Comparative Enzymology of Certain Insect Acetylsterases in Relation to Poisoning by Organophosphorus Insecticides

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Certain acetylsterases, particularly cholinesterases (ChE), have been repeatedly implicated in the poisoning of animals by organophosphate esters. Extensive comparative enzymology studies on these enzymes from vertebrate tissues have been made by Augustinsson (1948) and others. However, studies on the organophosphate-sensitive esterases of insects have been so limited and so varied in methods as to make critical comparisons impossible. The further development of selectively toxic organophosphorus insecticides may be largely dependent on the differences of specificity of acetylsterases among the insects (Metcalf & March, 1949; Beard, 1951) and between insects and mammals (Metcalf & March, 1950; Johnson, Fletcher, Nolan & Cassaday, 1952).

Accordingly, a survey was made to determine the properties of acetylsterases in different insects and in different organ systems of one insect. These studies were based on substrate specificity, activity on mixed substrates, and selectivity of three esterase inhibitors. Inhibition studies *in vivo* were made with poisoned insects to evaluate the physiological importance of the inhibited esterases. A preliminary report on this work has been presented (Casida, 1954).

METHODS AND MATERIALS

Insects

The insects used were: American cockroach, *Periplaneta americana* (L.); mite, *Acarus siro* L.; German cockroach, *Blattella germanica* (L.); leafhopper, *Paraphlepsius irroratus* (Say); aphid, *Macrosiphum pisi* (Kltb.); whitefly, *Trialeurodes vaporariorum* (Westw.); scale insect, *Toumeyella numismaticum* (P. & M.); milkweed bug, *Oncopeltus fasciatus* (Dall.); armyworm, *Prodenia eridania* (Cram.); waxmoth, *Galleria mellonella* (L.); carpet beetle, *Attagenus piceus* (Oliv.); bean beetle, *Epilachna varivestis* Muls.; granary weevil, *Sitophilus granarius* (L.); housefly, *Musca domestica* L.; and honey-bee, *Apis mellifera* L. All insects were from standard laboratory cultures except for the scales which were field collected. Insects were not differentiated as to sex unless specifically stated. Living insects were anaesthetized with CO₂ before dissection or homogenization. All organ systems were rapidly dissected free from other tissues under cooled bicarbonate buffer, quick-frozen and stored frozen (–10°) until assay: this was performed within 1 hr. of dissection.

Enzyme determinations

Tissue preparation. Insect tissues were rapidly weighed and then homogenized in an ice-cold Potter & Elvehjem (1936) homogenizer containing 4 ml. of the bicarbonate buffer. With the granary weevil a mortar and pestle were used instead of the homogenizer. The tissue homogenate was then filtered through a double thickness of cheesecloth and made up to 20 ml. with buffer. Samples (1.0 ml.) were assayed for esterase activity and a portion of the same homogenate was analysed for total N using a modified Nessler's solution after digestion with 5N-H₂SO₄ and a selenium catalyst (Johnson, 1941; Umbreit, Burris & Stauffer, 1949).

Manometric assay. Esterase activity was determined indirectly by CO₂ evolution from a bicarbonate buffer (0.025M-NaHCO₃, 0.15M-NaCl, and 0.040M-MgCl₂) in an atmosphere of 5% CO₂ and 95% N₂ (final pH 7.45) at 38° in 7 ml. Warburg flasks. The substrate was added to the side arm of the flask, and the inhibitor followed by the enzyme (tissue homogenate) to the main compartment. The enzyme was incubated with the flask contents for 30 min. at 28° and 30 min. at 38° before tipping in the substrate.

Substrates. The esters, in 0.4 ml. buffer, were tipped in from the side arm. The chlorides of acetylcholine (ACh), acetyl-β-methylcholine (MeCh) and benzoylcholine (BzCh) were used at a final concentration of 0.03M. Triacetin (TA) was used at 0.01M. The less soluble o-nitrophenyl acetate (NPA) was prepared by blowing from a pipette a 20-times concentrated stock solution in 95% ethanol under the surface of warm buffer solution to yield a uniform fine suspension which was 4.75% with respect to ethanol. This was then added to the flask to yield a final concentration of 0.003M. Mixed substrates were prepared by adding 0.2 ml. of a double concentration of each substrate to the side arm of the flask. For tissue blanks the substrate in the side arm was replaced by buffer. For substrate blanks the tissue sample was replaced by buffer.

Inhibitors used in vitro. Choline chloride was used at 0.10M final concentration. Eserine salicylate and tetraethyl pyrophosphate (TEPP) were added from stock CHCl₃ solutions to the bottom of the flask, the CHCl₃ removed with a stream of air, and the enzyme solution then immediately added to minimize losses of inhibitor by hydrolysis or volatilization. The TEPP was used at a final concentration of 10^{–7}M, and the eserine at 10^{–6}M. A 1 hr. incubation period before addition of the substrate was allowed for the combination of the enzyme with the inhibitor.

