Biochemistry of Resistance to Paraoxon in Strains of Houseflies

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The *in vivo* degradation of injected ^{3}H-paraoxon in one susceptible and two resistant strains of houseflies was examined. At a low dose, small differences were found in the time course of degradation, attributable to increased desarylation in the resistant strains. An unexpected observation was that desethylation was a major pathway in the susceptible strain. Studies on concentration-dependence of degradation showed approximately three-fold differences between degrading ability of susceptible and resistant strains under saturating conditions. These differences were considered adequate to account for the observed resistance.

The two factors most frequently postulated to account for the resistance of houseflies to organophosphates are differences in penetration and in metabolism. Several reports have shown that the acetylcholinesterase of susceptible and resistant strains do not differ (Bigley and Plapp, 1960; Forgash *et al.*, 1962; van Asperen and Oppenoorth, 1959) but Mengle and Casida (1960) suggested that resistant flies may contain a factor which protects the enzyme. Of particular interest is the suggestion of van Asperen and Oppenoorth (1959, 1960), that resistant flies contain a "mutant aliesterase" which acts as a phosphatase. But the work was conducted with insect homogenates and the amount of inhibitor lost was small compared with the actual dose of insecticide normally tolerated by the resistant strains. Also the evidence for degradation of the inhibitor was not demonstrated, as the loss of inhibitor was followed by loss of anticholinesterase activity (O'Brien, 1967). Three other research groups were unable to demonstrate substantial metabolic differences in degradation of toxicant when resistant and susceptible strains were compared *in vivo* (Forgash *et al.*, 1962; Krueger *et al.*, 1960; Mengle and Casida, 1960).

When several factors may be involved, such as activation, degradation, and penetration, interactions make it very difficult to establish the role of any one factor (Farnham *et al.*, 1965; Hollingsworth *et al.*, 1967; Matsumura and Hogendijk, 1964). We have avoided this problem by working with paraoxon, which requires no activation, and injecting it to avoid penetration effects. Some metabolic studies have yielded poor recoveries of applied toxicant, and small differences between strains are then of uncertain significance (Plapp *et al.*, 1961); we have therefore sought high recoveries as a first requirement for this study. Finally, we consider it essential to work with intact organisms; to compare strains under identical metabolic conditions, *i.e.*, with identical doses, and with a dose that does not give large mortalities in the susceptible strain; and to establish whether toxicant loss is due to degradation or to binding.

The penalties for meeting all the above conditions are that doses must be small, and therefore highly radioactive compounds are needed; and the ratio of toxicities for susceptible and resistant strains are very small when one injects compounds which do not require activation.

Materials and Methods. CHEMICALS. Paraoxon and ^{3}H-paraoxon were synthesized in this laboratory. The nonradioactive sample was prepared by reacting equimolar quantities of *p*-nitrophenol, sodium carbonate, and diethyl phosphorochloridate (Dauterman and O'Brien, 1964). After the reaction, the excess sodium chloride was removed by filtration and the mixture was washed three times with very dilute NaOH (pH 8), until only a pale yellow aqueous phase was obtained, and 12 times with distilled water to remove the last traces of sodium chloride and the yellow color from the solution. After removal of the organic solvent, the purity of the compound was established by IR spectra, thin-layer chromatography, and elemental analysis.

Analysis: $C_{10}H_{14}NO_6P$; calculated: C, 43.66; H, 5.09; N, 5.09; P, 11.26. Found: C, 42.7; H, 5.2; N, 5.4; P, 10.4.

^{3}H-Paraoxon was synthesized by condensing ^{3}H-ethanol (400 mc per mmole) with *p*-nitrophenyl phosphorodichloridate, using the method described by Kojima and O'Brien (1968). In this investigation, however, a higher yield of a pure product was obtained by using stoichiometric quantities of the two starting materials and adding pyridine as a catalyst to remove the hydrogen halide from the reaction. A 54% yield of ^{3}H-paraoxon was obtained having a specific activity of 18 mc per mole. The purity of the compound was checked by thin-layer chromatography, using a Packard Model 7201 radio chromatogram scanner, immediately after preparation and at frequent intervals throughout the investigation.

Desethyl paraoxon was synthesized by cleavage of the alkyl bond with sodium iodide (Cremlyn *et al.*, 1958; Hollingworth, 1966). A 20% yield of the sodium salt of the desethylated compound was obtained.

Analysis: $C_8H_9NO_6PNa$; calculated: P, 11.5; C, 35.7; H, 3.39. Found: P, 12.1; C, 35.9; H, 3.8.

Diethyl phosphate was obtained from Eastman Organic Chemicals. Ethyl phosphate was prepared by neutralizing the barium salt with the calculated quantity of 1*N* H_2SO_4. Final yield of this compound was 76% and the purity was checked by phosphorus analysis and paper chromatography.

