succinoxidase activity as compared with the controls, especially within the first minutes of the incubation, which we assume is indicative of greater mitochondrial damage.

Conclusions

The results of these experiments seem to indicate that as a consequence of the administration of the cerebral convulsants used in this work or electroshock, to animals, the mitochondria in the brain is damaged. This, in turn, could lead to the detachment and release of the CoA derivatives of the betaines into a new environment. These betaine esters are structurally similar to ACh, having the same quaternary ammonium nitrogen and carbonyl groups. In addition, the bond distance between these two functional groups in the two molecules is not very different. Hence, it is not surprising to find that the betaine esters are pharmacologically active substances. It is therefore possible that these betaine esters could attach themselves to the same receptors in brain as does ACh itself. Miller *et al.* (*22*) have observed that the topical application of ACh to the eserinized cerebral cortex can elicit the electrical activity normally associated with convulsions of the epileptiform type. It would appear therefore that betaine esters released in brain as a consequence of the administration of cerebral convulsants could act in a manner similar to that observed on the topical application of ACh to the cerebral cortex.

Table I. Damage of Brain Mitochondria in Vivo by Cerebral Convulsants as Assayed by Succinoxidase Activity

(μl. of oxygen consumed by mitochondria in 1 gram of fresh brain per minute)

Time, Min.	Agent	Control	Expr.	Diff.	Agent	Control	Expr.	Diff.
0–3	NH_4Cl	5.6	9.6	4.0	Camphor	7.1	11.6	4.5
3–6	(4.5 g.)[a]	6.2	9.0	2.8	(4.0 g.)[a]	6.0	7.1	1.1
6–9		7.8	8.6	2.4		5.3	6.4	1.1
9–12		5.8	7.3	1.5		4.9	4.9	0
0–3	Dieldrin	7.5	14.3	6.8	Electroshock	13.2	20.0	6.8
3–6	(7.0 g.)[a]	7.8	9.2	1.4	(5.0 g.)[a]	15.0	16.9	1.9
6–9		7.9	9.3	1.4		11.2	11.6	0.4
9–12		7.9	10.2	2.3		13.0	13.0	0

[a] Weight of fresh brain from which mitochondria were isolated.

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INSECT RESISTANCE TO INSECTICIDES

Biochemical Factors in the Acquired Resistance of Houseflies to Organophosphate Insecticides

D. C. MENGLE and J. E. CASIDA

Department of Entomology, University of Wisconsin, Madison, Wis.

THE RAPID DEVELOPMENT of resistance to certain organophosphates is proving to be a limiting factor in their use and a deterrent to the search for new insecticides of this type (*9, 10*). Houseflies have been most frequently used for investigations on the mechanism of this resistance. Although strains of houseflies have developed resistance to almost all the organophosphates, the resistance pattern varies with the strain (*11, 20, 22, 28, 31, 36, 48*, and others). The varying resistance pattern in different strains, along with genetical studies (*35, 37*), indicates that houseflies may have more than one mechanism of resistance to organophosphates. The resistance mechanism is even more complex, since selection with organophosphate insecticides can result in resistance to carbamates (*17, 18, 20, 29*) and *vice versa* (*32*). In addition, exposure to either phosphates or carbamates may result in high side resistance to chlorinated hydrocarbon insecticides (*27, 31, 32*), whereas the reverse situation does not hold (*27, 31*), although DDT-resistant flies developed organophosphate resistance faster than normal flies (*26*).

Many experimental approaches have been used in studying the mechanism of organophosphate resistance in houseflies. Attempts have been made to

The metabolic fate of radiolabeled Diazinon, malathion, and methyl parathion was studied in three housefly strains, two of which were resistant to organophosphates. Rate differences between the strains in cuticle penetration, phosphorothionate oxidation, or phosphate hydrolysis did not appear to explain the resistance. The lesser in vivo cholinesterase inhibition in resistant than susceptible flies treated with the same dose of the organophosphate probably results from some other mechanism than detoxification. Flies treated with the organophosphate immediately after decapitation were similar to whole flies in the symptomology of poisoning and degree of resistance. Evidence is presented for a "factor" in the thorax and/or abdomen which contributes to resistance by reducing rate of cholinesterase inhibition without destroying active antiesterase organophosphate.

correlate resistance with behavioristic avoidance (*19, 23, 44, 45*), lipoid content of the tarsi and thoracic ganglia (*41*), rate of insecticide penetration through the cuticle (*12, 25*), rate differences in oxidation of the phosphorothionates or persistence differences for the antiesterase phosphates formed (*25, 31, 36, 38*), rate differences in the hydrolysis of the phosphates (*6, 30, 31, 38*), the in vivo inhibition of cholinesterase (*38, 47*), and accumulation of acetyl choline (*39*), the activity (*5, 16, 31, 38*) or sensitivity (*6, 31, 38*) of the cholinesterase to organophosphates as assayed in vitro, and the level of aliesterase activity (*5, 6, 37*).

