Purification and Properties of Cholinesterases from Honeybees—*Apis mellifera* Linnaeus—and Boll Weevils—*Anthonomus grandis* Boheman

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A simple method is described for the purification of cholinesterases from honeybees and boll weevils. The effect of 12 different pesticides including carbamates, chlorinated hydrocarbons, and organophosphorus compounds on these enzymes is reported. Since the cholinesterase from each source shows different inhibition by various inhibitors, an increase in the specificity of enzyme systems for the determination of pesticides is possible.

In the last 10 years, the increasing importance of various pesticides (anticholinesterase agents) for insect control has attracted much attention to basic studies of insect cholinesterase (ChE), and extensive investigations on the mode of toxic action of pesticides toward this enzyme have been conducted (Bull and Lindquist, 1968; Hollingworth *et al.*, 1967; Jackson and Aprison, 1966; Kunkee and Zweig, 1963; Lord, 1961; Matsumura and O'Brien, 1966; Metcalf *et al.*, 1955). However, most of the studies on insect cholinesterases have been carried out with relatively crude preparations which contain esterases with overlapping substrate specificity (Arurkar and Knowles, 1968). Some of these esterases were active in hydrolyzing organophosphates and carbamates (Matsumura and Sakai, 1968). The presence of impurities or other esterases in the crude extract may therefore alter or modify the activity of this enzyme in the course of study. Furthermore, studies on the toxicity of various pesticides toward cholinesterases from different insects are equally incomplete, despite these numerous investigations. Because of these reasons, it seemed desirable to free these cholinesterases from the contaminants in the crude extracts and compare the sensitivity of these purified preparations against different pesticides.

A simple method for the purification of cholinesterases from honeybees as well as boll weevils is described, and the inhibitory studies on different pesticides toward these purified preparations are also presented.

MATERIALS AND METHODS

Pesticides. Stock solutions of various concentrations were prepared in dioxane. The pesticides were all of 99+% purity and were obtained from Polysciences Corp., Evanston, Illinois.

Substrates. The substrates, acetylcholine chloride (ACh), acetyl-β-methylcholine chloride (AMeCh), propionylcholine chloride (PrCh), butyrylcholine chloride (BuCh), succinylcholine chloride (SuCh), and benzoylcholine chloride (BzCh) were the best commercial grade available, and were used without further purification. *N*-methyl indoxylacetate was obtained from ISOLAB Inc., Elkhart, Ind.

Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Inc., and DEAE cellulose was obtained from General Biochemicals.

Measurement of Enzyme Activity. Cholinesterase activity of different preparations for various studies was assessed either fluorometrically, colorimetrically, or titrimetrically. The inhibitory effect of pesticides on enzyme activity was determined fluorometrically by the method of Guilbault *et al.* (1968) using *N*-methyl indoxyl acetate as substrate. A 10^{-2} *M* solution of *N*-methyl indoxyl acetate was prepared in methyl cellosolve and used as substrate for each cholinesterase. The other conditions for assay (buffer, pH, etc.) were the same as previously found optimal (Guilbault and Sadar, 1968). The rate of production of the highly fluorescent *N*-methyl indoxyl from the nonfluorescent ester *N*-methyl indoxyl acetate is measured at λ_{ex} = 430 mm and a λ_{em} = 500 mm. The concentration of each enzyme was selected, keeping in mind its activity and the rate of increase of fluorescence. Since their activities are not the same, a different concentration of each enzyme was picked which gave an appropriate rate. All concentrations are, however, taken from the linear portion of the curve drawn by plotting $\Delta F/\Delta t$ *vs.* enzyme concentration. The blank rate, *i.e.*, rate without any pesticide present, was always taken by adding 0.1 ml of dioxane, the solvent for pesticides. The substrate preference studies were accomplished titrimetrically by the technique of continuous titration with a Radiometer titrator, model Metrohm's 3M. As a rule, the total volume of the reaction mixture was 10 ml. Generally 3 ml of 0.2 *M* $MgCl_2$, 1 ml of 0.05 *M* Tris buffer, 4 ml of distilled water, and 1 ml of properly diluted enzyme solution were pipetted into the reaction vessel and brought to pH 7.4 with NaOH. One ml substrate, which was adjusted to pH 7.4 with NaOH prior to use, was then added to start the reaction. The amount of NaOH consumed *vs.* time was recorded after a constant rate was attained. The enzymic hydrolysis of acetylcholine was spot-checked by the modified method of Simpson *et al.* (1964). One enzyme unit is reported as 1 μmole substrate hydrolyzed per min for all the methods used in this experiment.

