THE EFFECT OF DDT AND CARBARYL ON HEMOLYMPH TREHALOSE OF THE AMERICAN COCKROACH *PERIPLANETA AMERICANA*, L.

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AN ABSTRACT

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Recent work on biochemical anomolies in insects resulting from insecticide poisoning suggested the possibility that carbohydrate levels in insects might be affected. Trehalose, the major transportable form of carbohydrates in insect hemolymph, seemed a probable metabolic indicator of insecticide intoxication.

The concentration of trehalose in the hemolymph of DDT [2,2-bis(p-chlorophenyl)-1,1,1-trichloroethane] and carbaryl (1-naphthyl N-methylcarbamate) poisoned American cockroaches (*Periplaneta americana* L.), waxworms (*Galleria mellonella* L.), and armyworms (*Pseudaletia unipunctata* Haworth) has been studied.

For determinations of trehalose in lyophilized hemolymph from poisoned and non-poisoned insects an anthrone colorimetric test was used. Glucose-trehalose transformations and fate in the fat body were studied by glucose-C^{14} injections.

In cockroaches, DDT and carbaryl were found to decrease the concentration and absolute amount of trehalose in the hemolymph. This effect was not observed for DDE or 1-naphthol.

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The lower trehalose level caused by DDT does not occur in headless cockroaches. There is no respiration peak with DDT in headless cockroaches. Injected glucose-C^{14} in cockroaches is largely converted to trehalose in 30 minutes. The equilibrium glucose $\rightleftharpoons$ trehalose in the cockroach hemolymph is to the right in unpoisoned cockroaches but favors glucose in DDT poisoned cockroaches. The equilibrium glucose $\rightleftharpoons$ trehalose is far to the right DDT, DDE and acetone control cockroaches without heads.

The presence of a positive head factor restricting trehalose synthesis is hypothesized to be due to response of cockroaches to DDT. This hypoglycaemic factor was not extractable by a perfusion method.

A hypothetical role of trehalose depletion in the mode of action of DDT is discussed.

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LITERATURE REVIEW

Trehalose Metabolism

Prior to 1956, trehalose [(α-D-glucosido)-α-D-glucoside] was considered a fungal and plant sugar. It had been reported only as a curiosity in the cocoons of weevils by Berthelot (1859). Trehalase, the enzyme hydrolyzing trehalose, had been identified as a constituent of desert scale excreta (Leibowitz, 1944) and Duspiva in 1954 recognized trehalose as a component of aphid honeydew as well as observing trehalase as a digestive enzyme of the aphids (Chefurka, 1965).

In 1937, Kuwana observed high levels of non-reducing substances in the hemolymph of silkworms fed glucose (Wyatt and Kalf, 1957). The identity of this non-reducing substance remained unknown until Wyatt and Kalf (1956; 1957) showed that trehalose is a major component of insect hemolymph.

Since 1956 there has been a concerted effort on the part of many scientists to 1) delineate the presence of trehalose in the animal kingdom, 2) evaluate its function, 3) determine its metabolic synthesis and catabolism, and 4) elucidate possible control mechanisms of trehalose concentrations in the hemolymph.

I. Occurrence of Trehalose in Invertebrates

Wyatt and Kalf (1957) reported the presence of trehalose in five orders of insects (Hemiptera, Coleoptera, Diptera, Hymenoptera, and Lepidoptera). In four lepidopterous insects trehlose comprised more than 90 percent of the total blood sugar. The larvae of the oriental silkworm, Bombyx mori had trehalose concentrations ranging from 306 to 419 mg trehalose per 100 ml plasma (mg percent). The highest concentrations of trehalose were found in the silk moth Telea polyphemus and ranged from 1036 to 1398 mg percent trehalose. Howden and Kilby (1956) reported trehalose levels between 800 and 1500 mg percent in the locust Schistocerca gregaria. Evans and Dethier (1957) reported concentrations of 200 to 3000 mg percent trehalose in the hemolymph of the blowfly Phormia regina. Duchateau and Florkin (1959) reported that adult bees (Apis sp.) had between 592 and 1203 mg percent threhalose. Steele (1963) found an average of 1070 mg percent trehalose in the American cockroach, Periplaneta americana. In contrast to these high trehalose concentrations, Barlow and House (1960) found that only 1 to 2 percent of the carbohydrates of the hemolymph of the larvae of the fly Agria affinis was trehalose, but glucose totaled 80 percent of the carbohydrates.

Fairbairn (1958) surveyed 71 invertebrate species from many groups for glucose and trehalose. Although most had trehalose, only some nematodes and the insects had more trehalose than glucose. Fairbairn and Passey (1957) located trehalose in the various organs and in the eggs of *Ascaris lumbricoides*.

Although trehalose is not a mammalian sugar, the enzyme trehalase has been found in humans and in rabbit plasma (Courtois *et al*., 1966) and has been isolated and characterized from pork kidney (Grossman and Sactor, 1968) and hog intestine (Dahlquist, 1960).

II. The Function of Trehalose in Insects

A. Activity and Trehalose Levels

Trehalose, as the predominant carbohydrate in the hemolymph of most insects, is comparable to glucose in mammalian systems, serving as a mobile source of energy (Wyatt and Kalf, 1957; Chefurka, 1965). The role of this carbohydrate has been studied during various activities of insects: 1) metamorphosis, 2) diapause, 3) flight, and 4) as a circadian fluctuation.

1. Fluctuations in Trehalose Levels during Metamorphosis

Trehalose plays a relatively minor role in the metamorphosis of insects (Wyatt and Kalf, 1957). There is a decrease by one-half in the hemolymph trehalose level from the pupal stage to the adult stage of the silkworm. However, the glycogen concurrently drops to one-fourth of its pupal level. Low respiratory quotient values in the blowfly, Lucilia cuprina indicate that carbohydrates are not greatly metabolized during the pupal stage, but that the main sources of energy are fats and amino acids (Crompton and Birt, 1967). However, trehalose is used in chitin synthesis. This metabolic pathway was elucidated by Candy and Kilby (1962).

2. Fluctuations of Trehalose during Diapause

Diapause hormone injected into silkworm pupae enhances *de novo* synthesis of trehalase (the enzyme hydrolysing trehalose to glucose) in the pupal ovaries. This results in higher ovarian glycogen concentrations (Yamashita and Hasegawa, 1967). Similar effects are seen with pupae whose supraeosophageal ganglia were excised and then the insects injected with ecdysone or cholesterol (Kobayashi *et al.*, 1967).

3. Fluctuations of Trehalose during Insect Flight

During flight, carbohydrates in the hemolymph are the chief source of mobile energy in insects (Williams *et al.*, 1943). These authors reported that the rate of decrease of glycogen in the fat body of *Drosophila* is parallel to the frequency of the wing-beat. Clegg and Evans (1961) extended this correlation to the trehalose of the hemolymph. High hemolymph trehalose concentrations allow high wing-beat frequencies. They hypothesized that the source of this trehalose is the fat body glycogen and dietary monosaccharides.

4. Circadian Fluctuations of Trehalose

Since the discovery of photoperiodic behavior in animals (Markovitch, 1924) numerous physiological phenomena have been associated with daily rhythms. Nowosielski and Patton (1964) reported peak hemolymph trehalose concentrations in the house cricket *Gryllus domesticus* at 3 hours before dawn (in a 12 hour day). Preliminary data from this laboratory indicate peaks in hemolymph trehalose concentration in the cockroach *Blaberus craniifer* at 3 hours and 12 hours after dawn and low points at 9 hours after dawn and 4 hours after dark (in a 16 hour day).

B. Digestion and Trehalose Levels

The absorption of various sugars by the gut and their incorporation into fat body glycogen and hemolymph trehalose was studied by Horie (1960) in the silkworm, *Bombyx mori*. He reported that sugars which increased the hemolymph trehalose level also increased the fat body glycogen level. Also, ingested trehalose was not as efficient at increasing hemolymph trehalose as were other sugars such as glucose or sucrose. Treherne (1958a, 1958b) studied gut absorption phenomena in the locust, *Schistocerca* *gregaria*. He found that sugars are absorbed by diffusion mainly in the midgut caecae and to a lesser extent in the ventriculus. Once in the hemolymph, the sugars are rapidly converted into trehalose which tends to maintain a steep glucose concentration gradient for movement of glucose into the hemolymph.

III. Trehalose Metabolism and Metabolic Control

A. Trehalase

The first enzyme of trehalose metabolism to be studied in insects was trehalase, the enzyme hydrolyzing trehalose to glucose (Frerejacque, 1941). Numerous investigators have isolated this enzyme from insects and purified it to varying extents (Howden and Kilby, 1956; Kalf and Rieder, 1958; Zebe and McShan, 1959; Saito, 1960; Friedman, 1960a, 1966b; Derr and Randall, 1966). Trehalase was found to be specific for trehalose and was suggested for use in a trehalose assay. The pH optimum is between 5.5 and 6.0, and it has a temperature optimum of 45°C. Its Km value varies from 1.3 to 6.7 x 10^{-4}M. It is not a disaccharide phosphorylase. Saito (1960) during his purification process found two forms of the enzyme. The enzyme is found mainly in the foregut epithelium (Zebe and McShan, 1959) and to a varying extent in other tissues (Saito, 1960) although no physiological function has been attributed to trehalase found at these sites (see Wyatt, 1961 for discussion).

B. Trehalose-6-phosphatase

Trehalose-6-phosphatase which hydrolyzes trehalose-6-phosphate to trehalose has been isolated and described from the blowfly Phormia regina by Friedman (1960b, 1966a). Its optimal conditions are pH 7.0 and 46°C. It has a divalent metal ion requirement best filled by Mg^{++} in a ratio of 1:1 to the substrate.

C. Glycogen - Trehalose Equilibrium System

The rest of the enzymes of trehalose metabolism in insects have been verified to be identical to those in the path of trehalose metabolism in yeast and mold (Leloir and Cabib, 1953; Candy and Kilby, 1961; Murphy and Wyatt, 1965). Synthesis of trehalose and glycogen are competitive (Leloir and Cardini, 1957; Murphy and Wyatt, 1965). Chefurka (1965) suggested that the equilibrium between glycogen and trehalose in insects is governed by phosphorylase a and b and cyclic 3',5'-AMP. This would be similar to the mammalian system (Sutherland *et al.*, 1962). These schemes are summarized from Chefurka (1965) in Figure 1. Treherne (1960) suggests that there is also an equilibrium between glucose and trehalose in the hemolymph.

IV. Control of Trehalose Metabolism

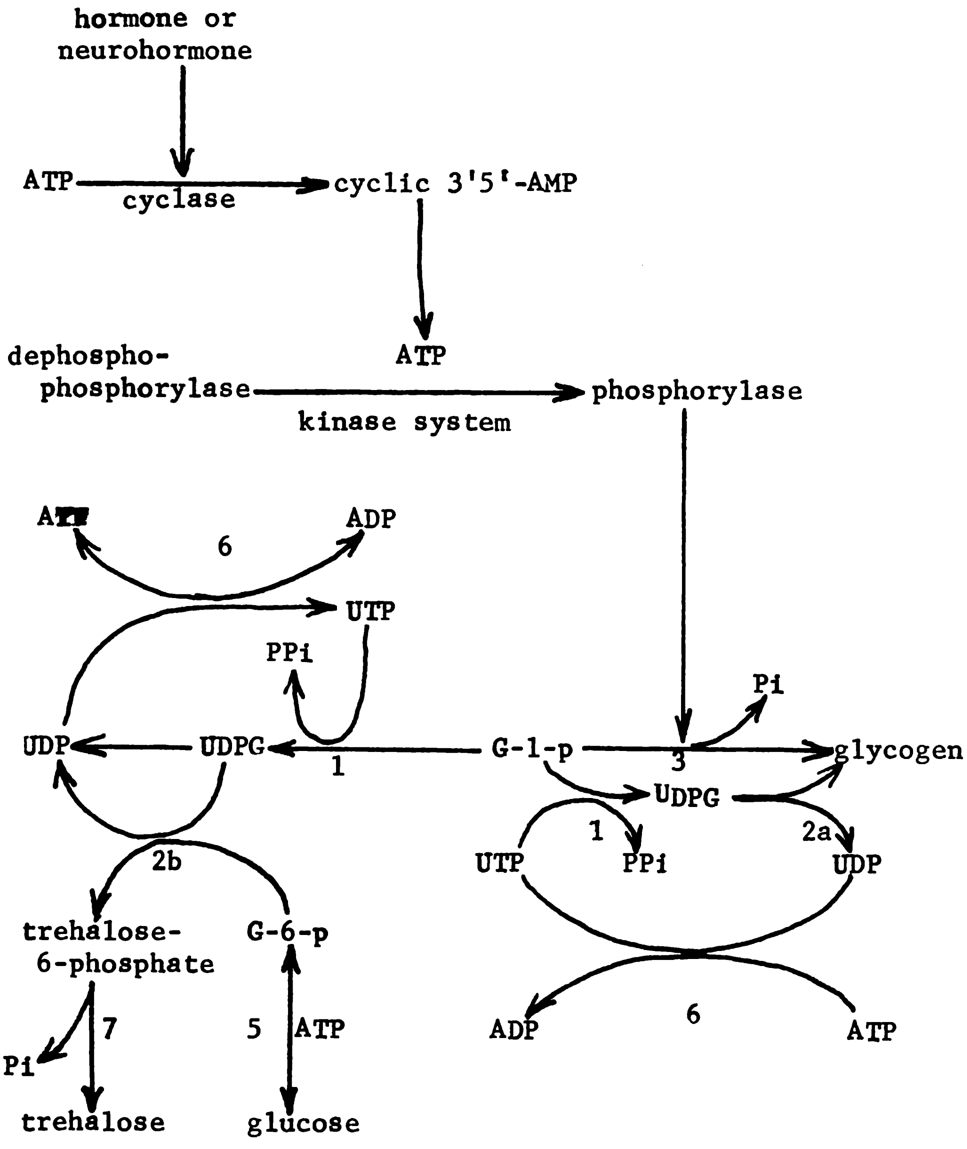
A. Feedback Inhibition of Trehalose Synthesis

Excess trehalose has been found to inhibit incorporation of glucose into trehalose and to stimulate its incorporation into glycogen in *in vitro* fat body incubations (Murphy and Wyatt, 1964; 1965). This inhibition was proposed to work by affecting the enzyme, trehalose phosphate synthetase (UDPG phosphotrehalose transglucosylase in Figure 1). The inhibition works allosterically by binding trehalose to a site separate from the catalytic site. This inhibition can be eliminated by mild protein denaturation.

