BRAIN CHOLINESTERASE ACTIVITY AND ITS INHERITANCE IN CATTLE TICK (*BOOPHILUS MICROPLUS*) STRAINS RESISTANT AND SUSCEPTIBLE TO ORGANOPHOSPHORUS ACARICIDES

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[*Manuscript received August* 30, 1967]

Summary

Brain acetylcholinesterase (AChE) activities of individual cattle ticks, *B. microplus*, of an organophosphorus-resistant strain were compared with those of a standard reference strain. When measured by a histochemical–densitometric method on photographic transparencies and by a biochemical method, brains from homozygous resistant adult female ticks had about 12% of the AChE activity of brains from homozygous susceptible ticks. Brains of hybrid adult females had about 78%, histochemically and biochemically, of the AChE activity of their susceptible parents, indicating that low AChE activity was incompletely recessive although the associated resistance to organophosphorus compounds had been shown previously to be incompletely dominant.

The ratios of brains with high and low AChE activities obtained for backcross and F_2 progenies led to the conclusion that brain AChE levels were controlled by a single pair of autosomal alleles.

The results of selection of a heterogeneous resistant strain for low brain AChE activity, together with the results of double backcrossing to ticks with low brain AChE activity, provide evidence that low brain AChE levels and resistance to organophosphorus compounds are pleiotropic effects of the same gene or are controlled by two closely linked genes.

I. Introduction

Resistance to organophosphorus acaricides in the cattle tick *Boophilus microplus* (Canestrini) was reported by Shaw and Malcolm (1964) and has been shown by Stone (1968) to be controlled by a single incompletely dominant gene. Homogenates of organophosphorus-resistant cattle tick larvae were found by Roulston (personal communication) and by Lee and Batham (1966) to have much lower acetylcholinesterase (AChE) activities than homogenates of susceptible larvae. The present investigation was started in an attempt to relate brain AChE activity and resistance of the various genotypes to organophosphorus compounds, using histochemical techniques.

II. Methods

The field history of the organophosphorus-resistant Ridgelands strain, R, which developed as a result of selection pressure with dioxathion and which exhibits varying degrees of cross-resistance to carbaryl, carbophenothion, diazinon, dioxathion, and formothion has already been reported (Roulston *et al.* 1968). Brains dissected from adult ticks of organophosphorus-resistant and susceptible strains, fixed in acetone, and tested histochemically by the indoxyl acetate

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method for esterases (Pearse 1960) showed a marked difference in AChE levels. High AChE activity was associated with susceptibility and low AChE activity with resistance. Following the demonstration that differences in AChE activity could be detected, R_c, a substrain of strain R selected almost continuously for dioxathion resistance, was selected for low brain AChE activity. Tests on larvae of substrain Rc, shown in Figure 1, indicate that there were up to 20% susceptibles in the population at that time. Thirteen engorged female ticks, each with its associated male, were picked off a steer. Brains from males dissected immediately after collection were tested histochemically for AChE while female brains were examined after the engorged females had oviposited for 6–7 days. Three females and three males (23%) had high brain AChE activity but only seven females with low brain AChE had mated with males of low brain AChE. Their progeny were used to establish substrain RR. Strain S, an acaricide-susceptible reference strain of high brain AChE activity, was used for crossing experiments with substrain RR.

