ever, incubation of Ruelene with whole blood and with plasma gave definite evidence of hydrolysis, as shown in Figure 4. Ruelene was added to freshly drawn blood and plasma at a concentration of 100 p.p.m., incubated at 37° C., extracted with acetone, and the extract concentrated and chromatographed on Whatman No. 1 paper. A modification of the solvent system of Kaplanis and Robbins was used (*6*)—namely, 80% aqueous acetonitrile containing 2% of NH_4OH. R_f values of known hydrolysis compounds chromatographed at the same time are shown in Figure 4. The major product of these incubations appeared to be the dimethylamido phosphoric acid, and the secondary product may be the substituted phenylmethyl phosphoric acid. Washed red cells did not degrade added Ruelene. More degradation occurred when the plasma was adjusted to pH 9 than at the normal pH of 7.3. Very little degradation occurred at pH 5.

The best indication of the magnitude of the metabolic breakdown of Ruelene was obtained by paper chromatography of urine. Figure 5 presents scans of paper chromatograms of whole urine, a chloroform extract of the same urine, and also a chloroform extract of whole blood. Acid-washed Whatman No. 1 paper was developed descendingly with a modification of the solvent system of Ebel (*4*), consisting of 75% of aqueous isopropyl alcohol and 2% of NH_4OH. The R_f values in parentheses are for spots visible on the radioautograph, but not apparent in the scan.

The urine contained four major P^{32} containing components and four minor components, one of which, at R_f 0.90, was presumably Ruelene. The chloroform extraction of urine concentrated the small amount of Ruelene present, and also extracted to a varying degree some of the other components.

Using the heavy paper chromatographic technique suggested by Brownell *et al.* (*2*), milligram amounts of the front-running major components of whole urine and urine extracts were isolated, purified, and tentatively identified by their infrared spectra as a salt of the substituted phenylmethyl phosphoric acid, and Ruelene, respectively.

The chloroform extract of whole blood contained primarily these two compounds, as well as traces of three of the more polar metabolites.

Figure 6 contains data from a more quantitative study of the urinary products, wherein paper chromatograms of urine, collected at the stated time intervals following dosage, were sectioned and counted in a liquid scintillation instrument. These data show that the least polar metabolite, *E*, decreased in relative amount with time, in favor of two of the more polar components.

Attempts to isolate and identify the three major components of the first two collections of this urine were only partially successful. Component *E* was isolated in good purity and again appeared, by infrared spectroscopy, to be a salt of the substituted phenylmethyl phosphoric acid. Components *A* and *B* could not be sufficiently separated to permit complete identification by infrared, but both appeared to have lost the substituted phenyl moiety.

In summary, it appears that Ruelene parasiticide fed to sheep is rapidly absorbed, and also rapidly hydrolyzed, in part to partial hydrolysis products which are eliminated via urine, and in part to inorganic phosphate, which enters the normal body processes.

Acknowledgment

The authors thank W. W. Muelder for preparing Ruelene labeled with P^{32}, E. H. Blair for preparing the hydrolysis products of Ruelene, R. A. Nyquist for interpreting the infrared scans, and D. R. Mussell for performing the anticholincsterase assays.

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Received for review April 13, 1961. Accepted August 2, 1961. Division of Agricultural and Food Chemistry, 138th Meeting, ACS, New York, September 1960.

INSECTICIDE METABOLISM IN PLANTS

Peroxidase and Ethylenediaminetetraacetic Acid–Ferrous Iron–Catalyzed Oxidation and Hydrolysis of Parathion

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MANY ORGANOPHOSPHATE ESTERS which have systemic insecticidal activity are oxidized within the plant. Such oxidations convert phosphorothionates to phosphates (*4, 5*) alkylthioalkyl groupings to their sulfinyl and sulfonyl derivatives (*1, 14*), and *N,N*-dimethylphosphoramides to *N*-methylphosphoramides through intermediate steps (*3, 10*). The rates of these oxidations vary greatly with the organophosphate, the plant species, and the physiological state of the plant (*14*). The mechanism of this oxidation in plants is not known. Since plants can oxidize parathion (*O,O*-diethyl *p*-nitrophenyl phosphorothionate) to para-oxon (*O,O*-diethyl *p*-nitrophenyl phosphate) (*5*), this phosphorothionate was selected as the substrate for the present studies.