Most of the resistance mechanisms proposed depend on rate differences in the penetration or hydrolysis of the organophosphate. A critical test of these hypotheses was made with radiolabeled insecticides of high specific activity. Another hypothesis was also considered whereby the resistance might be due to a factor protecting the cholinesterase of resistant flies from inhibition by the organophosphate.

Methods and Materials

Housefly strains. Three strains of organophosphate-resistant houseflies (*Musca domestica* L.) were investigated: R-A, the Rutgers A strain which developed resistance under Diazinon pressure (*20*); R-I, the Italian strain which developed resistance under Diazinon pressure (*11, 43*); and R-P, the Orlando Tropical P strain which developed resistance in the field under Diazinon, Dipterex, malathion, and parathion pressure and was further selected in the laboratory under parathion pressure (*28, 48*). The resistant flies were reared through the fourth generation in our laboratory without insecticide pressure. Susceptible (S) flies were the C.S.M.A. (1948) strain. The larval medium and rearing technique have been described (*46*). Adult female flies were used the first day after emergence. Prior to testing, these day-old flies were fed skim milk diluted with an equal volume of water.

Insecticides. Isotopic exchange (*13*) was used to obtain the labeled organophosphorothionates of high specific activity. Krueger and O'Brien's procedure (*24*) was used for preparing labeled malathion in 58% yield with a specific activity of 6000 c.p.m. per μg. Diazinon (*25, 40*) was obtained in 48% yield with a specific activity of 4900 c.p.m. per μg. The synthesis of methyl parathion-P^{32} (*40*) gave about 40% yield and a specific activity of 4000 c.p.m. per μg. Solvent extraction and partition chromatography were used for purifying the radiolabeled compounds (*40*) until they were identical in infrared spectra (10% solutions in carbon tetrachloride or carbon bisulfide, Baird infrared spectrophotometer, sodium chloride optics) with the authentic compounds. The LD_{50} values for flies 24 hours after injection were identical for the radiolabeled and the authentic nonlabeled compounds.

The nonlabeled insecticides used were of the highest purity obtainable from the manufacturers.

Administration of Insecticides and Toxicity Studies. Houseflies were injected through the mesoscutum with a glass needle drawn to an 0.3-mm. tip fitted on an 0.05-ml. microsyringe driven by a micrometer. Propylene glycol was used as the insecticide solvent, each fly receiving 0.08 μl. Topical applications on the ventral tip of the abdomen were made with acetone solutions of the toxicants and 1.3 μl. of acetone per fly (*33*). The mortalities occurring in controls treated with solvent alone were 3.6% at 4 hours and 5.8% at 24 hours with the topical application and 0.8% at 24 hours with injection. In a separate study the compounds were applied topically to whole flies or flies which had just been decapitated. Results from the decapitation studies are reported only at 4 hours after treatment, because at this time the control mortality was 9.7% and by 24 hours complete mortality had occurred. Following injection or topical application, flies were held in gauze-covered 1 × 3 inch shell vials with 10 flies per vial. A small amount of sucrose was added to the vials to prevent starvation. LD_{50} values as micrograms of toxicant per gram of fly were estimated from logarithm-probit mortality plots.

Cuticle Penetration. The rate of cuticle penetration was determined following topical application of acetone solutions of the labeled insecticides to the ventral tip of the abdomen. Methyl parathion was applied at 0.5 and 5.0 μg. per gram, malathion at 10 μg. per gram, and Diazinon at 4.0 μg. per gram. With the 4-hour penetration experiments, the mortality in the S strain was no more than 50%, except with the high methyl parathion level, which yielded complete mortality. The resistant strains (R-A and R-P) showed no symptoms of poisoning except with the high methyl parathion level, where hyperactivity and partial paralysis of the flies occurred within 4 hours.

The amount of cuticle penetration was determined by washing the surface of 1 gram of treated flies with highly refined kerosine (Deobase) at various intervals after topical application. The inside of the vial used to hold the flies was also washed, the washes were combined, and the total radioactivity was determined. The difference between the amount of insecticide recovered and the amount applied was assumed to have penetrated into or through the cuticle. All experiments were duplicated.

In vivo Detoxication Studies. The in vivo fate of the radiolabeled insecticides was determined following injection at the same insecticide levels used for the penetration studies. These dosages yielded no more than 50% mortality among susceptible flies and no poisoning symptoms with the resistant flies, with the exception of the 5.0 μg. per gram of methyl parathion which gave complete mortality of the S flies within 4 hours but only symptoms with the R-A and R-P strains.

For determining the nature of the metabolites at various time intervals after injection of the P^{32} insecticides, a procedure was used which allowed almost quantitative separation of the original phosphorothionate, its oxygen analog, and the hydrolysis products of either of these.

