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INSECT RESISTANCE TO INSECTICIDES

The Enzymatic Degradation of Parathion in Organophosphate-Susceptible and -Resistant Houseflies

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The metabolic fate of parathion and diazinon, in one susceptible and two organophosphate-resistant strains of houseflies, was studied by chromatography and radioisotope techniques. The main interstrain difference resulted from the superior ability of the resistant strains to degrade parathion to diethyl phosphorothionate. The enzyme involved in the degradation process in the strains S and Ka was purified about 30 times by acetone powder formation, DEAE-fractionation, and ethanol precipitation. The partially purified enzyme preparations obtained from all three strains hydrolyzed parathion and diazinon to diethyl phosphorothionate, but their activity in hydrolyzing paraoxon was relatively low. The importance of these interstrain differences in relation to organophosphate resistance is discussed.

STUDIES on insecticide resistance during the past decade have elucidated several mechanisms of resistance in many insect species (*3*). Biochemical aspects of organophosphate-resistance (OP-resistance) were first studied in the housefly (*Musca domestica*) by March (*14*), who found the malathion-resistant Stauffer strain to degrade malaoxon in vitro more rapidly than the susceptible individuals. Similarly, Oppenoorth and van Asperen (*21*) showed the importance of biochemical degradation of paraoxon, diazinon, and malaoxon by demonstrating that, at relatively low concentrations, the speed of disappearance of these toxic phosphates was much faster in homogenates of resistant strains than that in the susceptible strain. They employed a fly head cholinesterase bioassay technique. These authors had already shown by genetic and other means that OP-resistance was very often associated with an abnormally low level of aliesterase activity in the housefly (*1*). They concluded that the mutant gene which caused the lower aliesterase level was also responsible for the organophosphate-breakdown enzymes, and that, in fact, the aliesterase became modified to a phosphatase (*21, 22*).

Meanwhile, Matsumura and Brown (*16*) studied a malathion-resistant strain of *Culex tarsalis*, which showed no cross-resistance to any organophosphorus compounds other than malathion and its analogs (e.g., malaoxon), and found that it showed a high carboxyesterase activity against malathion. The carboxyesterase found in the resistant strain resembled the carboxyesterase of the susceptible strain in its function and properties, but it was more abundant, so that the resistant mosquitoes had the advantage of hydrolyzing malathion much faster than the susceptible ones (*17*).

While the balance of evidence at present favors the view that the biochemical mechanisms of resistance, e.g., the elimination of the toxicants by acquired enzymes, is the most effective mechanism of OP-resistance in many insect species, the mechanism in certain instances may be complex. For instance, in the housefly, interstrain differences—i.e., the resistant strain *vs.* the susceptible strain—were also found in the rate of cuticular absorption (*13*) and behavioristic avoidance (*8*).

Extensive studies made by Mengle and Casida (*19*), and Krueger *et al.* (*13*), did not reveal important interstrain differences in vivo in the rate of insecticide breakdown, cuticular penetration, phosphorothionate oxidation, or disappearance rate of the actual toxicant phosphate. The main difference which appeared to be related to resistance was in the rate of cholinesterase inhibition which was modified by a "factor" in the thorax and/or abdomen. Since no one has found any indication that the cholinesterase of the resistant housefly is different from the susceptible one in any respect, the "factor" causing the difference in the inhibition rate must be sought elsewhere.

So far, the metabolic fate of these insecticides in housefly strains has been studied mainly in vivo, where a possible difference in the detoxication activity at the vital site of the insects may not always be apparent. Moreover, no attempt has been made to compare the qualitative aspects of organophosphate metabolism in resistant and susceptible houseflies. In the present study, an

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effort was made to elucidate the biochemical fate of parathion and diazinon in vivo and in vitro, and to study the nature of the hydrolysis products in the hope that certain qualitative or quantitative interstrain differences in metabolism could be discovered. Such differences, if any, might contribute to the low rate of cholinesterase inhibition found in the resistant housefly individuals in vivo during the organophosphate poisoning process.

Materials and Methods

The housefly strains used in this study have been described (*1*, *22*). The strains Ka and C are resistant to parathion and diazinon, and the strain S is susceptible to organophosphates.

