The Properties of a Carboxylesterase from the Peach-Potato Aphid, *Myzus persicae* (Sulz.), and its Role in Conferring Insecticide Resistance

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Carboxylesterases from different strains of *Myzus persicae* were examined to try to understand their contribution to insecticide resistance. Preliminary evidence that they are involved comes from the good correlation between the degree of resistance and the carboxylesterase and paraoxon-degrading activity in aphid homogenates. Furthermore the carboxylesterase associated with resistance could not be separated from the insecticide-degrading enzyme by electrophoresis or ion-exchange chromatography. Homogenates of resistant aphids hydrolysed paraoxon 60 times faster than did those of susceptible aphids, yet the purified enzymes from both sources had identical catalytic-centre activities towards this substrate and also towards naphth-1-yl acetate, the latter being hydrolysed by both 2×10^6 times faster than paraoxon. These observations provide evidence that the enzyme from both sources is identical, and that one enzyme hydrolyses both substrates. This was confirmed by relating the rate of paraoxon hydrolysis to the rate at which paraoxon-inhibited carboxylesterase re-activated. Both had the same first-order rate constant (0.01 min^{-1}), showing clearly that the hydrolysis of both substrates is brought about by the same enzyme. Its K_m for naphth-1-yl acetate was 0.131 mM , and for paraoxon 75 pM . The latter very small value could not be measured directly, but was calculated from substrate-competition studies coupled with measurements of re-activation of the diethyl phosphorylated enzyme. Since the purified enzymes from resistant and susceptible aphids had the same catalytic-centre activity, the 60-fold difference between strains must be caused by different amounts of the same enzyme resulting from mutations of the regulator gene(s) rather than of the structural gene.

Populations of many pests have developed resistance to insecticides, which severely limits their practical usefulness. If a failure to control a pest is to be quickly corrected, it is important to establish rapidly whether the failure is due to resistance, necessitating the use of other insecticides, or to other factors such as weather conditions or poor application. Such confirmation usually relies on bioassays in which the effectiveness of insecticides is assessed under laboratory conditions.

The peach-potato aphid *Myzus persicae* is an important vector of plant viruses besides damaging plants directly when present in sufficient numbers. Organophosphorus and carbamate insecticides are therefore widely applied for its control, both in glass-houses and outdoors, particularly on sugar beet and potatoes. Although glasshouse populations have been resistant to these insecticidal esters for several years (Needham & Sawicki, 1971), field populations developed resistance only recently (Needham & Devonshire, 1975). Identifying resistant aphids by bioassay is very time-consuming, requires many

aphids, and results can be ambiguous, especially when resistance is slight and populations are heterogeneous, as commonly occurs in the field (Devonshire & Needham, 1975). Even very small infestations (less than one aphid per plant) can be economically significant, and before such aphids can be characterized by bioassay they must be reared in sufficient numbers in the laboratory.

Needham & Sawicki (1971) suggested a promising alternative to bioassays for detecting resistance, namely a biochemical assay based on the activity of carboxylesterases in homogenates of individual aphids. These enzymes were more active in resistant than in susceptible strains. Subsequent work has confirmed this good correlation (Devonshire & Needham, 1975; Needham & Devonshire, 1975), and shown that the increased activity is caused predominantly by changes in a single esterase (Beranek, 1974; Devonshire, 1975a), so that the assay can be made more sensitive by staining the enzymes after resolving them from other carboxylesterases by electrophoresis. Because individual

aphids can be characterized, this biochemical assay is particularly useful for examining heterogeneous field populations.

Homogenates of resistant aphids detoxify organophosphorus insecticides by hydrolysis more quickly than do those of susceptible aphids (Oppenoorth & Voerman, 1975), and it seemed possible that the hydrolysis of these phosphoric esters could be achieved by the same enzyme as that hydrolysing carboxyl esters, so explaining the significance of the increased carboxylesterase activity in resistant aphids. This possibility was therefore examined by studying the properties of the purified enzyme from resistant and susceptible aphids and the kinetics of hydrolysis of different substrates. These fundamental studies were undertaken to place the biochemical detection of resistant aphids on a firmer foundation, and to investigate the cause of such large differences between strains in the activity of the enzyme.

