Partial Purification of Rabbit Serum Arylester Hydrolase

James K. Zimmerman* and Thomas M. Brown

Arylester hydrolase has been partially purified from rabbit serum to a specific activity matching or exceeding all previous reports. Calcium ion is absolutely required for activity. The widely reported rapid loss of arylester hydrolase activity was overcome by combining this Ca^{2+} requirement with the presence of 0.02% sodium azide. A method has also been devised to store the enzyme for long periods of time (years). During size exclusion chromatography, the enzyme behaved as if it had a molecular weight of 180–200 kDa. SDS-polyacrylamide gel electrophoresis showed two bands of low molecular weight (40–45 and 47–54 kDa) directly correlated with enzymatic activity, suggesting a possible $\alpha_2\beta_2$ structure for the native enzyme.

INTRODUCTION

Arylester hydrolase (EC 3.1.1.2), commonly referred to as paraoxonase, has as substrates a range of toxic organophosphates and organophosphinates (Aldridge, 1953; Grothusen et al., 1986). Because of its target substrates, arylester hydrolase is an important enzyme in xenobiotic organophosphorus detoxication and in environmental detoxification. While nothing is known about the natural substrates of arylester hydrolase (Walker and Mackness, 1983; Chemnitius et al., 1983), the enzyme is found in high levels in mammals, especially in rabbit serum (Brealey et al., 1980; Zech and Zürcher, 1974) while it is found in low levels in birds (Brealey et al., 1980). Recently the gene for arylester hydrolase has been localized on human chromosome 7 and has been found to be of use as a genetic marker for cystic fibrosis (Eiberg et al., 1985).

Although several research groups have worked with arylester hydrolase from crude tissue or serum, only Main (1960) has published a complete purification (from sheep serum). Mackness and Walker (1983) have published a partial purification from the same source. Various estimates of the molecular weight have been made: 35 000–50 000 Da (sheep serum) (Main, 1960); greater than 200 000 Da (sheep serum) (Mackness and Walker, 1983); 500 000 Da (rabbit serum) (Zech and Zürcher, 1974).

This paper introduces a relatively rapid method for purifying rabbit serum arylester hydrolase to approximately the same specific activity as Main's preparation as well as conditions for maintaining enzymatic activity over an extended period of time.

MATERIALS AND METHODS

Paraoxon was purchased from Sigma Chemical Co. A stock solution was made by dissolving 114.4 mg of paraoxon in acetonitrile and bringing the volume to 1.0 mL (416 mM). Fresh rabbit blood was purchased from Blue Chip Farms, Taylors, SC. Poly(ethylene glycol) 4000 (PEG-4000), DEAE-Sepharose, and biological materials were purchased through Sigma. Ultrogel AcA34 resin was purchased from LKB instruments. GelBond PAG and Acrylaide were purchased from FMC Corp. Centricon 10 microconcentration tubes were purchased from Amicon Corp.

Two buffer systems were used extensively and are listed below. Buffer A was composed of 10 mM MOPS [3-(*N*-morpholino)propanesulfonic acid], pH 7.0, containing 2.5 mM Ca^{2+} and 0.02% sodium azide. Buffer B is the same as buffer A but with the addition of 100 mM NaCl.

Fractions containing arylester hydrolase activity in the effluent from chromatographic columns were detected by using a spot assay developed by us. A spot assay paraoxon solution was made by adding 25 μL of paraoxon stock (above) to 7.0 mL of 100 mM MOPS, pH 7.5, containing 2.5 mM Ca^{2+}. The vial was immediately placed in an ultrasonic bath for 2 min. Aliquots (200 μL) of the spot assay solution were added to each well of a white spot plate. Portions of 10–50 μL (depending on the anticipated activity) of the effluent tubes to be assayed were added to individual wells. After 5–30 min of observation, the color development of each well was graded. Active fractions showed a bright yellow coloration.

Quantitative enzymatic analyses were done by preparing a tube containing 100 μL of the sample (appropriately diluted with assay buffer when necessary) and adding 1.2 mL of a solution containing 100 mM MOPS, pH 7.5, and 2.5 mM Ca^{2+}. The reaction was initiated by the addition of 10 μL of stock paraoxon followed by mixing on a vortex mixer. The solution was then transferred to a 1.0-cm path length semimicro cuvette and the change in A_{405} with time measured in a continuous manner on a Cary 219 recording spectrophotometer. The extinction coefficient for *p*-nitrophenolate ion, under the experimental conditions, was determined to be 13.56 mM^{-1} cm^{-1}.

