	56 ± 2	3.9	+4	91	25		−7
p-Chloromercuribenzoate (p-CMB)	80	48 ± 1	2.9	−33	87	52 ± 1	2.7	−4	88	19		−30
p-Iodosobenzoate	82	68		−6	81	56 ± 2	5.0	+4	88	24		−11
Metal complexing agents												
Ethylenediaminetetraacetate, tetrasodium salt (EDTA)	81	72 ± 2	3.9	0	85	23 ± 5	27.4	−57	99	12 ± 2	30.0	−56
8-Hydroxyquinoline	78	49 ± 0		−32	85	47		−13	88	26		−4
2,2'-Dipyridyl	84	71		−1	82	55 ± 3	6.6	+2	92	25		−7
Carbamates												
Eserine sulfate	80	61 ± 9	19.7	−15	81	33		−39	88	12		−56
o-Chlorophenyl N-methylcarbamate (o-CPM)	89	67		−7	85	56		+4	91	23		−15
Sodium diethyldithiocarbamate	85	61		−15	80	49		−9	87	25		−7
Amino acid												
l-Histidine	81	71		−1	84	58 ± 3	7.2	+7	93	29 ± 2	9.7	+7
Others												
Phenylmercuric acid (PMA)	83	68 ± 3	6.2	−6	87	19		−65	91	7		−74
N-Bromosuccinimide (NBC)	81	63 ± 2	3.5	−13	85	33		−39	91	8		−70
Phenylhydrazine hydrochloride	75	60		−17	81	57		+6	91	25 ± 2	8.8	−7
Sodium azide	78	69		−4	82	56		+4	92	23 ± 1	8.7	−15
Sodium fluoride	79	67 ± 7	13.7	−7	83	61 = 2	3.6	+13	86	28		+4
Iodine	82	68		−6	84	56 ± 6	13.9	+4	87	24		−11
2-Diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF 525A)	83	72		0	84	57		+6	90	26 ± 2	8.4	−4
Thiourea	84	73		+1	84	55		+2	94	28 ± 1	3.2	+4
Control	78	72 ± 2	3.9		85	54 ± 2	4.1		93	27 ± 2	8.9	

[a] Reaction mixture incubated at 37.5° C. for 1 hour.
[b] Contained paraoxonase and desethylating enzymes.
[c] Final concentration, 1mM.
[d] R = recovery of radioactivity, %.
[e] D = degradation, %, ± standard error.
[f] V = coefficient of variability.
[g] Per cent inhibition (−) or activation (+).

chondrial enzyme, and affected to a lesser extent microsomal and soluble enzymes. Similarly, phenylmercuric acid inactivated strongly mitochondrial and microsomal enzymes, but had no effect on the soluble enzyme. The soluble enzyme was weakly inhibited by phenylhydrazine hydrochloride (which reacts with the carbonyl group of enzymes) but was unaffected by iodine, sodium azide, and sodium fluoride (which catalyzes the hydrolysis of paraoxon at a relatively high rate at a concentration of $1mM$ (Erdös and Boggs, 1961)). The mitochondrial enzyme was weakly inhibited by sodium azide and iodine and unaffected by phenylhydrazine hydrochloride. Microsomal enzyme activity was slightly activated by sodium fluoride and unaffected by sodium azide, iodine, and phenylhydrazine hydrochloride. All these enzymes were unaffected by 2,2-diethylaminoethyl 2,2-diphenylvalerate hydrochloride (SKF 525A), thiourea, and l-histidine.