The kinetic studies were based on the generally accepted reaction mechanism involving a Michaelis-type intermediate (Aldridge & Reiner, 1972):

For substrates, k_{+2} and k_{+3} are large, so that free enzyme is regenerated and the substrate is hydrolysed; for inhibitors, the acylated enzyme (EA) is either stable, or its rate of hydrolysis (k_{+3}) is very low. When the concentration of the Michaelis complex between enzyme and inhibitor is negligible, the conversion of free enzyme into acylated enzyme is measured by the second-order rate constant k_1 .

Experimental

Materials

Aphid strains. Aphids were collected from field crops and glasshouses usually after they had survived treatment with insecticides. The origin of most of the strains used has been described previously (Devonshire, 1975a); in addition, the most resistant strain (G6) was collected in 1976 from glasshouse-grown chrysanthemums where poor control by aldicarb (an oxime carbamate) had been reported. Laboratory cultures were maintained as clones without further selection by insecticides, and their resistance was assessed by topical application of measured drops of insecticide solutions in butanone (Needham & Devonshire, 1973).

Preparation of [^{14}C]paraoxon. [ethyl-1- ^{14}C]Parathion (diethyl 4-nitrophenyl phosphorothionate; 40mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was oxidized to paraoxon (diethyl 4-nitrophenyl phosphate) by using *m*-chloro-

perbenzoic acid. The benzene solvent was removed from a freshly opened vial of parathion (250 μCi , 6.25 μmol) and 500 μl of chloroform containing 4.3 mg (25 μmol) of *m*-chloroperbenzoic acid was added. After 10 min at room temperature (approx. 20°C), paraoxon was purified from the reaction mixture by t.l.c. on 0.25mm-thick Kieselgel 60 F₂₅₄ (Merck, Darmstadt, Germany) in 10% (v/v) hexane in chloroform (+2% ethanol as stabilizer). After elution from this first plate, paraoxon was rechromatographed on Kieselgel in 3% (v/v) methanol in chloroform (+2% ethanol), and recovered in 32% overall yield. It was radiochemically pure when co-chromatographed with authentic paraoxon on Kieselgel in eight different solvent systems.

Methods

Purification of carboxylesterase. The carboxylesterase associated with resistance was separated from other esterases in homogenates of susceptible and resistant aphids by gel filtration and ion-exchange chromatography, with the use of 0.02M-Tris/HCl, pH 8.5, as buffer throughout the purification. Aphids (3–6g) were homogenized at 0°C in 20ml of buffer containing 1% Triton X-100, and centrifuged at 1100g (r_{av} , 11cm) for 10min. The supernatant was filtered through glass wool to remove the wax, which separated as a layer on the surface, and low-molecular-weight material was removed by chromatography on a column (2.5cm×40cm) of Sephadex G-25. All the esterase activity was eluted unretarded and it was then chromatographed on a column (2.5cm×18cm) of DEAE-cellulose, eluted with a linear 0–0.35M-NaCl gradient in 500ml of buffer. The fractions containing the carboxylesterase involved with resistance were desalted and concentrated by using a hollow fibre 'Beaker Dialyser' (Bio-Rad Laboratories, Bromley, Kent, U.K.), and rechromatographed on a column (2.5cm×18cm) of DEAE-cellulose eluted with a linear 0–0.2M-NaCl gradient (500ml) in buffer to remove the remaining traces of esterase activity contributed by the adjacent peaks from the first ion-exchange column. The purified enzyme was stable for several months when stored at –20°C after replacement of the Tris buffer with 0.02M-phosphate($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$)buffer, pH 7.0.

Electrophoresis. Aphid homogenates and the purified carboxylesterase were examined in polyacrylamide gels by isoelectric focusing, gradient electrophoresis and zone electrophoresis in a discontinuous buffer system.

For isoelectric focusing, homogenate or purified enzyme was incorporated into the solution of acrylamide and *NN'*-methylenebisacrylamide [7.5% and 0.2% (w/v) respectively] containing 0.004% potassium ferricyanide, 0.0007% riboflavin and 1% ampholytes, pH 3.5–10 (Ampholine; LKB, South

Croydon, Surrey, U.K.), which was then photopolymerized in 70mm×3mm (internal diam.) glass tubes. The gels were electrofocused at 200 V for 30 min and then at 300 V for 2.5 h, with 0.2% H_2SO_4 at the anode and 0.4% triethanolamine at the cathode. They were either stained for esterase activity or cast into large-pore gel on the top of gel slabs for gradient or zone electrophoresis in a second dimension.