One gram of treated flies and approxi-

mately 7 grams of Celite were homogenized in 50 ml. of acetone in a cooled Lourdes blender. The homogenate was filtered and the residue with the Celite rehomogenized with 50 ml. of benzene. This second homogenate was filtered, and the filtrates were combined and extracted with 50 ml. of water. The layers were separated and aliquots were removed, evaporated, and counted for P^{32} content. The solvent was evaporated from the remaining organic solubles, and the residue was dissolved in hexane and chromatographed on an alumina column (*25*) with 150 ml. of benzene to elute the original phosphorothionate, 150 ml. of chloroform to elute the phosphate oxidation products, and finally with 100 ml. of methanol to ascertain that all the radioactivity had been eluted from the column.

When this procedure was used, more than 90% of the radioactivity was extracted from flies immediately after injection of the labeled compound, and subsequent recovery from the alumina column was also greater than 90%. The losses in these two steps probably contributed to the lack of complete recovery in determining the separate amounts of the various metabolites. All experiments were made in duplicate. Radioactive measurements were made on dried samples in an automatic gas flow counter with suitable corrections for self-absorption and isotopic decay.

The levels of oxygen analog derived from injected nonlabeled parathion, methyl parathion, and malathion (0.5, 0.5, and 10 μg. per gram, respectively) were also determined in separate experiments by extraction of the inhibitor and assay of the recovered anticholinesterase agents against housefly head cholinesterase. At 0, 1, and 4 hours after injection, the flies were immediately frozen in an acetone–dry ice mixture and subsequently homogenized in this same acetone. The acetone was then evaporated, equal volumes of water and chloroform were added, and the neutral phosphoric esters were extracted into chloroform. The anticholinesterase agents in aliquots of this chloroform were then assayed against fly head homogenates. The aliquot used was one predetermined to yield a maximum of 60 to 80% cholinesterase inhibition.

Rate Studies on Fly Cholinesterase Inhibition. The activity of fly head cholinesterase was determined at various times after topical application of the three organophosphates (*33*) with the S, R–A, and R–P strains. With this technique the interference from excess inhibitor (*3*) was minimal (*34*).

In vitro studies on the rate of cholinesterase inhibition followed the procedure of Aldridge and Davison (*1*).

The enzyme preparation in 1.60 ml. of bicarbonate buffer was placed in a 22-ml. double-side-arm Warburg flask. The organophosphate in 0.20 ml. of buffer was placed in one side arm and the substrate in 0.20 ml. in the second side arm. When the side arms were tipped, the final concentrations were $0.01M$ for acetyl choline, $1 \times 10^{-6}M$ for malaoxon, and $1 \times 10^{-7}M$ for paraoxon. After gassing with a 95% carbon dioxide–5% nitrogen mixture and equilibration at 25° C. in the Warburg bath for a total of 10 minutes, the first side arm was tipped to initiate the reaction of the inhibitor with the cholinesterase. When the desired time for inhibition had elapsed, the substrate was tipped in to terminate the inhibition reaction and initiate the assay of the residual cholinesterase activity.

All in vitro rate studies were made at 25° C. and results were calculated as b_{30} values (microliters of carbon dioxide evolved in 30 minutes). Enzyme sources were prepared from both susceptible (S) and resistant (R–A and R–P) flies and included homogenates of whole flies, heads from flies, and thoraces plus abdomens from flies. The enzyme concentration was standardized at 1.5 fly equivalents (or part thereof such as head, or thorax plus abdomen) per ml. The in vitro rate studies were varied by using the heads from one strain of flies as the cholinesterase source and the thorax plus abdomen from another strain as modifiers of the inhibition rate. In certain cases the homogenized thoraces plus abdomens were heated at 100° C. for 5 minutes and then cooled before use.

The stability of the organophosphate inhibitor on incubation with fly homogenates was also studied under the concentration, buffer, pH, and temperature conditions involved in the normal Warburg assay. After various incubation times of the fly homogenates with $1 \times 10^{-6}M$ malaoxon or $1 \times 10^{-7}M$ paraoxon at 25° C., 2.0-ml. aliquots were withdrawn and extracted with an equal volume of chloroform and the anticholinesterase agents recovered in the chloroform were assayed against a fresh fly head homogenate at 37° C. The aliquot size was standardized to give 50 to 60% cholinesterase inhibition. The difference between inhibitor recovered at zero time and that recovered with varying incubation times was assumed to represent the loss of the inhibitor under the conditions utilized in studying the in vitro rate of cholinesterase inhibition.

