ACETYLCHOLINESTERASE INSENSITIVITY IN THE BIARRA STRAIN OF THE CATTLE TICK *BOOPHILUS MICROPLUS*, AS A CAUSE OF RESISTANCE TO ORGANOPHOSPHORUS AND CARBAMATE ACARICIDES

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[*Manuscript received December* 20, 1967]

Summary

The penetration and metabolism of diazinon in cattle tick larvae of the susceptible Yeerongpilly strain and of the organophosphorus- and carbamate-resistant Biarra strain showed no difference that could account for the resistance of the Biarra strain to this acaricide. Several compounds known to synergize the effect of carbamates against houseflies did not increase the toxicity of carbaryl to larvae of the Biarra strain, suggesting that detoxification was not a factor in resistance.

Higher dosing of Biarra larvae was necessary to produce inhibitory effects on acetylcholinesterase activity similar to those produced in Yeerongpilly larvae for any of four acaricides tested. The acetylcholinesterase in homogenates of resistant larvae was inhibited by three organophosphates and a carbamate at a much slower rate than that of susceptible larvae. The ratios of rate constants for the first-order inhibition reactions indicated considerable enzyme insensitivity in the Biarra strain.

Acetylcholinesterase insensitivity to organophosphate and carbamate inhibitors seemed sufficient to indicate it as the major mechanism of resistance in the Biarra strain.

I. INTRODUCTION

It is accepted that organophosphorus and carbamate pesticides exert their toxic effect on arthropods by inhibition of cholinesterases and this has been confirmed for the cattle tick (Roulston, Schuntner, and Schnitzerling 1966). The appearance of an organophosphorus-resistant "M" strain prompted investigation by Lee and Batham (1966) who demonstrated greater insensitivity of acetylcholinesterase to inhibition *in vitro* in larvae of this strain than in larvae of a susceptible reference strain. Larvae of the resistant Ridgelands strain, similar in origin to the "M" strain, were shown by Schuntner, Roulston, and Schnitzerling (1968) to possess acetylcholinesterase less sensitive to inhibition *in vivo* compared with that in larvae of the susceptible reference Yeerongpilly strain. As no marked differences were observed in studies of penetration and metabolism of organophosphorus acaricides in these two strains, the authors concluded that relative insensitivity of acetylcholinesterase to inhibition in the Ridgelands strain was the cause of resistance.

Another strain of cattle tick, the Biarra strain, was shown by Roulston and Wharton (1967) to have higher levels and a wider spectrum of resistance to organophosphorus and carbamate compounds than the Ridgelands strain. The present paper describes the investigations made to determine the cause of this resistance in

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the Biarra strain of ticks. Comparisons of Biarra and Yeerongpilly larvae were made in studies of acaricide penetration and metabolism, effects of acaricide treatment on acetylcholinesterase *in vivo*, effects of organophosphates and a carbamate on acetylcholinesterase *in vitro*, and effects of potential synergists on carbamate toxicity. As it was reported that Ridgelands type larvae contained less acetylcholinesterase activity than susceptible larvae (Lee and Batham 1966; Schuntner, Roulston, and Schnitzerling 1968), a comparison of activities in Biarra, Ridgelands, and Yeerongpilly larvae was made.

II. Materials and Methods

(*a*) *Strains of Ticks*

The Yeerongpilly strain cultured without contact with chemicals for over 14 years was the reference susceptible strain. The resistant Biarra strain originated on a property in the Brisbane Valley, and has been in culture since March 1966. The strain was originally heterogeneous but larvae were selected with coumaphos prior to infesting cattle, and the larvae used in these studies included less than 1% susceptible individuals. The resistant Ridgelands strain was that described by Schuntner, Roulston, and Schnitzerling (1968). This strain was composed only of homozygous resistant ticks (Stone 1968). Larvae of all strains were used 8 to 16 days after hatching.