P^{32}-parathion and diazinon were synthesized from 3 to 10 mc. of P^{32}-phosphoric acid by first exchanging with P_2S_5 (*4*, *12*), then refluxing with ethanol to obtain *O,O*-diethyl phosphorodithioate, which was chlorinated to an intermediate *O,O*-diethyl phosphorochloridothionate by sulfuryl chloride (*6*). From this, parathion and diazinon were synthesized according to the method described by Krueger *et al.* (*13*). Average specific activity and yields for parathion and diazinon were in the order of 1 to 7 mc. per gram, and 20 and 25%, respectively. Paraoxon was obtained from P^{32}-parathion by means of peracetic acid oxidation at 35° C. for 12 hours, or synthesized from $P^{32}OCl_3$ obtained from $H_3P^{32}O_4$ (*10*). The sodium salt of *O,O*-diethyl phosphorothionate was obtained from *O,O*-diethyl phosphorochloridothionate by first distilling under reduced pressure and then heating with sodium hydroxide. *O*-Ethyl, *O*-hydrogen, *O*-*p*-nitrophenyl thionophosphate—designated as desethyl parathion—was obtained by the method described by Dauterman *et al.* (*7*) by substituting parathion for dimethoate. The sodium salt of *O,O*-diethyl phosphate was kindly supplied by American Cyanamid Co., and the other reference compounds were purified from commercially available samples. All solvent-soluble organophosphorus compounds were purified on a Celite column (*2*), and the purity was checked by refractive index measurement, paper chromatography (*5*, *23*), housefly bioassy, and phosphorus analysis. Salts were tested by the same paper chromatographic techniques and by measuring their melting points. Propyl paraoxon, *O,O*-di-*n*-propyl, *O*-*p*-nitrophenyl phosphate was kindly supplied by Agrochemie, N.V., Arnhem, Netherlands.

In Vitro Studies. The housefly material was homogenized in an ice-cooled blender (*22*) for 1 minute at the highest speed at a concentration of 3 flies per ml. of 0.2% NaCl solution. To 10 ml. of this homogenate, 200 μg. of parathion or diazinon in 0.1 ml. of ethanol was added. The reaction mixture was then shaken for 1 hour at 23° ± 0.5° C., and the reaction was stopped by the addition of 1 ml. of 10% trichloroacetic acid. This treatment lowered the pH of the solution to approximately 2. The resulting solution was twice extracted with 10 ml. of chloroform to remove unmetabolized insecticide. The chloroform extracts were washed with 20 ml. of 0.067*M* phosphate buffer, pH 7. Radioactivity was measured in the original solution and the washings separately, but the values were added together. They represent the total water-soluble metabolite fraction.

In Vivo Studies. Six-hundred flies were transferred to a small glass jar, the inner surface (approx. 130 sq. cm.) of which had been treated with 1 mg. of P^{32}-parathion. They were kept for 2 hours at 20° C., and then transferred into another jar and washed with 20 ml. of acetone. The first jar was separately washed with 20 ml. of acetone to recover the unabsorbed insecticide. The acetone-washed flies were extracted with 20 ml. of hot water (approx. 80° C.) and with 20 ml. of cold water to recover cuticular residues. They were then homogenized and separated into a water-soluble metabolite fraction and a chloroform-soluble fraction as above. The latter contained parathion and paraoxon, which were separated by an alumina column. A rather large part of the radioactivity (ca. 26 to 30%) was found to be unextractable, i.e., it stayed with the tissue debris.

Metabolites Analysis. Qualitative analysis of the water-soluble metabolite fraction was performed by Dowex column chromatography and paper chromatography, as described by Plapp and Casida (*23*). Radioactivity of all liquid samples was measured by a Geiger-Müller tube of dipping type (Tracerlab TGC 5), using 10 ml. of total liquid fractions or 6 ml. of column eluate fractions. The paper chromatogram was cut first longitudinally (3 cm. in width), and then laterally into 30 pieces, each representing 0.033 of R_f. The pieces were radiometrically assessed by means of an end-window tube (Philips 18505).

Table I. Degradation of P^{32}-Parathion in Vivo by Strain S and Ka[a]

(Results as % of administered parathion equivalent recovered in the fraction indicated)[b]

Total Recovery, μg.	External Residues: Unabsorbed	External Residues: Insect surface	Cuticular Residues	Internal Residues: Unextractable	Internal Residues: Water-soluble	Internal Residues: Parathion	Internal Residues: Paraoxon
			S Strain				
951.3	11.76	28.00	13.98	26.32	13.11	1.58	0.38
942.4	6.32	28.99	14.82	27.86	13.22	2.64	0.39
924.3	4.30	27.13	13.56	30.01	14.37	2.66	0.40
			Ka Strain				
1001.4	3.20	27.59	18.42	26.63	21.99	2.08	0.23
1044.6	2.45	27.60	19.85	30.31	22.02	1.97	0.26
978.7	4.19	28.95	14.75	26.30	21.37	2.07	0.24

[a] All insects were in knockdown state.
[b] Contact method: 600 flies with 1000 μg. of parathion at 20° C. for 2 hours.

Results

Level of Resistance. By the contact method, the levels of parathion-resistance of strains Ka and C over S were in the order of five- to 10-fold and 38-fold, respectively. The LD_{50} values in μg. per jar were 2.5 to 5 for Ka; 20 for C; and 0.52 for S. Except for Ka, the values obtained are in agreement with those previously reported (*22*). The dosage-mortality curve for Ka was not so sharp as those for the other strains, the slope for 10 to 90% mortality in probit unit ranging from 0.63 to 10 μg. per jar. This probably means that the Ka strain has come to contain a number of relatively susceptible individuals during the 2 years of breeding.

