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Studies of the Acetylcholinesterase from Houseflies (*Musca domestica* L.) Resistant and Susceptible to Organophosphorus Insecticides

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Acetylcholinesterase from the heads of insecticide-resistant and -susceptible houseflies (*Musca domestica* L.) was studied *in vitro*. The enzymes could not be distinguished electrophoretically, and their behaviour on polyacrylamide-disc-gel electrophoresis was influenced by the presence of Triton X-100 in both the homogenate and the gels. In the absence of detergent, the acetylcholinesterase was heterogeneous, but behaved as a single enzyme when it was present. By analogy with studies of acetylcholinesterase from other sources, these observations were attributed to aggregation of the enzyme when not bound by membranes. The enzyme from resistant flies was more slowly inhibited than the susceptible enzyme, bimolecular rate constants (k_i) differing by approx. 4–20-fold for a range of organophosphorus compounds. The kinetics of inhibition of acetylcholinesterase were consistent with the results of electrophoresis, i.e. they corresponded to those of a single enzyme in the presence of Triton X-100, but a mixture of enzymes in its absence. The susceptibility of the more sensitive components in these mixtures was determined.

Many insecticides act by inhibiting acetylcholinesterase, yet our knowledge of this enzyme and its inhibition is based mainly on studies with mammalian and electric-eel (*Electrophorus electricus*) enzymes (Aldridge & Reiner, 1972; Corbett, 1974). Better understanding of the differences between the enzymes in insect pests and man should enable selective and effective insecticides with low mammalian toxicity to be chosen and designed more rationally (Aldridge, 1971). Another major consideration, which is an increasing problem in insect control, is the appearance of strains which are resistant to insecticides. In insects such resistance is usually caused by more rapid degradation of the insecticides by the resistant strains (Devonshire, 1973). However, in spider mites, *Tetranychus urticae* (Smissaert, 1964; Smissaert *et al.*, 1970) and cattle ticks, *Boophilus microplus* (Roulston *et al.*, 1968), a modified acetylcholinesterase with decreased sensitivity to inhibition is a major cause of resistance. A similar resistance mechanism has only recently been detected in houseflies (Tripathi & O'Brien, 1973; Devonshire & Sawicki, 1974), although there have been many cases of resistance in this insect.

Kinetic investigations with membrane-bound enzymes are difficult to interpret because the system is biphasic, and studying acetylcholinesterase from nervous tissue is therefore complicated by the necessity to remove the enzyme from the cell membranes. Such extraction may yield a multiplicity of enzymes, and Tripathi *et al.* (1973) showed that these differed in their sensitivity to inhibition. These different forms have been attributed to isoenzymes (Habibulla & Newburgh, 1973; Tripathi *et al.*, 1973), aggregation of subunits (McIntosh & Plummer, 1973) or the presence of membrane components still bound to the enzyme (Grafius *et al.*, 1971; Brodbeck *et al.*, 1973).

The present paper describes the preparation from housefly heads of acetylcholinesterase which behaves as a single enzyme, and compares the properties of the enzymes extracted from strains of housefly with differing susceptibility to organophosphorus insecticides.

Experimental

Materials

Houseflies. Four strains of houseflies were used, of which two (608 and Cooper) were standard susceptible strains (Farnham, 1973). Strain $49r_2b$ had developed resistance to organophosphorus insecticides after treatment in the field, and a substrain (arD) had part of chromosome 2 from strain $49r_2b$ bred into a susceptible background (Sawicki, 1974). The acetylcholinesterase of strain arD is the same as that from strain $49r_2b$ (Devonshire & Sawicki, 1974).

Organophosphorus compounds. Omethoate* {*OO*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] phosphorothioate}, paraoxon* (diethyl 4-nitrophenyl phosphate) and its dimethyl* and di-isopropyl* homologues, malaoxon† {diethyl [(dimethoxyphosphinyl)-

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thio]butanedioate} and its *OO*-diethyl* homologue, and tetrachlorvinphos† {dimethyl [1-(2,4,5-trichlorophenyl)-2-chlorovinyl] phosphate} were analytical standards whose identity and purity were confirmed by n.m.r. (nuclear-magnetic-resonance) spectrometry and g.l.c.