Product inhibition was also recognized and studied by Friedman (1967a and b). He found that high concentrations of trehalose increase glucose-6-phosphate hydrolysis in fat body extracts of the blowfly, *Phormia regina*. This glucose-6-phosphate decrease may not be the same as observed by Murphy and Wyatt (1964) since in Friedman's extracts no phosphotransferase activity was observed.

B. Hormonal Control of Trehalose and Glycogen Synthesis

Steele (1961) extracted a hyperglycaemic factor from the corpus cardiacum of the American cockroach, *Periplaneta americana*. When injected into other cockroaches



1. UDPG pyrophosphorylase
2a. UDPG glycogen transglucosylase
2b. UDPG phosphotrehalose transglucosylase
3. Phosphorylase
4. Phosphoglucomutase
5. Hexokinase
6. UDP kinase
7. Trehalose phosphate phosphatase

Figure 1: Glycogen-trehalose equilibrium systems (from Chefurka, 1965).

this extract increased the hemolymph trehalose 150 percent within 5 hours, increased inorganic phosphate release in the hemolymph, and lowered the amount of glycogen in the fat body (Steele, 1963). Steele suggested that the hormone affected the phosphorylase activity. Ralph and McCarthy (1964) in similar experiments found that the hyperglycaemic function was present (in decreasing activity) in the corpus cardiacum, brain, corpus allatum and subeosophageal ganglion. Bowers and Friedman (1963) noted a hyperglycaemic hormone in the cockroach *Blaberus discoidalis*. Natalizi and Frontali (1966) found and purified two hyperglycaemic factors from the American cockroach, *Periplaneta americana* and the honey bee, *Apis mellifera*.

Steele (1963) hypothesized that the hormone factor was a peptide. Brown (1965) isolated two distinct hyperglycaemic factors from the cockroach by paper chromatography. They were both chymotrypsin sensitive and he hypothesized that they were low molecular weight polypeptides.

Friedman (1967a and b) found that the hyperglycaemic hormone did not work on blow flies (*Phormia regina*) fed *ad lib.*, but did work on starved blow flies. He hypothesized that the trehalose synthesis system normally works at full capacity as governed by the feedback inhibition of trehalose. When the trehalose concentration is low and there are plenty of sugars from the gut, these sugars are efficiently converted into trehalose. During starvation, however, glycogen breakdown is the rate-determining factor of trehalose formation; so Friedman hypothesized that the hormone acts on glycogen breakdown. Wiens and Gilbert (1967a) hypothesized several sites of action of the hyperglycaemic hormone, including: a) an increase of phosphofructokinase, b) inhibition of the pentose phosphate cycle, c) hexokinase activation, and d) trehalose-6-phosphate synthetase activation.

Carbaryl and DDT

I. Carbamate Insecticides

Prior to insecticidal use, carbamate compounds were used in Africa for ordeal trials and in modern medicine to treat muscle spasms, glaucoma, to lower blood pressure and for other uses. Use of carbamates as insecticides began with work by Gysin at Geigy Chemical Company about 1947 on N,N-dimethyl carbamates. About 1950 work on N-methyl carbamates was initiated by Metcalf in California (Kolbezen *et al.*, 1954). The most widely used carbamate to date, carbaryl (1-naphthyl methylcarbamate) was announced by Haynes *et al.* (1957).

The inhibition of cholinesterases by carbamates, due to their slowly-reversible binding with anionic sites, is responsible for their toxicity to vertebrates where blockage of nerve function results in rapid death. Inhibition of cholinesterase works by carbamylation of the enzyme (Wilson *et al.*, 1961). Inhibition by N-unsubstituted carbamates in such carbamylation is more readily reversible than the inhibition by dialkyl carbamates (Wilson *et al.*, 1960).

In insects, the action of carbamates is not as straightforward as in vertebrates for several reasons. The insect myoneural junction is probably not cholinergic (Wigglesworth, 1958; O'Connor *et al.*, 1965; O'Brien, 1967) and so is not affected by carbamates. In experiments where cholinesterase inhibition has been demonstrated, the potency of the compound as an insecticide does not correlate with its activity as a cholinesterase inhibitor (Kolbezen *et al.*, 1954; Casida *et al.*, 1960; Eldefrawi *et al.*, 1960).

No other mode of action has been seriously considered for carbamate insecticides (Casida, 1963; O'Brien, 1967).

II. DDT

A. History

Although DDT was first synthesized in 1874 by Zeidler, its insecticidal properties were not established until 1939 by P. Müller of the Geigy company. Widespread civilian use did not come about until after World War II. DDT's extremely widespread use is due to its cheapness, extremely wide spectrum of insecticidal activity, stability, and low acute mammalian toxicity. The stability of DDT leading to its accumulation as residues in the environment and its storage in animal adipose tissue has been the subject of controversy in recent years, causing DDT to lose some of its popularity. Possible chronic poisoning of animals by DDT has caused concern, especially in regard to hormonal balances and animal reproduction (McLean, 1968;

Wurster and Wingate, 1968).

B. DDT Mode of Action

Unlike the carbamates, there is no paucity of suggestions for the mode of insecticidal action of DDT. However, the theories are incomplete at best and restricted to one or two types of observations. The gross effects and symptomology observed during DDT poisoning will be discussed, followed by discussion of three general aspects of the mode of action: a) physical-chemical factors, b) biochemical and metabolic anomolies, and c) physiological explanations.

1. Symptoms

The most obvious symptoms of DDT poisoning are the tremors, hyperactivity, ataxia and eventual paralysis of the insects. Early work attempted to establish what part of the nervous system was affected. Yeager and Munson (1945) found that DDT could cause the symptoms in isolated legs of roaches, in intact legs where the roaches had no hearts, or in legs of roaches in which only the ganglia were treated with DDT. Tobias and Kollross (1946) did similar work with sectioned and decapitated insects. They found that with low concentrations of DDT the intact sensory-motor reflex arc was needed to demonstrate symptoms, although the motor nerves were affected directly by high DDT concentrations. Morrison and LeRoux (1954) found that the head was necessary for the lethal action of DDT in houseflies. Roeder and Weiant (1946) described the high-frequency trains of spike potentials caused by DDT in the axons of cockroach legs. They confirmed that sensory nerves, such as the large crural nerve of leg proprioceptors, were most sensitive to DDT. Welsh and Gordon (1947) and Gordon and Welsh (1948) described in detail the trains of impulses and tetanic muscle contractions from DDT poisoned crayfish.

Ludwig (1946) studied the effect of DDT on the Japanese beetle (*Popillia japonica*) and found that it caused a marked increase in respiration. Lord (1949) found a similar peak in respiration with *Oryzaephilus surinamenus* poisoned with DDT. A study by Harvey and Brown (1951) utilizing the German cockroach and a number of insecticides, showed that the roaches had a respiration peak 30 minutes after DDT poisoning.

Weight loss and water loss were other physiological parameters affected by DDT poisoning that were studied by Ludwig (1946). Patel and Cutkomp (1967) also described effects of DDT on the heart-beat of the American cockroach.

2. Physical-Chemical Theories of DDT Mode of Action

Because of obvious nervous system anomolies caused by DDT, many theories on mode of action have centered around an explanation of these symptoms. In 1947 Welsh and Gordon hypothesized that the cause of intoxication was probably a physical rather than a chemical lesion in the nervous system. Any such hypothesis, however, would have to consider the negative temperature coefficient of DDT (Vinson and Kearns, 1952).

In the early 1950's a considerable amount of work was done on the basic physiology of the insect nervous system. The sheath around all insect nerves was found to be an ion barrior (Hoyle, 1953). Although the potassium ion concentration in the hemolymph bathing the nerves is quite variable, the sheath keeps the inner concentration fairly constant. De-sheathing the last abdominal ganglion of roaches makes it much more susceptible to insecticide ions (Twarog and Roeder, 1957). O'Brien and Fisher (1958) and O'Brien (1959) used this ion barrier to explain why the many ionizable neurophysiological agents toxic to mammals (who have no such sheath) are much less toxic to insects.

Investigations of axonic transmission in insect nerves were made by Yamasaki and Narahashi (1958) and Narahashi and Yamasaki (1960). They found that DDT affected the negative after-potential of single nerve impulses causing a shoulder to form on the spike. This after-potential is associated with potassium ion efflux and it was therefore suggested that DDT affects the permeability of the nerve cell membrane (see O'Brien, 1967 for a discussion). Indeed, the effect of DDT on nerve permeability to ions had been studied as early as 1948 by Gordon and Welsh who suggested a complex of DDT with axon surface components. Gunther *et al*. (1954) and Gunther *et al*. (1958) studied the possibility that such complexes could be formed by Van der Waal's forces. Mullins (1955) studied the steriochemistry of a number of insecticides and analogues as a basis for describing such complexes.

O'Brien and Matsumura (1964) proposed that DDT might form a charge-transfer complex with the nerve membrane which upsets the charge balance and conductivity, thus causing the observed symptoms. Some evidence for such a complex was presented by Matsumura and O'Brien (1966a and b) and the effect on potassium and sodium ions was confirmed. Similar work with dieldrin was reported by Matsumura and Hayashi (1966).

From work on mammalian nerves it was similarly found that ion concentrations were important in DDT poisoning (Koster, 1947) and that poisoning symptoms were proportional to the amount of DDT found in the brain (Dale *et al*., 1963).

3. Biochemical Causes of DDT Intoxication

Since nervous disfunction is the most obvious symptom of DDT poisoning, the most logical place to look for biochemical changes is in the nerve. Tobias *et al*. (1946) noted that free acetylcholine in the nervous system of DDT-prostrate flies and roaches increased 200 percent while the bound acetylcholine decreases. However, the acetylcholine-forming enzymes were unaffected. Lewis (1953) also found higher acetylcholine levels in DDT prostrate houseflies. Lewis *et al*. (1960) found that the rise in acetylcholine in both physically-induced and DDT-induced prostration was similar. The magnitude of acetylcholine increase could be correlated to the degree of neuromuscular activity before prostration. They suggested that the rise was due to release of bound acetylcholine from the axons. The rise could not be attributed to increased activity of the acetylcholine synthesizing enzymes, choline acetylase or acetylkinase (Rothschild and Howden, 1961). Interpreting data of Sternburg and Hewitt (1962), Winteringham (1966) concluded that DDT poisoning increases acetylcholine turnover in the ventral nerve.

The high concentrations of amino acids in insect hemolymph lend themselves nicely to monitoring for possible changes as a result of insecticide poisoning. Winteringham (1958) reported accumulation of glutamine in the hemolymph. The rise in glutamine was hypothesized to be caused by transamination from other amino acids which are oxidized in the Krebs citric acid cycle. Corresponding to this report, Corrigan and Kearns (1958) reported a sharp drop in free proline with DDT poisoning while the other amino acids remained at constant levels in the hemolymph. Hoy and Gordon (1961) found that injecting proline to make up for what is lost does not alleviate DDT symptoms.

Cline and Pearce (1963) found that DDT interfered most with proline, formate and glycine metabolism in houseflies. Injected formate-C^{14} was converted more into uric acid and allantoin than into proline in DDT poisoned insects. It is interesting to note that carbamate insecticides did not affect formate metabolism. Corrigan and Kearns (1963) found that injected proline-C^{14} was oxidized to carbon dioxide three times faster in DDT-poisoned than control American cockroaches. Also, it was metabolized to glutamine-C^{14} which Winteringham (1958) had suggested might be an ammonia trap for amino acid oxidation. Corrigan and Kearns also suggested that the demand for oxidizable carbon shifted metabolism to proline. Cline and Pearce (1966) confirmed the drop in proline by radiotracer studies. They also found a decrease in radiolabeled trehalose after glucose-C^{14} injections in DDT-treated insects. Patel _et_ _al_. (1968) surveyed all the amino acids and found that their average concentrations decreased 22.7 percent in DDT-poisoned susceptible houseflies. In resistant houseflies, however, the total amino acids increased 5.5 percent.

Since 1960 there has been some detailed work on the effect of DDT on specific metabolic pathways. Winteringham *et al*. (1960) reported significant breakdown of ATP in DDT-poisoned flies. This drop was reversed by injecting glucose. Sparing the insect hypermotor activity during DDT poisoning by the use of anesthesia did not preserve the ATP level. At no level of poisoning were the entire energy reserves of the housefly exhausted. With DDT poisoning there is also a drop in α-glycerophosphate (Heslop and Ray, 1963). Agosin *et al*. (1961 and 1963) studied the influence of DDT on intermediary carbohydrate metabolism in *Triatoma infestans*. They found that DDT (as well as non-toxic DDE) inhibited anaerobic glycolytic pyruvate production by cell free preparations. Differences in enzyme inhibition were found between males and nymphs of *Triatoma infestans*. DDT enhanced incorporation of glucose into carbon dioxide, glycogen, and fatty acids while DDE did not. Glucose oxidation was 77 percent by the pentose phosphate pathway in DDT treated insects compared to 22 percent in normal insects. DDT increased the NADP level but not the NADP/NADPH ratio, possibly because of increased NAD-kinase. The increased NAD-kinase is thought to be related to detoxification and resistance

(Ilevicky et al., 1964). Increased glutathione turnover (glutathione is necessary for activity of DDT-dehydrochlorinase, a detoxifying enzyme) and protein synthesis is also related to DDT poisoning in resistant houseflies (Agosin et al., 1966).

