The Partial Purification and Properties of a Cholinesterase from *Blatella germanica* L.

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(Received 28 July 1960)

Enzymes hydrolysing acetylcholine have attracted much attention because acetylcholine is concerned with the functioning of the nervous system. The extensive literature on cholinesterases has been reviewed at intervals (Zeller, 1948; Koelle & Gilman, 1949; Augustinsson, 1950; Nachmansohn & Wilson, 1951; Whittaker, 1951; Davies, 1954; Bergmann, 1958; Davies & Green, 1958).

Although there is ample evidence that enzymes responsible for the hydrolysis of acetylcholine vary from species to species they can be divided roughly into types according to certain general properties (Augustinsson, 1957); one of these types is closely, but not exclusively, associated with nervous tissues. It is generally supposed that insect cholinesterases do not differ significantly from cholinesterases from other sources and that they are associated with the nervous system. However, most of the limited studies on insect cholinesterases have been made with crude breis of whole insects or parts of insects which may contain other esterases or substances that modify the activity of the cholinesterase. It is desirable to free the cholinesterase from contaminants and compare its properties with those of esterases from other sources. Any unusual properties of the enzyme might give an insight into the functioning of insect nervous tissues. In addition the properties of insect cholinesterase are of especial importance in relation to the mode of action of organophosphorus insecticides, which are generally considered to act by inhibiting cholinesterase of the nervous system. Doubts have been expressed that this is always the mode of action in insects, although it is well established that both cholinesterase and acetylcholine are associated with insect nervous tissues. The evidence has recently been reviewed by Winteringham & Lewis (1959).

Detoxication, rate of entry of poison and other similar factors may be expected to modify the toxicity of an insecticide, but not to the exclusion of effects due to the properties of the enzyme inhibited by the poison. A knowledge of insect cholinesterases should therefore assist the understanding of the toxic action of organophosphorus compounds even if cholinesterase inhibition is not invariably the primary action of this important group of insecticides.

The German cockroach (*Blatella germanica* L.) was chosen as the source of an insect cholinesterase, because it is easily reared and cholinesterase activity is readily demonstrated in extracts from it. The cockroach cholinesterase differs from those examined from other sources in several interesting ways.

METHODS AND MATERIALS

Measurements of esterase activity

With the Warburg apparatus it is possible to determine the activity of *B. germanica* cholinesterase with concentrations of acetylcholine as low as 1 mM and to obtain an indication of enzyme activity with substrate concentrations as low as 0.1 mM, because the rate of CO₂ evolution does not change appreciably until 90% of the substrate has been hydrolysed.

Because manometric methods of assay are not accurate at substrate concentrations as low as 0.1 mM some determinations of activity were made with an electrometric method in which the limiting factor is the detection of small changes of pH rather than the depletion of substrate (the volume of reaction mixture may be made large or the substrate replenished as the reaction proceeds). Results obtained with the electrometric method were consistent with those obtained with the manometric method at low substrate concentrations (0.1 mM-acetylcholine) and demonstrated high activity at even lower concentrations.

Colorimetric methods of assay were used to study effects of pH on activity. They are readily applicable to measurements of activity at different pH because the assay procedures can readily be made independent of pH.

All determinations of enzyme activity were made at 25°.

Manometric assay. This type of assay was made in Warburg manometers with a final concentration of 0.02M-NaHCO₃, or 0.02M-KHCO₃ where specified. No other salts were added unless stated. The apparatus was filled with CO₂+N₂ (5:95). Two sizes of reaction vessel were used, giving a total volume in the apparatus of approx. 8 ml. or 20 ml. When the smaller flasks were used the total volume of the reaction mixture was 1.5 ml. In the larger flasks the volume of the reaction mixture was 3 ml. unless the substrate concentration was less than 1 mM, when 6 ml. of reaction mixture was used. This procedure increases the accuracy of assay at low substrate concentrations. The amount of enzyme present was usually adjusted to give a rate of pressure change between 1 and 3 mm./min.

Electrometric assay. The enzyme-substrate reaction mixture was maintained at constant pH by titrating the acid liberated with 0.01N-NaOH delivered from an Agla

micrometer syringe. The reaction mixture contained no added salts unless this is specifically stated and the volume was usually 10 ml. It was stirred by means of a stream of CO_2 -free air. The titrations were done with the apparatus described by Burch (1954), except that the anode-load resistor of the pH comparator was increased to 25 000 ohms to reduce the drain on the batteries and so increase the stability of the apparatus.

