Purification and characterization of a carboxylesterase involved in insecticide resistance from the mosquito *Culex quinquefasciatus*

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A carboxylesterase (EC 3.1.1.1) involved in organophosphate insecticide resistance has been purified and characterized from the mosquito *Culex quinquefasciatus*. The monomeric enzyme has M_r of 67000 and a pI of 5.2. It hydrolysed medium-chain-length mono- and di-acylglycerols in addition to xenobiotic esters. Kinetic constants determined for four insecticides, temephos, chlorpyrifos, fenitrothion and propoxur indicate the rates of acylation and the affinities of binding of the insecticides to this carboxylesterase are important. This supports the major role of the A_2 carboxylesterase is the sequestration of the insecticide with a minor role in the slow turnover of the insecticide.

INTRODUCTION

In attempts to control *Culex quinquefasciatus* as a worldwide vector of diseases such as filariasis and encephalitis, mosquito field populations are sprayed with organophosphate insecticides. As a result of these control programmes, resistance to these compounds has subsequently appeared in the field populations [for a recent discussion, see the review by Brown (1986)]. The most commonly observed change that has been linked to resistance development is the increase in activity of carboxylesterases (EC 3.1.1.1) (Devonshire & Field, 1991). The putative mechanism involves an overproduction of this enzyme for the sequestration of, and cross-resistance to, various organophosphate and carbamate insecticides.

To date, none of the hydrolytic enzymes thought to be involved in insecticide resistance have been purified from mosquitoes. We believe that this should be the first step in the elucidation of the biochemical mechanisms involved in resistance, that is, subsequent characterization of the purified proteins will provide valuable information on the properties of the enzymes, highlighting differences in expression between strains and possibly even qualitative changes in this enzyme in different field populations. We therefore report the purification and characterization of a culicine carboxylesterase involved in insecticide resistance (Peiris & Hemingway, 1990a). This enzyme is sometimes referred to as the esterase 'A₂' because of its preferential hydrolysis of α - over β -naphthyl acetate [and not based on the Aldridge (1953) classification]. The increased esterase activity in the resistant strain of *C. quinquefasciatus* makes it an ideal source.

In addition to identifying quantitative changes in these enzymes, we are also interested in determining their possible physiological roles. Previous reports of an interaction of an insect esterase with endogenous substrates include studies on juvenile-hormone esterase and an esterase involved in pheromone synthesis (Rudnicka & Jones, 1987; Mane *et al.*, 1983). In vertebrates, carboxylesterases have been shown to catalyse the hydrolysis of many xenobiotic esters (Heymann, 1982). The carboxylesterases also exhibit high catalytic activity for lipids such as the acylglycerols and acylcarnitines (Mentlein *et al.* 1984, 1985a,b). Yet the precise biological role of the enzymes, apart from the detoxication of foreign compounds, remains undefined. This is the first report of the characterization of a purified insecticide-resistance-conferring insect carboxylesterase with endogenous substrates.

EXPERIMENTAL

Materials

Q-Sepharose Fast Flow, phenyl-Sepharose Fast Flow, and Sephacryl S-200 HR, PD-10 columns and Ampholine PAGplates pH 3.5–9.5 were purchased from Pharmacia (Uppsala, Sweden). Hydroxyapatite and the protein assay kit were purchased from Bio-Rad (Richmond, CA, U.S.A.). Biochemicals were purchased from Sigma (St. Louis, MO) except when stated otherwise. The NEFA C kit was purchased from Wako Pure Chemicals (Osaka, Japan). *OOO'*-tetramethyl *OO'*-thiodi-*p*-phenylene bis(phosphorothioate) (temephos) and *OO*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (chlorpyrifos) were purchased from British Greyhound (Birkenhead, Merseyside, U.K.). *OO*-dimethyl *O*-4-nitro-*m*-tolyl phosphorothioate (fenitrothion) was a gift from Sumitomo Chemical Co. (Osaka, Japan). Diethyl(dimethoxythiophosphorylthio)succinate (malathion) was a gift from Cheminova (Lemvig, Denmark). 2-isopropoxyphenyl methylcarbamate (propoxur) was a gift from ICI (Bracknell, Berks., U.K.). The source of the carboxylesterase reported here is a homozygous resistant mosquito (*Culex quinquefasciatus*) from Sri Lanka. This strain, PEL RR, was 31-fold more resistant to temephos than a susceptible strain (Peiris & Hemingway, 1990a).