4. Neurohormonal Explanations of DDT-Poisoning

Insects, like other organisms, have hormonal systems to integrate the diverse body functions. The centers of the insect system are the corpus cardiacum and the corpus allatum, nerve tissue just posterior to the supraeosophageal ganglion. Numerous functions have been attributed to secretions from these bodies, such as control of hemolymph sugar level, daily activity rhythms, heart-beat rate, ovary development, diapause and metamorphosis.

Sternburg and Kearns (1952) found that the blood of DDT-poisoned cockroaches, when injected into normal insects, produced DDT-poisoning symptoms. The injected blood did not contain sufficient DDT to cause the symptoms, so they concluded that the DDT had induced production of a neurotoxin which was capable of causing DDT-poisoning symptoms. This toxin caused multiple firings in ganglia and sensory nerve fibers. It was unstable in the hemolymph, but was dialyzable and stable in the dialysate (Shankland and Kearns, 1959). It was found that body stress, such as physical immobilization, forced movement, or electrical stimulation produced similar (although not necessarily identical) blood substances which also caused DDT-like symptoms in unpoisoned insects (Heslop and Ray, 1959). Such substances were also produced in the abdominal nerve cord (Sternburg _et al._, 1959). The production and release of these substances was observed histochemically in the corpus cardiacum (Hodgson and Geldiay, 1959). By parabiosis experiments, Colhoun (1960) concluded that the toxin was not the primary cause of the DDT death.

Isolation and identification of the toxins is difficult because of the small quantities present in the insects. Sternburg (1960) tried to solve this problem by using crayfish which produced similar toxins. He found that the toxin was not a known neurohumoral agent or a DDT metabolite. Hawkins and Sternburg (1964) identified it partially as an aromatic amine, possibly an ester. Patel and Cutkomp (1968) found that the substance was fluorescent and therefore easily detectable. It is not produced by insect treatment with certain organophosphates and therefore is not a dying tissue response.

Davey (1963) found that enforced activity of cockroaches also produced a cardiac stimulator in the hemolymph. The literature on such toxins is reviewed by Sternburg (1959; 1960; and 1963).

MATERIALS AND METHODS

I. Maintenance of Insects

In preliminary experiments, American cockroaches (*Periplaneta americana* L.) were maintained in battery jars with wooden separators at a constant 12 hour day for at least two weeks prior to use. In later experiments 16 hour day conditions were maintained. The cockroaches were fed dog food pellets and provided with water on cotton wicks. Only adult males were used in the experiments. The cockroaches were obtained from Rutgers University, Department of Entomology and Economic Zoology (preliminary experiments only), Ward's Natural Science Establishment, Inc. and the Department of Entomology, University of Illinois.

The greater waxworms (*Galleria mellonella* L.) were raised in continuous dark on an artificial diet consisting of a mixture of the following: 100 g honey, 72 ml glycerol, 40 ml water, 136 g pablum, 40 g brewers yeast, 20 g beeswax (Beck, 1960). The beeswax in acetone was added last and the acetone was allowed to evaporate at least 24 hours before use. Last instar larvae were used in all experiments. Care was taken not to use individuals beginning to pupate. This was done by not using the largest larvae which were beginning to darken. In dissections, these would be easily identified by the beginning of pupal body formation and muscle degeneration. The original waxworm colony was obtained from Central Bait Company, Bath, Michigan.

The armyworms (Pseudaletia unipuncta Haworth) were used as obtained from the Bioproducts Division, Dow Chemical Company, Midland, Michigan. They had been raised on bean plants but some were maintained on an artificial diet.

II. Intoxication Techniques

The insects used in experiments were poisoned by contact with treated panels, injections of acetone-insecticide solutions or topical treatment.

A. Some of the cockroaches were poisoned by exposure for 1 to 1½ hours on plywood panels evenly coated with an insecticide applied as an acetone solution. Ten ml of the p,p'-DDT (99%, City Chemical Corporation, New York) or p,p'-DDE (98%, Pesticides Repository, United States Public Health Service, Pesticide Research Laboratory, Perrine, Fla.) solutions were spread over the 100 square inch surface of the plywood panels. Control panels were treated with acetone. The panels were treated at least 18 hours before the exposure of the cockroaches in order to insure the complete evaporation of acetone.

To determine proper insecticide dosage, cockroaches were exposed to the panels for one hour and then kept in plastic containers (20.3 cm in diameter by 7.6 cm) with food pellets and water, where they were observed until death or up to 5 days.

DDT concentrations of 0, 1, 5, 10 and 50 mg per square inch were tested. When the cockroaches were on their back and unable to right themselves, they were considered dead. This corresponds to the "prostrate" classification of Heslop and Ray (1959). For the treatment of cockroaches from which hemolymph was taken for trehalose determinations, 5 mg DDT or DDE per square inch was used. The insects were confined to the panels by a large (8 inch) crystallizing dish. After being taken from the panels, they were held in 8 inch battery jars with moisture supplied by a damp paper towel until hemolymph collection.

B. The waxworms and some cockroaches were treated by insecticide injections. After anesthetization with carbon dioxide, the cockroaches were injected with up to 10 µl of the insecticide-acetone solution. A Hamilton 10 µl syringe or a one-forth cc tuberculin syringe was used with a microapplicator. A smaller quantity of solvent was later found to be more desirable for both animals. Cockroaches were injected in the membrane between the fourth and fifth sternites and waxworms were injected in the ventral aspect between the prolegs or in the proleg pads. The injections were made deep into the insects so that if there was some bleeding the insecticide was not lost.

C. Armyworms were treated with insecticide-acetone solutions topically, since it was found that they bled profusely from the slightest puncture.

D. The insecticide treatment of perfused dissections is described in Section IX.

III. Hemolymph Collection

A. Cockroaches

Hemolymph samples were obtained from cockroaches by centrifugation (Sternburg and Corrigan, 1959) or with capillary pipettes.

1. For the centrifugation method, the cockroaches were first anesthetized with carbon dioxide. The head and the tip of the abdomen were dipped in melted paraffin to close off the alimentary tract. The antennae and legs were then cut off close to the base. Groups of 3 to 5 cockroaches were placed head down in plastic containers with perforated bottoms. In some cases a perforated disk was just sealed to the bottom of a plastic centrifuge tube with paraffin. If necessary, empty space in the plastic containers was filled with wax-covered cheese cloth wads

to prevent crushing the cockroaches at the bottom of the plastic containers. The tubes were then centrifuged at 3000 rpm for 10 minutes. The entire process of anesthetizing, waxing, cutting, and centrifuging took between 30 and 45 minutes for 20 cockroaches. The total volume of hemolymph (approximately 0.25 ml for each group of 5 cockroaches) was recorded and a sample was placed in a small vial and frozen until lyophilized.

2. The pipette method of hemolymph collection is faster and less destructive to the test animals during collection. The cockroaches are similarly anesthetized with carbon dioxide. Then the tips of the antennae, tarsi, cerci and sometimes styli and phallomeres are cut off. As droplets of hemolymph form they are collected in a disposable Pasteur capillary pipette. Often squeezing or massaging the insects increases the volume of hemolymph obtained. The volume of hemolymph collected may be approximated by calibrating the pipettes or by determining the wet weight. The volume collected from a single cockroach varies from 5 µl to more than 60 µl (often depending on insecticide treatment) with a representative value of about 30 µl.

B. Waxworms and Armyworms

Hemolymph from waxworms and armyworms was collected only with Pasteur capillary pipettes. After carbon dioxide anesthetization, the insects are gently bent in half. Then a puncture is made or a proleg is cut off and the exuded hemolymph collected. Squeezing is often necessary to obtain adequate hemolymph from the waxworms. However, armyworms bleed very rapidly from punctures with very little pressure applied. With pressure, the gut of armyworms will be extruded. Each waxworm will produce up to 60 µl of hemolymph, with 30 µl as a usual amount. Each armyworm produces up to 90 µl with 70 µl as a normal amount.

C. Preparation of Hemolymph

Preparation of the hemolymph followed procedures of Wyatt and Kalf (1957). Weighed, frozen hemolymph samples were first lyophilized for 5 to 24 hours. They were then reweighed and the amount of water lost was calculated. The dry hemolymph (or a sub-sample) was placed in a graduated centrifuge tube with 2.0 ml of 60% ethanol. The tubes were capped and heated at 75 to 80° for 30 minutes. With some samples, for convenience, the hemolymph samples were lyophilized in pre-weighed cups shaped from aluminum foil. In these cases, the whole cups with the entire amount of dried hemolymph were unfolded into the ethanol in the centrifuge tube. After the heating, more ethanol was added to replace that which had evaporated. If the ethanol solution was then cloudy or had particles floating on the surface, it was centrifuged.

Between 0.1 and 0.2 ml of this ethanol solution was used for the anthrone test to determine trehalose levels. With radioactive samples, similar volumes were taken and placed in scintillation vials with 15 ml of the PPO-POPOP fluor without solublizers and 20 µl were used for spotting thin layer plates.

IV. Anthrone Test for Trehalose

Anthrone reagent was prepared by the method of Carrol, Longley and Roe (1956). The reagent was made in 200 ml batches by first mixing 56 ml water and 144 ml of concentrated H_2SO_4 and allowing it to cool to 85 or 90°; 100 mg anthrone (9,10-dihydro-9-oxoanthracene) and 2.00 g thiourea were then mixed into the acid solution. The reagent solution was cooled rapidly to 30°, stored in the refrigerator, and must be used within two weeks.

This anthrone reagent was used in determining trehalose levels in ethanolic hemolymph extracts by the method of Wyatt and Kalf (1957). The 60% ethanolic hemolymph extracts were measured into test tubes. Appropriate standards in similar volumes of 60% ethanol were used for the standard curve. These samples were heated with boiling water or steam and carefully reduced to dryness with a stream of air. The ethanol must be evaporated because of interference with the test. To each dry sample, 0.2 ml of

0.1 N H_2SO_4 was added. The tubes were loosely capped with bacterial test tube caps and placed in boiling water for 10 minutes. This acid treatment hydrolyzed sucrose and glucose-1-phosphate, if present. Similar 10 minute treatment with 0.2 ml of 6 N NaOH followed to destroy any reducing carbohydrates present.

To each tube, 4.0 ml of cold anthrone reagent was added, the tube was thoroughly shaken, capped, and placed in boiling water for 10 minutes. Optical density (O.D.) at 620 mμ versus the reagent blank was determined on a Spectronic 20 (Bausch and Lomb, Inc., Rochester, New York). The reagent blank when read versus distilled water was between O.D. 0.130 and 0.180 for fresh anthrone reagent, properly made.

If the anthrone is cooled too much before or after the 10 minute boiling a precipitate, presumably sodium sulfate, occurs and affects readings. Dilution of the anthrone reagent at any time will also cause a precipitate. A standard curve was determined with each anthrone test. Reciprocal extinction coefficients for the anthrone test for trehalose average between 0.130 and 0.180 mg trehalose per O.D. unit.

V. Thin Layer Chromatography

The thin layer plates giving the best results were 1/3 mm Silica Gel H (E. Merck AG) on 20 x 20 cm glass plates. They were allowed to partially dry and were then placed in an oven at 100° for 2 hours. They were developed with the direction of application of the silica gel in a 0.02% EDTA (ethylene diamine tetraacetic acid; salts of the acid are more soluble in water) in 1.0 N HCl solution to bind any heavy metal ions and clear the plates of water-soluble organic compounds. This treatment was found necessary to prevent subsequent streaking of the carbohydrates. The plates were then reactivated at 100° for at least 2 hours. This heating produced a yellow line corresponding to the EDTA - HCl solvent front. The plates were stored in a $CaCl_2$ desiccator until used. The carbohydrates were spotted on the plates to run at right angles to the direction of application of the silica gel. Solvent systems used were:

I. Methanol:NH_4OH:water (6:1:3) (Bandursky and Axelrod, 1951)

II. Methanol:HCOOH (88%):water (80:15:5) (Bandursky and Axelrod, 1951)

III. Methyl cellosolve:methyl ethyl ketone:3 N NH_4OH (7:2:3) (Mortimer, 1952)

The thin layer plates in solvent III were developed twice, being left in the desiccator overnight between runs. This solvent was replaced when it began to turn yellow.

The following methods were used for detection of carbohydrates:

1. Concentrated H_2SO_4 spray, heat at 100° until charred for detection of all carbohydrates.
2. Silver nitrate spray (Trevelyan *et al.*, 1950) for reducing sugars.
    a. Spray with $AgNO_3$ reagent made by adding one ml of a saturated, aqueous $AgNO_3$ solution to 200 ml acetone. Add water dropwise to redissolve the precipitate.
    b. Spray with 0.5 N NaOH in aqueous ethanol, made by diluting 1.0 N NaOH with ethanol.

Plates with radioactive spots were either autoradiogramed or separate areas scraped into vials with a razor blade and counted in the scintillation counter as described in the next section.

VI. Radioactive Carbon-14 Procedures

Radioactive glucose used was uniformly labeled with carbon-14 at 196 μc/mmole. The radioactive glucose was in an aqueous solution with 3% ethanol at a concentration of 0.05 mc/ml. Generally 2 μl of this solution, or 222,000 dpm, were injected into test animals. For injections, a 10 μl Hamilton syringe was used. Waxworms were injected through the pad of a proleg or through the ventral cuticle of the posterior quarter of the insect. Cockroaches were injected into the membrane between the fourth and fifth sternal plates.

