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ESTERASES FROM THE MIDGUT AND GASTRIC CAECUM OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* (L.) ISOLATION AND CHARACTERIZATION

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SUMMARY

Seven esterases were isolated from the midgut and gastric caecum of the American cockroach, *Periplaneta americana* (L.), and were shown to be homogeneous by molecular sieve column chromatography and polyacrylamide gel electrophoresis. Molecular weights were 58 000, 130 000, 135 000, 230 000, and $\gg$ 230 000. Linear Lineweaver–Burk plots were obtained, and the K_ms were about 10^{-4}–10^{-5} M.

Four of the esterases were further purified and characterized by their activation energy (E_a), thermal stability, inhibition by organophosphate, and substrate specificity. Arrhenius plots for these esterases were double sloped, with E_a between 6.3 and 8.5 kcal/mole at higher temperatures and between 11.6 and 14.5 kcal/mole at lower temperatures. Nonlinear inhibition plots were obtained by using 2,2-dimethyldichlorovinyl phosphate, paraoxon, and diisopropylfluorophosphonate. The presence of substrate partially protected the enzymes from organophosphate inhibition. The esterases were unable to hydrolyze acetyl DL-carnitine, acetyl-CoA, phosphocreatin, methyl formate, ethyl formate, methyl propionate, *p*-nitrophenyl phosphate, and Tween 80. The nonlinear Arrhenius plots and inhibition plots were taken as evidence that the esterases were molecular aggregates, and the enzymes were further classified as B esterases.

INTRODUCTION

Carboxylesterases are a group of hydrolytic enzymes that are classed as hydrolases EC 3.1.1 and are widely distributed in nature. These enzymes may be divided into three broad classes: cholinesterases, lipases, and the simple or nonspecific esterases. The role of the nonspecific esterases has been obscured because the esterases studied were not purified and because they have a broad substrate specificity.

Insect esterases appear to have a role in food digestion [1], insecticide resist-

Abbreviations: DDVP, 2,2-dimethyldichlorovinyl phosphate.

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ance [2–4] and fat metabolism [5, 6], but little attempt has been made to isolate and characterize these enzymes. Cook and Forgash [7] used starch gel electrophoresis to partially characterize the esterases in the organs and tissues of American cockroaches, *Periplaneta americana* (L.), as nonspecific esterases (5 organophosphorus-resistant esterases, 6 aliesterases) and cholinesterase. The major sites of the organophosphorus-resistant esterases were in the alimentary tract and hemolymph. Colhoun [1] found that tri-*O*-tolyl phosphate interfered with the normal function of the alimentary tract, and Cook and Forgash [7] showed that tri-*O*-tolyl phosphate was a more effective inhibitor of esterases in vivo than in vitro.

Cook et al. [8] used starch gel electrophoresis and found 6 esterases in the midgut and gastric caecum of the American cockroach. Heat-stable esterases were located in the epithelium of the midgut and gastric caecum but not in the foregut and hindgut.

In this paper, we report the isolation by column chromatography of esterases from the midgut and gastric caecum of the American cockroach. The esterases were characterized by determining their inhibition by organophosphates, thermal stability, activation energies, and substrate specificity.

MATERIALS AND METHODS*

Insects

Adult cockroaches 0–4 months old [9] were used for all experiments. No attempt was made to compare the esterases of the different sexes because all esterase bands observed in the cockroach are present in both sexes with only slightly different levels of activity [8].

Tissue preparation

The proventriculus, midgut, and gastric caecum of 30–100 adults were dissected and immediately placed in ice-cold water for homogenization (30 guts per 10 ml water). The tissues were homogenized with a Sorvall Omnimixer, centrifuged at 10 000 × *g* for 10 min, and the pellet was resuspended in 10 ml of water. Homogenization was repeated until 4 supernatant fractions were obtained that were then combined for further purification. All purification steps were carried out at 4 °C.

Assays

Protein was determined spectrophotometrically by measuring the absorbance at 280 and 260 nm [10] or by the method of Lowry et al. [11].

Esterases were assayed by the method of Gomori [12]. A tube containing 0.42 mM 1-naphthyl acetate, 2.4% acetone, 40 mM sodium phosphate buffer (pH 7.0) and enzyme solution in a total volume of 2.5 ml was incubated for 10 min at 29 °C. Then 0.5 ml of Fast Garnet GBC (0.075 g in 15 ml of 10% (w/v) sodium lauryl sulfate) was added to the reaction tube, mixed and the absorbance read at 560 nm after 15 min. The enzymatic reactions were linear to 0.400 absorbance and for 20 min. When the concentration of 1-naphthyl acetate was varied, 8.8% acetone (final concn) was used to solubilize this substrate. Acetone concentration between 2.4 and 8.8% and sodium

* Mention of a proprietary product or company name in this paper does not constitute an endorsement by the United States Department of Agriculture.

phosphate concentration between 14 and 110 mM did not affect either the azo-coupling reaction or the enzymic reaction [13]. A unit of enzyme activity was defined as the amount of enzyme that produced 1 μmole of naphthol in 1 min under the standard assay conditions.

The organophosphorus inhibitors were vacuum-distilled before use. Water-soluble organophosphorus inhibitors were prepared in water, 2,2-dimethyldichlorovinyl phosphate (DDVP) was solubilized in 2% acetone, and eserine (physostigmine) was solubilized in 2% ethanol.