Enzyme assays

During purification, esterase activity was assayed at 405 nm by using 1 mM-*p*-nitrophenyl acetate in 50 mM-phosphate buffer, pH 7.4, at 22 °C. Unless stated otherwise, all enzyme assays were performed at 22 °C. Diethyl succinate and malathion carboxylesterase activities were assayed by the method of Talcott (1979). Esterase activity for the naphthyl acetate esters was measured at 235 nm (Mastropaolo & Yourno, 1981). Palmitoyl-CoA hydrolysis was measured as described by Berge & Farstad (1979). With the exception of palmitoyl-CoA and the lipid carnitine salts, which are soluble in aqueous solutions, all stock solutions of lipid substrates were prepared in chloroform. Before use, an aliquot of a chloroform substrate solution was dried under nitrogen then resuspended by sonication in 0.01 % (v/v) Triton X-100 in 50 mM-phosphate buffer, pH 7.4, to give a concentration of the lipid substrate of 0.5 mM. The lipid carnitine salts were dissolved in water and then diluted into 0.01 % Triton X-100 in 50 mM-phosphate buffer, pH 7.4. Each of the 0.5 mM-substrate stock solutions (0.1 ml) and 0.9 ml of purified carboxylesterase (9 µg of protein/ml final concn.) or 50 mM-

phosphate buffer, pH 7.4, were mixed and incubated for 10 min at 28 °C. The enzymic hydrolysis was stopped by the addition of 10 μ l of 10 mM-paraoxon, and the NEFA C kit was used to determine non-esterified-fatty-acid concentration.

Kinetic constants

All specific activities are given in units/mg of protein. A unit corresponds to the hydrolysis of 1 μ mol of substrate in 1 min under the assay conditions used. Michaelis constants and maximum specific activity (V_{max}) were calculated by non-linear regression (Duggleby, 1981). For the inhibition kinetics, stopped-time inhibition assays were performed using *p*-nitrophenyl acetate as the substrate. The purified A_2 enzyme was incubated with a series of concentrations of the respective insecticide and at various times aliquots were withdrawn and residual activity was determined by measuring the rate of *p*-nitrophenyl acetate hydrolysis. The activities were divided by those measured in the absence of insecticide (control). The re-activation experiments were performed by incubating the enzyme with the respective insecticide for 15 min so that the enzyme was more than 90% inhibited. The unbound insecticide and enzyme-insecticide complex were separated on a Nick spin column (Pharmacia), the manufacturer's instructions being followed. Aliquots were then removed to measure the *p*-nitrophenyl acetate esterase activity of the re-activating enzyme, as well as a control, over time. The pseudo-first-order inhibition rate constants were calculated and used to determine the various inhibition constants (Aldridge & Reiner, 1972).

Purification of the carboxylesterase

Unless stated otherwise, the purification steps were performed at 4 °C. Fourth-instar larvae were snap-frozen in liquid N_2 and stored at -70 °C until used. The larvae (15 g) were homogenized in 100 ml of 25 mM-Bistris propane buffer, pH 6.5, containing 15 mM-DL-dithiothreitol. The homogenate was centrifuged at 10000 *g* for 10 min, and the supernatant was applied to a Q-Sepharose Fast Flow column (4.4 cm $\times$ 4 cm) equilibrated with the homogenization buffer. The enzyme was eluted with a 10-bed-volume salt gradient (0–0.5 M-NaCl in the Bistris propane buffer), and elution profiles were determined for esterase activity, protein and conductivity. The esterase activity was eluted in one peak, and the salt concentration of the pooled fractions was adjusted to 3 M-NaCl. This sample was then applied to a phenyl-Sepharose Fast Flow column (2.2 cm $\times$ 13.2 cm) equilibrated with 25 mM-Bistris propane buffer, pH 6.5, containing 10 mM-DL-dithiothreitol and 3 M-NaCl. The adsorbed esterase activity was eluted with a 5-bed-volume decreasing salt gradient (3–0 M-NaCl in the equilibration buffer) with elution continued isocratically once the gradient ended. The esterase peak of interest was eluted first and the fractions were combined and dialysed against dry sucrose. Further de-salting and buffer exchange into the hydroxyapatite buffer was performed on PD-10 columns according to the manufacturer's instructions. The sample was applied to an hydroxyapatite column (2 cm $\times$ 6.4 cm) equilibrated with 10 mM-phosphate buffer, pH 6.8, containing 10 mM-DL-dithiothreitol. The esterase activity was eluted with a 5-bed-volume gradient of the phosphate buffer (10–200 mM, pH 6.8). The hydroxyapatite chromatography was performed at 22 °C. The esterase activity was eluted as a single peak and the fractions were combined and dialysed against dry sucrose at 4 °C. Removal of sucrose and buffer exchange was performed on PD-10 columns equilibrated with 50 mM-phosphate buffer, pH 6.8.

