

A MECHANISM OF RESISTANCE TO ORGANOPHOSPHORUS ACARICIDES IN A STRAIN OF THE CATTLE TICK *BOOPHILUS MICROPLUS*

By C. A. SCHUNTNER,* W. J. ROULSTON,* and H. J. SCHNITZERLING*

[Manuscript received July 14, 1967]

Summary

Radioactive coumaphos, diazinon, *cis*- and *trans*-dioxathion, and dioxene derivative of dioxathion individually gave similar results for penetration and metabolism in larvae of an organophosphorus-resistant and an organophosphorus-susceptible strain of cattle tick. The phosphorothionates, coumaphos and diazinon, were metabolized to their respective oxygen analogues, diethyl thiophosphate and diethyl phosphate. The dioxathion components, phosphorothiolothionates, were metabolized additionally to diethyl dithiophosphate. The production of cholinesterase-inhibiting oxidation metabolites from the dioxathion components was shown indirectly by the considerable lowering of acetylcholinesterase activity in treated larvae and by assays for enzyme inhibitors in larvae treated with *trans*-dioxathion.

The ratios of the percentage of acetylcholinesterase remaining in the susceptible and resistant strains 6 hr after treatment with the five acaricides showed a marked correlation with resistance factors. Lower sensitivity of the enzyme *in vivo* accounts for the resistance of the resistant strain to these organophosphates.

I. INTRODUCTION

Studies of arthropod resistance to organophosphorus pesticides have frequently shown that survival of resistant strains has been aided by decreased rates of penetration and increased degradation of internal toxicant. Bigley (1966) showed that parathion-resistant strains of houseflies had an increased capacity to detoxify paraoxon, the toxicant, compared with susceptible strains. Mengle and Casida (1960), working with a strain of flies resistant to parathion, malathion, and diazinon, and also Farnham, Lord, and Sawicki (1965), working with a strain resistant to diazinon and diazoxon, revealed more complex mechanisms whereby the resistant strains were protected by increased detoxification, decreased rates of penetration, and possibly other factors. The demonstration by van Asperen, Mazijk, and Oppernorth (1964) of an enzyme capable of hydrolysing *in vivo* inhibitors, in a resistant strain of housefly, clarified the concept of increased detoxification as a resistance mechanism. Increased hydrolysis of internal malathion and malaoxon by a carboxyesterase and phosphatase in a resistant strain of *Culex tarsalis* was shown by Matsumura and Brown (1961) to be the mechanism of resistance to malathion in this mosquito. The work of Smissaert (1964) and Voss and Matsumura (1964) showed that organophosphorus-resistant spider mite survival was associated with either enhanced hydrolysis of *in vivo* inhibitors or with acetylcholinesterase which was insensitive to inhibitors.

* Division of Entomology, CSIRO, Veterinary Parasitology Laboratory, Yeerongpilly, Qld. 4105.

Roulston, Schuntner, and Schnitzerling (1966) demonstrated that metabolism of coumaphos in a susceptible strain of the Australian cattle tick, *Boophilus microplus* (Canestrini), followed the usual pattern of oxidation and hydrolysis and that acetylcholinesterase was the target. Lee and Batham (1966), working with strains of similar origin to those used in the present study, showed that their resistant strain contained acetylcholinesterase which was insensitive to several inhibitors *in vitro* compared with the enzyme of the susceptible strain. We have observed the same phenomenon in our Ridgeland strain (Schuntner *et al.*, unpublished data). A study of the metabolism of organophosphorus acaricides by such strains of tick, and observation of acetylcholinesterase levels *in vivo*, should demonstrate whether a simple or complex mechanism of resistance is operative. Accordingly a comparative study was made of penetration, metabolism, and anti-acetylcholinesterase effects of five radioactive organophosphorus acaricides in the susceptible strain and the resistant strain at doses intended to produce little mortality of susceptible larvae within the experimental period.

II. MATERIALS AND METHODS

(a) Tick Larvae

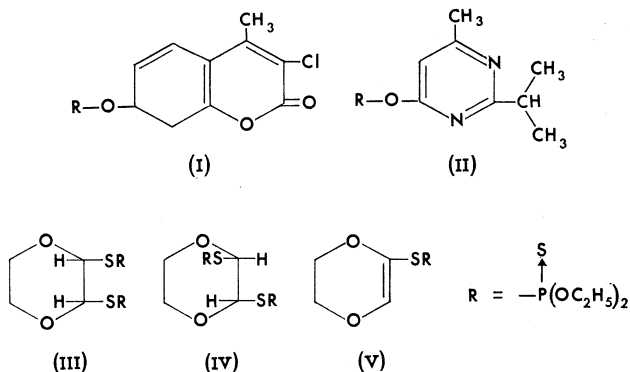
The Yeerongpilly strain cultured without contact with acaricides for more than 14 years was the reference susceptible strain.

The resistant Ridgeland strain originated on a cattle property adjacent to one which was the field source of the strain described by Shaw and Malcolm (1964) and subsequently referred to as the "M" strain by Lee and Batham (1966) and Shaw (1966). These resistant strains appeared in the field following the use of dioxathion. The Ridgeland strain has been maintained at this laboratory under continuous selection for resistance to dioxathion.

Larvae of both strains were used in experiments 10–14 days after hatching. In any one experiment susceptible and resistant larvae were of the same age.

(b) Chemicals

Coumaphos (I, 3-chloro-4-methyl-7-coumarinyl diethyl phosphorothionate) labelled with ^{32}P (Radiochemical Centre, Amersham, England) was shown to be radiochemically pure by paper chromatography in the system used for metabolite analysis and spectroscopically pure by infrared analysis. The specific activity was 5.5 mc/g at the commencement of experiments.