Colorimetric assays. These assays were all made in 0.1M-buffer. Samples were removed from the enzyme-substrate mixture at various time intervals to determine the degree of hydrolysis. For cholinesterase the amount of choline esters remaining in the samples was determined by the method described by Hestrin (1949). The hydrolysis of phenyl esters was measured by a modification of the method of Collins (1945) to determine the free phenol in each portion. The reaction was stopped by adding an equal volume of diazotized 0.03% (w/v) *p*-nitroaniline in 0.1N-HCl. A buffer of an equimolar mixture (Na_2CO_3 - NaHCO_3) was added to adjust the pH to approx. 10 (25 vol. of 0.1M-buffer were used for phenyl acetate and 5 vol. of 0.5M for phenyl propionate), and the colour was read 1 min. later at 470 $m\mu$ with a Unicam SP.350 spectrophotometer.

Units of activity. Unless otherwise specified, all activities have been given as μ moles of substrate hydrolysed in 15 min. by 1 ml. of the enzyme purified to stage 5 (see below). During the purification procedure the cholinesterase was measured manometrically. One unit of cholinesterase activity was defined as the amount of enzyme which would liberate 1 μ l. of CO_2 in 15 min. at 25° from a medium containing 10 mM- MgCl_2 , 20 mM- NaHCO_3 and 2.75 mM-acetylcholine chloride. Specific activity was defined as the units of activity of cholinesterase/ml. divided by the extinction of the solution at 280 $m\mu$ measured for a 1 cm. light-path.

Substrates. Substrate solutions were usually prepared in water, but 5% (v/v) acetone was used to increase the solubility of the poorly water-soluble substances, phenyl acetate, phenyl propionate, phenyl butyrate, tripropionin and tributyrin. The pH of substrate solutions was adjusted when necessary with 0.1N-NaOH. The solutions were usually freshly prepared, but some acetylcholine chloride solutions were stored frozen at -16°. Acetylcholine chloride was obtained from Roche Products Ltd. Acetylcholine perchlorate, triacetin, tripropionin, tributyrin, ethyl acetate, ethyl propionate, ethyl butyrate and phenyl acetate were purchased from British Drug Houses Ltd. The ethyl esters were redistilled and the phenyl acetate was freed from phenol by refluxing with acetic anhydride and redistilling. Propionylcholine perchlorate and butyrylcholine perchlorate were synthesized by refluxing the appropriate acid anhydride with equimolar portions of choline chloride for 1 hr. The ester was precipitated with dry ether and filtered off, converted into the perchlorate by the method of Bell & Carr (1947) and recrystallized from ethanol. Phenyl propionate and phenyl butyrate were synthesized by the method of Chattaway (1931), with excess of the appropriate anhydride. The product when distilled was virtually phenol-free. Dimethylaminoethanol acetate was prepared according to Jones & Major (1930).

Insects. Adult males were selected from cultures of *B. germanica* maintained at 29° and fed on a mixture of rolled oats, dried yeast and dried milk (8:1:1). There was no attempt to control humidity and water was supplied to the

culture. Houseflies were selected as adults (3-4 days old) from normal insecticide-susceptible cultures of *Musca domestica* L. kept at 27° and 50% relative humidity. The adult flies were fed on dry powdered full-cream milk and sugar with a supply of water and fresh milk diluted with an equal volume of water. Larvae were reared in a gel consisting of powdered milk (100 g.), dried brewer's yeast (100 g.) and agar (20 g.) in 1000 ml. of water.

Cholinesterase preparations

Cholinesterase from *Musca domestica*. No attempt was made to purify the cholinesterase from houseflies. Tests on this material were made on extracts prepared by homogenizing heads of equal numbers of male and female flies with water in a power-operated all-glass homogenizer.