Analysis: $C_2H_6O_4P$; calculated: P, 24.6. Found: P, 24.3.

Insects. Three housefly strains were used, the organophosphorus resistant SKA and Ru strains whose origin have been described (Forgash, 1967; Sawicki and Green, 1964), and the Wilson susceptible strain maintained free of insecticide pressure in this laboratory. Resistance was maintained by treating each alternate generation of the Ru strain and every generation of the SKA strain with diazinon using the method described by Sawicki and Farnham (1964).

Inhibition of Acetylcholinesterase. A comparison of the initial rate of inhibition of the target enzyme was made using a similar manometric technique to that described by Mengle and Casida (1960). The enzyme source was prepared by homogenizing 3-day-old female houseflies, 1.5 flies per ml, in

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Table I. The Susceptibility of the Wilson (susceptible), Ru (resistant), and SKA (resistant) Strains

Strain	Compound	Application Method	LD_{50} μg/g at 24 hr	Limits defined by ± SE	Slope	Resistance Factor
Wilson	Paraoxon	Topical	2.32	1.49–3.66	5.1	...
Ru	Paraoxon	Topical	9.75	6.76–14.1	6.3	4
Wilson	^{3}H-Paraoxon	Injection	0.83	0.61–1.55	4.9	...
Ru	^{3}H-Paraoxon	Injection	1.40	0.81–2.48	4.2	1.7
SKA	^{3}H-Paraoxon	Injection	1.30	0.74–2.20	4.3	1.6
Wilson	Malaoxon	Topical	7.6	3.88–14.9	3.4	...
Ru	Malaoxon	Topical	57.6	37.2 –89.4	5.2	8
Wilson	Malaoxon	Injection	0.64	0.38–1.09	4.3	...
Ru	Malaoxon	Injection	1.38	1.07–1.78	8.9	2

bicarbonate buffer pH 8.5, 0.025*M*, containing 0.04*M* $MgCl_2$, and 0.15*M* NaCl. The homogenate was filtered through a double layer of cheesecloth. Enzyme and inhibitor were incubated for time periods extending from 0 to 20 min before the reaction was stopped by tipping in acetylcholine bromide. Final concentrations during the inhibition phase were $5 \times 10^{-8}M$ for paraoxon and $5 \times 10^{-9}M$ for malaoxon. Results were calculated by the linear regression method of Aldridge, Berry, and Davies (1949). The inhibition of cholinesterase *in vitro* was also examined by attempting to determine the I_{50} value for the inhibition of acetylcholinesterase in whole body homogenates, using a series of concentrations of malaoxon and paraoxon. The enzyme was prepared in this case by homogenizing 3-day-old female houseflies, 5 flies per ml, in phosphate buffer pH 7.2, 0.067*M*. One milliliter of the enzyme preparation was added to a tube containing an equal volume of inhibitor. After 10 min incubation at 25° C, the reaction was terminated by adding 3 ml of acetylcholine bromide $4 \times 10^{-3}M$. After 30 min the remaining acetylcholine was assayed (Hestrin, 1949).

Bioassay. Topical application tests were performed by applying a 2-μl drop of insecticide dissolved in analytical grade acetone to the mesonotum of the insect. Injection experiments were conducted by inserting a 30-gauge needle, mounted on an Agla micrometer syringe, into the thorax. The stock solutions of the inhibitor ($10^{-2}M$ in acetone) were diluted with phosphate buffer pH 7.2, 0.1*M*, to the appropriate concentration. The maximum concentration of acetone never exceeded 3% in the test solution. In all bioassay experiments at least five concentrations were tested, as well as control samples. In preliminary tests, 25 to 30 flies were used per concentration, this number being extended to 40 to 50 flies per concentration in all later experiments, which included bioassay tests conducted in conjunction with biochemical experiments. Results were analyzed by an IBM 360/65 computer (Dixon, 1967).

Paraoxon Degradation. The time course of degradation was followed by injecting 50 houseflies and homogenizing at times up to 90 min. They were injected in groups of 50 for long time periods, groups of 5 for 7–30 min, and of 2 for lesser periods. A constant dose (0.63 μg/g) equivalent to the LD_5 value for the susceptible strain was used in the treatment of all flies. Labeled compounds were extracted by homogenizing the insects in acetonitrile, then in water, and repeating the process with a final homogenization in an acetone methanol mixture (1:1). The method used was essentially similar to that described by Hollingworth *et al.* (1967), although reduced volumes were necessary in this investigation because of the low dose rates. The water was first used to wash out the glass containers and cheesecloth tops. Most of the organic solvent was removed by gentle evaporation and the resulting aqueous solution partitioned with either benzene or chloroform, both of which had been shown to extract greater than 90% of labeled paraoxon added to an homogenate. The residue was digested (Eldefrawi, 1966) to account for labeled compound bound to protein.