Organophosphate esters for poisoning in vivo. The designations for the phosphate insecticides used in Table 6 are as follows: E600, diethyl p-nitrophenyl phosphate; DFP,

diisopropyl phosphofluoridate; 13/59, dimethyl 2:2-trichloro-1-hydroxyethyl phosphate; 2046, dimethyl 1-carbomethoxy-1-propen-2-yl phosphate. The 50% ChE inhibition levels for purified bovine acetylcholinesterase (AChE) were: for E 600, 2×10^{-8} M; for DFP, 10^{-8} M; for 13/59, 5×10^{-6} M; and for 2046, 8×10^{-8} M. Compounds 13/59 and 2046 were purified by column chromatography on silica gel until only one peak appeared, which did not change in antiChE activity on repeated passage through the column.

Presentation of results. Results are expressed as μ l. CO₂ evolved in the first 30 min. after adding the substrate. In all cases, the results were corrected for non-enzymic ester hydrolysis and gas exchange from the tissue homogenates in the absence of the substrate. All values represent the

average of 3-6 determinations depending on the variability of the results. The percentage activity of mixed substrates was obtained by dividing the CO₂ evolution value from the mixed-substrate determinations by the sum of the values for each of the substrates separately and multiplying by 100.

RESULTS

Substrate specificity

Table 1 shows the substrate specificity of certain esterases from various insects. The activity of the various organ systems of the American cockroach against the five esters is shown in Table 2. The insect preparations with the highest relative activities for

Table 1. *Substrate specificity of certain esterases from various insects*

Tissues from various species were homogenized in bicarbonate buffer and a 1.0 ml. sample was added to each flask to yield the indicated amount of tissue per ml. Substrates (0.4 ml.) were tipped in from the side arm to give a final molar concentration of 0.03 for ACh, BzCh and MeCh, 0.01 for TA and 0.003 for NPA. Mixed substrates were prepared by adding 0.2 ml. of a double concentration of each substrate to the side arm of the flask. The enzyme was incubated with the flask contents (1.6 ml.) for 30 min. at 28° and 30 min. at 38° before tipping in the substrate. The CO₂ evolution in 95% N₂ and 5% CO₂ was followed manometrically and corrected for tissue and substrate blanks. A known sample of each insect preparation was analysed for total N by nesslerization.

Order and common name*	Tissue (mg./ml.)	Total N (μ g./ml.)	Substrate specificity (μ l. CO ₂ /30 min.)					% activity of mixed substrates	
			ACh	BzCh	MeCh	TA	NPA	ACh + TA	ACh + NPA
Acarina									
Mite	Whole adults 7.5	220	13	6	8	28	151	115	116
Orthoptera									
German cockroach	Adult heads 1.88	140	40	17	105	175	147	98	114
Homoptera									
Leafhopper	Whole adults 13.7	120	38	3	46	24	144	54	53
Aphid	Whole adults 122	2200	37	17	3	309	145	102	105
Whitefly	Whole adults 1.36	43	41	6	7	8	69	94	65
Scale insect	Whole adult females 50	980	27	3	21	69	132	100	131
Hemiptera									
Milkweed bug	Adult heads 1.88	100	17	5	36	26	122	93	95
Lepidoptera									
Armyworm	Larval heads 1.98	280	10	4	12	71	112	107	120
Waxmoth	Larval heads 2.50	260	9	4	8	14	104	91	109
Waxmoth	Adult heads 2.50	98	18	6	52	12	141	96	55
Coleoptera									
Carpet beetle	Whole larvae 25	580	26	38	32	69	137	80	129
Bean beetle	Adult heads 2.1	120	17	10	21	15	59	71	89
Granary weevil	Whole adults 25	490	15	10	14	37	160	94	118
Diptera									
Housefly	Eggs 25	980	10	6	2	16	121	77	129
Housefly	Larvae 32	600	32	5	9	45	128	87	114
Housefly	Pupae 29	880	93	8	37	76	164	69	84
Housefly	Adult heads 1.25	57	130	13	48	78	178	61	48
Hymenoptera									
Honey-bee	Adult heads 1.5	150	67	15	69	33	131	39	53
Purified bovine AChE†	0.150 μ g.	18	113	10	51	11	165	94	47

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† Winthrop-Stearns Inc., New York. Purified bovine cholinesterase plus stabilizer.

hydrolysing particular esters were as follows: ACh, adult housefly heads, whole whiteflies and the malpighian tubules and muscles of the cockroach; MeCh, whole leafhoppers, cockroach ventral nerve cords, and the heads of German cockroaches, milkweed bugs, adult waxmoths and honey-bees; BzCh, whole aphids, carpet-beetle larvae and several cockroach organs; TA, whole aphids, scale insects and carpet-beetle larvae, the heads from adult German cockroaches and larval armyworms, and the cockroach gastric caecae; NPA, whole mites, whiteflies, adult granary weevils, housefly eggs, and the foregut, gastric caecae, fat body and nerve cord of the American cockroach.

substrates are hydrolysed by the same sites on one enzyme, then inhibitors would be expected to reduce the hydrolysis of both substrates by the same extent; however, the degree of inhibition of two enzymes acting on two esters would be different.