Gel filtration

A Sephacryl S-200 HR column (1.5 cm $\times$ 32 cm) equilibrated

with 50 mM-phosphate buffer, pH 7.2, was calibrated, at 4 °C, in individual experiments using Blue Dextran (M_r 2000000), and standard proteins (M_r 12400–200000). A 1 ml sample volume was applied with a flow rate of 0.066 ml/min.

Protein determination

Protein concentration was estimated with the method of Bradford (1976), with bovine serum albumin as the standard protein.

PAGE

Electrophoresis of native protein samples was performed in polyacrylamide gels at 4, 6, 8 and 10% acrylamide in Tris/glycine buffer, pH 8.9, by the method of Davis (1964). To estimate the M_r of the carboxylesterase, electrophoresis was performed with standard proteins (M_r 14200–272000) in the same vertical slab gels (Hedrick & Smith, 1968). The gels were stained for esterase activity with 0.04% (w/v) α - and β -naphthyl acetate and 0.1% (w/v) Fast Blue B in 100 mM-phosphate buffer, pH 7.4. Coomassie Blue R250 was used to stain for protein according to the LKB protocol.

SDS/PAGE was performed, with standard proteins (M_r 26000–180000), using a Bio-Rad Miniprotein II electrophoresis unit and a 4–20% gradient acrylamide gel as described by Laemmli (1970). On a similar gradient gel using BSA, a Coomassie Blue-stained band could be detected when 0.025 μ g of protein was loaded (results not shown).

Isoelectric focusing

Isoelectric focusing was performed on LKB Ampholine PAGplate pH 3.5–9.5 gels focused at 30 W constant power, at 10 °C, for 1.5 h.

RESULTS AND DISCUSSION

Purification

The purification procedure, involving sequential chromatography on Q-Sepharose, phenyl-Sepharose and hydroxyapatite columns, is straightforward and efficient (Fig. 1). Using the outlined procedure, approx. 500 μ g of purified esterase A_2 can be obtained within 48 h from 15 g wet weight of larvae. The final esterase preparation appeared to be homogeneous as determined by SDS/PAGE, and any contaminating protein must be less than 0.6% of the total protein (Fig. 2). In addition, on non-denaturing gels stained for esterase activity, only the A_2 carboxylesterase was observed. Data for a single purification procedure is given in Table 1, and the three column-chromatography profiles shown are from the same experiment (Fig. 1). Multiple preparations of the A_2 esterase routinely gave approx. 20–30% final recovery of enzyme activity and an approx. 200-fold purification. The resistant strain Pel RR had two carboxylesterases with increased activity, esterase A_2 and esterase B_2 , compared with a susceptible strain (Fig. 3). The esterase B_2 was eluted with the A_2 enzyme from the ion-exchange column, since both esterases had similar pI values. The B_2 enzyme was then separated from esterase A_2 on the phenyl-Sepharose column in a second, smaller, esterase-activity peak (Fig. 1b). The data (means $\pm$ S.D.) are from several experiments and from several different purifications of the enzyme.

It was critical in the purification to maintain a minimum dithiothreitol concentration of 10 mM. In the absence of dithiothreitol, pigments in the mosquito homogenate oxidized rapidly (within minutes) to produce a black-coloured solution in which the esterases quickly lost activity. High dithiothreitol concentration prevented this. After purification the enzyme was

Fig. 1. Elution profiles for protein, conductivity and esterase activity using 1 mM-*p*-nitrophenyl acetate as substrate

See the text for parameters used. (a) Q-Sepharose chromatography; (b) phenyl-Sepharose chromatography; (c) hydroxyapatite chromatography

stable, with no decrease in specific activity after 5 weeks if stored in the presence of 10 mM-DL-dithiothreitol and 10% (v/v) glycerol at 4 °C.