Parathion Metabolism In Vivo. Distribution, degradation, and activation are shown in Table I. Removal of external and cuticular insecticide residues often reveals small internal interstrain differences otherwise masked by the bulk of these residues (*15*, *16*, *18*). The internal accumulation of paraoxon was slightly lower in strain Ka, possibly as a result of a higher production of water-soluble metabolites in this strain. The difference in degradation activity, i.e., between internal water-soluble metabolite fractions, was statistically significant at the level of $t = 0.01$. No significant difference was observed either in the rate of penetration or in the total uptake.

The greatest care should be exercised in interpreting these results. The applied dose of parathion was so high that both S and Ka flies were knocked down at the very beginning of the 2 hours of exposure, but possibly the condition of the Ka flies was better than that of the S flies, and the difference in degradation rate observed could be a consequence of resistance and not its cause. In vitro studies may, therefore, give more reliable results.

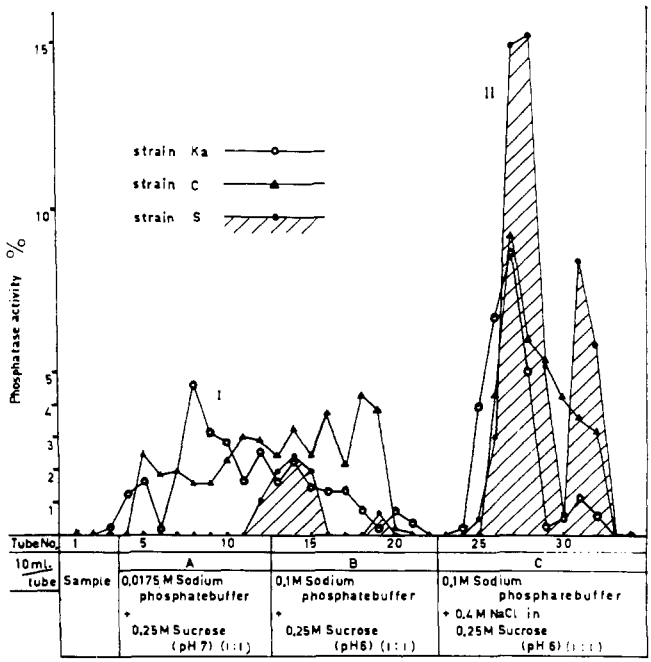

Figure 1. DEAE-cellulose column chromatographic separation of parathion-breakdown enzymes of the acetone powder extract preparations from one susceptible (S) and two resistant (Ka and C) strains of houseflies

Degradation of Parathion by Homogenates and Partially Purified Enzyme Preparations. Preliminary experiments showed the parathion-degrading capacity of Ka and C homogenates to be significantly higher than that of S homogenates. In particular, the presence of large amounts of tissue debris was thought to interfere with the accuracy of the measurement, and suggested the use of less concentrated homogenates and a partial purification of the enzymes responsible for the degrading activity in susceptible and resistant strains. A description of the purification follows.

Removal of the debris at a relatively low speed of centrifugation (500 × G, 1 minute) did not improve the situation, as a great part of the activity was retained in the precipitation. An acetone extraction method was, therefore, employed. The flies were homogenized in dry acetone at −15° C., and the powder, obtained after removal of the acetone by centrifugation, decantation, and reduced pressure above silica gel in a vacuum desiccator (all of which took 5 minutes and raised the temperature to about 0° C.), was suspended in an ice-cold solution containing 0.2% NaCl and 0.125*M* sucrose. After mixing in the same homogenizer at low speed for 10 minutes, the debris was precipitated by low-speed centrifugation (1550 × G, 5 minutes). More than 90% of the total activity was found in the supernatant solution, which was designated as the acetone powder extract.

The acetone powder extract was then subjected to DEAE-cellulose fractionation as follows. DEAE-cellulose [Serva Entwicklungslabor, Heidelberg, prepared as described (*17*)] was added at a ratio of 2.5 wet grams per 10 ml. of acetone powder extract. After vigorous shaking, the unabsorbed protein was separated from the cellulose by centrifuging at 3000 r.p.m. for 5 minutes. This fraction was designated as DEAE-fraction I. The precipitated cellulose was mixed with 2% NaCl in sucrose. A large amount of protein went into solution, and was again separated from the cellulose by centrifugation and designated as DEAE-fraction II. The remaining cellulose was simply suspended in 4% NaCl in sucrose and used as an enzyme preparation (DEAE-fraction III). Each fraction was adjusted to make an equal dilution as the starting concentration of the enzyme (30 fly equivalents in 10 ml.), and the enzyme activity was tested as described (Table II). The interstrain difference in breakdown activity was highest in DEAE-fraction I.

The DEAE-fraction I was further purified by ethanol precipitation. The fraction was cooled to 0° C., and ethanol added to make a 20% ethanol concentration. Precipitates were allowed to form over a period of 1 to 2 hours at 0° C. and then collected by centrifugation. The sediment was mixed with 0.2% NaCl in sucrose, and the ethanol concentration in the supernatant was increased to 30%. After standing overnight at −15° C., a second precipitate had formed and was similarly collected and dissolved in the same medium. The highest interstrain difference and most of the activity was found in the latter solution, i.e., the fraction containing the proteins precipitated between 20 and 30% ethanol concentrations (ethanol precipitate).