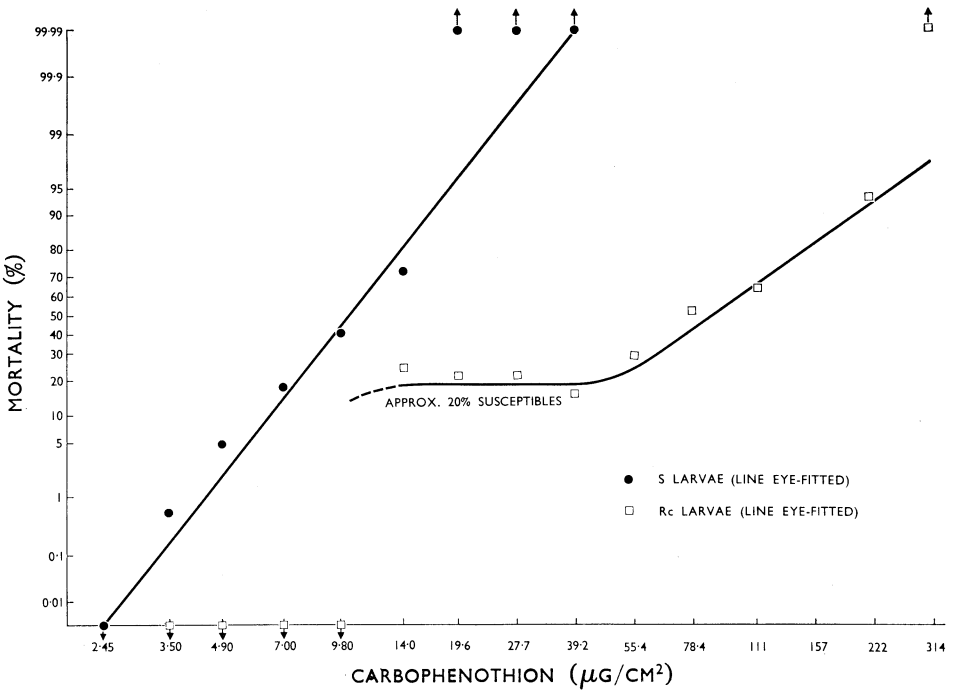

Fig. 1.—Mortality of R_c and S larvae of *B. microplus* enclosed in carbophenothion packets. Each percentage mortality is based on 150–280 larvae.

The methods of culture, toxicological testing, and the crossing technique were as described by Stone (1968), brains from F_1, F_2, and backcross adults resulting from some of the crossing experiments outlined by Stone (1968) being tested for AChE activity in order to determine F_2 and backcross ratios. The following notation was used to identify the progeny of crosses by their parentage, the female parent being always given first:

F_1: RS and SR;
F_2: F_2 RS;
Backcross: RS/R and R/RS.

Double backcrosses with selection for low brain AChE were made after the initial backcrosses, the progeny being backcrossed twice to RR with selection for low brain AChE. The resulting engorged females were tested for brain AChE activity after partial or complete oviposition. The progeny of "high"× "low" brain AChE types and of "low"× "low" brain AChE types were

backcrossed again with RR types. Some of the engorged females resulting were tested for brain AChE after completing oviposition but no males were so tested.

Brains were dissected preferably from freshly plucked medium-sized to semi-engorged female ticks about 0·5 cm in length which were less distended with blood and allowed a much cleaner, neater dissection. However, brains were also obtained from fully engorged females, females which had partially or fully completed oviposition, and from male ticks. If necessary ticks were stored at 10°C while awaiting dissection. The tick was embedded in black paraffin wax leaving only the ventral part of the integument visible. This was removed by means of an iris knife and iris scissors after the tick had been covered with ice-cold 1% sodium chloride solution (saline). The brain was then removed with the minimum amount of attached tracheal or gut tissue. The pedal, cheliceral, and other nerves were cut as far away from the brain as possible whereas the oesophagus and pharynx were severed as close as possible to the brain.

After washing in ice-cold saline, brains were fixed in ice-cold A.R. acetone for at least 1 hr. After a very brief rinse in distilled water, brains were incubated at room temperature (21–24°C) for at least 6 hr in 5-bromoindoxyl acetate medium (Pearse 1960) or for 1–2 hr in the "direct-colouring" thiocholine medium of Karnovsky and Roots (1964). Overnight fixation (16 hr) or incubation (24 hr) were occasionally used for greater convenience without evidence of loss of AChE activity or colour localization.

Where inhibition studies were required to distinguish between esterases, brains were given a pre-incubation period of 1 hr in an aqueous solution of the specific inhibitor, e.g. eserine, iso-OMPA, or 284C51, and all except the irreversible inhibitor iso-OMPA were included in the incubating medium (Pearse 1960).