Results

Degree of Resistance. The degree of resistance—i.e., the 24-hour LD_{50} of the resistant strain divided by the LD_{50} of the susceptible strain—of the R–A, R–P, and R–I strains did not vary greatly when determined by topical application or injection of the insecticides. With

Table I. Characteristics of Housefly Strains Studied

Characteristic	Housefly Strain S	R–A	R–P	R–I
Av. wt. adult ♀ mg.	22	22	22	20
ChE activity of heads $-b_{30}$/head at 37° C.	41	42	39	35
Sensitivity to Compds.[a]	LD_{50} μg./g.	Degree of Resistance[b]		
Topical, 24 hr.				
Diazinon	3.6	37	15	17
Malaoxon	1.8	8	10	4
Malathion	15	9	7	7
Methyl parathion	0.9	6	7	4
Paraoxon	0.5	3	6	44
Parathion	0.7	9	16	44
Isolan	15	13	10	..
Sevin	45	25	10	..
Injected, 24 hr.				
Diazinon	6	30	6	21
Malathion	12	6	11	3
Methyl parathion	0.5	11	13	16

[a] Replications. After approximate LD_{50} values were found in survey runs, final values were determined with 1.2 to 1.4 factor dosage increments and 20 flies at each dose. Final values for topical LD_{50} were based on 2 to 4 separate dosage series about the LD_{50} for the S and 1 to 3 series for the R strains. A single series was used in arriving at final injected LD_{50} values.

[b] $\frac{LD_{50} \text{ resistant strain}}{LD_{50} \text{ susceptible strain}}$.

Table II. LD_{50} Values for Strains 4 Hours after Topical Treatment of Houseflies with and without Decapitation[a,b]

Insecticide	Whole Flies S	R–A	R–P	Decapitated Flies S	R–A	R–P
Diazinon	4	175	385	4	150	360
Malaoxon	1.9	18	20	1.8	17	20
Malathion	14	180	120	12	180	100
Methyl parathion	0.9	6	7	0.9	6	7
Paraoxon	0.6	2	4	0.6	2	4
Parathion	0.9	8	12	0.8	7	11
Isolan	45	210	175	45	210	140
Sevin	65	1400	670	65	1400	670

[a] LD_{50} as μg./g. disregarding weight loss on decapitation.

[b] After approximate LD_{50} values were found in survey runs, final values were determined with 1.2 to 1.4 times dosage increments and 20 flies at each dosage. Reported values are based on 1 to 3 dosage series.

topical application the resistance was almost as great with the oxygen analogs (malaoxon and paraoxon) as with their corresponding phosphorothionates (malathion and parathion). The resistance pattern in each of the strains (Table I) was similar to that recorded in the literature (*11*, *20*, *28*, *43*, *48*).

Flies decapitated just prior to treatment were similar to intact flies in their susceptibility to the toxicants based on LD_{50} values 4 hours after topical application (Table II). The symptoms of poisoning at 4 hours after treatment were the same for whole or decapitated flies on the basis of the proportion of normal, hyperactive, paralyzed, and dead individuals. The site of action involved in initiating the poisoning symptoms and that governing the degrce of resistance to these insecticides appears to be primarily localized in the thorax and/or abdomen.

These studies were made on flies from the second through the fourth generations without insecticide pressure. Little loss in the degree of resistance occurred by the fourth generation with the R–A, R–P, and R–I strains, whereas March (*31*) has noted that in other strains the phosphate resistance drops off rapidly once removed from insecticide pressure.

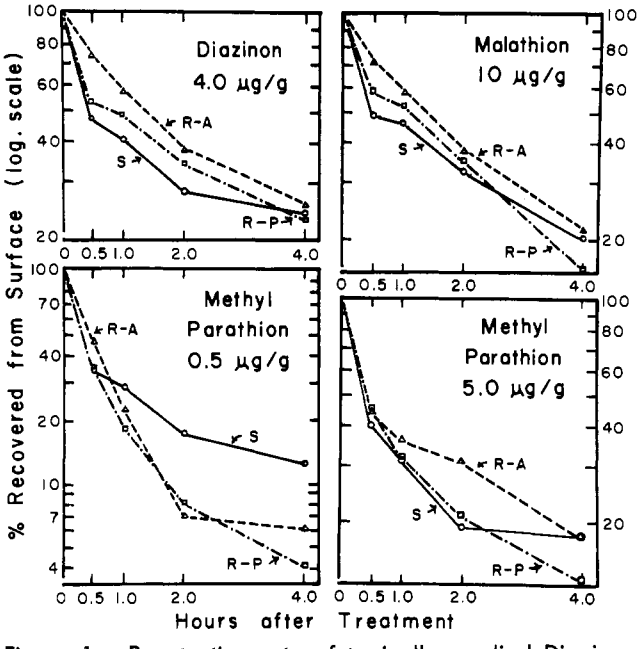

Figure 1. Penetration rate of topically applied Diazinon, malathion, and methyl parathion into three housefly strains

Penetration as a Factor in Resistance. The penetration rate was not a major factor contributing to the resistance based on the tolerance of the resistant flies studied to the toxicants following either topical application or injection (Table I). Penetration following topical application in this study may have been greatly aided by the solvent or disruptive action of the acetone on the cuticular constituents.