Gradient electrophoresis was performed for 20 h at 200 V on commercially available (Universal Scientific Ltd., London E13 0QU, U.K.) gels with an acrylamide gradient from 2.5% to 28%, after pre-running in the same electrode buffer as used for zone electrophoresis. Discontinuous zone electrophoresis was performed on gel rods and slabs as described in the Explanation of Plate 1, and by Devonshire (1975a). When individual aphids on gel slabs were examined, homogenates were added to pockets cast in the surface of an additional gel layer containing the same buffer as the running gel, but only 2.5% acrylamide.

Carboxylesterase assay. Individual aphids were weighed on a Beckman LM-500 micro-balance and homogenized in 20mM-phosphate ($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) buffer, pH 7.0, by using a glass homogenizer with a Teflon pestle. Portions of the uncentrifuged homogenates were incubated at 25°C with naphth-1-yl acetate (0.25 mM) in a total volume of 3.0 ml of buffer. After 30 min, Fast Blue B salt (tetra-azotized *o*-dianisidine/ ZnCl_2) (0.3%) in aq. 3.5% (w/v) sodium dodecyl sulphate (0.5 ml) was added, and the A_{605} of the resulting complex measured spectrophotometrically 15 min later. Under these conditions A_{605} was proportional to enzyme activity (Devonshire, 1975a).

Fig. 1. Effect of acetone on the activity of carboxylesterase E-4

Experimental details are given in the text.

The purified carboxylesterase was assayed similarly, but with the addition of bovine serum albumin (1 mg/ml) to the assay to stabilize the enzyme. In the absence of albumin, the activity of the enzyme decreased during the assay, especially with high concentrations of substrate, but in its presence the assay remained linear for up to 30 min provided that no more than 10% of the substrate was hydrolysed. The activity of the enzyme was sensitive to the concentration of acetone in the assay; this is important, because both substrate and inhibitors were added as acetone solutions. Activity was greatest with 1% acetone in the incubation (Fig. 1), and this concentration was therefore used in all assays.

Inhibition of carboxylesterase. Pseudo-first-order rate constants for the inhibition of esterases by organophosphorus compounds are usually measured by incubating together the enzyme and inhibitor for various times and then measuring the residual enzyme activity by a continuous assay after stopping further inhibition by diluting the mixture with a large excess of substrate (Aldridge & Reiner, 1972). However, the carboxylesterase assay chosen for this work, because of its good sensitivity, is destructive, and many point assays would be required to measure the residual activities of the inhibited enzymes. Therefore inhibition was measured in the presence of substrate, so that each measurement of A_{605} represented a point on the first-order inhibition curve. High concentrations of inhibitor and short incubation times were used to minimize the error caused by any re-activation of the enzyme. Pseudo-first-order rate constants were determined from these curves by the least-squares method of linear regression by using a maximum-likelihood computer program (Ross, 1975). Bimolecular rate constants were calculated from these (Main & Iverson, 1966), with correction for the presence of substrate in the inhibition mixture as described by Hart & O'Brien (1973).

Re-activation of inhibited carboxylesterase. Recovery of esterase activity after removing excess inhibitor is usually measured by a series of continuous assays of short duration compared with the rate of reactivation. First-order rate constants are then determined by plotting $\log(\text{fraction inhibited})$ against time of re-activation. Because of the rapid recovery of enzyme activity in the present work, the limited amount of enzyme available and the destructive nature of the assay, such a procedure was not practicable, and re-activation was measured in the presence of substrate.

Enzyme and inhibitor were mixed, left for 10 min so that the enzyme was more than 90% inhibited, and then separated on a column (2.5 cm×38 cm) of Sephadex G-25. Naphth-1-yl acetate was added to the inhibited enzyme, and the production of naphth-1-ol monitored in 1 ml samples at 25°C for up to 30 min.

The assays were started within 10 min of applying the inhibited enzyme to the column. Tangents to the resulting curve would give a measure of enzyme activity by that time, but for better accuracy the curves were analysed by computer by using the maximum-likelihood program. The curves were fitted according to the following derivation.