In addition to the DEAE-cellulose fractionation described above, the crude acetone powder extract (containing 2 grams of housefly material) was also subjected to DEAE-cellulose column chromatography. The method employed was essentially that of Matsumura and Brown (*17*), with stepwise elution as indicated in Figure 1, except that the eluent contained 0.125*M* of sucrose. The role of sucrose is not fully understood, but there are some indications that it protected the parathion-breakdown enzymes against denaturation and/or deactivation. Ten-milliliter portions of the eluate were collected in test tubes and used for the standard phosphatase assay. Figure 1 shows that there are at least two groups of phosphatases—the one which is eluted by a low salt concentration, peak I, probably identical with DEAE-fraction I; and a second peak which is eluted by higher salt concentrations, peak II, probably containing components of the DEAE-fraction II. In accordance with the earlier findings on DEAE-fractions, peak I activity was much higher in Ka and C preparations, whereas peak II activity was much higher in S preparations. The recovery of the total enzymic activity was rather poor (19 to 25%), indicating some denaturation of enzymes on the column, or presence of particular enzymes which could not pass the column. A similar chromatographic procedure was also applied to the ethanol-precipitate fraction of Ka and S, but in this case only 5 ml. of each 10-ml. eluate fraction was used for phosphatase assay. The separation pattern (Figure 2) was less complicated, as might be expected, since the applied samples were obtained from the DEAE-fractions I by ethanol pre-

Table II. Effect of Acetone Extraction and DEAE-Cellulose Fractionation[a] on Parathion-Breakdown Enzymes

Activities expressed as percentage of total parathion[b] applied.

Fraction	Strain S	Ka	C
Acetone powder extract	7.7	14.6	14.4
DEAE-fraction I	1.5	3.7	10.6
DEAE-fraction II	4.6	4.1	3.3
DEAE-fraction III	1.2	1.0	2.6
Debris from acetone powder extraction	0.6	1.1	0.9

[a] The sum of DEAE-fractions in Ka is considerably less than the activity of the acetone extract, possibly because of denaturation or inactivation of the enzymes during purification.
[b] 200 μg.

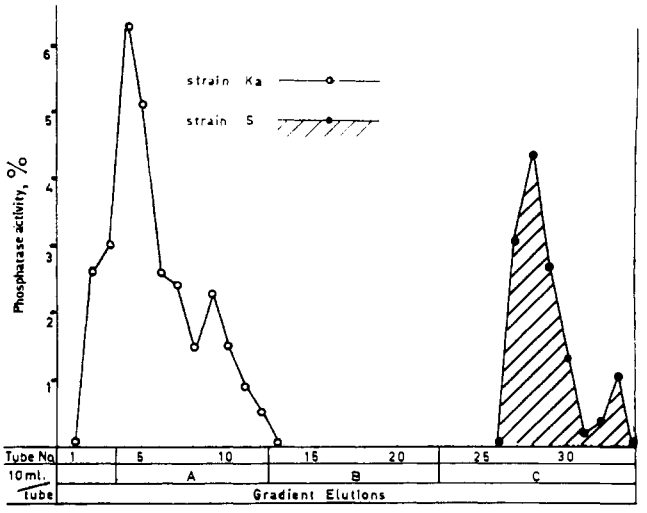

Figure 2. DEAE-cellulose column separation of partially purified, parathion-breakdown enzymes (ethanol precipitate fraction) of the susceptible (S) and the resistant (Ka) houseflies

Chromatographic conditions as in Figure 1

cipitation. The Ka sample showed only peak I, whereas the S sample showed no activity at this position but some of the peak II position.

Table III summarizes the results in the purification procedure. The values given for "DEAE-column" refer to peak I eluate for Ka and to peak II eluates for S. The average % yields of each step, calculated from the total phosphatase activity of the original homogenates, were 71, 28, 19, and 7 for S, and 64, 35, 21, and 6 for Ka. According to Table III, none of the purification steps led to a significant increase in the relative interstrain difference. However, Figures 1 and 2 clearly show that the Ka flies, and possibly C flies, contain a parathion-degrading enzyme which is absent, or at a very low level, in strain S, i.e., the DEAE-column peak I enzyme (s). It is difficult to decide which part of the total interstrain difference between the whole homogenates is due to this enzyme, because of the considerable loss of activity that occurred in the different steps of purification. If the resistant strains are compared to the susceptible strain, the results of DEAE-column chromatography on crude acetone powder extracts (Figure 1) indicate an increased peak I activity and a lowered peak II activity. This suggests that the peak I enzyme in the resistant strains could be produced at the cost of the peak II enzyme, originally present in the susceptible strain from which it was derived. However, this is speculation and more evidence would be required for confirmation.

Characterization of Breakdown Enzymes. No detailed studies on the effects of time and substrate concentration have been performed, but some experiments with crude homogenates indicated the production of metabolites to be roughly proportional to incubation time and substrate concentration, i.e., the per cent degradation is linear with time and independent of substrate concentration in the experimental range. It seems likely that the Km value of the reaction of breakdown enzyme with parathion is rather high, i.e., higher than $10^{-4}M$.