Results from penetration rate studies with radiolabeled Diazinon, malathion, and methyl parathion following topical application to the ventral tip of the abdomen are given in Figure 1. The data are plotted as a logarithmetic function of the percentage of applied insecticide recovered against hours, on the assumption that a certain constant fraction of the insecticide penetrated during each equal time interval. By averaging the data for the three fly strains and considering the slope of the line from 0.5 to 4 hours, it was found that the time for half penetration with methyl parathion was 45 minutes at 0.5 and 100 minutes at 5.0 µg. per gram, with Diazinon it was 110 minutes at 4.0 µg. per gram, and with malathion it was 130 minutes at 10 µg. per gram. The results with methyl parathion at 5.0 µg. per gram cannot be directly compared with the other compounds and dosages, since the susceptible flies were paralyzed and died during the course of the experiment. Extrapolation of these curves to zero time indicated that 40 to 48% of the methyl parathion and 30 to 32% of the Diazinon and malathion had penetrated at a faster rate, possibly through the action of the solvent.

Diazinon and malathion penetrated more rapidly into the susceptible than into the R–A and R–P strains, the major difference occurring within the first 30 minutes. This is in agreement with a published report comparing the S and R–A strains with Diazinon at 30 µg. per gram (*25*). The strain differences with methyl parathion were less clear-cut. Insufficient data are available to make precise correlations of degree of resistance (Table I) and differences in penetration rate (Figure 1), but radiotracer penetration studies do not implicate penetration as the major factor contributing to the resistance.

Detoxication of Organophosphate as a Factor in Resistance. Studies on the rate of degradation and excretion for the radiolabeled insecticides following injection into the different fly strains are presented in Table III. Recovery of the applied radioactivity was good, considering that unextractable radioactivity remaining in the fly residue and that not eluting from the alumina column in the positions for the unhydrolyzed phosphorothionate or phosphate were not included in the percentage recovery figures. Averaging all data gave recoveries of

Table III. Degradation and Excretion of Radiolabeled Phosphorothionates after Injection into Three Housefly Strains

(Results as % of administered dose recovered in fraction indicated)

Insecticide, Dose and Fraction	Hours after Injection of Phosphorothionate											
	Susceptible				Resistant A				Resistant P			
	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
Diazinon, 4 µg./g.												
Org. sol., P = S	72	57	40	23	72	50	35	18	77	40	30	18
Org. sol., P = O	3.7	6.2	3.7	3.0	2.5	5.0	3.7	3.2	5.0	6.2	3.7	2.5
Water sol.	25	48	38	35	28	48	50	45	23	48	48	43
Excreted	0.1	0.2	5	20	0.1	1	5	23	0.1	1	8	20
Malathion, 10 µg./g.												
Org. sol., P = S	63	45	23	17	61	53	23	8	65	50	25	9
Org. sol., P = O	0.6	0.6	0.6	1.1	0.2	0.3	0.7	0.7	0.3	0.9	0.5	0.8
Water sol.	22	33	51	35	29	43	65	21	22	33	47	20
Excreted	1	2	3	30	3	5	10	51	2	3	5	36
Methyl parathion, 0.5 µg./g.												
Org. sol., P = S	36	24	22	18	34	30	24	24	46	36	30	28
Org. sol., P = O	6	2	2	2	4	2	4	4	6	4	2	2
Water sol.	10	62	44	18	40	58	50	26	16	80	52	24
Methyl parathion, 5.0 µg./g.												
Org. sol., P = S	36	26	22	16	30	24	18	16	32	26	22	18
Org. sol., P = O	6	5	2	2	6	5	4	2	6	3	2	2
Water sol.	26	60	56	52	28	58	42	42	24	62	52	52
Excreted	2	16	14	24	2	28	38	40	4	24	36	42

95% for Diazinon, 85% for malathion, 96% for the high level of methyl parathion, and 73% for the 0.5 μg. per gram of methyl parathion dosage where excretion data were not available for consideration. The percentage recovery, particularly with the low level of methyl parathion, varied considerably within this experiment, as indicated by the following ranges calculated from Table III: Diazinon 81 to 111%, malathion 66 to 101%, and methyl parathion (0.5 μg. per gram) 38 to 120% and (5.0 μg. per gram) 66 to 115%.

The rates of destruction of the injected phosphorothionate and formation of water-soluble metabolites (hydrolysis products) were not greatly different between the susceptible and resistant strains. Only with the high dose of methyl parathion were large excretion differences evident and at this dose the paralysis of the susceptible flies probably accounted for their low excretion compared with the active resistant flies.