Electrophoresis. Disc electrophoresis was accomplished according to the method of Davis (1964) using acrylamide gel cast in glass tubes. The applied current was maintained at 3 mA per column until the dye front approached 3 mm from the end of the column. Cholinesterase activity was detected by the method of Hunter and Burstone (1960), using α-naphthyl acetate as substrate and Fast Blue as the coupler. The demonstration of protein after electrophoresis was according to the method of Chrambach *et al.* (1967). For inhibition studies after electrophoresis, the gel cylinders were incubated in 10^{-5} *M* eserine for 40 min, then the enzyme activity was demonstrated as described.

Estimation of Protein. Protein concentrations in different preparations were estimated by the method of Folin-Ciocalteu as outlined by Litwack (1963) using egg albumin as reference.

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Table I. Ammonium Sulfate Fractionation of Crude Enzyme Extract from Bees and Boll Weevils

Saturation (%)	Volume (ml)	Enzyme Activity (Units/ml)	Total Units	Protein (mg/ml)	Specific Activity	Recovery (%)
			Bees			
0	94	0.556	52.3	0.752	0.739	
0–20	55	0.059	3.2	1.067	0.054	5.8
20–40	31	0.896	26.9	0.200	4.48	51.5
40–60	24	0.688	16.5	0.115	5.99	31.6
60–80	8	0.033	0.264	0.035	0.940	0.001
80–100	82	0	0	0.003	0	0.0
			Boll Weevil			
0	185	0.497	91.95	1.241	0.401	100
0–20	90	0.033	2.97	1.832	0.018	3.2
20–40	30	0.922	27.7	0.730	1.26	30.1
40–60	30	1.265	38.0	0.521	2.43	41.3
60–80	30	0.172	5.16	0.164	0.105	5.6
80–100	10	0.003	0.035	0.082	0.004	...

The specific activity of the preparation was calculated based on the enzyme units/mg protein obtained by this method.

Assay of Pesticides. To 3.0 ml of phosphate buffer, 0.1 *M*, pH 7.0, and 0.1 ml of 10^{-2} *M* *N*-methyl indoxyl acetate was added 0.1 ml of dioxane (the solvent used for the pesticides). The fluorescence was adjusted to zero and then 0.1 ml of the appropriate enzyme stock solution was added. The rate of change in fluorescence with time, $\Delta F/min$, was recorded. This rate was labeled as the blank rate or the rate with no pesticide present. To 3.0 ml of buffer and 0.1 ml substrate was added 0.1 ml of a solution of the pesticide to be assayed and the fluorescence was adjusted to zero. Then 0.1 ml of the enzyme solution was added and $\Delta F/min$ recorded. The percent inhibition was calculated as below:

$$\% \text{ inhibition} = \frac{(\Delta F/\text{min})_{\text{no inhibition}} - (\Delta F/\text{min})_{\text{inhibition}}}{(\Delta F/\text{min})_{\text{no inhibition}}} \times 100$$

The concentration of pesticide was determined from a calibration plot of % inhibition *vs.* concentration of the pesticide. All assays were performed at 25° ± 0.1° C.

Cholinesterase Preparation. Unless otherwise stated, all the purification steps were done under temperature control between 0–4° C. The enzymes were prepared and purified as follows: Normal adult male bees (3–6 weeks old, 120 mg/insect) were selected from cultures of a hybrid strain evolved from crossing Italian with Caucasian honeybees (APC strain), and normal boll weevils (10 days old 10 mg/insect) were selected as adults from Castelberry Endrin Strain. The latter were reared on a normal synthetic boll weevil diet as modified by Earle *et al.* (1959), regardless of their sex and developmental stages.

