were made to determine the possible occurrence of γ-butyrobetaine which Hosein (17) concluded was a factor in dieldrin poisoning of the rat. While attempting to find this substance, Colhoun and Spencer (11) found that the esters of the betaine were more likely to cause toxic effects if found in the nervous system. However, neither γ-butyrobetaine nor its esters were present in the nerve cords of dieldrin-treated roaches or those of normal roaches. The occurrence of any one of these substances in the nerve cord of treated roaches would suggest that dieldrin is capable of inducing the metabolism of a substance not normally found. This principle, although exciting, has no factual foundation in chlorinated hydrocarbon poisoning in insects at present. However, the possibility suggests a biochemical approach to solving the mechanism of chlorinated hydrocarbon in the central nervous system of insects alongside the search for a physical effect thought to occur in the peripheral nervous system (14).

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Effect of Insecticides on Neurophysiological Activity in Insects

JAMES STERNBURG

Department of Entomology, University of Illinois, Urbana, Ill.

During the course of DDT poisoning, a neuroactive substance accumulates in the blood of American cockroaches and crayfish. Although its structure is unknown, it is not a metabolite of DDT, as its release by nervous tissue can be effected by electrical stimulation alone or by the action of TEPP. Chemical and chromatographic evidence indicates it is not a known neurohumoral agent. The chemical structures of this and other physiologically active compounds must be determined before their role in the mode of action of insecticides is understood.

MUCH of our understanding of the events occurring during neurotransmission in insects is based on a comparison of the properties of insect nerves with known properties of vertebrate nerves. In many ways, insect nerves respond to chemicals much as do the nerves of vertebrates. For example, physostigmine, a reversible inhibitor of cholinesterase, and the organophosphates, irreversible inhibitors of cholinesterase, disrupt synaptic transmission in insects. These synapses are located in the ganglia of the central nervous system and it is here that high concentrations of cholinesterase are located. Acetylcholine and choline acetylase, the other components required for cholinergic transmission, are also found for the most part within the ganglia (3). The presence of these substances clearly indicates that at least some phases of neurotransmission in insects are cholinergic. Disruption of normal neuro- activity by anticholinesterases confirms this. The intense stimulation of central nervous activity by pilocarpine in insects (13, 16) also lends support to the presence of cholinergic pathways, as pilocarpine is believed to act by stimulating cholinergic vertebrate effectors.

But there are some disturbing differences. Curare and atropine are known in vertebrates to block neuromuscular junctions and sympathetic ganglia from the action of either

exogenous acetylcholine or electrical stimulation. These are cholinergic sites in vertebrates, and it is thought that curare and atropine desensitize these sites postsynaptically to either exogenous or endogenous acetylcholine. In insects, however, neither curare nor atropine has any effect when injected into cockroaches (13) nor do they affect synaptic transmission through the sixth abdominal ganglion of the cockroach (14). As it is known that insect neuromuscular junctions lack cholinesterase (27) and thus are presumably noncholinergic, it is not surprising that injected doses are ineffective here, but the failure to affect sensory-central synaptic transmission is difficult to explain, unless it is due to the failure of atropine or curare to penetrate to a critical site within the ganglion.

For ionized compounds failure to penetrate may be due to an ion-impermeable sheath around the nerve cord (9). As shown by O'Brien (11), ionized inhibitors of cholinesterase do not readily penetrate the extremely thin sheath that covers the nerves and ganglia of insects, whereas nonionized inhibitors penetrate rapidly. Acetylcholine itself is surprisingly ineffective when injected into insects, and several milligrams may be injected into a cockroach weighing 1 gram with no ill effects. When applied directly onto the last abdominal ganglion of the cockroach, acetylcholine is without consistent effect on synaptic transmission, even at concentrations of 1.0% (14). However, Twarog and Roeder (23) have shown that if the protective sheath surrounding the ganglion is ruptured, $10^{-3}M$ concentrations of acetylcholine will often cause a partial block of synaptic transmission, and that this is potentiated by physostigmine, with $10^{-4}M$ acetylcholine then causing bursts, after discharge and block. As Twarog and Roeder point out, this is still a relatively high concentration, and although the evidence favors interpreting these synapses to be cholinergic, the question cannot as yet be considered answered.