Radioactive tissue was handled according to the Beckman Bio-solve BBS series solublizers instructions: 0.2 ml of fat body homogenates were heated at 90° for 20 minutes with 0.2 ml of 10% NaOH in scintillation vials. Two ml of BBS-2 solublizer or enough to neutralize the base were added to the vial prior to the addition of 15 ml of scintillation fluor [5.5 g PPO (2,5-diphenyloxazole) and 0.1 g POPOP (1,4-bis-2-(5-phenyloxazolyl)-benzene) per liter of solvent: 2:1 toluene:ethylene glycol monomethyl ether]. Up to 65 percent counting efficiency was obtained by this method.

Ethanolic hemolymph extracts were placed in 15 ml PPO-POPOP scintillation fluor without evaporation of the ethanol and without solubilizers. Chloroform extracts of the fat body homogenates were placed in scintillation vials. The chloroform was completely evaporated before the fluor was added. No solublizer was used. Aliquots of 0.2 to 0.4 ml of fat body rinse-water were counted in 15 ml of fluor without solublizers.

Silica gel thin layer plate scrapings were placed directly in scintillation vials. Because these plates had been sprayed with concentrated H_2SO_4 and charred for detection of spots, a solublizer was used to increase counting efficiency. BBS-1 or BBS-3 solublizers worked equally well. Addition of 0.5 ml solubilizer resulted in counting efficiencies of up to 80%. However, charring caused a loss of about one-third the radioactive material. These experiments were done assuming that for any given plate, radioactivity loss due to charring was uniform.

Radioactive spots on thin layer plates were also detected as autoradiograms with Kodak No-Screen medical X-ray film (NS-54T film). The film was placed in contact with the thin layer plate for up to 5 days, depending on the amount of radioactivity present. When the film was developed, 100 dpm could be detected after 24 hours exposure as a dark spot on a light background.

A Nuclear-Chicago Mark I Liquid Scintillation System was used for radioactivity counting. Counting efficiencies were determined by using a quenched series and the B/C channels ratio (narrow-band carbon-14: wide-band carbon-14 windows) or the external standard ratios of A/C (tritium window: carbon-14 window).

VII. Glucose Carbon-14 Metabolism Studies

The fate of uniformly labeled glucose-C^{14} with and without poisoning in cockroaches was studied by the procedures described above.

The cockroaches were poisoned by 1½ hours exposure to insecticide-treated panels, anesthetized by carbon dioxide, and injected with 2 µl of randomly labeled glucose-C^{14} (about 222,000 dpm). At times varying from 5 minutes to 9 hours after the radioactivity injections, hemolymph was collected by the capillary pipette method. The cockroaches were immediately frozen on dry ice after the bleeding. The wet weight of the hemolymph was recorded and the samples were then lyophilized. After dissolving the trehalose in ethanol, 0.2 ml was counted in the scintillation counter, 0.2 ml was used for an anthrone test to determine trehalose, and 0.02 ml was spotted on a thin layer plate. The spots on the thin layer plate were detected with H_2SO_4 spray and charring. These spots were scraped and the carbon-14 detected with the scintillation counter.

The frozen cockroaches were individually defrosted and the fat bodies dissected out under saline (11.0 g NaCl, 1.4 g KCl, 1.1 g $CaCl_2$ per liter). They were rinsed in distilled water and homogenized in one ml distilled water in a glass homogenizer (Rochester Scientific Company, Rochester, New York). Aliquots of 0.2 to 0.4 ml of these rinses were counted with the scintillation counter. The total volume of the homogenate was recorded and 0.2 ml was placed in a scintillation vial. After treatment with base and solubilizer, 15 ml fluor were added to the scintillation vial. A 0.2 ml aliquot was placed in a plastic cup, lyophilized and weighed to determine the dry weight of the fat body. A half ml cold chloroform was added to the remaining homogenate. It was then homogenized and this homogenate centrifuged to produce a fatty layer between the chloroform and water layers. A 50 µl aliquot of the chloroform was counted in the scintillation counter, the fatty layer was discarded, and the aqueous layer was similarly re-extracted with chloroform until it was clear (2 to 3 extractions). The volume of the final aqueous extract was determined. A 0.1 to 0.2 ml aliquot of this aqueous extract was counted in the scintillation counter and 0.02 ml was spotted on a thin layer chromatogram. The spots on this developed chromatogram were visualized by spraying with H_2SO_4 and charring after which they were scraped into vials and counted in the scintillation counter.

VIII. Ligation of Cockroaches

For cockroaches, ligatures were made while the animals were anesthetized with carbon dioxide by tying thread around the neck posterior to the cervical sclerites. Leakage of glucose-C^{14} in the hemolymph from the body into the head showed that this ligature was not tight enough. In order to overcome this flow, the head was cut off after the ligation was made.

IX. Perfusion Technique (Kater, 1968)

The head and thorax of living, anesthetized cockroaches were positioned on a cork, the top of the cranium was cut off with a razor blade, and the sclerites and membranes over the cervex region were removed. Exposed tracheae were cut away to expose the corpora cardiaca (CC) and the corpora allata (CA). The dissection was kept continually moist with saline (11.0 g NaCl, 1.4 g KCl, 1.1 g $CaCl_2$ per liter). To collect a perfusate, a disposable Pasteur capillary pipette was placed adjacent to the CC and CA and 50 μl of saline was dropped on the area over a 15 minute period and collected in the capillary pipette.

A perfusate resulting from DDT treatment was obtained by drenching the exposed dissection surface of the cockroach with a one mg DDT per ml corn oil solution for a period of 5 to 10 minutes. The oil and insecticide were rinsed off with copious amounts of saline although small droplets remained. The amount of DDT getting into the perfusate was not detectable by thin layer chromatography using rhodamine B as a chromogenic reagent (silica gel H thin layer plates, hexane:ether 9:1 as solvent). However, a peak was detected by gas chromatography, but the elution time did not correspond to either DDE or DDT. These perfusates were frozen and stored on dry ice until assayed, generally within a few hours.

To assay the perfusate, 8 μl were injected into a cockroach along with 2 μl of the glucose-C^{14}. In the first series of experiments the cockroaches were bled 5 hours after the injections and the fat body then excised. These were analyzed for radioactivity and carbohydrates as described in section VI.

In later experiments, only the hemolymph was analyzed and this was collected two hours after injections.

RESULTS AND INTERPRETATIONS

I. Procedural Results

A. Panel Toxification

A DDT concentration on panels of 5 mg per square inch was selected as the poisoning concentration for the American cockroaches. After one hour on these panels the insects showed typical DDT symptoms (tremors, convulsions, and ataxia), but remained active for at least 12 hours after poisoning. This was adequate time for obtaining the hemolymph samples, but did not permit complications resulting from reduced water and food intake and a changed day:night ratio.

B. Anthrone Tests

1. Figure 2 shows the spectral transmittance of trehalose reacted with anthrone reagent. The high plateau in the spectrum between 610 and 630 mμ was utilized in this assay.

2. Results of the reaction of anthrone reagent with other sugars and compounds is shown in Table 1.

3. The salts of the minimal saline used in the experiments were tested with glucose and the anthrone

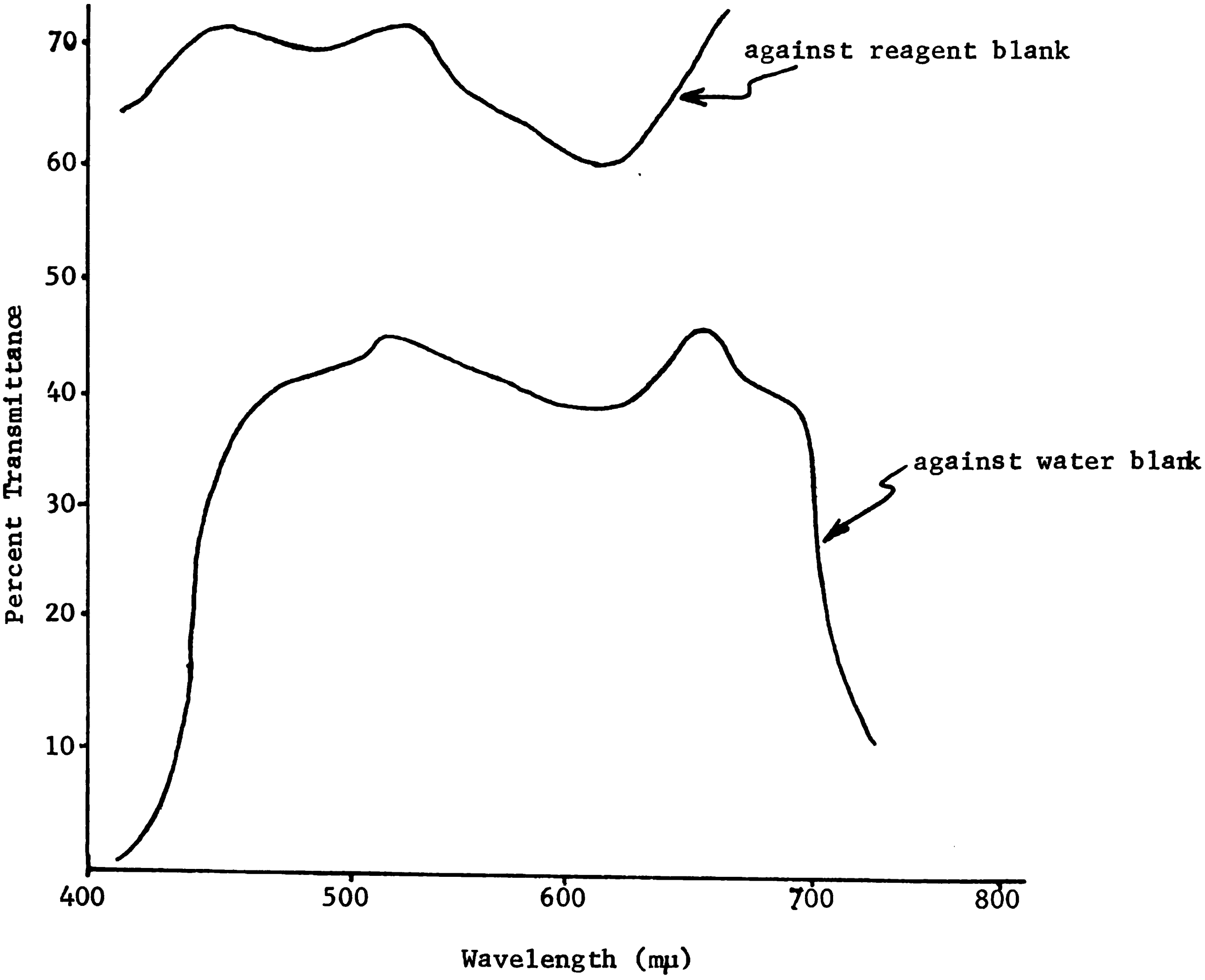


Figure 2: Spectral transmittance of the anthrone reaction with trehalose (0.050 mg).

Table 1: Reaction of Compounds with Anthrone Reagent (620 mμ)

Compound	Extinction coefficient	Comments
glucose	5.7	
sucrose	8.5	
trehalose	5.3	
UDPG	1.7	
glucose-1-phosphate	+	reaction present but not quantitative as run
glucose-6-phosphate	+	reaction present but not quantitative as run
tryptophane	-	pink reaction
glutamic acid	-	no reaction
glycerin	-	no reaction

Table 2: Salt Effects on the Anthrone Reaction with Glucose

	Extinction coefficient
glucose	5.3
glucose + NaCl (11 mg)	6.5
glucose + KCl (1.4 mg)	5.5
glucose + $CaCl_2$ (1.1 mg)	5.6

reagent to determine quantitative interference. NaCl, KCl and $CaCl_2$ in concentrations present in the saline solution increased the readings as shown in Table 2.

4. The data from the anthrone tests of hemolymph samples for trehalose were calculated as mg trehalose per 100 ml whole hemolymph (or 100 g whole hemolymph if volume data were not available) (abbreviated mg %) and as mg of trehalose per gram of lyophilized hemolymph (abbreviated mg/g). Values for trehalose based on the wet weight of hemolymph were judged to be less reliable than those based on the dry weight because of the problems of water balance in the insects. With water available, the insects might ingest extra water during the experiments or dehydrate at differing rates. Such changes were observed to occur to some extent. Poisoned insects provided with water during the test tended to have more hemolymph available than did non-poisoned insects. Also, the density of this hemolymph was lower. The data presented in Table 3 (a and b) show this increased volume and decreased density. The data are for hemolymph collected from cockroaches by centrifugation, a method of collection giving fairly constant volume yields. Data based on such "dilution" of the hemolymph trehalose were avoided by determining trehalose concentrations based on the dry weight.

Physiologically, however, the concentration of trehalose based on whole hemolymph is more valid. This is

Table 3a: Hemolymph Volume by Centrifugation of DDT-Treated and Control Cockroaches

Treatment	Hemolymph volume (ml/animal)	
	2 hrs.	11 hrs.
acetone	.042	.030
DDT	.046	.062

Table 3b: Density of Hemolymph Collected by Centrifugation of DDT Treated and Control Cockroaches

Treatment	Hemolymph density (g dry weight/100 ml)	
	2 hrs.	11 hrs.
acetone	7.7	9.7
DDT	7.6	7.7

the concentration that the body tissues "see" in osmotic and feedback mechanisms in the production of trehalose by the fat body and uptake of trehalose by muscles.