Physical characterization

The monomeric M_r of the purified enzyme estimated from

Fig. 2. SDS/PAGE of the purified carboxylesterase

Lane A contains the purified esterase A₂ (4 µg total protein) and lane B contains the M_r markers.

Table 1. Purification of the carboxylesterase

The purification was monitored by the assay of 1 mM-*p*-nitrophenyl acetate hydrolysis.

Step	Specific activity (units/mg)	Protein (mg)	Purification (fold)	Recovery (%)
10000 g supernatant	1.81	391	—	—
Q-Sepharose	34.4	16.7	19.0	81
Phenyl-Sepharose	222	1.85	123	58
Hydroxyapatite	427	0.507	235	31

Fig. 3. Esterase-activity-stained native-PAGE gel

Equal amounts of protein were loaded for homogenates from the resistant strain Pel RR (lanes A and B) and from a susceptible strain (lanes C and D).

SDS/PAGE is 67200 ± 1800 . On PAGE using various acrylamide-gel concentrations native carboxylesterase had an estimated M_r of 60000 ± 9000 . The M_r estimated by Sephacryl S-200 chromatography was 56000. This data supports the hypothesis that the native form of this carboxylesterase is monomeric. This contrasts with the previous report of a dimeric form of the partially purified enzyme (Fournier *et al.*, 1987). There may be a number of reasons for this apparent discrepancy. First, the earlier study used only one method, namely acrylamide-concentration-gradient PAGE, to determine the M_r of an esterase in a heterogeneous population. Secondly, there may be

Table 2. Effect of inhibitors on the activity of the purified carboxylesterase

The individual inhibitors were preincubated with the purified enzyme for 30 min at 22 °C. Esterase activity was then measured with 1 mM-*p*-nitrophenyl acetate in the presence of each inhibitor.

Inhibitor	Concentration (mM)	Inhibition (%)
Paraoxon	0.1	99.4 ± 0.95
Bis-(<i>p</i> -nitrophenyl) phosphate	0.1	99.4 ± 0.87
EDTA	1	3.2 ± 1.6
HgCl ₂	1	99.5 ± 0.80
Eserine	0.01	95.1 ± 5.3

association–dissociation of carboxylesterases, and this has been shown to be concentration- and pH-dependent (Heymann *et al.*, 1971; Scott & Zerner, 1975). The pI of pH 5.2 was determined by isoelectric focusing on Ampholine PAGplates. This pI is typical for carboxylesterases, being in the general range of pH 4.7–6.5 (Heymann, 1982). The carboxylesterase is glycosylated, as shown by the thymol/H₂SO₄ staining for glycoprotein detection (Gerard, 1990). Glycosylation is characteristic of the vertebrate carboxylesterases (Junge *et al.*, 1974; Hashinotsume *et al.*, 1978; Oehm *et al.*, 1982; Robbi & Beaufay, 1983).

Inhibitor confirmation of enzyme classification

Inhibitor studies were performed to confirm the esterase classification. Inhibition by 0.1 mM-paraoxon demonstrated the enzyme is a B-type serine hydrolase according to the classification of Aldridge (1953) (Table 2). Bis-(*p*-nitrophenyl) phosphate, which has been shown to be a specific carboxylesterase inhibitor in rats (Brandt *et al.*, 1980), also inhibited the purified mosquito enzyme. Only a slight inhibition was observed in the presence of EDTA, this indicating that there is no metal-ion requirement for catalysis. Inhibition by HgCl₂ implicates the involvement of a thiol group in catalysis, or more likely, in conformational stability, because dithiothreitol addition was necessary to maintain esterase activity in storage. The acetylcholinesterase inhibitor eserine also inhibited the carboxylesterase. This result is perhaps unsurprising in light of the putative sequestering role for carboxylesterase in protecting the target enzyme acetylcholinesterase.