Esterase activity was classified initially by eye according to the intensity of the coloured deposit of copper ferrocyanide (direct-colouring thiocholine method), or of indigo (indoxyl acetate method), and ranged from the most positive (+++++) to completely negative (−). Very low activity was shown as (±). A more quantitative approach was attempted by comparing the integrated densities of photomicrographic colour transparencies of brains using a Joyce–Leobl Chromoscan integrating densitometer with a yellow-green filter. Scans were usually performed by a single sweep with a 1-cm slit, so as to include all or very nearly all of the stained regions. Only transparencies obtained from the same film were compared directly. Extraneous colour in the transparencies due to factors other than hydrolysis of the substrate was corrected for by deducting the integration readings obtained on photographing brains incubated in a blank medium containing no ester.

The AChE activities of individual brains were determined biochemically by an adaptation of the method of Ellman *et al.* (1961) in which whole brains were fixed in acetone and then incubated for 100 min at 37°C (Schuntner, personal communication).

III. Results

(*a*) *Identification of Brain Esterases*

By the use of specific inhibitors in conjunction with the indoxyl acetate method, it was shown that high brain esterase activity occurred in S strains and that this was almost completely inhibited by 2×10^{-6}M eserine which inhibits all cholinesterases, and by 1×10^{-4}M 284C51 which specifically inhibits AChE. By contrast 1×10^{-4}M iso-OMPA, which completely inhibits cholinesterase (ChE), failed to produce any detectable inhibition of brain esterases. Thus, practically all of the esterase activity shown in the acetone-fixed brain of *B. microplus* of strain S by the indoxyl acetate method appeared to be AChE. Because of the successful use of inhibitors in conjunction with the indoxyl acetate method to demonstrate the presence of AChE, this method was used in studies on the inheritance of AChE.

Further evidence of the presence of AChE in brains of S strains was obtained by the use of the thiocholine method which yielded excellent results for the detection of cholinesterases and showed that there was strong activity towards acetylthiocholine iodide. There was much weaker activity towards butyrylthiocholine iodide and this was additional evidence that the cholinesterase detected was AChE and not ChE (Dixon and Webb 1964).

(*b*) *Comparison of AChE Activity from Brains of RR, Hybrid, and S Strains*

Brains from RR and hybrid (RS + SR) Strains were tested for AChE activity by the indoxyl acetate method. RR strains were usually very low in AChE (± or −) compared with S strains (++++ or +++++). Hybrid brains appeared to be very high in AChE (++++ or +++++) — intensity of coloration could not be readily distinguished visually from that for S strains. It appeared, therefore, that high brain AChE activity was at least partially dominant and autosomal as there was little difference in AChE activity between RS and SR strains. Occasionally brains from the original parental strain R from which substrain RR was derived showed high AChE activity but this was attributed to the presence of hybrid and susceptible ticks which were known to occur in the former heterogeneous population (Stone, unpublished data).

The use of the thiocholine method confirmed the above results showing that AChE activity was high in brains of S and SR strains and much lower in R brains. The means of the corrected densitometer–integrator readings for three transparencies for brains of each of S, SR, and RR strains incubated for 2 hr were 758 (675–880), 538 (523–565), and 68 (57–81) respectively. The corresponding values obtained for four brains of each of S, RS+SR, and RR strains incubated on a different occasion for 1·4 hr were 504 (460–543), 417 (360–495), and 83 (77–92) respectively. Thus, hybrid and RR strains were found to have 71–82 and 9–16% respectively of the brain AChE activity of S strains. The level of brain AChE activity in hybrids was greater than the sum of half the activities of the two parental type brains (54–58%) which supports the earlier finding that high brain AChE is incompletely dominant over low brain AChE activity.

The biochemical method used on individual brains revealed that the mean optical densities after incubation for 1·7 hr of 7, 8, and 8 ticks respectively of S, RS+SR, and RR strains were 1·42 (1·17–1·69), 1·12 (0·93–1·27), and 0·18 (0·14–0·23). These were the combined results for two separate experiments and indicated that brains of the hybrid and RR strains had 78 and 12% respectively of the AChE activity of S strains. This is in general agreement with the histochemical findings.