From the results of these studies, there appears to be only one enzyme responsible for the hydrolysis of both ACh and NPA by whole leafhoppers and whiteflies, heads of adult houseflies and honey-bees and purified bovine AChE. Only the inhibition of esterases by choline *in vitro* differentiated the enzymes attacking ACh and NPA in adult waxmoth heads, and in the various parts of the American cockroach including the nerve cord. Differentiation

Table 2. *Substrate specificity of esterases from various organs of the American cockroach*

Tissues were homogenized in bicarbonate buffer and assayed under conditions identical with those shown for Table 1.

Organ system	Tissue (mg./ml.)	Total N (μ g./ml.)	Substrate specificity (μ l. CO ₂ /30 min.)					% activity of mixed substrate	
			ACh	BzCh	MeCh	TA	NPA	ACh + TA	ACh + NPA
Whole insect	29	410	4	3	10	21	157	67	97
Blood	1.5	23	13	4	2	2	24	55	65
Cuticle	2.4	33	9	7	4	6	34	92	87
Digestive system:									
Foregut	4.1	74	11	4	5	11	140	87	95
Midgut	1.3	18	12	8	3	11	93	78	95
Hindgut	5.6	55	14	6	3	8	78	66	79
Gastric caecae	0.65	18	10	8	9	17	140	82	94
Salivary glands	1.0	28	10	8	5	2	33	60	74
Fat body	5.7	190	12	5	7	9	171	82	89
Malpighian tubules	0.32	16	17	7	3	7	39	50	65
Muscles	1.3	57	30	3	10	3	71	41	49
Nerve cord	1.7	47	28	7	68	13	181	71	50
Reproductive glands:									
Female	3.3	82	11	7	2	3	70	113	98
Male	1.3	39	10	6	5	2	45	80	72
Tracheae	0.37	18	10	5	5	2	37	66	60

Mixed substrates and inhibition studies in vitro

As evidence of the homogeneity of enzymes hydrolysing ACh, TA and NPA from the different insect sources, studies were made with mixed substrates (Tables 1 and 2) and with three inhibitors (Tables 3 and 4). Where two or more enzymes hydrolyse two different substrates, a preparation containing both enzymes should display a total activity equal to the sum of the separate activities, whereas a single enzyme hydrolysing both substrates should yield less than an additive activity on mixed substrates, unless both substrate concentrations are considerably below the K_s (Augustins-son, 1948). The substrate concentrations were above the K_s with the housefly enzymes and probably also with the majority of the other enzymes. Where two

of the ACh and NPA esterases in cockroach nerve is of particular interest, since Hopf (1954) considered the hydrolysis of ACh and NPA to be due to the same enzyme in the locust nerve cord. Only one enzyme appeared to be involved in ACh and TA hydrolysis in the whole leafhopper, the heads of adult houseflies and honey-bees, and the malpighian tubules of the cockroach. The triacetinase activity of many cockroach organs was so low that the efficiency of the inhibitors could not be evaluated and so no values are given for these cases in Table 4.

Some of the more sensitive enzymes to all of the inhibitors were found in whole leafhoppers and whiteflies and the heads of houseflies and honey-bees (Table 3). The triacetinase of the cockroach nervous system was very sensitive to eserine and TEPP. Enzymes of a very low degree of sensitivity to

inhibitors were found in whole aphids, scales and mites. The poor efficiency of TEPP in inhibiting the esterases in certain of the crude insect preparations might have been due to the presence of a phosphoryl phosphatase enzyme which destroyed a portion of the inhibitor before it could react with the acetyl-esterase (Augustinsson & Heimbürger, 1954; Mounter, 1954). The enzymes from the larval heads of waxmoths and armyworms were insensitive to choline, as were those from the adult heads of the milkweed bug.

sensitive to inhibition by all three inhibitors. Choline and eserine inhibited to a large extent where one enzyme appeared to hydrolyse both the ACh and non-choline esters. This indicated the presence on those enzymes showing this mixed substrate inhibition of two active sites for combination with the acetyl ester, since choline is known to compete for the anionic site and eserine to block both the anionic and esteratic sites of cholinesterase (Myers, 1951). It is interesting to note that eserine at 10^{-6} M inhibited NPA esterase in those insect tissues

Table 3. *Efficiency of eserine, tetraethyl pyrophosphate and choline as inhibitors of certain insect esterases in vitro*

Assay conditions and tissue levels for various species were identical with those shown in Table 1. Enzyme and inhibitor were incubated together for 30 min. at 28° and 30 min. at 38° before assay. Final inhibitor concentrations were 10^{-7} M for TEPP, 10^{-6} M for eserine and 10^{-4} M for choline.