Substrate specificity

Substrate specificities were examined with several xenobiotic and possible physiological substrates. Michaelis constants were determined for several general esterase substrates, *p*-nitrophenyl acetate ($V_{\max}$ 328.6 ± 9.3 µmol/min per mg; K_m 98.9 ± 9.34 µM) and diethyl succinate ($V_{\max}$ 648.2 ± 238.5 nmol/min per mg; K_m 4.65 ± 1.95 mM). The two substrates used to originally define the esterase A₂ were also examined, α-naphthyl acetate ($V_{\max}$ 80.9 ± 10.0 µmol/min per mg; K_m 2.71 ± 0.51 mM) and β-naphthyl acetate, although constants could not be determined for β-naphthyl acetate because the substrate was not soluble at the concentrations necessary for the determination. However, the maximum specific activity (38.0 µmol/min per mg) measured was for β-naphthyl acetate at 1 mM. No activity could be detected when using malathion as a substrate. In addition, palmitoyl-CoA (no activity detected), acetylthiocholine (0.0438 µmol/min per mg) and butyrylthiocholine (0.1382 µmol/min per mg) were also tested as substrates. Interestingly, although the carboxylesterase was inhibited by eserine, it did not

appear to have significant activity with the two commonly used cholinesterase substrates.

The insecticides temephos (the A₂ enzyme was isolated from a strain selected with temephos), chlorpyrifos, fenitrothion, propoxur and malathion were used in inhibition kinetic studies. The insecticides are not true irreversible inhibitors of the A₂ carboxylesterase, but rather they are poor substrates whose sluggish reaction is accounted for by rate-determining deacylation. A slow recovery of A₂ carboxylesterase activity follows rapid inhibition (< 20 min) and the generally accepted reaction mechanism is:

where EI is the Michaelis complex and EI' is the acylated enzyme. In Table 3, $K_a = (k_{-1} + k_2)/k_1$, i.e., the Michaelis constant K_m for the insecticide and k_a is the second-order rate constant for the formation of the acylated enzyme (Aldridge & Reiner, 1972). Linear pseudo-first-order kinetics were obtained for the inhibition of the A₂ carboxylesterase in the insecticide–interaction experiments. This would indicate the absence of a possible contaminant which rapidly hydrolysed the insecticides. The A₂ esterase re-activation kinetic experiments, to determine k_3 , also support the ability of the A₂ carboxylesterase to bind and hydrolyse the insecticides, albeit at a low rate. Malathion was used to determine whether an interaction with the A₂ enzyme could now be observed with the higher sensitivity of the *p*-nitrophenyl acetate assay. However, no interaction of the A₂ esterase with malathion could be detected. The kinetic constants determined for the four other insecticides studied indicate that the k_a and K_a appear to be important in determining the A₂ enzyme's role in resistance. The rates of binding and affinities of binding of the insecticides observed support the role of the A₂ carboxylesterase to be mainly sequestration followed by the slow turnover of the insecticide. This mechanism has been reported for an aphid esterase characterized for insecticide resistance (Devonshire & Moores, 1982). However, the aphid esterase had been shown also to be present in greater amounts (~ 60-fold) in the resistant aphids compared with the susceptible aphids (Devonshire, 1977). Qualitative differences in the A₂ esterase's interactions with the insecticides will be important as a 30-fold resistance to temephos is observed without an observable increase in carboxylesterase quantity, either A₂ or B₂ as both are similar in size on SDS gels (results not shown).

To date only xenobiotic substrates have been used to measure insecticide resistance-conferring esterases; however, several possible physiological substrates were also investigated and the results for the substituted glycerol substrates are shown in Table 4. In addition, both 1-stearoyl-2-arachidonoylglycerol and β-oleoyl-γ-stearoyl 1-α-phosphatidylcholine were used as substrates, but no fatty acid hydrolysis was measurable. The present study demonstrates this mosquito carboxylesterase also has acylglycerol lipase activity which has been reported for vertebrate carboxylesterases (Mentlein *et al.*, 1984, 1985a,b; Ketterman *et al.*, 1989). The substrate preference of the mosquito enzyme appeared to be medium-chain-length mono- and diacylglycerols, although all the monoacylglycerols tested were hydrolysed to some extent. However, two acylcarnitines (decanoyl-, 0.24% hydrolysis; palmitoyl-, no hydrolysis detected) tested were very poor substrates. The present study therefore provides further support for the involvement of carboxylesterase in lipid–fatty acid metabolism in addition to a detoxication role. As has been reported for other carboxylesterases, the mosquito enzyme also hydrolysed diacylglycerols, which are potent activators of protein kinase C.