Other chemicals. Acetylthiocholine iodide, butyrylthiocholine iodide and 5,5′-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma (London) Chemical Co., London S.W.6, U.K., Triton X-100 was from Koch–Light Laboratories, Colnbrook, Bucks., U.K., physostigmine sulphate was from Hopkin and Williams, Chadwell Heath, Essex, U.K., and all other chemicals were of the highest purity available from BDH, Poole, Dorset, U.K., or Fisons, Loughborough, Leics., U.K.

Enzyme preparation. For preliminary experiments, individual housefly heads were homogenized (1 head/ml) in 0.1M-phosphate buffer, pH7.5 (KH_2PO_4–NaOH), or in 1% (w/v) Triton X-100 in the same buffer, and the acetylcholinesterase activity was measured. For kinetic studies, larger quantities of the enzymes were prepared from 200–2000 houseflies of mixed sexes which were decapitated after being frozen by shaking in a sieve (8-mesh) with pellets of solid CO_2 (Moorefield, 1957). Heads, legs and wings passed through the sieve. The heads were removed and homogenized at 0°C either in 0.05M-phosphate buffer, pH7.5 (KH_2PO_4–NaOH) by using a Silverson blender at its slowest speed (20%, w/v, homogenate), or in 0.1M-phosphate buffer, pH7.5 (KH_2PO_4–NaOH), containing 1% Triton X-100, by using a glass homogenizer with a polytetrafluoroethylene (PTFE) pestle (5%, w/v, homogenate). The homogenates were centrifuged for 60min at 5°C and 175000*g* ($r_{av.}$ 6.26cm). In the presence of Triton, a lipid zone formed on the surface, and the supernatant was withdrawn from beneath this with a syringe. With both resistant and susceptible strains the supernatant contained only approx. 15% of the total acetylcholinesterase activity in the absence of Triton, compared with 90–100% when the detergent was present. These supernatants were used without further purification for studying the acetylcholinesterase and its inhibition.

Methods

Electrophoresis. The enzyme preparations were examined by disc gel electrophoresis on 5% (w/v) polyacrylamide running gels in the buffer systems described by Williams & Reisfeld (1964). Triton X-100 (0.1%, w/v) was incorporated into some of the gels. Sucrose was added to the samples to a concentration of 10%, and 15μl of each sample was layered on the top of the spacer gels. After 2h of electrophoresis at 2mA/tube (10V/cm) the gels were stained at 20°C for 2.5h, with acetylthiocholine as substrate in the presence of Cu^{2+} (Lewis & Shute, 1966). The gels with white bands of copper thiocholine were photographed as described by Oliver & Chalkley (1971). Electrophoretic mobilities (*m*) were measured relative to the movement of the buffer interface in the small-pore gel. In gels without detergent, this was indicated by a marker dye, Bromophenol Blue, incorporated into the samples. However, in gels with 0.1% Triton, the dye trailed behind the interface which was clearly visible as a refractive boundary. The position of this interface was marked on the glass running tubes immediately after electrophoresis, and in calculating *m*, allowance was made for the swelling of the gels during staining.

Enzyme assay. Acetylcholinesterase activity was measured at 25°C and pH7.5 by the technique of Ellman *et al.* (1961) with acetylthiocholine iodide as substrate. The final concentrations of substrate and 5,5′-dithiobis-(2-nitrobenzoic acid) in the assay mixture were usually 1mM. Activity was completely inhibited by 10μM-physostigmine, and acetylthiocholine was hydrolysed faster than butyrylthiocholine.

Inhibition in the absence of substrate. Solutions of inhibitor (500μl) and enzyme (500μl) in buffer were mixed at 25°C, and 100μl portions added at intervals of 10s to cuvettes containing 2.9ml of substrate plus 5,5′-dithiobis-(2-nitrobenzoic acid). The absorbance at 412nm was recorded for 2–5min. With enzymes prepared in the presence of Triton, activity was constant during the assay because the 30-fold dilution with substrate prevented further inhibition, and there was no reactivation of inhibited enzyme. However, in the absence of detergent, inhibition continued after dilution and residual enzyme activity at the time of dilution was estimated from the initial slope of the plot of absorbance against time. Pseudo-first-order rate constants and bimolecular rate constants were calculated as described by Main & Iverson (1966) and their accuracies computed by the least-squares method of linear regression with a maximum-likelihood programme.

