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Distribution of Cholinesterases in Insects*

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The study of toxicology and other related fields has been largely based on in vitro *techniques. These methods have provided quantitative information on the effects of inhibitors on enzymes, but none on the localized effects of inhibitors on selected sites of action within the animal. Histochemical study of frozen sections does provide data on the site of action of toxicants. The utility of histochemistry in conjunction with* in vitro *methods is discussed.*

The substrates acetylthiocholine and phenyl thioacetate were utilized in demonstrating cholinesterase. Neither substrate penetrated well into freshly dissected nerve cord preparations, but both compounds were hydrolysed by sectioned tissue. The leaving group of phenyl thioacetate was demonstrated to be benzenethiol. In general, acetylthiocholine was hydrolysed slightly more rapidly by insect cholinesterases. A unique cholinesterase was found in motor end-plates of cricket muscle, which hydrolyses acetylthiocholine and which was inhibited by physostigmine. No other insect muscle preparation showed this activity. Topical application of insecticides showed that a vital site of action in flies is the peripheral area of the thoracic ganglia, and that in crickets the brain and nerve cord are involved at knock-down. Kinetic data indicate that acetylthiocholine has a greater affinity than does phenyl thioacetate for a variety of enzyme sources. Ultrastructural evidence shows that cholinesterases that hydrolyse acetylthiocholine are membrane-bound. Phenyl thioacetate was found to be useful as a model in designing new insecticides.

The presence of cholinesterase (ChE), acetylcholine, and choline acetyltransferase in insect nervous tissue has been well established (Koelle, 1963). Inhibition of ChE is considered to be the principal mode of action of organophosphorus and carbamate insecticides, but little agreement exists as to the level of inhibition needed to cause death (O'Brien, 1967). It is apparent from the literature that some compounds are toxic with little inhibition of total ChE, while others inhibit the enzyme to a much greater extent without toxic effects (Brady & Sternburg, 1967).

However, most studies of the mode of action of organophosphorus and carbamate insecticides have been limited to the use of *in vitro* or *in vivo* techniques. Biochemical studies of this nature have provided us with quantitative information on the action of these inhibitors on ChE, but the mass-brei approach gives no data on the distribution of the ChE enzymes at the cellular, tissue, and organ-system levels. Therefore, localized effects of these compounds may be restricted by the mixing of tissues during their preparation for enzyme assay, and unduly high inhibitions may occur in homogenates of whole insects as a result of poison that, *in vitro*, is far removed from the site of action (Molloy, 1961). Histochemical methods, on the other hand, do not have these particular disadvantages. Hence, the question arises as to whether such inhibition as does occur is highly localized and complete, in which case death might occur by a localized area ceasing to function, or whether the inhibition is generalized and only partial through the nervous system (Booth & Metcalf, 1970a).

This paper deals with the histochemical and kinetic behaviour of two acetyl thioester substrates with insect cholinesterase and anticholinesterase activity at localized sites in insect tissue.

MATERIALS AND METHODS

Substrates

Acetylthiocholine was obtained commercially from Mann Chemical Co. Phenyl thioacetate was prepared

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by refluxing benzenethiol with acetyl chloride for several hours. The excess acetyl chloride was removed by distillation and the product distilled under reduced pressure (b.p. 99–100°C (6 mm), n_D^{30} 1.5655).

Inhibitor

The insecticide *O,O*-dimethyl *S*-phenyl phosphorothioate was synthesized by the method of Murdock & Hopkins (1968) and purified by Florisil column chromatography as described by Patchett & Batchelder (1961). The inhibitor was chemically pure as determined by thin-layer chromatography and infrared spectroscopy.

Enzymes

Bovine erythrocyte acetylcholine acetyl-hydrolase (3.1.1.7) (AChE) was obtained from Nutritional Biochemical Corp. Particulate NAIDM housefly (*Musca domestica*) head and cricket (*Acheta domestica*) head AChE were purified, bacterial (*Bacillus subtilis*) protease obtained from CalBiochem being used to render them soluble. The protein mixture was then subjected to DEAE and Sephadex G-200 column chromatography. Approximately 500-fold and 150-fold purification was obtained for the housefly and cricket-head acetylcholinesterases, respectively.

Insects

The principal insects used in this study were crickets (*Acheta domestica*), houseflies (*Musca domestica*), cockroaches (*Periplaneta americana*), and honey-bees (*Apis mellifera*). The following insects were also used in comparative-distribution studies of ChE: *Phormia* sp., *Sarcophaga* sp., *Tenebrio* sp., *Oncopeltus* sp., *Manduca* sp., and *Anopheles* sp.

Histochemical and electron-microscopic methods

The histochemical studies were conducted according to the procedures described by Booth & Metcalf (1970a, 1970b) and Booth & Whitt (1971). The procedures for localizing ChE by electron microscopy were modifications of histochemical methods (Booth & Metcalf, 1970a, 1970b) and of routine methods of fixation, dehydration, and embedding.

Kinetic measurements

Acetylthiocholine and phenyl thioacetate were used as substrates for determining ChE activity as described by Ellman et al. (1961). The K_m values were determined by Lineweaver–Burk plots.

RESULTS

Histochemistry

The hydrolysis of acetylthiocholine and the chemistry of the thiocholine technique for demonstrating ChE have been studied in considerable detail. It has been reported that when acetylthiocholine was hydrolysed, the liberated moiety was confirmed to be thiocholine (Malmgren & Sylven, 1955). No evidence is available on the nature of the products of hydrolysis of phenyl thioacetate. A partially purified aliquot of honey-bee ChE (100 μlitres) was incubated with phenyl thioacetate for 60 minutes and was subjected, with appropriate standards, to silica gel GF 254 thin-layer chromatography using two different solvent systems. The results of these experiments indicate that the R_F of the benzenethiol standard is identical to that of the hydrolysed sample of phenyl thioacetate and bee enzyme (Table 1).