Diazinon (II, diethyl 2-isopropyl-6-methyl-4-pyrimidinyl phosphorothionate) was labelled with ^{14}C in the ethoxy group, and was purified by extracting a solution of the acaricide in

spectroscopic grade n-hexane twice with 50% (v/v) aqueous methyl cyanide and once with water to remove polar impurities. The hexane solution was dried over sodium sulphate and stored in a refrigerator. Paper chromatography in the appropriate system and ultraviolet spectroscopy showed the product to be pure. The specific activity was 3.48 mc/g.

[³²P]Dioxathion (Radiochemical Centre, Amersham) was obtained as the technical mixture of components (Arthur and Casida 1959). The *cis* (III) and *trans* (IV) isomers of dioxathion (1,4-dioxan-2,3-diyl bis(*O,O*-diethyl phosphorothiolothionate) and the dioxene derivative (V) were separated from the technical product by gradient elution with benzene-n-hexane from silicic acid and identified by infrared spectroscopy (Schuntner and Schnitzerling 1967). The specific activity at commencement of experiments was 14.95 mc/g.

Reference compounds for the probable *in vivo* hydrolytic metabolites from the acaricides, e.g. diethyl phosphoric, diethyl thiphosphoric, and diethyl dithiophosphoric acids, were purified by silicic acid chromatography (Schnitzerling and Schuntner 1965), and their *R_F* values were determined in the paper chromatographic systems employed for this study as was done by Roulston, Schuntner, and Schnitzerling (1966).

The oxygen analogue of coumaphos, prepared by bromine oxidation of coumaphos (Roulston, Schuntner, and Schnitzerling 1966), and the oxygen analogue of diazinon (diazoxon) were also used as chromatographic and counter-current distribution reference compounds respectively. As *trans*-dioxathion (IV) was the component present in the largest proportion in the technical mixture it was used in an attempt to produce an *in vitro* inhibitor using three methods: bromine oxidation of the compound in aqueous ethanol; catalytic oxidation in the model system used by Nakatsugawa and Dahm (1965) to oxidize parathion to paraoxon; irradiation of thin films of the compound by the unfiltered emission from a low-pressure mercury lamp for periods up to 24 hr.

Acetylthiocholine iodide (99% pure) was obtained from Koch-Light Laboratories, Ltd. 5,5'-dithiobis-2-nitrobenzoic acid ("research" grade) was obtained from Aldrich Chemical Co. Inc.

(c) *Acaricidal Treatment of Larvae*

Larvae were dipped in aqueous colloidal suspensions of acaricides, as detailed by Roulston, Schuntner, and Schnitzerling (1966). Triplicate subsamples of about 100 larvae each were taken at appropriate times for knockdown and mortality counting.

(d) *Extraction of Radioactive Compounds*

External and internal radioactive compounds in treated larvae were usually removed by acetone washes and cold methanol extraction respectively (Roulston, Schuntner, and Schnitzerling 1966). Some samples of larvae treated with *trans*-dioxathion were extracted with cold methyl cyanide which was directly used as the lower-phase sample in a countercurrent distribution system. Methanol extracts for paper chromatographic separation were evaporated at room temperature to small volume before spotting. Duplicate samples were used for each occasion shown in Figures 1-5.

(e) *Paper Chromatography Systems*

System I: Silicone 550 (stationary solvent); methyl cyanide-water (85 : 15 v/v) (mobile solvent) (Chamberlain, Gatterdam, and Hopkins 1960).

System II: Polyethylene glycol 400 (stationary solvent); upper phase of n-hexane-benzene-methanol-ammonia, sp. gr. 0.880 (65 : 24 : 10 : 1 v/v) (mobile solvent) (Kaplanis, Hopkins, and Treiber 1959).

(f) *Separation of Metabolites*

Extracts of coumaphos-treated larvae were chromatographed using system I to resolve hydrolysis products and system II to separate the oxygen analogue. Similarly extracts of diazinon-treated larvae were chromatographed in system I to resolve hydrolysis products and unchanged diazinon. The oxygen analogue was estimated by the counter-current system employed by Schuntner and Thompson (1966) to estimate diazoxon *in vivo*.

The same method was used for all three components of dioxathion. Extracts of treated larvae were separated with system I into the major hydrolysis products and unchanged acaricide. The *in vivo* inhibitors on chromatograms from *trans*-dioxathion treatments were detected using larval esterases as a modification of the enzyme-spray technique of McKinley and Johal (1963). Separation of the *in vivo* inhibitor was also attempted using methyl cyanide and n-hexane as solvent pair in a counter-current distribution apparatus.

TABLE 1

ACETYLCHOLINESTERASE ACTIVITY, MORTALITY, AND KNOCKDOWN IN RIDGELANDS AND YEERONGPILLY LARVAE 2 AND 6 HR AFTER ACARICIDAL TREATMENT