Order and common name	% inhibition on substrate indicated								
	Eserine			TEPP			Choline		
	ACh	TA	NPA	ACh	TA	NPA	ACh	TA	NPA
Acarina									
Mite	30	.5	5	9	49	47	6	3	+1
Orthoptera									
German cockroach	83	74	16	76	97	56	12	10	18
Homoptera									
Leafhopper	65	72	59	86	88	64	80	64	65
Aphid	17	22	+73	1	+5	+8	12	10	9
Whitefly	71	58	55	45	45	41	37	24	42
Scale insect	43	11	5	24	31	6	25	5	2
Hemiptera									
Milkweed bug	46	39	23	58	65	31	+5	+4	15
Lepidoptera									
Armyworm	19	17	11	23	64	25	+5	0	6
Waxmoth larvae	21	27	11	+9	29	22	+7	0	8
Waxmoth adult	53	51	58	10	39	21	+45	43	55
Coleoptera									
Carpet beetle	53	61	+1	47	66	28	44	16	7
Bean beetle	31	52	18	19	66	30	30	62	+2
Granary weevil	51	20	10	19	52	30	+28	7	11
Diptera									
Housefly egg	83	73	3	80	64	4	53	63	0
Housefly larvae	70	35	12	69	29	18	38	30	5
Housefly pupae	82	67	27	88	74	40	57	72	18
Housefly adult	83	95	75	93	96	81	67	82	50
Hymenoptera									
Honey-bee	66	84	46	82	94	61	73	92	46
Purified bovine AChE	42	47	26	74	84	65	55	93	60

Insect sources in Tables 1-4 were divided for further consideration into those showing reduced (less than 75 % of the activity on the mixed as compared with the separate substrates), intermediate (75-100 %) and those showing an additive effect (100 %) with mixed substrates (Table 5). Enzyme sources showing reduced activity on either ACh-TA or ACh-NPA mixtures were generally more

showing reduced activity on ACh and NPA but did not generally inhibit the NPA esterase where an additive effect occurred (Table 5). It can also be seen from Tables 2 and 4 that those cockroach tissues displaying the greatest reduced activity on ACh and NPA (nerve cord, muscles and tracheae) were also those in which the eserine gave the greatest inhibition of the NPA esterase.

Table 4. *Efficiency of eserine, tetraethyl pyrophosphate and choline as inhibitors in vitro of certain esterases from various organs of the American cockroach*

Assay conditions and tissue levels for different organs were identical with those shown in Table 2. Enzyme and inhibitor were incubated together for 30 min. at 28° and 30 min. at 38° before assay. Final inhibitor concentrations were 10^{-7} M for TEPP, 10^{-6} M for eserine and 10^{-1} M for choline.

Organ system	% inhibition on substrate indicated								
	Eserine			TEPP			Choline		
	ACh	TA	NPA	ACh	TA	NPA	ACh	TA	NPA
Whole insect	72	31	12	81	96	75	32	25	9
Blood	76	—	13	77	—	29	56	—	0
Cuticle	18	97	31	40	90	41	7	80	17
Digestive system:									
Foregut	38	58	6	28	100	63	57	17	23
Midgut	71	49	5	34	89	61	26	35	20
Hindgut	60	44	7	35	83	46	39	30	8
Gastric caecae	20	42	13	35	78	64	6	40	19
Salivary glands	68	—	21	74	—	33	62	—	15
Fat body	69	70	17	71	76	84	41	34	4
Malpighian tubules	44	38	24	77	94	61	34	45	10
Muscles	80	—	38	74	—	39	75	—	24
Nerve cord	53	100	45	87	100	80	22	95	72
Reproductive glands:									
Female	35	—	21	59	—	28	21	—	14
Male	39	—	33	65	—	43	39	—	22
Tracheae	36	—	52	67	—	44	41	—	20

Table 5. *Inter-relationship of inhibitor sensitivity and activity on mixed substrates with insect esterases hydrolysing ACh and NPA*

Results are based on those of Tables 1 and 3.

% activity of mixed substrates (ACh + NPA)	No. of insect tissues	Inhibition (%)					
		Eserine		TEPP		Choline	
		ACh	NPA	ACh	NPA	ACh	NPA
<75	6	63	53	65	56	45	53
75-100	3	53	23	55	34	27	10
>100	10	47	0	34	23	15	6.5

Table 6. *Properties of esterases hydrolysing ACh and NPA from different developmental stages of the housefly*

Results are based on those of Tables 1 and 3.