Mason (*13*) has presented evidence in support of the view that peroxidase, functioning as an oxidase, activates molecular oxygen toward aromatic substrates. The hydroxylating system of Udenfriend *et al.* (*18*) involving ascorbic acid, ferrous iron, and (ethylenedinitrilo)-tetraacetic acid (ethylenediaminetetraacetic acid, EDTA acid) functions in a somewhat similar manner. The EDTA-Fe^{+2} complex is a good synthetic model for peroxidase. In the presence of ascorbic acid, peroxidase is inactive, but

Peroxidase and the ethylenediaminetetraacetic acid–ferrous iron (EDTA-Fe^{+2}) complex were investigated for their ability to catalyze the oxidation and hydrolysis of parathion in the presence of an active hydrogen donor. Peroxidase catalyzed a 10% oxidation of parathion to para-oxon, while 12% conversion was maximum for the EDTA-Fe^{+2} complex. In addition to this oxidative conversion, peroxidase catalyzed the hydrolysis of 36% of the parathion and the EDTA-Fe^{+2} complex hydrolyzed 66%. Para-oxon was more stable than parathion to this hydrolytic attack. The EDTA-Fe^{+2} complex catalyzed 12% hydrolysis of para-oxon, whereas peroxidase catalyzed the hydrolysis of 6%. Peroxidases in plants may play a role in the metabolism of parathion and related phosphorothionates.

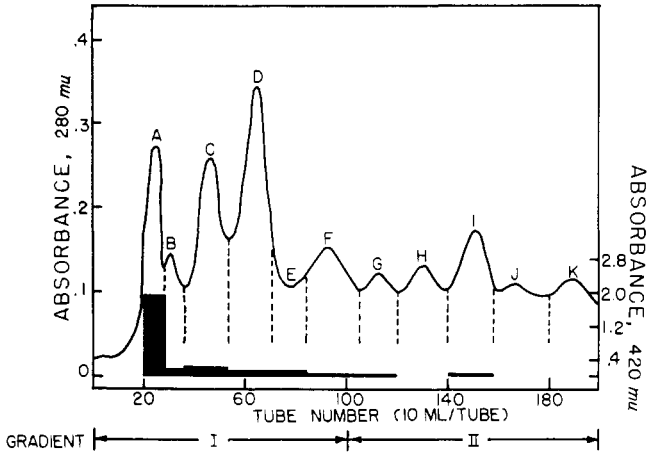

Figure 1. Separation of bean hypocotyl proteins

100 ml. of dialyzed bean hypocotyl extract containing 3 mg. of protein per ml., chromatographed on DEAE-cellulose column. Fractions between broken lines combined and 20 μg. of protein from each fraction assayed for peroxidase activity. Solid bars denote peroxidase activity after 3 minutes. See Figure 2 for assay conditions. Composite of six chromatograms, gradient elution program
I. 0.02M glycine to 0.02M glycine + 0.04M K phosphate, pH 6.7
II. 0.02M glycine + 0.04M K phosphate, pH 6.7, to 0.02M glycine + 0.1M K phosphate, pH 5.7, + 0.1M NaCl

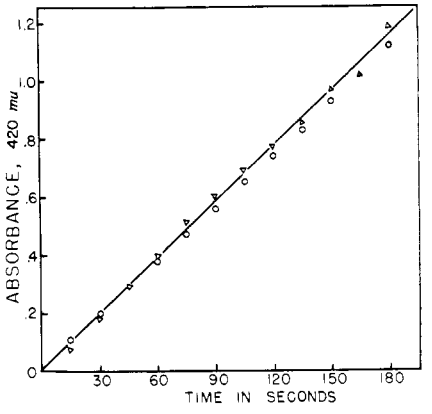

Figure 2. Peroxidase activity of bean hypocotyl protein chromatographing as fraction A and commercial horseradish peroxidase

△ 10 μg. of bean hypocotyl protein, fraction A
○ 5 μg. of commercial horseradish peroxidase
20 μmoles of guaiacol, 200 μmoles Na phosphate, pH 6.0, 0.3 μmole H_2O_2 in 7 ml. Read in 18-mm. test tube at 420 mμ

it functions as an oxidase in the presence of dihydroxyfumaric acid (DHF). Inhibition studies suggest that both ferrous iron and hydrogen peroxide are involved in the over-all activation and transfer of molecular oxygen toward substrates. Fenwick (*8*), using the model system of Udenfriend *et al.* (*18*), oxidized schradan (octamethylpyrophosphoramide) to an active inhibitor of cholinesterase.