Cholinesterase from *Blattella germanica*. When whole adult *B. germanica* males are ground in a mortar with water and the resulting suspension is centrifuged, 50-60% of the cholinesterase activity remains in the supernatant solution. Power-operated apparatus for preparing homogenates, such as the Waring Blendor, often inactivate the cholinesterase, probably by local heating. The crude enzyme is partly inactivated by heating to 40° for 5 min. The natural pH of the suspension is 6.0-6.5. Only slightly larger proportions of the cholinesterase activity remain in the supernatant when the pH of the suspension is raised to 9.0 or when salts are added. When sodium taurocholate is added to the suspension at pH 6.5, almost all of the activity remains in the supernatant on centrifuging. Dialysis of this supernatant removes much ultraviolet-absorbing material, and if the dialysis fluid is kept at pH 8 or above the cholinesterase activity remains in solution. This dialysis facilitates fractionation by $(\text{NH}_4)_2\text{SO}_4$ precipitation. The cholinesterase can be precipitated almost completely by $(\text{NH}_4)_2\text{SO}_4$ whereas the greater part of the other esterase activity remains in solution (Table 1). Most of the cholinesterase activity can be extracted from the $(\text{NH}_4)_2\text{SO}_4$ precipitate with a solution of NaHCO_3 , followed by an alkaline $(\text{NH}_4)_2\text{SO}_4$ solution; a considerable purification is obtained in this way. Between 15 and 30% of the cholinesterase activity cannot be extracted from the precipitate and may represent a different enzyme, but this has not been examined. When the main portion of the cholinesterase (stage 3, Table 1) is dialysed for a prolonged period in cellophan sacs much activity is lost. This does not happen with collodion membranes but then the enzyme is diluted to an inconvenient extent. However, adequate dialysis may be obtained in a short time by using cellophan sacs and agitating. After dialysis the preparation is frequently opalescent because of suspended matter not readily removed by prolonged centrifuging. When viewed under the microscope the preparation contains spherical droplets which are presumed to be liquid fat. Treatment with protamine sulphate facilitates subsequent fractional precipitation with acetone, although it does not appear to give any purification (Table 1). However, when the enzyme is assayed under optimum conditions (1 mM-acetylcholine and 0.1 M-NaCl) the activity appears to increase almost two-fold, and in stages 4 and 5 the yield and purification is nearly twice that given in Table 1. Fractionation with acetone gives a product that can be stored frozen at -16° for at least a year without loss of activity. The enzyme is sensitive to repeated freezing and thawing and was therefore stored in small batches.

Stage 1 (extraction). Adult male *B. germanica* were extracted in a pestle and mortar by grinding (four insects/ml.) with a 0.4% solution of sodium taurocholate in water. The solution was strained through muslin and centrifuged (2000 g for 50 min.). The supernatant was strained through a cotton-wool plug after as much fat as possible had been removed from the surface. The solids were rejected.

Stage 2 (dialysis). The supernatant was dialysed at 5° against 12 vol. of 5 mM- Na_2HPO_4 for 6 hr. with stirring, followed by dialysis against 12 vol. of 2 mM- Na_2HPO_4 overnight. The contents of the dialysis sacs were then centrifuged (2000 g for 40 min.) and any fat was removed from the surface of the supernatant. The solids were discarded.

Stage 3 (ammonium sulphate precipitation). Ammonium sulphate was added slowly with stirring to the supernatant (0.225 g./ml.). After standing for 2 hr. at 20° the mixture was centrifuged (4000 g for 60 min.). The supernatant contains considerable esterase activity but hydrolyses acetylcholine only slowly; it was retained for further examination of other esterases. The precipitate contained most of the cholinesterase of the preparation and was extracted by suspending in 0.2 M- NaHCO_3 [1 ml. for each 20 ml. of supernatant from stage 2 treated with $(\text{NH}_4)_2\text{SO}_4$] for 1 hr. at 20°. The mixture was centrifuged (14 000 g for 15 min.) and the supernatant decanted off. The solids were re-extracted by suspending for 1 hr. in each of two successive portions of a solution containing 62.5 mM- NaHCO_3 and 750 mM- $(\text{NH}_4)_2\text{SO}_4$. The mixtures were centrifuged and the extracts combined.

Stage 4 (dialysis and protamine sulphate). The combined extracts were dialysed for 1 hr. with rocking at 0° against 10 vol. of 10 mM-sodium citrate buffer, pH 6.6. The dialysis was then repeated with a fresh portion of sodium citrate buffer. The product was then treated with 0.4 vol. of 2% (w/v) protamine sulphate (ex-salmon roe; L. Light and Co. Ltd.) at pH 6. It was allowed to stand for 1 hr. at 20° and was then centrifuged (2000 g for 30 min.). The solids were rejected. The supernatant was dialysed for 30 min. against two successive portions of 5–6 vol. of water at 0°.