(*c*) *AChE Activity in Brains from Backcross Ticks*

Tests on brains of adult ticks, the progeny of backcrossing hybrid (high AChE) and homozygous resistant (low AChE) ticks, indicated that there was no significant departure from an expected 1 : 1 ratio of high AChE to low AChE activity (Table 1).

This provides strong evidence that brain AChE activity in *B. microplus* is controlled by a single pair of alleles.

(*d*) *AChE Activity in Brains from F_2 Ticks and a Discriminating Dosage Test on F_2 Engorged Females*

Tests on brains of 25, 20, and 20 F_2 female ticks showed that there was no significant difference between the observed and expected number of ticks with high and low AChE activity based on a 3 : 1 ratio (Table 1). This result gives further proof of the monofactorial nature of the inheritance of AChE activity in the brain of the cattle tick.

TABLE 1

OBSERVED AND EXPECTED NUMBER OF TICKS WITH HIGH (*H*) AND LOW (*L*) BRAIN ACETYLCHOLINESTERASE ACTIVITY

AChE activity was determined histochemically in unengorged females approx. 0·5 cm long after acetone fixation for 1–6 hr and incubation for 6–24 hr

Strain	No. of Ticks				
	$H_{obs.}$	$H_{exp.}$	$L_{obs.}$	$L_{exp.}$	χ^2
R/RS	12	12	12	12	0
R/RS	6	8·5	11	8·5	1·47 †
(R/RS+RS/R)*	9	8·5	8	8·5	0·06 †
F_2 (RS+SR)	15	18·75	10	6·25	3·00
F_2RS	13	15	7	5	1·07 †
F_2RS	16	15	4	5	0·26 †

* Ovipositing females. † Differences not significant.

F_2 engorged females derived from the same batch of ticks, RR, and S engorged females were treated with a carbophenothion dosage of 221 $\mu g/cm^2$ on filter papers. This dosage was intended to discriminate between resistant homozygotes and hybrids on the one hand and susceptibles on the other. The quantal responses for F_2, RR, and S engorged females were 35·7, 0, and 91·1% respectively while the expected F_2 response was 22·8% for a population comprising three-quarter resistant and one-quarter susceptible ticks. The observed response was not significantly different from the expected. This suggests that the batch of females tested for brain AChE had the expected F_2 proportions of resistants and susceptibles.

(*e*) *Selection of Substrain R_c for Low Brain AChE Activity*

Substrain RR after two generations of culturing following the initial selection for low brain AChE was tested for the proportion of adults with low brain AChE. Of 41 adults tested (37 females and 4 males) all showed low brain AChE.

As shown in Figure 2 no test of the homogeneity of substrain RR with respect to carbaryl-, dioxathion-, carbophenothion-, or formothion-resistant individuals has shown the presence of any susceptible individuals. Thus, selection for low brain

AChE resulted in a substrain which consisted of homozygous resistant ticks at the third generation of culturing following only one initial selection.

(f) Resistance Testing of Double Backcrosses Selected for Low Brain AChE

Figure 3 shows the expected effects of double backcrossing hybrid ticks to fully resistant low brain AChE types with selection for low brain AChE. It can be seen that the progeny of such a backcross would all be fully resistant if the gene for resistance to organophosphorus compounds was either identical with or closely linked