(*b*) *Acaricides and Synergists*

The following acaricides were used for treating larvae in dosage mortality tests and in studying their effects on acetylcholinesterase *in vivo*:

Diazinon: 2-isopropyl-6-methylpyrimidinyl diethyl phosphorothionate;
Dursban: 3,5,6-trichloro-2-pyridyl diethyl phosphorothionate;
Coumaphos: 3-chloro-4-methyl-7-coumarinyl diethyl phosphorothionate;
Carbaryl: 1-naphthyl *N*-methylcarbamate.

[*ethoxy*-^{14}C]Diazinon* (3·48 mCi/g) was used for dosing larvae in penetration and metabolism studies. Carbaryl and the oxygen analogues of diazinon (diazoxon), Dursban (Dursbanoxon), and coumaphos (coroxon) were used as inhibitors to study the kinetics of inhibition of acetylcholinesterase *in vitro*.

The following compounds† were tested as synergists of carbaryl:

(1) 6-propyl-5-(5,8,11-trioxydodecanyl)-1,3-benzodioxole (piperonyl butoxide);
(2) 5-acetoxy-1,3-benzodioxole;
(3) 5-formyl-1,3-benzodioxole (piperonal);
(4) 5-allyl-1,3-benzodioxole (Safrole);
(5) 5-acetoxymethyl-1,3-benzodioxole (piperonal acetate);
(6) 5-octylsulphinylisopropyl-1,3-benzodioxole (Sulphoxide);
(7) 5-(3,6,9-trioxyundecan-10-yl)-1,3-benzodioxole (Sesamex);
(8) 5-carboxy-1,3-benzodioxole (piperonylic acid);
(9) 5-methoxycarbonylmethyl-1,3-benzodioxole;
(10) 5-dimethylamino-6-nitro-1,3-benzodioxole;
(11) 5-methylamino-6-nitro-1,3-benzodioxole;
(12) 5-bromo-6-methoxy-1,3-benzodioxole;
(13) 5-nitro-1,3-benzodioxole;
(14) 6-chloro-5-nitro-1,3-benzodioxole;
(15) 4,5,6,7-tetrachloro-1,3-benzodioxole.

* Supplied by Geigy (A'asia) Pty. Ltd.

† Compounds (1)–(9) were supplied by Dr. Beroza, Entomology Research Division, U.S.D.A. Beltsville, Maryland, and compounds (10)–(15) were supplied by Dr. C. F. Wilkinson, Department of Entomology, Comstock Hall, Cornell University, Ithaca, New York.

(c) *Acaricidal Treatment of Larvae*

Larvae were dosed by dipping in aqueous suspensions of the acaricides (Roulston, Schuntner, and Schnitzerling 1966) for dosage mortality tests, and for studies on penetration, metabolism, and the effects of acaricide dosing on acetylcholinesterase *in vivo*. In toxicity tests LC_{50} values* were determined from the plots of log concentration of acaricide against the probit of mortality after 24 hr. In tests for synergism the potential synergist was added to the suspensions of carbaryl in the ratio of 5 : 1 synergist to acaricide.

(d) *Penetration and Metabolism of [^{14}C]Diazinon*

Larvae were dipped in 0·0004% w/v [^{14}C]diazinon. Rates of penetration and metabolism were determined by methods previously described (Schuntner, Roulston, and Schnitzerling 1968).

(e) *Acetylcholinesterase Activity*

This was determined in homogenates which were prepared by grinding ticks in glass-distilled water (0·1 g/ml) using an ice-cooled all-glass homongenizer.

(i) *Determination of Activity*

The activity of homogenates towards acetylthiocholine (0·001M) was determined by the colorimetric method of Ellman *et al.* (1961) at 27°C and pH 7·0. Homogenates of Yeerongpilly, Ridgelands, and Biarra larvae were diluted with 0·1M phosphate buffer (pH 7·0) to contain 0·70, 3·45, and 1·40 mg of larvae/ml respectively. The activity towards acetylcholine (0·02M) was determined by a pH-stat method at 27°C in which liberated acetic acid was titrated with CO_2-free sodium hydroxide to pH 7·0. Homogenates of larvae of all strains were diluted with CO_2-free glass-distilled water to contain 12 mg/ml.