Samples obtained as the aqueous extract 90 min after injection were examined to determine qualitative differences in the metabolic pathways for resistant and susceptible strains.

Metabolite Identification. Separation of the polar metabolites was achieved using an ion exchange technique (Plapp and Casida, 1958). Twenty-milliliter fractions were eluted from the column and 2-ml aliquots were counted with 18 ml of a counting solution (Bray, 1960), using a Packard Tricarb liquid scintillation counter Model 3375. Peaks were identified by including in separate experiments known quantities of nonradioactive candidate metabolites. Each fraction was then analyzed for total phosphorus by the method of Rockstein and Herron (1951).

RESULTS

Resistance Level. Despite constant selection with diazinon, the degree of resistance to injected paraoxon remained at a low level (Table I). The values obtained for the Ru strain were in agreement with those previously reported (Mengle and Casida, 1960). However, the level of resistance shown by the SKA strain was lower than the value of seven-fold reported by El Basheir (1965). Bioassay tests were conducted in conjunction with all biochemical experiments.

A constant level of resistance was maintained by the SKA strain to injected paraoxon for the 12 generations during which the experiments described here were conducted. The level of resistance to injected paraoxon shown by the Ru strain varied from a three-fold factor in early experiments to a level of 1.7 after 60 generations.

Comparison of Acetylcholinesterase. The total cholinesterase activity was the same in the Ru and Wilson strains; the figure calculated for the Ru strain with standard deviation was 258 ± 8μl CO_2 per hr and for the Wilson strain 264 ± 14.

The manometric technique showed no significant difference in initial rate of cholinesterase inhibition by malaoxon and paraoxon in these two strains (Figures 1 and 2) in contrast to the previous report by Mengle and Casida (1960). One of the resistant strains used, Ru, was the same in both investigations and showed a comparable level of resistance to the same organophosphates. Bimolecular reaction constants were calculated from these manometric results (Aldridge, 1950)

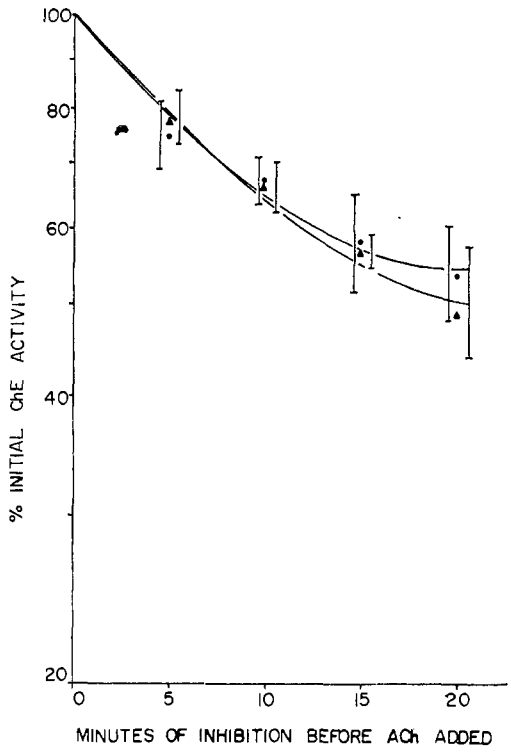

Figure 1. Rate of inhibition of acetylcholinesterase from whole housefly homogenates by malaoxon 5 × $10^{-9}M$ at 25° C. Mean of four experiments showing standard deviations for each point. ● Susceptible strain ▲ Resistant strain

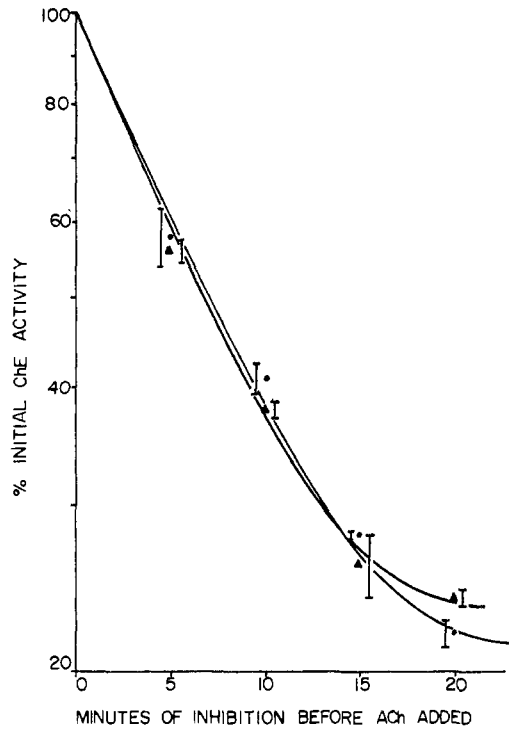

Figure 2. Rate of inhibition of acetylcholinesterase from whole housefly homogenates by paraoxon 5 × $10^{-8}M$ at 25° C. Mean of three experiments showing standard deviations for each point. ● Susceptible strain ▲ Resistant strain