Inhibition studies were carried out by incubating inhibitor, 100 mM phosphate buffer (pH 7.0), and enzyme solution (total volume 1 ml) for 10 min at 29 °C prior to the addition of 1-naphthyl acetate (final concn 40 mM and containing the same concentration of inhibitor as the solution to which it was being added) to give a total volume of 2.5 ml. The remainder of the procedure was the same as for the esterase assay. The presence or absence of phosphatases was determined by using the Sigma phosphatase kit [14].

pH stat esterase-lipase assay

Tween 80 (0.2–0.42 mM) was used to dissolve 1-naphthyl propionate and 1-naphthyl butyrate. The substrate solution (20 ml) was combined with 5 ml of $1.5 \cdot 10^{-5}$ M phosphate buffer (pH 7.0), 4.5 ml of 0.021 M $CaCl_2$, and 0.42 M NaCl and enough water to give a volume of 30.5 ml after the addition of the enzyme.

Triglycerides were prepared as an oleate–gum arabic emulsion by the combined methods of Marchis-Mouren et al. [15] and Benzonana and Desnuelle [16]. Three μl of oleic acid was added to 500 ml of distilled water with 0.75 ml of 0.1 M NaOH, giving a pH of slightly greater than 9.0. After 24 h, the pH was adjusted to 9.0 with HCl prior to the addition of 1020 μmoles of substrate and 0.66 g of gum arabic to 12 ml of the oleate solution. The mixture (kept cold) was sonicated at maximum intensity for 1 min with a 500-W Fisher ultrasonic probe with a 9.5-mm tip. Cholesteryl esters were dissolved in a minimum volume (1 ml) of chloroform and then added to the oleate–gum arabic medium for sonication. Emulsified substrate (0.1–2.0 ml) was added to sufficient water plus 3 ml of 1 M NaCl and 1 ml of 0.015 M $CaCl_2$ to make the total volume 30.5 ml upon the addition of the enzyme.

In all assays, the temperature of the reaction vessel was held at 29 °C and the vessel was continuously flushed with a stream of pure dry N_2. The pH of the solution was adjusted to 9.0 (in some cases, pH 7.0 was used) with 5 mM NaOH. After the rate of autohydrolysis was determined, 0.2 ml of the enzyme solution was added, and the rate of the reaction was followed by titrating the acid produced with 5 mM NaOH. The reaction was linear to a product concentration of 0.05 mM when 1-naphthyl acetate (0.42 mM) was used as a substrate. All reaction velocities were determined from the initial velocity portion of velocity–time plots.

Electrophoresis

Starch gel electrophoresis was carried out by using the method of Smithes [17], and the gels were stained for esterase according to the method of Markert and Hunter [18]. Polyacrylamide gel disc electrophoresis was carried out with a Canalco Model 200 apparatus as described by the manufacturer [19].

Column chromatography

The anion exchanger DEAE-Sephadex (A-25, Pharmacia) was prepared as described by the manufacturer [20] to give a packed column 2.5 cm × 35 cm in 0.02 M phosphate buffer (pH 6.0, or in some cases pH 5.0 was used). After the sample was placed on the column and 100 ml of 0.02 M phosphate buffer (pH 6.0) was passed through the column, the esterases were eluted by using a salt gradient calculated by the method of Peterson and Sober [21]. The column had a flow rate of 20 ml/h, and the eluant was collected in 6-ml fractions and assayed for protein and esterase activity.

Sephadex G-200 was prepared as described by the manufacturer [22] in 0.05 M phosphate buffer (pH 7.5) and packed under a 10-cm hydrostatic head to give a 1.5 cm × 65 cm column with an upward flow of 4 ml/h. Samples (less than 0.5 ml) were added to the bottom of the column, followed by 0.5 ml of 10% sucrose in the eluting buffer (0.05 M phosphate buffer (pH 7.5) containing 1 mg/ml bovine serum albumin (Sigma Fraction V)). Columns were calibrated with Dextan blue, catalase, γ-globulin, bovine serum albumin, ovalbumin, and cytochrome *c* (15 mg/ml) [23].

RESULTS AND DISCUSSION

Acetone precipitation of the esterase activity

10 vol. of acetone (−10 °C) were added to the combined centrifugate (spec. act. 0.069) and the resulting precipitate was removed by centrifugation and dried at −10 °C. From 6 different preparations, the average weight of precipitate per midgut-proventriculus-gastric caecum was 17 mg. 4 ml of cold Tris or phosphate buffer (0.01 M, pH 7.5) were added to the dry acetone precipitate for each 10 guts in the preparation. The centrifugate was decanted and fresh buffer added to the pellet. This procedure was repeated, and the 2 extracts (98% of the extractable esterase activity) were combined for further purification and were stable indefinitely when stored frozen at −10 °C. Because removal of lipids improved the ion-exchange chromatography, the acetone treatment was continued. $MnCl_2$ (final concn 0.04 M) treatment resulted in labile esterase activity that could not be stabilized with salts, substrates, or bovine serum albumin.

$(NH_4)_2 SO_4$ fractionation

A saturated $(NH_4)_2 SO_4$ solution was slowly added to the buffer extract (41 mg protein/ml) of the acetone precipitate to give a solution 30% saturated with $(NH_4)_2 SO_4$ and stirred gently for 30 min. The solution was centrifuged at 10 000 × *g* for 10 min, and the pellet was taken up in a minimum volume of 0.01 M phosphate buffer (pH 7.5) and had a specific activity of 0.09. Additional solid $(NH_4)_2 SO_4$ was added to the centrifugate, and the precipitated protein fractions were collected at 49, 66, 81, and 92% saturation and had specific activities of 0.05, 0.26, 1.9, and 1.3, respectively.