Protein concentrations were measured by the biuret method when possible or, for dilute solutions, by a fluorescamine assay.

Biuret measurements were made by adding 200 μL of sample to 1 mL of biuret reagent (Zimmerman and Brown, 1977), mixing, and measuring the absorbance at 540 nm. Bovine serum albumin was used as a standard.

For fluorescamine assays a stock solution of 11.2 mg of fluorescamine was dissolved in, and brought to 50 mL with, spectroscopic grade, anhydrous, acetone. The assay consisted of adding 100 μL of sample to 1.4 mL of 0.05 M boric acid buffer, pH 8.5, in an acid-washed tube. To this was added 0.5 mL of fluorescamine stock and the mixture placed on a vortex mixer. The contents of the tube were transferred to an acid-washed cuvette, and the fluorescence was measured on a Perkin-Elmer Model 65-40 fluorescence spectrophotometer using 390 nm as the excitation wavelength and 475 nm as the emission wavelength. Both excitation and emission slit widths were 10 nm. Bovine serum albumin was used as a standard.

Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (1970). Gels were 7.5 or 10% acryl-

Department of Biological Sciences, Clemson University, Clemson, South Carolina 29634-1903 (J.K.Z), and Department of Entomology, Clemson University, Clemson, South Carolina 29631 (T.M.B.).

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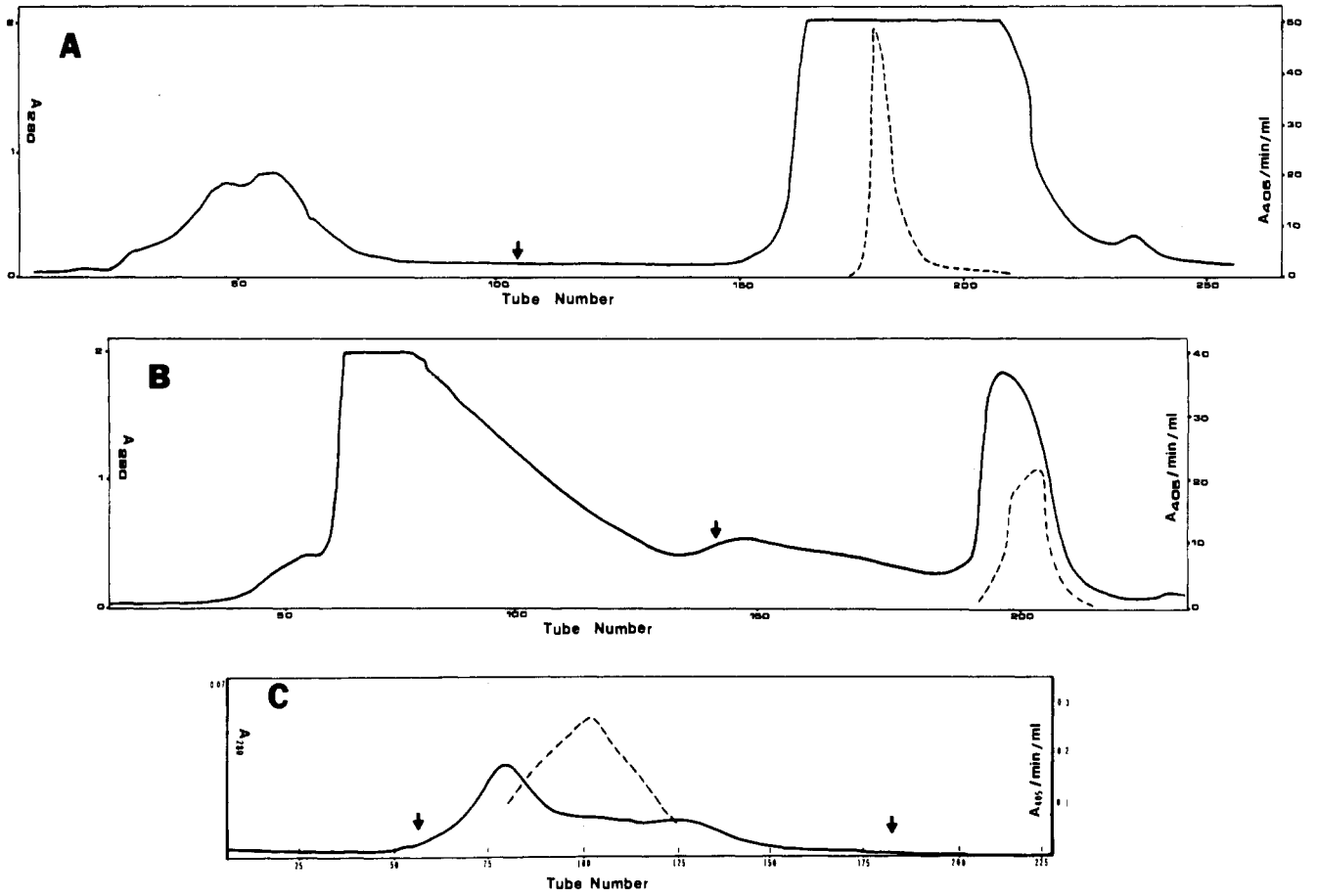