Strain	Treatment	Acetylcholinesterase Activity (%) [*]		Mortality (%) [†]		Knockdown (%) [‡]	
		After 2 hr	After 6 hr	After 2 hr	After 6 hr	After 2 hr	After 6 hr
Ridgeland Yeerongpilly	} Coumaphos (0.0004%)	35 ± 1.2	2.4 ± 0.8	0.24	9.5	0.84	89
		42 ± 0.9	0.8 ± 0.2	0.72	4.2	1.6	96
Ridgeland Yeerongpilly	} Diazinon (0.0005%)	52 ± 1.7	22 ± 1.8	0	3.2	0	0.30
		50 ± 2.2	0.9 ± 0.2	0.49	22	5.6	78
Ridgeland Yeerongpilly	} <i>cis</i> -Dioxathion (0.00045%)	36 ± 2.9	20 ± 2.0	0	0.24	0	0.24
		30 ± 1.3	0.5 ± 0.2	0.25	6.3	0	94
Ridgeland Yeerongpilly	} <i>trans</i> -Dioxathion (0.0016%)	58 ± 3.5	20 ± 1.6	0.22	0.72	0	0
		40 ± 2.2	0.9 ± 0.2	0.28	4.7	0	95
Ridgeland Yeerongpilly	} Dioxene derivative (0.0014%)	83 ± 2.0	28 ± 1.1	0.52	1.1	0	0
		57 ± 1.7	1.9 ± 0.3	0.18	24	0	54

^{*} Activity in acaricide-treated larvae as a percentage of the activity in untreated control larvae.

[†] Percentage of treated larvae showing no movement in a sample of about 300.

[‡] Percentage of treated larvae showing only occasional leg motion in a sample of about 300.

(g) Radioactivity Determinations

The radioactivity of aqueous colloids of ³²P-labelled acaricides used to treat larvae and of the run-off fluids was counted directly in an annular Geiger-Müller detector which operated an automatic scaler. Similar samples of ¹⁴C-labelled acaricides were made miscible in a 0.3% toluene solution of diphenyl oxazole, with absolute ethanol, for liquid-scintillation counting.

All paper chromatograms were scanned in an automatic 4π scanner.

(h) Acetylcholinesterase Assay

Aqueous homogenates of untreated control and acaricide-treated larvae were prepared by grinding 0.2-g samples in 2 ml of ice-cold water using an all-glass rotary homogenizer. These were diluted with 0.01M phosphate buffer, pH 7.0, to give final concentrations in the incubation mixture of 0.75 mg larvae/ml for Yeerongpilly larvae and 3.0 mg/ml for Ridgeland larvae. Acetylcholinesterase activity was estimated by the colorimetric method of Ellman *et al.* (1961). Approximately equal control enzyme activities were found for homogenates of both strains using the above dilutions.

Protection of acetylcholinesterase against excess inhibitor by homogenization of acaricide-treated larvae in substrate solution was avoided as an unnecessary complication. No differences

in enzyme activities were obtained by homogenization of treated larvae in the presence or absence of $10^{-2}M$ acetylthiocholine, nor was there any increase in inhibition in nonprotected homogenates on standing. Plapp and Bigley (1961) reported similarly for cholinesterase activities in homogenates of organophosphate-poisoned houseflies.

III. RESULTS

Mortality and knockdown of larvae subjected to the five acaricidal treatments are recorded in Table 1. In these experiments, Yeerongpilly larvae were not affected until 2.5–3 hr after treatment, when they rapidly developed signs of toxicosis. Larvae suffering knockdown were very sluggish but frequently showed some revival

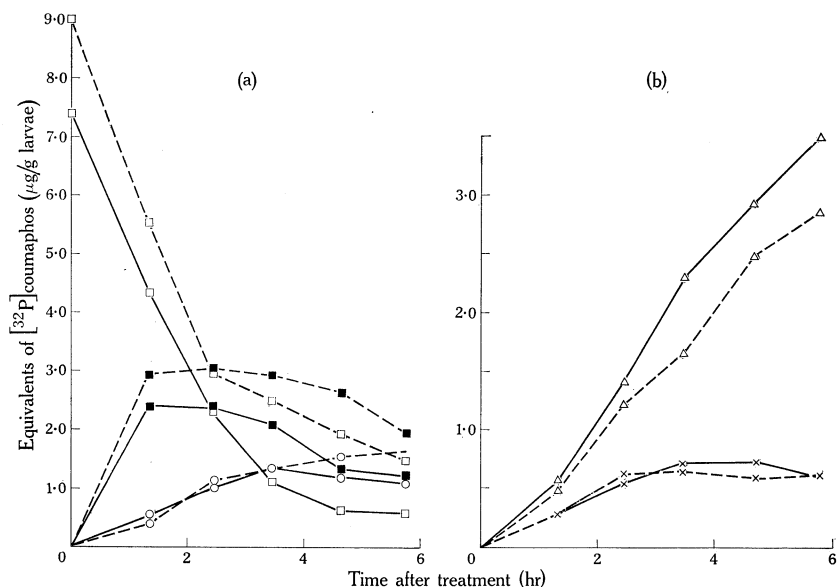


Fig. 1.—(a) Penetration and metabolism of $[^{32}P]$ coumaphos in Ridgeland (—) and Yeerongpilly (---) larval ticks after dipping in 0.0004% coumaphos colloid, as indicated by paper chromatographic separations. (b) Production of hydrolytic metabolites. □ “Outside” coumaphos. ■ “Inside” coumaphos. ○ Oxygen analogue of coumaphos. × Diethyl thiophosphate. △ Diethyl phosphate.

before dying. Samples showing a high percentage knockdown a few hours after treatment invariably had a high mortality within 24 hr. Also shown in Table 1 are the acetylcholinesterase activities, assayed at the same times as mortalities were estimated, and recorded as percentages of the activity in untreated control samples.