Starch gel electrophoresis of the precipitated fractions showed that there was no separation of the individual esterases with $(NH_4)_2 SO_4$. Because those fractions precipitating above 50% satn with $(NH_4)_2 SO_4$ had the highest specific activity (average of 1.08, 11-fold purification), subsequent preparations were made at 50% satn by the addition of a saturated solution of $(NH_4)_2 SO_4$. The resulting precipitate was collected; solid enzyme grade $(NH_4)_2 SO_4$ was added to the centrifugate to make the

solution 90% saturated, and the resulting precipitate was collected. The 50–90% precipitate was stored frozen in a minimum volume of 0.01 M phosphate buffer (pH 7.5), and was used as the source of the esterases in subsequent purification steps.

DEAE-Sephadex column chromatography

The 50–90% satd $(NH_4)_2 SO_4$ fraction (usually 340 mg of protein with 37.5 units of esterase activity) was applied to the DEAE-Sephadex column at pH 6.0 and eluted with a pH and NaCl gradient as shown in Fig. 1. The pH gradient was necessary for resolution of the esterases because only 2 esterase fractions were resolved on the column when the pH was held constant at 7.5. However, when this combination of pH and salts gradients was used, 4 esterase and 6 protein fractions were eluted from the column and labeled as shown in Fig. 1.

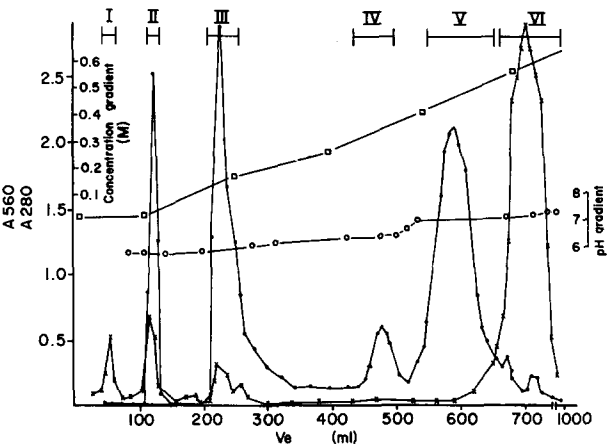

Fig. 1. DEAE-Sephadex column chromatography of the esterase fraction precipitating between 50 and 90% satn with $(NH_4)_2SO_4$ as described in the text. ○, pH gradient; □, NaCl–phosphate buffer gradient; ×, protein $A_{280\ nm}$; ●, esterase $A_{560\ nm}$. Tubes under each peak were combined and labeled DEAE-I, DEAE-II, etc.

Apparently, the isoelectric point of fractions DEAE-I and -II lies near pH 6.0 because lowering the initial column pH from 7.5 to 6.0 resulted in DEAE-I and -II being resolved. DEAE-I was eluted in the void volume and DEAE-II was eluted before there was any effective increase in pH or salt concentration.

Enzyme preparations that had been precipitated with acetone before fractionation with $(NH_4)_2 SO_4$ were unstable after ion-exchange chromatography and were not stable when frozen. However, the addition of bovine serum albumin (Sigma, Fraction V, 1 mg/ml) stabilized most of the esterases. Bovine serum albumin either heated or unheated had no effect on the assay for esterase activity.

DEAE-I and -VI (Fig. 1) were not further isolated because they consistently had no esterase activity, and DEAE-IV was not characterized because it had low activity and was labile. From the slope of the peak, DEAE-III appeared to contain several esterases. Therefore, this fraction from several columns was pooled and rechromatographed on DEAE-Sephadex packed in 0.02 M phosphate (pH 5.0) and

eluted with the same salt gradient described above. DEAE-III was resolved into 3 fractions that were labeled as shown in Fig. 2. Fractions IIIb and IIIc were not isolated further or characterized because of their low activity, instability, and incomplete resolution.

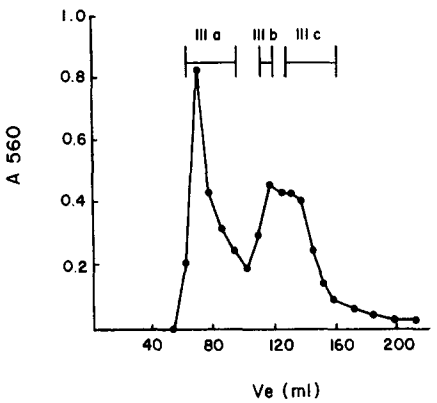

Fig. 2. DEAE-Sephadex column chromatography of the esterase fraction DEAE-III. Tubes were combined as indicated and labeled DEAE-IIIa; DEAE-IIIb; DEAE-IIIc.

Sephadex G-200 column chromatography

The homogeneity of the esterase fractions (DEAE-II, -IIIa, and -V) was determined by molecular sieve column chromatography and disc electrophoresis (starch gel was ineffective in resolving the esterase activities [13]). Aliquots of DEAE-II (Fig. 1) were chromatographed on Sephadex G-200 and electrophoresed on polyacrylamide gel. Three esterase fractions were obtained by molecular sieve chromatography (DEAE-II-G-200-I, -II, and -III), but only 2 bands were observed on polyacrylamide gel. This was not surprising because the enzymes had eluted together from DEAE-Sephadex, an indication that the proteins were similarly charged. DEAE-IIIa (Fig. 2) was also chromatographed on Sephadex G-200 and separated into 2 active fractions (DEAE-IIIa-G-200-I and -II). When DEAE-V was chromatographed on Sephadex G-200, it also was resolved into 2 fractions, DEAE-V-G-200-I and -II, which gave single bands on disc electrophoresis. DEAE-V-G-200-I was not characterized further because it was very labile and could not be stabilized with bovine serum albumin.