	Egg	Larva	Pupa	Adult head
Activity (μ mole/mg. total N/hr.)				
ACh	0.91	4.8	9.4	203
NPA	11.0	19.2	16.6	278
Activity on mixed substrates (%)				
ACh + NPA	129	114	84	48
Inhibition <i>in vitro</i> (%)				
ACh-eserine	83	70	82	83
ACh-TEPP	80	69	88	93
ACh-choline	53	38	57	67
NPA-eserine	3	12	27	75
NPA-TEPP	4	18	40	81
NPA-choline	0	5	18	50

A shift in the acetylcholinesterase pattern during the stages of development was evident in the housefly (Table 6). Two enzymes appeared to hydrolyse ACh and NPA in the egg, but a progressive shift occurred during development and in the adult head only one enzyme was evident for hydrolysis of the two substrates. The ACh esterase activity increased greatly between the egg and larval stage and continued to increase in activity more rapidly than the NPA esterase activity as the fly matured. The sensitivity of the fly NPA-esterase enzymes to eserine, TEPP and choline progressively increased during development, so that in the head of an adult fly the sensitivity of the ACh and NPA esterases were almost identical, indicating the presence of but one enzyme.

Properties of aphid esterases

The properties of pea aphid esterases were studied in more detail because of their apparent differences from other insect esterases and the selective toxicity for aphids that has been reported with certain

activity towards NPA. Fractionation with ammonium sulphate precipitated the NPA esterase more readily than the ACh-hydrolysing enzyme. Tetraisopropyl pyrophosphate was more than 100 times as effective in blocking the ACh than the NPA esterase. Activity/pS curves (Fig. 1) indicate an excess substrate inhibition with BzCh but not with ACh while no activity was displayed towards MeCh. This is an analogous situation to that occurring with horse-serum esterases (Augustinsson, 1948). An apparent excess substrate inhibition occurred with the NPA esterase. A partial explanation may be that the physical properties of the system were altered by the large amount of insoluble NPA which appeared as oily droplets at the higher concentrations. The properties of the aphid cholinesterase were more similar to those of 'pseudocholinesterases' than with most other insects.

Relative activity of insect enzymes hydrolysing choline esters

Fig. 1. Hydrolysis of ACh, BzCh, MeCh, TA and NPA by homogenates of whole aphids. The esterase activity, expressed as $\mu\text{l. CO}_2/30 \text{ min.}$, is plotted against $-\log$ (substrate concentration) (pS). Aphids used at 85 mg./ml.

organophosphate insecticides (Ludvik & Decker, 1947, 1951; Bottger & Yerington, 1952; Hopf, 1954). Whole aphid homogenates were found to hydrolyse ACh, BzCh, TA, NPA, ethyl *n*-butyrate, Na β -glycerophosphate and tyrosine ethyl ester but not MeCh. No evidence was obtained, by adding fresh or boiled aphid juice to a fly ChE source, that the low activity of these preparations is due to interference by an antiChE agent. Such natural inhibitors appear partially to explain the low ChE activity of certain insect preparations (Lord & Potter, 1953). Results with mixed substrates and inhibitors indicated that the enzymes hydrolysing ACh, NPA and TA were distinct in pea aphids. An ice-cold ethanol or acetone fractionation inactivated the ACh-hydrolysing enzyme without affecting the

Certain insect cholinesterases, particularly those from housefly brains, have been reported to be extremely active (Metcalf & March, 1950), comparing favourably in activity with the ChE in the electric organs of *Torpedo* sp. and *Electrophorus electricus* (Nachmansohn & Rothenberg, 1945). This study confirmed the very high concentration or activity of ChE in certain insect parts and the reproducibility of the acetylcholinesterase activity values obtained from any given insect source. The quantitative results obtained here can be compared with the results of other workers for the ACh-hydrolysing enzymes; these enzymes hydrolysed 1.01–2.05 $\mu\text{moles ACh/mg./hr.}$ with bee brain, 11 with housefly brain, and 0.7–0.84 with American cockroach ventral nerve cord (Richards & Cutkomp, 1945; Chadwick & Hill, 1947; Metcalf & March, 1950; Chamberlin & Hoskins, 1951). Corresponding values from this study were 0.74 $\mu\text{mole ACh/mg./hr.}$ for cockroach nerve cord, 22 for fly brain and 0.80 for bee brain.

Table 7 gives the specific activity of the enzymes hydrolysing choline esters in various insect parts when expressed on a total nitrogen basis. The most active insect enzyme assayed was the housefly head on ACh. However, when a whole housefly was compared with a whole whitefly, the whole whitefly was far more active on ACh than the housefly. The highest specific activity on MeCh was in the cockroach ventral nerve cord and on BzCh in the malpighian tubules. The malpighian tubules have previously been known to be very active metabolically and to contain high concentrations of certain other enzymes (Patton, 1953). The relatively high specific activity of cockroach blood observed

here is of particular interest since earlier workers reported little or no ChE activity in insect compared with mammalian blood.