The rate of re-activation of inhibited enzyme is assumed to be first-order, i.e. at time t :

$$\ln(\text{fraction inhibited}) = -kt + c$$

where k is the first-order rate constant of re-activation. Therefore:

$$\text{fraction inhibited} = e^{(-kt+c)}$$

Thus:

$$\frac{dA}{dt} = a[1 - e^{(-kt+c)}]$$

where A is the amount of naphthol produced (measured as A_{605}) and a is the activity of uninhibited enzyme. Therefore:

$$\frac{dA}{dt} = a - ae^c e^{-kt}$$

Integrating:

$$A = at + \frac{ae^c}{k} \cdot e^{-kt} + d$$

Therefore:

$$\frac{A}{a} - t = f + \frac{e^c}{k} \cdot e^{-kt}$$

where c , d and f are constants. Values for $\frac{A}{a} - t$ were

calculated from the available data, and fitted to a standard form of exponential curve, $Y = C + Be^{-kt}$, by the computer program. This gave two estimates of k , one directly and the other indirectly, since $B = e^c k^{-1}$ and e^c is the fraction inhibited when observations began.

Measurement of [^{14}C]paraoxon degradation *in vitro*. Weighed groups of adult apterous aphids were homogenized in 0.02 M-phosphate ($\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$) buffer, pH 7.0 (2.5 mg/ml), and incubated at 25°C with 1.25 μM -[^{14}C]paraoxon. Products of hydrolysis were measured in 1 ml samples by liquid-scintillation spectrometry (Needham & Devonshire, 1973) of the aqueous phase after extracting unchanged paraoxon into 3 $\times$ 1 ml of chloroform as described by Oppenoorth & Voerman (1975). The concentration of paraoxon in the incubations was such that the enzyme was saturated and its V_{max} attained, and the fraction of substrate degraded was always less than 20%, so that the hydrolysis rate was constant throughout the incubations. However, the amount of ^{14}C in the aqueous phase increased rapidly

immediately the homogenate and paraoxon were mixed, and this was shown to result from an initial rapid binding of the insecticide to high-molecular-weight water-soluble material, because this fraction of the radioactivity was eluted unretarded when the homogenate was chromatographed on Sephadex G-25. The ratio of enzyme activity to amount of bound paraoxon at the time of mixing was similar for all five strains examined, despite a 57-fold range of enzyme activity between strains, indicating that the intercept is accounted for by the insecticide binding specifically to the paraoxon-degrading enzyme rather than non-specifically to other components of the homogenate.

Results and Discussion

Relationship between resistance and the activities of the carboxylesterase and paraoxon-degrading enzyme

There was good correlation between the degree of insecticide resistance and the activity of these enzymes (Table 1), suggesting that the insecticide-hydrolysing enzyme is responsible for resistance, and that measuring the carboxylesterase activity will give a good indication of the degree of resistance. Paraoxon was degraded approx. 60 times faster by homogenates of the most resistant strain than by the susceptible, and naphth-1-yl acetate approx. 35 times faster. In the three most resistant strains (TIV, PirR and G6), there is no significant contribution to the total esterase activity by enzymes other than esterase E-4 (Plate 1), whereas in strains USIL and MSIG this enzyme represents approximately one-sixth and one-half respectively of the total activity. This was estimated from the profiles of the carboxylesterase activity after ion-exchange chromatography. Thus the activity of carboxylesterase E-4 in strain G6 is considerably more than 35 times its activity in susceptible aphids.

The good proportionality between the activities of the carboxylesterase and the paraoxon-degrading enzyme (Fig. 2) suggests that the hydrolysis of both substrates is brought about by the same enzyme.

Location of paraoxon-degrading enzyme after electrophoresis

Homogenate containing the equivalent of three aphids (strain PirR) was run on each of eight gel rods (Devonshire, 1975a), which were then cut into slices approx. 1.2 mm thick by using apparatus similar to that described by Chrambach (1966). The slices of one gel were stained for esterase activity and the corresponding slices from the remaining seven gels were pooled and incubated for 4 h at 25°C in 1 ml of 20 mM-phosphate buffer, pH 7.0, containing [^{14}C]paraoxon (2.5 μM). Only the first 16 slices were so

Table 1. *Resistance, esterase activity and paraoxon degradation for several strains of M. persicae*

Values are means $\pm$ S.D. for the numbers of experiments in parentheses. Resistance factor is defined as LD₅₀ of strain/LD₅₀ of susceptible strain (see Needham & Devonshire, 1973). Substrate concentration for esterase activity measurements was 0.25 mM.