In the present study, the peroxidase system described by Mason (*13*) and the model system of Udenfriend *et al.* (*18*) were investigated for their ability to oxidize parathion. Since the peroxidase from plant sources may differ in respect to oxidase activity, bean hypocotyl peroxidase was extracted, purified by chromatography on diethylaminoethylcellulose (DEAE - cellulose) columns, and compared in activity to commercial horseradish peroxidase (HRP).

Material and Methods

Radiosynthesis. Radioactive parathion was prepared from phosphoric-32 acid via phosphorus pentasulfide, diethyl phosphorodithioic acid, and diethyl phosphorochloridothionate (*15*). The specific activity was 1500 c.p.m. per microgram. Bromine water oxidation (*7*) of radioactive parathion yielded radioactive para-oxon as the only chloroform-soluble product. Identification was based upon co-chromatography with known para-oxon on Florisil (Floridin Co., Warren, Pa.) columns (*16*).

Extraction and Chromatographic Purification of Bean Hypocotyl Peroxidase. Six-day-old etiolated bean (variety Stringless Green Pod) hypocotyls were obtained by sprouting the seed at room temperature between two layers of damp paper toweling. One hundred grams of the growing sprouts were ground at 4° C., using Virtis homogenizer, in 100 ml. of a freshly prepared 0.05*M* phosphate buffer at pH 7.4, containing 0.057*M* ascorbic acid, 0.36*M* sucrose, and 0.002*M* $MgCl_2$. After grinding, the brei was filtered through four layers of cheesecloth and centrifuged immediately in a Spinco ultracentrifuge at 105,000 × g for 2 hours. The clear protein solution was dialyzed for 24 hours against a 60-fold volume change of the grinding buffer with the ascorbic acid concentration lowered to 0.0057*M*. At this time the protein solution was dialyzed against a 40-fold volume change of 0.005*M* phosphate buffer at pH 7.4 containing 0.36*M* sucrose. The solution was clear and light yellow in color after the final dialysis. Protein precipitation resulted if storage at 4° C. was longer than 2 days prior to chromatography.

Twenty-five to 30 grams of DEAE-cellulose (Brown Co., Berlin, N. H.), coarse grade with a capacity of 0.78 meq. per gram, was washed and 2.5 × 40 cm. chromatographic columns were prepared according to Mandeles (*12*). The temperature of the packed columns was maintained at 6° C. by circulating precooled ethylene glycol through a water jacket surrounding the column.

Just prior to chromatography, 300 to 400 mg. of protein in 100 ml. of solution was adsorbed on top of the column and washed in with small portions of 0.02*M* glycine. Two linear gradients were used to elute the hypocotyl protein, as indicated in Figure 1. Volumes

Table I. Activity of Anticholinesterase Agent (Para-oxon) Formed from Parathion during Incubation with Peroxidase–Hydrogen Peroxide and EDTA-Fe^{+2}–Ascorbic Acid

Experiment	System	Cholinesterase Inhibition, %
1	HRP	5
2	H_2O_2	5
3	H_2O_2 + HRP	18
4	H_2O_2 + HRP + guaiacol	7
5	EDTA + Fe^{+2} + ascorbic acid	50
6	EDTA + Fe^{+2} + ascorbic acid + catechol	27
7	EDTA + Fe^{+2} + hydroquinone	4

Reaction mixture. Expts. 1–4. 1 μg. parathion, 200 μmoles phosphate buffer, pH 6.0, 0.3 μmole H_2O_2 (Expts. 2, 3, 4), 5 μg. HRP (Expts. 1, 3, 4) and 6.0 μmoles guaiacol (Expt. 3) in total volume of 2.0 ml. Incubated at 25° C. for 1 hour without shaking. Expts. 5, 6, 7. 1 μg. parathion, 200 μmoles phosphate buffer, pH 6.7, 16 μmoles EDTA acid, 0.5 μmole Fe^{+2}, 28 μmoles ascorbic acid (Expts. 5, 6), 28 μmoles catechol (Expt. 6), and 28 μmoles hydroquinone (Expt. 7) in 2.0 ml.