Inhibition in vivo of esterase activity of various cockroach organs by four organophosphate insecticides

Enzymes with varying properties hydrolyse ACh and NPA in the various organ systems of the cockroach (Tables 2 and 4). American cockroaches display different symptoms of poisoning with different organophosphates (Chamberlin & Hoskins, 1951). American cockroaches were poisoned with different phosphate esters (2–500 $\mu\text{g./g.}$) to reach about the same stage of poisoning 1 hr. after treatment. Various organ systems were then assayed for esterase inhibition *in vivo*. Compound E 600 has been previously shown to be present in large amounts in the blood and foregut with very little in the nerve (Fernando, Roan & Kearns, 1950); it has

been found in this laboratory that 2046 accumulates in the hindgut with fairly large amounts in the nervous tissue. The values for the inhibition of blood esterase shown in Table 6 are at best an approximation, because clotting of the blood on bleeding the cockroach necessitated homogenization and blood clotting *in vivo* occurred with E 600 as had been previously reported (Roan, Fernando & Kearns, 1950) but not as much with the other materials.

Table 8 shows the results of inhibition *in vivo*. Compound 2046 appeared to be the most specific esterase inhibitor *in vivo* affecting no esterase more than 40 % except the nerve acetylcholinesterase which it inhibited to a greater degree than did any of the other phosphates on any of the esterases at this stage of poisoning. DFP and 13/59 proved quite similar in their inhibition patterns even though the phosphofluoridate was used at 50 $\mu\text{g./g.}$ while the trichlorohydroxyethyl phosphate was used at

Table 7. *Relative activity of different insect parts in hydrolysing esters of choline*

Insect	Part	$\mu\text{moles/mg. total N/hr.}$		
		ACh	BzCh	MeCh
American cockroach	Blood	50	16	8
	Malpighian tubules	95	39	16
	Ventral nerve cord	54	13	131
Housefly	Adult heads	203	20	75
Waxmoth	Adult heads	16	5	46
Whitefly	Whole adults	86	13	15
Purified bovine AChE		561	50	253

Table 8. *Effect of poisoning with four organophosphate esters on the esterase activity of various organ systems from the cockroach*

Dissected tissues from three adult male cockroaches were homogenized and a sample equivalent to the tissue from one cockroach was added to each flask. All percentage inhibition figures represent the average of four to nine comparisons of control with treated cockroaches and are based on 30 min. determinations at 38° with 0.03M ACh and 0.003M NPA.

Organ system*	mg. tissue/flask	Substrate	% esterase inhibition <i>in vivo</i> in cockroaches poisoned with†			
			E 600	DFP	13/59	2046
Blood	6.5	NPA	55	18	9	+36
Fat body	20.6	ACh	56	39	28	22
		NPA	29	81	54	26
Foregut	17.7	NPA	48	60	56	+9
Muscle	53.6	ACh	43	7	7	29
		NPA	42	36	30	11
Nerve	5.0	ACh	67	77	77	85
		NPA	49	73	71	37
Salivary glands	3.7	NPA	60	2	35	30
Tracheae	1.8	NPA	50	33	11	33

* No significant inhibition occurred *in vivo* from any of the phosphates with the following organ-substrate combinations: ACh and blood, cuticle, foregut, malpighian tubules, salivary glands and tracheae; NPA and malpighian tubules.

† Organophosphates were applied in the following amounts per cockroach to the pronotum in 0.05 ml. acetone; E 600, 2 $\mu\text{g.}$; DFP, 50 $\mu\text{g.}$; 13/59, 500 $\mu\text{g.}$; and 2046, 50 $\mu\text{g.}$ These amounts caused all cockroaches to display agitation at about 10 min. and be prostrate at about 30 min., while still exhibiting tremors of the appendages after 1 hr. All poisoned cockroaches were dissected 1 hr. after administration of the phosphate ester.

500 $\mu\text{g./g.}$ Compound E 600 was the most toxic and the most general esterase inhibitor of the four *in vivo*.

The cockroaches were poisoned with different types of phosphate insecticides and in greatly varying amounts, but were all treated to yield the same apparent stage of poisoning. The only enzyme which was consistently inhibited to a large extent (67–85 %) at this stage of poisoning was the acetylcholinesterase of the nerve cord.