Aphid strain	Approximate resistance factor to dimethoate	Total esterase activity (μ mol of naphth-1-ol/h per mg of aphid)	Paraoxon degraded (pmol/h per mg of aphid)
USIL	1	0.21 $\pm$ 0.03 (31)	0.26 $\pm$ 0.12 (6)
MSIG	8	0.42 $\pm$ 0.07 (25)	1.32 $\pm$ 0.45 (4)
FrenchR	15	0.96 $\pm$ 0.12 (13)	—
TIV	100	1.85 $\pm$ 0.56 (35)	4.04 $\pm$ 0.40 (2)
PirR	250	3.87 $\pm$ 1.04 (20)	7.07 $\pm$ 0.70 (5)
G6	500	7.13 $\pm$ 1.46 (27)	14.80 $\pm$ 0.12 (2)

Fig. 2. Rates of hydrolysis of paraoxon and naphth-1-yl acetate by aphid homogenates

The points refer to the enzyme activities of five of the clones shown in Table 1.

incubated, because a preliminary experiment showed that the paraoxon-degrading activity was located entirely within this distance from the origin. After extraction of unchanged paraoxon from each incubation into chloroform, and correction for its control hydrolysis (in the presence of gel containing no enzyme), radioactivity was only found in the incubation of slices 10, 11 and 12. This corresponded exactly to the position of the most active carboxylesterase band (E-4) in the stained gel slices, and provided further preliminary evidence that this carboxylesterase is the same enzyme as that degrading the paraoxon.

Purification of carboxylesterase

Carboxylesterase E-4 was separated from other esterases before examination of the kinetics of its reaction with naphth-1-yl acetate and paraoxon. Fig. 3 shows the elution profiles of aphid carboxyl-

Fig. 3. Elution of carboxylesterases from DEAE-cellulose chromatography of homogenates of susceptible USIL (○) and slightly resistant MSIG (●) aphids

For this, 500 aphids (178 mg of strain USIL, 150 mg of strain MSIG) were homogenized in 8 ml of 0.02 M-Tris/HCl, pH 8.5 (at 20°C), containing 1% Triton X-100. After centrifuging at 1100g for 10 min, the supernatants were chromatographed on columns (2.5 cm $\times$ 18 cm) of DEAE-cellulose, eluted with linear NaCl gradients (—, 0–0.35 M) in the same buffer (500 ml) without detergent; 10 ml fractions were collected.

esterases from DEAE-cellulose. Fractions were concentrated in a Minibeaker Dialyser (Bio-Rad Laboratories) and examined by zone electrophoresis. The enzymes eluted at NaCl concentrations of 0.11 M, 0.14 M and 0.28 M corresponded to esterase bands E-1, E-4 and E-7 (Plate 1) respectively, and band E-4 contained all the paraoxon-degrading activity. The enzyme eluted at 0.22 M-NaCl was probably esterase

E-6, although it could not be detected after electrophoresis of the concentrated fractions because it lost activity. The instability of this particular enzyme is also evident when the elution profiles are compared with esterase zymograms (Plate 1) of aphid homogenates. On the latter, enzyme E-6 is the most active esterase from susceptible aphids, and is almost as active as enzyme E-4 from aphids of strain MSIG, whereas after ion-exchange chromatography it contributes only a small proportion of the total activity.

To purify enzyme E-4 from many aphids, the fractions containing this enzyme were pooled, concentrated and rechromatographed on a column (2.5 cm $\times$ 18 cm) of DEAE-cellulose by elution with a shallower NaCl gradient (0–0.25 M in a total volume of 500 ml of Tris/HCl). After this second ion-exchange chromatography, the homogeneity of the enzyme was checked by electrophoresis in two dimensions. The first dimension was isoelectric focusing in a gel

rod, and this was followed by either zone electrophoresis or gradient electrophoresis in a gel slab. In all three systems, the carboxylesterase was present entirely in a single area of activity, and the subsequent kinetic studies used this purified enzyme, mainly prepared from the PirR strain.