Female crickets were dissected from the dorsal side, the alimentary tract was pushed aside, and the incubation solution, containing acetylthiocholine or phenyl thioacetate, was placed in the animals. The results indicate that acetylthiocholine is hydrolysed hardly at all by the intact nerve cord. No other tissue in the animal appeared to cleave this substrate. When the nerve cord was dissected out, it showed

Table 1

Thin-layer chromatography R_F values for chemically pure standards and for phenyl thioacetate plus honey-bee enzyme, using two solvent systems

Solvent system	R_F values			
	benzenethiol	phenyl thioacetate	thioacetic acid	phenyl thioacetate + enzyme
carbon tetrachloride + ethyl acetate, 50:1, v/v	0.67	0.38	0.24	0.67, 0.38
n-hexane + diethyl ether, 100:3, v/v	0.62	0.20	0.12	0.62, 0.20

small dark areas of enzyme activity, which were probably the result of damage occurring during the process of dissection. In addition, the exposed neural fibres that had been cut were stained for ChE. The copper mercaptides appeared for a short distance from the severed edges of these nerves. Fig. 1 shows the dark " spots " of enzyme activity, and the arrow indicates staining of a severed nerve. When the same dissection processes were applied to another animal using phenyl thioacetate, a somethat different pattern appeared. When phenyl thioacetate was incubated in an open-dissected cricket, a number of tissues hydrolysed the aromatic compound. The fat body, the malpighian tubules, peripheral areas of the nerve cord, and the surfaces of the alimentary tract picked up activity. Fig. 2 shows the peripheral area of the nerve cord stained for enzyme activity. When the nerve cord was dissected out, the intensity of the stain was found to increase along its length, and severed nerves picked up the activity in much the same pattern as was seen with acetylthiocholine.

The substrate phenyl thioacetate is hydrolysed rather rapidly by honey-bee brain (Fig. 3). Recently it has been shown in honey-bees that acetylthiocholine and phenyl thioacetate give very similar patterns of ChE distribution when selective inhibitors are used (Booth & Metcalf, 1970b). However, different insects seem to show a good deal of variation in specificity to this aromatic substrate. Cricket brains, for example, hydrolyse phenyl thioacetate at a lower rate than do honey-bee brains (Fig. 4). Other organisms, such as the cockroach, the milkweed bug, and the tobacco hornworm also hydrolyse it more slowly than acetylthiocholine. However, the cricket had a very unusual pattern of enzyme activity in the muscle, particularly for acetylthiocholine. Of all the insects that we have studied thus far, the cricket is the only organism that gives a positive reaction in the bundles of virtually all muscles of the body. Fig. 5 shows a section of muscle from the head region of the cricket stained with acetylthiocholine, showing intense ChE activity. This result has been found to be highly reproducible in all the crickets that have been sectioned. It is completely inhibited by 10^{-6} M physostigmine. The same region of the brain from a close relative, *Periplaneta*, was treated in an identical manner, but no activity whatsoever could be detected (Fig. 6).

The inhibitor *O,O*-dimethyl *S*-phenyl phosphorothioate, whose leaving group (benzenethiol) is the same as that of phenyl thioacetate, was prepared in our laboratory. This compound was applied topically to crickets and houseflies and the physical activity of the animals was followed in conjunction with histochemical effects on the central nervous system. Phenyl thioacetate was hydrolysed only slightly by the nerve cord of crickets, hence we traced the effects of the toxicant using acetylthiocholine. Fig. 7 and 8 show the metathoracic ganglion of the cricket before and after topical application of the inhibitor to the tip of the abdomen. It can be seen that there was total inhibition in the nerve cord proper, with peripheral inhibition in the ganglion. The cricket was knocked-down—i.e., it could not right itself. Fig. 9 and 10 show the mesothoracic ganglia of the control and knocked-down crickets, respectively. The inhibition again was complete in the interganglionic connectives but greater peripheral inhibition occurred in the mesothoracic ganglion than in the metathoracic ganglion. Fig. 11 and 12 show the prothoracic ganglia of the control and knocked-down crickets, respectively. In this case deeper penetration of the ganglion by the toxicant could be observed, with a slight amount of activity in the neuropil. We also monitored the effects of the compound in the brain and found that at knock-down the brain ChE was always completely inhibited. This pattern of enzyme inactivation appeared to be reproducible when correlated with the physical state of the animal. Whenever there was less enzyme inhibition in the ganglia, commissures, and brain the animal was still able to make co-ordinated walking movements. The time for knock-down using a dose of 140 μg/g was usually 35–40 minutes. When the animals appeared to be only " affected "—i.e., when they showed nervous movement, the antennae moving rapidly and the animal walking about—brain ChE was always normal, slight peripheral inhibition was evident in the prothoracic ganglion, and the inhibition became progressively less evident towards the posterior part of the central nervous system. The activity in the muscle was always inhibited at knock-down.

The housefly, *Musca domestica*, was treated topically on the abdomen with a dose of 500 μg/g. In this case both acetylthiocholine and phenyl thioacetate could be monitored because both substrates were hydrolysed quite well, although the former was always cleaved more uniformly throughout the tissue. Fig. 13 and 14 show the fused thoracic ganglia of the control and knocked-down flies, respectively. It should be noted that it is the peripheral ChE that is inhibited when the animal is affected in this way.

Table 2

K_m values for AChE from housefly heads, cricket heads, and bovine erythrocytes with acetylthiocholine and with phenyl thioacetate (0.1 M phosphate buffer, pH 8.0, 25°C)

Substrate	K_m value (M) for:		
	housefly-head AChE	cricket-head AChE	bovine erythocyte AChE
acetylthiocholine	2.88×10^{-5}	1.67×10^{-4}	1.43×10^{-4}
phenyl thioacetate	1.49×10^{-3}	1.43×10^{-3}	1.04×10^{-3}

The inhibition pattern in the animal treated with phenyl thioacetate was the same, the only difference being that the control showed little activity in the peripheral area (Fig. 15 and 16). The brain ChE for either substrate was always the same as the control when the flies were in a hyperactive state after treatment. However, when more time (1 hour at this dose) was allowed to elapse between treatment and histochemical sectioning, the poison penetrated into the central part of the brain (Fig. 17 and 18) and the thoracic ChE was always completely inhibited.

