1958

Studies on the mode of action of insecticides

Chandra Prakash Pant

Iowa State College

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Zoology Commons

Recommended Citation

https://lib.dr.iastate.edu/rtd/2262

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
STUDIES ON THE MODE OF ACTION OF INSECTICIDES

by

Chandra Prakash Pant

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Entomology

Approved:

Signature was redacted for privacy.
In Charge of Major Work

Signature was redacted for privacy.
Head of Major Department

Signature was redacted for privacy.
Dean of Graduate College

Iowa State College
1958
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>REVIEW OF LITERATURE</strong></td>
<td>6</td>
</tr>
<tr>
<td><strong>MATERIALS AND METHODS</strong></td>
<td>17</td>
</tr>
<tr>
<td>General</td>
<td>17</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>20</td>
</tr>
<tr>
<td>Succinic Dehydrogenase</td>
<td>24</td>
</tr>
<tr>
<td>Over-all Glycolysis</td>
<td>27</td>
</tr>
<tr>
<td>Distribution of P(^{32})-Labeled Intermediates</td>
<td>31</td>
</tr>
<tr>
<td><strong>RESULTS AND DISCUSSION</strong></td>
<td>39</td>
</tr>
<tr>
<td>Cytochrome Oxidase</td>
<td>39</td>
</tr>
<tr>
<td>Succinic Dehydrogenase</td>
<td>48</td>
</tr>
<tr>
<td>Over-all Glycolysis</td>
<td>54</td>
</tr>
<tr>
<td>Distribution of P(^{32})-Labeled Intermediates</td>
<td>57</td>
</tr>
<tr>
<td><strong>SUMMARY</strong></td>
<td>65</td>
</tr>
<tr>
<td><strong>LITERATURE CITED</strong></td>
<td>68</td>
</tr>
<tr>
<td><strong>ACKNOWLEDGMENTS</strong></td>
<td>78</td>
</tr>
</tbody>
</table>
INTRODUCTION

Very little is known on the precise mode of action of the majority of insecticides. A large amount of work has been done on this subject and several hypotheses have been advanced from time to time. The earliest theories proposed in this connection were mostly derived from the general Overton-Meyer concept of lipoid solubility. The partition coefficient of a chemical between water and the lipoid material was correlated with its biological activity. This hypothesis, however, had shortcomings from the experimental point of view because there was inadequate lipoidal material to choose from for laboratory studies. Ferguson (1939) showed that the use of thermodynamic indices (chemical potential and activity), was useful in predicting the biological activity of chemicals. It was shown that the partition coefficient and other physico-chemical properties of chemicals were related to their thermodynamic activity. Although these hypotheses related toxicity to certain physico-chemical properties, they did not explain any mechanism of the toxic action.

The importance of the shape and size of an entire molecule in relation to toxicity (e.g. DDT and its analogues and the isomers of BHC or 1,2,3,4,5,6-hexachlorocyclohexane) have also been considered by several authors (Riemschneider and Otto 1954, Mullins 1955). Factors such as molecular weight, lipoid solubility, type, number, and position of particular groupings, and finally the configuration of the entire molecule have been shown to be critical in imparting the toxic properties to a molecule.

Gunther et al. (1954) postulated the "Structural Topography Theory"
in connection with the mechanism of action of DDT type compounds. The insecticidal properties of these compounds were directly correlated to the magnitudes of their van der Waals' attractive forces for protein. This suggested that these insecticides interact with a protein-like substance, presumably an enzyme.

It appears that the insecticide molecule as a whole serves a twofold purpose in exerting the toxic effect. Firstly, during the transport of the molecule through the cuticle and the internal tissues of the insect, and secondly, after reaching the site of action, the whole molecule must fit into the active site of the enzyme. Thus the insecticide may produce biochemical disturbances which finally lead to the death of the organism. Since the reactions involved in energy metabolism are the most vital of all the physiological processes, it is likely that insecticides act at one or more points in this chain of reactions. The energy required for life processes is released in the cell by chemical degradation of metabolites in a step by step process. Even a small disturbance in this chain of reactions can produce very serious effects on the overall metabolic picture.

Much emphasis has been given in the past on the effect of an insecticide on one particular enzyme system in energy metabolism. It is quite likely that more than one enzyme system is affected by an insecticide. If so, this could produce an accumulative effect on the whole pattern of energy metabolism.

The majority of the workers in the field of insecticide biochemistry have used in vitro techniques for studying the effects of insecticides on enzyme systems. However, the addition of a chemical (insecticide) to an
enzyme preparation does not necessarily follow the normal sequence of events in in vivo insecticidal action. In the present study, therefore, it was decided to study the effects of insecticides in vivo.

The important routes of energy utilization in insects involve glycolytic and oxidative pathways of carbohydrate catabolism. The energy derived from these reactions is transferred to the high energy bonds of the phosphorylated intermediates. Finally the organism utilizes this energy for the muscular and other activities necessary for the maintenance of life.