DISCUSSION

The presence of cholinesterase has been established in enough insects for its distribution to be assumed to be universal. Earlier studies had demonstrated ChE activity in the following arthropod orders: Orthoptera, Homoptera, Hemiptera, Coleoptera, Diptera, Hymenoptera, Scorpionida and Aranae (Gautrelet, 1938; Corteggiani & Serfaty, 1939; Mikalonis & Brown, 1941; Truhaut & Vincent, 1947, 1948; Duspiva, 1951; O'Brien, 1953; O'Brien & Spencer, 1953; Roan & Maeda, 1953; Casida, Chapman, Stahmann & Allen, 1954). ChE occurs primarily in the nervous system, but was also found in large amounts in the wing muscles of a grasshopper (Means, 1942) and in the leg muscles and intestine of a predacious diving beetle (Stegwee, 1951). In the American cockroach the enzyme is approximately twice as concentrated in the central nervous system as in larger peripheral nerves (Richards & Cutkomp, 1945) and it can readily diffuse out of ventral nerve cord preparations from certain insects (Mikalonis & Brown, 1941). Earlier reports of almost no activity in insect blood (Bacq, 1935, 1946; Corteggiani & Serfaty, 1939; Stegwee, 1950, 1951) compared with high activity in vertebrate blood have been disproven by work on blood of the rice-stem borer (Yushima & Chino, 1953) and this study with the cockroach. Other cockroach tissues shown to be high in ChE content are the malpighian tubules, salivary glands, gastric caecae, midgut, tracheae and muscles. In the flour beetle (O'Brien, 1953) and in the waxmoth and housefly the ChE activity is greater for the adult than for earlier developmental stages. The ChE activity in the adult honey-bee is dependent on age, showing an increase during the first week after emergence and then decreasing until very old age (Rockstein, 1950). In the embryological development of grasshopper eggs the ChE first appears in the prediapause stage and its increase of activity thereafter is correlated with the development of the nervous system (Tahmisian, 1943). Although lepidoptera eggs (Lord & Potter, 1951) and fly eggs are only slightly active on ACh, they are very active on substituted phenyl acetates. A somewhat analogous situation occurs with sea-urchin eggs

(Augustinsson, 1948). At least in the case of the housefly this ester hydrolysis is apparently due to two enzymes in the egg and early stages and only one in the adult.

Diverse substrate specificity patterns appear among the insects. Excess substrate inhibition with ACh but not with MeCh or BzCh occurred with fly, bee and cockroach heads (Metcalf & March, 1950; Babers & Pratt, 1950, 1951), whereas the nervous system of a water-scavenger beetle displayed excess substrate inhibition with both ACh and MeCh (Stegwee, 1950). Aphids, on the other hand, are not inhibited by excess ACh but are by excess BzCh in a manner similar to that reported by Augustinsson (1948) for a purified horse-serum preparation. Mixed substrate studies have shown that the same enzyme splits ACh and MeCh in housefly heads, while two are involved in bee heads (Babers & Pratt, 1951). Housefly heads will attack a great diversity of aliphatic, choline and phenyl esters (Metcalf, March & Spratt, 1953). The results presented here indicate that ACh, TA and NPA may be hydrolysed by one, two or three enzymes depending on the insect organ system and its developmental stage. In certain cases the ACh and NPA esterases can be separated by ethanol (O'Brien, 1953) or acetone fractionation and by inhibitor and mixed-substrate studies. A remarkable diversity occurs in the ability of insect esterases to hydrolyse choline and aliphatic esters, and in their response to mixed substrates and inhibitors. The insect cholinesterases appear to be a group of related enzymes with widely varying properties among themselves and in comparison with mammalian cholinesterases. These cholinesterase specificity differences appear to be great enough to suggest the feasibility of synthesis of organophosphate anticholinesterases showing high selective toxicity, as has previously been suggested by several workers (Metcalf & March, 1949, 1950; Johnson *et al.* 1952).

Whether or not the blockage of nerve conduction through ChE inhibition is the primary mechanism of organophosphate insecticide poisoning has been a subject of considerable controversy. Much of this has been due to the observed resistance of the insect nerve to conduction blockage by perfusing ions (Tobias, 1948*a, b*; Roeder, 1948*b*; Krupp, Lendle & Stapenhorst, 1952) and choline esters (Tobias, Kollros & Savit, 1946; Hopf, 1952) which are introduced into the body cavity. Careful dissection and micro-injection experiments have shown this resistance to be due to the lipoidal membranes surrounding the insect nerves (Richards, 1943) which are relatively impermeable to ionized materials (Hoyle, 1952, 1953). The threshold potency of several anticholinesterases, including DFP and TEPP, in inactivating cockroach nerve