Table IV shows the results obtained with the "ethanol precipitate fraction" with diazinon and paraoxon as substrates. This preparation degraded diazinon slightly faster than parathion, and also hydrolyzed paraoxon, but at a much lower rate.

Oppenoorth and van Asperen (*21*, *22*), using crude homogenates of Ka and C flies, found that paraoxon and diazoxon at very low concentrations are rapidly degraded by an enzyme, hereafter designated as the "oxonase," which is strongly and irreversibly inhibited by low concentrations of *n*-propyl paraoxon. To determine whether the parathion- and diazinon-degrading enzyme found in this study (the "thionase") could be identical with the oxonase, the effect of *n*-propyl paraoxon on thionase activity was investigated. In these experiments, DEAE-fraction I was used, and *n*-propyl paraoxon was added immediately before the addition of the substrate, i.e., parathion. The rate of inhibition was studied by comparing the activity in the presence of inhibitor with that of a zero-inhibition control (untreated) and that of a complete inhibition control in which the enzyme had been destroyed by boiling. The results (Table V) show that the I_{50} of *n*-propyl paraoxon against the "thionase" activity is in the order of 10^{-5} to $10^{-6}M$ in all strains tested. Also, paraoxon at $10^{-5}M$ concentration exerts little or no inhibitory effect on the thionase activity of crude homogenates. Although the situation is rather complex, as probably several thionases are involved, there can be little doubt that the oxonase contributes very little to the parathion- and diazinon-degrading activity reported in the present study; thionase and oxonase activities are likely due to different enzymes.

The Nature of the Metabolites. Separation of the water-soluble metabolites produced in vivo was attempted

Table III. Partial Purification of Parathion-Degradation Enzyme

Fractions	Strain	Av. Enzyme[a] Activity per Unit, % Recov.	Protein per Unit, Mg.	Specific Activity, % Recov. per Mg.	Approximate Purification, -fold
Homogenate	S	7.34 ± 0.01	80.7 ± 4.7	0.091	1
	Ka	13.28 ± 0.81	84.3 ± 4.9	0.158	1
Acetone powder extract	S	5.18 ± 1.59	26.9 ± 4.6	0.193	2.1
	Ka	8.53 ± 1.41	28.3 ± 4.5	0.301	1.9
DEAE-fraction I	S	2.03 ± 0.38	8.3 ± 1.3	0.245	2.7
	Ka	4.59 ± 0.95	7.1 ± 0.6	0.646	4.1
Ethanol precipitate	S	1.42 ± 0.60	3.10 ± 0.93	0.458	5.0
	Ka	2.85 ± 1.20	2.16 ± 0.21	1.319	8.3
DEAE-column	S	0.55 ± 0.26	0.21 ± 0.04	2.619	28.8
	Ka	0.83 ± 0.53	0.15 ± 0.03	5.533	35.0

[a] Average of 5 runs, not including one experiment which failed to recover any enzyme activity through possible denaturation of the enzyme. One unit represents 30 flies equivalent protein per 10 ml. of preparation.

Table IV. Substrate Specificity of Partially Purified Parathion-Breakdown Enzyme[a]

Strain	Substrate[b] Diazinon	Substrate[b] Paraoxon
S	2.49	0.57
Ka	5.20	0.92
C	5.83	0.92

[a] Experiments performed on the "ethanol precipitate" fraction.

[b] Diazinon substrate concentration 1 mg. per unit. Paraoxon substrate concentration 50 μg. per unit. The results are expressed in % recovery as metabolites.

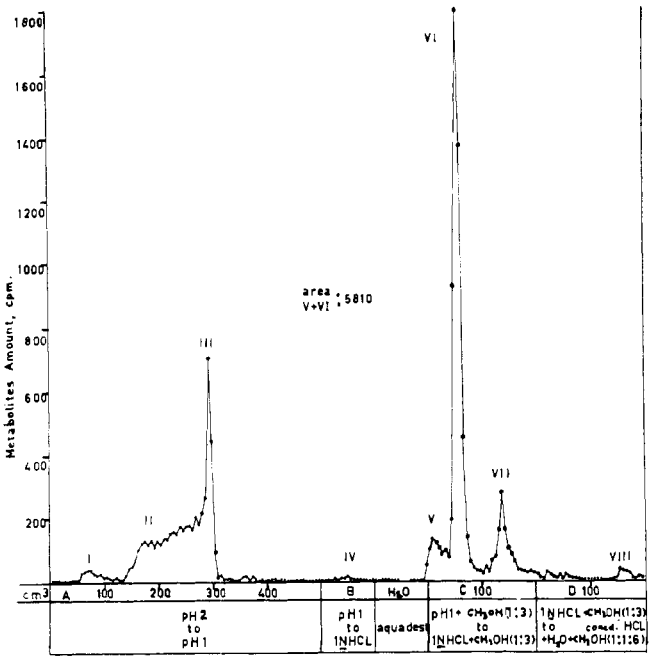

Figure 3. Dowex column chromatographic separation of in vivo parathion metabolites of the resistant houseflies (Ka)