The localization of enzymes that hydrolysed acethylthiocholine and phenyl thioacetate was determined at the ultrastructural level. We have found that cholinesterases that hydrolyse acetylthiocholine are membrane-bound (Fig. 18), whereas those that hydrolyse phenyl thioacetate are not.

Kinetics

In an attempt to determine the *in vitro* differences in affinity of several cholinesterases for acetylthiocholine and phenyl thioacetate, we determined the K_m values. Table 2 shows the results of these studies for AChE from houseflies, crickets, and bovine erythrocytes. The enzymes from the different sources show very slight differences in affinity for phenyl thioacetate, but housefly-head ChE shows significant affinity for acetylthiocholine. However, each enzyme shows large differences in its affinity for the two substrates.

DISCUSSION AND CONCLUSIONS

This report has provided histochemical and kinetic information on the specificity of acetylthiocholine and phenyl thioacetate for a variety of ChE sources. We have attempted to point out the advantages and disadvantages of *in vitro* methods in studying the mode of action of organophosphorus compounds. The mass-brei techniques have many disadvantages, such as the destruction of structural barriers and the release of endogenous inhibitors. Other limitations have become apparent following the accumulation of information on the many complex factors that may change the behaviour of enzymes in intact structures. Therefore, it is frequently impossible to extrapolate from observations of the behaviour of enzymes in solution to their behaviour in intact cells (Nachmansohn, 1970).

The data in Table 1 show that when phenyl thioacetate is hydrolysed by honey-bee ChE, the leaving group is benzenethiol. This compound is an important intermediate in the manufacture of insecticides, herbicides, and pharmaceuticals, and is now an important histochemical reagent. In insects it has been shown that an *S*-glucosylation occurs both *in vitro* and *in vivo* (Gessner & Acara, 1968). It appears that *S*-methylation, oxidation, and conjugation are the principal steps in the metabolism of benzenethiol in rats (McBain & Menn, 1969). Therefore, it is apparent that a number of different chemical groups may couple with the thiol moiety. Furthermore, during histochemical staining with phenyl thioacetate, coupling apparently takes place with copper ions to give a dark mercaptide reaction product. The inhibitor used in this study, *O,O*-dimethyl *S*-phenyl phosphorothioate, has benzenethiol as a leaving group when ChE is phosphorylated, but there is no interference with the histochemical process since complete inhibition can be observed with this organophosphorus compound.

It was hoped that the lipid-soluble phenyl thioacetate molecule would penetrate intact nerve sheath barriers, which acetylthiocholine has not been able to do well. Winton et al. (1958) showed that, along

Fig. 1. Isolated nerve cord preparation of the cricket *Acheta domestica* stained for ChE; the substrate was acetylthiocholine. Note that hydrolysis is " spotty ". The arrow shows a severed nerve with good activity.

Fig. 2. Preparation of nerve cord similar to that shown in Fig. 1 ; phenyl thioacetate was used as the substrate. Peripheral hydrolysis is generally shown over the entire cord.

Fig. 3. Honey-bee brain ChE, using phenyl thioacetate.

Fig. 4. Cricket brain ChE, using phenyl thioacetate.

Fig. 5. Cricket-head muscle showing motor end-plate ChE activity, using acetylthiocholine.

Fig. 6. Cockroach, *Periplaneta americana,* head muscle stained for ChE with acetylthiocholine. No ChE activity is present.

Fig. 7. Control ChE of metathoracic ganglion of cricket, using acetylthiocholine.

Fig. 8. Metathoracic ChE from the ganglion of the cricket at knock-down after treatment with *O,O*-dimethyl *S*-phenyl phosphorothioate (DMPT), using acetylthiocholine.

Fig. 9. Control ChE from the mesothoracic ganglion of the cricket, using acetylthiocholine.

Fig. 10. Mesothoracic ChE from the ganglion of the cricket at knock-down after treatment with DMPT, using acetylthiocholine.

Fig. 11. Control ChE from the prothoracic ganglion of the cricket, using acetylthiocholine.

Fig. 12. Prothoracic ChE from the ganglion of the cricket at knock-down after treatment with DMPT, using acetylthiocholine.

Fig. 13. Control ChE from the thoracic ganglion of a housefly (*Musca domestica*), using acetylthiocholine.

Fig. 14. Thoracic ChE from the ganglion of a housefly at knock-down after treatment with DMPT, using acetylthiocholine. Note the strong peripheral inhibition.

Fig. 15. Control ChE from the thoracic ganglion of a housefly, using phenyl thioacetate.

Fig. 16. Thoracic ChE from the ganglion of a housefly at knock-down after treatment with DMPT, using phenyl thioacetate. Peripheral inhibition is evident.

Fig. 17. Control ChE from the left half of a fly brain, using phenyl thioacetate.

Fig. 18. Left half of a fly brain showing ChE after treatment with DMPT for an extended period after knock-down. Complete inhibition of the central part of the brain is evident.

Fig. 19. An electron micrograph of membrane-bound ChE (arrows), using acetylthiocholine. M, mitochondria; N, nucleus.