The effect of metallic ions on organophosphate and fluorophosphate hydrolysis by mammalian and insect enzymes has been investigated by several workers, However, it is complicated by overlapping substrate specificities and by the complex nature of these enzymes. Rabbit serum paraoxonase was inhibited by mercury, copper, and nickel at 1 μM (Aldridge, 1951, 1953b). Main (1960a, b) found that sheep serum purified paraoxonase preparation (purified 385 times) was moderately inhibited by Mn^{2+} and Ba^{2-} (Ba^{2+} inhibition depends on the method of analysis), and that the DFP hydrolyzing activity of this preparation was strongly inhibited by Mn^{2+}. On the other hand, DFP-hydrolyzing enzymes of hog kidney purified fraction (Mounter and Chanutin, 1953, 1954), and sheep and horse serum (Main, 1960b) were activated by Mn^{2+}. Similar activation was observed in other species and tissues, but not in serum (Mounter, 1955). The DFP-hydrolyzing activity of rabbit serum (Main, 1960b; Mounter, 1954) and of certain bacteria (Mounter et al., 1955a) was inhibited by Mn^{2+}. Main (1960a, b) suggested that sheep, horse, and other mammalian sera contained enzymes hydrolyzing DFP and paraoxon, and that the complex effect of Mn^{2+} on the enzymes of various species could be explained by the presence of varying amounts of DFP hydrolyzing enzyme and DFP-hydrolyzing paraoxonase in these tissues.

In the present investigation, although rat liver enzymes were inhibited by SH-inhibitors, some were very weakly inhibited, and it would be premature to assume that the enzyme site contains a sulfhydryl group. Disodium ethylenediaminetetraacetate inactivates completely or partially the hydrolysis of paraoxon by a purified human arylesterase (fraction IV-1) and by sera of pig, horse, rabbit, guinea pig, and sheep, but it does not inhibit the degrading activity of paraoxon by a purified human albumin (fraction V or crystallized) or by serum albumin of other animals such as rabbit, pig, cow, and horse (Erdös et al., 1959). Ethylenediaminetetraacetate also inhibits a purified sheep serum paraoxonase (93% inhibition at $1mM$; Main, 1960a), insoluble DFP hydrolyzing (fraction I) of rat and hog livers (Mounter, 1955), and DFP hydrolyzing enzymes of certain bacteria (Mounter et al., 1955a). But it did not inhibit enzymic desalkylation of methyl parathion in soluble fraction of rat liver at a concentration of $0.1mM$ (Fukami and Shishido, 1963). Mounter et al. (1953) found that 8-hydroxyquinoline and phenylhydrazine inhibited DFP-

hydrolyzing enzyme of hog kidney. The former affected the hydrolysis of paraoxon, DFP, TEPP, and p-NPA by rabbit serum enzyme (Mounter, 1954). On the other hand, 2,2'-dipyridyl and l-histidine at $1mM$ activates soluble DFP hydrolyzing enzymes (fraction S, A, and A-1) of rat and hog livers (Mounter, 1955) and a purified DFP-hydrolyzing enzyme of hog kidney (Mounter et al., 1953; Mounter and Chanutin, 1954), particularly in the presence of Mn^{2+}.

Aldridge (1951, 1953b) reported that paraoxon-hydrolyzing enzyme in rabbit serum was sensitive to eserine sulfate at 0.8 and $4mM$ and sodium fluoride at $100mM$. Sodium fluoride was a poor inhibitor of enzymic desalkylation of methyl parathion in soluble fraction of rat liver, causing only 12% inhibition at 100 mM (Fukami and Shishido, 1963).

O'Brien (1961) found that SKF 525A inhibited the degradation of paraoxon in mice and American cockroaches, Periplaneta americana in vivo. In the present in vitro studies, however, the paraoxon-degrading enzymes of rat liver were not inhibited by it.

The observation of effects of metallic ions and inhibitors on paraoxonases and desethylating enzyme of rat liver suggested the possibility that these enzymes belong to a different group from those of the well-known hydrolases (Figure 4 and 5).

Nature of Metabolites in Unpurified Fractions. The R_f's of potential metabolites were obtained experimentally by chromatography of known materials. Desethyl paraoxon was not available and therefore was prepared as follows. Plapp and Casida (1958b) showed that alkaline hydrolysis of paraoxon by $25mM$ KOH produces only 12% of the desethyl paraoxon at 28° C. for 20 hours. Therefore, the authors hydrolyzed 10 μmoles of H^3-paraoxon in $25mM$ KOH at 28° C. for 20 hours, then extracted three times with chloroform (separate experiments showed that this treatment removed all H^3-paraoxon). In the aqueous phase, only two radioactive products were found with R_f's 0.60 (94%) and 1.0 (6%) in a 2-propanol–ammonium hydroxide system, and 0.27 (96%) and 1.0 (4%) in an acetonitrile–water–ammonium hydroxide system. The peak with an R_f of 1.0 therefore must be desethyl paraoxon, for it is the only possible ethyl containing hydrolysis product (only ethyl-containing compounds are detected because it is the ethyl group that is labeled) and is distinct from peaks of monoethyl phosphate and diethyl phosphate, whose R_f's are, respectively, 0.07 and 0.60, in a 2-propanol–ammonium hydroxide system.