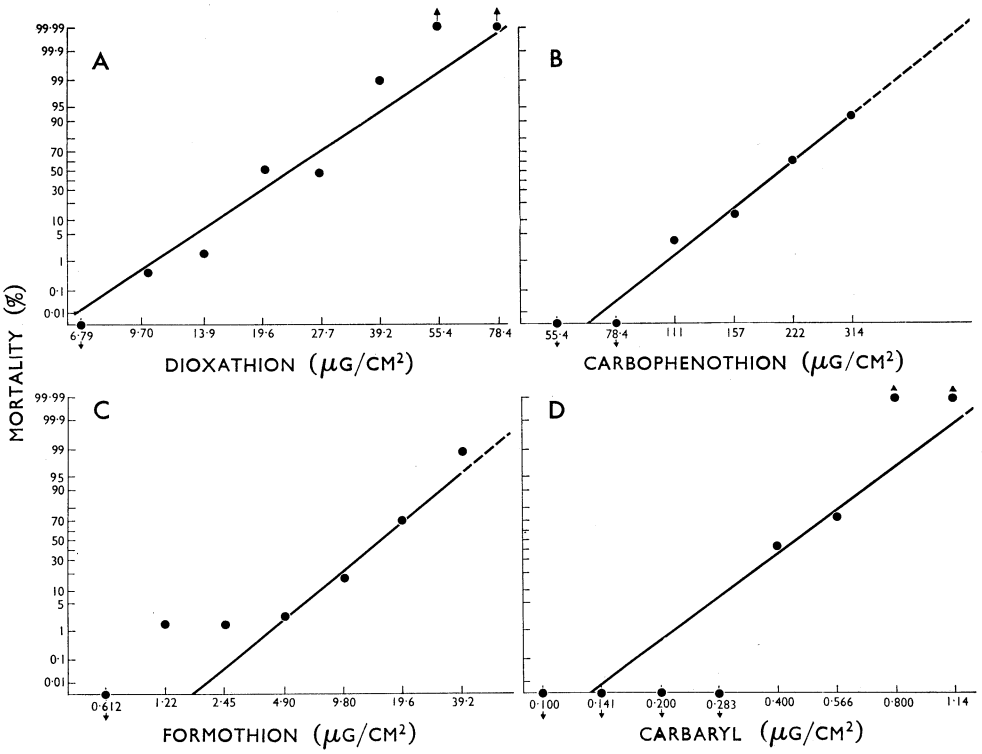

Fig. 2.—Mortality of RR larvae of *B. microplus* enclosed in packets. Each percentage mortality is based on 140–310 larvae. *A*, 10-day-old larvae selected for three generations; *B*, 14-day-old larvae, selected for one generation; *C*, 16-day-old larvae, selected for three generations; *D*, 28-day-old larvae, selected for three generations.

with the gene for low brain AChE. Only half the batches of larvae would be fully resistant and the other half would consist of equal numbers of fully and partially resistant phenotypes if the genes were unlinked.

The larval progeny of 22 engorged females obtained by backcrossing with selection were tested in formothion packets. Mortalities obtained at a discriminating dosage of 19·6 μg/cm^2 were 0% for 13 batches, 1–4% for six batches, and 7, 9, and 16% for the remaining three batches. The expected mortality for fully resistant phenotypes was 0–4% (RR mortality). Thus, 19 batches of low $\times$ low brain AChE

larvae consisted only of individuals which were about as resistant as RR larvae while the remaining three batches may have contained some individuals which were not as fully resistant. However, mortality in the latter three batches was significantly different from the mortality expected (30%, Stone 1968) for a 1 : 1 ratio of fully to partially resistant phenotypes. Thus, the results support the hypothesis that the gene *OP* for resistance to organophosphorus compounds (Stone 1968) is either identical with or closely linked to the gene for low brain AChE.