cord ChE is closely correlated with their efficiency in producing synaptic facilitation and block (Roeder, 1948a). The degree of correlation between ChE inhibition *in vitro* for different organophosphates and insecticidal toxicity is high (Metcalf & March, 1949; Chamberlin & Hoskins, 1951) but not absolute (Chefurka, 1953). Discrepancies in this correlation may be due largely to highly active impurities in the organophosphates giving erroneous inhibition values *in vitro* (Aldridge & Barnes, 1952); to metabolic conversion of the organophosphates into more active anticholinesterases in the insect nerve (O'Brien & Spencer, 1953; Metcalf *et al.* 1953) or other tissues; to the detoxication of certain organophosphates within insects based on the work in this laboratory; and to the apparent inability of certain organophosphates to penetrate into the nerve (O'Brien & Spencer, 1953) to inhibit the internal cholinesterases of the nerve to the degree necessary for conduction blockage (Wilson & Cohen, 1953). The inability to demonstrate cholinesterase activity in certain adult insects may be due either to ethanol inactivation of the enzyme during preparation (O'Brien, 1953) or to natural ChE inhibitors which are released when the insect tissue is homogenized (Lord & Potter, 1953). The suggested implication of a phenylesterase in the primary mechanism of organophosphate poisoning of insects (Lord & Potter, 1950, 1951, 1953; Hopf, 1952, 1954) does not appear to be applicable for the adult American cockroach, since the ACh esterase of the nerve was the only esterase consistently inhibited over 67% *in vivo* in cockroaches at the prostrate stage of poisoning from various organophosphates. However, before the nervous system is developed in the early developmental stages of various insects (Tahmisian, 1943; Lord & Potter, 1953) or other animals (Augustinsson, 1948; Karczmar & Koppanyi, 1953) and the cholinesterase system is not physiologically important, the inhibition of enzymes capable of hydrolysing other acetate esters may indeed be the primary mechanism of poisoning. The hypothesis most consistent with the divergent experimental data is that in those cases where the cholinesterase systems of insects are sufficiently well developed to be of physiological importance, the inhibition of nerve cholinesterase and subsequent blockage of conduction due to acetylcholine accumulation is the primary mechanism of insecticidal action of organophosphates.

SUMMARY

1. A survey was made of the enzymes hydrolysing acetylcholine (ACh), benzoylcholine (BzCh), acetyl- β -methylcholine (MeCh), triacetin (TA) and *o*-nitrophenyl acetate (NPA) in various insects and the organ systems of the American cockroach.

Substrate specificity patterns were found to vary greatly among different insects and among the organs of one insect.

2. The properties of these esterases from the various insect sources were studied by determining activities on mixed substrates and through the use of eserine, tetraethyl pyrophosphate and choline as inhibitors *in vitro*. The hydrolysis of ACh, TA and NPA may be due to one, two or three enzymes depending on the source of insect esterases. Large differences in sensitivity to the various inhibitors occurred with the various insect esterases. Both the quantity and properties of the esterases of the housefly and waxmoth shift during development.

3. Pea aphids were shown to contain cholinesterases attacking ACh and BzCh but not MeCh. Excess substrate inhibition occurred with BzCh but not ACh. Very high activity was observed on TA and NPA and lower activity on a group of other ester substrates. The activity towards ACh could be separated from that towards NPA by solvent or salt fractionation or by the use of selective inhibitors.

4. Enzymes very active in hydrolysing choline esters were observed in many insect parts. Noteworthy are the high activity of housefly heads and whole whiteflies for ACh, of cockroach malpighian tubules for BzCh and of cockroach ventral nerve cord for MeCh. A concentration of the triacetinase of the cockroach in the gastric caecae and a high activity of the blood for choline esters was also observed.

5. The relative physiological importance of ACh esterase and NPA esterase in organophosphate poisoning of the adult cockroach was evaluated. Studies with four organophosphates, two substrates, and eight organ systems showed that the only enzyme consistently inhibited to a large extent (67–85%) at the prostrate stage was the ACh esterase of the nerve cord.

6. No evidence was obtained contrary to the hypothesis that the primary cause of organophosphate insecticide poisoning is due to blockage of the nerve acetylcholine–cholinesterase system in those insects where this system is physiologically important. Considering the specificity differences occurring in the cholinesterase part of this system among the insects and between insects and mammals, a further development of organophosphate insecticides utilizing these differences to achieve the desired degree of selective toxicity seems feasible.

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A Kinetic Study of the Reactions on some Disulphides with Sodium Sulphite

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The reactivities of disulphide bonds in different compounds show considerable variation. For example, the rates of decomposition of simple cystine derivatives with alkali vary widely (Schöberl & Hornung, 1938) and oxidized glutathione (GSSG)

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reacts very much faster with silver nitrate than does cystine (Cecil, 1950).

This paper describes kinetic studies of the reactions of some disulphides with sodium sulphite. Clarke (1932) and Lugg (1932) showed that the reaction is $\text{RSSR} + \text{Na}_2\text{SO}_3 \rightleftharpoons \text{RSNa} + \text{RSSO}_3\text{Na}$. Sodium sulphite has since been used extensively for