Table II. Nature of Products Formed from Parathion and Para-Oxon during Incubation with EDTA-Fe^{+2}–Ascorbic Acid

Compound	Hydrolysis Products, %	Parathion, %	Para-oxon, %
Parathion	66	22	12
Para-oxon	12	0	88

Reaction mixture. 1 μg. radiolabeled parathion or para-oxon, 200 μmoles phosphate buffer, pH 6.7, 16 μmoles EDTA acid, 0.5 μmole Fe^{+2}, and 28 μmoles ascorbic acid in 2.0 ml.

Table III. Activity of Anticholinesterase Agent (Para-oxon) Formed from Parathion during Incubation with Different Peroxidase–Hydrogen Donor Systems

	Peroxidase System			% Cholinesterase Inhibition, Incubation Time, Minutes	
Expt.	μg. LRP	μmole Fe^{+2}	H-donor	10	60
1	50	...	DHF	16	41
2	...	0.72	DHF	25	...
3	...	...	DHF	9	...
4	50	...	DHM	...	41
5	...	0.72	DHM	...	54
6	...	...	DHM	...	25
7	50	...	RnH_2	...	15
8	...	0.72	RnH_2	...	24
9	...	...	RnH_2	...	12
10	50	...	IAA	...	0

Reaction mixture. 1 μg. parathion, 200 μmoles acetate buffer, pH 4.7, 16 μmoles hydrogen donor, and HRP or Fe^{+2} as indicated in 2.0 ml.

of 500 ml. were used in both the mixing chamber and the reservoir. The flow rate was adjusted to 3 ml. per minute. When the initial gradient system was exhausted, it was replenished by the next succeeding gradient system without interruption of flow.

A fraction collector equipped with a flow-through spectrophotometer which recorded transmittance at 280 mμ enabled continuous measurements to be made of the effluent. Ten-milliliter fractions were collected and maintained at 6° C. by coolant circulating between the tubes. The gradient device consisted of two 500-ml. graduated polyethylene cylinders connected at the bottom through a loop of 5-mm. i.d. polyethylene tubing. The gradient solution was taken off through a separate 5-mm. i.d. tubing connected through the bottom of the mixing vessel. A pump controlled the flow rate, while a synchronous motor equipped with a polyethylene paddle mixed the gradient. The gradient was cooled to 6° C. by a cooling coil prior to entering the top of the jacketed column which was maintained at the same temperature.

After chromatography the fractions were combined as indicated in Figure 1 and dialyzed at 4° C. for 24 hours against a 20-fold volume change of 0.01*M* phosphate buffer at pH 7.2. After dialysis the protein fractions were concentrated by ultrafiltration as described by Sober *et al.* (*17*) to less than 1/10th of their original volume at 4° C., analyzed for nitrogen using Nessler's solution (*19*), and stored at −10° C. for further use.

Peroxidase Assay. Twenty micrograms of protein (N × 6.25) from each fraction were assayed for peroxidase activity. Spectrophotometric measurements of the enzyme-substrate complex at 420 mμ were made according to the method of Devlin (*6*) using hydrogen peroxide and guaiacol. Five micrograms of commercial horseradish peroxidase (Worthington Biochemicals Co., Freehold, N. J.) were assayed by the same method and compared in activity to 10 μg. of bean hypocotyl peroxidase from the most active fraction.

Oxidation Procedure. One microgram of labeled or nonlabeled parathion dissolved in 0.2 ml. of chloroform was placed in the main compartment of a 15-ml. Warburg flask. The chloroform was evaporated off by the aid of a gentle stream of air. Two milliliters of buffer containing the desired hydrogen donor and peroxidase or EDTA-Fe^{+2} was added to the main compartment of the flask. The reaction mixtures are indicated in Tables I to V. The flask was shaken for 1 hour at 25° C. in an atmosphere of air.