Insects were killed in a −20° C deep freezer and every 400 bees or 150 boll weevils were ground in a mortar and pestle with 100 ml distilled water containing 4 ml butanol and 0.4 gm sodium taurocholate. The slurry was left in the cold room with stirring for autolysis for 62 hr. The mixture was strained through glass wool and centrifuged at 6000 g for 30 min. Since the preliminary trial indicated that about 80–90% of the ChE activity remained in the supernatant, the precipitate was therefore rejected and the supernatant was again strained through glass wool to remove wax particles. The filtrate was dialyzed against 20 volumes of 0.003 *M* phosphate buffer (pH 7.0). Dialysis was accomplished with two changes of buffer at 12-hr intervals. The dialyzed contents were then centrifuged at 6000 g for 30 min. Any fat particles formed on the top were removed from the supernatant by filtration. The filtrate was kept for ammonium sulfate fractionation and the precipitate which had only trace amounts of ChE activity was discarded. From this stage on, a small amount of a 0.01 *M* choline solution was regularly added to every preparation to increase the enzyme stability during purification.

The dialyzed enzyme solution was fractionated with ammonium sulfate at 0 to 20, 20 to 40, 40 to 60, 60 to 80, and 80 to 100% saturation. After each addition of ammonium sulfate, the mixture was allowed to stand for 1 hr then centrifuged at 6000 g for 30 min. The precipitates from each fractionation were dissolved in a small amount of 0.005 *M* phosphate buffer (pH 7.0), then dialyzed against 20 volumes of distilled water. The enzyme activity and protein concentration of these fractions were determined. Dialyzates of 20 to 40 and 40 to 60% saturation were combined, then divided into 3 portions of which 2 parts were separately chromatographed on the same Sephadex G-100 column and the remaining portion was used for enzyme and protein assays, as well as for electrophoretic analysis.

A Sephadex column was prepared by swelling Sephadex G-100 in 0.003 *M* phosphate buffer (pH 7.0) and packing it in a Sephadex K 15/19 column. The sample was carefully applied to the top of the column and elution was started with 0.003 *M* phosphate buffer, after a complete penetration of the sample into the upper surface. Fractions of 2 ml were collected on a GME fraction collector and analyzed for protein concentration with Gilson UV absorption meter. Fractions of enzyme activity were pooled and concentrated to one-third the original volume in a freezer dryer for further DEAE-cellulose chromatography.

DEAE-cellulose was immersed in 1*N* NaOH and washed repeatedly with distilled water until a neutral pH was reached. The slurry was equilibrated with 0.003 *M* phosphate buffer (pH 7.0) and then packed onto a 1.5 × 60 cm column. After the application of the sample, the column was washed with 0.003 *M* phosphate buffer and linear gradient elution was then started. The sample collection, analysis, and concentration were the same as described in the above paragraph. This final product was used for electrophoresis and other studies.

RESULTS

Results of ammonium sulfate fractionation of the crude extracts from bees and boll weevils are shown in Table I. The bulk of the enzyme activity for both preparations were found to be present in fractions between 20–40 and 40–60%

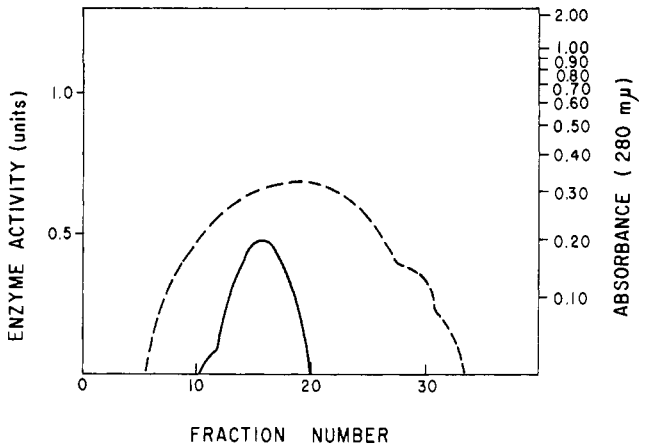

Figure 1. Chromatography of $(NH_4)_2SO_4$ fractionated boll weevil enzyme solution on Sephadex G-100