As insect ganglionic tissue is rich in both acetylcholine and cholinesterase, it is logical to conclude that neurotransmission is at least in part cholinergic. The site of action of the organophosphates appears to be those areas containing cholinesterase, localized within ganglia, whereas DDT has been shown to act primarily on sensory nerves and associated structures (17, 26). Although these sites undoubtedly are distinct, certain characteristic effects produced by these two classes of toxicants suggest similar disruptions of normal nervous behavior. The visible symptoms of poisoning by DDT clearly indicate involvement of the nervous system. As shown by Roeder and Weiant (17), the sensory nerves are most sensitive to DDT, with little or no direct action occurring on central or motor nerves. Yet prostration and paralysis eventually do take place, indicating that more profound changes occur than mere excitation of afferent nerves.

Although the visible symptoms of DDT prostration and final paralysis are suggestive of cholinesterase inhibition, no inhibition of this enzyme by DDT can be shown, either in vivo or in vitro. Yet, during prostration bound acetylcholine within the central nervous system increases two to three times above normal (2, 21). This increase is not great, nor does it occur during the earlier stages of poisoning, and may be of only secondary importance. However, the increase in free acetylcholine following complete inhibition of cholinesterase by an organophosphate is ultimately no greater (2).

Electrophysiological studies by Roeder and his associates have shown that multiple afterdischarges in giant fibers follow electrical stimulation of cercal nerves in DDT-prostrate cockroaches, and that similar discharges may occur during phosphate poisoning (15). Here again, the similarity is striking, and further indicates the impairment of a common system. This intriguing situation suggests that common factors may in some way be involved in a sequence of events initiated at different sites by DDT and an organophosphate antiesterase.

Release of a Neuroactive Substance

During DDT poisoning a toxic substance has been found to accumulate in the blood of American cockroaches [Periplaneta americana (L.)] (18–20). Chemical analysis indicated the absence of significant amounts of DDT in such blood. Yet, when injected into either susceptible or DDT-resistant houseflies, blood from DDT-prostrate cockroaches initiated typical symptoms of DDT poisoning. Furthermore, blood from DDT-prostrate cockroaches was found to cause an increase in the spontaneous nervous activity of an isolated central nerve cord obtained from a normal cockroach. After a brief period of high excitation, the activity usually decreased and blocked suddenly. As DDT itself has no such direct action on the central nervous system, it was concluded that something other than DDT was responsible for these effects.

Concomitant with instability of the sensory nerves, DDT-poisoned cockroaches become hyperexcitable. At this time, the first traces of a neuroactive substance appear in the blood. As the visible symptoms of poisoning by DDT progress through intoxication to prostration, the amount of the neuroactive substance in the blood increases. Conversely, if advantage is taken of the negative temperature coefficient of DDT (25), the amount of neuroactive substance present decreases rapidly and by the time recovery is complete—in a half hour or so—no neuroactive substance can be detected in the blood.

The neuroactive substance is not a metabolite of DDT, but is probably a natural component of the insect's body, as it can be produced without the use of DDT by electrical stimulation (19). Such treatment results in the appearance of a neuroactive substance in the blood similar chromatographically to that induced by the action of DDT. Under certain conditions, what appears to be the same neuroactive substance can be detected in the saline perfusing isolated central nerve cords. When a central nerve cord, with the cercal nerves and cerci still attached, is placed in physiological saline, and the cerci are suspended above the saline and treated topically with DDT, the perfusing saline gradually accumulates a neuroactive substance. Application of DDT suspensions or emulsions directly to isolated central nerve cords fails to lead to toxic perfusates, agreeing with Roeder's earlier observation that DDT has no direct action on the central nervous system.

Release of the neuroactive substance occurs concomitantly with excessively high central nervous activity (19). The initial effect of tetraethyl pyrophosphate (TEPP) on the electrical activity of an isolated cockroach nerve cord consists of a wild burst of spontaneous activity, varying in duration with the concentration of TEPP applied. Although TEPP rapidly inhibits the cholinesterase of isolated nerve cords, whereas the neuroactive substance has no known effect on cholinesterase, the similarity in the extremely high level of spontaneous activity following treatment with either substance suggested that neuroactive material might also be released following treatment with TEPP.