Inhibition in the presence of substrate. Enzyme (1ml) was added to substrate (1.5mM) and 5,5′-dithiobis-(2-nitrobenzoic acid) (1.5mM) in buffer (2ml) containing various concentrations of inhibitor, and the absorbance was recorded for 1–3min. The resulting curves, analysed by using the maximum-likelihood programme, had first-order kinetics. The first-order rate constants were used to calculate the bimolecular rate constants, correcting for the presence of substrate as described by Hart & O'Brien (1973).

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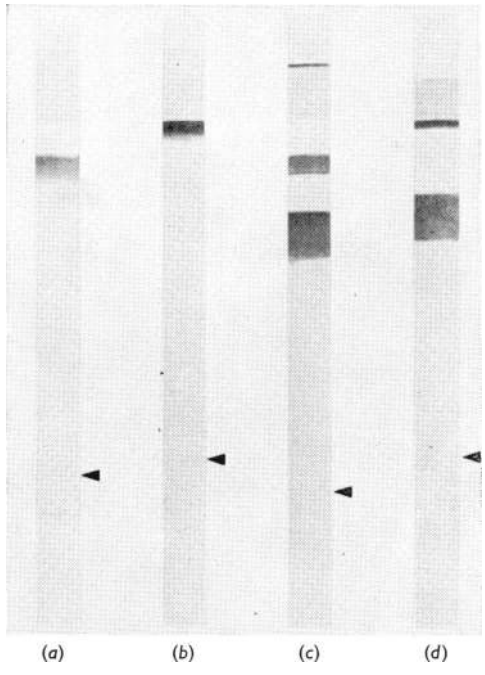

EXPLANATION OF PLATE 1

Electrophoresis of acetylcholinesterase from houseflies of the Cooper strain on polyacrylamide gels

The running and staining conditions are described in the text. Detergent-solubilized enzyme equivalent to 0.3 head (approx. 0.3 mg) or enzyme prepared in the absence of Triton equivalent to 1.2 head (approx. 1.2 mg) was added to the gels. (*a*) Enzyme in 1% Triton, gel containing 0.1% Triton; (*b*) enzyme in 1% Triton, normal gel; (*c*) enzyme in buffer, gel containing 0.1% Triton; (*d*) enzyme in buffer, normal gel. Arrows indicate the buffer interface.

Results

Electrophoresis

The acetylcholinesterase from resistant and susceptible houseflies could not be distinguished electrophoretically. Enzymes prepared in the presence of 1% Triton X-100 behaved as a single moiety whether or not Triton was incorporated into the gels (Plate 1). However, in gels containing no Triton its electrophoretic mobility (m) was low ($m = 0.02$) compared with that in gels containing 0.1% Triton ($m = 0.12$). When detergent was omitted from both homogenate and gel, the enzyme migrated as two components with approximately equal activity with $m = 0.02$ and $m = 0.28$. However, when the homogenate without detergent was run on gels containing 0.1% Triton, the faster band was unaffected but the slower band was replaced by one with $m = 0.12$ (Plate 1).

Substrate hydrolysis

Preliminary experiments established that the acetylcholinesterase activity was more closely related to the number of heads than to the weight of heads. There was no systematic difference in the weight of heads from different strains, although weight varied slightly with generation depending on the conditions of rearing. Although the heads of female flies were about one-third heavier than the heads of male flies there was no difference between sexes in either activity of the extracted enzyme or its sensitivity to inhibition.