——— Enzyme activity
- - - - - Protein

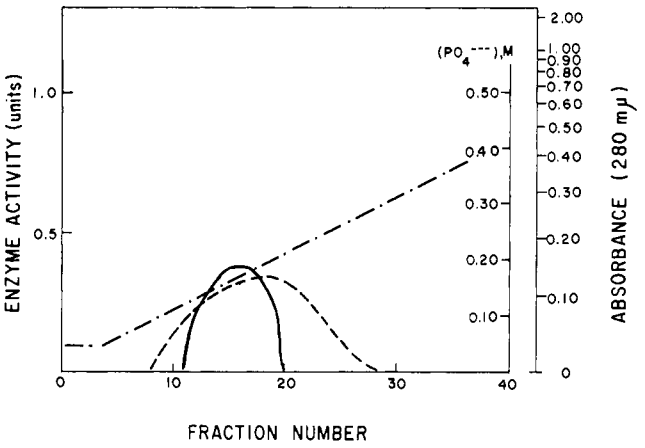

Figure 3. Gradient elution diagram of bee cholinesterase from DEAE-cellulose of pooled fractions from Sephadex G-100 chromatography

——— Enzyme activity
- - - - - Protein
-·-·-·- Phosphate buffer

saturation. The recovery of enzyme activity in these two fractions was as high as 83% for the bee enzyme and 71% for the boll weevil enzyme. About sevenfold purification was obtained when *N*-methyl indoxyl acetate was the substrate.

A plot of the results for both bee and boll weevil enzymes from chromatography of the combined fraction from ammonium sulfate fractionation on G-100 is illustrated in Figure 1 and Figure 2, respectively. The elution patterns of these two cholinesterases are very similar. A smooth single peak of cholinesterase activity, partially overlapping a wide spread protein peak, was obtained. Of the activity applied to the column, more than 90% could be recovered after chromatography. It can also be seen from this figure that the enzyme appeared in the early fractions while the nonenzymic protein was separated and removed from the preparation in the tailing portion of the protein eluted. The combined fractions of the enzyme peak had a threefold increase in specific activity.

A plot of the results of the purification of bee cholinesterase on DEAE-cellulose column is given in Figure 3. There is only one enzyme peak, and this peak falls in between the peak of 280 mμ-absorbing material. The recovery of the applied enzyme activity was in the neighborhood of 50%, and twofold purification was achieved after chromatography. The fractions 15, 16, and 17, which had the highest activity, were pooled and concentrated in a freeze dryer for disc electrophoresis studies. This purified preparation was found to be very unstable. An 80% loss of activity was observed during a 4-day storage at 4° C. Because of this finding, boll weevil cholinesterase was not further purified on DEAE-cellulose.

Figure 4 shows the zymograms resulting from separation of various preparations from bees at different purification stages in acrylamide gel electrophoresis. Of all the esterase zones, only one zone was inhibited by eserine. The mobility of the enzymic zones was found to coincide with the protein bands.

To classify the cholinesterases from both insects, the preparations from G-100 chromatography were characterized by the observed hydrolysis rate with several substrates, and the results are shown in Figures 5 and 6. For comparison, both the insect cholinesterases hydrolyzed acetylcholine ester much faster than butyrylcholine ester under optimal substrate concentration. Generally, the hydrolysis rate and substrate inhibition decreases with increase of carbon atoms in the acid portion of the substrate with the exception of acetyl-β-methylcholine ester. The optimal substrate concentration for bee cholinesterase appeared to be at 10^{-3} *M* for most of the substrates, while a little higher optimal substrate concentration was found for boll weevil enzyme.

Table II summarizes the purification of cholinesterases from both insects, starting with crude extracts. In order to compare it with data obtained by other investigators, both *N*-methyl indoxyl acetate and acetylcholine were used as substrate for enzyme assay. The overall enrichment is given in terms of specific activity. When acetylcholine was the substrate, the fold of purification was about one-third that of *N*-methyl indoxyl acetate.

Data on the stability of the two enzyme preparations is presented in Figure 7. The stability was determined by noting the rate of hydrolysis of *N*-methyl indoxyl acetate in ΔF/min. The boll weevil enzyme is fairly stable and maintains over 80% of its activity after 10 days in a refrigerator. The bee cholinesterase, however, is unstable and loses about 50% of its activity in 3 days at 4° C.