Figure 1. Chromatographic separations: (A) Recorder tracing of first DEAE-Sepharose chromatography. The column measured 4.5 × 25 cm. The monitor was set at 2 AUFS with 99 drops/tube; the path length of the flow cell of the monitor was 0.5 cm. The flat reading at 2 AU corresponds to the signal giving off-scale. At the arrow a linear gradient of 400 mL of buffer A (see Materials and Methods) to 400 mL of buffer A + 0.4 M NaCl was initiated. The dashed line is the measured arylester hydrolase activity. (B) Recorder tracing of second DEAE-Sepharose chromatography. The column measured 4.5 × 25 cm. The monitor settings were the same as in (A). The flat readings at 2 AU correspond to the signal giving off-scale. At the arrow a linear gradient of 400 mL of buffer B (see Materials and Methods) to 400 mL of buffer B + 0.4 M NaCl was initiated. The dashed line indicates the measured arylester hydrolase activity. (C) Recorder tracing of Ultrogel AcA34 chromatography. The column size was 2.5 × 76 cm. The monitor settings were 40 drops/tube and 0.1 AUFS. The monitor flow cell had a 0.5 cm path length. The dashed line indicates the measured arylester hydrolase activity. The two arrows indicate the elution volumes of the void volume (Blue Dextran) and total included volume (ammonium sulfate).

amide, cast on GelBond PAG at a thickness of 0.75 mm, and cross-linked with Acrylaide. Samples were prepared by mixing (in a 1.5-mL polyethylene microcentrifuge tube) one volume (usually 50 μL) of protein sample with one volume of a solution of 0.2 M Tris, pH 8.0, 8 M urea, 2% SDS, and containing 6 mg/mL dithiothreitol. This mixture was placed over a steam bath. At the end of 4 min, an aliquot of 0.2 M Tris, pH 8.0, 8 M urea, 2% SDS, and 92 mg/mL of iodoacetamide, equal to half the volume of the protein aliquot, was added and the mixture left in the steam bath for another 2 min. After the mixture cooled, 5 μL of bromphenol blue in 10 mM HCl was added as a marker. Gels, 12 × 14 cm, were run at 10 °C at a constant amperage of 17.5 mA/gel. When the bromphenol blue tracking dye reached the bottom of the gel, the gels were silver stained according to the method of Neilsen and Brown (1984), increasing only the time of the silver nitrate wash from 20 to 30 min to compensate for the presence of GelBond PAG.

Purification. Fresh rabbit blood was obtained by decapitation at a commercial rabbit slaughtering house. The blood was allowed to clot at room temperature for approximately 2 h. The clotted material and the serum were funnelled through cheesecloth and the serum made 2.5 mM Ca^{2+} and 0.02% in NaN_3. All subsequent steps were performed at 4 °C. The filtrate from the previous step was centrifuged for 10 min at 700*g* to remove any nonclotted red blood cells. The light reddish supernatant was carefully and gently aspirated into a vacuum flask and saved.

Dry PEG-4000 was added to the supernatant (14 g/100 mL of supernatant) and the tannish solution stirred overnight. The solution was then centrifuged at 16300*g* for 30 min. The resulting clear ruby red supernatant was easily decanted. Eleven grams of solid PEG-4000 per 100 mL of supernatant was added with stirring. The mixture was stirred 3 h and then centrifuged at 16300*g* for 30 min. The reddish supernatant was easily poured off and discarded. The gummy, tannish precipitate was slowly resuspended in buffer A.