Stage 5 (acetone precipitation). After dialysis the solution was cooled again to 0° and acetone at –5° was added slowly with stirring whilst the temperature was decreased to –5°. When the acetone concentration was 25% (v/v) the mixture was allowed to stand for 1 hr. at –5° before centrifuging (1500 g for 30 min.). The solids were rejected. Further acetone was added to the supernatant until the

concentration of acetone was 50% (v/v); the mixture was allowed to stand for 1 hr. before centrifuging at –5° (1500 g for 30 min.). The precipitate was stored overnight at –16° to allow any acetone to evaporate. The precipitate was then taken up in 2.5 mM-sodium citrate buffer, pH 6.6 (1 ml. for each 10 ml. of stage 4 treated). The preparation was used for all experiments on the properties of the *B. germanica* cholinesterase. The final product contained 32% of the cholinesterase in the initial extract and the purification was at least 40-fold in terms of absorption of light at λ 280 m μ . Details of yield and purification at each stage are set out in Table 1.

Electrophoresis

Electrophoresis experiments with *B. germanica* cholinesterase preparations were done at +0.5° in thin sheets of starch gel prepared according to Smithies (1955). A voltage of 4.5 v/cm. (approx.) was applied for 17 hr. The ionic strength of the buffer solutions was 0.05.

The position of esterase activity was located in the gel with five substrates which were sprayed on the gel supported on strips of filter paper. Indoxyl acetate was applied as a 0.1% (w/v) solution in benzene. It is hydrolysed by esterases to give indoxyl, which gives rise to a blue fluorescence and, on oxidation by air, indigo. Fluorescein acetate was also applied as a 0.1% (w/v) solution in benzene. Esterases hydrolyse it to give the strongly fluorescent fluorescein. The hydrolysis of aqueous 0.5% (w/v) acetylthiocholine sprayed on the gel was observed after incubating for 1 hr. at room temperature by spraying with an ethanolic 0.1% (w/v) solution of 2,6-dichlorophenolindophenol. The thiocholine liberated by enzyme activity reduces the intensely blue oxidation-reduction indicator to a colourless compound. The enzymic hydrolysis of phenyl acetate and phenyl propionate was detected by formation of a red pigment from the phenol liberated. The substrates were sprayed on the gel as 0.1% (v/v) solutions in benzene. One hour later the gel was sprayed with diazotized 0.3% (w/v) *p*-nitroaniline in 0.1 N-HCl and the gel immediately made alkaline by exposing to ammonia vapour.

RESULTS

The rate of hydrolysis of acetylcholine by the purified preparation of *B. germanica* cholinesterase was proportional to the amount of enzyme used

Table 1. *Blattella germanica* cholinesterase preparation

For full details of the preparation see the text. The hydrolysis of phenyl acetate and triacetin was determined manometrically in the presence of NaHCO_3 alone. The hydrolysis rates are given relative to the hydrolysis of acetylcholine in the presence of 0.01 M- MgCl_2 .

Stage	Volume (ml.)	Total activity (units)	Specific activity	Relative rate of hydrolysis	
				3.7 mM- Phenyl acetate	18 mM- Triacetin
(1) Extraction	585	84 000	1.6	5.7	1.8
(2) Dialysis	640	83 000	5.2	5.2	1.8
(3) Ammonium sulphate precipitation					
(a) Supernatant	640	12 000	1.5	17	8.4
(b) Precipitate	97	45 500	22	1.2	0.21
(4) Dialysis and protamine sulphate	165	31 000	24	2.0	0.31
(5) Acetone precipitation	23	27 000	64	1.7	0.33

over a tenfold range, whether estimated manometrically or by electrometric titration.

Variation of hydrolysis of acetylcholine with substrate concentration and the effects of anions and cations

Manometric assay. It was not possible to determine a Michaelis constant for the *B. germanica* cholinesterase with acetylcholine as substrate. In the absence of salts other than sodium bicarbonate maximum activity was exhibited at the lowest substrate concentrations used (0.1–0.3 mM). The activity fell with increasing substrate concentrations and at 0.1 M the rate of hydrolysis of acetylcholine was about one-tenth of the rate at 1 mM. Sodium chloride increased the rate of hydrolysis of acetylcholine at substrate concentrations above 1 mM, under all conditions examined. The effect increased with the salt concentration and M-sodium chloride approximately doubled the activity of the enzyme. In the presence of M-sodium chloride a maximum activity was observed with about 1 mM-acetylcholine and at substrate concentrations less than 0.5 mM the activity of the enzyme was decreased. Inhibition was not observed with lower concentrations of salt and enzyme activity did not fall as the concentration of acetylcholine chloride was decreased to 0.1 mM when the concentration of sodium chloride was less than 0.1 M.