DDVP (Vapona) was found to be a potent inhibitor of boll weevil cholinesterase, 5×10^{-6} *M* causing a 50% inhibition of the enzyme activity with no preincubation (Figure 8) [All I_{50}'s reported herein were obtained by noting the concentra-

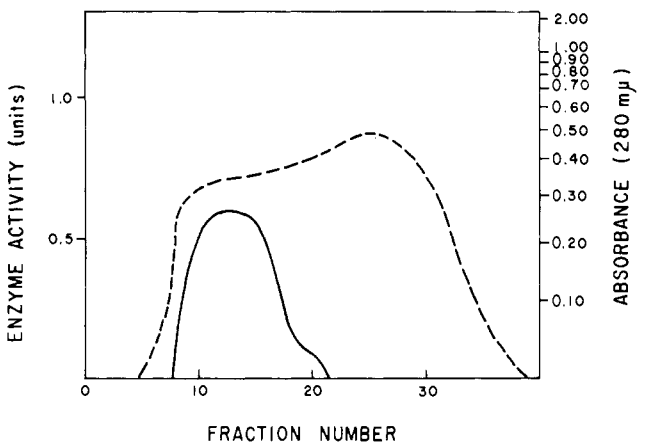

Figure 2. Chromatography of $(NH_4)_2SO_4$ fractionated bee enzyme solution on Sephadex G-100

——— Enzyme activity
- - - - - - Protein

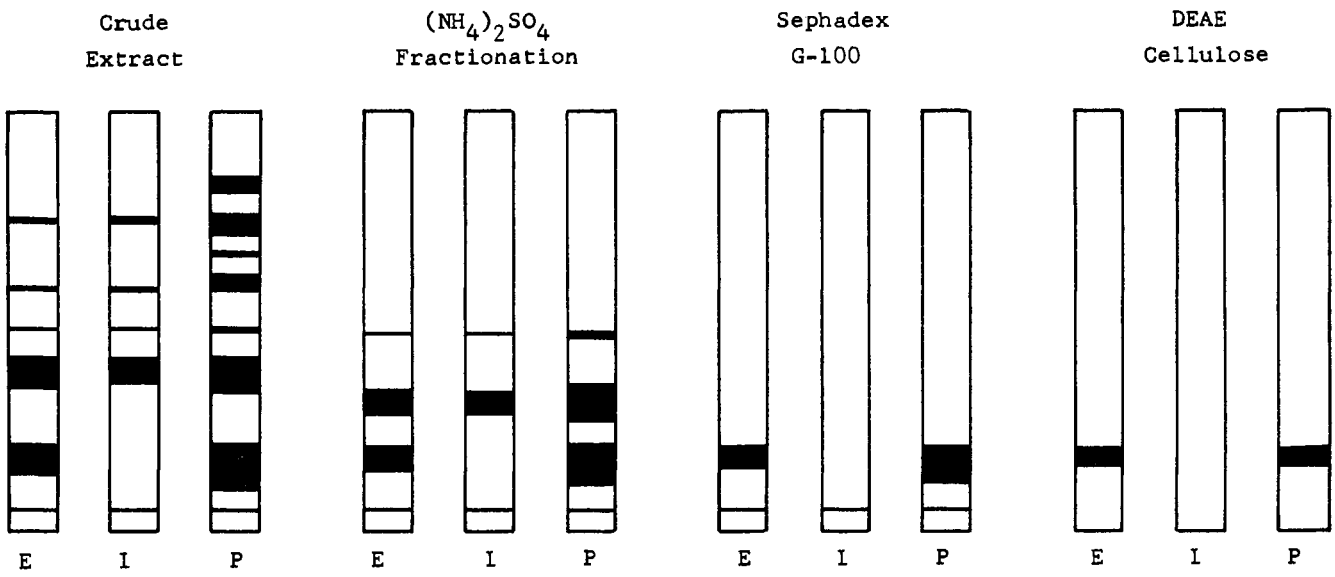

Figure 4. Polyacrylamide gel electrophoresis pattern of bee cholinesterase preparation at different purification stages