Hydrolysis of naphth-1-yl acetate and its inhibition by paraoxon

Carboxylesterase activity could not be measured at substrate concentrations that allow the enzyme to operate at its maximum rate ($V_{\max}$), because of the limited solubility of naphth-1-yl acetate. Instead, it was usually assayed in 0.25 mM-substrate, and its activity corrected to $V_{\max}$ when appropriate (e.g. in the calculation of catalytic-centre activity). The K_m of the enzyme for naphth-1-yl acetate was 0.131 ± 0.013 mM (S.E.M., seven independent determinations), and the ratio $V_{\max}/V_{0.25\text{mM}}$ was 1.54 ± 0.03 .

Inhibition of the enzyme by paraoxon was measured in the presence of substrate (Fig. 4) by using a range of naphth-1-yl acetate concentrations (0.25–0.5 mM) and of paraoxon concentrations (1–10 nM). The second-order rate constant (k_i) calculated from these inhibition studies was 0.133 ± 0.008 nM $^{-1}$ min $^{-1}$ (S.E.M., nine independent determinations). The k_i values from the individual determinations were independent of the concentration of substrate, or of the duration of the assays (5–15 min), indicating that there had been no significant re-activation of the inhibited enzyme during the longer incubations, as these would have then given a lower value for the k_i .

The bimolecular rate constant relates to the overall conversion of active enzyme into inactive phosphorylated enzyme. This type of reaction commonly occurs via a Michaelis-type intermediate, the dissociation of the complex and the phosphorylation step being defined by the constants K_a and k_{+2} . In individual experiments it was not possible to determine these accurately, because of the small proportion of enzyme present as the Michaelis complex. However, estimates of $K_a = 15.1 \pm 5.4$ nM and $k_{+2} = 1.6 \pm 0.5$ min $^{-1}$ were obtained by analysing all 58 inhibition experiments together as a single graph by using the maximum-likelihood computer program.

Fig. 4. Hydrolysis of naphth-1-yl acetate (0.5 mM) in the presence of paraoxon

Experimental details are given in the text. The concentrations of paraoxon and the corresponding pseudo-first-order rate constants (min $^{-1}$) were: $\circ$, 3 nM, 0.076 ± 0.005 ; $\triangle$, 4 nM, 0.093 ± 0.007 ; $\square$, 5 nM, 0.105 ± 0.008 ; $\bullet$, 6 nM, 0.126 ± 0.006 ; $\blacktriangle$, 8 nM, 0.165 ± 0.012 ; $\blacksquare$, 10 nM, 0.188 ± 0.016 . Standard errors refer to the computed best fit of each curve. ----, Activity of uninhibited enzyme.

Re-activation of paraoxon-inhibited carboxylesterase

Carboxylesterase activity recovers completely from paraoxon inhibition several hours after removal of excess inhibitor on Sephadex G-25, i.e. paraoxon phosphorylates the carboxylesterase, but the phosphorylated enzyme is not stable, and slowly hydrolyses to regenerate active enzyme. In other words, paraoxon is a substrate, albeit a poor one compared with

EXPLANATION OF PLATE I

Electrophoresis of aphid homogenates on 7.5% polyacrylamide gels containing 0.2% Triton X-100

Individual aphids were homogenized in 15 μ l of water containing (by wt.) 10% sucrose and 0.5% Triton X-100, and 10 μ l samples were electrophoresed on gel rods in the discontinuous buffer system of Williams & Reisfeld (1964), but omitting the sample gel and spacer gel. After 1.5 h at approx. 10 V/cm, gels were stained for esterase activity in the dark at 20°C for 45 min. The stain was prepared by adding 30 mM-naphth-1-yl acetate in acetone (1 ml) to 0.2% (w/v) Fast Blue RR salt (diazotized 4-benzoylamino-2,5-dimethoxyaniline/ ZnCl_2) in 0.2 M-phosphate buffer, pH 6.0 (50 ml). Electrophoretic mobilities (m) were measured relative to the buffer interface in the gel, and esterases E-3 and E-5 were detected in susceptible aphids only after staining for 90 min.

Fig. 5. Hydrolysis of naphth-1-yl acetate by uninhibited enzyme (Δ), and by the same amount of paraoxon-inhibited enzyme ($\circ$) after removal of excess inhibitor on a Sephadex G-25 column. Experimental details are given in the text.