(ii) *Inhibition of Activity* in vivo

Yeerongpilly and Biarra larvae were treated with diazinon, Dursban, coumaphos, and carbaryl at various doses. The acetylcholinesterase levels in homogenates were determined subsequently at various intervals over a period during which there was no mortality of larvae.

Carbaryl is an inhibitor *per se* and larvae treated with this acaricide were washed with acetone to remove the sometimes large external residue prior to homogenization. Earlier experiments had shown no difference in acetylcholinesterase activity of homogenates of organophosphorus- or carbaryl-treated larvae whether homogenized in water or in 0·01M acetylthiocholine solution. Therefore substrate was not used in the homogenizing medium.

(iii) *Inhibition of Activity* in vitro

Solutions of the inhibitors were prepared in ethanol containing 2% Triton X-100 and added to homogenates of Biarra and Yeerongpilly larvae in the ratio of 1 : 100. The residual acetylcholinesterase activity towards acetylthiocholine was measured at 2-min intervals over a period of 12 min, at 27°C. The log of residual activity was plotted against time of inhibition and the bimolecular rate constants (k_2) were calculated according to Aldridge (1950).

III. Results

(a) *Penetration and Metabolism of [^{14}C]Diazinon*

The results of the study of penetration and metabolism of [^{14}C]diazinon in Yeerongpilly and Biarra larvae are shown in Figure 1. They clearly show no marked difference in penetration of acaricide, oxidation to diazoxon *in vivo*, or hydrolysis to the non-toxic diethyl thiophosphate and diethyl phosphate which could reasonably account for the 64-fold resistance (see Table 2) of Biarra larvae to diazinon.

* Concentration of acaricide required to kill 50% of larvae.

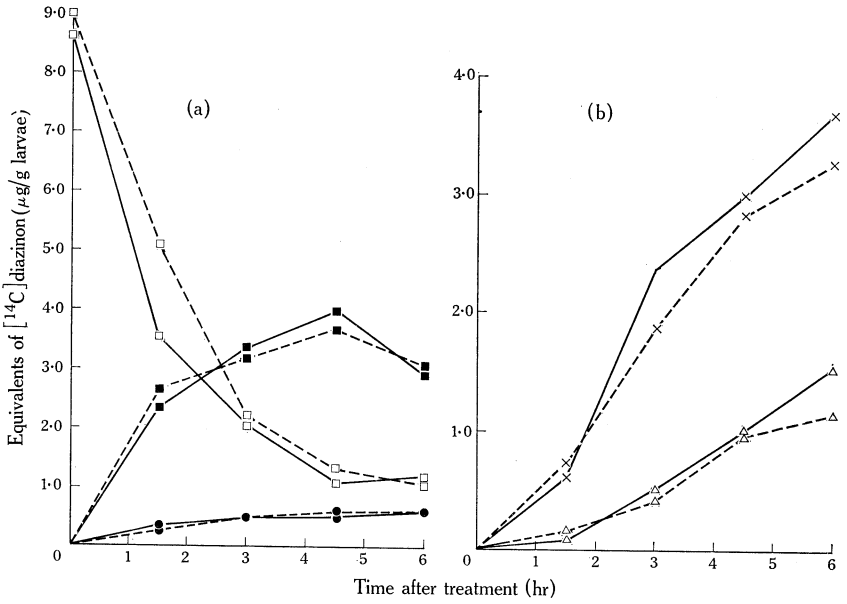

Fig. 1.—(*a*) Penetration and metabolism of [^{14}C]diazinon in Biarra (——) and Yeerongpilly (- - -) larvae after dipping in 0·0004% diazinon colloid as indicated by paper chromatography and countercurrent distribution. □ Diazinon outside larvae. ■ Diazinon inside larvae. ● Diazoxon. (*b*) Production of hydrolytic metabolites. × Diethyl thiophosphate. △ Diethyl phosphate.