The present work was undertaken to study the effect of a series of insecticides, representing a wide spectrum of chemical groups, on some of the important enzymes and enzyme systems. Hydrocyanic acid, ethylene dibromide or 1,2-dibromoethane, ethylene dichloride or 1,2-dichloroethane, carbon disulfide, DDT or 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane, Dipterex or O,O-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate, and pyrethrum extract were selected for study from a large number of insecticides and fumigants. Among the important enzyme systems in energy metabolism, cytochrome oxidase, succinic dehydrogenase, and the over-all glycolytic enzymes in the thoracic muscle tissue of the house fly, Musca domestica L., were selected for study. Figure 1 gives the location of these enzymes in the intermediary metabolism of glucose. In order to study the effect of these insecticides on the over-all energy metabolism, the distribution of phosphorylated intermediates was studied by labeling the phosphorus pool of house flies with P^{32} and subsequently conducting chromatographic separations of labeled compounds from muscle extracts of insecticide-treated and untreated flies.
Figure 1. Summary of the intermediary metabolism of glucose showing the enzymes and enzyme systems studied (enclosed in red)
An attempt has been made in the following pages to review at first some papers on the intermediary metabolism of the carbohydrates including glycolytic and aerobic mechanisms, presence of adenosine triphosphate (ATP), cytochromes and oxidative phosphorylation in insects. Second, the literature on the mode of action of insecticides has been treated according to the mechanisms of toxic action. These include such subjects as the effects of insecticides on oxygen uptake, respiratory quotient, cytochrome oxidase, succinic dehydrogenase, cholinesterase, carbonic anhydrase, oxidative phosphorylation, distribution of metabolites, and the physical basis of toxicity of insecticides.

A survey of the literature on the intermediary metabolism of carbohydrates in the insects has been published by Gilmour (1953) and Rockstein (1957). Early work on the occurrence of the Embden-Meyerhof cycle in the insects came chiefly from studies of respiratory metabolism and changes in compounds such as glycogen, glucose and lactic acid. Davis and Slater (1926, 1928) and Slater (1927) showed that the amount of lactic acid produced in the American cockroach, *Periplaneta americana* (L.), was not sufficient to account for all the glycogen consumed. The work of Bodine (1928) suggested the occurrence of typical glycolysis in the male nymphs of the red-legged grasshopper, *Melanoplus femur-rubrum* (DeG.) and the differential grasshopper, *Melanoplus differentialis* (Thos.). Blanchard and Dinulescu (1932) and Gilmour (1940, 1941a, 1941b) reported further work on the glycolytic mechanisms in insect tissue. The more recent work of Chefurka (1954) has conclusively demonstrated the presence of the
essential enzymes of vertebrate glycolysis in the insect tissue, viz., hexokinase, isomerase, phosphofructokinase, aldolase, phosphotriose-isomerase, alpha-glycerophosphate dehydrogenase, triosephosphate dehydrogenase, enolase, and lactic dehydrogenase. Chefurka (1955) has also demonstrated the occurrence of a direct oxidative pathway of carbohydrate metabolism in the house fly. McGinnis et al. (1956) showed the presence of glycolytic enzymes in the soluble fraction of homogenates from adults and larvae of the black blow fly, Phormia regina (Meig.). Zebe and McShan (1957), however, have shown that the flight muscles of insects generally possess low amounts of lactic dehydrogenase and surprisingly high amounts of alpha-glycerophosphate dehydrogenase. These results have been interpreted to indicate that there is a system in the insects which accomplishes an immediate and direct breakdown of the metabolites to supply large amounts of energy required during flight. Chefurka (1957) studied glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in extracts of the thoracic flight muscles of the house fly. Both were found exclusively in the soluble fraction of the flight muscles. The activity of both dehydrogenases was dependent on active sulfhydryl groups. These studies confirmed the occurrence of a hexose monophosphate oxidative pathway in the house fly thoracic muscles.

Barron and Tahmisian (1948) recognized the presence of the Krebs cycle in insects. It was shown that the known intermediates of the Krebs cycle enhanced oxygen uptake by American cockroach muscle. Meyerhof (1928), Meyerhof and Lohmann (1928a and 1928b), and Baldwin and Needham (1934) reported that concentrations of arginine phosphate were present in muscle tissues of a crustacean and the insects Calliphora and Lucilia.
Thus arginine phosphate is the invertebrate analogue of the vertebrate phosphagen, creatine phosphoric acid.

Albaum and Kletzkin (1948) have conclusively demonstrated the presence of adenosine triphosphate (ATP) in the fruit fly, *Drosophila melanogaster* (Meig.); the compound possessed the same physical, chemical, and physiological properties as vertebrate ATP. Calaby (1951) confirmed these findings and showed that the ATP in the hind femoral and thoracic tissues of the locust, *Gastrimargus musicus* Fabr., was identical with that from rabbit muscle.