Peaks V and VI represent O,O-diethylphosphorothionate (see text)

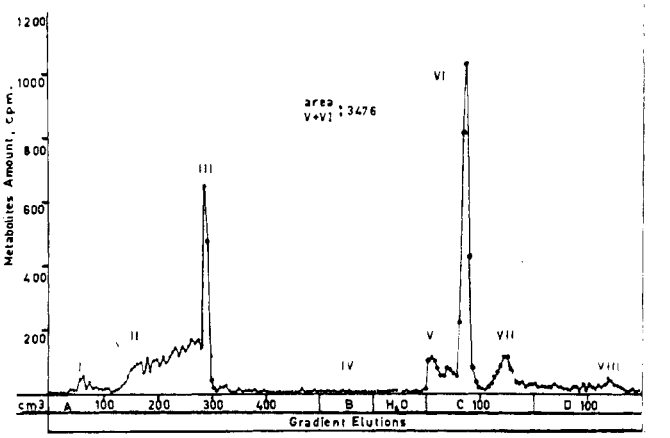

Figure 4. Dowex column separation of in vivo parathion metabolites of the susceptible houseflies (S)

Conditions are same as Figure 3; area under peaks V and VI was calculated and indicates the interstrain difference in the amount of this metabolite (see Figure 3 for comparison)

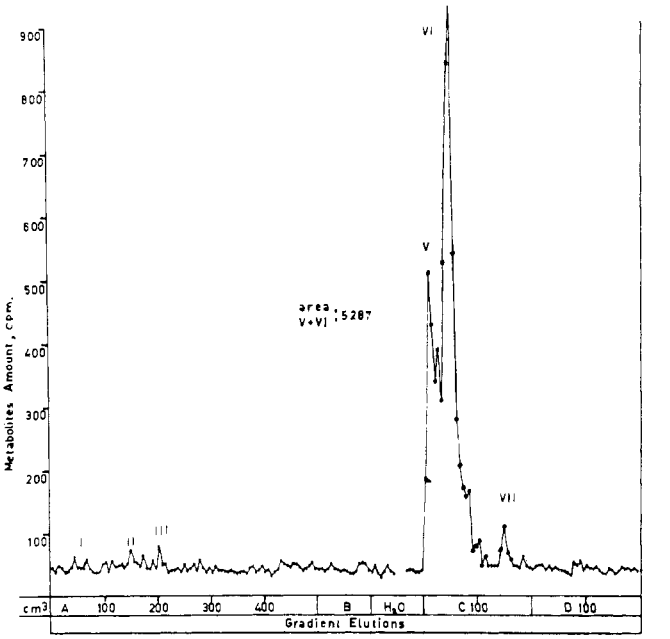

Figure 5. Dowex column separation of in vitro parathion metabolites of the resistant houseflies (Ka)

Conditions are same as in Figure 3

Table V. Inhibition[a] of DEAE-Fraction I Enzymes by *n*-Propyl Paraoxon

Expressed as percentage of original activity

	Molar Concentration of Inhibitor				
Strain	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
S	0	45	75	100	100
Ka	0	11	61	75	100
C	0	44	100	100	100

[a] Average of one to three determinations. Inhibitor was added immediately before enzyme assay.

by Dowex column chromatography (Figures 3 and 4). At least 6 peaks were found in both S and Ka experiments, and reference compounds were used for identification. Peaks I, II, and III represent paraoxon metabolites (phosphoric acid, monoethyl phosphate, and diethyl phosphate, respectively). Peak IV represents thionophosphoric acid, though the actual amount recovered in the present experiments was very small. The peaks V and VI were identical with those obtained with diethyl phosphorothionate. Probably peak V is due to degradation (or isomerization) of diethyl phosphorothionate under the strongly acid conditions during storage and on the column, and consists of monoethyl phosphorothionate, thionophosphoric acid, and possible isomers—e.g., diethyl phosphorothiolate. The most significant interstrain difference was found in the amount of diethyl phosphorothionate produced (peak VI), whereas the quantities of paraoxon metabolites produced by S and Ka flies did not differ significantly.

The water-soluble products from in vitro experiments were analyzed similarly. The elution patterns (Figures 5 and 6) were much simpler; only two peaks (V and VI), again representing diethyl phosphorothionate and possible artificial degradation products, were found. The absence of the paraoxon metabolites is quite understandable, as conversion of parathion into paraoxon is not expected to take place under the in vitro experimental conditions. The quantitative differences are reflected by the areas covered by peaks V and VI, as indicated in the figures.

The in vitro degradation products, after concentration, were also analyzed by paper chromatography. The results indicated that thionophosphoric acid or monoethyl phosphorothionate, which should run at low R_f (0.07 and 0.33, respectively), do not occur in appreciable amounts and that diethyl phosphorothionate must be the main degradation product.

Dowex column chromatography of a freshly prepared sample, using less acid eluents than described in the *Methods* section, revealed a single peak for the water-soluble metabolites produced from diazinon by the ethanol precipitate fraction of Ka. This cochromatographed exactly with diethyl phosphorothionate as the main degradation product of both parathion and diazinon under in vitro conditions.