TABLE 1

ACETYLCHOLINESTERASE ACTIVITIES OF YEERONGPILLY, BIARRA, AND RIDGELANDS LARVAL HOMOGENATES

Comparison of activities towards 0·02M acetylcholine (pH-stat) and 0·001M acetylthiocholine (colorimetric) at 27°C

Strain	Acetylcholine Hydrolysed (A) (μmoles/g/hr)*	Acetylthiocholine Hydrolysed (B) (μmoles/g/hr)†	Ratio A/B (%)
Yeerongpilly	219	421·6	
	242	425·2	
	209	440·9	
Mean	223	429·2	52·0
Ridgelands	20·9	56·7	
	19·4	62·1	
	22·7	55·4	
Mean	21·0	58·1	36·1
Biarra	23·4	158·0	
	19·0	162·6	
	26·8	157·5	
Mean	23·1	159·4	14·5

* Ratios of acetylcholine hydrolysed by Ridgelands and Biarra strains relative to Yeerongpilly strain are 9·4 and 10·4% respectively.

† Ratios of acetylthiocholine hydrolysed by Ridgelands and Biarra strains relative to Yeerongpilly strain are 13·5 and 37·1% respectively.

(*b*) *Acetylcholinesterase Activity in Larval Homogenates*

The acetylcholinesterase activities of homogenates of Yeerongpilly, Ridgelands, and Biarra larvae towards acetylcholine and acetylthiocholine are shown in Table 1. With acetylcholine as substrate, Ridgelands and Biarra homogenates were equally active, but only one-tenth as active as Yeerongpilly homogenate. Towards acetylthiocholine Biarra homogenate was about one-third and Ridgelands about one-seventh as active as Yeerongpilly homogenate. In each of the strains the activity was markedly lower towards acetylcholine than towards acetylthiocholine, the difference being least in Yeerongpilly and greatest in Biarra homogenate.

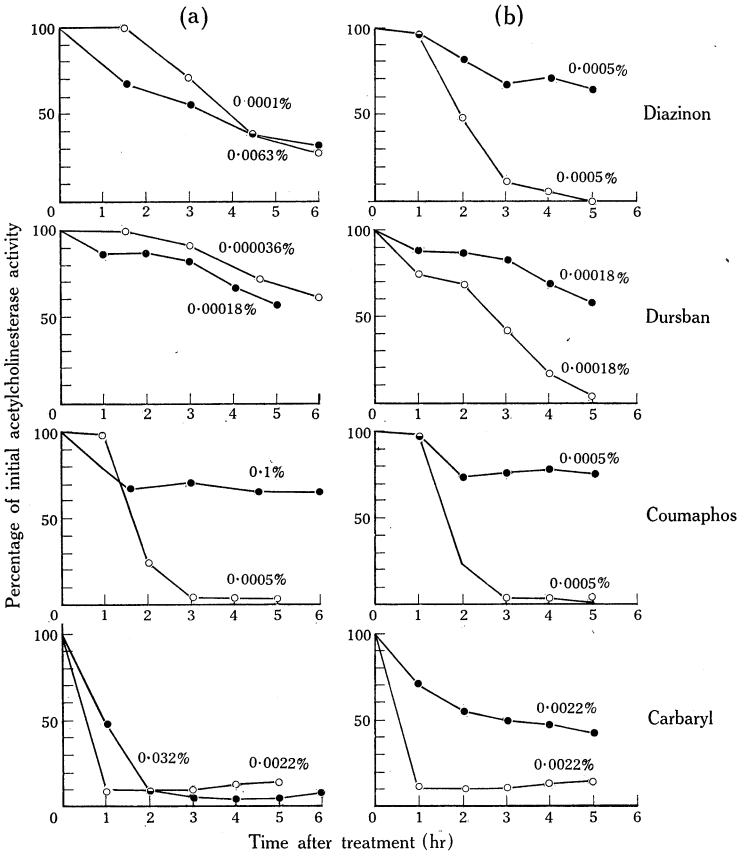

Fig. 2.—Effect of approximately equi-effective doses (*a*) and equal doses (*b*) of diazinon, Dursban, coumaphos, and carbaryl on the acetylcholinesterase activity *in vivo* of Biarra (●) and Yeerongpilly (○) larvae. For coumaphos 0·1% is the highest stable colloidal concentration possible.