Acetylcholine was hydrolysed at the same rate whether used as the perchlorate or chloride in the presence of sodium bicarbonate alone. The effects

of sodium sulphate on the hydrolysis of acetylcholine perchlorate were closely similar to those of sodium chloride on the hydrolysis of acetylcholine chloride.

In low concentrations (0.1 M and 0.01 M) magnesium chloride increased the activity of *B. germanica* cholinesterase at acetylcholine concentrations above 1 mM, but at lower substrate concentrations it was inhibitory. Higher concentrations of magnesium chloride (0.33 M) inhibited to some extent at all substrate concentrations but very markedly at low ones, giving an optimum substrate concentration of about 0.5 mM-acetylcholine chloride.

Acetylcholine chloride was hydrolysed by *B. germanica* cholinesterase in potassium bicarbonate at the same rates as in sodium bicarbonate. The effects of potassium chloride closely resembled those of sodium chloride.

Electrometric assay. The Michaelis constant of *B. germanica* cholinesterase could not be determined by the electrometric-titration technique. With no added sodium chloride the activity of the enzyme increased as the substrate (acetylcholine) concentration was decreased to 100 μ M and stayed the same to 10 μ M, the lowest value at which measurements could be made. Increasing concentrations of sodium chloride up to 0.1 M increasingly activated the enzyme at pH 7.5 and no optimum substrate concentration was observed (Fig. 1).

Hydrolysis of esters other than acetylcholine

Variation of activity with substrate concentrations. *B. germanica* cholinesterase hydrolysed a variety of esters other than acetylcholine. The esters, propionylcholine, *N*-dimethylaminoethanol acetate and acetyl- β -methylcholine, which are closely related to acetylcholine and were rapidly hydrolysed by the *B. germanica* cholinesterase, all showed optimum substrate concentrations, with inhibition at high substrate concentrations and a decrease in activity at very low substrate concentrations (Table 2). Butyrylcholine and benzoylcholine were hydrolysed only very slowly compared with acetylcholine. Phenyl acetate, phenyl propionate and triacetin were hydrolysed by the cholinesterase at rates which increased with their concentration. There was no inhibition with excess of substrates and the maximum rates observed were comparable with the highest rate with acetylcholine under similar conditions (Table 2). Ethyl acetate, ethyl propionate, ethyl butyrate, tripropionin, tributyrin, phenyl butyrate and *O*-acetyl ethanolamine were hydrolysed at less than 10% of the rate of hydrolysis of acetylcholine. Acetone, which was used to increase the solubility of some of these esters, had no effect on the rate of hydrolysis of acetylcholine under the conditions used.

Fig. 1. Effect of sodium chloride on the variation of *B. germanica* cholinesterase activity with acetylcholine chloride concentration: electrometric-titration assay. Δ , No salts; $\bullet$, 0.1 M-NaCl; $\circ$, 0.01 M-NaCl.

Variation of activity with pH. Activity-substrate concentration curves with acetylcholine chloride were obtained at a number of pH values by the electrometric-titration method. Similar curves were obtained at all pH values above pH 7; under these conditions the activity increased as the substrate concentration was decreased to 100 μ M and remained constant as it was decreased to 10 μ M. Below pH 7, maximal activity was observed at substrate concentrations of about 1 mM. Activity increased with increasing pH up to pH 8, when

difficulty was experienced in making satisfactory determinations.

With the colorimetric methods the variation of activity with pH was determined at optimum substrate concentrations for acetylcholine, propionylcholine, phenyl acetate and phenyl propionate. Activity increased with pH to a value between 7 and 8; with acetylcholine and phenyl acetate it remained constant up to pH 10, but propionylcholine showed maximum activity between pH 7 and 8 and phenyl propionate between pH 8 and 9 (Fig. 2).

Properties of the enzyme preparation

Stability. *B. germanica* cholinesterase is only slightly inactivated by heating to 40° for 3 min. and is almost completely inactivated by heating to 50° for 3 min. The hydrolyses of acetylcholine, phenyl acetate, phenyl propionate and triacetin, assayed manometrically, are equally affected by heat treatment (Table 3).