The resuspended material was added to a 4.5 × 25 cm column of DEAE-Sepharose previously equilibrated in buffer A. When all of the dark red solution had entered the gel, the column was washed with buffer A until the A_{280} again reached base-line values. The breakthrough material was a deep red while the column showed definite red, orange, and blue bands. The column was eluted with a linear gradient consisting of buffer A in the mixing chamber and buffer A plus 400 mM NaCl in the feed chamber. Several colored peaks were observed. Arylester hydrolase activity eluted late in the gradient appearing just after (and overlapping) a peak of dark red material and just before (and almost completely overlapping) a deep blue peak

Table I. Purification of Arylester Hydrolase

fraction	vol, mL	total act., μmol/min	total protein,[a] mg	sp act., μmol/min per mg	% yield	purificn
serum	200	182	14300	0.0127	100	1
14–25% PEG	50	77.8	8540	0.0091	42.7	0.72
first DEAE-Sepharose	85	119	5050	0.0236	65.4	1.85
second DEAE-Sepharose	98	106	335	0.317	58.4	24.9
80% ammonium sulfate	7.5	46			25.3	
ultrogel[b] AcA34	1140	27.5	42.8	0.642	15.1	50.5

[a] For all but the Ultrogel AcA34 effluent, the protein concentration was determined by using a biuret assay. For the AcA34 effluent a fluorescamine assay was required. [b] Corrected for volume used. A 0.5-mL portion of the ammonium sulfate resuspension was applied, resulting in a pool of 76 mL.

(Figure 1A). Very small differences from run to run varied the overlap of the three peaks.

The pooled material was dialyzed overnight vs. several changes of buffer A and then applied to a column of DEAE-Sepharose previously equilibrated with buffer B. After the material was loaded, the column was washed with buffer B until the A_{280} began to return to base-line values. The effluent of this isocractic elution showed two peaks, the first being light orange, and then, clearly separated from the first peak, was a bright red peak that tailed considerably. A very small amount of arylester hydrolase activity was found in this peak. After the isocratic wash the column still showed a greyish blue band at the top. Arylester hydrolase activity was eluted with a linear gradient running from buffer B with no added NaCl to buffer B with 0.4 M NaCl added (Figure 1B). The peak containing essentially all of the arylester hydrolase activity also contained other protein components and a green color (See Table I for specific activity). Grothusen et al. (1986) have used material from this stage of purification to measure substrate specificity and kinetic parameters. A single band of esterase activity was observed on isoelectric focusing gels using a 2-naphthyl acetate stain; no esterase bands were observed with an 1-naphthyl acetate stain.

To concentrate the enzyme, the pooled fractions with arylester hydrolase activity were brought to 80% ammonium sulfate saturation by the addition of four volumes of saturated ammonium sulfate previously buffered at pH 7.0 and containing 2.5 mM Ca^{2+}. This mixture was allowed to stir overnight and then centrifuged at 48000*g* for 60 min. The clear white precipitate was resuspended in a minimal volume of buffer B. Material in this concentrated form loses activity only slowly over a period of months.

For later studies a final step was included immediately before use. A 1-mL portion of the 80% ammonium sulfate resuspension was applied to a 2.5 × 76 cm column of Ultrogel AcA34 previously equilibrated in buffer B. After the sample entered the gel, the column was eluted with buffer B. Arylester hydrolase activity eluted as a broad peak between two other peaks (Figure 1C). The relative size of the two contaminating peaks varied from preparation to preparation, but the contaminant peaks were always sharper than the arylester hydrolase peak.

Physical Methods. Fluorescence scans were made using a Perkin-Elmer Model 65-40 fluorescence spectrophotometer. Excitation and emission maximal wavelengths of 282 nm (excitation) and 334 nm (emission) were determined in the prescan mode as described in the instructional manual. All emission scans were done in the constant-ratio mode using the excitation wavelength of 282 nm and emission and excitation slit widths of 5 nm.