(*c*) *Inhibition* in vivo *of Acetylcholinesterase Activity of Acaricide-treated Larvae*

The results of this experiment are shown in Figure 2. A very marked difference in inhibition of acetylcholinesterase was obvious in the two strains dosed equally by any one of the four acaricides. Five hours after treatment little enzyme activity remained in Yeerongpilly larvae compared to Biarra larvae. Concentrations of

acaricide which produced approximately equal inhibitory effects on the acetylcholinesterase activity of larvae of both strains were markedly different. The ratios of these concentrations (Biarra/Yeerongpilly) are shown in Table 2.

There was a reasonable correlation of these ratios with resistance factors (LC_{50} Biarra/LC_{50} Yeerongpilly). Figure 2 shows that there was reversal of inhibition of acetylcholinesterase in larvae of both strains dosed with carbaryl. This type of response *in vivo* is consistent with the known reversibility of carbamate-inhibited acetylcholinesterase *in vitro*.

TABLE 2

BIARRA RESISTANCE FACTORS: INHIBITION DATA FOR BIARRA (B) AND YEERONGPILLY (Y) LARVAE *in vivo* AND *in vitro*

Acaricide	Resistance Factor*	Concn. Ratio†	k_2‡(B)	k_2‡(Y)	k_2(Y)/k_2(B)
Diazinon	64	63	$8{\cdot}9\times10^{2}$	$7{\cdot}6\times10^{4}$	85
Dursban	8·3	5	$1{\cdot}9\times10^{5}$	$2{\cdot}3\times10^{7}$	126
Coumaphos	1000	>200	$6{\cdot}7\times10^{2}$	$1{\cdot}7\times10^{5}$	248
Carbaryl	14·5	14·5	$2{\cdot}0\times10^{3}$§	$4{\cdot}6\times10^{5}$§	228§

* LC_{50} Biarra/LC_{50} Yeerongpilly.

† Ratio of acaricide concentrations (obtained from Fig. 2) which produced approximately equal inhibitory effects on acetylcholinesterase *in vivo* in the two strains (B/Y).

‡ Bimolecular rate constant (Aldridge 1950) for acetylcholinesterase inhibition by oxygen analogue of acaricide *in vitro* (litre $mole^{-1}$ min^{-1}).

§ An approximate value only, due to reversibility of inhibition by carbaryl.

(*d*) *Kinetics of Acetylcholinesterase Inhibition* in vitro

Diazoxon, Dursbanoxon, coroxon, and carbaryl were used as inhibitors of acetylcholinesterase in homogenates of Biarra and Yeerongpilly larvae. The experimental conditions employed minimized loss of activity in controls during the experimental period and the loss was never greater than 5%.

The results depicted in Figure 3 show essentially first-order reaction rates for the irreversible inhibition of enzyme activity in homogenates of both strains by the organophosphorus inhibitors, and some deviation from such rates by the reversible inhibition due to carbaryl. By extrapolation of the lines to zero time the ordinate was intercepted at 100% activity for diazoxon and Dursbanoxon, and at 90% for coroxon inhibition of Yeerongpilly enzyme. By extrapolation of the corresponding lines for Biarra enzyme the ordinate was intercepted at 70–80%. These results were interpreted as indicating a possible heterogeneity of the acetylcholinesterase activity, with 20–30% of the enzyme being present in a more sensitive form. The bimolecular rate constants are presented in Table 2 where a comparison may be made of the k_2 Yeerongpilly/k_2 Biarra ratios and resistance factors for the corresponding acaricides. Although the two sets of values did not correlate well, the relative insensitivity of Biarra acetylcholinesterase to inhibition *in vitro* was clearly demonstrated.