Discussion

Both in vivo and in vitro, the OP-resistant strains Ka and C have a significantly higher activity, in degrading

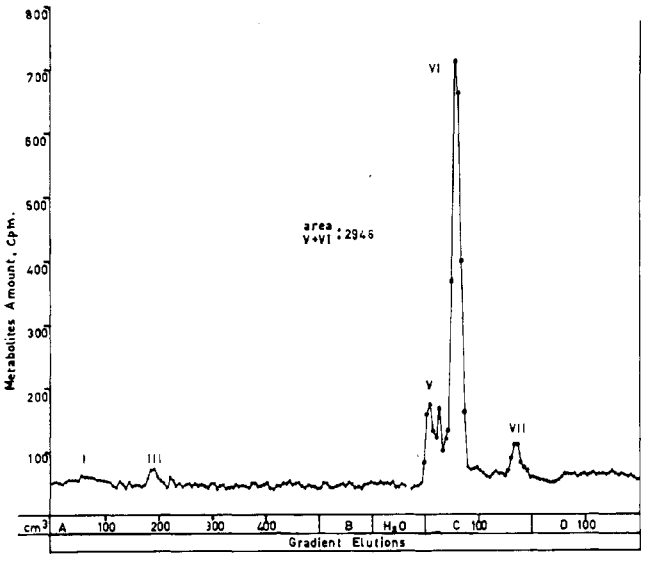

Figure 6. Dowex column of in vitro parathion metabolites of the susceptible house flies (S)

Conditions are same as in Figure 3

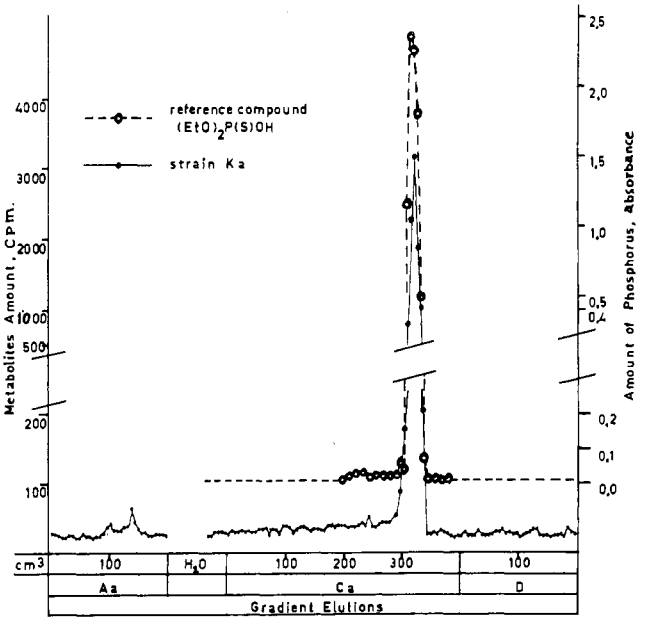

Figure 7. An improved Dowex column separation of in vitro diazinon metabolites of the partially purified enzyme preparation (ethanol precipitate fraction) of the resistant houseflies (Ka)

To avoid the secondary breakdown of the metabolites during the chromatographic separation, the gradient elution program was modified as follows—Aa: pH 2 (100 ml.) to pH 1 (100 ml.); Ca: pH 2, CH_3OH (1:3) (350 ml.) to 1N HCl-CH_3OH (1:3) (100 ml.)

parathion and diazinon to diethyl phosphorothionate, than the susceptible strain S. The organophosphate resistance in these strains may be at least partly due to this difference in these phosphorothionate-degrading activities.

However, difficulties arise if this conclusion is compared to that arrived at by Oppenoorth and van Asperen (*1, 21, 22*), who demonstrated that OP-resistance in the strains Ka and C is due to the activity of paraoxon- and diazoxon-degrading enzymes produced by so-called low aliesterase genes. As resistance in these strains has further been shown to be largely caused by a single genetic factor (*20, 22*), it is difficult to reconcile these two conflicting opinions. The strikingly different sensitivity to *n*-propyl paraoxon of the thionases demonstrated in this paper, and the oxonase found and studied by Oppenoorth and van Asperen, exclude the possibility that these enzymes are identical. The synergistic effects obtained with *n*-propyl paraoxon in diazinon poisoning certainly favor the oxonase-theory. Yet, the results reported in this paper make it difficult to neglect the difference in thionase activity as a cause of resistance. A tentative explanation may be that both mechanisms of resistance do occur. If monofactorial inheritance of the resistance is assumed, they should be controlled by the same chromosomal locus. However, the statement of monogenetic control of resistance must be interpreted carefully. It could, in fact, mean that the resistance is mainly due to a single factor, but that one or more quantitatively less important factors are also acting.