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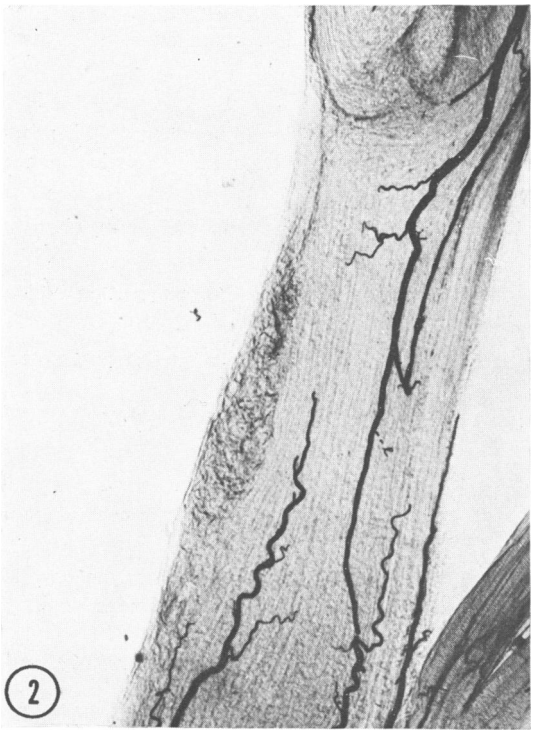
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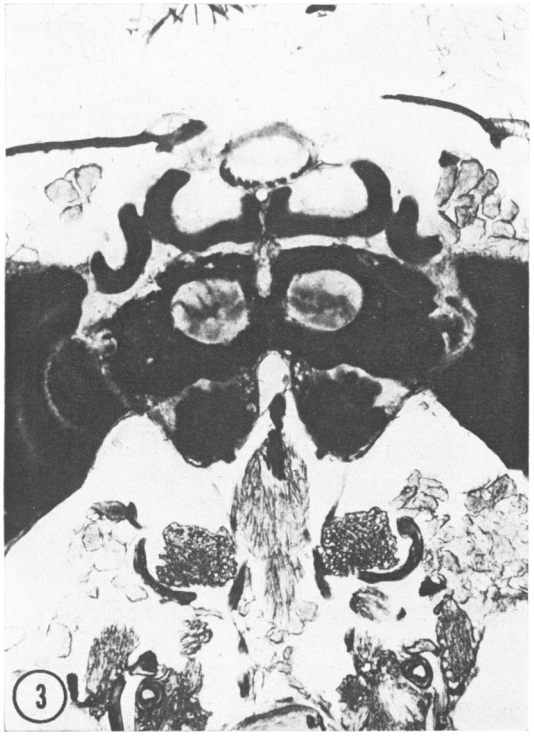
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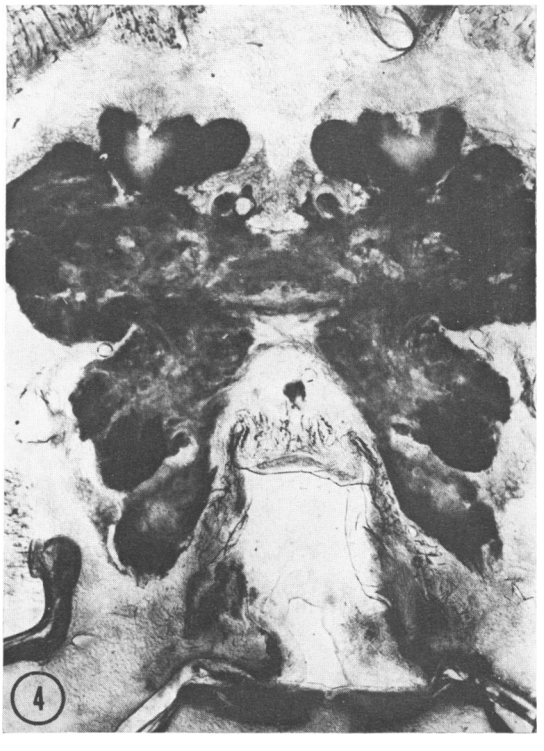
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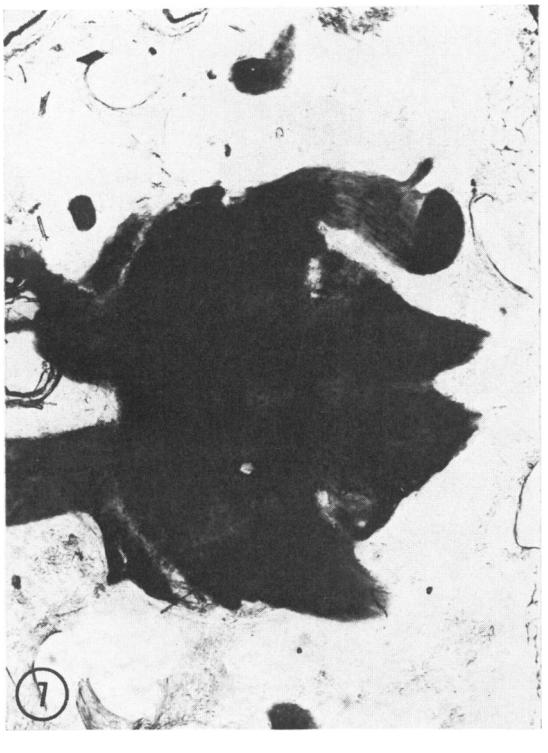
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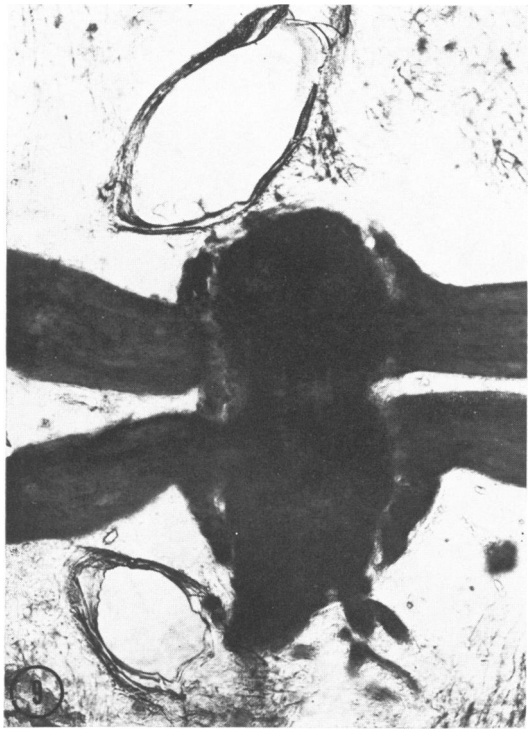