RESULTS AND DISCUSSION

Purification. Arylester hydrolase has been purified 50-fold from rabbit serum with yields varying from 14 to 30% (Table I). The enzyme resulting from any purification of arylester hydrolase, from any source, must be compared with that of Main (1960) since this work has provided by far the highest specific activity of any arylester hydrolase studied. The simple and relatively rapid procedure described here routinely provides yields with a specific activity near those obtained by Main (1960) for arylester hydrolase from sheep serum and greater than that reported by Mackness and Walker (1983) from sheep serum. The highest specific activity recovered in any Ultrogel AcA34 pool was 1.15 μmol/min per mg, slightly greater than Main's. To obtain this activity, a variation of the concentration step was performed on a very small DEAE-Sepharose column but this method was unsuitable for overall recovery of activity. A direct comparison of the specific activities of the current preparation with that of Main (1960) is not possible because Main's assay contained ethanol and phosphate, had not deliberately added Ca^{2+}, was done at 37 °C, and was from a different animal source. The current assay had no ethanol, MOPS replaced phosphate when phoshate was found to be an inhibitor, Ca^{2+} was found to be necessary for optimal activity, and the assay was done at 25 °C. The specific activity values in Table I are underestimates, since the concentration of substrate used (3.2 mM final concentration) is only 5 times the K_m value for arylester hydrolase (0.609 mM; Grothusen et al., 1986) under our experimental conditions but the paraoxon concentration could not be increased because of limited solubility. Table I shows the results from the current purification procedure. The apparent loss of activity in the 14–25% PEG-4000 fraction is because PEG inhibits the reaction strongly. This is confirmed by the "gain" in total units seen after the first DEAE-Sepharose column. The major purification occurs in the second DEAE-Sepharose column and the Ultrogel AcA34 fractionation. The major loss occurs during the resuspension of the 80% ammonium sulfate pellet to minimize the volume for passage over the Ultrogel AcA34 column (it is of note that the pooled arylester hydrolase from the AcA34 effluent often had a higher activity than could be expected from the average of the velocities of individual tubes). In this respect Mackness and Walker (1983) reported four very closely spaced peaks in their preparative gel electrophoresis of sheep serum arylester hydrolase and Kojima and O'Brien (1968) reported at least four enzyme forms in rat liver. We have seen occasional evidence for two peaks of arylester hydrolase activity in the AcA34 effluent.

Even though the colorless arylester hydrolase pool at this stage matches, or exceeds, the highest reported specific activity for arylester hydrolase, densitometer scans at 525 nm of AcA34 effluent material show the preparations to be only 20–50% pure. Accurate quantitative analysis of these silver stains is not possible because, in the system of Neilsen and Brown (1984), different proteins show different colors.

The final step of the current purification (the Ultrogel AcA34 column) is not yet satisfactory. While adequate

separation was achieved using the AcA34 column, the fact that it is a size fractionation method limits the volume of material that can be applied to the column in any one run. Concentration of the material by ultrafiltration, using any of a number of filters, resulted in a severe loss of activity, as does concentration by ammonium sulfate precipitation (see above). Centricon 10 tubes do concentrate the protein without major inactivation, but at a limit of 2 mL/tube this method is impractical on a preparative scale. Several alternative methods have been tried. A concanavalin A column failed to bind any of the residual proteins; the use of a thiol agarose column to find the free SH group (Kojima and O'Brien, 1968) bound some protein, but not arylester hydrolase; and a Matrex Red column bound an impurity but the arylester hydrolase was in the breakthrough volume, not the property desired for a final step. When arylester hydrolase was applied to two separate Sepharose affinity columns, using compounds known to be competitive inhibitors of arylester hydrolase (Lenz et al., 1973), arylester hydrolase bound very tightly, was highly active, but could not be eluted even with paraoxon. Other methods are being tested, but because of the relatively large volume of material, the desired column matrix should selectively bind (and therefore concentrate) arylester hydrolase while allowing the two remaining impurities to pass through.

During the development of the assay system it was found that the commonly used phosphate ion (Main, 1960; Lenz et al., 1973; Chemnitius et al., 1983; McIlvain et al., 1984) is a potent arylester hydrolase inhibitor and was replaced with MOPS. Very recently McIlvain et al. (1984) also found that phosphate inhibited the assay and they replaced phosphate with either borate or HEPES.