Identification of Reaction Products. For the cholinesterase inhibition studies, the flask was removed from its manometer and 3 ml. of distilled water added. The entire contents of the flask were decanted into a 15-ml. centrifuge tube and the parathion and para-oxon in solution partitioned between equal volumes of chloroform and water. One-tenth milliliter of the chloroform layer, containing para-oxon formed from 0.02 μg. of parathion and the residual unoxidized parathion, was pipetted into a test tube, the chloroform was evaporated off, and 0.8 ml. of 0.9% NaCl and 200 μg. of purified bovine acetylcholinesterase (Winthrop Laboratories, New York, N. Y.) dissolved in 0.2 ml. of 0.9% NaCl were added. The enzyme and inhibitor were incubated for 1 hour at 38° C. and the volume was brought up to 10 ml. with 0.9% NaCl at the end of the incubation period. After further temperature equilibration and pH adjustment to 7.4, 0.5 ml. of 3% acetylcholine bromide was added and the hydrolysis rate of this compound was followed by means of an automatic recording titrator (Titrator TTTI and Titrigraph Type SBR2/SBU1, Radiometer, Copenhagen, Denmark), at 38° C. as described by Larsson and Hansen (*11*). The acid which was liberated was neutralized by addition of 0.02*N* NaOH at such a rate that the pH was maintained at 7.4. The consumption of alkali was recorded as a function of time. The first-order reaction was followed for 10 minutes.

For the radioactive studies, the flask was removed from its manometer and the contents were partitioned between equal volumes of water and chloroform. The layers were separated and the water layer was again extracted with an equal volume of chloroform. The hydrolysis products remained in the aqueous phase while both parathion and para-oxon were extracted by the chloroform. Adsorption chromatography with 1.0 × 12 cm. Florisil columns was used to separate parathion and para-oxon derived from the EDTA-Fe^{+2}–ascorbic acid and the peroxidase–hydrogen donor systems. The chloroform extracts were evaporated to dryness and dissolved in *n*-hexane, added to the top of the column, and eluted according to the method of Seume and O'Brien (*16*). Thirty 5-

Table IV. Effect of pH and Mn^{+2} on Peroxidase-Catalyzed Oxidation of Parathion

Expt.	pH	Mn^{+2}, Mmole	Cholinesterase Inhibition, %
1	4.7	...	33
2	4.7	0.03	21
3	6.7	...	10
4	6.7	0.03	19

Reaction mixture. 1 μg. parathion, 4 μg. HRP, 16 μmoles DHF, (Expts. 1, 2) 200 μmoles acetate buffer, (Expts. 3, 4) 200 μmoles phosphate buffer, and Mn^{+2} as indicated in 2.0 ml.

Table V. Products Formed from Parathion and Para-oxon during Incubation with Peroxidase and a Hydrogen Donor

Peroxidase System		Parathion			
μg. HRP	H-donor	Hydrolysis products, %	Parathion, %	Para-oxon, %	Para-oxon, Hydrolysis Products, %
0	DHF	7	90	3	...
0	DHM	6	92	2	...
5	DHF	16	78	6	0
5	DHM	15	79	6	0
50	DHF	36	54	10	6
50	DHM	36	54	10	6
50	...	0	100	...	...

Reaction mixture. 1 μg. radiolabeled parathion or para-oxon, 16 μmoles hydrogen donor, HRP as indicated, and 200 μmoles acetate buffer, pH 4.7 in 2.0 ml.

ml. fractions were collected. Identification was based on co-chromatography with known compounds.

Results

Chromatographic Purification of Bean Hypocotyl Peroxidase. Figure 1 represents the degree of protein separation achieved by chromatography on DEAE - cellulose columns. Individual peak height was found to vary from preparation to preparation according to the rate of protein synthesis within the growing hypocotyl. Protein recovery (N × 6.25) for the over-all process (chromatography, dialysis, and ultrafiltration) with extracts from hypocotyl tissue was 10%. The protein (N × 6.25) found in fractions A, B, C, D, E, F, G, H, I, J, and K after ultrafiltration was, respectively, 1.3, 0.8, 1.0, 1.4, 2.5, 2.6, 4.1, 5.9, 4.6, 3.3, and 6.4 mg. The fractions were assayed for peroxidase activity. Absorbance values after 3 minutes for fractions A through K are given in Figure 1. Based on the weight of the original extract, a 300-fold purification was achieved. Figure 2 compares the activity of 10 μg. of protein from fraction A with that of 5 μg. of commercial horseradish peroxidase.