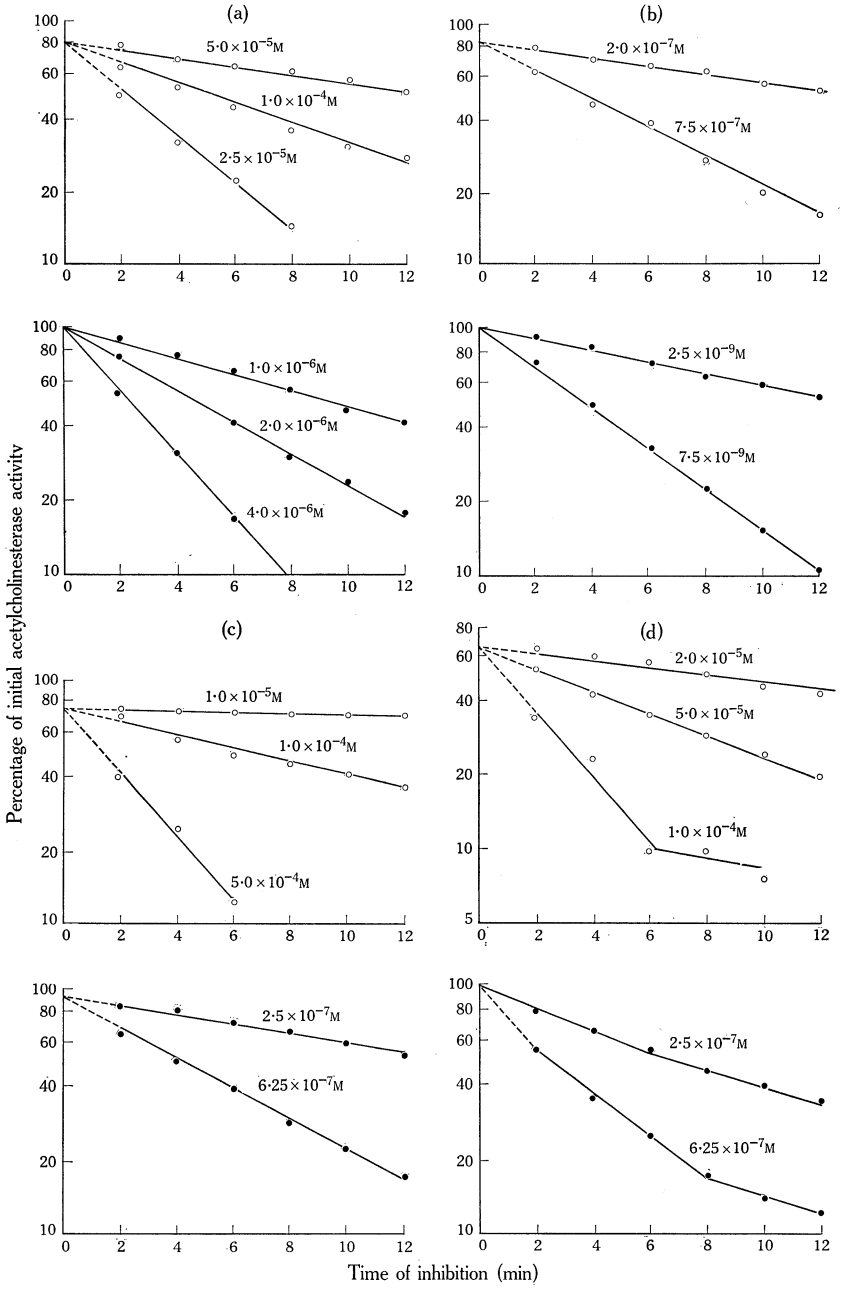

Fig. 3.—Effect of various inhibitors on the acetylcholinesterase activity *in vitro* of Biarra (○) and Yeerongpilly (●) larvae. (*a*) diazoxon; (*b*) Dursbanoxon; (*c*) coroxon; (*d*) carbaryl.

(*e*) *Synergism*

Fifteen derivatives of 1,3-benzodioxole were tested [see Section II(*b*)] but none synergized the toxicity of carbaryl to Biarra larvae.