The molecular weights for the 7 esterase fractions obtained from the molecular sieve columns were determined from the K_{av} vs log molecular weight plot (Fig. 3) and summarized in Table I. The molecular weights reported here are of the same order of magnitude as those reported by La Motta et al. [24] for a different esterase family, serum cholinesterase, that was shown to exist in 5 interconvertible forms. Benöhr and Krisch [25] reported that bovine liver carboxyl-esterase was a dimer having a molecular weight of 167 000 and monomer subunits of 85 000, and Ecobiochon [26] reported subunits of 55 000 for the same enzyme. Several workers [27–29] studied pig liver esterase and found the enzyme had a molecular weight of about 160 000 with active subunits (equivalent weight) of about 80 000. Ox liver esterase [30, 31], horse liver esterase [32], and chicken liver esterase [33] had equivalent weights that were similar

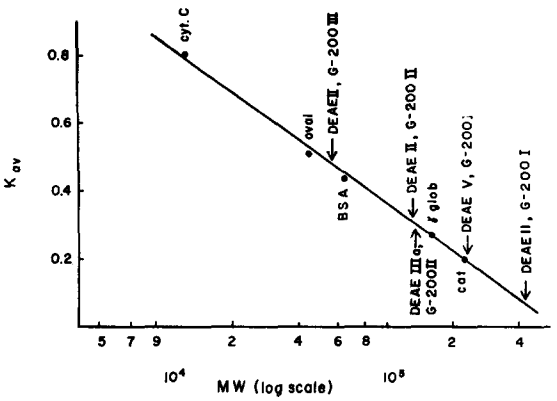

Fig. 3. Calibration curve for Sephadex G-200 and Bio-Rad P-200 molecular sieve columns showing the K_{av} and molecular weight for several purified esterases as indicated by arrows.

to pig liver esterase. The large molecular weights observed for DEAE-II-G-200-I, DEAE-IIIa-G-200-I, and DEAE-V-G-200-I and -II from the cockroach indicated that these esterases might be in the form of a molecular aggregate. The fractions from the molecular sieve columns were not considered to be mixtures because polyacrylamide gel electrophoresis showed only one esterase component.

TABLE I

PHYSICAL AND KINETIC PROPERTIES OF THE ESTERASES

Fraction	K_{av}	Mol. wt.	V (esterase units)	K_m (mM)
DEAE-II				
G-200-I	0.075*	420 000*	0.028	0.027
G-200-II	0.31	130 000	0.030	0.040
G-200-III	0.48	58 000	0.033	0.10
DEAE-IIIa				
G-200-I	0.038*	520 000*	0.0286	0.027
G-200-II	0.30	135 000	0.0329	0.128
DEAE-V				
G-200-I	0.001*	—**	—	—
G-200-II	0.19	230 000	0.0358	0.064

* Assumes linearity of the K_{av} vs log mol. wt at higher mol. wt than catalase.
** K_{av} so small that the molecular weight cannot be determined.

Determination of K_m and V

The K_m and V for esterases obtained from the molecular sieve columns were determined from the slope and intercept of Lineweaver–Burk plots. The enzyme concentration was adjusted to give a corrected absorbance (sample A_{560nm} − blank A_{560nm} = corrected A_{560nm}) of 0.300 when the substrate was 0.42 mM. Thus, in these assays, V was useful only to make relative comparisons of the data within Table I because it

was dependent on the enzyme concentration that was standardized and which gave initial reaction velocity measurements at all substrate concentrations used. The Lineweaver–Burk plots were linear over a 25-fold range of substrate concentration. The larger molecular weight esterases have a smaller K_m than do the esterases of lower molecular weight (Table I), indicating that the larger molecular weight esterases have a much greater affinity for the substrate.

Thermal stability and activation energy

The stability of the esterases (DEAE-II-G-200-I, -II, and DEAE-IIIa-G-200-II) toward heat was carried out by heating an aliquot of the enzyme in 10 mM phosphate (pH 7.5), containing 1 mg/ml of bovine serum albumin in a water bath for 10 min prior to determination of the esterase activity under standard conditions. The stability was in the order: DEAE-II-G-200-I > DEAE-II-G-200-II > DEAE-IIIa-G-200-II (Table II). It is likely that DEAE-II-G-200-I and -II were components of esterase E6 [7, 8] because in both cases, the enzymes were stable when heated at 55 °C.

TABLE II

THERMAL STABILITY OF THREE ESTERASES

The enzymes were heated at the indicated temperature for 10 min prior to the standard esterase assay.

Temperature (°C)	Percentage esterase activity remaining after heat treatment of fractions:		
	DEAE-II-G-200-I	DEAE-II-G-200-II	DEAE-IIIa-G-200-II
4	100	97	100
15	99	100	100
29	98	98	96
40	100	99	89
55	97	85	67
70	60	24	30

The activation energy of each of the 3 esterases was then determined at temperatures between 4 and 70 °C and the observed enzymic activity was adjusted for inactivation of the enzymes (Figs 4 and 5). It is recognized that heat denaturation may not proceed at the same rate in the presence and absence of substrate and that it is difficult to determine if corrections for enzyme inactivation will bring the temperature optimum and activation energy closer to that which is correct or will introduce a greater error. However, in the studies reported here, correction for enzyme inactivation applies to only 1 point (Figs 4 and 5) for DEAE-II-G-200-I and -II and only 2 points for DEAE-IIIa-G-200-II because of their heat stability (see Table II). Because these corrected points fall on the plot, the inactivation in the presence and absence of substrate must be similar. DEAE-II-G-200-I had an optimum temperature (60 °C) in the range studied, but both the other enzymes showed a continuous increase in velocity as the temperature increased. Each of the 3 enzymes yielded an Arrhenius plot having 2 linear portions and concave downward (Fig. 5). The E_a (Table III) shows that an increase in temperature resulted in a decrease in the energy of activation.