We also confirmed the work of McIlvain et al. (1984) and Mackness and Walker (1983) concerning the requirement for Ca^{2+}. In earlier stages of this work an ammonium sulfate cut was included and without the presence of added Ca^{2+} the yields were drastically reduced. The yield at the PEG fractionation step is also enhanced if Ca^{2+} is added although it is not as critical at this point because the PEG cut occurs as the first purification step and Ca^{2+} is naturally present in serum. For high yields and to maintain activity, the presence of Ca^{2+} throughout the preparation is imperative. As further indication of the requirement for Ca^{2+}, the addition of 5 mM EGTA completely abolished arylester hydrolase activity.

One of the frustrating problems in working with arylester hydrolase is its reported instability in the latter stages of purification (Main, 1960; Zech and Zürcher, 1974; Zech and Wigand, 1975; Chemnitius et al., 1983). Main (1960) had some success in stabilizing the activity by freezing the enzyme in a weak bicarbonate solution. We have succeeded in stabilizing the activity by rapid dropwise freezing in liquid nitrogen, allowing the excess nitrogen to boil off, and storing the beads at −80 °C. As observed by Main, an immediate loss of activity occurs, but the remaining activity is maintained, in our case for over 1 year. This method of preservation can be used at any stage in the preparation but is most effective when the total proten concentrations are high. Highly concentrated ammonium sulfate suspensions at 4 °C also retain activity for long periods of time (months). Inclusion of 0.02% sodium azide in all solutions greatly reduced the rapid loss of arylester hydrolase activity reported by others.

Main (1960), Mackness and Walker (1983), and others have indicated that they believe that during purification a cofactor is lost. We have tried to add back fractions separated from those fractions containing arylester hydrolase activity and have had no success at restoring activity. The addition of 10 mg/mL of BSA (to increase total protein concentration and to coat all glass surfaces) did not affect the activity but did slow the rate of inactivation. Because Mackness and Walker (1983) indicated that arylester hydrolase activity is located in the HDL_2 fraction of sheep serum and may be an activity of one of the apo A proteins, the effect of phosphatidylcholine was examined. There was no effect on enzyme activity over the range of 20–100 μM phosphatidylcholine.

In a search for a natural physiological activity of arylester hydrolase, the enzyme was also tested for tyrosine phosphatase activity. Serine phosphatase and threonine phosphatase activities were used as controls. In none of the cases was any phosphatase activity found.

Physical Studies. Rabbit serum arylester hydrolase seems to differ in its physical properties from those reported by Main (1960) for sheep serum arylester hydrolase. The rabbit protein does not show strong blue fluorescence reported by Main.

Reduced and alkylated SDS-PAGE of the enzyme after silver staining showed two closely spaced bands with molecular weights of 40 000–45 000 and 47 000–54 000 that directly correlated with arylester hydrolase activity. The results were the same under nonreducing conditions. On SDS-PAGE of the effluent ofthe AcA34 column, fractions containing these two bands are found to elute between peaks with estimated molecular weights of 150 000–165 000 and 170 000–190 000. The position of elution of arylester hydrolase activity from the AcA34 column gave an estimated M_r of 180 000–200 000. This information is consistent with native arylester hydrolase having an $\alpha_2\beta_2$ structure. Main (1960) estimated the molecular weight of sheep serium arylester hydrolase to be between 35 000 and 50 000 Da based on a single, uncorrected sedimentation coefficient measurement of 3.69 S. Mackness and Walker (1983) state that the molecular weight of the enzyme from sheep serum is greater than 200 000 based on its behavior on Sephadex G-200, but no K_d nor standard curve was given. Zech and Zürcher (1974) claim a molecular weight of 500 000 for rabbit serum arylester hydrolase, also based on its elution behavior on Sephadex G-200, but the K_d value of 0.063 reported is far too small to obtain a reliable molecular weight estimate.

CONCLUSION

This paper presents a relatively rapid purification of rabbit serum arylester hydrolase with good recovery of enzymatic activity. Crucial factors include the presence of Ca^{2+} at all steps and the inclusion of low levels of azide ion as a bacteriostat in all solutions. Activity at all stages can be maintained for long periods of time by rapid, dropwise freezing and storage at −80 °C.

The enzyme characterized in this report appears to be substantially different than the arylester hydrolase purified by Main (1960) from sheep serum. There are differences in estimated molecular weights as well as in fluorescence characteristics. It may be that the differences in separation procedures have resulted in the characterization of different isozymes. So little is known about the enzyme(s) that give arylester hydrolase activity that a true comparison must wait.