(a) Penetration and Metabolism of $[^{32}P]$ Coumaphos

$[^{32}P]$ Coumaphos penetrated both Yeerongpilly and Ridgeland larvae at almost equally rapid rates during the first 2.5 hr after treatment [Fig. 1(a)]. Thereafter penetration into Yeerongpilly larvae was at a slower average rate than that into Ridgeland larvae judging from the loss of “outside” coumaphos. The coumaphos “inside” rose to a peak at about 1.5 hr after treatment and then declined more

rapidly in Ridgeland larvae. The oxygen analogue of coumaphos, shown by Roulston, Schuntner, and Schnitzerling (1966) to be the *in vivo* toxicant in *B. microplus* larvae dosed with coumaphos, was produced in both strains at virtually the same rate for the first 3 hr. Then the amount in Ridgeland larvae diminished slightly while that in Yeerongpilly larvae showed a slight increase. The internal production of hydrolytic metabolites is shown in [Fig. 1(b)] where both strains have similar amounts of diethyl thiophosphate and of diethyl phosphate up to 2.5 hr after treatment. After this time some diminution of rate is evident in the Yeerongpilly strain for the hydrolysis product, diethyl phosphate.

TABLE 2

CORRELATION OF THE RATIOS (RIDGELANDS STRAIN: YEERONGPILLY STRAIN) OF ACETYLCHOLINESTERASE ACTIVITY (AS A PERCENTAGE OF UNTREATED CONTROL) IN LARVAE 6 HR AFTER ACARICIDAL TREATMENT WITH CORRESPONDING RESISTANCE FACTORS*

Treatment	(A) Ratio of Acetylcholinesterase Activities	(B) Resistance Factor	Ratio A/B
Coumaphos	2.9	1.3	2.2
Diazinon	25	12	2.1
<i>cis</i> -Dioxathion	37	19	1.9
<i>trans</i> -Dioxathion	23	7.6	3.0
Dioxene derivative	15	6	2.5

* The resistance factor is the ratio of the LD₅₀ values for Ridgeland to Yeerongpilly strains. LD₅₀ values were obtained from log probit plots of mortalities 24 hr after dipping larvae in acaricidal colloids. Duplicate samples were treated at concentrations to give 0-100% mortality. Approximately 300 larvae were subsampled from each duplicate for mortality counting.

(b) *Acetylcholinesterase Inhibition by, and Larval Response to, [³²P]Coumaphos*

There was greater reduction in acetylcholinesterase activity in Ridgeland than in Yeerongpilly larvae 2 hr after treatment with [³²P]coumaphos. At 6 hr, both strains had very low levels of enzyme activity, with the greatest reduction in Yeerongpilly strains. There was considerable knockdown in both strains and some mortality, this being higher in the Ridgeland strain. This disagrees with the resistance factor of 1.3 obtained from 24-hr mortality data (Table 2) but in view of this low level of resistance and the relatively high dose necessary to give some mortality within 6 hr the experimental results appear less contradictory.

(c) *Penetration and Metabolism of [¹⁴C]Diazinon*

It is shown in Figures 2(a) and 2(b) that, qualitatively, penetration and metabolism of [¹⁴C]diazinon proceeds as for coumaphos in the two strains. A striking quantitative difference was in the minor amount of metabolism to diethyl phosphate in diazinon-treated larvae, whereas in those treated with coumaphos diethyl phosphate was the major hydrolysis product. Diazoxon, the oxygen analogue of diazinon, has been shown by Schuntner and Thompson (1966) to be the only potent esterase inhibitor produced in diazinon-treated larvae *in vivo*. Figure 2(a) shows a slightly

greater rate of diazoxon production in the Ridgeland strain up to 2 hr whereas the hydrolytic products [Fig. 2(b)] are at about the same level in the two strains at this time. After 2 hr the rate of hydrolysis was lower in the Yeerongpilly strain than in the Ridgeland strain.

(d) *Acetylcholinesterase Inhibition by, and Larval Response to, [^{14}C]Diazinon*

The acetylcholinesterase levels 2 hr after treatment were about half the control levels in both strains, with only 0.49% mortality in Yeerongpilly strains. At 6 hr the Yeerongpilly larvae had less than 1% of control enzyme activity with still only 22% mortality but the remainder were knocked down. The Ridgeland larvae retained 22% of their enzyme activity and suffered only slight mortality.

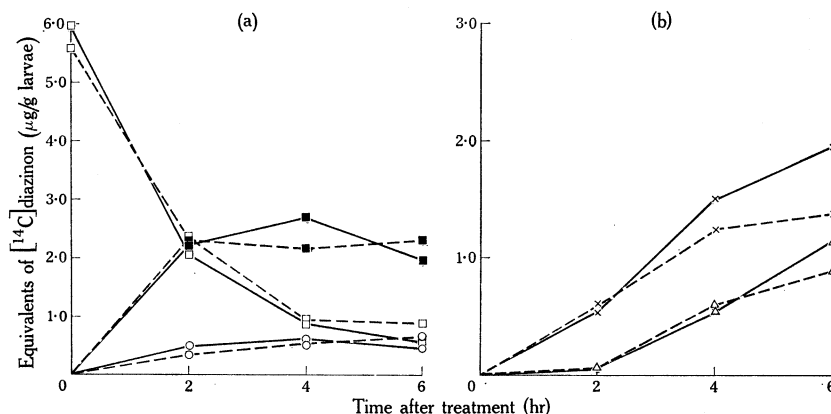


Fig. 2.—(a) Penetration and metabolism of [^{14}C]diazinon in Ridgeland (—) and Yeerongpilly (---) larval ticks after dipping in 0.0005% diazinon colloid, as indicated by paper chromatography and countercurrent distribution. (b) Production of hydrolytic metabolites. □ “Outside” diazinon. ■ “Inside” diazinon. ○ Oxygen analogue of diazinon (diazoxon). × Diethyl thiophosphate. △ Diethyl phosphate.