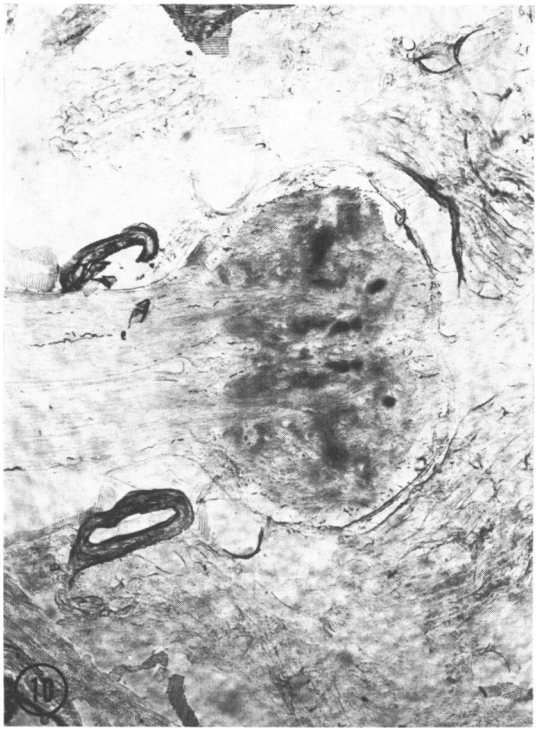

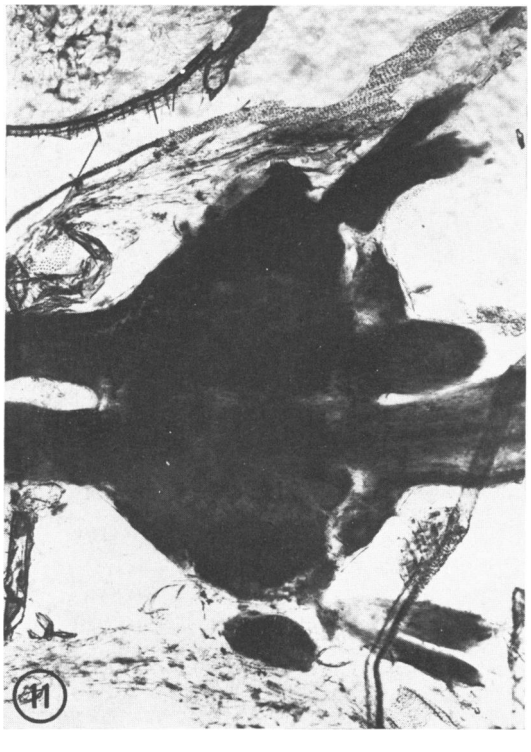

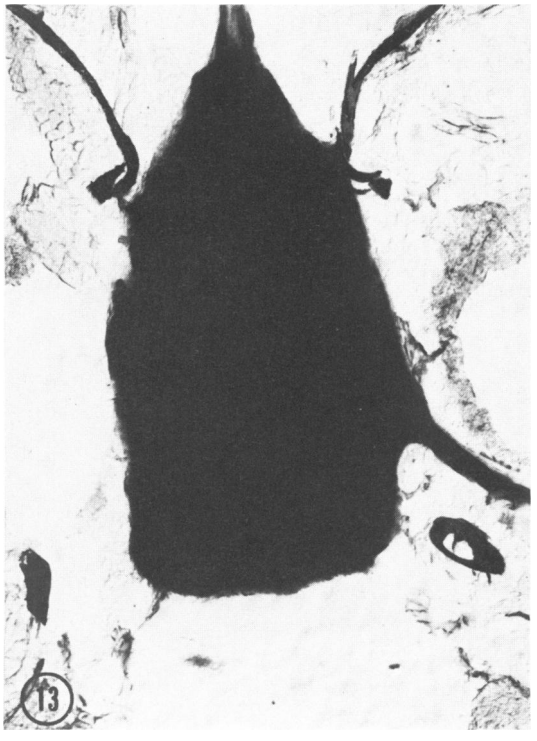
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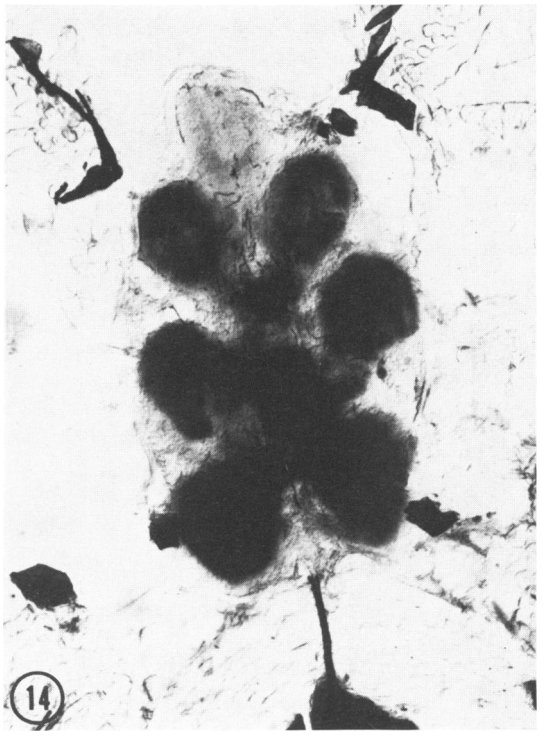
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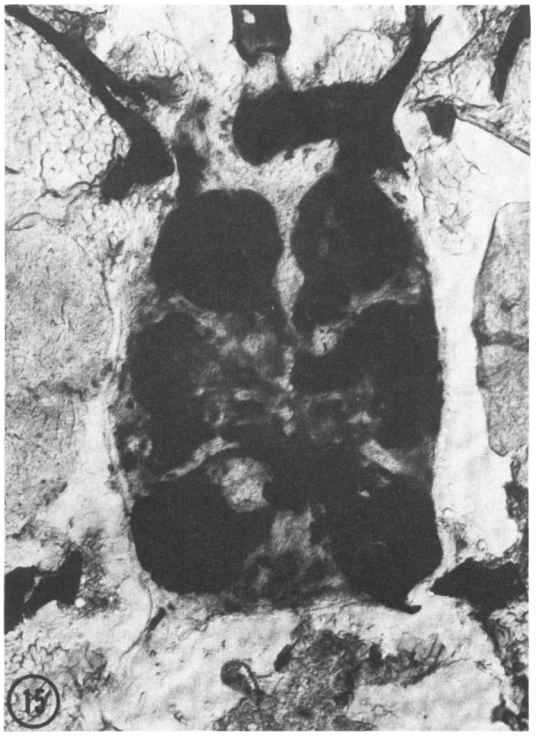
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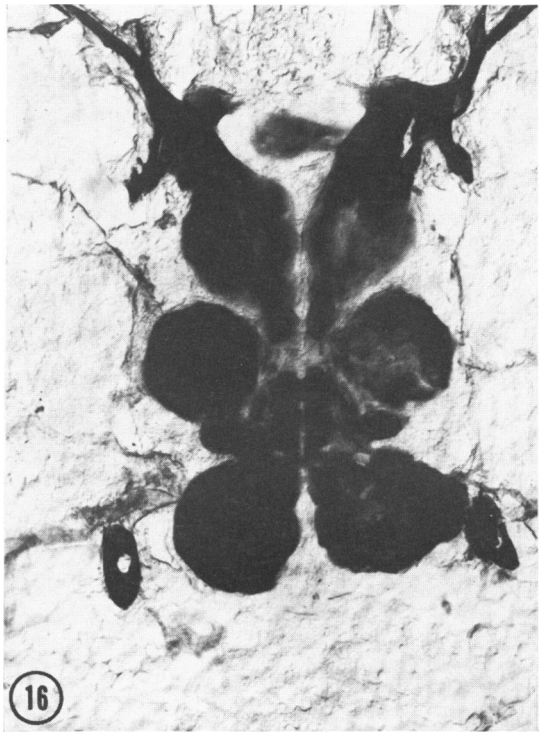
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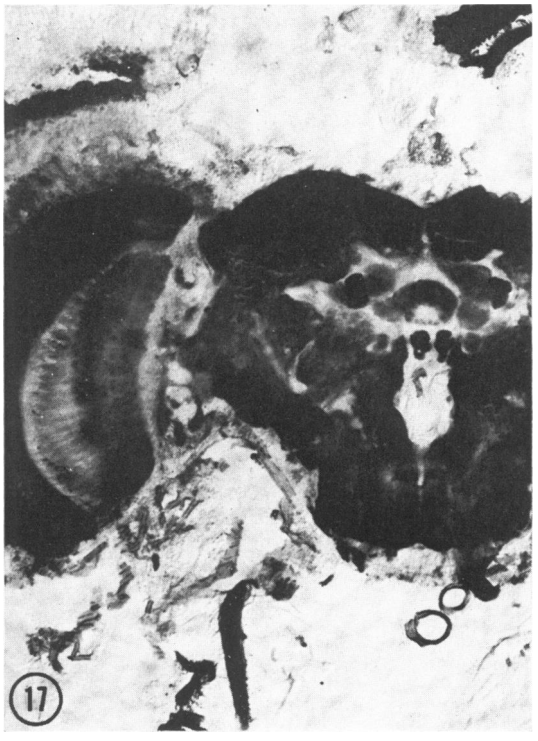