Table 2. First-order rate constants (h^{-1}) for re-activation of paraoxon-inhibited carboxylesterase E-4

The values for k , B and e^c were computed from three experiments (see under 'Methods'), and the s.e. for each is based on the computed best fit of the line.

k	$B (= e^c k^{-1})$	e^c	$e^c/B (= k)$
0.41 ± 0.02	2.05 ± 0.07	0.832	0.41
0.62 ± 0.02	1.48 ± 0.04	0.912	0.62
0.46 ± 0.02	1.96 ± 0.09	0.899	0.46

Mean 0.50

naphth-1-yl acetate, for the carboxylesterase. However, this slow hydrolysis is significant to resistance because of the extremely small amount of insecticide (approx. 1 ng) required to kill an aphid. The first-order rate constant for the hydrolysis of phosphorylated enzyme (Table 2) was calculated (see under 'Methods') from the progress of this recovery (Fig. 5). The good agreement between the direct and indirect estimates of k , and the small computed errors associated with these values, indicate that the data fit the equation well.

Catalytic-centre activity of enzyme E-4

The great variation between strains in the activity of the enzyme could result from either changes in the regulatory gene(s) causing more enzyme to be

Fig. 6. Rates of [^{14}C]paraoxon hydrolysis in 0.02M-phosphate buffer, pH7.0 ($\circ$), and by the purified carboxylesterase E-4 from susceptible (strain USIL: $\square$) and resistant (strain PirR: Δ) aphids in the same buffer. Experimental details are given in the text.

Catalytic-centre activities for paraoxon were calculated from the molar concentration of enzyme (intercept) and enzyme activity (slope). The corresponding rates of naphth-1-yl acetate hydrolysis were $15.8 \mu\text{mol/h}$ per ml for strain USIL and $10.4 \mu\text{mol/h}$ per ml for strain PirR, from which catalytic-centre activities for this substrate were calculated.

produced, or from mutations of the structural gene, altering the catalytic-centre activity of the enzyme without changing its electrophoretic behaviour. Such a change in the nature of the enzyme could be caused by a small change in the active centre, and need not be accompanied by a detectable difference in its electrophoretic properties; for example, the acetylcholinesterases from resistant and susceptible houseflies are indistinguishable electrophoretically, but differ markedly in their kinetic properties (Devonshire, 1975b). The catalytic-centre activities of the purified carboxylesterases from resistant and susceptible aphids were therefore measured to distinguish between these possibilities.

The rate of [^{14}C]paraoxon degradation by the purified enzymes was measured, and the enzyme concentration in the incubations determined from the amount of bound paraoxon at zero time (Fig. 6). The catalytic-centre activity of the enzyme from resistant aphids was calculated from the data of three such experiments, but only one determination (Fig. 6)

Table 3. *Catalytic-centre activities (min^{-1}) of carboxylesterase E-4 from susceptible (USIL) and resistant (PirR) aphids (S.E.M., three independent determinations)*
Experimental details are given in the text.

Strain	$10^2 \times$ catalytic-centre activity with Paraoxon	$10^{-4} \times$ catalytic-centre activity with naphth-1-yl acetate
USIL	0.9	1.9
PirR	1.1 ± 0.1	2.05 ± 0.13

was made with the enzyme from the susceptible strain, because of the limited amount of material available. However, this single determination agrees well with the others (Table 3), and it is therefore clear that resistant strains have greater activity of enzyme E-4, because they have more of the same enzyme than susceptible aphids, rather than the same amount of a more active enzyme. The enzyme from both strains hydrolysed naphth-1-yl acetate 2×10^6 times as fast as paraoxon, and this good agreement between strains in the ratios of catalytic-centre activity for both substrates further supports the hypothesis that they are hydrolysed by the same enzyme.

The first-order rate constant for the hydrolysis of diethyl phosphorylated carboxylesterase and the catalytic-centre activity of the paraoxon-degrading enzyme are the same (0.01 min^{-1}), clearly demonstrating that carboxylesterase E-4 hydrolyses both naphth-1-yl acetate and paraoxon. The rate-limiting reaction is the hydrolysis of phosphorylated enzyme (k_{+3}), because the phosphorylation step (k_{+2}) is approx. 160 times as fast ($k_{+2} = 1.6 \pm 0.5 \text{ min}^{-1}$, $k_{+3} = 0.01 \text{ min}^{-1}$).