Under normal physiological conditions, an isolated thoracic and abdominal central nerve cord will maintain a continual rhythmic background of spontaneous nervous impulses for many hours. If the preparation includes an intact cercus, electrical stimulation of the cercal nerve will cause a normal postsynaptic response in the giant fibers of the abdominal region. If the abdominal region is stimulated directly, the response to axonal stimulation of the giant fibers can be observed. Application of $10^{-3}M$ TEPP to such a preparation results in a complete block of postsynaptic responses to cercal stimulation within 30 seconds. At the same time, spontaneous activity increases enormously for a brief period, then blocks suddenly, usually in less than 1 minute. Responses to direct axonal stimulation do not block, nor do they fail subsequently. If the nerve cord is

left in the initial TEPP saline, spontaneous activity remains more or less blocked, and only rarely returns within 15 minutes. However, if the saline containing the initial dosage of TEPP is removed, and the nerve washed four or five times with fresh $10^{-3}M$ TEPP, the spontaneous activity rapidly returns, generally within 2 to 5 minutes. The recovered spontaneous activity appears in general to be normal, and remains so for at least 5 hours, the longest time any have been monitored. The response to axonal stimulation continues during this time, and appears to be normal. Postsynaptic response to cercal stimulation, blocked by the first dosage of TEPP, does not return.

If the initial perfusate of TEPP is reapplied to the nerve cord, spontaneous activity again increases greatly above normal, then gradually declines to a low level or in some cases blocks again. As before, washing with fresh $10^{-3}M$ TEPP restores the spontaneous activity to a normal level.

During the entire experiment, the concentration of TEPP has been maintained at a nearly constant molarity $(10^{-3}M)$. Calculated hydrolysis of TEPP during this time (2 hours) does not reduce the concentration below ca. $8 \times 10^{-4}M$ (22). No attempts were made to determine possible enzymatic hydrolysis of TEPP. However, replacement of the bathing solution with fresh TEPP saline caused no change in activity. The reappearance of spontaneous activity thus cannot be explained as due to hydrolytic destruction of the TEPP. The rapid return of spontaneous activity after removal of the initial dosage of TEPP, but with the concentration of TEPP maintained at a constant level, and the ability of the initial perfusate of TEPP to excite a cord again after recovery of spontaneous activity, indicate that a substance other than TEPP must be present in the initial TEPP perfusate. Because only nervous tissue is present, this must come from the nervous system, and presumably is liberated during the initial burst of high spontaneous activity following the application of TEPP.

The cholinesterase of nerve cords subjected to the treatment just described is completely inhibited within a few minutes after the start of the experiment and remains inhibited. Return of spontaneous activity to an essentially normal level, but with no recovery of cholinesterase activity, must mean that the acetylcholine-cholinesterase system is not necessary for the maintenance of spontaneous activity within the central nerve cord. Presumably the substance liberated during the initial burst of high activity is responsible for the block of activity. This does not mean that the cholinesterase system is not important. Normal synaptic transmission certainly

depends on this system, and inhibition of cholinesterase ordinarily abolishes transsynaptic responses.

Comparison with Other Active Substances

In recent years there have been other reports of physiologically active compounds present in various tissues of the cockroach. Some of these have cardiac activity. Unger (24) and Gersch, Unger, and Fischer (6) found several substances, active on isolated cockroach hearts, present in the blood, the central nervous system, and the corpora cardiaca. Colhoun (2) reported several active materials present in the blood of DDT- or TEPP-poisoned cockroaches, most with cardiac activity, but he also reported neuroactivity for blood from DDT-poisoned roaches. Ozbas and Hodgson (12) have reported that a neuroactive substance can be extracted from the corpora cardiaca of normal cockroaches, but that after electrical stimulation these glands are depleted of their substance (8). Because of differences in bioassay methods used by different workers, it is difficult to determine how many compounds are actually involved in these reports. By employing the procedures used by Unger for chromatographic separation of the cardiac active substances, it has been possible to compare them biologically with the neuroactive substance found during DDT poisoning. Briefly, it can be stated that the substances reported by Unger were located on the chromatograms and found to have cardiac activity but no detectable neuroactivity, nor did the neuroactive substance in the blood after DDT poisoning appear to have any effect on the heart. In regard to the neuroactive substance present in the corpora cardiaca, comparison of its R_f in a chromatographic system (1-butanol–acetic acid–water:4:1:5) for which the R_f is known for the DDT-induced neuroactive substance, has revealed that they are not the same compound. The corpus cardiacum substance has an R_f of about 0.3, whereas the DDT-induced substance has an R_f of 0.6 in this system.