Table 1 shows the K_m for acetylcholinesterase from different strains in the presence and absence of 1% Triton X-100, and the $V_{max.}$ in the presence of 1% Triton X-100. Although in uncentrifuged homogenates the enzyme activities in the presence or absence of detergent were similar, after centrifuging at 176000g only approx. 15% of the activity remained in the supernatant in the absence of Triton X-100, compared with 90–100% in the presence of detergent. The heads of susceptible flies had 2–3 times the enzyme activity ($V_{max.}$) of resistant flies, and the 608 strain had significantly greater activity than the Cooper strain. Enzymes prepared in the absence of detergent had a lower K_m than those containing 1% Triton X-100, and the K_m of the enzyme from resistant flies was greater than those from susceptible flies. In the presence of detergent, the K_m of the enzyme from the Cooper strain was slightly but consistently greater than that from the 608 strain, although this difference

Table 1. *K_m and $V_{max.}$ (±S.E.M.) for acetylcholinesterase from resistant and susceptible houseflies (at 25°C)*

Experimental details are given in the text.

Strain	K_m (mM) +Triton	K_m (mM) −Triton	$V_{max.}$ (+Triton) (μmol of acetylthiocholine hydrolysed/h per head)
Resistant			
arD	0.154±0.009	0.067±0.005	1.36±0.05
Susceptible			
Cooper	0.121±0.004	0.039±0.005	2.29±0.07
608	0.115±0.003	0.049±0.006	3.39±0.09

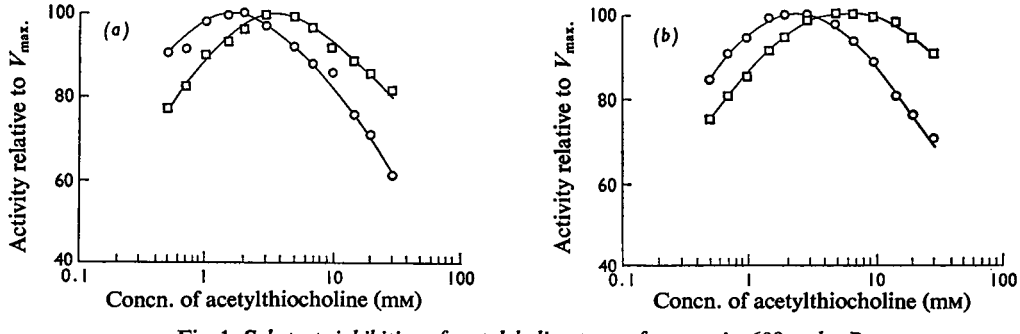

Fig. 1. *Substrate inhibition of acetylcholinesterase from strains 608 and arD*

(*a*) Triton-solubilized enzymes assayed at 25°C in phosphate buffer, pH7.5. ○, Strain 608 ($K_m = 0.115 \pm 0.003$ mM, $V_{max.} = 3.4$ μmol/h per head); □, strain arD ($K_m = 0.154 \pm 0.009$ mM, $V_{max.} = 1.4$ μmol/h per head). (*b*) As (*a*), but with the addition of 0.1 M-NaCl. Strain 608, $K_m = 0.128 \pm 0.008$ mM, $V_{max.} = 3.4$ μmol/h per head; strain arD, $K_m = 0.155 \pm 0.010$ mM, $V_{max.} = 1.4$ μmol/h per head.

Table 2. *Bimolecular rate constants (k_i) for inhibition of acetylcholinesterase from resistant- and susceptible-housefly heads measured in the absence of substrate*

Experimental details are given in the text. The values are means±S.E.M.

	$10^{-3}\times k_i$ ($M^{-1}\cdot min^{-1}$)		
		Susceptible	
	Resistant	608	Cooper
Omethoate	1.77±0.04	19.9± 0.32	20.5± 0.53
Methyl paraoxon	21.6 ±1.0	—	90.1± 4.9
Paraoxon	139 ±6.5	578 ±22	—
Isopropyl paraoxon	9.23±0.43	76.3± 3.5	80.6± 6.0
Malaoxon	47.3 ±3.6	867 ±35	601 ±21
Ethyl malaoxon	94.3 ±3.5	932 ±12	—
Tetrachlorvinphos	24.7 ±0.2	—	172 ±26