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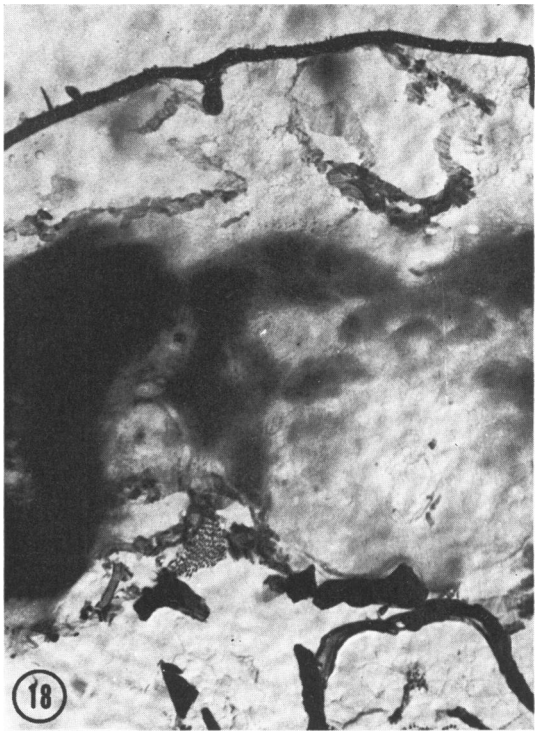

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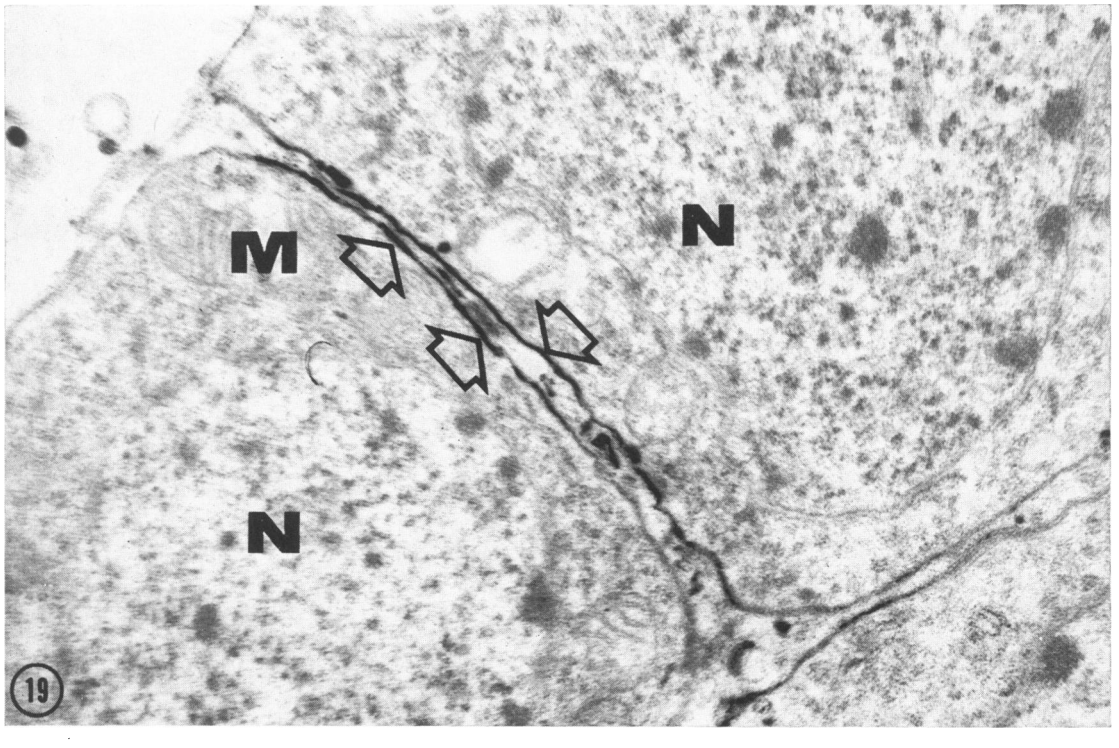