C. Paper and Thin Layer Chromatography

Good separation of all sugars used was obtained only with solvent III as listed in Table 4 or with a combination of solvents I and II in two directions. The movement of various sugars in these solvents is also shown in Table 4. With these systems, solvent separation occurred with paper (Whatman #1) and so could not be used. Thin layer plates made with cellulose powder were unsatisfactory because of difficulties encountered in the $AgNO_3$ and H_2SO_4 charring detection methods.

D. Scintillation Counting Efficiency

The quenched series used for efficiency determinations had 255,000 dpm of carbon-14 per vial. The windows on the three channels were: A. D125 0 - 9.9 v., B. E700 0.9 - 9.9 v. and C. E700 0 - 9.9 v. The counting efficiency curves from the B/C ratio and A/C external standard ratio are shown in Figure 3. Although the window widths were changed during this research, similar curves were calculated for each new setting.

Table 4: Thin layer Chromatography Rg[1] values

Thin layer plates: silica gel H (without Ca^{++})

pre-run in aqueous 0.02% EDTA in 1 N HCl, activated at 100° for 2 hours.

Solvent I: Methanol:NH_4OH:H_2O (6:1:3)

II: Methanol:HCOOH:H_2O (80:15:5)

III: Methyl cellosolve:methyl ethyl ketone:3 N NH_4OH (7:2:3) (run twice)

	Rg		
Compound	I	II	III
glucose	1.00	1.00	1.00
trehalose	1.03	.95	.93
glucose-1-phosphate	.46	1.16	.42
glucose-6-phosphate	.87	1.16	.26
glucose-1,6-diphosphate	-	-	.19
uridine diphosphate glucose	.95	1.24	1.16
fructose	-	-	1.16
mannose	-	-	.97

[1] Rg = migration compared to that of glucose

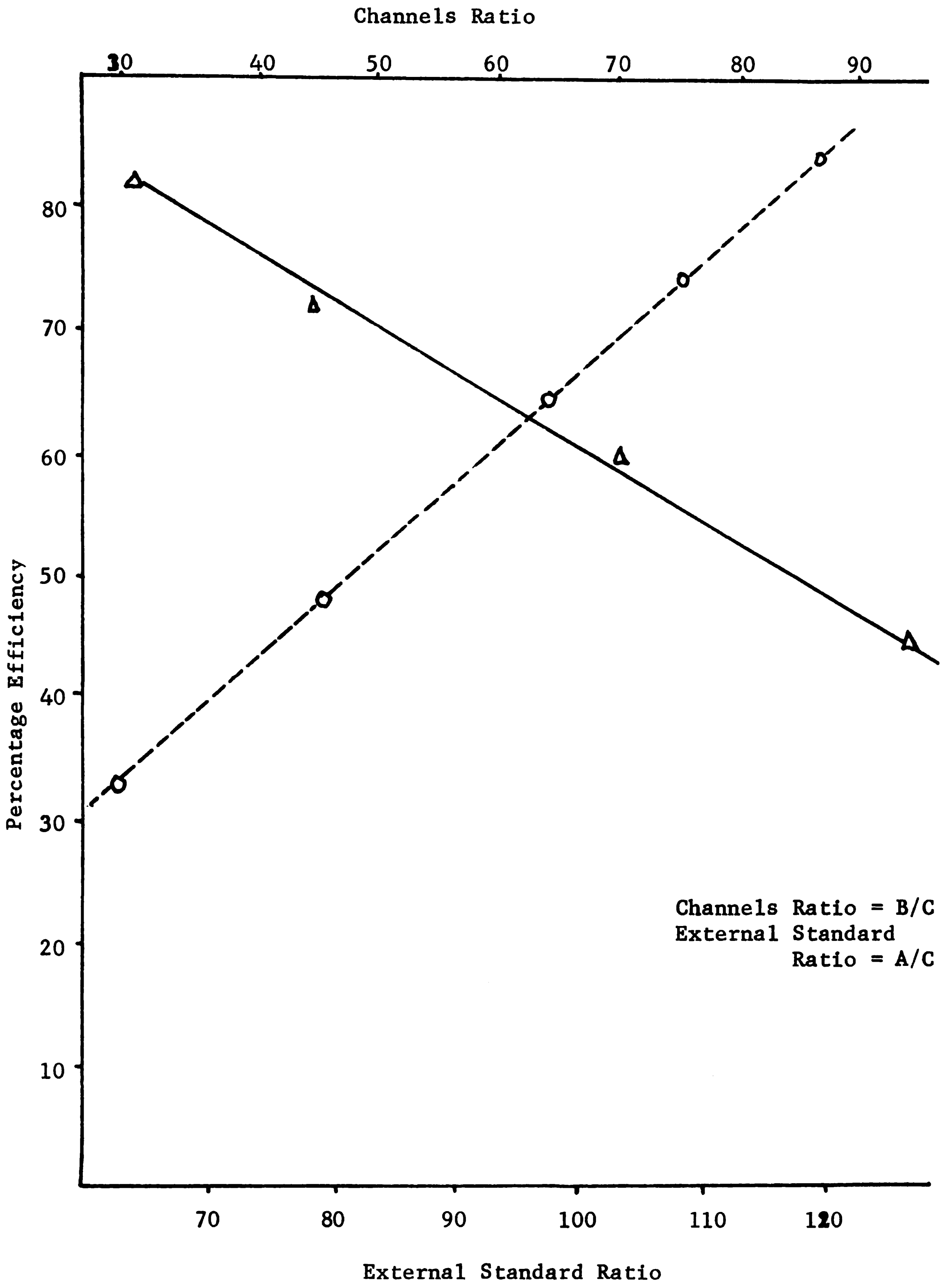


Figure 3: Scintillation Counting efficiency curves.

E. Thin Layer Plate Charred Spot Scraping

Scrapings from thin layer plates counted on the scintillation counter indicated a loss of about 35 percent of the radioactivity during the charring process. This loss varied depending on the amount of sulfuric acid spray and length of heating time but was assumed constant within a single plate.

II. Experimental Results

A. Trehalose Concentrations

The trehalose concentration in the hemolymph of the American cockroach decreases markedly as a result of DDT poisoning (Table 5). DDE, an insecticidally inactive DDT analogue, slightly increases the trehalose level in cockroach hemolymph while 1-naphthol, the insecticidally inactive hydrolysis product of carbaryl does not (Table 6).

Tests with waxworms did not show such a consistent decrease in trehalose concentration as a result of DDT poisoning or DDE treatment (Table 7), but carbaryl and 1-naphthol gave a consistent increase of the hemolymph trehalose levels (Table 7).

Last instar armyworms reared on bean leaves throughout their entire life cycle showed no drop in trehalose hemolymph concentrations with DDT poisoning or DDE treatment (Table 8).

Table 5: Effect of DDT and DDE on Hemolymph Trehalose Concentrations of American Cockroaches

Treatment[1]	3½-4½ hrs.		11½-12½ hrs.	
	mg/g[2]	mg %[2]	mg/g	mg %
acetone	32.4	1132	74.6	833
DDE	84.9	1160	70.1	918
DDT	77.4	833	10.2	188

[1] 8 µg insecticide/5 µl acetone, injected.

[2] mg/g = mg trehalose per g dry hemolymph.
mg % = mg trehalose per 100 g whole hemolymph.

Table 6: Effect of Carbaryl and 1-naphthol on Hemolymph Trehalose Concentrations of American Cockroaches

Treatment	Time (hrs.)	mg/g	mg %
acetone	1½	117	1460
carbaryl[1]	1	101	1780
	2½	65	853
	5	33	294
		After 4 hrs.	
acetone		130	960
1-naphthol[2]		162	1280
carbaryl[2]		73	564

[1] 0.7 µg carbaryl/3.5 µl acetone, injected.

[2] 200 µg 1-naphthol/5 µl acetone, topically.
200 µg carbaryl/5 µl acetone, topically.

Table 7: Effect of Selected Chemicals on the Hemolymph Trehalose Concentration of Waxworm

Treatment	2 hrs.		5 hrs.	
	mg/g	mg %	mg/g	mg %
acetone[1]	72	1130	87	1310
DDE[1]	45	830	70	1290
DDT[1]	71	1240	68	1160
	1 hr.		3 hrs.	
acetone[2]	49	840	-	-
carbaryl[2]	56	1270	51	830

[1] topical treatment of 100 μg insecticide/1 μl acetone. Acetone treatment is with 1 μl.

[2] 1 μg carbaryl/5 μl acetone, injected. Acetone treatment is 5 μl injected.

Table 8: Effect of DDT and DDE on Hemolymph Trehalose Concentration of Armyworm

Treatment[1]	2 hrs.		6 hrs.	
	mg/g	mg %	mg/g	mg %
acetone	76	1510	56	1250
DDE	68	1400	62	1300
DDT	75	1520	55	1090

[1] 0.5 mg insecticide/5 μl acetone, applied topically.

B. Factors Influencing Trehalose Levels

The remaining experiments were performed to determine the physiological cause of the trehalose changes. Since these changes were most pronounced in cockroaches treated with DDT, this preparation was used in most of the tests.

Theoretically, the decrease in hemolymph trehalose concentration can occur by two routes: 1) excessive respiration, or 2) by a biochemical lesion in the trehalose equilibrating mechanisms.

1. Excessive Respiration

Rapid utilization or excessive use of the trehalose by cells, such as in muscles, can cause a temporary decrease in the available trehalose (Evans and Dethier, 1957). This undoubtedly occurs in the poisoning. DDT causes increased respiration in cockroaches to a sharp peak, after which it decreases to zero at death (Ludwig, 1946; Lord, 1949, 1950; Harvey and Brown, 1959). Increased utilization of trehalose, the transported energy source, presumably occurs during this period. However, this probably is not the only cause of the trehalose drop. In healthy insects the rapid utilization of trehalose as a result of muscular exertion is followed by increased production of trehalose through an equilibrating mechanism (Clegg and Evans, 1961). In the cockroach poisoning experiments the cause of trehalose depletion is probably not solely an exhaustion of the supply, since the depletion is gradual over a 12 hour period and there is an apparent initial increase in trehalose levels (Figure 4) in both poisoned and unpoisoned insects. If exhaustion were the sole cause, this initial increase would not be expected. Also the depletion would be more rapid since a slow depletion could be easily compensated for by the trehalose equilibration mechanisms. A complete exhaustion of glycogen, the ultimate source of trehalose, does not occur (Ludwig, 1946; Winteringham, 1960).

2. Metabolic Lesions

The second possible cause of decreased trehalose levels in the hemolymph is a biochemical lesion in the trehalose equilibrating system. For instance, one of the enzymes might be inhibited by DDT or such an enzyme might be inhibited by a toxin which is released as a result of DDT (Sternburg and Kearns, 1952). Alternatively, the metabolic disorder might result from an upset in the hormonal system which regulates the proportion or direction of various enzymatic pathways concerned with trehalose synthesis and glycogen storage.

a. The glucose-C^{14} injection experiments were done to test some of the above possibilities. If one or more of the enzymes in the trehalose synthesis pathway were inhibited, the accumulation of the precursor compound