Oxidative and Hydrolytic Activity of Bean Hypocotyl Peroxidase–Hydrogen Donor System. Twenty micrograms of protein from fractions A through K were assayed for their ability to catalyze the conversion of parathion to para-oxon. The reaction mixture contained 1 μg. of parathion, 16 μmoles of dihydroxymaleic acid (DHM), 20 μg. of the desired protein, and 200 μmoles of acetate buffer at pH 4.7 in a total volume of 2.0 ml. Of the fractions tested, only fraction A was able to catalyze the oxidation of parathion to para-oxon as determined by cholinesterase inhibition studies. Fraction A also had the highest peroxidase activity, as indicated in Figure 1. Although this does not rule out other oxidative systems, it does indicate that peroxidase from the bean plant is capable of functioning in vitro as an oxidase under the conditions of the experiment to convert parathion to para-oxon.

Ten micrograms of bean hypocotyl peroxidase from fraction A were found to be almost as active as 5 μg. of a commercial horseradish peroxidase in catalyzing the oxidation of radioactive parathion. Figure 2 shows that they were equivalent in peroxidase activity when assayed with hydrogen peroxide and guaiacol. Under the conditions cited in the preceding paragraph, 10 μg. of hypocotyl peroxidase catalyzed 4% oxidation and 14% hydrolysis of radioactive parathion, while 5 μg. of horseradish peroxidase catalyzed 6% oxidation and 15% hydrolysis.

Cholinesterase Inhibition Studies *vs.* Phosphorus-32 Analysis in Detecting Para-oxon. In the initial studies, acetylcholinesterase was used to detect and estimate para-oxon. When the procedure described above was used, 0.001, 0.002, and 0.003 μg. of para-oxon inhibited, respectively, 40, 60, and 80% of the cholinesterase activity. The efficiency of the extraction and recovery procedure for para-oxon from water as determined by cholinesterase inhibition was 80%. The inhibition studies were found to be highly reproducible within studies, and the microgram quantities detected compared closely with those obtained using radioactive parathion. Thus for EDTA-Fe^{+2}–ascorbic acid oxidation of parathion, inhibition studies showed that 11% was converted to para-oxon in contrast to 12% by phosphorus-32 analysis. Five micrograms of peroxidase catalyzed a 5% conversion as determined by inhibition studies and 6% for phosphorus-32 analysis, while peroxidase at the 50-μg. level converted 7.5 and 10%, respectively. The cholinesterase assay method for para-oxon was found extremely useful in the exploratory phases, but radioactive studies were necessary to differentiate other reaction constituents such as parathion and hydrolysis products.

Oxidative Activity of Peroxidase and Hydrogen Peroxide. Small quantities of parathion were oxidized in the presence of peroxidase and hydrogen peroxide (Table I). Peroxidase or hydrogen peroxide alone was unable to oxidize parathion. The addition of guaiacol substantially decreased the amount oxidized by reducing the hydrogen peroxide to water.

Oxidative and Hydrolytic Activity of EDTA-Fe^{+2}–Ascorbic Acid System. The EDTA-Fe^{+2}–ascorbic acid system (Tables I and II) was capable of converting 11 to 12% of the parathion in the oxidizing system to para-oxon. Table II shows that the system is also capable of hydrolyzing both parathion and para-oxon, para-oxon being considerably more stable to hydrolysis. Catechol (Table I) was found to inhibit the oxidation, and hydroquinone was unable to replace ascorbic acid as a hydrogen donor.

Oxidative and Hydrolytic Activity of Horseradish Peroxidase–Hydrogen Donor System. Peroxidase (Table III) catalyzed the aerobic oxidation of parathion when supported by active hydrogen donors. Dihydroxyfumaric acid and dihydroxymaleic acid (Table III) were equally active, triose reductone (RnH_2) was active to a lesser extent, while indoleacetic acid (IAA) was completely inactive. Ferrous iron can replace peroxidase in catalyzing the oxidation of parathion, although the ferrous iron is much more active in the enzyme than as inorganic iron. High control values (hydrogen donor alone) probably indicate that trace amounts of iron are present in the system. Manganous ions inhibited the oxidation of parathion by the peroxidase–hydrogen donor system at pH 4.7 and enhanced this oxidation at pH 6.7 (Table IV). Peroxidase is more active as an oxidase at pH 4.7. Peroxidase (Table V) catalyzed not only the oxidation of parathion, but also the hydrolysis of the phosphorothionate ester. The peroxidase–hydrogen donor system was capable of hydrolyzing para-oxon (Table V) when high levels of peroxidase were used.