Table II. Bimolecular Reaction Constants (k_i) for Cholinesterases from Susceptible Wilson and Resistant Ru Strains

All Figures Calculated from Inhibition Observed at 10 Min

Compound	Strain	k_i min^{-1} liter $moles^{-1}$	Technique
Paraoxon	Ru	2.31×10^6	manometric
	Wilson	2.19×10^6	manometric
Malaoxon	Ru	8.1×10^6	manometric
	Wilson	7.8×10^6	manometric
	Ru	1.54×10^6	colorimetric
	Wilson	1.62×10^6	colorimetric

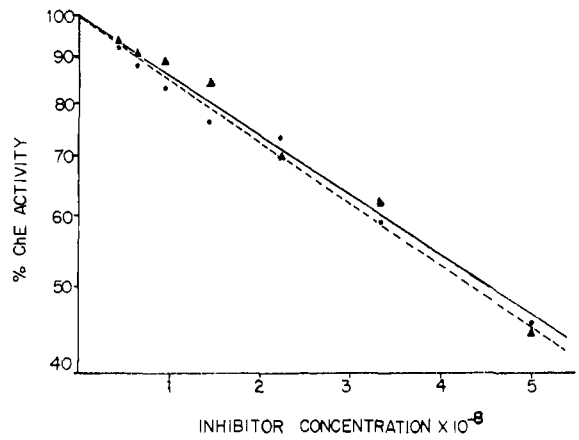

Figure 3. *In vitro* inhibition of acetylcholinesterase from whole housefly homogenates by malaoxon in 10 min at 25° C. ● Susceptible strain ▲ Resistant strain

and also for malaoxon from the colorimetric method (Table II). The two methods do not give identical values and the difference is probably due to the fact that a final concentration of 1.33 flies per ml was used during the inhibition phase in the manometric technique as compared to 2.5 flies per ml in the colorimetric technique. Although the expected straight line relationship between inhibitor concentration and log of acetylcholinesterase activity was obtained when malaoxon was used as the inhibitor (Figure 3), an anomalous result was obtained when paraoxon was used in the colorimetric assay method (Figure 4).

The difference between the two methods, and also the fact that with paraoxon the colorimetric date (Figure 4) are different for head and whole body, is probably due to the existence in the trunk of (a) large amounts of aliesterase (van Asperen, 1959) whose presence has been shown by van Asperen and Oppenoorth (1960) to protect acetylcholinesterase against inhibition (b) phosphatases, whose existence is discussed below, and (c) a variety of proteins which bind paraoxon nonspecifically. Although housefly cholinesterase is less susceptible to inhibition by paraoxon than aliesterase, leading to the protective effect discussed above, Bigley and Plapp (1969) showed the relative susceptibility of these two enzymes was reversed when malathion was used as an inhibitor. Despite these complications, this colorimetric technique, when used for malaoxon, confirmed the manometric result. Differences in the rate of inhibition of the target enzyme do not appear to contribute to organophosphorus resistance in this species. Nevertheless, one cannot yet eliminate the possibility that some subfraction of the enzyme is of particular importance, yet contributes little to the total which is measured.

Recovery of Applied Dose. Recoveries obtained from the supernatant and residual fractions using the extraction and digestion process described are shown in Table III. The results

Table III. Percentage of Applied Radioactivity Recovered Following Injection

Time after Injection Min	Recovery in Supernatant	Recovery in Digested Residue	Total Recovery
0	92	4	96
5	94	4	98
10	93	8	101
15	95	8	103
20	79	11	90
25	90	4	94
45	85	14	99
60	80	18	98
90	86	11	97

represent the means of two experiments conducted with the susceptible strain. In subsequent experiments on the time course of the degradation reaction, results were discarded if recovery of applied dose in the supernatant did not exceed 75%. Although eight solvent systems were investigated for use in this extraction technique, only the one quoted gave consistent high recoveries of applied radioactivity.