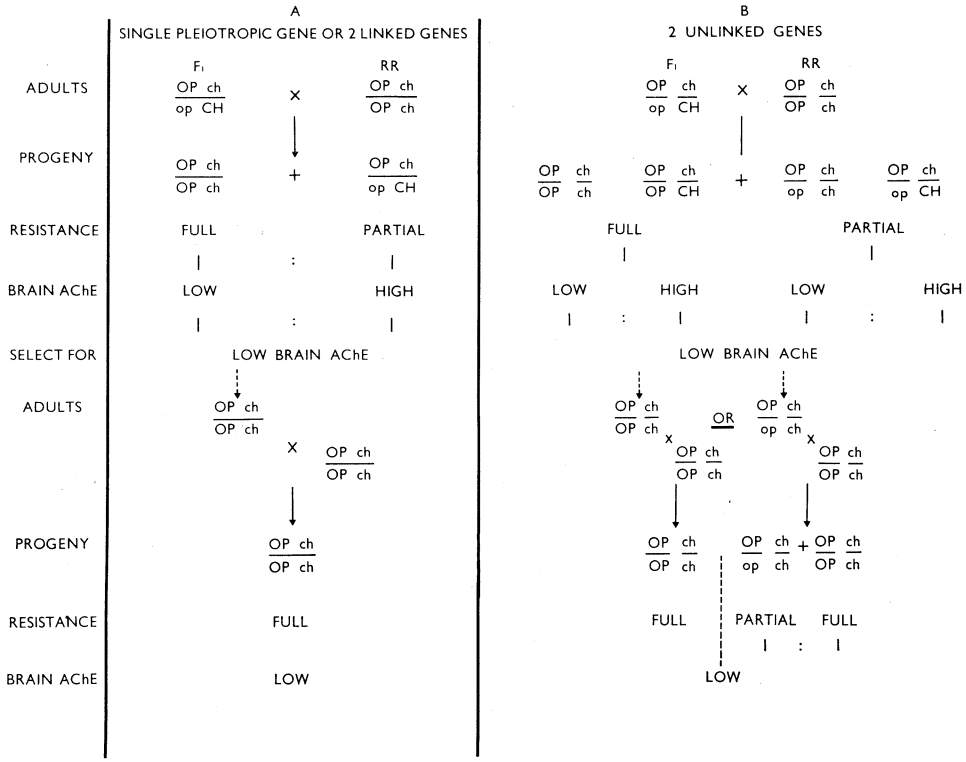

Fig. 3.—Expected effects of double backcrossing hybrid to fully resistant *B. microplus* with low brain AChE activity, with selection for low brain AChE.

IV. Discussion

The above results provide strong evidence that low brain AChE activity in *B. microplus* is due to a single autosomal gene (here referred to as *ch*) which is incompletely recessive with respect to its allele for high brain AChE activity (*CH*).

It was shown by Stone (1968) that resistance to organophosphorus compounds in the cattle tick is controlled by the incompletely dominant autosomal gene *OP* and its corresponding allele for susceptibility, *op*. The obvious association between homozygosity for resistance and low brain AChE suggests that there may be two closely linked genes for these characters or that they are pleiotropic effects of the same gene, but it would be very difficult to differentiate between these two possibilities in the absence of more comprehensive knowledge of the genetics of the cattle tick.

The association between resistance to organophosphorus compounds and AChE inheritance in cattle ticks is similar to that described for house flies (*Musca domestica*) and red spider mites (*Tetranychus urticae*). Oppenoorth (1959) found that low ali-esterase activity was associated with resistance to organophosphorus compounds in house flies, and was controlled by a single gene which appeared to be neither dominant nor recessive with respect to its allele controlling high ali-esterase activity and susceptibility to organophosphorus compounds. Resistance to these compounds was incompletely dominant so hybrids resembled the resistant parent in their response to chemicals but were intermediate between the two parents in ali-esterase activity. Smissaert (1964), working with an organophosphorus-resistant strain of red spider mite, in which resistance was controlled by a single, incompletely dominant gene, showed that low AChE activity was associated with resistance and high AChE activity with susceptibility. Subsequently Smissaert (personal communication) has found that AChE activity of F_1 mites is about two-thirds that of the susceptible parent indicating that low AChE is neither dominant nor recessive.

Hereditary differences in esterase activity have also been studied by workers in fields quite unrelated to that of insecticide resistance. Sawin and Glick (1943), for example, showed that an incompletely recessive gene is responsible for low atropinesterase activity in rabbits while Johnson (1964) proved that the lack of an esterase zone in *Drosophila melanogaster*, as shown by starch gel electrophoresis, was inherited as a simple autosomal recessive trait.

Thus, where the inheritance of esterase deficiency in animals has been studied (Sawin and Glick 1943; Oppenoorth 1959; Johnson 1964; Smissaert 1964; and the present investigation) it has never proved to be dominant and is usually partially recessive to normal high esterase activity. This appears to be in agreement with the generalization that mutant effects are usually recessive (Wagner and Mitchell 1964). The same authors reported that enzyme deficiencies usually seem to be at least partially recessive, particularly those that affect humans such as alcaptonuria, phenylketonuria, etc.