(e) *Penetration and Metabolism of cis-[^{32}P]Dioxathion*

Little or no difference between the strains was found in the penetration of *cis*-dioxathion into the larvae [Fig. 3(a)] and in the rates of metabolism to diethyl dithiophosphate, diethyl thiophosphate, and diethyl phosphate [Fig. 3(b)]. It is noteworthy that dithiophosphate was produced at a slower rate than was thiophosphate. The latter compound and the still further oxidized diethyl phosphate would no doubt be produced in part by oxidation prior to hydrolysis.

(f) *Acetylcholinesterase Inhibition by, and Larval Response to, cis-[^{32}P]Dioxathion*

Treatment of Ridgeland and Yeerongpilly larvae reduced the activity of acetylcholinesterase to 36 and 30% of control levels after 2 hr with no visible sign of poisoning. However, at 6 hr the enzyme activity had been almost eliminated from Yeerongpilly larvae, 94% of which were knocked down. The Ridgeland larvae retained almost 20% of enzyme activity at this stage and showed no visible sign of poisoning.

(g) *Penetration and Metabolism of trans-[³²P]Dioxathion*

Deposition and penetration of *trans*-dioxathion were both similar in the two strains [Fig. 4(a)] and there was no consistent difference between strains in the rate of hydrolysis to diethyl dithiophosphate [Fig. 4(b)]. Values for the oxidized hydrolysis products were consistently slightly lower for the Yeerongpilly strain. The relatively high proportion of dithiophosphate in the total of metabolites when compared with the corresponding proportion in *cis*-dioxathion-treated larvae [Fig. 3(b)] is of interest. The lower toxicity of the *trans* isomer is probably due in part to its being more rapidly hydrolysed than the *cis* isomer.

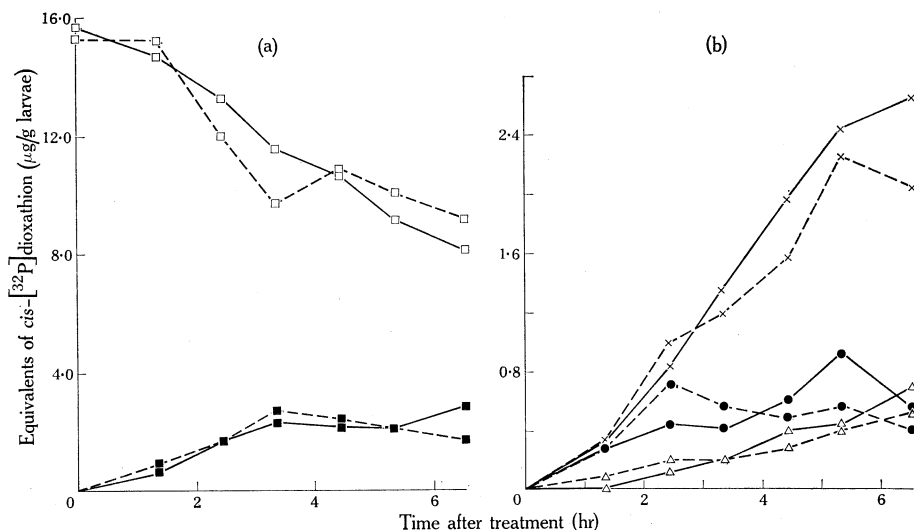


Fig. 3.—(a) Penetration and metabolism of *cis*-[³²P]dioxathion in Ridgeland (—) and Yeerongpilly (---) larval ticks after dipping in labelled 0.00045% *cis*-dioxathion colloid, as indicated by paper chromatographic separations. (b) Production of hydrolytic metabolites. □ “Outside” *cis*-dioxathion. ■ “Inside” *cis*-dioxathion. ● Diethyl dithiophosphate. × Diethyl thiophosphate. △ Diethyl phosphate.

(h) *Acetylcholinesterase Inhibition by, and Larval Response to, trans-[³²P]Dioxathion*

The treatment of larvae with *trans*-dioxathion produced results for acetylcholinesterase inhibition and mortality very similar to those obtained by treatment with the *cis* isomer except for a slower inhibition of enzyme activity. This was more marked in Ridgeland larvae which retained 58% of enzyme activity at 2 hr compared with 40% in the Yeerongpilly strain.

(i) *Penetration and Metabolism of [³²P]Dioxene Derivative*

The [³²P]dioxene derivative of dioxathion gave penetration and metabolism results for the two strains [Figs. 5(a) and 5(b)] which are scarcely distinguishable, apart from a slightly lower production of diethyl thiophosphate by the Yeerongpilly

strain which was more marked as incubation proceeded. As with *trans*-dioxathion treatment, there was a predominance of diethyl dithiophosphate in the hydrolysis metabolites.

(j) *Acetylcholinesterase Inhibition by, and Larval Response to, [³²P]Dioxene Derivative*

The inhibition of acetylcholinesterase was slower with this treatment than with any of the other treatments. The enzyme activity in treated Ridgeland's larvae was