Only one radioactive metabolite (R_f 0.60, presumably diethyl phosphate) was found with washed mitochondrial, crude microsomal, and crude soluble fractions (Figure 6). Radioactivity at the origin of the chromatograms was obviously not phosphoric acid as a metabolite, for phosphoric acid could not be radioactive. It might be due to metabolites complexed with proteins, which were not moved from the applied area by the developing solvent systems.

Ammonium Sulfate Fractionation of the Crude Soluble Fraction. There was some indication of extra enzyme precipitation at 80% (even though, paradoxically, the supernatant had negligible activity between 60 and 100%), so the possibility of different enzymes at different per cent saturations was explored. When the data were expressed in terms of specific activity, it suggested that the prepara-

Figure 4. Hydrolases of organophosphates and fluorophosphates in mammals

Table header row (Figure 4):

Hydrolase	Animal tissue	Fraction	Substance (Ca Sr Ba Mn Co Mg Hg Cd Ni Cu Zn G Di Ph Hi PM pC NB Es 8H ED)	Reference
Paraoxon	Rat liver	Mitochon.		1
		Microso.		
		Soluble		
	Sheep	Serum		2
	Rabbit	Serum		3
	Human	Serum		4
Dichlorvos	Rat liver	Mitochon.		5
		Soluble		
Diazoxon	Rat liver	Mit. Mic.		6
Diazinon	Rat liver	Soluble		7
M. parathion	Rat liver	Soluble		
DFP	Rat kidn.	Soluble		8
	Rat liver	Soluble		
		Insoluble		9
	Hog kidn.	Soluble		10
	Hog liver	Soluble		
		Insoluble		
Tabun	Human	Serum		11
	Hog kidn.	Soluble		

Legend: ■ Activation ⊠ Inhibition ▤ No effect

G. Reduced glutathione; Di. 2,2'-Dipyridyl; Ph. o-Phenanthroline; Hi. l-Histidine; PM. Phenylmercuric acid; pC. p-Chloromercuribenzoate; NB. N-Bromosuccinimide; Es. Eserine sulfate; 8H. 8-Hydroxyquinoline; and ED. Ethylenediamine tetraacetic acid, tetrasodium or disodium salt. (1) This paper, (2) Main (1960a. b), (3) Aldridge (1951, 1953b, c), (4) Erdös and Boggs (1961). (5) Hodgson and Casida (1962), (6) Suwanai and Shishido (1965), (7) Fukami and Shishido (1963). (8) Mounter (1955), (9) Mounter et al. (1953), (10) Mounter et al. (1955b), (11) Augustinsson (1954), and Augustinsson and Heimbürger (1955)

Figure 5. Hydrolases of organophosphates and fluorophosphate in insects

Table header row (Figure 5):

Hydrolase	Animal tissue	Fracti.	Substance (Ca Sr Ba Mn Co Mg Hg Cd Ni G Cy DL Di Ph PM pC)	Reference
Paraoxon	House fly	Homog.		1
Dichlorvos	House fly	Homog.		
TEPP	House fly	Homog.		
DFP	House fly	Homog.		
M. parathion	H.B. larvae	Soluble		2
	S.W.L.M	Soluble		
	A. cockroa.	Soluble		
Diazinon	A. cockroa.	Soluble		3
Diazoxon	A. cockroa.	Soluble		
P=O compounds	Lepidopter.	Soluble		4
P=S compounds	Lepidopter.	Soluble		