Discussion

Peroxidase and the EDTA-Fe^{+2} complex were active in catalyzing the oxidation of parathion to para-oxon when supported by active hydrogen donors. The amount of parathion recovered as

para-oxon in either system was small. This is in agreement with in vivo studies with wheat plants administered parathion, where only small quantities of para-oxon were recovered based on cholinesterase inhibition studies (*5*). Schradan is converted to a more potent anticholinesterase agent by the EDTA-Fe^{+2}-ascorbic acid system (*8*), presumably through an initial oxidative attack at a nitrogen. The oxidation of thioether groups to their sulfinyl and sulfonyl derivatives is rapid in plants (*14*), while phosphorothionates and *N,N*-dimethyl phosphoramidates usually oxidize more slowly. It might then be anticipated that the peroxidase–hydrogen donor system would also oxidize the thioether grouping.

In addition to oxidation, peroxidase and the EDTA-Fe^{+2} complex catalyzed the hydrolysis of parathion and para-oxon. The large amount of hydrolysis products from parathion may explain the low para-oxon yields. The ratio of hydrolysis products to para-oxon formed has been found to be fairly constant for the peroxidase-catalyzed reactions. On the basis of this observation and the greater stability of para-oxon to hydrolysis by those oxidative systems, the thiono sulfur must play an important role in the hydrolysis reaction. In contrast, parathion is considerably more stable to hydrolysis by alkali than para-oxon. This has been explained on the basis of the lower electronegativity of sulfur than of oxygen. The approach of the hydroxyl ion for nonenzymatic hydrolysis depends on the relative electronegativities of the central phosphorus atom and of the attached substituents, on the degree to which the transition state is stabilized, and on steric hindrance by groups surrounding the phosphorus atom (*9*).

The results presented are consistent with the assumption of Buhler and Mason (*2*) that oxygen is activated as a free perhydroxyl radical by the peroxidase–hydrogen donor system. Free perhydroxyl radicals are formed by the reaction of molecular oxygen and the hydrogen donor (*2*) as distinguished from the EDTA-Fe^{+2}–ascorbic acid system in which free hydroxyl radicals are produced by the reaction of ascorbic acid with hydrogen peroxide generated in the reaction (*2, 18*). Free perhydroxyl or hydroxyl radicals may bring about the formation of an activated intermediate of parathion which rapidly decomposes by displacement of sulfur or of an ethoxy or *p*-nitrophenyl radical. Characterization of the hydrolysis products from parathion would establish the site of hydrolysis and aid in explaining how a free radical causes both oxidation and hydrolysis.

The results obtained with peroxidases in vitro suggest that peroxidases in plants may play a role in the metabolism of phosphorothionates in vivo.

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Received for review March 31, 1961. Accepted July 12, 1961. Approved for publication by the Director of the Wisconsin Agricultural Experiment Station. Study supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and by grants from the U. S. Atomic Energy Commission [Contract No. AT(11-1)-64, Project No. 14], U. S. Public Health Service, and National Institutes of Health (Contract RG-5304 and Regional Research Project NC-33).

GRAIN FUMIGANT DETERMINATION

Polarographic Determination of Methyl Bromide, Ethylene Dibromide, Acrylonitrile, Chloropicrin, and Carbon Tetrachloride in Air

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To expedite a factorial investigation (*7*) of the concept that wheat may behave as a chromatographic column toward fumigant gases (*3, 5*), polarographic methods were developed for the measurement of methyl bromide, ethylene dibromide, acrylonitrile, chloropicrin, and carbon tetrachloride in concentrations as low as 10^{-5} *M* with a precision within ±5.1% in air samples taken in duplicate, and within ±0.3% in aliquots of a given sample in solution. These fumigants were applied singly and in combination with carbon tetrachloride to the surface of $7^1/_2$-foot columns of wheat, following which the composition of the gas-air mixture that emerged from the bottom was determined as a function of time. This report presents details of the sampling and analytical methods that were used.

Aspects of Research Methods

In the initial stages of development of the methods, carbon tetrachloride was determined spectrophotometrically by the Ramsey method (*18*), employing the Fujiwara pyridine-alkali reaction. However, since the cumulative time required to fit several thousand samples of unknown concentrations to the relatively narrow range of optimum measurement (0.1 to 1.0 mg. of carbon tetrachloride) was considerable, the Ramsey method was replaced by the direct polarographic method of Kolthoff *et al.* (*13*), modified as described herein.

Methods for acrylonitrile (*8*) and chloropicrin (*11*) determination were