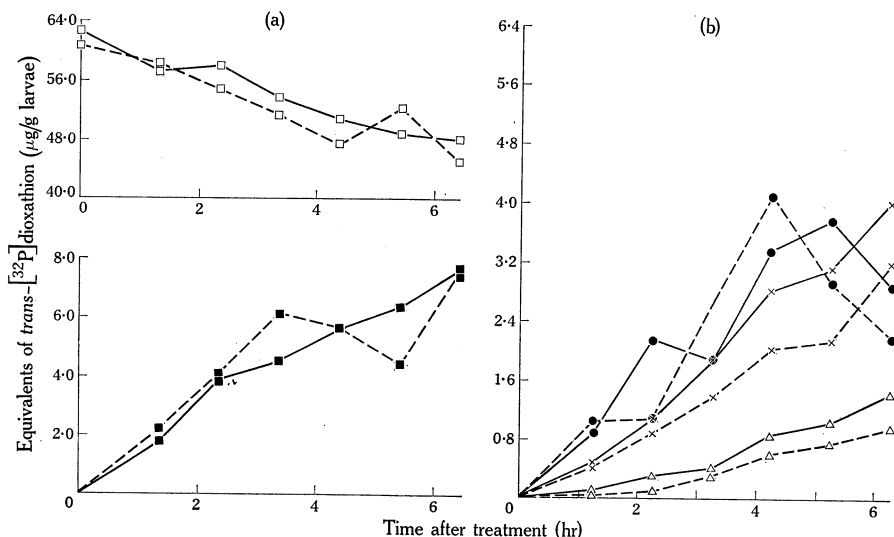


Fig. 4.—(a) Penetration and metabolism of *trans*-[³²P]dioxathion in Ridgeland's (—) and Yeerongpilly (---) larval ticks after dipping in labelled 0.0016% *trans*-dioxathion colloid, as indicated by paper chromatographic separations. (b) Production of hydrolytic metabolites. □ "Outside" *trans*-dioxathion. ■ "Inside" *trans*-dioxathion. ● Diethyl dithiophosphate. × Diethyl thiophosphate. △ Diethyl phosphate.

83% of controls 2 hr after treatment, and at the same time Yeerongpilly larvae retained 57% of activity with virtually no mortality or knockdown. By 6 hr, however, only about 2% of enzyme activity remained in the Yeerongpilly larvae which showed considerable knockdown and mortality. The Ridgeland's larvae retained 28% of enzyme activity at 6 hr and had a mortality of only 1% and no knockdown.

(k) *Correlation of Resistance Factors and Residual Acetylcholinesterase Activity*

In Table 2, column A, are shown the ratios (Ridgeland's strains: Yeerongpilly strains) of the percentage acetylcholinesterase activity remaining in larvae 6 hr after treatment with the five radioactive chemicals. Column B gives the resistance factors for Ridgeland's to Yeerongpilly strains. The ratios of the values for column A to column B (shown in column C) are approximately of the same order. It is evident that the ratio of the percentage of acetylcholinesterase activity remaining in Ridgeland's and in Yeerongpilly larvae shows some correlation with resistance factor.

(l) *Recovery of Radioactive Materials*

For each sampling occasion in Figures 1-5, the radioactive equivalents recovered as unchanged acaricide and metabolites were totalled (Table 3). The recoveries from the two strains were reasonably close for all acaricides except coumaphos for which the recovery was inexplicably low in the Ridgeland strain. Generally the two strains may be regarded as equally dosed.

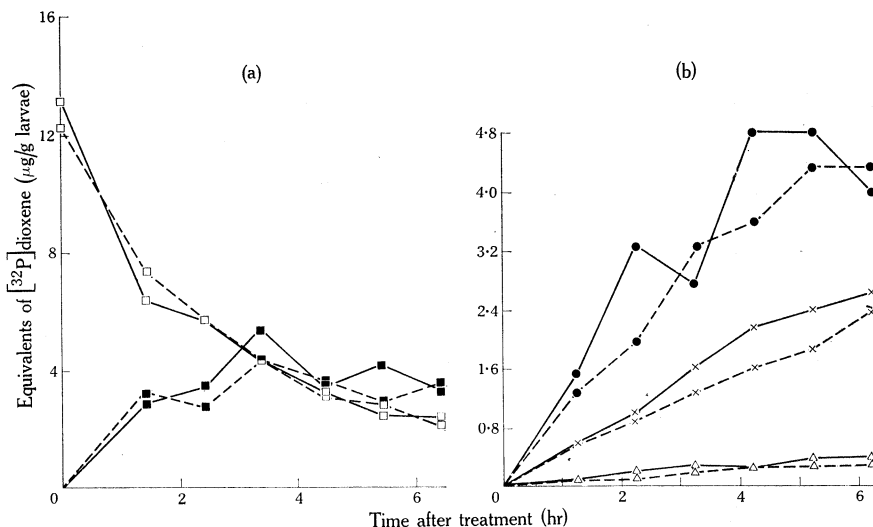


Fig. 5.—(a) Penetration and metabolism of [^{32}P]dioxene derivative of dioxathion in Ridgeland (—) and Yeerongpilly (---) larvae after dipping in labelled 0.0014% dioxene derivative colloid, as indicated by paper chromatographic separations. (b) Production of hydrolytic metabolites. □ “Outside” dioxene derivative. ■ “Inside” dioxene derivative. ● Diethyl dithiophosphate. × Diethyl thiophosphate. △ Diethyl phosphate.

(m) *Inhibitors from trans-Dioxathion*

Only qualitative results were obtained in the search for *in vivo* inhibitors produced in larvae treated with *trans*-dioxathion. Using the enzyme-spray technique, a narrow band of inhibition was demonstrated near the solvent front on paper chromatograms of extracts of treated larvae. This band was not resolved from the parent acaricide and with such an R_F value was no doubt heavily laden with larval extractives. Control chromatograms of extracts of untreated larvae gave similar, although less, inhibition, making interpretation uncertain. Counter-current distribution of methyl cyanide extracts of treated larvae gave more definite evidence of an inhibitor when, after 30 transfers, the tubes were assayed for esterase inhibitors. The inhibition value obtained was too great to be accounted for by the known amount of *trans*-dioxathion present in the same tubes. Here again the inhibitor was not separated from the parent compound. The percentage inhibition was calculated from control values obtained from a similar countercurrent distribution of an extract of untreated larvae.