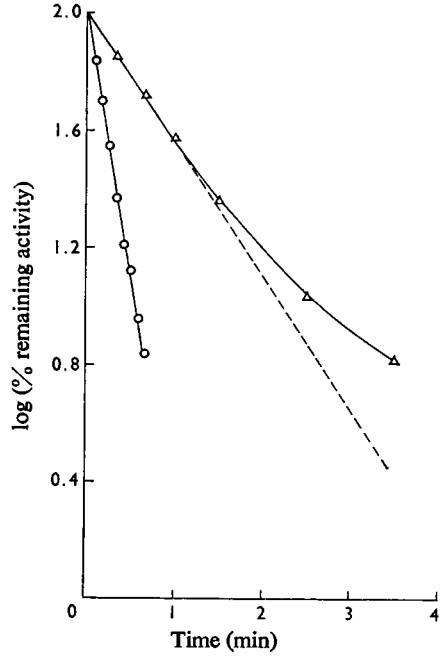

Fig. 2. *Inhibition by malaoxon of acetylcholinesterase from strain arD in the presence and absence of 1% Triton X-100*

Details are given in the text. ○, Enzyme prepared in 1% Triton X-100; 0.1 mM-malaoxon. △, Enzyme prepared in buffer; 10 μM-malaoxon.

is not significant at the 95% confidence level. The enzymes were inhibited in the presence of high substrate concentrations, and $V_{max.}$ was attained by the enzyme from the resistant strain at a greater substrate concentration than was that from the susceptible (608) strain (Fig. 1*a*). The addition of 0.1 M-NaCl increased

Table 3. *Inhibition by malaoxon of acetylcholinesterase from resistant- and suceptible-housefly heads in the presence and absence of Triton X-100*

Experimental details are given in the text. The values are means±S.E.M.

	$10^{-3}\times k_i$ ($M^{-1}\cdot min^{-1}$)	
Strain	1% Triton X-100	No Triton X-100
Susceptible		
Cooper	601 ±21	2540± 80
608	867 ±35	3780±380
Resistant		
arD	47.3± 3.6	100± 4

the K_m of the strain-608 enzyme and also the substrate concentration at which $V_{max.}$ occurred in both strains, although there was no change in $V_{max.}$ (Fig. 1*b*). These properties are characteristic of acetylcholinesterase (Augustinsson, 1963).

Inhibition in the absence of substrate

Most of the inhibition data were obtained with the Triton-solubilized enzyme preparation (Table 2) because it migrated as a single component on disc gel electrophoresis and behaved kinetically as a single moiety during inhibition studies. In the absence of Triton, two enzyme components were detected on electrophoresis, and the pseudo-first-order inhibition lines were slightly curved, suggesting a mixture of enzymes with different susceptibility to inhibition (Fig. 2). However, the initial slopes of these lines were used to calculate the bimolecular rate constants for inhibition of the more sensitive and predominant component by malaoxon in the absence of Triton (Table 3).

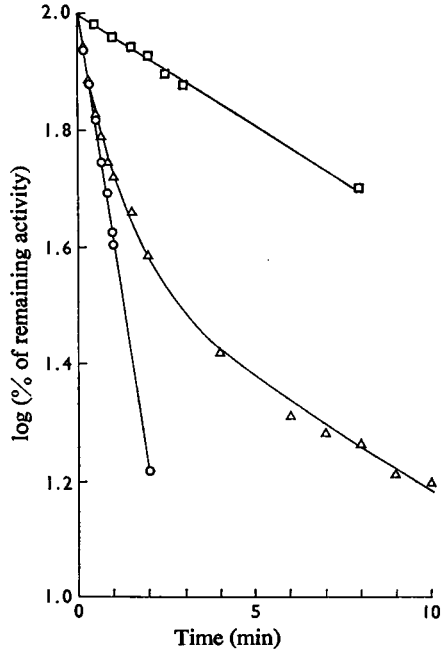

Fig. 3. *Inhibition by malaoxon of a mixture of 'resistant' and 'susceptible' acetylcholinesterase*

○, Strain 608; □, strain arD; △, mixture (approx. 2:1) of strains 608 and arD. Details of the experiment are given in the text.

Table 4. *Affinity (K_a) and phosphorylation (k_2) constants for the inhibition of acetylcholinesterase from resistant- and susceptible-housefly heads by isopropyl paraoxon*

Experimental details are given in the text. The values are means±S.E.M.