Table 3. Kinetic constants for the interaction of A_2 carboxylesterase with the insecticides temephos, chlorpyrifos, fenitrothion, propoxur and malathion

Enzyme activity was measured by the assay of 1 mM-*p*-nitrophenyl acetate hydrolysis. Results are means $\pm$ S.D. for three to five separate determinations.

Insecticide	$10^2 \times k_2$ (min ⁻¹)	$10^{-2} \times k_a$ (M ⁻¹ ·min ⁻¹)	$10^5 \times K_a$ (M)	$10^4 \times k_3$ (min ⁻¹)
Temephos* (29)†	7.1 $\pm$ 2.5	37.7 $\pm$ 13.1	1.9 $\pm$ 0.3	4.2 $\pm$ 3.1
Chlorpyrifos* (37)†	14.6 $\pm$ 5.3	26.9 $\pm$ 15.6	6.1 $\pm$ 2.0	26.3 $\pm$ 0.1
Fenitrothion* (4)†	2.6 $\pm$ 1.7	1.9 $\pm$ 1.2	19.5 $\pm$ 14.2	7.4 $\pm$ 4.1
Propoxur† (2)†	11.4 $\pm$ 2.8	4.6 $\pm$ 0.4	25.1 $\pm$ 8.4	62.4 $\pm$ 14.0
Malathion*§ (7)†	—	—	—	—

* Organophosphate.

† The value in parentheses is the resistance ratio of LD₅₀ concentrations from resistant-versus-susceptible larval bioassay experiments.

‡ Carbamate.

§ The enzyme was not inhibited at 400 μ M, which is the maximum limit of solubility for malathion.

Table 4. Percentage hydrolysis of 50 μ M-acylglycerol substrates

The monoacylglycerol fatty acid is at C-1 and the fatty acid positions of the diacylglycerols are indicated. Percentage hydrolysis for a 10 min incubation in 50 mM-phosphate buffer, pH 7.4, at 28 °C (9 μ g of carboxylesterase/ml; 1 ml final volume). The reaction was stopped with 10 μ l of 10 mM-paraoxon and non-esterified fatty acids were measured. Percentage hydrolysis is mol of fatty acid released/mol of substrate. Abbreviation: N.D., not detected.

Acyl chain length	Fatty acid...	Hydrolysis (%)		
		Mono-	Di-	Tri-
C ₈		38.3	(1,3) (1,2)	78.8 48.1
C ₁₀		27.2		
C ₁₂		28.1		
C ₁₄		N.D.		
C ₁₆		N.D.	(1,3)	N.D.
C _{18, cis-9}		2.9	(1,2)	4.0
C _{18, trans-9}		N.D.		

General discussion

The putative mechanism of resistance to organophosphate insecticides involves the overproduction of non-specific esterases (Fournier *et al.*, 1987; Mouchès *et al.*, 1987). The esterases have been attributed to closely linked loci, 'A' and 'B'. The overproduction of the 'B' esterases has been shown to be due to gene amplification (Mouchès *et al.*, 1986; Raymond *et al.*, 1989). The esterases A_2 and B_2 have always been found together throughout at least three continents (Raymond *et al.*, 1991). Recently it has been proposed that the amplified A_2 and B_2 esterases have arisen from a single initial event and through migration have spread worldwide (Raymond *et al.*, 1991). If this is true, then all amplified A_2 and B_2 esterases would be the same. However, the question of whether resistance may be attributed to a qualitative change in the carboxylesterase has, to date, not been explored with a purified enzyme. The carboxylesterases, both A_2 and B_2 , in the resistant strain Pel RR in the present study had no

detectable increase in protein quantity, although the strain was 30-fold resistant to the selecting insecticide. Preliminary evidence of pesticide cross-resistance in larval bioassays supports the contrary hypothesis that amplification has arisen several different times, amplifying several different A_2 and B_2 esterases (Hemingway *et al.*, 1990; Peiris & Hemingway, 1990b). In the present study we report the purification and characterization of an esterase A_2 . The characterization of purified enzymes with physiological substrates as well as insecticide kinetic constants should demonstrate more clearly if the latter hypothesis is true. As a premise we postulate that qualitative changes in the carboxylesterase will be more readily demonstrated by the use of more specific physiological substrates than with the use of general esterase substrates.

A. J. K. was supported by The Wellcome Trust, K. G. I. J. by Sumitomo Chemical Company and J. H. by The Royal Society.

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Received 30 October 1991/21 April 1992; accepted 28 April 1992