The three methods of oxidation employed to produce an *in vitro* inhibitor from the parent compound gave the following results which suggest that oxidation of *trans*-dioxathion produces a readily hydrolysed inhibitor. Bromine oxidation yielded some hydrolysis products which were demonstrated by paper chromatography in system I, but no inhibitor was demonstrated when tested by the Gomori esterase assay method, and the aqueous catalytic system gave similar results. Irradiation of thin films of the acaricide by ultraviolet light yielded a product with readily detectable inhibition capacity, and also some water-soluble products. The total "inhibition yield" gave little prospect of obtaining enough product for purification, characterization, and comparison with the *in vivo* inhibitor.

TABLE 3

TOTAL RADIOACTIVE ACARICIDES RECOVERED FROM RIDGELANDS (R) AND YEERONGPILLY (Y) STRAINS ON SUCCESSIVE SAMPLING OCCASIONS

Recoveries given as μg -equivalents per gram larvae

Sampling Occasion	Coumaphos		Diazinon		<i>cis</i> -Dioxathion		<i>trans</i> -Dioxathion		Dioxene Derivative	
	R	Y	R	Y	R	Y	R	Y	R	Y
1	8.1	9.6	5.8	5.6	16.0	16.7	60.4	61.8	11.3	12.4
2	7.6	9.0	6.1	5.4	16.3	15.7	65.6	61.1	13.4	11.2
3	7.6	9.1	6.0	5.9	15.9	14.3	62.6	61.8	14.2	13.2
4	6.9	9.1			15.8	15.5	63.7	59.7	13.6	12.1
5	7.0	9.5			15.1	15.4	63.0	62.4	14.1	12.0
6					14.7	13.8	63.5	58.5	12.5	12.4

IV. DISCUSSION

Metabolism of phosphorothionates is generally recognized as involving oxidation to the corresponding oxygen analogues which are the active agents *in vivo*, and hydrolysis of parent compound and oxygen analogue to non-toxic phosphorus esters (O'Brien 1960). Such oxidation has been demonstrated in *B. microplus* larvae treated with coumaphos (Roulston, Schuntner, and Schnitzerling 1966), and in larvae treated with diazinon (Schuntner and Thompson 1966). The qualitative demonstration of an *in vivo* inhibitor in larvae treated with dioxathion components, and the almost complete inhibition of acetylcholinesterase in such larvae, strongly suggests that the phosphorothiolothionates are also metabolized to more potent cholinesterase inhibitors.

The studies of [^{32}P]coumaphos- and [^{14}C]diazinon-treated Yeerongpilly and Ridgeland larvae showed no interstrain difference in rates of penetration of acaricides, oxidation to toxic metabolites, or hydrolysis of either the parent compounds or oxygen analogues which would favour survival in the resistant strain. Comparison of acaricidal penetration and metabolism are most valid during the earlier portions of the incubation periods, before toxic effects are apparent. The similarity in the case of coumaphos is not surprising in view of the low resistance factor. The metabolism of diazinon for which the resistance factor was 12.4 was so similar in

the two strains that one must conclude that the resistance is not due to any gross metabolic differences.

Similar results were obtained for Ridgeland and Yeerongpilly larvae treated with the dioxathion components. Interstrain differences in penetration, hydrolysis of internal acaricides, and hydrolysis of oxidized compounds *in vivo* seem too small to confer any obvious mechanism of resistance. The possibility exists that the slightly greater production of oxidized hydrolysis products in Ridgeland larvae treated with *trans*-dioxathion and the dioxene derivative indicates enhanced hydrolysis of inhibitor at or near the target. An interesting feature of the metabolism of dioxathion components was the slow hydrolysis of the *cis* isomer to diethyl dithiophosphate when compared with the hydrolysis of the *trans* isomer and dioxene derivative. The predominant metabolite of the *cis* isomer was the oxidized product diethyl thiophosphate, and it seems possible that steric hindrance to enzymic hydrolysis of the C-S bond may be caused by the proximity of the *cis* ester groups, allowing oxidation to proceed preferentially. The metabolic similarity of the susceptible and resistant tick larvae contrasts with the interstrain differences usually reported for insecticide penetration and metabolism in susceptible and organophosphorus-resistant houseflies.

Acetylcholinesterase determinations made 2 hr after treatment of larvae showed that considerable reduction in enzyme activity was effected before the slightest signs of toxicosis appeared, and also indicated a difference in rates of inhibition *in vivo* in the two strains. Apart from the coumaphos-treated larvae there was a slower *in vivo* response to acaricides by the Ridgeland strain acetylcholinesterase, which is consistent with the idea of a resistant enzyme system. The low mortalities at 6 hr for all treatments made it unnecessary to correct the cholinesterase values for the possible lack of contribution of dead larvae in assays of homogenates, as the interstrain differences are far too great to be affected appreciably. The approximate correlation of resistance factors and ratios of remaining enzyme activity in Ridgeland and Yeerongpilly larvae 6 hr after treatment (Table 2) is in general accord with the findings of Lee and Batham (1966). These authors studied inhibition *in vitro* in homogenates of similar strains of larvae to those used here, and demonstrated a correlation between rates of *in vitro* inhibition and resistance factors for several organophosphorus oxons and a carbamate. The demonstration of an organophosphorus-resistant acetylcholinesterase in our Ridgeland strain and in the similarly derived "M" strain of Lee and Batham (1966), while contrasting with the apparent lack of such an insensitive target enzyme in houseflies, has a parallel in one type of spider mite resistance (Smitsaert 1964). The similarity is strengthened by the low levels of acetylcholinesterase in resistant cattle ticks, which were found by Lee and Batham and by the present authors to be 20-25% of the enzyme level in susceptible ticks. Smitsaert reported that the resistant spider mites containing insensitive acetylcholinesterase had only 33% of the amount of activity in susceptible strains.