No great difference occurred between the strains in the percentage of the injected dose that existed as radiolabeled oxygen analog at any time after treatment (Table III). Possible differences in in vivo levels of oxygen analogs were also examined by injecting the S, R–A, and R–P flies with 0.5 μg. per gram of parathion or methyl parathion and 10 μg. per gram of malathion and recovering the unhydrolyzed materials by extraction with acetone and partitioning into chloroform from water for subsequent assay of extracted cholinesterase inhibitors against fresh fly head homogenates. Little or no strain difference was found in the amount of inhibitor recovered from the S, R–A, and R–P flies treated with parathion or methyl parathion and the S and R–P flies treated with malathion. The extracts from flies homogenized immediately after treatment yielded no cholinesterase inhibition with the aliquot assayed. Parathion at 1 hour yielded 80% inhibition and at 4 hours yielded 30% inhibition, and corresponding values with methyl parathion were 80% at 1 hour and 30% at 4 hours, and with malathion were 65% at 1 hour and 50% inhibition at 4 hours with each of the S and R strains studied. A greater instability for the oxygen analogs was indicated by these studies on recovery of the anticholinesterase agents than was found with the radiotracer experiments (Table III).

Cholinesterase Inhibition as a Factor in Resistance. This study confirmed a previous report (*17*) that the cholinesterase inhibition in the heads of organophosphate-poisoned flies correlates with resistance. The data from methyl parathion applied topically at 5.0 μg. per gram (Figure 2) illustrate the lesser inhibition and milder symptoms occurring in the resistant strains. Studies with methyl parathion at 0.5 μg. per gram, malathion at 10 μg. per gram, and Diazinon at 4.0 μg. per gram showed less than 5% inhibition at any time after treatment with the R–A and R–P strains, while the greatest degree of inhibition occurred at 0.5 hour with the S strain, when values of 9, 24, and 16% inhibition, respectively, for the three organophosphates were observed.

The initial rate of in vitro inhibition by malaoxon and paraoxon was greater for the cholinesterase in homogenates of whole susceptible flies than in homogenates of whole resistant flies (Figure 3), even though the initial cholinesterase activity was nearly the same in both cases. This rate difference with strain was not evident when only the heads were studied, confirming previous reports (*31*, *38*). When the experiments illustrated in Figure 3 were repeated using the heads of S flies in both cases combined with either the S or R thoraces plus abdomens, similar rate differences were noted. In such studies about 75% of the cholinesterase activity was contributed by the heads, and the remainder by the combined thoraces plus abdomens. The cholinesterase in homogenates of heads was inhibited more rapidly than that in homogenates of whole flies, a difference which was greatly reduced or lost by boiling the thoraces plus abdomens before adding to the fresh head homogenate. Comparisons were also made between boiled and unboiled thoraces plus abdomens from S or R flies mixed with fresh heads from S flies. In both cases (S and R flies) the esterase was more rapidly inhibited by the organophosphate in the presence of the boiled thoraces plus abdomens, and the difference in rate resulting from boiling was greater with the R than with the S flies.

The difference in inhibition rate of the cholinesterase in homogenates of S and R flies was not due to more rapid hydrolysis of the inhibitor by the R-fly homogenates, since comparable amounts of the inhibitor could be recovered by chloroform extraction of the S or R fly homogenate–organophosphate incubation mixtures (Table IV). It appears probable that in resistant flies there is a higher concentration of some "factor" in the thorax and/or abdomen capable of reducing the rate of cholinesterase inhibition by organophosphates without hydrolyzing the inhibitor.

Discussion

Several differences were found between the resistant and susceptible houseflies which might contribute to the survival of the resistant forms when exposed to certain organophosphate insecticides.

Rate differences in cuticle penetration may be a contributing factor (*12*, *25*, *31*), since a somewhat slower penetration of Diazinon and malathion occurred with the resistant than with the susceptible flies (Figure 1). Penetration did not appear to be the most critical factor, since similar degrees of resistance and resistance patterns resulted with topical

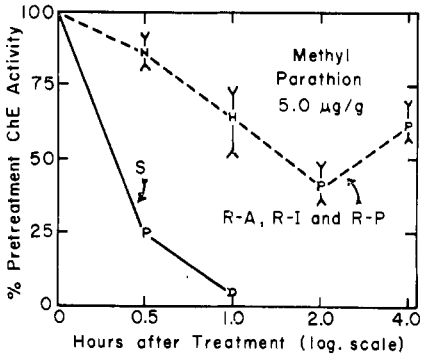

Figure 2. Brain cholinesterase activity and symptomology of surviving houseflies after topical treatment with methyl parathion

Symptoms for over 50% of the flies at the selected posttreatment times. N normal, H hyperactive, P paralyzed, D dead. Results on cholinesterase inhibition with resistant flies indicated as the range for the three strains. Each point with each strain is based on 2 to 4 replicates.