IV. Discussion

There is little doubt that the Biarra enzyme is less sensitive to inhibition than the Yeerongpilly enzyme *in vivo* since with equal dosing of both strains by the four acaricides there was always less inhibition of acetylcholinesterase activity in Biarra larvae than in Yeerongpilly larvae (Fig. 2). The correlation between resistance factors and the ratios in Biarra and Yeerongpilly larvae of acaricide concentrations producing approximately equal inhibitory effects on acetylcholinesterase activity *in vivo* (Table 2) suggests that acetylcholinesterase is the target system in the poisoning of ticks of both strains by the organophosphorus compounds and carbaryl and that insensitivity of acetylcholinesterase in Biarra larvae to inhibition *in vivo* is causally related to resistance. Although insensitivity to inhibition *in vivo* does not necessarily mean that an enzyme system is insensitive to inhibition *in vitro*, the kinetic studies of the inhibition of acetylcholinesterase *in vitro* by the inhibitors corresponding to acaricides used in studies *in vivo* amply confirmed such insensitivity in the Biarra enzyme system compared with the Yeerongpilly system. The acetylcholinesterase from the Ridgelands strain was found to have a sensitivity to these inhibitors intermediate between the enzymes of the Biarra and Yeerongpilly strains (Roulston *et al.*, unpublished data). The Yeerongpilly/Biarra ratios for k_2 values do not correspond closely with resistance factors for the parent acaricides. Considering the number of steps involved between deposition of pesticide and inhibition of acetylcholinesterase *in vivo*, as discussed by Hollingworth, Fukuto, and Metcalf (1967), it is not surprising that some discrepancies appear in such a comparison.

Other factors that could have contributed causally to resistance in Biarra larvae were those of penetration and metabolism. These were compared in Yeerongpilly and Biarra larvae using [^{14}C]diazinon and little difference was found. The rates of penetration and metabolism varied little between strains and the metabolites diazoxon, diethylthiophosphoric acid, and diethylphosphoric acid were found in similar quantities in larvae of both strains. The lack of marked differences between Yeerongpilly and Biarra larvae concerning any aspect of penetration and metabolism of [^{14}C]diazinon indicated that neither of these factors was a major cause of resistance of Biarra larvae to this acaricide. Diazinon, coumaphos, and Dursban are closely related structurally. Thus it is most likely that the pattern of penetration and metabolism of coumaphos and Dursban would follow closely that of diazinon in Yeerongpilly and Biarra larvae. A detoxification mechanism could have contributed to the resistance of Biarra larvae to carbaryl. This possibility was investigated by the use of synergists of carbaryl. An increased response would have been expected if detoxification had been a defence mechanism (Metcalf 1967). Several derivatives of 1,3-benzodioxole which strongly synergized carbamates against resistant houseflies with this type of defence (Georghiou and Metcalf 1961; Georghiou, Metcalf, and March 1961) failed to synergize carbaryl against Biarra larvae, indicating that detoxification was an unlikely defence mechanism.

The organophosphorus resistant "M" strain (Lee and Batham 1966) and the similar Ridgelands strain (Schuntner, Roulston, and Schnitzerling 1968) contained lower native levels of acetylcholinesterase than susceptible strains; hence it is not surprising that Biarra enzyme activity differed from that of Yeerongpilly by exhibiting 60% less activity towards acetylthiocholine (Table 1). Enzymes from each of the three strains hydrolyse acetylcholine more slowly than acetylthiocholine. In this respect all three strains differ markedly, which suggests that their acetylcholinesterase systems are different. The relative rates of hydrolysis of acetylcholine and acetylthiocholine shown in Table 1 are inversely related to the resistance of the strains. Thus there is a possibility that the factors responsible for these substrate–specificity variations may also be involved in determining the degree of insensitivity to inhibition.

The overall picture emerges of Biarra ticks possessing an acetylcholinesterase system relatively insensitive to organophosphate and carbamate inhibitors. The evidence points to this insensitivity being the major, if not the only, cause of the resistance of Biarra ticks to organophosphorus and carbamate acaricides.

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