Massey et al. [34] obtained nonlinear Arrhenius plots for amino acid oxidase

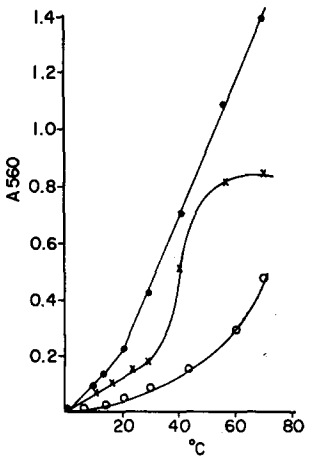

Fig. 4. Effect of temperature on the velocity of 3 esterases in the standard assay with the temperature varied between 4 and 70 °C. Absorbance measurements were corrected for enzyme inactivation. ×, DEAE-II-G-200-I; ●, DEAE-II-G-200-II; ○, DEAE-IIIa-G-200-II.

similar to those observed for cockroach esterases. They found that amino acid oxidase underwent a temperature-dependent conformational change as observed by changes in the sedimentation constant, difference spectra, and fluorescence and that the critical temperature for these phenomena coincided with the break in the Arrhenius plots.

It is not possible from our data to relate the intercept of the Arrhenius plots (Fig. 5, Table III) to the critical temperature for a conformational change, but the

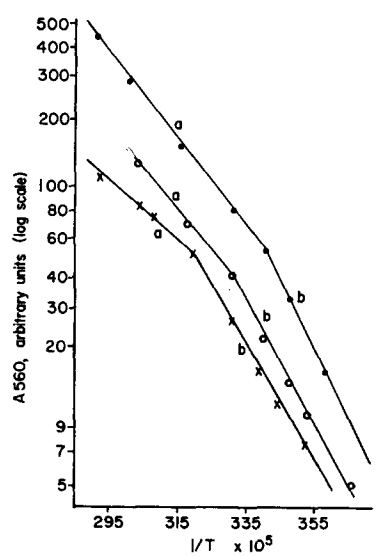

Fig. 5. Arrhenius plots of 3 esterases. Velocity is plotted on log scale in arbitrary absorbance units that were corrected for enzyme denaturation and adjusted to same order of magnitude for plotting. ×, DEAE-II-G-200-I; ○, DEAE-II-G-200-II; ●, DEAE-IIIa-G-200-II (see Table III for value of E_a from Slopes a and b).

TABLE III

THERMAL PROPERTIES AND INHIBITOR RATE CONSTANTS FOR THE PURIFIED ESTERASES
Data are obtained from Figs 2–5.

Fraction	E_a (kcal/mole)		Arrhenius intercept (°C)	DFP		Paraoxon		DDVP	
	a	b		$k_i \times 10^{-3}$ ($M^{-1} \cdot min^{-1}$)	I_{50} ($M \cdot 10^7$)	$k_i \times 10^{-3}$ ($M^{-1} \cdot min^{-1}$)	I_{50} ($M \cdot 10^7$)	$k_i \times 10^{-3}$ ($M^{-1} \cdot min^{-1}$)	I_{50} ($M \cdot 10^7$)
DEAE-II									
G-200-I	6.320	11.668	40	1.76	300	33.8	2	11.3	40
G-200-II	7.600	13.198	29	14.2	40	28.1	10	1.8	300
G-200-III	–	–	–	35.1	6	21.7	10	28.1	20
DEAE-IIIa									
G-200-II	8.473	14.532	20	–	–	–	–	–	–

shape of the Arrhenius plots indicates that more than one enzyme form is contained in each fraction. The Arrhenius plots were consistent with the suggestion that the esterases might be molecular aggregates, and decreasing the temperature could dissociate the aggregates into less active subunits, as observed by Antonini et al. [35] for D-amino acid oxidase.

If the esterases of each fraction are single enzymes with each possessing a single active center, then linear first order inhibition kinetics would be expected. Conversely, if the esterases are molecular aggregates or other enzyme forms having multiple active centers, then nonlinear inhibition kinetics might be expected. Inhibition studies were used to investigate these possibilities and to further characterize the esterases.

Determination of k_i and I_{50}

Preliminary studies were carried out on fraction DEAE-II before chromatography on Sephadex G-200. No inhibition was found with *p*-hydroxymercuribenzoate (an A esterase inhibitor and C esterase activator [36–38])at concentrations between 10^{-3} and 10^{-7} M, and less than 7% activation was observed at 10^{-3} and 10^{-4} M. It was concluded that none of the esterases in DEAE-II were A or C esterases. Eserine (a cholinesterase inhibitor [39] at 10^{-5} to 10^{-8} M) inhibited this fraction (DEAE-II) only 9% at 10^{-6} M and only 13% at 10^{-5} M, which indicated the absence of cholinesterase. Therefore, these inhibitors were not used on the more highly purified fractions.