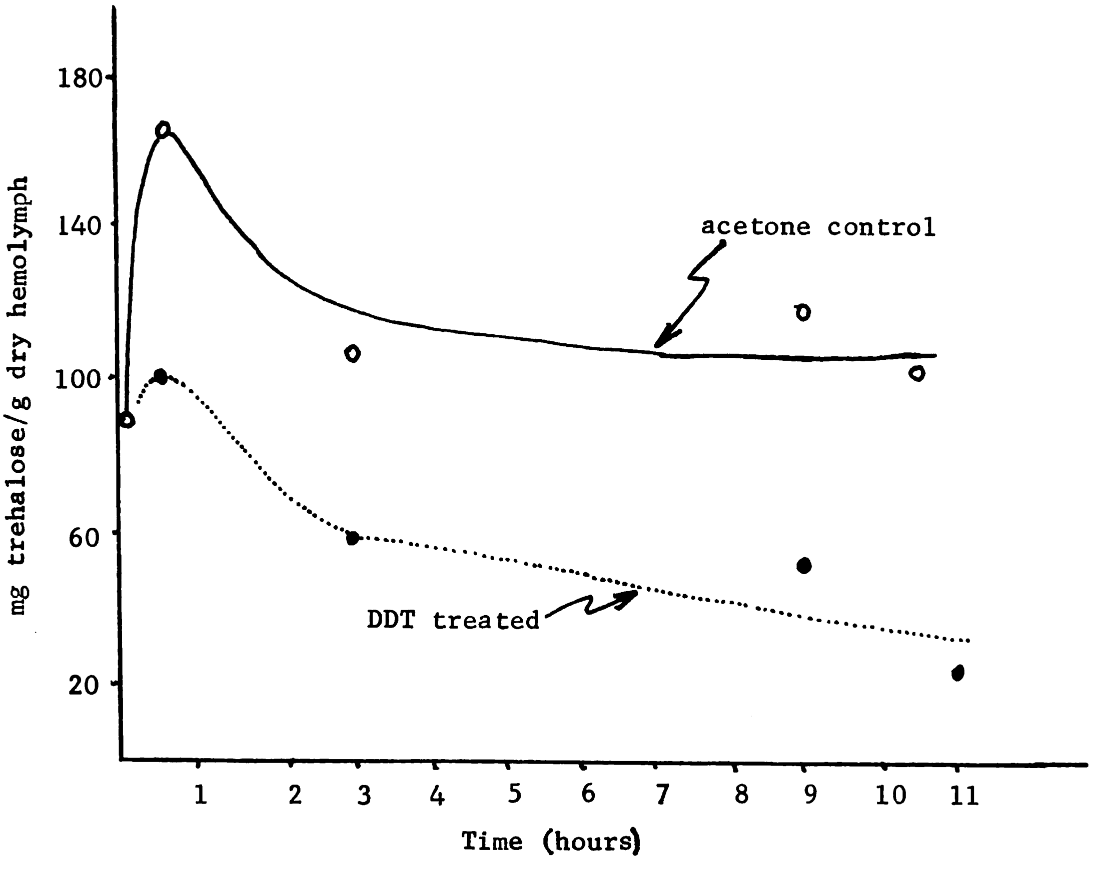
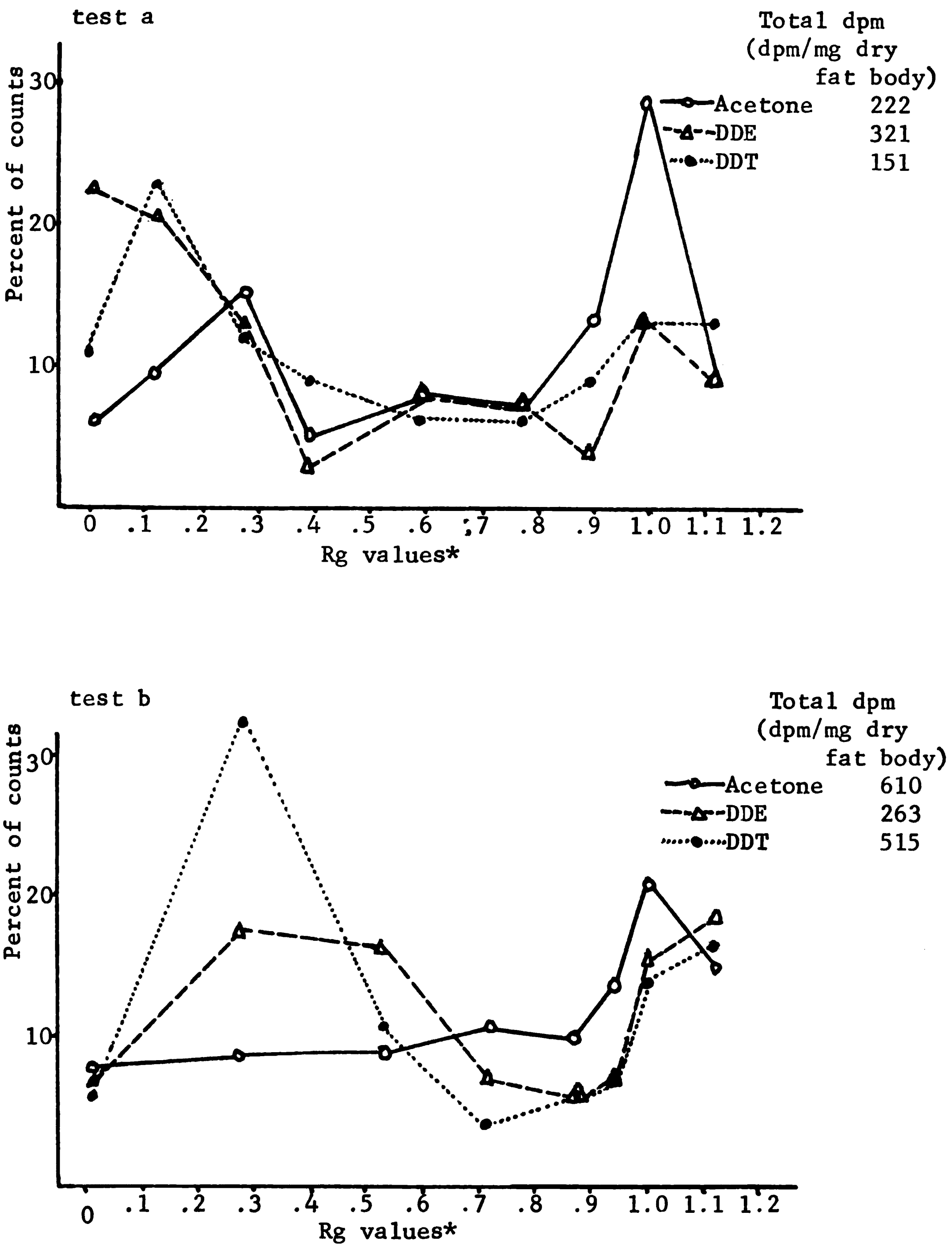


Figure 4: Effect of DDT on hemolymph trehalose concentrations with time.

might be expected. To detect the presence of such accumulations an extract of the fat body homogenate was chromatographed and regions of the chromatogram checked for radioactivity. These results are shown in Figures 5a and 5b. There is very little agreement between the two experiments shown. This is probably due to differences in extraction technique and the freshness of the fat body extract when chromatographed. The acetone treated fat bodies have more glucose and trehalose radioactivity and less radioactivity nearer the origin of the chromatogram. These general differences indicate that DDT (as well as DDE) probably does not have an effect on specific target enzymes but affects the whole system of trehalose metabolism.

b. Another place to look for metabolism of the glucose-C^{14} is the hemolymph. The conversion of injected glucose to trehalose in non-poisoned insects appears to be essentially complete after 30 minutes (Table 9a and 9b). This conversion in poisoned insects is not as complete and seems to reverse itself after 30 minutes. The experiments are inconclusive with regard to whether this reversal is caused by increased uptake of trehalose by tissues or an enzymatic breakdown of trehalose into glucose in the hemolymph.

c. Recent work on the role of trehalose in insect carbohydrate metabolism has shown the existence of



*Solvent system: Methyl cellosolve:methyl ethyl ketone:3 N NH_4OH (7:2:3) (run twice). See Table 4 for sugars corresponding to the Rg values

Figure 5: The effect of DDT and DDE on glucose-C^{14} metabolites in fat body extracts.

Table 9a: Effect of DDT and DDE on the Metabolic Fate of Glucose-C^{14} in Hemolymph of the American Cockroach

Treatment	Test	dpm/mg dry hemolymph glucose	trehalose	ratio: glucose/trehalose
acetone	a	270	584	.45
	b	406	2440	.17
	c	520	1200	.43
DDE	a	980	923	1.0
	b	364	1190	.31
	c	906	1250	.73
DDT	a	388	406	1.0
	b	411	378	1.1
	c	1650	700	1.8

Test a - hemolymph collected 5 hours after poisoning for 1½ hours on panels.

b - hemolymph collected 9½ hours after poisoning for 1½ hours on panels.

c - hemolymph collected 5 hours after poisoning by topical application of 24 µg DDT (or DDE) in 15 µl acetone.

Table 9b: **Effect of DDT and DDE on Metabolic Fate of Glucose-C^{14} Hemolymph of the American Cockroach with Time**

		5 min.			30 min.		
		dpm/mg dry hem.			dpm/mg dry hem.		
Treatment	Test	glucose	trehalose	ratio gl/tr	glucose	trehalose	ratio gl/tr
acetone	a	3200	600	5.3	300	1900	.16
	b	400	2880	.14	870	5300	.16
DDE	a	-	-	-	-	-	-
	b	1510	3660	.41	1870	5420	.34
DDT	a	2200	1100	2.0	700	2500	.28
	b	2250	3440	.65	3390	7890	.43

a,b - in both tests cockroaches poisoned by 1½ hours on treated panels.

a hyperglycaemic hormone released from the corpora cardiaca (CC) (Steele, 1961, 1963). This concept, plus ideas on hemolymph toxins produced with DDT poisoning (Sternburg and Kearns, 1952) and the importance of the head in DDT poisoning in flies (Morrison and LeRoux, 1954) led to experiments in which the influence of the CC was eliminated during poisoning. This was done by tying the neck region of the cockroach to prevent bleeding and removing the head. Three differences were observed between such insects and insects with heads, when they were poisoned with DDT: a) hemolymph trehalose concentrations, b) glucose-C^{14} metabolites in the hemolymph, and c) insect respiration.

i) Headless cockroaches treated with DDT show a slight increase in the hemolymph trehalose concentration rather than the decrease noted in whole insects (Table 10a). Even the control concentrations of trehalose are above the trehalose concentrations of cockroaches with heads. The headless cockroaches treated with carbaryl, however, continue to show the decrease in hemolymph trehalose found in the whole insect (Table 10b).

ii) The proportion of the injected glucose-C^{14} converted to trehalose is as high or higher in the DDT treated, headless cockroaches than in the acetone treated cockroaches (Table 11). The variation between the absolute values of the three tests is high, but the values of

Table 10a: Effect of DDT and DDE on the Hemolymph Trehalose Concentration of Headless American Cockroaches

Treatment[1]	mg trehalose/g dry hemolymph 5 min.	 5 hrs.	 10 hrs.
acetone	127	76	127
DDE	141	163	143
DDT	154	165	173

[1] Treatment by 1½ hours on insecticide panels. Time measured from glucose-C^{14} injection.

Table 10b: Effect of Carbaryl and 1-Naphthol on the Hemolymph Trehalose Concentrations of Headless American Cockroaches

Treatment[1]	mg trehalose/g dry hemolymph (4 hrs.)
acetone	125
1-naphthol	139
carbaryl	34

[1] 0.6 mg insecticide/15 µl acetone, applied topically.

Table 11: Effect of DDT and DDE on the Metabolic Fate of Glucose-C^{14} in Hemolymph of Headless American Cockroaches

Treatment	Test	glucose	trehalose	glucose/trehalose
		dpm/mg dry hemolymph		
acetone	a	960	1230	.78
	b	273	2469	.11
	c	42	1480	.03
DDE	a	1070	2000	.54
	b	399	4942	.08
	c	185	2180	.08
DDT	a	530	2280	.23
	b	377	4560	.08
	c	113	1940	.05

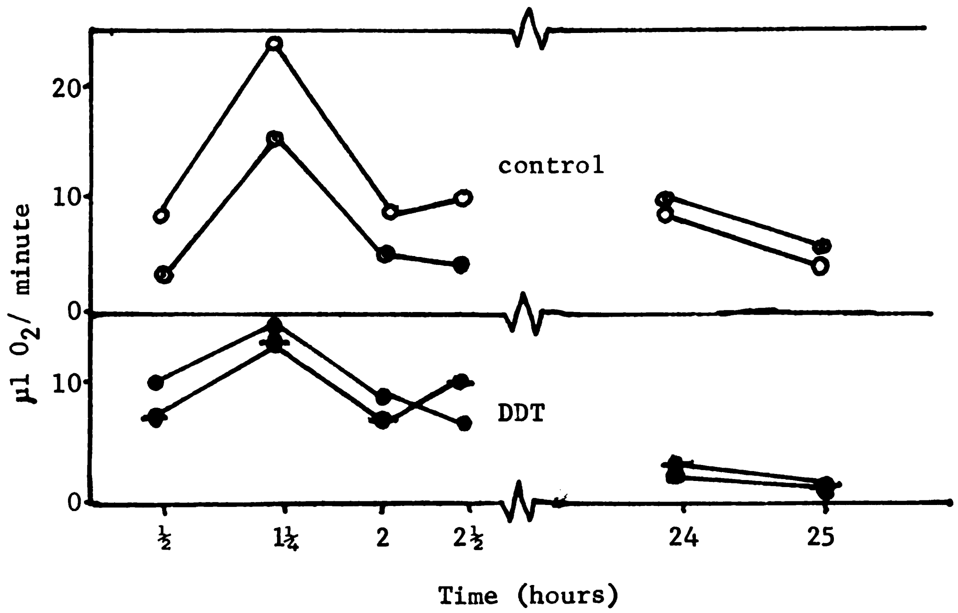
Tests a - hemolymph collected 5 hours after poisoning for 1½ hours on panels

b - hemolymph collected 9½ hours after poisoning for 1½ hours on panels

c - hemolymph collected 5 hours after poisoning by topical application of 24 µg DDT (or DDE) in 15 µl acetone the glucose:trehalose ratio tend to be lower than in the similar tests with whole cockroaches.

iii) Respiration curves of headless cockroaches with and without DDT treatment are shown in Figure 6. There seems to be no peak in the respiration of the DDT treated cockroaches, but such a peak does occur in whole insects poisoned with DDT (Harvey and Brown, 1951).

d. The above experiments with headless insects indicate that some factor from the head is involved in the drop in hemolymph trehalose concentration, the reduction of the glucose:trehalose ratios in the hemolymph, and the increased respiration found in whole, poisoned cockroaches. The perfusion experiments described in the Methods section were an attempt to isolate this factor. In all three tests, a hyperglycaemic factor was found which produced a slight increase in the trehalose levels of treated bioassay cockroaches. However, DDT did not prevent the release of this factor or produce a decrease in the trehalose level of the bioassay animals (Table 12). There was a slight increase in the glucose: trehalose ratio, similar to that which occurs in DDT poisoned cockroaches (Table 12).