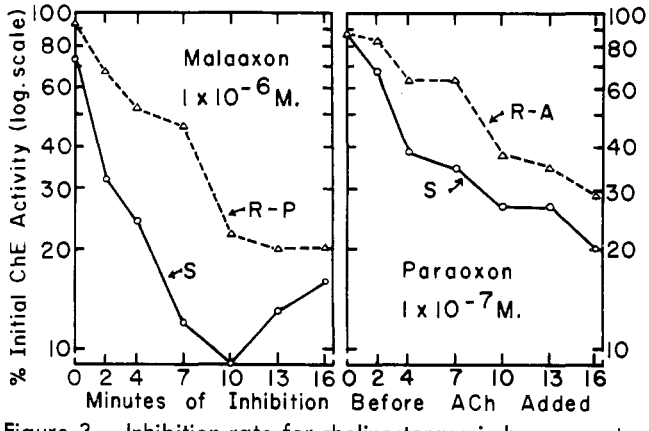

Figure 3. Inhibition rate for cholinesterase in homogenates of whole S and R flies by malaoxon and paraoxon at 25° C.

Each point based on 4 determinations

Table IV. Extent of Malaoxon and Paraoxon Breakdown on Incubation in pH 7.45 Bicarbonate Buffer with Homogenates of Resistant and Susceptible Houseflies

Substrate and Fly Strain	% ChE Inhibition from $CHCl_3$ Extract Made after Indicated Minutes of Incubation			
	0	5	20	80
Malaoxon, $1 \times 10^{-6} M$				
S	63	61	63	57
R-P	61	60	63	59
Paraoxon, $1 \times 10^{-7} M$				
S	54	48	49	51
R-A	51	48	49	50

application and injection of the organophosphate (Table I), although the resistance to injected toxicants found in the present study was greater than that reported in the literature (*11*, *31*, *38*).

Possible differences in efficiency of phosphorothionate oxidation to form the antiesterase phosphate analogs cannot account for the resistance, because the tolerance to the oxygen analogs is of the same order of magnitude as that to their phosphorothionates (Table I; *31*, *36*, *38*). Radiotracer studies on phosphorothionate oxidation failed to show strain differences in rate of loss of the phosphorothionate or formation of the phosphate analog (Table III), confirming the bioassay results.

The rate of in vivo hydrolysis following administration of the radiolabeled phosphorothionate was not greatly different between the resistant and susceptible flies (Table III). As comparable levels of the oxygen analogs were recovered with all the strains (Table III), it appears likely that the oxygen analogs were degraded no more rapidly by the resistant than the susceptible flies. No strain difference was noted in the total anticholinesterase activity recovered by chloroform extraction from flies treated with three phosphorothionates including parathion (see results), confirming the lack of difference noted in rate of paraoxon destruction by a susceptible and resistant strain by a slightly different analytical method (*30*).

The brain cholinesterase from resistant and susceptible flies is similar in in vitro susceptibility to inhibition by organophosphates (*31*, *38*; see Results), but this same cholinesterase is much more greatly inhibited in vivo by the same organophosphate dose in susceptible than in resistant strains (Figure 2; *10*, *47*) to yield a greater acetylcholine accumulation in the susceptible than in the resistant flies (*39*). The head can be removed from the fly without altering the symptoms of organophosphate poisoning or the degree of resistance (Table II), indicating that the site of action involved in initiating the poisoning symptoms and that governing the degree of resistance to these insecticides are primarily localized in the thorax and/or abdomen.

With whole fly homogenates, a slightly larger amount of certain organophosphates is required to inhibit the cholinesterase from susceptible than from resistant flies, possibly because of the aliesterase which is present in higher concentration in the susceptible forms preferentially reacting with these organophosphates (*6*). Treatment of head or whole fly homogenates by a method which partially inactivates the aliesterase (pH 8.3, 37° C., 1 hour) increases the susceptibility of the cholinesterase in these homogenates to organophosphates, again presumably by removing the aliesterase which preferentially reacts with the organophosphate. The aliesterase activity and susceptibility relationships tend to favor the survival of the susceptible rather than the resistant strains (*6*). This discrepancy has been explained by the assumption, with no more than meager correlative evidence, that mutation of a single gene in the resistant forms has resulted in the modification of the aliesterase to a phosphatase capable of destroying the organophosphate (*6*).