Strain	K_a (μM)	k_2 (min^{-1})
Susceptible		
608	26.6± 2.2	2.1±0.1
Cooper	22.5± 2.5	1.6±0.1
Resistant		
arD	2090 ±920	17.7±6.6

Table 5. *Inhibition by malaoxon of acetylcholinesterase prepared (in 1% Triton X-100) from resistant- and susceptible-housefly heads measured in the presence and absence of 1 mM-acetylthiocholine*

Experimental details are given in the text. Values are means±S.E.M.

	$10^{-3}\times k_i$ ($M^{-1}\cdot min^{-1}$)	
Strain	−Substrate	+Substrate
Susceptible		
Cooper	601 ±21	608 ±51
Resistant		
arD	47.3± 3.6	43.5± 2.5

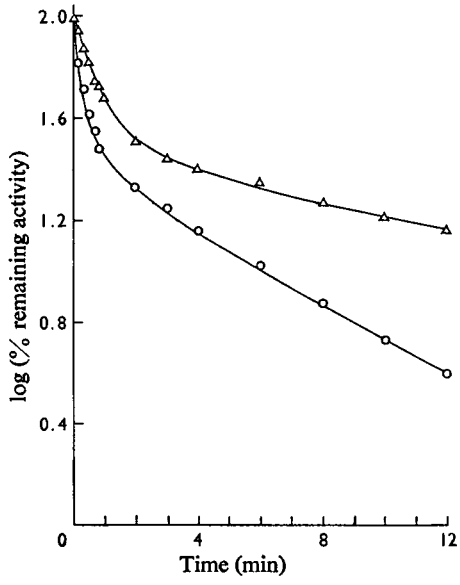

Fig. 4. *Inhibition by malaoxon of acetylcholinesterase from the F_1 generation of the cross strain 608×strain arD*

△, 1.5μM-malaoxon; ○, 4.0μM-malaoxon. Further details are given in the text.

Enzymes prepared from resistant and susceptible houseflies were mixed and the inhibition kinetics studied to confirm that the differences in sensitivity were genuine and not merely a result of some other difference between homogenates, e.g. the inhibitor might have been consumed more rapidly in the resistant homogenate by metabolism or by binding to other esterases. Fig. 3 shows that in such mixtures the enzymes behaved independently because two phases of inhibition were detected, corresponding to the two types of enzyme.

Because inhibition was so rapid, only the bimolecular rate constants could be accurately calculated for most of the inhibitors tested. However, with isopropyl paraoxon, the phosphorylation rate was much lower, and it was possible to calculate the affinity and phosphorylation constants for this inhibitor (Table 4). The tendency for the inhibition by isopropyl paraoxon to reach a limiting rate was not due to the limited solubility of this inhibitor because the concentrations used were well below its solubility.

Inhibition in the presence of substrate

Points from the absorbance curves obtained on adding enzyme to a mixture of acetylthiocholine and inhibitor were fitted by the least-squares method to an exponential function by using the maximum-likelihood programme. This gave the first-order rate constants and their associated standard errors. The standard errors were small (1–3%), confirming that under these conditions the rate of inhibition closely approximated to first-order. The bimolecular rate constants calculated from these inhibition data were the same as those measured in the absence of substrate (Table 5).

Nature of the acetylcholinesterase in the F_1 generation obtained by crossing strain arD with susceptible houseflies

Fig. 4 shows the progress of inhibition of the acetylcholinesterase from these progeny by malaoxon. Two phases of inhibition are apparent, with rates corresponding to the two types of enzyme. A computer analysis of five such curves (fitting the data to the equation $v = A+Be^{-kt}+Ce^{-lt}$, i.e. a combination of two first-order reactions) gave an estimate of the relative activity of each type of enzyme. The ratio of the activities of the susceptible to the resistant enzyme was 2.09 ± 0.06 whether resistant males were crossed with susceptible females, or vice versa.