Recently, Milburn, Weiant, and Roeder (10) have reported that the application of corpus cardiac extract to abdominal ganglia leads to increased rhythmic efferent activity in the phallic nerves, evidently because of the suppression of the inhibitory influence of the subesophageal ganglion. They found that the DDT-induced substance did not cause rhythmic bursts of activity to appear in efferent nerves, although both the DDT-induced substance and corpus cardiacum extracts caused increased central nervous activity in the abdominal cord. They concluded that the DDT-induced substance was not the same as the active material from the

corpora cardiaca, thus confirming the difference found by chromatographic separation.

Partial Isolation and Identification

From Cockroaches. The neuroactive substance from blood of DDT-prostrate cockroaches has been partially isolated by chromatography (19). After development of the chromatograms, the active substance was located by extracting sections of the chromatogram with physiological saline and determining the effect of these extracts on the spontaneous activity of isolated cockroach nerve cords. By using several different chromatographic systems in sequence, each time rechromatographing only the biologically active section, good separation of the neuroactive substance from other components of the blood was obtained. Because of losses in biological activity entailed during the many manipulations involved, and because of the difficulty in obtaining large samples of cockroach blood, only a few chemical tests have been tried on this substance from roaches, and only one of these has been consistently positive. Diazotized *p*-nitraniline gave a red color coinciding with the biologically active section of chromatograms of toxic blood extracts. Normal blood extracts failed to give this color at the R_f for the active material.

Although little was learned concerning the chemical nature of the neuroactive substance, its R_f in various chromatographic systems was compared with the R_f's of a number of compounds believed to play a role in neurotransmission. It was immediately apparent that it was not acetylcholine, epinephrine, norepinephrine, serotonin, histamine, nor γ-aminobutyric acid. This was not surprising, as none of these compounds disrupt the normal spontaneous activity of isolated nerve cords from cockroaches.

From Crayfish. At best, cockroach blood is difficult to obtain in quantity. One hundred roaches will yield only 10 ml. of blood. As it was unlikely that more than a few micrograms were present per milliliter, it was necessary to find a better source. A search was made therefore for a DDT-susceptible arthropod easily obtained in quantity and possessing a larger volume of blood. The requirements were met by crayfish [*Orconectes virilis* (Hagen) and *O. propinquus* (Girard)], large individuals yielding 3 to 5 ml. of blood. Crayfish were poisoned by immersion for 15 minutes in water containing a suspension of 100 p.p.m. of DDT. They were then transferred to trays lined with wet paper towels, and held until prostrate (3 to 8 hours). This precaution was necessary to prevent premature death, presumably from anoxia after movements of the gills became erratic due to DDT poisoning.

Blood from DDT-prostrate crayfish, processed in the same manner as that

Table I. Organic Spot Test Analysis of Neuroactive Substance from DDT-Prostrate Crayfish Blood[a]

Test[b]	Result[c]	Remarks
Ignition with CaO	Positive	Contains nitrogen
Diazotized sulfanilic acid	Positive	Phenol or aromatic amine
Diazotized p-nitraniline	Positive	Phenol or aromatic amine
Concd. HCl + p-dimethylamino-benzaldehyde	Negative	Not pyrrole, unless lacking CH α or β to cyclic NH
EtOH + HCl + p-dimethylamino-benzaldehyde	Positive (yellow)	Primary aromatic or aliphatic amine
Ninhydrin	Negative	Not primary or secondary aliphatic amine
1,2-Naphthaquinone-4-sulfonate	Negative	No active CH_2 or NH_2 unless hindered by negative groups in ortho or para positions
Sodium nitroprusside + acetaldehyde	Negative	Not secondary aliphatic amine
Hydroxylamine (Hestrin test)	Positive	Ester of carboxylic acid
$NaN_3 + I_2$	Negative	Not thioketone or thiol
Millon test	Negative	Not phenol, unless di-o- or di-m-substituted
HNO_2 in concd. H_2SO_4	Negative	Not phenol, unless p-substituted