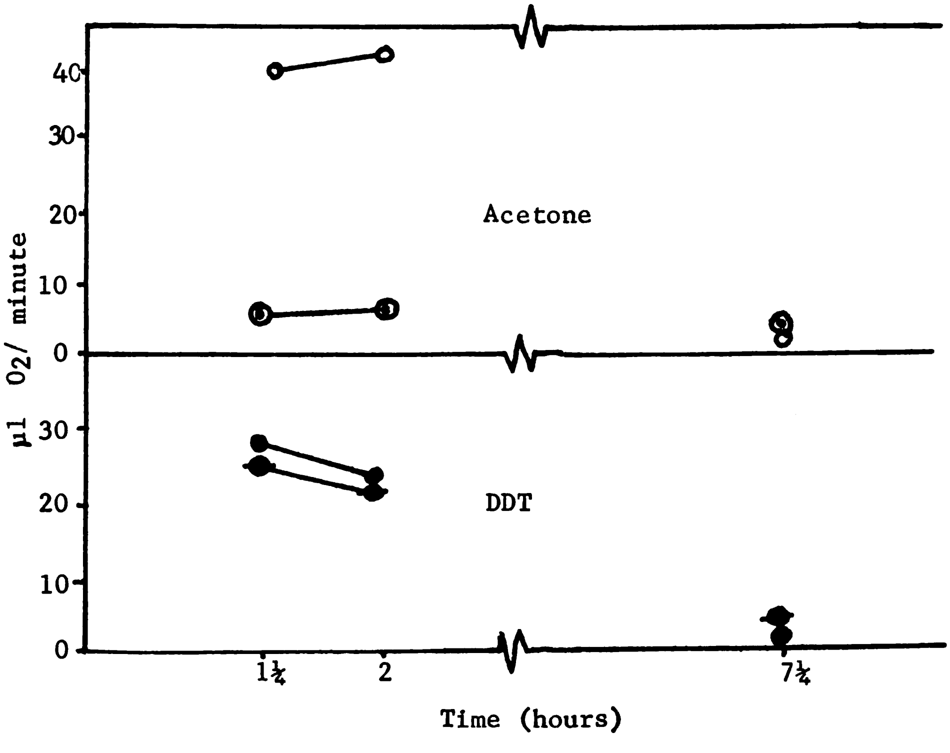


Figure 6: Respiration of headless cockroaches with and without DDT poisoning.

Table 12: Bioassay of Corpora Cardiaca Perfusions Before and After DDT Treatment

Experiments 1 and 2

Treatment	mg trehalose/g dry hemolymph	
Saline	18.7	15.9
Perfusion 1 (0 - ¼ hr.)	39.1	17.8
Perfusion 2 (3/4 - 1¼ hr.)	21.4	20.2
Perfusion 3* (10 min after DDT treatment)	18.6	14.9

Experiment 3

Treatment	mg/g	glucose (dpm)	trehalose (dpm)	glucose / trehalose
Saline	17.7	30	2860	.011
Treatment with DDT*				
Perfusion 1 (5 - 20 min.)	39.0	290	4240	.069
Perfusion 2 (45 min. - 1 hr.)	26.0	500	3980	.130
Perfusion 3 (2 hr. - 2¼ hr.)	19.0	15	1830	.008

*Flooded with solution of 10 mg DDT/ 10 ml corn oil.

DISCUSSION

I. Cause of Death

A. Vertebrates

Since body organization and maintenance of the normal physiology of vertebrates is so sophisticated, relatively minor abnormalities will cause death. Temporary cessation of the heart or nervous system function is sufficient to cause death. Slight chemical changes in the blood such as changed pH, glucose levels, and osmotic concentrations will affect all parts of the body causing rapid death. As a result, the acute effect of insecticides in vertebrate systems is fairly easy to document. Carbamates, as cholinesterase inhibitors, inactivate the respiratory muscles resulting in fairly rapid death. In this case the symptoms of poisoning: convulsions, ataxia and paralysis are directly related to the actual cause of death, asphixiation caused by respiratory failure.

With DDT, poisoning of the mammalian system is not as clear. The exact biochemical-biophysical site of the DDT effect is not known, but death probably still is caused by nervous disfunction and, as with the carbamates, by cardiac or respiratory failure and asphixiation. In this case also, the symptoms are direct indications of the eventual cause of death.

B. Insects

The insect organism, though highly organized and regulated, is far inferior to vertebrates with respect to precision of control. For example, the hemolymph trehalose levels in control insects in the experiments in this research varied from 833 to 1132 mg percent in the American Cockroaches (Table 6). Levels reported in the literature varied even greater. Evans and Dethier (1957) gave trehalose concentrations of the blowfly, Phormia regina, as varying from 200 to 3000 mg percent.

Insects can be greatly abused before acute death processes occur. Beheaded cockroaches will live several days. As much as 10 µl of acetone may be injected into cockroaches with only temporary ill effects.

Such natural variation and survival of abuse make it difficult to propose a cause of death for insects; analogy to the mammalian model does not work and symptomology gives almost no clue as to the lesion or cause of death.

What is a cause-effect relationship and how is it determined? Hypothetically, an insecticide may cause an obvious symptom in an insect, but is it possible to determine whether that symptom is a primary cause of death or a remote secondary effect of some hidden train of events? In DDT poisoning, nervous disfunction is the obvious symptom. But demise of the nervous system (if this is the sole effect of DDT) will not kill the rest of the cells in the insect. Asphixiation will not occur because respiration is largely passive. Death will not occur if the heart beat stops since the heart function is irrelevant on a short term basis. If one were to define death simply as demise of the nervous system, i.e. prostration, one would still not define what killed the cells of the insect other than those in the nervous system. Similarly, there is no good evidence that the increased respiration of DDT poisoned insects is directly related to the increased nervous activity occurring in DDT poisoning, so these two major symptoms of DDT poisoning are related only by circumstance. At present it is not possible to say that the nervous disfunctions are a direct cause of the toxins found in the insect blood during DDT poisoning. In summary, it seems unlikely with insects that any single or simple cause-effect relationship will explain death by poisoning.

Past theories on the mode of action of DDT have been restricted to one or two types of observations and then writers either ignored the rest of the data or relegated them to a classification of "secondary effects."

A more logical approach to determining mode of action would be to look at all the symptoms and anomalies described. In those cases where no cause-effect relationship can be found between two fragments of data, they should be considered as parallel evidence until additional information is available.

In the following discussion I will first explain the effects of DDT on trehalose metabolism described in this research and will then attempt to incorporate this data into the mass of DDT literature for a hypothetical model of DDT mode of action.

II. Insecticidal Effects on Trehalose Metabolism

DDT (but not DDE) in cockroaches decreases the amount of trehalose in the hemolymph and its concentration (Table 5). This is in agreement with data of Cline and Pearce (1966) who found that injected glucose-C^{14} is metabolized to trehalose to a lesser extent in DDT-poisoned insects than in unpoisoned insects.

In comparison with the acetone-treated insects, there appears to be no correlation between the rate of trehalose decrease in DDT-poisoned insects and observed symptoms of DDT-poisoning. In treatment of the cockroaches, any type of injection or excessive handling seemed to cause a rise in trehalose levels, presumably because of the normal stress responses (Heslop and Ray, 1959). Using the acetone-treated insect as a base for such response, there appears to be no peak of trehalose decrease in the DDT-poisoned cockroaches which could be correlated with the peak in respiration for DDT-poisoned insects reported in the literature (Ludwig, 1946; Lord, 1949; Harvey and Brown, 1951).

To the accuracy of the methods used, there appeared to be no accumulation of a metabolite in DDT-poisoned insects, indicating that no specific enzyme in trehalose metabolism was inhibited by DDT. Testing for individual enzymes by biochemical methods would probably give more definitive results, however, since many enzymes of carbohydrate metabolism have been found to be inhibited by DDT (Agosin *et al.*, 1961).

When the influence of the head on the rest of the cockroach was eliminated, the hemolymph trehalose level did not decrease with DDT poisoning (Table 10). This indicates that some factor in the head mediates the influence DDT has on trehalose levels. Also, the equilibrium in the hemolymph of glucose ⇌ trehalose, which is normally to the right in unpoisoned insects, was farther to the right in unpoisoned headless insects. In whole DDT-poisoned insects the equilibrium maintains nearly equal quantities of glucose and trehalose, but in headless poisoned insects the equilibrium again falls far to the right (Tables 9, 11). This indicates that the influence on the trehalose level is due to a positive factor rather than the lack of a factor. Since the equilibrium is farther to the right in unpoisoned, headless cockroaches than in the unpoisoned, whole cockroaches, this head factor or some other factor is normally present in low concentration in the whole insects.

The literature on carbohydrate metabolism indicates that the two main forms of control of trehalose metabolism in the fat body are feedback control and hormonal control on individual enzymes or on the cyclic 3,5-AMP-phosphorylase a and b system (Murphy and Wyatt, 1964, 1965; Chefurka, 1965; Steele, 1961; Weins and Gilbert, 1967b). The hormonal system is probably responsible for trehalose uptake in the DDT-poisoned whole cockroaches, although a toxin or complicated inhibitor effect should not be ruled out as a possibility.

An additional possibility of control for trehalose levels outside the fat body would be increased uptake by cells as a result of some other hormone. If this were the case, these data would fit in nicely with the information on the increased respiration in DDT-poisoned insects. This information would also tie in nicely with the data on circadian rhythms. Specifically, Harker (1960) has shown that a head factor can increase the activity of insects in conjunction with circadian rhythms.

This hypothesis of an additional activity factor would also be supported by the data from the experiment showing that headless, DDT-treated cockroaches do not appear to have increased respiration in comparison with acetone-treated insects (Figure 5). In these cases the high respiration factor has been removed with the head.

To summarize, this hypothesis (which has very little experimental evidence), states that in DDT poisoning a head factor inhibits trehalose release by the fat body. Simultaneously, this same factor or a different factor from the head increases respiration resulting in an overall decrease in trehalose, restricted conversion of glucose to trehalose, and increased respiration in the intact animal, but not in the headless animal.

The variability of the total counts of carbon-14 in the hemolymph (Table 9) does not allow any interpretation of whether DDT increases the uptake of carbohydrates. The hypoglycaemic factor was not isolated by the perfusion technique attempted (Table 12) although indications of the hyperglycaemic hormone of Steele (1961) were observed.

Trehalose levels in whole cockroaches treated with carbaryl were similar to those treated with DDT, since the hemolymph trehalose level decreased. Such a decrease was not observed for 1-naphthol.

There is no effect by DDT on trehalose concentration in waxworm larvae or armyworm larvae. This may indicate differences in physiology for these insects, either as species or as immature forms.

III. A Hypothetical Mode of Action for DDT

The following is a discussion attempting to integrate the information obtained in this research with the body of knowledge of DDT poisoning in insects. Such a discussion is somewhat hypothetical and not justifiable as an explanation of DDT poisoning. The correlations are tenuous and often without substantiating experimental evidence. However, such a discussion is necessary as a tentative model upon which to base further experimentation.

In the literature review of DDT poisoning, the research described is divided into three areas: physical-chemical, biochemical, and physiological investigations. Very little effort has been made to correlate them. This section will discuss the possibility that these three types of data may be integratable.

Workers on the DDT-poisoned insect nervous system at present are pursuing the possibility of the existence of a complex between DDT and nerve cord components, possibly the membrane (O'Brien and Matsumura, 1964; Matsumura and O'Brien, 1966 a and b). They have suggested that such a complex disrupts the electrical balance of the membrane in some unknown way to cause the train of impulses, the negative after-potential shoulders characteristic of DDT poisoning, and interfere with the permeability of the membrane to K^+ and Na^+.

The increased acetylcholine levels observed in the axons (Tobias _et al._, 1946; Lewis _et al._, 1960) could be related to the increased ion permeability. There has been no suggestions for the function or source of this acetylcholine except that it is from a bound form. In any case, however, if permeability to ions is increased with DDT poisoning the permeability of a polar compound such as acetylcholine will also be affected.

This permeability might also be correlated with the many types of toxins and hormones that have been described in the hemolymph after DDT poisoning. Since the corpus cardiacum, the supposed source of these substances, is itself nervous tissue the increased permeability of the nerve tissue very likely will release the many hormones stored in the corpus cardiacum indiscriminately and in large amounts.

The third factor of DDT poisoning is the biochemical anomolies. DDT poisoning upsets the glycolytic metabolism to some extent by inhibition of various enzymes (Agosin _et al._, 1961, 1963). Amino acid metabolism is upset (Corrigan and Kearns, 1958), ATP levels decrease (Winteringham _et al._, 1960) and, as shown above, trehalose metabolism is affected. Also, at least in resistant insects there is increased protein synthesis (Agosin, et al., 1966). All these changes are likely to result from or cause increased metabolism. The breakdown of proline and tyrosine and the increase in glutamine and phenylalanine (Corrigan and Kearns, 1963) can conceivably be attributed to transaminations for the purpose of carbon oxidation to support the higher rate of respiration and protein synthesis as observed in resistant houseflies for enzyme induction (Ilevicky et al., 1964). In addition, O'Brien (1967) remarked that these factors, as well as the increased allantoin and uric acid production, could be an attempt by the insect to increase its ability to excrete materials including the toxic compounds.

The decreased ATP and trehalose would serve similar purposes, to satisfy the increased respiration, muscular activity, and detoxification and excretion attempts. Hypothetically then, this would ultimately lead to utilization of all mobile reserves of energy available to the insect, although much glycogen in the fat body may still be present. Hence, the insect dies of cellular starvation.

In summary then, the hypothetical scheme is that DDT by combining with the nerve cord induces tremors and the random release of neurohumeral substances which a) induce more tremors, b) induce higher activity and respiration, and c) inhibit trehalose metabolism. The increased metabolism and detoxification and excretion attempts by the insect result in upset and decreased amino acid levels, decreased ATP, and decreased trehalose. Ultimate death of the cells is by starvation because of a lack of mobile reserves, although the prostration symptom may be caused by the original nervous disfunction.

SUMMARY

1. DDT [2,2-bis(p-chlorophenyl-1,1,1-trichloroethane] and carbaryl (1-naphthyl N-methylcarbamate) markedly decrease the quantity and concentration of trehalose in the hemolymph of the American cockroach, *Periplaneta americana* (L). This effect is not observed with DDE or 1-naphthol.
2. Such a decrease in trehalose is not observed with either insecticide in greater waxworm larvae (*Galleria mellonella* L.) or armyworm larva (*Pseudaletia unipunctata*, Haworth).
3. The lower trehalose level caused by DDT poisoning does not occur in headless cockroaches.
4. There is no respiration peak with DDT poisoning in headless cockroaches.
5. Injected glucose-C^{14} in cockroaches is largely converted to trehalose in 30 minutes. The equilibrium of glucose $\rightleftharpoons$ trehalose in the cockroach hemolymph is to the right in unpoisoned cockroaches but favors glucose in DDT-poisoned cockroaches.
6. The equilibrium of glucose $\rightleftharpoons$ trehalose is far to the right in DDT, DDE and acetone control cockroaches without heads.
7. The presence of a positive head factor restricting

trehalose synthesis is hypothesized to be due to the response of cockroaches to DDT.