The pH of the purified preparation can be changed over the range 5-10 without loss of activity as assayed manometrically. About 25% activity was lost when the pH was lowered to 4.2.

Mixed-substrate experiments. *B. germanica* cholinesterase always hydrolysed mixtures of acetylcholine together with phenyl acetate, phenyl propionate, phenyl butyrate or triacetin at rates intermediate between the rates of the separate

Fig. 2. Variation of *B. germanica* cholinesterase activity with pH for different substrates: colorimetric assay. ○, 0.3 mM-Acetylcholine chloride; △, 0.6 mM-propionylcholine perchlorate; ●, 20 mM-phenyl acetate; ▲, 20 mM-phenyl propionate. Buffer solutions: pH 6-8, sodium phosphate; pH 8-10, sodium borate.

Table 2. *Esters hydrolysed by Blatella germanica cholinesterase*

Manometric assays in the presence of 0.1 M-sodium chloride.

Substrate	Highest experimental rate of hydrolysis		Inhibition by excess of substrate	V_{max} (approx.) (μ moles/15 min./ml.)	K_m (approx.) (mM)
	μ moles/15 min./ml.	Substrate concn. (mM)			
Acetylcholine	74	1	+	.	.
Propionylcholine	45	1	+	.	.
Butyrylcholine	3	1	+	.	.
Acetyl- β -methylcholine	56	26	+	.	.
N-Dimethylaminoethanol acetate	23	2	+	.	.
Phenyl acetate	91	18	-	110	3
Phenyl propionate	36	20	-	40	2
Phenyl butyrate	4	10	-	.	.
Triacetin	15	100	-	15	14

Table 3. *Heat inactivation*

Percentage activity remaining after heating for 3 min. Manometric assay in the presence of 0.1 M-NaCl.

		Temperature ...	40	43	47	50°
Substrate						
Test 1	1 mM-Acetylcholine chloride		84	71	35	35
			.	.	.	25
	10 mM-Phenyl acetate		84	70	35	29
Test 2			.	.	.	22
	1 mM-Acetylcholine chloride		.	100	37	24
	17 mM-Phenyl propionate		.	81	46	30
	67 mM-Triacetin		.	96	43	28

Table 4. *Mixed-substrate experiments*

Rates of hydrolysis were determined manometrically in the presence of 0.1 M-NaCl.

	Total rate of acid liberated (μ moles/15 min./ml. of enzyme)		
	With acetylcholine chloride		
	Alone	0.5 mm	20 mm
Non-choline esters			
16.5 mm-Phenyl acetate	39	30	12
16.6 mm-Phenyl propionate	22	25	15
7.1 mm-Phenyl butyrate	6	18	.
67 mm-Triacetin	8	21	9
None	.	24	5
Choline esters			
10 mm-Butyrylcholine chloride	1	2	.
9.7 mm-Benzoylcholine chloride	4	5	.
None	.	28	.

Table 5. *Inhibition by physostigmine*

Percentage activity remaining after incubation of enzyme and inhibitor for 30 min. before manometric assay in the presence of 0.1 M-NaCl.

Concn. of physostigmine (0.1 μ mm)	29	15	9	7.3
Substrate				
1 mm-Acetylcholine chloride	31	47	76	72
10 mm-Phenyl acetate	34	45	.	71
17 mm-Phenyl propionate	31	46	.	77
67 mm-Triacetin	36	52	70	59

Table 6. *Inhibition by paraoxon*

Percentage activity remaining after incubating the enzyme with inhibitor for 30 min. before manometric assay in the presence of 0.1 M-NaCl.

Substrate ...	1 mm-Acetyl- choline chloride	10 mm-Phenyl acetate
Concn. of paraoxon (μ mm)		
44	20	27
22	29	44
11	61	61
5.5	76	76
2.7	100	86

components. High concentrations of acetylcholine inhibited the hydrolysis of the other substrates (Table 4). Butyrylcholine and benzoylcholine, which are hydrolysed only very slowly, inhibited the hydrolysis of acetylcholine (Table 4).