E—without eserine I—with eserine P—protein

tion of pesticide that effected a 50% decrease in the rate of hydrolysis of *N*-methyl indoxyl acetate (overall concentration 3×10^{-4} *M*). The rate was calculated by measuring the change in fluorescence after 2 min. [No preincubation of enzyme and inhibitor was used.] A plot of % inhibition *vs.* DDVP concentration is linear from 5×10^{-7} *M* to 1×10^{-5} *M* with no preincubation. Good inhibition of boll weevil ChE occurs after about 5 min (90% inhibition for a 1.5×10^{-6} *M* DDVP). Inhibitions of 21.8%, 49.7%, 79.8%, and 96.8% were obtained at 0, 2, 4, and 10 min, respectively. Using 5 min preincubation, from 5×10^{-8} to 1×10^{-6} *M* DDVP can be specifically determined with this enzyme. Sevin and aldrin show a slight inhibition (maximum 25%) at high concentrations (10^{-4} *M*) (Figure 8). No inhibition was observed by methoxychlor, DDT, lindane, dieldrin, and heptachlor at any concentration.

Organophosphorus compounds: DDVP, methyl parathion, and the carbamate Sevin were found to be good inhibitors of bee cholinesterase (Figure 9) (I_{50} for DDVP 8.3×10^{-7} *M;* Sevin, 1.5×10^{-6} *M*). Chlorinated pesticides: aldrin, heptachlor, lindane, and methoxychlor show slight inhibition (15–25%); DDT and dieldrin show no inhibition.

DISCUSSION

Isolation of Enzymes. As shown in Tables I and II, the high recovery of enzyme activity makes the ammonium sulfate

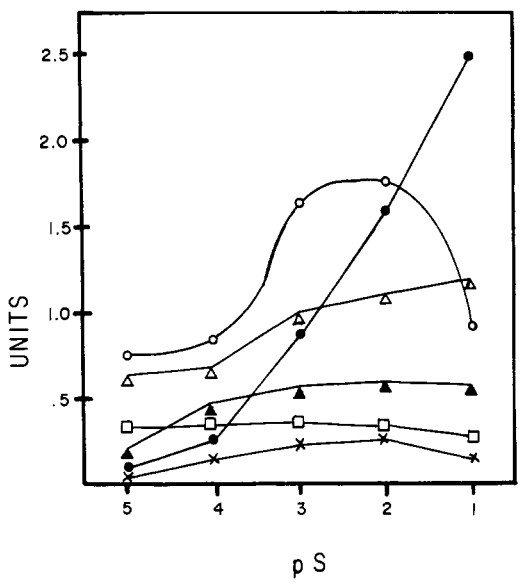

Figure 5. Substrate activity curves for the concentrated bee cholinesterase from Sephadex G-100 chromatography

○—○ Acetylcholine
●—● Acetyl-β-methylcholine
△—△ Propionylcholine
▲—▲ Butyrylcholine
□—□ Succinylcholine
X—X Benzoylcholine

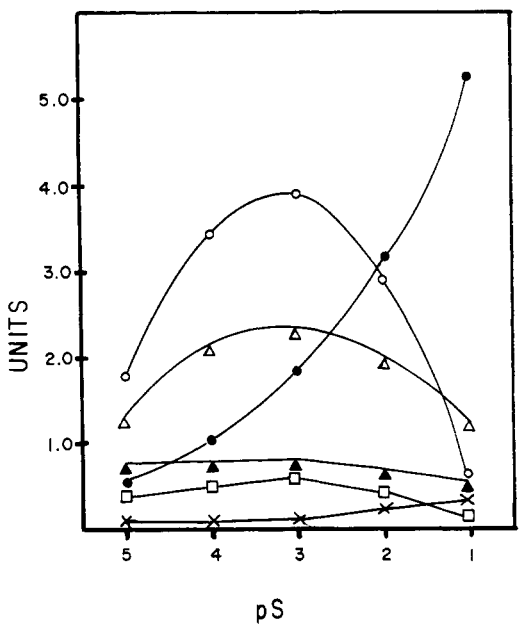

Figure 6. Substrate activity curves for the concentrated cholinesterase of boll weevil from Sephadex G-100 chromatography

○—○ Acetylcholine
●—● Acetyl-β-methylcholine
△—△ Propionylcholine
▲—▲ Butyrylcholine
□—□ Succinylcholine
X—X Benzoylcholine