Legend: ■ Activation ⊠ Inhibition ▤ No effect

G. Reduced glutathione; Cy. Cysteine; DL. DL-Homocysteine; Di. 2,2'-Dipyridyl; Ph. o-Phenanthroline; PM. Phenylmercuric acid; and pC. p-Chloromercuribenzoate. H. B. larvae. Horn beetle larvae (Mid gut); S. W. L. M. Silk worm larvae (mid gut and fat body); A. cockroa. American cockroach (fat body); and Lepidopter. Lepidopterous caterpillars (intestines of Agrotis segetum, Antheraca pernyi, Lymantria dispar and Mamestra bassicae). (1) Krueger and Casida (1961), (2) Fukami and Shishido (1963), (3) Suwanai and Shishido (1965). (4) Jarczyk (1966)

tions contained at least two components having different specific enzyme activities. One precipitated between 30 to 100% saturation, and another was not precipitated between 30 to 50% saturation, and precipitated between 60 to 100% saturation. Two fractions therefore were prepared by 40% saturation of the soluble fraction of liver with ammonium sulfate; the supernatant was called S 40 and the precipitate, P 40. Table VI compares the specific activity of these with other liver fractions, against paraoxon and parathion.

The nature of the metabolites was examined, using the above fractions and also the precipitate (P 80) and supernatant (S 80) from 80% saturation. Desethyl paraoxon was found in chromatograms after incubation of paraoxon with S 40, P 40, and P 80 fractions. The percentage of breakdown products present as desethyl paraoxon was 23 in S 40, 9 in P 40, and 4 in P 80. S 80 had no paraoxon-degrading activity. When these values are multiplied by the activity of each fraction (as read in Figure 7) the relative desethy-
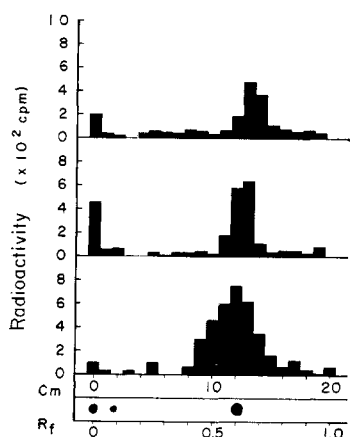
Figure 6. Radioactivity of water-soluble metabolites of H³-paraoxon on paper chromatograms

Reaction mixture incubated at 37.5° C. for 1 hour. Top. 33% washed mitochondria, pH 7.0; Middle. 20% crude microsomes, pH 7.7; Lower. 20% crude soluble fraction, pH 8.8

Figure 7. Ammonium sulfate fractionation of 20% crude soluble fraction of rat liver

Total activity of H³-paraoxon-degrading enzymes in supernatant and precipitate fractions after dialysis. Reaction mixture incubated at 37.5° C. for 1 hour at pH 8.8. Substrate was 3.3 μM in a final concentration. Each point represents a different sample saturated to the indicated extent. Crude soluble fraction stored at 0–5° C. for 2.5 days without dialysis. ○ Supernatant; ● Precipitate; — Enzymic activity; — — — Protein

lating activities were 3.2 for S 40, 2.3 for P 40, and 1.5 for P 80. These values were found when the products were analyzed by chromatography in 2-propanol–ammonium hydroxide system. The values when an acetonitrile–water–ammonium hydroxide system was employed differed in detail, but presented the same picture, the respective percentages were 12, 7, and 6, and the respective desethylating activities were 1.2, 1.8, and 2.2. These findings do not support the view that high ammonium sulfate concentrations precipitated a fraction enriched in desethylating enzyme. But because the total activity (of supernatant plus precipitate) with 80% ammonium sulfate is only two thirds of that with 40% ammonium sulfate, denaturation apparently occurs with high ammonium sulfate concentrations, so that this is an undesirable enrichment procedure.

Solubilization of Particulate Enzymes. Washed mitochondrial and crude microsomal paraoxonases could be solubilized by sodium desoxycholate at a final concentration of 5mM (2 mg. per ml.). Table V gives the paraoxonase activity of fractions treated with and without sodium desoxycholates. This treatment led to the appearance in the soluble phase of all of the activity of microsomes and 80% of the activity of mitochondria.

The soluble fractions are claimed to be soluble only under the centrifugal conditions that precipitated their respective subcellular sources. The authors do not exclude the possibility that high speed centrifugation might precipitate these soluble fractions.