Time Dependence of Degradation. The comparison between the Wilson and Ru strain (Figure 5) was conducted over a period of 90 min because it was not realized before the experiment that both strains possessed the ability to degrade the low dose used very rapidly. A similar comparison was made for the Wilson and SKA strain with a more detailed examination of the period between 0 time and 15 min (Figure 6). The resistant strains are moderately more efficient at degrading the inhibitor than the susceptible strain. After 10 min the susceptible strain had reduced the level of parent compound to 25% of the applied dose, whereas the level in Ru and SKA strains had dropped to 17.5% and 16% in the same time. However, it would not be correct to assume that the slightly superior degradation rate would be sufficient to explain even the twofold level of resistance to this injected compound. There is some indication from these results that the nature of the enzymic reaction may be different in the two strains. The initial elimination of the parent compound to 30% of its original value occurred in approximately 5 min in the Wilson and Ru strains, but only half this time in the SKA strain. However, once this level of parent compound had been reached, the Ru flies appeared to degrade the inhibitor compound more rapidly than either of the other strains. The apparent two-phase nature of the elimination reaction shown by all three strains is probably due to an initial rapid loss of parent compound caused by a combination of enzymic degradation and adsorption of the parent compound by other protein molecules. The difficulty experienced in recovering the injected radioactive compound both in this investigation and in many other reports would support this conclusion.

Concentration Dependence of Degradation. One is experimentally forced to compare strains under conditions which are essentially nonlethal for the susceptible strain. Yet the metabolic differences in strains might well be substantial only at very high toxicant levels; *i.e.*, the ability to degrade a low dose might be similar, yet the ability to degrade a high dose (lethal to susceptibles) might be different. Since one cannot examine this point directly, we attempted to extrapolate to saturating conditions by using the Lineweaver-Burk treatment, designed to extrapolate to saturating conditions in single-enzyme systems. Various doses were applied to 50 insects and the loss of parent compound was measured at

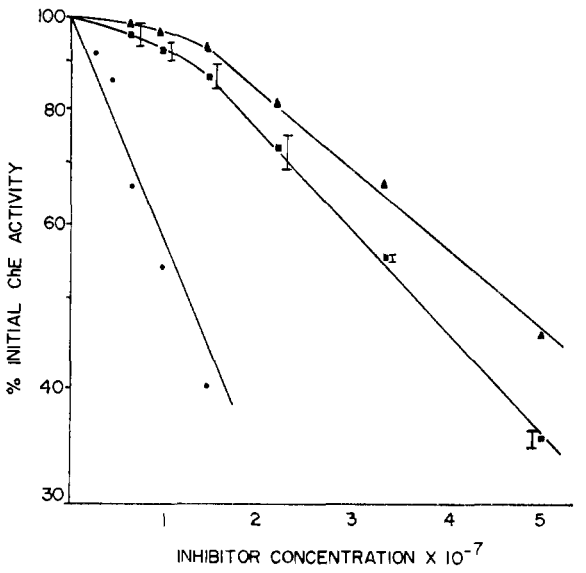

Figure 4. *In vitro* **inhibition of acetylcholinesterase from housefly homogenates by paraoxon in 10 min at 25° C. Mean of three replications with standard deviation ▲ Resistant strain ■ Susceptible strain ● Susceptible strain–head homogenate**

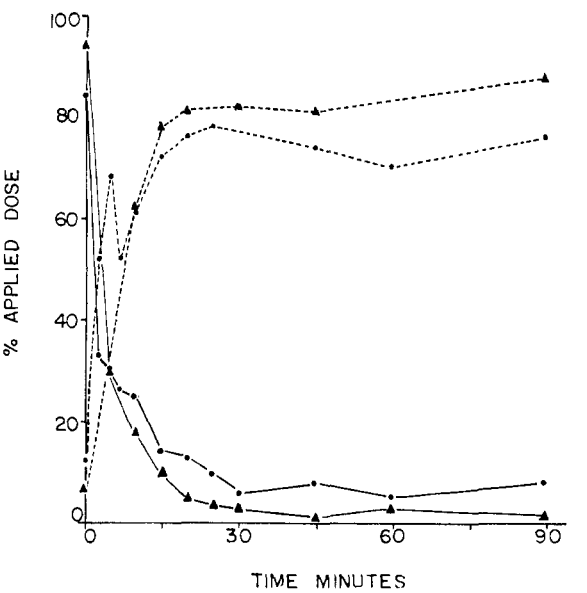

Figure 5. Time course of the enzymic degradation of ^{3}H-paraoxon by Wilson and Ru strains. Except for the 3- and 7-min readings which were taken from one experiment only, all other points for the susceptible strain were derived from the mean of three experiments. The data for the resistant Ru strain were from two experiments. ● Susceptible strain ▲ Resistant strain, ——Parent compound, - - -Metabolite fraction

10 min (Figure 7). Surprisingly enough, this complex degradation system *in vivo* showed a relation between dose and degradation of the same form as that observed in single-enzyme systems.