Chromatography of esterase fraction DEAE-II on Sephadex G-200 gave 3 fractions: DEAE-II-G-200-I, -II and -III, which were individually incubated for 10 min with each of the organophosphorous inhibitors (paraoxon, DDVP and DFP) in the presence of 0.1 M phosphate buffer (pH 7.5), and 0.1 mg/ml bovine serum albumin prior to the addition of the substrate, which also contained the inhibitor to prevent dilution of the inhibitor from the enzyme. The results were plotted based on the equation of Aldridge [40] and Hartley and Kilby [41] except that the inhibitor concentration was varied and the time was held constant. $E + I \xrightarrow{k_i} EI$ where $dx/dI = k_i\, t\,(a-x)$ and the slope $m = -k_i\, t/2.303$ (Figs 6–8). Straight-line plots were obtained with all 3 inhibitors over a small concentration range with DEAE-II-G-200-I and -III (Figs 6 and 8), but a curve was obtained for DEAE-II-G-200-II with paraoxon and DDVP as the inhibitors (Fig. 7). Fractions I and III had curved lines when the concentration range was increased, which indicated multiple active centers for all 3 esterases.

Another series of experiments was carried out in which the enzymes were added to a mixture of substrate and inhibitor without preincubation of the inhibitor and the enzyme. The presence of substrate partially protected the enzymes from the inhibitors (DDVP, DFP, paraoxon). The protection of the enzyme by substrate from organophosphate inhibition suggested that this type of inhibitor binds at or near the active center.

The rate constant k_i for DDVP calculated from a tangent to the midpoint of the curve (Fig. 7) was 1800 $M^{-1} \cdot min^{-1}$. The rate constants for the other inhibitor studies were calculated from linear plots (Figs 6 and 8) and showed that the esterases had significantly different rates of inhibition toward these inhibitors (DFP, paraoxon) (Table III).

Singh and Mehrotra [42] studied the simple esterases in whole-body homo-

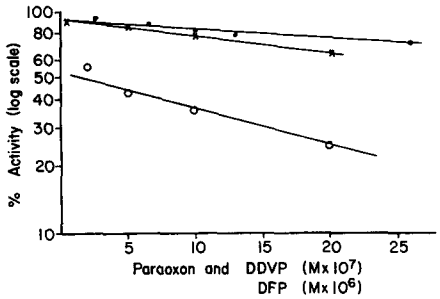

Fig. 6. Effect of organophosphate concentration on inhibition of esterase DEAE-II-G-200-I as described in text. ○, paraoxon; ●, DDVP; ×, DFP.

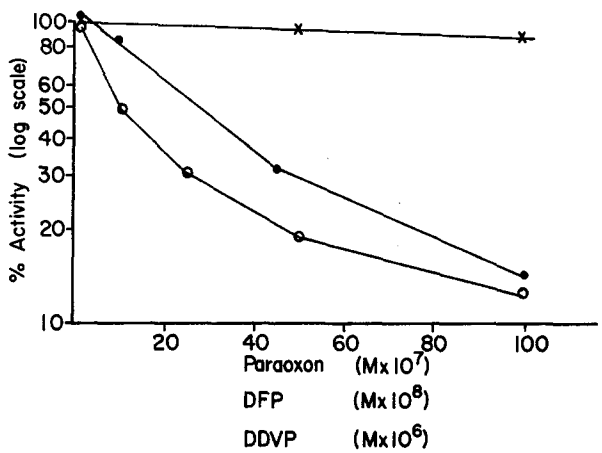

Fig. 7. Effect of organophosphate concentration on inhibition of esterase DEAE-II-G-200-II as described in text. ○, paraoxon; ●, DDVP; ×, DFP.

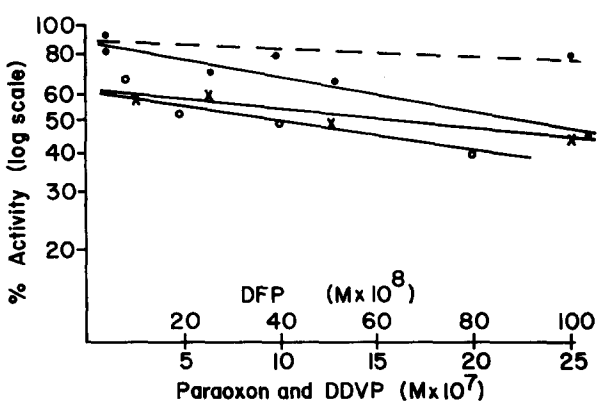

Fig. 8. Effect of organophosphate concentration on inhibition of esterase DEAE-II-G-200-III as described in text. Paraoxon (○), DDVP (●), DFP (×), DDVP and 1-naphthyl acetate (—) were simultaneously added to the enzyme without preincubation of the enzyme and inhibitor.

genates of larvae of the maize stem borer and used DFP and diazoxon as inhibitors. They plotted their results as log–log plots and obtained straight lines over wide concentration ranges. However, when we plotted their data as a semilog plot, curves were obtained that were similar to those obtained from the cockroach esterases (Fig. 7). They reported composite or average k_i values with DFP as the inhibitor that ranged from $8.4 \cdot 10^4$ to $4.4 \cdot 10^7$ $M^{-1} \cdot min^{-1}$. These k_i values were higher than those found for cockroach gut esterases (Table III) and may be the result of species differences or of a complexity of esterases with varied rates of inhibition in the whole-body homogenates used.