Enzymic Degradation of Paraoxon and Parathion. The relative rates of enzymic degradation of paraoxon and parathion were examined on the crude and partially purified enzyme fractions of rat liver (Table VI). Paraoxon was always degraded more rapidly than parathion by all fractions. It was degraded more than twice as fast in all fractions on the basis of specific activity.

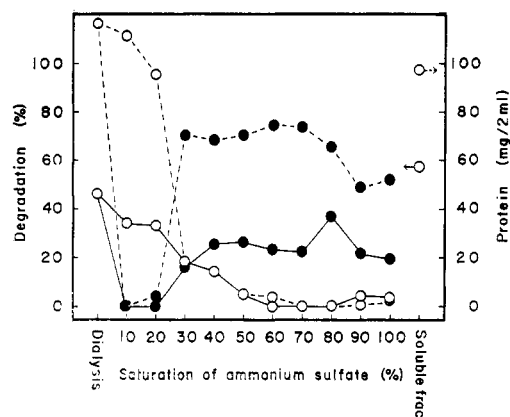In the present investigation, no attempt was made to

Table V. Solubilization of Microsomal and Mitochondrial Paraoxonases by Sodium Desoxycholate Treatment[a]

Enzyme Source	Degradation, %
20% crude microsomes, pH 7.7	
Untreated	41
Soluble fraction of treated	42
Insoluble fraction of treated	4
20% washed mitochondria, pH 7.0	
Untreated	10
Soluble fraction of treated	8
Insoluble fraction of treated	2

[a] Reaction mixture incubated at 37.5° C. for 1 hour. Recovery of radioactivity in above experiments was between 80 and 104%.

show whether or not the enzymes responsible for the degradation of paraoxon and parathion in the rat liver were the same. However, the above relative activities toward the two compounds agree with in vivo observations of Plapp *et al.* (1961) on the housefly, and of Kojima *et al.* (1963a) on the rice stem borer, and with in vitro studies of Fredriksson (1961) on skin from man, cat, and rabbit, of Mounter (1954) on rabbit serum, and of Mounter and Tuck (1956) on microorganisms. Fukami and Shishido (1963) showed that a soluble fraction of rat liver hydrolyzed methyl paraoxon faster than its thiono analog. Recently, Jarczyk (1966) found that analogs of paraoxon were degraded more rapidly than the analogous phosphorothionates by the midgut of lepidopterous caterpillars. In contrast with these results, Metcalf *et al.* (1956) observed that homogenates of bee abdomen hydrolyzed parathion more readily than paraoxon.

The enzymic activity of paraoxon and parathion degradation in crude soluble fraction was lost, to the extent of about

Table VI. Enzymic Degradation of H^3-Paraoxon and H^3-Parathion by Rat Liver Fractions[a]

Enzyme Source	Protein, Mg./2 Ml.	Substrate	Recovery of Radioactivity, %	Enzymic Activity Degradation, %	Specific activity[b]
33% washed mitochondria, pH 7.0	56.7 ± 16.2[c]	Paraoxon	95 ± 6[c]	30 ± 2[c]	1.76
		Parathion	102 ± 15	8 ± 6	0.46
20% crude microsomes, pH 7.7	51.1 ± 5.0	Paraoxon	84 ± 0	54 ± 2	3.52
		Parathion	100 ± 12	20 ± 3	1.30
20% crude soluble fraction, pH 8.8, not dialyzed[d]	94.2 ± 11.9	Paraoxon	84 ± 8	52 ± 10	1.88
		Parathion	124 ± 11	21 ± 9	0.74
20% crude soluble fraction, pH 8.8, dialyzed	93.8 ± 15.7	Paraoxon	85 ± 10	44 ± 5	1.68
		Parathion	129 ± 7	16 ± 4	0.59
P 40 fraction, pH 8.8	53.7 ± 5.2	Paraoxon	94 ± 1	24 ± 2	1.53
		Parathion	116 ± 7	7 ± 2	0.44
S 40 fraction, pH 8.8	13.0 ± 3.2	Paraoxon	95 ± 2	13 ± 5	3.56
		Parathion	111 ± 5	6 ± 8	1.60

[a] Reaction mixture incubated at 37.5° C. for 1 hour.   [c] Standard error.
[b] × 10^{-2} μmole per mg. N per hour.   [d] Stored at 0° to 5° C. for 40 hours.