[a] Purified by chromatographing ethanol extract of lyophilized dialyzate of blood on paper with 1-butanol–acetic acid–H_2O::4:1:5, rechromatographing active substance found at R_f 0.6 on paper with benzene–H_2O–butanol–methanol::1:1:1:2, and using material found from R_f 0.90 to 0.95 for tests. Aliquots equivalent to 10 ml. of blood used for each test, replicated 3 times.

[b] According to procedures outlined by Feigl (5).

[c] Controls consisted of normal crayfish blood processed in same manner. Results all negative.

used for cockroach blood, was found to have neuroactive properties when applied to isolated nerve cords from either crayfish or cockroaches, causing first excitation and then depression of spontaneous activity (7). One difference was noted; the substance from crayfish was more active on nerves of crayfish than of cockroaches, and conversely, the substance from cockroaches was more active on the nerves of cockroaches than of crayfish. In other respects, their properties appeared to be identical. They had identical R_f's in all chromatographic systems tried. Both were soluble in water and ethanol, and insoluble in carbon tetrachloride and ether. Both were rapidly inactivated by mild alkaline conditions, and by exposure to acetone. For these reasons, it is believed that although they may not be identical, they are at least closely related chemically.

From 1 liter of blood from DDT-prostrate crayfish, enough neuroactive material was obtained to test for the presence of a number of functional groups. The tests used (5), the results obtained, and the interpretation of each test are summarized in Table I. Blood from nonpoisoned crayfish was processed in the same manner for controls, which were negative. Briefly, the chemical tests indicate that the neuroactive substance may be a primary aromatic amine, and possibly an ester.

It has not been possible to obtain enough of the substance liberated during the action of TEPP on an isolated nerve cord to run all of the tests listed. Chromatographically the TEPP-produced material appears to be the same substance as that found in blood from DDT-prostrate cockroaches. It is rapidly destroyed by weak alkali, and is biologically inactivated by acetone. After spraying with diazotized p-nitraniline, a faint red spot has appeared on the chromatograms coinciding with the biologically active section.

Discussion

To date, the evidence indicates that the DDT-induced substance is the same as that liberated by an isolated nerve cord following application of TEPP. The neuroactive substance seems to appear during periods of excessive nervous activity, whether initiated by disruption of the acetylcholine-cholinesterase system with an antiesterase or by excessive afferent bombardment of synaptic areas induced by DDT.

In the case of an antiesterase, the great increase of neuroactivity is presumably due to the sudden failure of cholinesterase to remove free acetylcholine released at the synapses, thus upsetting the normal balance at postsynaptic regions. But this explanation cannot apply to DDT, a compound that does not inhibit cholinesterase. Nevertheless, sensory-central synapses become unstable during DDT poisoning. It is unlikely that the instability is due to DDT itself. First, chemical analysis of even several grams of nerve cords from DDT-prostrate roaches fails to reveal the presence here of DDT. Second, the instability is reversible. By applying the proper dosage of DDT, it is possible to get a cockroach into a condition where it can be prostrated or recovered within a temperature range of a few degrees. Under these conditions, the instability at synaptic regions will

be present or absent, depending on whether the roach is prostrate or recovered. Spontaneous trains of nerve impulses within the central nervous system are also present or absent, correlating with early prostration or recovery.

In a cockroach or crayfish that has advanced to a stage of DDT poisoning no longer reversible, the spontaneous activity of the central nervous system is depressed or nearly absent. Yet if the nerve cord from such an animal is carefully dissected out and washed with physiological saline, a higher level of spontaneous activity will return. Either washing removes whatever it is that caused depression of normal activity, or the cessation of abnormal afferent phenomena due to amputation of sensory nerves permits physiological recovery of the central nervous system.