Despite much speculation regarding possible detoxifying enzymes involved in resistance, only three reports to date have presented data favoring such a hypothesis. In one of these studies (*38*), the degree of in vivo cholinesterase inhibition following injection of parathion or paraoxon was interpolated from a standard curve prepared with fly homogenates and paraoxon in vitro to derive the degree of detoxification. A large difference in detoxification rate was found between the R and S strains. This indirect analytical procedure yielded a far greater apparent efficiency in oxidation of the phosphorothionate (85%) than radiotracer studies (Table III; *24*, *25*). Further, when R or S flies were injected with parathion, no difference was noted in the amount of paraoxon that could be recovered by chloroform extraction of the flies at various times after treatment (see results). When whole fly homogenates (*6*) or sections of decapitated flies (*31*) are incubated with such antiesterases as malaoxon, paraoxon, Diazoxon [*0,0*-diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphate], or DDVP (*0,0*-dimethyl 2,2-dichlorovinyl phosphate) and the persisting antiesterases after 1 to 4 hours determined by their inhibitory activity on an added fly head cholinesterase source, lesser amounts of anticholinesterase agents are recovered from the resistant strains. The time and substrate concentration relationships (*6*, *31*) indicate, but do not necessarily establish, the involvement of an enzymatic detoxification reaction. In any case, the detoxifying system in resistant flies is probably unable to reduce the total amount of organophosphate rapidly enough to a harmless level (*6*).

An alternative mechanism to detoxification might be a "factor" present in higher concentration in the resistant forms which protects the cholinesterase from inhibition by the organophosphate. Such a factor might act by reducing the rate of the inhibition process or increasing the rate of reactivation of the inhibited cholinesterase. Evidence for the existence of such a factor is presented in the results section and Figure 3. The nature of this factor in the thorax and/or abdomen of resistant flies is not known.

Several mechanisms are evident by which a factor might protect cholinesterase both in vivo and in vitro from inhibition by organophosphates without destroying the organophosphate. The first mechanism would be one of localization where the organophosphate was unavailable for reaction with the cholinesterase because of differences in lipide concentration or characteristics, extent of protein binding, or loss of the organophosphate by reaction with proteins other than cholinesterase. Another mechanism would be a direct protection of the cholinesterase as by substrates or competitive inhibitors, so that the active sites could not be approached by the organophosphate. A third mechanism would involve an enzymatic (*8*) or nonenzymatic factor serving to dissociate or destroy the inhibitor bound to cholinesterase before the more difficultly reversible stage of phosphorylation occurred. Finally, the factor might act as a reactivator for the phosphorylated cholinesterase. The potentiality of such mechanisms has been shown directly or indirectly in studies involving fly cholinesterase (*2–4*, *33*, *34*, *42*) and many other cholinesterase sources, but they have usually not been applied specifically to the problem of insect resistance to organophosphate insecticides.

The side resistance of organophosphate-resistant houseflies to carbamate insecticides indicates a possible involvement of a similar resistance mechanism. On the basis of studies concerning carbamate insecticide degradation by mammalian enzymes (*7*, *14*, *15*, *21*), it appears unlikely that similar detoxification mechanisms are involved for the phosphate and carbamate antiesterase agents. However, some factor altering the mechanism of cholinesterase inhibition might be effective against both types of inhibitors in contributing to resistance.

Further work is in progress on the nature of the factor in resistant flies which reduces the rate of cholinesterase inhibition by certain organophosphates, and on the correlation of this factor with resistance.

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INSECTICIDE RESIDUES IN MILK

Excretion of Co-Ral in the Milk of Dairy Cattle

R. D. RADELEFF, Animal Disease and Parasite Research Division

H. V. CLABORN, Entomology Research Division, U. S. Department of Agriculture, Kerrville, Tex.

Co-Ral, *O* - (3 - chloro - 4 - methylumbelliferone) *O,O* - diethyl phosphorothioate, known also as Bayer 21/199, has shown considerable promise as a systemically active insecticide against the cattle grubs *Hypoderma lineatum* (DeVill.) and *H. bovis* (Deg.) (*1, 2*) and as a contact insecticide against a number of other external parasites (*5*). Recent reports indicate that it may have activity against certain helminths (*3*).

The use of Co-Ral on lactating dairy cattle could be recommended only if Co-Ral were absent from their milk. The present series of studies was initiated to determine the amount of Co-Ral which might appear in milk of cattle sprayed with the material.

Material Used

Through the courtesy of the Bayer Chemical Co., Leverkusen, Germany, and the Chemagro Corp., Kansas City, Mo., Co-Ral labeled with phosphorus-32 was made available to the Animal Disease and Parasite Research Division. This material was chromatographically demonstrated to be of at least 99.5% purity and at the time of shipment demonstrated a specific activity of 4.6 mc. per gram.

The radioactive material was diluted with ordinary Co-Ral and made up into a 20% emulsifiable concentrate by dissolving the chemical in a mixture of 65 parts of xylene, and 10 parts of Triton X-100.

This emulsifiable concentrate was then diluted to 0.5 and 0.75% concentrations with tap water immediately before application to the cows.