From the figures were computed values of V_{max} and Km, as in enzyme studies, by an IBM 360/65 computer version of the weighted regression technique of Wilkinson (1961). The use of the term Km does not imply that we believe we are necessarily measuring a single parameter for some one enzyme; it is used purely by analogy, and could involve contributions from various sources. The V_{max} is the more important parameter, and indicates the maximal potential

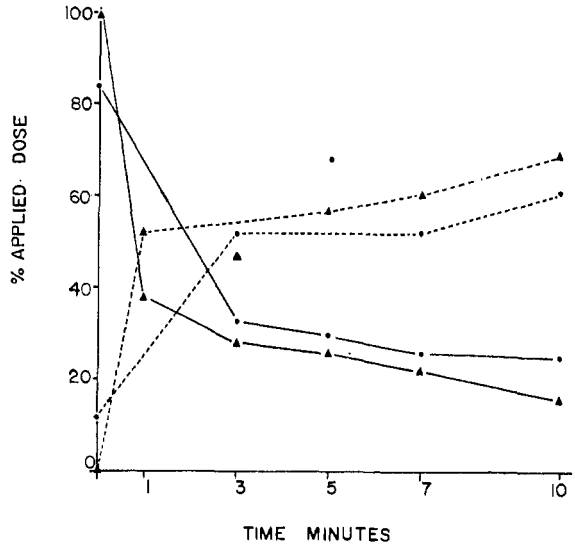

Figure 6. Time course of the enzymic degradation of ^{3}H-paraoxon by Wilson and SKA strains. Data for the SKA strain derived from one experiment only. ● Susceptible strain ▲ Resistant strain, ——Parent compound, – – –Metabolite fraction

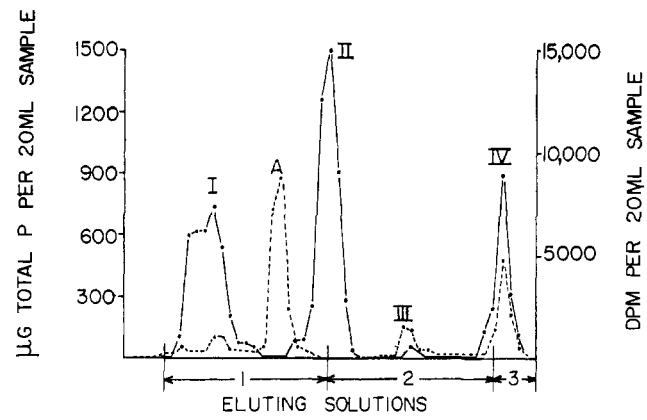

Figure 8. Ion exchange chromatography of the metabolite fraction extracted from 100 houseflies (Wilson strain), 90 min after injection with ^{3}H-paraoxon. Cochromatography with cold ethyl *p*-nitrophenyl phosphate, (IV) and ethyl phosphate (A). Radioactivity as solid lines, phosphorus analysis as dashed line

of the whole system to degrade inhibitor *in vivo*. The Km and V_{max} values are indicated on the graphs.

Identification of Metabolites. Ion-exchange cochromatography with candidate metabolites of the aqueous phase remaining, following extraction of insects 90 min after injection with ^{3}H-paraoxon, showed four metabolite peaks (Figure 8). Peak I did not correspond to any of the synthesized candidate metabolites and appeared as a single fraction which could be eluted from the column at pH values as high as 6.0. Its identity is discussed below. The second peak was identified as diethyl phosphate and peak IV as desethyl paraoxon. No attempt was made to identify peak III because of the small percentage of total activity involved. Ethyl phosphate did not correspond to any of the radioactive peaks, but it was eluted from the column at the same pH value as an unknown phosphorus-containing compound occurring in all experiments (peak A, Figure 8). Later investigation showed this phosphorus peak is present even when untreated flies are homogenized and chromatographed.

In considering the identity of peak I, it was possible that it could be acetaldehyde (Donninger *et al.*, 1966) or ethanol, derived from desalkylation. However, the 2,4-dinitrophenyl hydrazone derivative did not cochromatograph with that of acetaldehyde or formaldehyde on thin-layered or gas chromatography; and the 3,5-dinitrobenzoate derivative gave only traces which cochromatographed with that of ^{3}H-ethanol on tlc (Randerath, 1960).

It seemed possible that the primary metabolic product might have been further metabolized. We therefore compared the behavior of the radioactivity in the extract with that in an extract derived in an identical way, but without resin treatment, from flies treated with ^{3}H-ethanol. Indeed the radioactivity in these two extracts behaved in similar ways as follows.