Chiu et al. [43] and Aldridge [44] used paraoxon as an inhibitor of cholinesterase activity and found k_i values of $1.2 \cdot 10^5$ $M^{-1} \cdot min^{-1}$ and $1.1 \cdot 10^6$ $M^{-1} \cdot min^{-1}$. Main [45] reported that the k_i value for DFP with cholinesterase was $4.18 \cdot 10^6$ $M^{-1} \cdot min^{-1}$.

The lower k_i values for the simple esterases when compared with cholinesterases might reflect a greater affinity of the simple esterases for organophosphate inhibitors. This greater sensitivity of the simple esterases than the cholinesterases for organophosphorous inhibitors is in agreement with the findings of Van Asperen [46], who found that house fly "ali-esterases" were inhibited 83% but cholinesterases were inhibited only 27% by DDVP.

The nonlinear inhibition kinetics reported here are also similar to those reported by Main [47], who used the organophosphate inhibitors DFP and amiton (*O,O*-diethyl *S*-2-diethylaminoethyl phosphorothiolate, hydrogen oxalate salt) and found that cholinesterases from horse serum, human serum, and bovine erythrocytes displayed nonlinear inhibition kinetics. He concluded that the concave upward inhibition plots reflected a reversible aggregated enzyme system with allosteric behavior and suggested that each slope in log % activity vs time plots (with constant inhibitor concentration) represented the different rates of inhibition for each form of the enzyme. Kitz et al. [48] used carbamate as an inhibitor and also found that cholinesterase had allosteric properties, as demonstrated by concave upward inhibition plots of log % activity vs time. Pig liver esterase studied by Barker and Jencks [49] and by Levy and Ocken [50] had nonlinear first order kinetics similar to those observed for the cockroach esterases and a monomer–dimer relationship with the monomer existing in both an active and an inactive form.

I_{50} was also calculated from the inhibition experiments and is tabulated in Table III. Because the actual esterase weight, the number of active centers used, and the number of molecules of enzyme added to the inhibition assay could not be determined, I_{50} represented a number that could be used only to make relative comparisons of the data in Table III.

The inhibition data reported here and the Arrhenius plots discussed earlier indicated that the cockroach gut esterases probably exist as thermally-dependent molecular aggregates with different rates of hydrolytic activity in the associated and dissociated forms. From the inhibition data (inhibition by paraoxon and lack of activation or inhibition by *p*-hydroxymercuribenzoate), these cockroach gut esterases cannot be classified as aryl, A, or C esterases. It is possible to describe these enzymes as B esterases according to Aldridge [36, 37] or as aliesterases, but the different sensitivities to the inhibitors tested necessitates subdivision.

Substrate specificity

The substrate specificity of the esterases was studied by using a crude homegenate, and no activity was observed with acetyl DL-carnitine, acetyl-CoA, phosphocreatin, methyl formate, ethyl formate, methyl propionate, Tween 80, or triacetin at substrate concentrations between 0.08 and 4.2 mM. Also, Tween 80 did not inhibit the esterase activity toward 1-naphthyl acetate, 1-naphthyl propionate, and 1-naphthyl butyrate. The naphthyl esters were hydrolyzed more readily than cholesteryl palmitate, and the least reactive substrates tested were tripropionate and tripalmitate (Table IV).

TABLE IV

SUBSTRATE SPECIFICITY AT pH 7.0 OF THE CRUDE HOMOGENATE
Combined centrifugates from 4 homogenizations of the cockroach gut are used.

Substrate	Concn (mM)	Relative activity
1-Naphthyl acetate	0.42	100
1-Naphthyl propionate	0.42	133
1-Naphthyl butyrate	0.42	128
Tripropionate	2.8	3.1
Tripalmitate	2.8	0.6
Cholesteryl palmitate	0.61	25

The hydrolysis reaction with 0.42 mM 1-naphthyl acetate was linear spectrophotometrically and titrametrically until 20 μM acetate was formed, but the hydrolysis reaction of triacetate, tripropionate, and tripalmitate continued to be linear titrametrically after 150 μM product had formed. The titrametric curve for the hydrolysis of cholesteryl palmitate was similar to that for 1-naphthyl acetate, which suggested the possibility of product inhibition by cyclic alcohols but not with glycerol.

The purified esterase DEAE-II-G-200-II was studied by using tributyrate, tripalmitate, cholesteryl acetate, cholesteryl palmitate, and 1-naphthyl acetate as substrates. The results in Table IV do not show a clear substrate specificity. The relative rates of hydrolysis at 2.6 mM were tributyrate (100), cholesteryl palmitate (56), cholesteryl acetate (8), and tripalmitate (3). No phosphatase activity was found in this purified fraction.

It is customary and popular to give a common name and classification to every esterase visualized on electrophoresis, but the lack of inhibition specificity and substrate specificity for the highly purified fraction DEAE-II-G-200-II showed this to be unwise. The esterases described here do not fit well into any proposed classification system although based on the substrates hydrolyzed, they would be classed as EC 3.1.1. These enzymes might be classed as esterases rather than lipases because they hydrolyzed soluble substrates, i.e. 1-naphthyl acetate and triacetin more effectively, but significant activity upon emulsified cholesteryl acetate, cholesteryl palmitate, and tributyrate was also observed. The distinction between esterase and lipases based on the idea that lipases are specific for long chain fatty acid glyceryl esters and that esterases are active on short chain esters of other alcohols does not hold with these enzymes. Based on the sensitivity to paraoxon and the lack of inhibition or activation with

p-hydroxymercuribenzoate, these esterases might be classed as aliesterases or B esterases, but the different sensitivities to the organophosphate inhibitors requires additional criteria for accurate classification. Therefore, it is suggested that further naming and classifying of these esterases be left until the physiological substrate is known.