In addition to reversal of physiological phenomena, certain biochemical events are known to occur reversibly. During DDT prostration, the free proline in the blood and central nerve cord is selectively depleted to about one fourth the normal level (4). By using C^{14}-proline it was found that as proline was depleted, there was a corresponding rise in C^{14}-glutamine. If the temperature was raised so that the roach recovered, the proline and glutamine contents were restored to a normal level. The significance of this observation is not known, but it emphasizes the ease with which recovery to a more or less normal state may take place.

The presence or absence of the neuroactive substance in the blood also correlates with DDT prostration and recovery (19). It first appears during the early hyperactive stage of poisoning, increases to a maximum level during prostration, and then rapidly disappears if the cockroach is placed at a higher temperature to permit recovery. A subsequent drop in temperature, leading to a second prostration, results in the reappearance of the neuroactive substance in the blood.

Under certain experimental conditions there is evidence that DDT-prostrate cockroaches are less sensitive than normal to injected dosages of an anticholinesterase (1, 19). Tetramethyl pyrophosphate (TMPP) is an irreversible inhibitor of cholinesterase, nearly equal in potency to TEPP. Because of its rapid rate of hydrolysis in water [98% in 3 hours for an 0.02M solution (21)] excess TMPP that has not reacted with cholinesterase rapidly neutralizes itself. In normal cockroaches injected with 6 γ of TMPP in 2 μl. of physiological saline, about 90% of the cholinesterase within the central nervous system was found inhibited 3 hours later. When DDT-prostrate cockroaches were injected with 6 γ of TMPP, there was less than 10% inhibition of cholinesterase.

It is not known whether this protection is due to the accumulation of a substance that competes with TMPP for active sites on cholinesterase, to a change in membrane permeability barring the entrance of TMPP into esteratic sites, or to the failure of the circulatory system to transport TMPP through the body.

The in vivo protection against TMPP may also be cited as an example of a reversible phenomenon. DDT-prostrate cockroaches tolerated an injected dosage of TMPP that inhibited 90% of the cholinesterase of a normal cockroach, but if the DDT-prostrate cockroaches were allowed to recover even briefly by raising the temperature before injection of TMPP, no protection occurred.

The exact role in the mode of action of DDT for the events discussed is not known. Perhaps some of them are merely secondary, and are a result rather than a cause of poisoning. The primary action of DDT on sensory nerves still remains unexplained. Before there is a complete understanding of the mode of action of compounds affecting neuro-transmission in insects, the basic bio-chemistry and physiology of insect excitatory tissue must be more thoroughly investigated. The chemical structures of compounds apparently involved with either cardiac or neuroactivity must be determined before their role under normal conditions can be understood. Only then can their part, if any, in the mode of action of pesticides be determined.

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Synergistic and Antagonistic Actions of Insecticide-Synergist Combinations and Their Mode of Action

YUN-PEI SUN and ELMER R. JOHNSON

Agricultural Research Division, Shell Development Co., Modesto, Calif.

Synergistic and antagonistic actions of pyrethrin synergists in combination with many organophosphorus and chlorinated insecticides have been studied on several species of insects and mites. The highest increase in toxicity to houseflies was 38.7 times for a mixture containing 1% sesamex and methyl 3-[ethoxy(p-dimethylaminophenyl)phosphinyl-oxy]crotonate. Pyrethrin synergists also reduced the toxicity of some organic thiono-phosphorus and cyclodiene insecticides. Toxicity results as well as colorimetric and enzymic analyses indicate that synergistic or antagonistic action caused by a pyrethrin synergist appears to be mainly due to the inhibition of certain biological oxidations which either activate or detoxify the compounds.

IN THE STUDY of joint action of insecticides, a true synergistic action resulting from a mixture of two or more insecticides is rarely found. Although high increases in toxicity to houseflies of pyrethrins, allethrin, etc., in combination with pyrethrin synergists (or activators) are well known, only small increases in toxicity have been reported on other species of insects.

Many pyrethrin synergists have been discovered, but little progress has been made on the study of their mode of action. This report includes not only the studies on the synergistic and antagonistic actions of pyrethrin synergists to vinyl phosphates, phosphonates, and other insecticides, but also the elucidation of a possible mode of action.

Materials and Methods

With the exception of common insecticides and synergists, chemical names