Fractional distillation of the extracts, 135 ml for the 500 ^{3}H-paraoxon treated insects and 43.5 ml for the 200 ^{3}H-ethanol-treated flies, demonstrated the existence of three components: the first fraction distilled (fraction A, 10 ml)

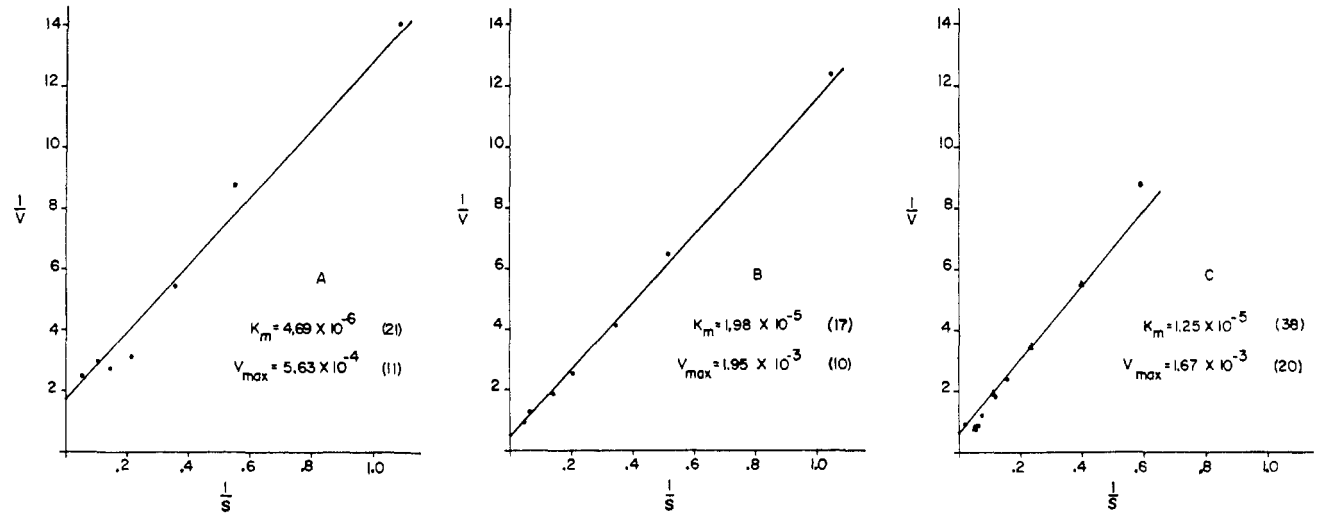

Figure 7. Reciprocal plot of substrate concentration *vs.* velocity of degradation reaction. S: mµ moles ^{3}H-paraoxon applied per g of insect (Km in molar units) V: mµ moles product formed per min per g insect (V_{max} in moles per min per kg). (A) Wilson (susceptible strain), (B) Ru (resistant strain), and (C) SKA (resistant strain) with points plotted from two separate experiments. Figures in parentheses represent percentage standard error for the calculated parameters

contained a disproportionately high amount of radioactivity (5% for the paraoxon treated and 27% for the ethanol treated). Further distillation gave 120 and 26 ml, respectively, for each treatment of fraction B, containing another 42 and 40%; towards the end of this period of distillation little radioactivity was in the distillate. The nonvolatile residue (fraction C) contained the residual 53% in 5 ml for the ^{3}H-paraoxon treated and 32% in 7.5 ml for the ^{3}H-ethanol treated insects.

Each of these fractions was investigated separately by partitioning in a butanol-buffer system. Repartitioning of each phase of the system was continued until either a constant figure was obtained for the butanol-buffer partition coefficient or the number of counts obtained reached a low level of significance. We shall use the term "paraoxon" for the butanol-buffer coefficient for the ^{3}H-paraoxon metabolites, and "ethanol" for the corresponding value for the ^{3}H-ethanol metabolites. After repeated partitioning of fraction A, the butanol soluble components achieved a coefficient essentially the same as for unmetabolized ^{3}H-ethanol, *i.e.*, 1.25. Thus the paraoxon value was 1.40, and the ethanol value 1.30. Fraction B gave pH-independent values in both cases: at pH 2.2 the value for the aqueous phase was 0.28 for paraoxon and 0.32 for ethanol; at pH 7.0 the values were 0.31 and 0.27. By contrast fraction C showed a pH-dependent effect: partition coefficients for the aqueous phase of 0.07 and 0.08 were obtained at pH 7.0 for paraoxon and ethanol, respectively; of 0.07 and 0.07 at pH 4.7 and 0.17, and 0.19 at pH 2.2.

The material in fraction C was considerably less water-soluble at low pH than at high pH values, indicating an acid pKa value less than 4.7. Because fraction C was nonvolatile, the possibility that it was a conjugate was explored. After digestion of an aliquot in 6*N* HCl for 3 hr, approximately 10% of the radioactivity for ^{3}H-ethanol treated flies could be extracted with butanol; further back extraction of this component with buffer pH 7.0 gave a final partition coefficient butanol-buffer for the butanol soluble component of 1.30 in good agreement with the figure of 1.25 found for ^{3}H-ethanol. The implication is that digestion of fraction C yielded a modest amount of ethanol.