8. This hypoglycaemic factor was not extractable by a perfusion method.
9. A hypothetical role of trehalose depletion in the mode of action of DDT is discussed.

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APPENDIX

Several series of experiments were performed which proved to be irrelevant to the logical trends in this thesis. The procedures used and some results are included here because of their physiological interest.

A. Procedures

1. Ligation

In DDT and carbaryl poisoning experiments with waxworms some hemolymph and fat body samples were taken from insects under ligation. Rubber bands (Staal, 1967) were used for the ligaturing. A series of small holes was burned into the rubber band. A hole was stretched over the end of a glass tube about 3 to 4 mm internal diameter. The head of a waxworm under carbon dioxide anesthetization was placed in this end of the glass tube and the rubber band was slipped off around the neck to make a tight ligature.

2. Respirometry

The fat body of waxworms was used in respirometry experiments. Two ml Grace's media (Grace, 1962; Grand Island Biological Company, Grand Island, New York), 0.67 ml antibiotic-antimycotic mixture (Grand Island Biological

Company) and 2 µl of uniformly labeled glucose-C^{14} (222,000 dpm) were used as the medium and placed in flasks of a Gilson Differential Respirometer (Gilson Medical Electronics Company, Middleton, Wisconsin). Twenty percent KOH was placed in the center wells to absorb carbon dioxide. Fat bodies were excised from uninjected waxworms and DDT-, carbaryl-, and acetone-injected waxworms and placed in the medium. The oxygen uptake and carbon-14 dioxide production was measured. Some difficulty was experienced in these preparations with bacterial and fungal infections and results seemed more to reflect microorganism contamination than fat body respiration.

In later experiments, a sterile technique was used coupled with a less complete medium. The medium was similar to that of Murphy and Wyatt (1965) and consisted of 40 mM KCl, 15 mM $MgCl_2$, 15 mM $MgSO_4$, 5 mM K Malate, a K_2HPO_4 buffer at pH 6.5 and 60 mM glucose. This was sterilized by filtering it through a millipore type filter (Sterilizing Filter Sheet, type ST-1 23837, Hercules Filter Corp., Hawthorne, New Jersey). The final medium had one part antibiotic-antimicotic to 4 parts total saline-antibiotic solution; the concentration of the saline part being made so that the molarity would be as listed above after dilution with the antibiotic solution. Sterility of this medium was checked by plating on agar and found to be good even with the fat body added. Fat bodies were extracted after incubation in this medium and samples weighed and placed in the scintillation counter. Similarly, samples of the medium and carbon dioxide trap were counted in the scintillation counter.

3. Organ Culture

The medium of Murphy and Wyatt used in the respirometry was also used in the organ culture except that radioactive chemicals were not used and varying amounts of different sugars were tested. Samples of the medium with incubation varying from one-fourth to 53 hours were tested for trehalose by the anthrone method. DDT and DDE in acetone solutions were tested similarly in these experiments either by injection into the waxworms before the fat body was removed or by placing the insecticide directly into the medium. Acetone in all cases was used as the control.

B. Results and Interpretation

1. Ligation

The hemolymph trehalose concentration of ligatured waxworms decreases markedly with time, whether poisoned with DDT or not (Figure 7). This may be attributed to lack of the hyperglycaemic hormone control on the fat body (Steele, 1961), although feedback mechanisms would have been expected to function (Murphy and Wyatt, 1965;

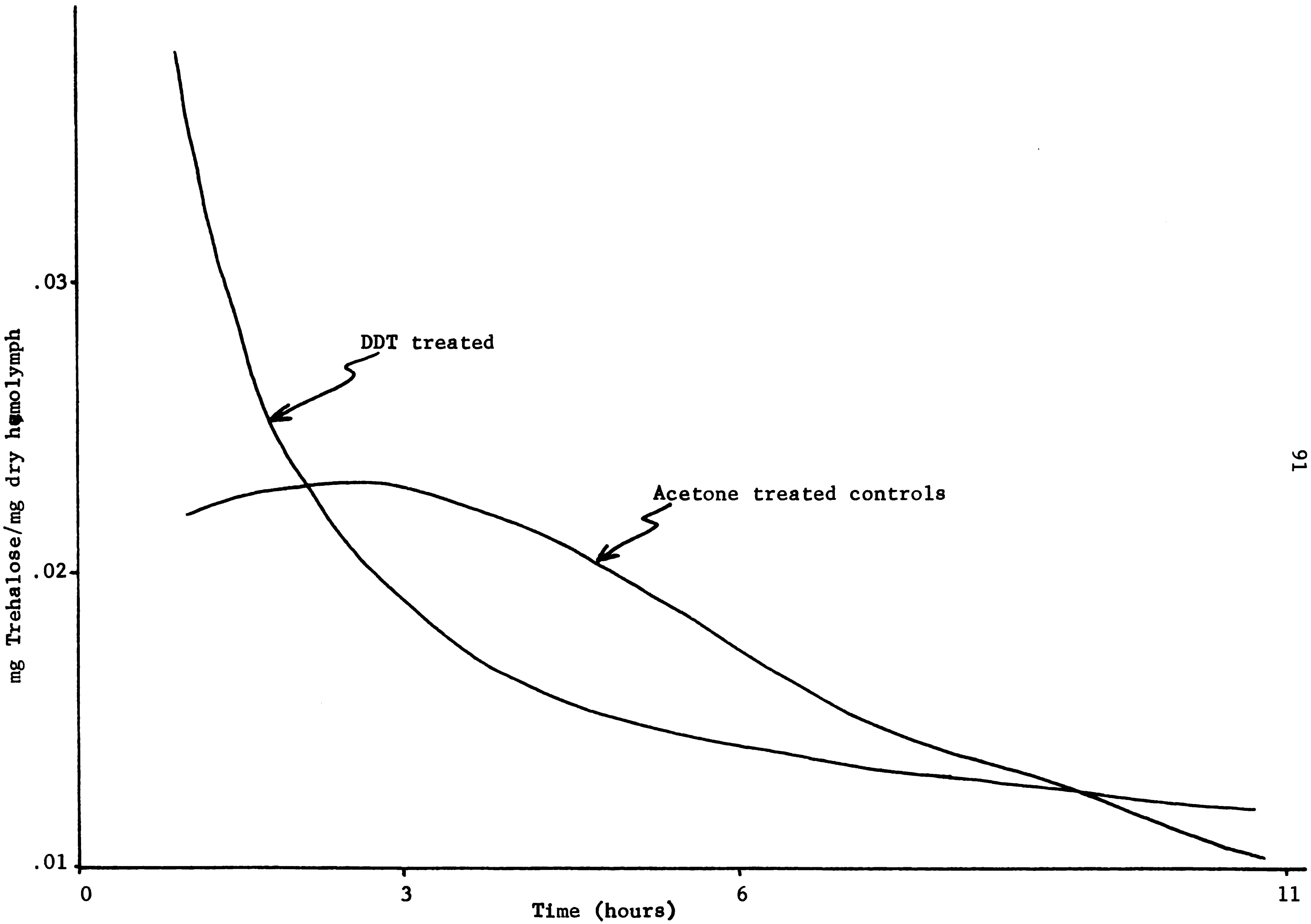

Figure 7: Trehalose levels in ligatured waxworms.

Friedman, 1967 a and b). These results are contrary to results received from cockroaches and probably indicate a different physiology both as immature forms and as representatives of a different order.

2. Respirometry

The fat body of waxworms did not appear to respire at a greater rate as a result of DDT or carbaryl addition to the medium (Figure 8). However, more precise measurements particularly as to volume of oxygen consumed per gram of fat body should be made to determine exact results.

3. Organ Culture

The production of trehalose by the waxworm fat bodies was measured in these experiments as mg of trehalose per mg of lyophilized fat body. In these experiments, an added sugar was necessary for optimum trehalose production. Glucose-6-phosphate alone and a combination of glucose-6-phosphate and uridine diphosphate glucose (UDPG) were better than glucose alone for trehalose production (Table 13). This can be explained by the fact that trehalose-6-phosphate is synthesized by UDPG and glucose-6-phosphate and trehalose-6-phosphate is immediately broken down to form trehalose. On the other hand glucose must be converted to glucose-6-phosphate before it can be used in trehalose synthesis. ATP here would have probably increased trehalose production from glucose. The differences

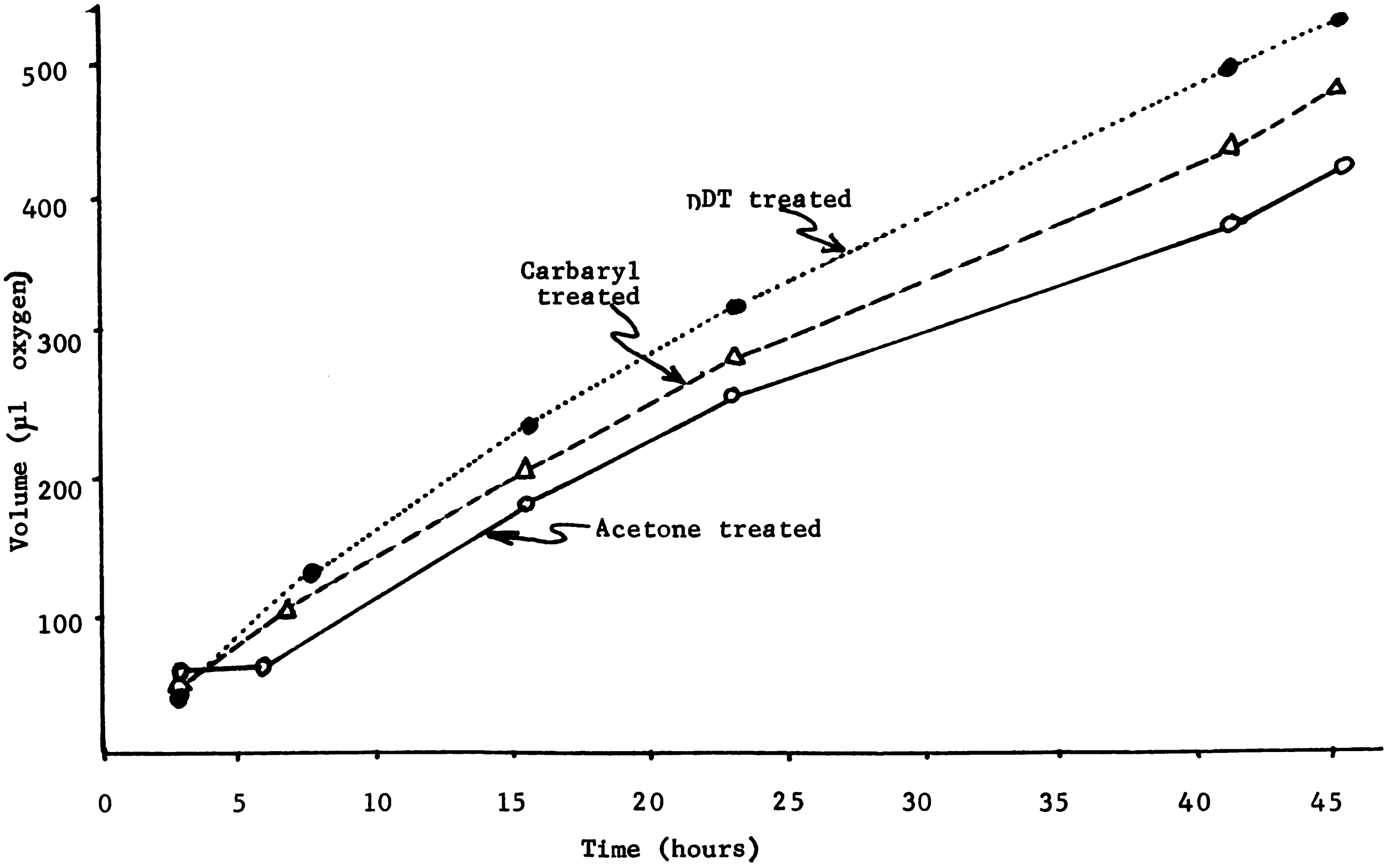

Figure 8: Respiration of waxworm fat bodies with and without insecticide poisoning.

Table 13: Waxworm Fat Body Trehalose Production with Various Sugars and Insecticides

Sugar	Treatment[1]	mg trehalose/ mg dry fat body/hour
glucose	none	.0144
none	acetone	.0004
glucose	acetone	.0128
glucose	DDT	.0118
none	acetone	.089
G-6-P + UDPG	acetone	.28
G-6-P + UDPG	DDT	.43
none	acetone	.042
G-6-P	acetone	.064
G-6-P	DDT	.054

[1] 40 μg insecticide/5 μl acetone, injected into animal 2 hrs. before dissection.

in the trehalose production with or without DDT are probably not significant. If samples of the medium are taken over a period of time, the trehalose concentration keeps increasing (Table 14). This type of preparation might be utilized for synthesis of trehalose-C^{14}.

Table 14: Trehalose Production of Waxworm Fat Bodies with Time

		mg trehalose/mg dry fat body		
Sugar	Treatment[1]	5 hrs.	24 hrs.	53 hrs.
glucose	acetone	.0100	.0261	.0891
glucose	DDE	.0109	.0294	.135
glucose	DDT	.0040	.0239	.106
glucose	DDT	.0067	.0286	.127

[1] 40 μg insecticide/5 μl acetone, injected into animal 2 hrs. before dissection.