REFERENCES

1 Colhoun, E. H. (1960) Can. J. Biochem. Physiol. 38, 1363–1376
2 Matsumura, F. and Voss, G. (1965) J. Insect. Physiol. 11, 147–160
3 Van Asperen, K. and Oppenoorth, F. J. (1959) Ent. Exp. Appl. 2, 48–57
4 Van Asperen, K. and Oppenoorth, F. J. (1960) Ent. Exp. Appl. 3, 68–83
5 Stevenson, E. (1969) J. Insect Physiol. 15, 1537–1550
6 Gilbert, L. I., Chino, J. and Domroese, K. A. (1965) J. Insect Physiol. 11, 1057–1070
7 Cook, B. J. and Forgash, A. J. (1965) J. Insect Physiol. 11, 237–250
8 Cook, B. J., Nelson, D. R. and Hipps, P. P. (1969) J. Insect Physiol. 15, 581–589
9 Beckendorf, G. W. and Stephen, W. P. (1970) Biochim. Biophys. Acta 201, 101–108
10 Kalckar, H. M. (1947) J. Biol. Chem. 167, 461–475
11 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
12 Gomori, G. (1953) J. Lab. Clin. Med. 42, 445–453
13 Hipps, P. P. (1972) Ph.D. Thesis, North Dakota State University, Fargo
14 Sigma Tech. Bull. 104 (1963) Sigma Chem. Co., St. Louis
15 Marchis-Mouren, G., Sarda, L. and Desnuelle, P. (1959) Arch. Biochem. Biophys. 83, 309–319
16 Benzonana, G. and Desnuelle, P. (1968) Biochim. Biophys. Acta 164, 47–58
17 Smithies, O. (1955) Biochem. J. 61, 629–641
18 Markert, C. L. and Hunter, R. L. (1959) J. Histochem. Cytochem. 7, 42–49
19 Tech. Bull. Disc Electrophoresis (1965) Canal Industrial Corp., Rockville, MD
20 Sephadex Bull. Ion Exchangers (1966) Pharmacia Fine Chemicals, Piscataway, N.J.
21 Peterson, E. A. and Sober, H. A. (1959) Anal. Chem. 31, 857–862
22 Sephadex-gel Filtration in Theory and Practice (1966) Pharmacia Fine Chemicals, Piscataway, N.J.
23 Andrews, P. (1964) Biochem. J. 91, 222–233
24 La Motta, R. V., Woronink, C. L. and Reinfrank, R. F. (1970) Arch. Biochem. Biophys. 136, 448–451
25 Benöhr, H. C. and Krisch, K. (1967) Hoppe-Seyler's Z. Physiol. Chem. 348, 1102–1114
26 Ecobichon, D. J. (1969) Can. J. Biochem. 47, 799–805
27 Barker, D. L. and Jencks, W. P. (1969) Biochemistry 8, 3879–3897
28 Horgan, D. J., Dunstone, J. R., Stoops, J. K., Webb, E. C. and Zerner, B. (1969) Biochemistry 8, 2006–2013
29 Borguth, W., Kirsch, K. and Niemann, H. (1965) Biochem. Z. 341, 149–156
30 Runnegar, M. T. C., Scott, K., Webb, E. C. and Zerner, B. (1969) Biochemistry 8, 2013–2018
31 Runnegar, M. T. C., Webb, E. C. and Zerner, B. (1969) Biochemistry 8, 2018–2026
32 Boursnell, J. C. and Webb, E. C. (1949) Nature 164, 875
33 Augusteyn, R. C., de Jersey, J., Webb, E. C. and Zerner, B. (1969) Biochim. Biophys. Acta 171, 128–137
34 Massey, V., Curti, B. and Ganther, H. (1966) J. Biol. Chem. 241, 2347–2357
35 Antonini, E., Brunori, M., Bruzzesi, M., Chiancone, E. and Massey, V. (1966) J. Biol. Chem. 241, 2358–2366
36 Aldridge, W. N. (1953) Biochem. J. 53, 110–117
37 Aldridge, W. N. (1953) Biochem. J. 53, 117–124
38 Bergmann, F., Segal, R. and Rimon, S. (1957) Biochem. J. 67, 481–486
39 Augustinsson, K. B. (1960) in The Enzymes (Boyer, P. D., Lardy, H. and Myrbäck, K., eds), 2nd edn, Vol. 4, Part A, pp. 521–540, Academic Press, New York
40 Aldridge, W. N. (1950) Biochem. J. 46, 451–460
41 Hartley, B. S. and Kilby, B. A. (1952) Biochem. J. 50, 672–678

42 Singh, H. N. and Mehrotra, K. N. (1970) J. Insect Physiol. 16, 2385–2399
43 Chiu, Y. C., Main, A. R. and Dauterman, W. C. (1969) Biochem. Pharmacol. 18, 2171–2177
44 Aldridge, W. N. (1953) Biochem. J. 54, 442–448
45 Main, A. R. (1964) Science 144, 992–993
46 Van Asperen, K. (1958) Nature 181, 355–356
47 Main, A. R. (1969) J. Biol. Chem. 244, 829–840
48 Kitz, R. J., Braswell, L. M. and Ginsburg, S. (1970) Mol. Pharmacol. 6, 108–121
49 Barker, D. L. and Jencks, W. P. (1969) Biochemistry 8, 3879–3897
50 Levy, M. and Ocken, P. R. (1969) Arch. Biochem. Biophys. 135, 259–264