Breeding studies with aphids are restricted by the difficulty of rearing them through the sexual phase, but preliminary experiments (Blackman *et al.*, 1977) have shown that the progeny of crosses between FrenchR and susceptible aphids fall into two groups with esterase activities and degrees of resistance intermediate between those of the parents. It was not clear from those studies whether the increased activity is the result of two independent loci or of several alleles at a single locus. However, it is evident from the present studies that the gene(s) involved are regulatory rather than structural. Mutations in regulatory genes causing such large differences (57-fold) in the amount of enzyme produced have not been positively identified previously in insects, even in the most intensively studied species *Drosophila* (Courtright, 1976).

The wide variation in the activity of the enzyme might also have been influenced by a naturally occurring chromosome translocation (Blackman & Takada, 1975) common to all very resistant glass-house populations so far examined (Blackman *et al.*, 1977). This could have separated the structural and

regulator genes on to different chromosomes, so allowing enzyme production to proceed unrestricted. Aphids with this translocation also lack esterase E-5, which is only present in the susceptible and slightly resistant (MSIG and FrenchR) strains.

The K_m of the enzyme hydrolysing paraoxon could not be measured directly, because of the limited sensitivity of the radioassay (Oppenoorth & Voerman, 1975), but the present data allow an indirect estimate because $K_m = k_{+3}/k_i = 75 \text{ pM}$ (Aldridge & Reiner, 1972). This approach may be applied to any similar substrate with a very small K_m and does not depend on the availability of radiolabelled substrate. The small K_m of this enzyme indicates that it will hydrolyse insecticide efficiently, even at the very small concentrations present in treated insects.

This confirmation that the ability to degrade insecticides and the carboxylesterase activity of E-4 are conferred by the same enzyme puts on a sound basis the use of carboxylesterase assays to detect and measure resistance of *M. persicae* to insecticides. As this is the only cause of resistance identified in this aphid, it should be possible to predict from the substrate specificity of the enzyme the likelihood of there being resistance to any given insecticide.

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References

- Aldridge, W. N. & Reiner, E. (1972) *Enzyme Inhibitors as Substrates*, *Frontiers of Biology* (Neuberger, A. & Tatum, E. L., eds.), vol. 26, North-Holland, Amsterdam and London.
- Beranek, A. P. (1974) *Entomol. Exp. Appl.* **17**, 129–142.
- Blackman, R. L. & Takada, H. (1975) *J. Entomol. Ser. A* **50**, 147–156.
- Blackman, R. L., Devonshire, A. L. & Sawicki, R. M. (1977) *Pestic. Sci.* **8**, 163–166.
- Chrambach, A. (1966) *Anal. Biochem.* **15**, 544–548.
- Courtright, J. B. (1976) *Adv. Genet.* **18**, 249–314.
- Devonshire, A. L. (1975a) *Proc. Br. Insectic. Fungic. Conf. 8th* **1**, 67–73.
- Devonshire, A. L. (1975b) *Biochem. J.* **149**, 463–469.
- Devonshire, A. L. & Needham, P. H. (1975) *Proc. Br. Insectic. Fungic. Conf. 8th* **1**, 15–19.
- Hart, G. J. & O'Brien, R. D. (1973) *Biochemistry* **12**, 2940–2945.
- Main, A. R. & Iverson, F. (1966) *Biochem. J.* **100**, 525–531.
- Needham, P. H. & Devonshire, A. L. (1973) *Pestic. Sci.* **4**, 107–111.
- Needham, P. H. & Devonshire, A. L. (1975) *Pestic. Sci.* **6**, 547–551.

Needham, P. H. & Sawicki, R. M. (1971) *Nature (London)* **230**, 125–126
Oppenoorth, F. J. & Voerman, S. (1975) *Pestic. Biochem. Physiol.* **5**, 431–443

Ross, G. J. S. (1975) *Proc. Sess. Int. Stat. Inst.* **40th** **2**, 585–593
Williams, D. E. & Reisfeld, R. A. (1964) *Ann. N. Y. Acad. Sci.* **121**, 373–381