It must not be inferred, however, that mutant alleles associated with resistance necessarily always produce enzyme deficiencies; Sternburg, Kearns, and Moorefield (1954) found that DDT-resistant houseflies (presumably a naturally occurring mutant type) contained much more of the enzyme DDT-dehydrochlorinase than susceptible (wild type) flies.

Although a full discussion of the biochemistry or physiology of resistance to organophosphorus compounds in the cattle tick is not possible from results presented in this paper, it seems desirable to remark on one aspect which has become apparent as a direct result of this investigation. This is that low brain AChE activity, although associated with resistance, can hardly be regarded as a cause of resistance since the partially resistant hybrids have high brain AChE activity. Van Asperen (1964) came to a similar conclusion that low ali-esterase activity was not necessarily connected with and not in itself a cause of resistance to organophosphorus compounds in houseflies since Franco and Oppenoorth (1962) had found a susceptible strain which contained some flies of low activity. Smissaert (1964) felt that low AChE activity in organophosphorus-resistant *Tetranychus urticae* was a side effect and that

the mechanism of resistance was a decreased sensitivity of the cholinesterase at the site of action of the organophosphorus acaricide. Lee and Batham (1966) reported that organophosphorus-resistant *B. microplus* possessed at least one cholinesterase which reacted more slowly with organophosphorus acaricides than did the cholinesterase of the susceptible strain.

The brain and attached nerves represent the bulk of the nervous tissue of the cattle tick. Although it is not known to what extent AChE occurs in other tissues, most of the AChE [by analogy with other arthropods (Colhoun 1963)] would be in the nervous system. Thus it seems reasonable to assume that AChE activity in brains of ticks represents most of the AChE activity. Similarly, the histochemical test seems reliable. Acetone fixation may require some justification since it destroys some non-specific esterases. However Lord, Molloy, and Potter (1963) have shown that acetone fixation does not affect the activity of cholinesterase in the thoracic ganglion of *Musca domestica* and in fact increases the permeability of the ganglion to the substrate (acetylcholine or acetylthiocholine), such an increase in permeability being necessary for the successful use of the more specific acetylthiocholine method. This illustrates an exception to the general rule proposed by Wylie (1965) that fixation *after* incubation is recommended in histochemistry. Although in the indoxyl acetate method the substrate penetrated well into the unfixed brain of the cattle tick, the prior fixation in acetone resulted in a clearer demonstration of the presence of AChE.

The thiocholine histochemical method of Karnovsky and Roots (1964) for cholinesterases was tried only after most of the experimental work had been completed in order to confirm the results obtained by the indoxyl acetate method. It is clear from the results that such confirmation was provided and the thiocholine method proved to be the most reliable in practice.

The high AChE activity and the apparent absence of ChE in the brain of the cattle tick is similar to the situation in houseflies (Colhoun 1963). The demonstrated inability (Stone, unpublished data) of a susceptible engorged female tick to oviposit after treatment with a certain dosage of an organophosphorus acaricide may be associated with the inhibition of brain AChE as a result of the acaricidal treatment, this concept being generally accepted as the mode of action of an organophosphorus acaricide. However, the small amount of AChE present in the brain of the homozygous resistant tick raises the question of the significance of AChE in the nervous system. Ticks of the *ch ch* genotype have proved to be individuals of apparently normal viability and activity despite their being atypical with regard to AChE activity.

V. Acknowledgments

The author is indebted to the following colleagues of the Division of Entomology, CSIRO: Dr. R. J. Tatchell, who advised on various aspects of histochemical technique, Dr. R. H. Wharton for encouragement and advice, and Mr. C. A. Schuntner who carried out the densitometric determinations. Various colleagues criticized the manuscript and provided help in its preparation. The technical assistance of Misses W. Ward and N. J. Youlton is acknowledged with gratitude.

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