Diethyl phosphorothionate has been found to be the main degradation product in both susceptible and resistant strains. This suggests a thionophosphorylation of the degradation enzyme followed by a dethionophosphorylation, a reaction sequence which is comparable to that occurring in the reaction of paraoxon with the A-esterase of rabbit serum (*9*). In those cases where subsequent dephosphorylation does not occur, as when the organophosphates act as inhibitors, thionophosphorylation occurs far more slowly than phosphorylation, so that phosphorothionates are poor inhibitors. Indeed, rabbit plasma paraoxonase has negligible activity against parathion (*11*). In the purified thionase reported here, by contrast, activity is far greater against parathion than against paraoxon, suggesting a rather radical difference in reaction mechanisms as compared with other hydrolases.

A further question is how many phosphorothionate-degrading enzymes are involved. The DEAE-cellulose chromatographic separation reveals the occurrence of at least two enzymes, or more probably two groups of enzymes, one prevailing in the susceptible stain S (group II) and one prevailing in the resistant strains Ka and C (group I). This could be explained by assuming one single group I enzyme in Ka and C as a consequence of gene mutation. However, the available evidence does not allow any definite conclusion.

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PLANT METABOLISM OF INSECTICIDES

Identification of Metabolites of Zectran Insecticide in Broccoli

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The metabolism of Zectran in broccoli is reported. In addition to a small amount of Zectran, the following metabolites were found to be present in the broccoli flower: 4-dimethylamino-3,5-xylenol, 2,6-dimethylhydroquinone, 2,6-dimethyl-p-benzoquinone, and 4-dimethylamino-3,5-dimethyl-o-benzoquinone. The xylenol was found in both free and conjugated form, but the hydroquinone derivative was detected only as a water-soluble conjugate. Evidence is presented which suggests that metabolites of this pesticide have been incorporated into lignin.

ZECTRAN (registered trademark of The Dow Chemical Co.), 4-dimethylamino-3,5-xylyl methylcarbamate, is a highly active material for control of a large number of arthropod pests of plants (*5, 6, 8, 10, 12, 14*). It is toxic also to mollusks including some of the economically important plant-eating slugs and snails (*7*). Its potential use on food crops necessitated a study of its metabolism in plants.

Very little is known concerning the actual identity of carbamate degradation products in plants (*4*). The purpose of this investigation is to identify the metabolites formed when broccoli is treated with Zectran.

Materials and Methods

Application of Zectran. Green sprouting broccoli was grown from seed in a sandy loam–sponge rock (1:1 v./v.) soil mixture in 3-gallon cans with drainage. After 63 days, at the appearance of the first flowers, Zectran insecticide was applied to the plant by dissolving 4 mg. of 4-dimethylamino-3,5-xylyl-α^3-3-C^{14}_2-methylcarbamate in 50 μl. of corn oil and streaking the stem immediately below a flower cluster. After 10 days, the broccoli was harvested.

Counting Procedure. All fractions were counted with an end-window Geiger-Müller tube at infinite thickness for 1024 counts. All of the count rates in this report are in gram-counts per minute. This unit is arrived at by multiplying the net count rate of an infinitely thick sample taken from any given fraction by the total weight in grams of that fraction. This is an arbitrary measure of the total activity in each fraction and served to determine the distribution of radioactivity.

Chromatography. Crude extracts were purified on large-scale paper chromatograms (Whatman No. 3 MM) since, in most cases, the radioactive metabolites were associated with large amounts of interfering material, and initial chromatograms did not usually give well defined bands. Whatman No. 1 paper was used in all other cases. In all identification work, the known compound was run simultaneously with the radioactive metabolite. R_f values and solvent systems used are given in Table I.

Fractionation Procedure. To assess

Table I. R_f Values of Metabolites Found in Broccoli

Metabolites	Solvent System[a] I	II	III	IV	V	VI	VII
4-Dimethylamino-3,5-xylyl methylcarbamate	0.72	0	...	0.78	...	...	...
4-Dimethylamino-3,5-xylenol	0.36	0	...	0.47	0.19	...	...
2,6-Dimethylhydroquinone	...	0.21	...	...	0.53	...	...
2,6-Dimethyl-*p*-benzoquinone	...	0.74	0.97	0.92	0.90	...	...
4-Dimethylamino-3,5-dimethyl-*o*-benzoquinone	0.92	0	0.40	0.97	...	...	...
2,6-Dimethyl-*p*-benzoquinone-2,4-dinitrophenylhydrazone	...	...	...	...	..	0.12	0.19
4-Dimethylamino-3,5-dimethyl-*o*-benzoquinone-2,4-dinitrophenylhydrazone	...	...	...	...	...	0.06	...

[a] I: Isoamyl alcohol–formic acid–water (12:1:7 v./v.). II: Benzene saturated with formic acid. III: Benzene–acetic acid–water (1:2:1 v./v.). IV: Chloroform–acetic acid–water (2:2:1 v./v.). V: Methanol–water (1:1 v./v.) saturated with dibutylphthalate as the mobile phase and dibutylphthalate as the stationary phase. VI: *n*-Heptane saturated with dimethylformamide as the mobile phase and dimethylformamide as the stationary phase. VII: *n*-Heptane saturated with methanol as the mobile phase and methanol as the stationary phase.