Discussion

The behaviour of acetylcholinesterase solubilized from nervous tissue depends on the conditions of its preparation. Thus multiple forms have been reported on electrophoresis (Grafius *et al.*, 1971; Brodbeck *et al.*, 1973; McIntosh & Plummer, 1973; Tripathi *et al.*, 1973), the patterns obtained depending on the method of extraction (McIntosh & Plummer, 1973). These authors showed that Triton X-100 solubilized the enzyme very well, this being a genuine effect and not merely activation of the enzyme. The interconvertibility of the different forms and the ratios of their molecular weights supported the hypothesis that they arose by aggregation. Levinson & Ellory (1974) showed by irradiation inactivation that the acetylcholinesterases from electric eel and erythrocytes behaved as monomers when membrane-bound and that the higher-molecular-weight forms in solubilized preparations were aggregates of this monomer. In Triton X-100 they found the enzyme to behave predominantly as a dimer.

Although the molecular weights of the different forms found after electrophoresis in the present work were not determined, their interconvertibility is consistent with the hypothesis that they arise by aggregation. If this is correct, the two components observed in the absence of Triton would correspond to high- ($m = 0.02$) and low- ($m = 0.28$) molecular-weight forms (Plate 1, gel *d*). On running this preparation on gels containing Triton (Plate 1, gel *c*), the low-molecular-weight fraction was unchanged, but the high-molecular-weight component dissociated to a form of intermediate aggregation ($m = 0.12$) with the same mobility as the single form obtained when detergent was present during both homogenization and electrophoresis (Plate 1, gel *a*). When the solubilized enzyme was run on gels with no detergent it was completely converted into the high-molecular-weight form (Plate 1, gel *b*).

This electrophoretic behaviour and the inhibition kinetics of the enzymes (Figs. 2 and 3) indicate that in the presence of Triton they behave as single soluble entities, but are heterogeneous in the absence of detergent. For this reason, the Triton-solubilized enzymes were used for most of the work described. The enzymes from the different strains differed in their affinity for substrate (Table 1) and in their response to large substrate concentrations (Fig. 1). However, the greatest difference was in susceptibility to inhibition (Table 2); the resistant enzyme was from fourfold (with paraoxon) to 18-fold (with malaoxon) less sensitive than the two susceptible enzymes. There was a small difference between the two susceptible enzymes in sensitivity to malaoxon, although the 608 and the Cooper strains were equally susceptible to this and the other insecticides tested (R. M. Sawicki, personal communication). With isopropyl paraoxon (Table 4), the decreased sensitivity of the resistant enzyme is caused by a large (approx. 80-fold) decrease in affinity (K_a) partly offset by a smaller (approx. 9-fold) increase in phosphorylation rate (k_2).

These results were compared with those obtained with enzymes prepared in the absence of detergent (Table 3) and also when inhibition was measured in the presence of substrate (Table 5). Malaoxon was used for these comparisons because the differences between the susceptibility of the different enzymes to this compound were greater than for any other compound tested. Bimolecular rate constants (k_i) were the same whether or not substrate was present during inhibition (Table 5). However, the rate of inhibition was lower in the presence of Triton (Table 3), and this effect was greater on the susceptible enzymes. Therefore the difference between strains in resistance to inhibition in the absence of detergent was approximately twice as high as when measured with enzyme preparations containing 1% Triton.

On crossing strains arD and 608, the acetylcholinesterase of the progeny had the properties of a mixture of susceptible and resistant enzymes behaving independently, with activities in the ratio 2:1 (Fig. 4). This could be explained if equal molar quantities of each enzyme were present in the offspring and the

susceptible enzyme had twice the catalytic-centre activity of the resistant enzyme. This suggestion is consistent with the finding that the heads of resistant flies have approximately one-half the acetylcholinesterase activity of those of susceptible flies (Table 1). In ticks, a similar decreased activity in resistant strains with less sensitive acetylcholinesterase has been observed (Roulston *et al.*, 1968), and this has been shown to be due to a lower catalytic centre activity of the resistant enzyme (Schnitzerling & Nolan, 1975).

Resistance caused by a modified acetylcholinesterase has been observed most frequently in ticks and mites, in which differences in sensitivity of the enzyme of the order of 1000-fold have been reported. The smaller differences between the housefly enzymes described here confer only slight resistance (less than 20-fold) when isolated genetically, but can interact with other resistance mechanisms resulting in high levels of resistance (Devonshire & Sawicki, 1974).

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