Table II. Summary of the Purification of Bee and Boll Weevil Cholinesterases

Stage	Protein (mg/ml)	Acetylcholine Chloride: Specific Activity	Acetylcholine Chloride: Fold of Purification	Acetylcholine Chloride: Yield % Activity	*N*-Methyl Indoxylacetate: Specific Activity	*N*-Methyl Indoxylacetate: Fold of Purification
		Bee			Bee	
Crude extract	1.630	0.354	1.0	100	0.028	1.0
Filtrate after dialysis	0.725	0.739	2.1	91	0.074	2.6
$(NH_4)_2SO_4$ Fractionation	0.163	4.94	13.9	75	0.915	32.7
Sephadex G-100 chromatography	0.053	12.7	35.9	61	3.49	124.6
DEAE-cellulose chromatography	0.015		68.2	30	...	...
		Boll Weevil			Boll Weevil	
Crude extract	1.241	0.208	1.0	100	0.024	1.0
Filtrate after dialysis	0.697	0.401	1.9	93	0.043	1.8
$(NH_4)_2SO_4$ fractionation	0.163	2.07	19.9	66	0.833	34.7
Sephadex G-100 chromatography	0.074	7.98	38.4	58	2.67	115.0

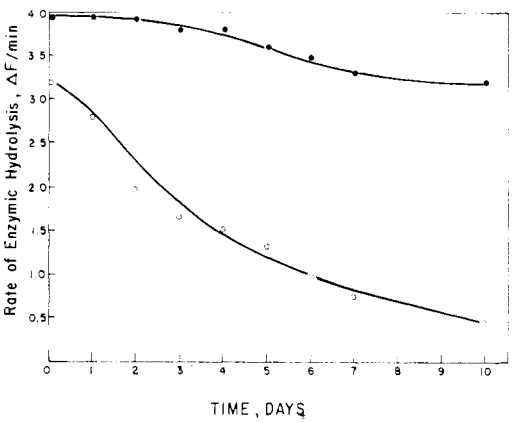

Figure 7. Stability of purified boll weevil and bee cholinesterase preparations with time. Rate of hydrolysis of *N*-Methyl indoxyl acetate, ΔF/min, by the enzyme preparation used as measure of enzymic activity

●—● Boll weevil
○—○ Bee

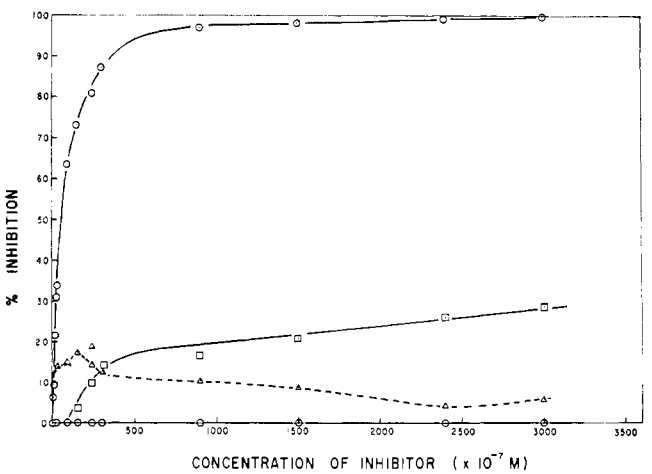

Figure 8. Plot of % inhibition of boll weevil cholinesterase *vs.* concentration of pesticide

⊙—⊙ DDVP
△—△ Aldrin
⊡—⊡ Sevin
○—○ Lindane, dieldrin, DDT, heptachlor

fractionation a very useful step for the purification of cholinesterase from insects because of the limited source of material available. The electrophoretic analysis also indicates that some esterases and nonenzymic proteins were actually removed from the preparation by fractionation.

The success of Sephadex G-100 chromatography as a means of purification is amply demonstrated (Figures 1 and 2). About two-thirds of the nonenzyme protein could be removed from the preparation by using this simple technique. In addition, the recovery rate was relatively high in comparison to DEAE-cellulose column chromatography. The electrophoretic patterns also show the effectiveness of this technique in selective removal of the impurities from the preparation, though there still appeared another minor esterase zone.