It is concluded that, in view of the similar metabolic results for the two strains and the marked difference in acetylcholinesterase sensitivity *in vivo* and *in vitro*, resistance of the Ridgeland strain can be satisfactorily explained by insensitivity of acetylcholinesterase to inhibitors.

V. ACKNOWLEDGMENTS

The authors wish to thank Miss G. Mulligan and Mr. P. G. Thompson for assistance in Laboratory experiments; and Geigy (Australasia) Pty. Ltd. for the supply of diazoxon and [^{14}C]diazinon.

VI. REFERENCES

- ARTHUR, B. W., and CASIDA, J. E. (1959).—Biological activity and metabolism of Hercules AC-528 components in rats and cockroaches. *J. econ. Ent.* **52**, 20–7.
- ASPEREN, K. VAN, MAZIJK, M. VAN, and OPPERORTH, F. J. (1964).—Relation between electrophoretic esterase patterns and organophosphate resistance. *Entomologia exp. appl.* **8**, 163–74.
- BIGLEY, W. S. (1966).—Inhibition of cholinesterase and aliesterase in parathion and paraoxon poisoning in the house fly. *J. econ. Ent.* **59**, 60–5.
- CHAMBERLAIN, W. F., GATTERDAM, P. E., and HOPKINS, D. E. (1960).—Metabolism of [^{32}P]-Delnav in cattle. *J. econ. Ent.* **53**, 672–5.
- ELLMAN, G. L., COURTNEY, K. D., ANDRES, V., and FEATHERSTONE, R. M. (1961).—A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharm.* **7**, 88–95.
- FARNHAM, A. W., LORD, K. A., and SAWICKI, R. M. (1965).—Study of some of the mechanisms connected with resistance to diazinon and diazoxon in a diazinon-resistant strain of house flies. *J. Insect Physiol.* **11**, 1475–88.
- KAPLANIS, J. M., HOPKINS, D. E., and TREIBER, C. H. (1959).—Dermal and oral treatments of cattle with phosphorus-32 labelled Co-ral. *J. Agric. Fd Chem.* **7**, 483–6.
- LEE, R. M., and BATHAM, P. (1966).—The activity and organophosphate inhibition of cholinesterases from susceptible and resistant ticks (Acari). *Entomologia exp. appl.* **9**, 13–24.
- MATSUMURA, F., and BROWN, A. W. A. (1961).—Biochemistry of malathion resistance in *Culex tarsalis*. *J. econ. Ent.* **54**, 1176–85.
- MCKINLEY, W. P., and JOHAL, P. S. (1963).—Esterase inhibition technique for detection of organophosphorus pesticides. *J. Ass. off. agric. Chem.* **46**, 840–2.
- MENGLE, D. C., and CASIDA, J. E. (1960).—Biochemical factors in the acquired resistance of house flies to organophosphate insecticides. *J. Agric. Fd Chem.* **8**, 431–7.
- NAKATSUGAWA, T., and DAHM, P. A. (1965).—Parathion activation enzymes in the fat body microsomes of the American cockroach. *J. econ. Ent.* **58**, 500–9.
- O'BRIEN, R. D. (1960).—“Toxic Phosphorus Esters.” (Academic Press, Inc.: New York.)
- PLAPP, F. W., and BIGLEY, W. S. (1961).—Inhibition of house fly aliesterase and cholinesterase under *in vivo* conditions by parathion and malathion. *J. econ. Ent.* **54**, 103–8.
- ROULSTON, W. J., SCHUNTNER, C. A., and SCHNITZERLING, H. J. (1966).—Metabolism of coumaphos in larvae of the cattle tick *Boophilus microplus*. *Aust. J. biol. Sci.* **19**, 619–33.
- SCHNITZERLING, H. J., and SCHUNTNER, C. A. (1965).—Column chromatography of acid esters of phosphoric acid in a non-aqueous system. *J. Chromat.* **20**, 621–3.
- SCHUNTNER, C. A., and SCHNITZERLING, H. J. (1967).—Separation of [^{32}P]dioxathion components on a silicic acid column. *J. Chromat.* **27**, 272–5.
- SCHUNTNER, C. A., and THOMPSON, P. G. (1966).—Detection and estimation of [^{14}C]diazoxon *in vivo* by countercurrent distribution and inhibition assay. *Biochem. biophys. Acta* **115**, 225–7.
- SHAW, R. D. (1966).—Culture of an organophosphorus-resistant strain of *Boophilus microplus* (Can.) and an assessment of its resistance spectrum. *Bull. ent. Res.* **56**, 389–405.
- SHAW, R. D., and MALCOLM, H. A. (1964).—Resistance of *Boophilus microplus* to organophosphorus insecticides. *Vet. Rec.* **76**, 210–1.
- SMISSAERT, H. R. (1964).—Cholinesterase inhibition in spider mites susceptible and resistant to organophosphate. *Science, N.Y.* **143**, 129–31.
- VOSS, G., and MATSUMURA, F. (1964).—Resistance to organophosphorus compounds in the two-spotted spider mite: two different mechanisms of resistance. *Nature, Lond.* **202**, 319–20.