We therefore conclude that fraction 1 behaves precisely as the metabolites from ^{3}H-ethanol and indeed contains a little ^{3}H-ethanol. Presumably it is all derived from desethylation of paraoxon. An interesting possibility is that the glutathione-dependent dealkylating enzyme (Fukami and Shishido, 1966) is involved; it transfers methyl groups to glutathione (Hollingworth, 1969). But so far, it has only been shown to be very effective for dimethyl phosphates.

A comparison of the relative quantity of each metabolite produced by the three strains is shown in Table IV. The resistant strains, already shown to have an increased ability to detoxify paraoxon, apparently achieve this result with an increased hydrolysis of the P—O—aryl bond, since diethyl phosphate is the only metabolite of which more is produced by the resistant strains. The SKA hydrolyzes paraoxon with very little desalkylation in contrast to the Wilson strain (and to a lesser extent the Ru strain) where desalkylation is a major metabolic pathway.

Table IV shows that peak I (ethanol and derivatives) is always greater than peak III (desethyl paraoxon). Consequently, the desethylation cannot consist simply of a one-step cleavage of paraoxon to desethyl paraoxon, for then the two peaks would be equal. It may be that some of the desethyl paraoxon is cleaved further to ethyl phosphate; or perhaps some of the paraoxon (or conceivably, diethyl phosphate) undergoes simultaneous bis-desethylation. Simultaneous bis-demethylation has been reported recently for alkaline and also enzymic degradation of bromophos (Stenersen, 1969).

Table IV. Metabolites[a] Recovered from Susceptible and Resistant Strains 90 Min after Injection with ^{3}H-paraoxon

	Wilson (susceptible)	Ru (resistant)	SKA (resistant)
Total metabolite fraction	78 ± 3.3	77	71
Column recovery	87 ± 2.9	91	86
No I (ethanol and derivatives)	53 ± 5.3	42	10
No II (diethyl phosphate)	30 ± 2.4	45.5	83
No III (unidentified)	2.0 ± 1.6	1.5	0.5
No IV (desethyl paraoxon)	15 ± 5.5	11	6.5

[a] Total metabolite fraction expressed as percentage of applied dose. Individual metabolites expressed as percentage of material recovered from the column. Wilson strain figures represent mean of four experiments, Ru mean of two experiments, and SKA mean of two experiments.

DISCUSSION

At the low dose rate (0.63 μg per g; the LD_5 for the susceptible strain), at which the time dependence and product identity data were necessarily obtained, resistant strains degrade paraoxon at a rate somewhat greater than susceptible. But Figure 7 shows (by extrapolation) that at higher doses the difference in degradation rates is much greater. Thus the V_{max} values differ 3.5-fold for Ru and 3.0-fold for SKA. From Figure 7 one may compute that at a dose of 1.40 μg per g (the LD_{50} for the Ru strain) there is a 1.3-fold difference in degradation rate between this strain and the susceptible Wilson strain. Similarly, at the LD_{50} figure for the SKA resistant strain, a 1.2-fold difference exists in the degradation rate between the susceptible and resistant strains. This difference in degradation of an injected dose seems adequate to account for the resistance factors for injected doses of 1.7-fold and 1.6-fold for the resistant strains.

The apparent Km's for the degradation systems in the strain differ; they are greater by 4.2-fold and 2.7-fold for the resistant strains as compared with the susceptible. The implication is that resistance is not due to a proportional increase in the total degradation system. The finding is compatible with the disproportionate increase of dearylating as compared with the desalkylating pathway.

Our data therefore agree with the conclusions of Oppenoorth and Van Asperen (1961) that metabolic differences account for resistance in these strains (our Ru strain has the same origin as their K strain). Our data differ from theirs obtained with homogenates in showing substantial progressive loss *in vivo* by the susceptibles; they found no progressive loss *in vitro*. They reported an unusually low Km of $10^{-6}M$ for the degradation by resistant strains (Oppenoorth, 1965); we find more usual values in the $10^{-5}M$ range. Their data cannot distinguish between degradation and binding of paraoxon, whereas ours specifically indicates degradation as the source of loss. In general, we feel that data on intact flies is more relevant than that on homogenates, either unfortified (Oppenoorth and van Asperen, 1961), or fortified with cofactors (Krueger and Casida, 1961). We concur with the majority of workers that differences in or protection of cholinesterase is not a factor in resistance.

It is widely recognized that resistance ratios which may

be large with topical applications may be very small with injected applications (Bigley, 1966; Oppenoorth, 1958; Table I). This does not necessarily mean that the strains differ in their rapidity of penetration. Thus a two-fold difference in metabolizing activity could have a disproportionality large protective effect if, in both strains, the topical dose penetrated at identical rates, sufficient to overwhelm the degrading system of the susceptible strain.

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