The homogeneity of the final product demonstrated by electrophoretic analysis suggests that DEAE-cellulose chromatography is a valuable complementary step to the purification procedure. However, the poor stability of the preparation

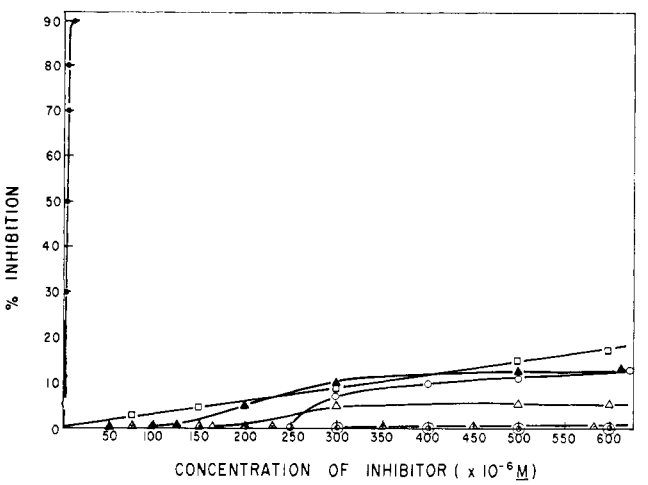

Figure 9. Plot of % inhibition of bee cholinesterase *vs.* concentration of inhibitor

●—● Sevin, DDVP
□—□ Methoxychlor
○—○ Aldrin
▲—▲ Heptachlor
△—△ Lindane
△—△ DDT
⊙—⊙ Dieldrin

discourages us from using it as a final purification step. The reason is not known. The loss of activity may be due either to the loss of cofactors which are required for the enzyme activity, or to the removal of certain proteins which cause the conformational change of the enzyme molecule, since some proteins have been reported to be effective in stabilizing the cholinesterase activity (Fleisher *et al.*, 1955).

Electrophoretic separation of the crude extract has clearly indicated that at least six esterases were present in the crude preparation. Of these esterases, only one can be classified as cholinesterase. Based on the following facts, we would classify the enzyme preparation obtained from both insects as acetylcholinesterase rather than pseudocholinesterase: the enzyme shows typical substrate inhibition with acetylcholine; the hydrolysis rates with acetylcholine were much faster than with butyrylcholine; the enzyme hydrolyzes acetyl-β-methylcholine at a faster rate than benzoylcholine.

As a result of these studies, a simple procedure for obtaining highly purified product in a good yield has been developed. The fold of purification of our preparation after Sephadex G-100 chromatography had an average of 120. The total enrichment of the enzyme is somewhat better than those preparations obtained from other insects (Dauterman *et al.*, 1962). The electrophoretic analysis also shows that insect ChE thus purified is pure enough for most of the basic investigations.

Inhibition by Pesticides. Many workers have reported enzymatic determination of pesticides. Guilbault and Kramer (1963, 1964) used both lipase and cholinesterase enzymes to determine very low concentrations of certain pesticides. Giang and Hall (1964) used acetylcholine chloride as substrate for cholinesterase to measure percent inhibition by organophosphorus insecticides. Although the above quoted papers and many more provided a sensitive method of analysis, the major emphasis seems to have been on organophosphorus compounds. Moreover, all these methods lack specificity. Guilbault and Sadar (1969) have already reported inhibition of lipase by chlorinated insecticides such as aldrin, lindane, heptachlor, DDT, and a carbamate Sevin. A sensitive method for analysis of all the above mentioned insecticides in addition to bismuth beryllium and methyl parathion has been proposed Guilbault *et al.* (1969). Unfortunately, these methods are of limited use because of a lack of specificity.

Since it is known that various animals and insects respond in different ways to different insecticides or pesticides, we decided to attempt the isolation of pure enzymes from different sources and study their inhibition by different pesticides.

Some specificity of possible use in the assay of pesticides was observed with bee and boll weevil cholinesterase. The boll weevil is specifically inhibited by DDVP, the bee by DDVP and Sevin. Neither enzyme was seriously inhibited by chlorinated pesticides. In addition, good sensitivities are observed. As little as 5×10^{-8} *M* DDVP (0.01 μg/ml) can be assayed with either boll weevil or bee cholinesterase with 5 min preincubation. Because of the instability of the enzymes, however, better analytical results can be obtained if no preincubation is used.

From the results of this study it can be concluded that insect cholinesterases offer promise of selectivity and sensitivity for the assay of pesticides.

ACKNOWLEDGMENT

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