15 to 19% of the total activity, by dialysis against a buffer of pH 8.8 (Figure 7 and Table VI). Main (1960a) reported that sheep serum paraoxonase did not lose activity by prolonged and repeated dialysis against water at pH 7.0. Fukami and Shishido (1963), however, found that desmethylating activity in soluble fraction of rat liver and midgut of horn beetle larva was lost by dialysis or after standing at 4° C. for 24 hours in air.

General Discussion. This study shows the differences in the distribution and characteristics of paraoxon-degrading enzymes of rat liver. However, the authors have not shown that any one of the fractions contained only one enzyme degrading paraoxon. The most pure DFP-ase fraction of Mounter (purified 65 to 100 times) was shown by Cohen and Warringa (1957) to contain two additional unknown components, one green (which they call G) and the other yellow (Y), which activated the purified DFP-hydrolyzing enzyme, and by Bergmann et al. (1957) to contain an additional enzyme, which they called C-esterase. Main (1960a, b) demonstrated that even highly purified paraoxonase preparation contained DFP-hydrolyzing enzyme. Moreover, the activating and inhibiting effects of metallic ions and other compounds vary with the enzyme sources and different substrates (Mounter and Chanutin, 1953, 1954) and with the possible presence of natural activators and inhibitors (Cohen and Warringa, 1957). Furthermore, the authors will show in a subsequent paper that purification of the paraoxonase in the soluble phase profoundly changes the response to metallic ions, inhibitors, and cofactors. With these reservations in mind, the following conclusions can be drawn about the nature of the crude enzymes of rat liver (neglecting effects small enough to be of a doubtful significance).

There are in liver at least three paraoxonases, defined as enzymes which hydrolyze paraoxon to diethyl phosphate and p-nitrophenol. The mitochondrial enzyme has a pH optimum of 7.0, is stimulated by Ca^{2+}, and inhibited by tetrasodium salt of ethylene diaminetetraacetate, but not by 8-hydroxyquinoline. It is strongly inhibited by the metallic cations Hg^{2+}, Cd^{2+}, Cu^{2+}, and Ni^{2-} at a concentration of

$1 mM$. The enzyme is sensitive to eserine sulfate, N-bromosuccinimide, phenylmercuric acid, and p-chloromercuribenzoate, but is relatively insensitive to SH-reagents such as iodosobenzoate. The microsomal enzyme has a pH optimum of 7.7, is stimulated by Ca^{2+}, and inhibited by tetrasodium ethylenediaminetetraacetate. It is similar in sensitivity to metallic cations, SH-reagents, and inhibitors to the mitochondrial enzyme. The enzyme is relatively sensitive to 8-hydroxyquinoline, but not to p-chloromercuribenzoate. The crude soluble enzyme has a pH optimum of 8.8, is stimulated by Ca^{2-}, and unaffected by tetrasodium salt of ethylenediaminetetraacetate and phenylmercuric acid. It is inhibited by Cd^{2+}, Zn^{2+}, Hg^{2+}, Co^{2+}, iodoacetate, p-chloromercuribenzoate, 8-hydroxyquinoline, phenylhydrazine hydrochloride, and eserine sulfate.

In addition, an enzyme responsible for degrading paraoxon to desethyl paraoxon and ethanol is present in crude soluble fraction; its properties have not been explored.

One of the long-term objectives of this and related studies was to establish some unity among the bewildering diversity of reported phosphotriesterases—i.e., enzymes cleaving triesters of phosphorus acids and thioacids. Instead, the authors have found at least four different enzymes in one tissue of one species acting on a single compound, and Figures 4 and 5 imply that similar situations exist for other tissues and other compounds, with virtually no cases of identity. Yet is is surely extravagant to suggest that the liver cell contains as many different enzymes as are implied by these findings. The authors suggest that probably only a few phosphotriesterases really exist; that many compounds are hydrolyzed by each enzyme, being affected by a single catalytic site, but binding to a variety of zones around that site. The variations in sensitivity to activators and inhibitors probably reflect variations in effect upon such binding.

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