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neurones and ganglia of *Periplaneta*, in areas where the nerve sheath was intact, acetylthiocholine did not penetrate and no hydrolysis occurred. Using phenyl thioacetate, they found the copper mercaptide of this substrate along the length of the nerve cord and ganglionic areas. The reaction was observed beneath the lipid–connective material of the nerve sheath. Fig. 1 and 2 show this surface reaction in crickets for acetylthiocholine (in spots) and phenyl thioacetate. However, the latter did not penetrate into the inner portion of the nerve cord proper. Both substrates readily hydrolysed severed nerves and small nerves. Phenyl thioacetate was hydrolysed by the malpighian tubules, the fat body, and the gut surface, while acetylthiocholine was not.

Booth & Metcalf (1970b) found that phenyl thioacetate was hydrolysed to a considerable extent by honey-bee brain ChE (Fig. 3); the distribution pattern was essentially the same as that found for acetylthiocholine. Subsequently it was shown, by electrophoresis and the use of selective inhibitors, that honey-bee brains contain at least 3 ChE isoenzymes. The region of the deutocerebrum was found to contain an esterase insensitive to organophosphorus compounds. Fig 4 shows the hydrolysis of phenyl thioacetate by cricket brains. The enzyme activity was less uniform in these preparations than in those of the honey-bee, but some areas of the brain—e.g., the corpora pedunculata—were extremely active. Using acetylthiocholine on the cricket brain, we observed a pattern similar to that shown for the honey-bee in Fig. 3.

With cricket preparations, the most surprising observation was the intense ChE activity in muscle sections (Fig. 5). This preparation was most active on acetylthiocholine, and was highly reproducible. We thought at first that it was an artifact or possibly a nonspecific esterase, but subsequent incubation with 10^{-6}M physostigmine inhibited this enzyme completely. Mouse intercostal muscle was then dissected out to determine if the substrates were contaminated, and by using exactly the same conditions as for the cricket preparation highly specific motor end-plates could be produced histochemically. An identical muscle section of the American cockroach treated under the same conditions produced no activity (Fig. 6). Wigglesworth (1958), using acetylthiocholine, found no evidence of ChE at the motor nerve endings in *Rhodnius prolixus*, and Colhoun (1959) was unable to demonstrate ChE in denervated roach muscle. However, ChE has been demonstrated histochemically in the muscle receptor organs and—more tentatively—in the motor nerve endings of a crustacean, *Homarus americana*, by Maynard & Maynard (1960). The work of the latter authors was apparently the first histochemical demonstration of ChE at the nerve endings of an arthropod. Wigglesworth (1959) reported that ChE seemed to be confined to the nervous system of insects.

Hamori (1961) studied the ChE of muscle from different species of Orthoptera, Coleoptera, Hymenoptera, and Diptera, using thiocholine analogues and 1-naphthyl acetate, and showed that a type of esterase was present in insect motor axons and terminals. However, he concluded that the esterase could not be considered as a specific ChE, since at pH 5 neither acetylthiocholine nor butyrylthiocholine was hydrolysed at the periphery of the muscle. Furthermore, at pH values of 6.2 or higher a positive reaction appeared in the motor fibres only with butyrylthiocholine. No enzyme reaction occurred with acetylthiocholine. Hence, Hamori concluded that an enzyme of the ChE type was localized in the motor axons (using butyrylthiocholine) and that, using 1-naphthyl acetate, large amounts of an enzyme were found in terminal arborizations (end-plates), possibly participating in different types of neuromuscular synapse. No direct role of these enzymes in neuromuscular transmission was suggested, however. Furthermore, he suggested that the enzyme could not be a specific AChE since it gave no reaction with acetylthiocholine.

Orlov (1924) has shown that motor nerve axons can cross over several nerve bundles and eventually terminate in Doyere's hillocks, endings, or simply end-plates. It is conceivable, from the description of Orlov, that the tiny dark pockets of enzyme activity seen in Fig. 5 might be motor end-plates (Doyere's hillocks) that contain a specific ChE that hydrolyses acetylthiocholine. Only slight activity could be detected with phenyl thioacetate and absolutely no reaction with butyrylthiocholine was observed in cricket muscle. In fact, only a slight amount (possibly 1%) of activity with butyrylthiocholine was observed in the brain and ganglion of the nerve cord. From careful observation of our cricket preparation under oil (970×) we have concluded that this reaction is not likely to be the result of esterase activity of the tracheoles, since these structures could be seen as clear unstained entities. Flattum et al. (1967) concluded that the site of action of (+)-tubocurarine in the house cricket was not the neuromuscular junction, since stimulation of the crural nerve at knockdown resulted in muscular contraction in the same

threshold range as that of normal crickets, since flaccid paralysis did not occur, and since succinylcholine chloride did not potentiate and endrophonium [1] did not antagonize the effects of (+)-tubocurarine. Further studies will be necessary to determine whether this enzyme is involved in neuromuscular synapses or other secondary sites.