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Registry No. Ca, 7440-70-2; arylester hydrolase, 9032-73-9.

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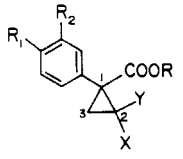
Synthesis and Biological Activity of DDT–Pyrethroid Insecticides

George Holan, Wynona M. Johnson,* Christopher T. Virgona, and Reimund A. Walser

Thirty-five examples of DDT–pyrethroid insecticides have been synthesized. The halo-substituted cyclopropyl rings were formed by the addition of thermally or phase-transfer generated halocarbenes, to suitably substituted 2-aryl acrylates **3**. It was found that the 1-aryl-2,2-difluorocyclopropane-1-carbonyl chlorides were susceptible to ring cleavage to give substituted butyrates as products. In contrast, the 1-aryl-2,2-dichlorocyclopropane-1-carbonyl chlorides underwent ring-opening–ring-closure reactions as demonstrated by the racemization of these compounds during esterification reactions. Structure–activity studies revealed that for optimum insecticidal activity the substituents required at C-2 of the cyclopropane ring were two fluorine, one chlorine and one fluorine, or two chlorine atoms. These studies also revealed that substitution by fluorine at the 4-position of the 3-phenoxybenzyl moiety increased the toxicity of these compounds to *Heliothis punctigera* (Wall) but not to *Lucilia cuprina* (Weid.) or *Blatella germanica* (L.)

The rational design of a group of insecticides that possesses combined DDT–pyrethroid structures, e.g. **1**, has



1

X = Y = F, Cl, Br
X = F, Y = Cl
X = F, Y = Br
X = Cl, Y = Br
R_1 = OEt, Cl, F, etc., R_2 = H
R_1R_2 = $-OCH_2O-$
R = pyrethroid alcohol component

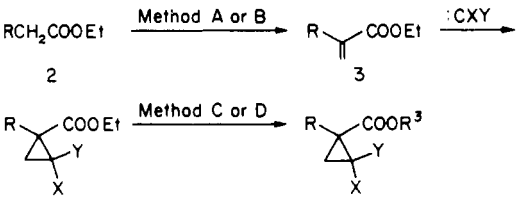
been reported previously (Holan et al., 1979). The biological activities of DDT and pyrethroid compounds have been compared to some of these new structures (Holan et al., 1978). This paper describes the preparation of some of these structures and extends the above work by describing the synthesis and biological activity of several new series of insecticidal DDT–pyrethroid esters.

It is hoped that the systematic structure–activity analysis will assist in the design of new highly active and selective insecticides, as well as in the future elucidation of the shape and nature of the neuroreceptor(s) for the DDT–pyrethroid compounds.

EXPERIMENTAL SECTION

General Procedures. Microanalyses were performed by Microanalytical Services, Amdel, Melbourne. Melting points were determined with a Mettler FP5/FP51/FP52. Proton (^1H) NMR spectra were recorded at 60, 90, or 250 MHz with Varian Associates EM 360, EM 390, or Bruker WH 250 spectrometers, respectively. Tetramethylsilane was used as an internal standard, and deuteriochloroform was used as solvent. IR spectra were recorded on Perkin-Elmer 710B or 783 spectrometers. High-performance liquid chromatography (HPLC) was carried out with a Waters Associates M-6000 pump with a Model U6K valve injector. Eluates from the column were monitored with a Model 440 UV detector. The chromatographic columns used were (A) 250 × 21.2 mm Du Pont SIL and (B) 300 × 3.9 mm μ-Porasil. Optical rotations were obtained on a Perkin-Elmer 241 polarimeter using solution of 1% in $CHCl_3$ unless otherwise stated. Supplementary material containing spectral and analytical data is available (see paragraph at end of paper regarding supplementary material).

Scheme I



Method A (1) $(EtOOC)_2$, NaOEt (2) HOAC (3) HCHO, K_2CO_3

Method B HCHO, K_2CO_3, DMF

Method C (1) 1N NaOH/EtOH (2) $SOCl_2$, pyr (3) R^3OH, pyr

Method D (1) 1N NaOH/EtOH (2) Et_3N, R^3Br, acetone

Division of Applied Organic Chemistry, CSIRO, Melbourne, Victoria 3001, Australia.

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