Sensitivity to inhibitors. *B. germanica* cholinesterase is inhibited by low concentrations of paraoxon and physostigmine. The enzyme and inhibitor were incubated together for 30 min. in a medium containing 0.1 M-sodium chloride and 0.02 M-sodium bicarbonate. The substrate was then added and the residual activity assayed manometrically. Physostigmine inhibited the hydrolysis of acetyl-

Fig. 3. Variation of housefly-head-cholinesterase activity with acetylcholine chloride concentration and the effect of added sodium chloride: manometric assay. ●, NaHCO₃ alone; ▲, 0.1 M-NaCl.

choline, phenyl acetate, phenyl propionate and triacetin equally (Table 5).

Paraoxon inhibited the hydrolysis of acetylcholine and phenyl acetate to the same extent (Table 6).

Electrophoresis. In all tests with purified extracts of *B. germanica* cholinesterase only a single diffuse band of esterase activity was observed. Under a given set of conditions the position of the activity was the same irrespective of the substrate used to detect it. In all tests movement of activity was small and to the anode. At pH 6, with both sodium phosphate and sodium succinate buffer, movement was less than 2 cm. In phosphate buffer, pH 7.4, enzyme activity was located between the starting point and 3 cm. towards the anode. With veronal buffer, pH 8.6, esterase activity was detected in a band between 1 and 4 cm. from the point of application.

Variation of activity with substrate concentration for housefly-head cholinesterase

Assayed under conditions identical with those for *B. germanica* cholinesterase, the effect of acetylcholine concentration on the activity of housefly-head cholinesterase differed considerably (Fig. 3). Optimum substrate concentrations were observed manometrically in the presence of sodium bicarbonate alone as well as with 0.1M-sodium chloride present.

DISCUSSION

Substances that modify the activity of cholinesterases have been reported in a number of preparations, including some from insects (Augustinsson, 1948; Lord & Potter, 1953; Smallman & Wolfe, 1954; Wolfe & Smallman, 1956). In the final stage of the preparation this kind of substance may be considered to be virtually absent because the hydrolysis of acetylcholine is proportional to the amount of enzyme over a tenfold range.

No claim can be made that the enzyme is pure, but considerable amounts of inactive materials were removed in addition to quantities of other esterases which do not hydrolyse acetylcholine. Heat-inactivation (Table 3) and inhibition by physostigmine and paraoxon (Tables 5 and 6) suggest that the preparation contains only one major esterase component responsible for the hydrolysis of triacetin, phenyl acetate and phenyl propionate [known to be hydrolysed by cholinesterases (Whittaker, 1951; Mounter & Whittaker, 1953)] as well as for hydrolysis of choline esters. Mixed-substrate studies and electrophoresis on starch gel support this conclusion. In Table 1 the highest ratio of the hydrolysis rates of acetylcholine/triacetin and acetylcholine/phenyl acetate occur at stage 3b, which could be interpreted as suggesting that the enzyme is more pure with respect to other esterases at this stage than at later stages. However, at stage 3b there are considerable quantities of salts in the preparation, which affect the activity of the enzyme. These salts are absent from later stages of the preparation and probably account for the apparent decrease of purity in terms of esterase activity.

The effects of salt (potassium chloride, sodium chloride, sodium sulphate and magnesium chloride) on the relationship between the activity of the *B. germanica* esterase and acetylcholine concentration resemble those reported for other cholinesterases and cannot be attributed to a single ion (Myers, 1952; Chadwick, Lovell & Egner, 1953; Van der Meer, 1953; Wolfe & Smallman, 1956). However, inhibition by high (0.33M) concentrations of magnesium does distinguish the *B. ger-*

manica cholinesterase. Inhibition by high acetylcholine concentrations (Fig. 1) clearly places the esterase in the group of cholinesterases usually associated with nervous tissues (Augustinsson, 1957). The other choline esters hydrolysed (Table 2) also fit this classification. The butyryl and benzoyl esters of choline (Table 4) are hydrolysed only slowly and inhibit the hydrolysis of acetylcholine. In this, the enzyme from *B. germanica* resembles erythrocyte cholinesterase (Aldridge, 1950). The effects of pH on the activity of *B. germanica* cholinesterase are atypical (see review by Davies & Green, 1958), because no pH optimum was observed with acetylcholine (Fig. 2). Apart from a slight fall in activity at pH 10 these results resemble those obtained by Bergmann, Rimón & Segal (1958) for acetylthiocholine rather than acetylcholine. These workers interpreted their results as indicating that the electric-eel cholinesterase forms hydrogen bonds with acetylcholine but not the thio ester. If this is the true explanation it is difficult to explain why the *B. germanica* enzyme shows optimum activity to propionylcholine between pH 7 and 8 but not to acetylcholine. The effects of pH on the hydrolysis of phenyl acetate and phenyl propionate by *B. germanica* cholinesterase correspond to those reported by Bergman *et al.* (1958).