When the cricket was treated topically on the tip of the abdomen with *O,O*-dimethyl *S*-phenyl phosphorothioate at a dosage of 140 μg/g, we were able to trace the effects of the poison on the ChE of both the central nervous system and the muscle. At 25 minutes after treatment only peripheral ChE of the prothoracic ganglion and nerve cord were inhibited, but the animal was still able to make co-ordinated walking movements. However, at knock-down (40 minutes after treatment) the inhibition of the ganglia of the nerve cord was progressively worse from the metathoracic ganglion to the mesothoracic ganglion to the prothoracic ganglion (Fig. 7–12). However, the neurones and brain proper were always completely inhibited, as were the muscle end-plates at this point. Inhibition of various parts of the central nervous system is predictable from the physical symptoms of poisoning.

The housefly, when treated topically with the organophosphorus compound at a dosage of 500 μg/g, showed a different pattern of inhibition. At knock-down, using either acetylthiocholine or phenyl thioacetate, the peripheral ChE of the thoracic ganglion was always inhibited (Fig. 14 and 16), but inhibition of brain ChE was never observed. The principal difference in the distribution of ChE in the thoracic ganglion of the control animals was that higher activity of peripheral enzyme was observed when acetylthiocholine was used than when phenyl thioacetate was used. These data correlate well with those of our previous work on houseflies, in which peripheral ChE was inhibited by a variety of inhibitors (Booth & Metcalf, 1970b). The thoracic ganglion controls segmental reflex activity, and it is not surprising to find the locomotor apparatus nonfunctional when peripheral ChE is inhibited. Other workers have found that peripheral ChE inhibition occurs frequently (Molloy, 1961; Lord et al., 1963; Farnham et al., 1966). If longer periods of time are allowed to elapse after initial poisoning, it is found that the central portion of the fly brain is eventually inhibited (Fig. 17 and 18).

[1] International nonproprietary name (INN) for ethyl (*m*-hydroxyphenyl)dimethylammonium chloride.

It is apparent from *in vitro* studies that no one level of ChE inhibition causes knock-down and/or death in all insects. At the present time ChE inhibition seems to be the best explanation for the toxic effects produced by organophosphorus insecticides, but it is likely that localized sites of inhibition are more important than quantitative estimates of ChE inhibition (Zettler & Brady, 1970).

Few data are available on the ultrastructure of thiocholine-hydrolysing enzymes in insects. Fig. 19 is an electron-micrograph of a honey-bee preparation showing that ChE is localized in the membranes of the nervous system of this insect (see arrows). This information agrees with findings on the ultrastructure of ChE in mammalian tissue (Nachmansohn, 1970). The enzymes that hydrolyse phenyl thioacetate have not thus far been detected in membranes. Neither acetylthiocholine nor phenyl thioacetate penetrates membranes well enough to be an ideal ultrastructural substrate for ChE, although investigations with these interesting molecules are continuing. For example, various halogenated alkyl thioesters have been found to inhibit choline acetyl-transferase in flies but not in mammals (Yu & Booth, 1971).

In addition we are currently investigating phenyl chlorothioacetate as a selective inhibitor of insect choline acetyl-transferase. It may also be useful as a histochemical aromatic substrate for ChE. Thus, phenyl thioacetate has served as a model substrate for the development of some rather selective inhibitors that may be useful in pest control programmes.

Although *in vitro* methods have their limitations, they are the only convenient way of studying the kinetics of enzymes that hydrolyse acetylthiocholine and phenyl thioacetate (Table 2). From the K_m values given in Table 2 it can be seen that (1) there was no significant difference in the affinity of cricket-head ChE and bovine erythrocyte ChE for acetylthiocholine, but the affinity of housefly-head ChE for acetylthiocholine was approximately 10 times that of the other two enzyme sources; (2) the affinity of housefly-head ChE for acetylthiocholine was about 100 times its affinity for phenyl thioacetate, while the affinity of both cricket-head and bovine erythrocyte ChE for acetylthiocholine was 10 times their affinity for phenyl thioacetate; and (3) it would appear that acetylthiocholine has a better fit to most enzymes than does phenyl thioacetate, possibly owing to the quaternary amine, which is conceivably drawn to the proposed anionic site of ChE.

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DISCUSSION

AUGUSTINSSON: This is, to my knowledge, the first time that a phenyl thioester has been used as a substrate in such studies. These esters probably involve instability problems: have they been used in quantitative determinations of esterase activity by the Ellman method?

BOOTH: We have used the Ellman method routinely in our laboratory for studying both phenyl thioacetate and thiocholine analogues on purified and on crude breis of insect and mammalian cholinesterase. We find phenyl thioacetate to be almost as stable as the thiocholine analogues in Ellman's buffer. The specificity of enzymes for phenyl thioacetate varies according to their source, and provides for some interesting comparisons with the thiocholines. For the honey-bee, acetylthiocholine is hydrolysed slightly better than is phenyl thioacetate.

ALDRIDGE: You have mentioned that a histochemical technique is useful in insect toxicology. How do you evaluate the situation when the insect has been treated with a carbamate? I presume that your technique involves incubation with substrate for prolonged periods. This will change the steady state of the reaction of enzyme and inhibitor. How do you interpret your findings in the light of this problem?

BOOTH: We incubate sections of insects treated with carbamates for the same length of time (1 hour) as those treated with organophosphorus compounds. In spite of the reversibility of inhibition by carbamates, it is likely that the steady state is much different in intact tissue than *in vitro*. Under the conditions of our experiments, the peripheral enzyme is always inhibited at knock-down after treatment with carbamates. The correlation of data for intact tissue with those for in vitro studies will continue to be difficult owing to the complete change in environment of the enzyme.

BARNES: Was it possible to produce histochemical evidence of the inhibition of cholinesterase in the central neuropil by using inhibitors such as DFP?

BOOTH: Yes. Using the diisopropyl homologue of parathion, paraoxon, and oxime phosphates, the inhibitors do in fact penetrate the neuropil. At knockdown, however, one usually sees peripheral inhibition of the thoracic ganglion of the housefly first, and the toxicant subsequently penetrates the neuropil. This is not to say that this pattern follows for all species of insect. In the cricket, for example, the brain seems to be affected at about the same time as the nerve cord.