Comparison with esterases from other insect species is of interest but must be made with care because almost without exception crude preparations have been used. The cholinesterase from *B. germanica* (Table 2) differs from that in the housefly head, which attacks butyrylcholine at an appreciable rate and propionylcholine about as fast as acetylcholine (Metcalf, March & Maxon, 1955; K. Van Asperen, personal communication). In hydrolysing acetyl- β -methylcholine at a maximum rate about as great as for acetylcholine *B. germanica* cholinesterase resembles the enzyme from the bee brain rather than those from the housefly (Metcalf & March, 1950; Babers & Pratt, 1951; Metcalf *et al.* 1955), the melon fly and the oriental and Mediterranean fruit flies (Roan & Maeda, 1954), which are comparatively inactive towards acetyl- β -methylcholine, or, on the other hand, the enzymes from *Hydrophilus piceus* L. and *Periplaneta americana* L., which hydrolyse acetyl- β -methylcholine much faster than acetylcholine (Stegwee, 1951). Relative to acetylcholine, triacetin is hydrolysed much more slowly by the cockroach enzyme than by extracts of housefly brain, bee brain or eggs of *Bombyx mori* (Metcalf & March, 1950; Babers & Pratt, 1951; Staudenmayer, 1955; Van Asperen, 1958), but because these data refer to unpurified preparations no accurate comparisons can be made.

The properties, including the range of esters hydrolysed, of *B. germanica* cholinesterase resemble

those from a variety of sources including insects, but it is clearly distinguished by the low values of the optimum substrate concentrations for choline esters (Augustinsson, 1948; Augustinsson, 1949; Whittaker, 1953; Smallman & Wolfe, 1954; Metcalf *et al.* 1955; Lundin, 1959). The optimum value of 26 mM for acetyl- β -methylcholine is among the lowest values reported and the values for acetyl- and propionyl-choline are at least one-tenth of those usually reported for cholinesterases. With acetylcholine the fall in activity with decreasing substrate concentration usual for cholinesterases could not be observed. There is no reason to suppose that this is an artifact of our manometric techniques, because the result was confirmed by the electrometric method (Fig. 1). Further, the data obtained with housefly heads (Fig. 3) under the same conditions of manometric assay as for the cockroach enzyme agree closely with those obtained by other workers (Metcalf & March, 1950; Babers & Pratt, 1951; Van Asperen, 1958).

Most of the properties of the cholinesterase from *B. germanica* are typical of cholinesterases associated with nervous tissue and it is likely that the enzyme represents a preparation of the cholinesterase that has been demonstrated histochemically by F. Molloy (personal communication) in the nervous system of *B. germanica*. The unusual properties of the enzyme may represent a species difference which is reflected in the relative susceptibility of different species to organophosphorus insecticides. The very high affinity of the enzyme for acetylcholine implies that it would be protected from inhibition (by organophosphorus compounds) by much lower substrate concentrations than is usual. But conditions *in vivo* may be such that the amount of acetylcholine present is not sufficient to protect the enzyme, and further investigation of the cockroach nervous system may show that the unusual properties of the enzyme are necessary for it to function adequately in the milieu of the nervous tissues.

SUMMARY

1. A purified preparation of cholinesterase was obtained from *Blatella germanica*. The overall purification was 40-fold and the yield 32%.
2. The enzyme has a high affinity for acetylcholine and is inhibited by high concentrations of this and analogous esters.
3. Various salts activate the enzyme at high concentrations of acetylcholine and under some circumstances inhibit at low substrate concentrations.
4. The enzyme hydrolyses phenyl and triglyceryl esters as well as choline esters. Esters of acetic acid and propionic acid are hydrolysed much more rapidly than those of butyric acid.
5. The enzyme showed optimum activity to

acetylcholine and the acetyl and propionyl esters of phenol over a wide pH range.

6. The properties of the enzyme are characteristic of the type of cholinesterases frequently associated with nervous tissues and are compared with those reported for cholinesterases from insect and other sources.

I have to thank Mr S. R. B. Solly for his assistance in carrying out some of the experiments described in this paper.

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