The presence of cytochromes in insects was established by Keilin (1925). Keilin and Hartree (1938, 1940) did further work on the isolation and characterization of cytochromes. Harvey and Beck (1953) found that the leg muscle of the male American cockroach contained an active succinoxidase system. No evidence for the absence of any of the components of the typical mammalian succinoxidase system was reported.

Lewis and Slater (1953, 1954) and Sacktor (1954) demonstrated that insect mitochondria were associated with oxidative phosphorylation.

Thus, conclusive evidence for the existence of anaerobic and aerobic mechanisms, similar to those found in the higher vertebrates for the metabolism of carbohydrates, is limited to information obtained from a few species of insects. It is nonetheless highly probable that these systems exist in the majority of the insects.

A number of extensive reviews on the mode of action of insecticides have appeared in the literature recently (Metcalf 1948, Winteringham and Barnes 1955, Kearns 1956, Dahm 1957, Fukuto 1957, and Spencer and O'Brien 1957). This subject has also been treated exhaustively in various text-
books (Brown 1951, Shepard 1951 and Metcalf 1955). Albert (1951) has discussed in some detail the scientific principles of selective toxicity in connection with chemotherapy. Sexton (1953), in an excellent account of the chemical constitution and biological activity of chemicals, showed that the whole system of metabolic processes was very delicate and complex, and many points of attack were open to any drug which was designed to interfere with the normal working of the system. Winteringham (1957), while discussing the comparative biochemical aspects of insecticidal action stated that there was evidence of differences in the sensitivity of enzymes common to both insects and mammals and that biochemically selective insecticides would be the outcome of the exploitation of such enzymes. There is a large amount of literature on the phenomenon of narcosis and Quastel (1955) has reviewed the biochemical aspects of narcosis. The physicochemical action of inhibitors has been discussed in a very comprehensive treatise by Johnson, Eyring and Polissar (1954).

Several workers in the past have studied the physiological effects of insecticides. As early as 1911, Shafer described interesting experiments designed to study the action of insecticides. It was concluded from these studies that the vapors of gasoline, kerosene, and carbon disulfide after absorption into the insect body, were mainly effective due to their tendency to prevent oxygen consumption by the insects.

When the larvae of the Japanese beetle, Popillia japonica Newm., garden wireworms, Pheletes agonus Say, and adult Colorado potato beetles, Leptinotarsa decemlineata (Say) were given arsenicals by mouth, Fink (1926) found that oxygen consumption was lessened and the respiratory quotient, \( \frac{CO_2}{O_2} \), was raised. The arsenic apparently interfered with
some step in the oxidative process. Parfentjev and Devrient (1930) also demonstrated that oxygen consumption by the American cockroach was decreased when arsenic was injected into the hind coxa.

Tischler (1935) showed that derris had a marked effect on insect respiration. Injection of an extract into grasshoppers, silkworms, and corn earworms caused cessation of the respiratory movements and oxygen consumption dropped 42 per cent of normal. Lord (1949, 1950) and Harvey and Brown (1951) studied the effect of a number of insecticides on the respiration of insects and observed a marked stimulation of the rate of oxygen uptake by Blatella when chlorinated hydrocarbon insecticides (e.g. DDT, methoxychlor, lindane and toxaphene), the dinitro compounds, pyrethrins, nicotine, azobenzene and organic phosphorus insecticides (e.g. parathion and TEPP) were injected through the intersegmental membrane behind the fifth abdominal ventrum. Ryania, rotenone, and organic thiocyanates had a marked depressing effect on respiration.

Parfentjev and Devrient (1930) reported that when 0.05 ml. of a 0.025 M potassium cyanide solution was injected into roaches, no changes in respiration were produced. Keilin (1933) conclusively demonstrated that the toxic action of hydrocyanic acid in animals was due to inactivation of metal-containing respiratory enzymes.

Wolsky (1938) found that carbon monoxide inhibited the respiration of Drosophila melanogaster pupae in proportion to its concentration. Graham (1946) in a comprehensive study of the aerobic oxidation and anaerobic glycolysis concluded that respiration in the muscular tissue of larvae of the codling moth, Carpocapsa pomonella (L.), was inhibited by varying amounts of sodium azide, sodium cyanide, sodium arsenite,
sodium fluoride and sodium iodoacetate. In general, inhibition was pro-
portional to the logarithm of the inhibitor concentration. Glycolysis
was accelerated by cyanide inhibition of oxidation, which provided a
compensatory mechanism for the insect to obtain energy.

Läuger et al. (1946) in their early work on the mode of action of
DDT stated that oxygen consumption was increased almost five times when
house flies were treated with DDT. Very little change in oxygen consump-
tion was noted when flies were treated with pyrethrum. When rabbits were
fed DDT at the rate of one and two grams per kilogram of body weight the
blood sugar and lactic acid increased seven to eight hours after treat-
ment. In experiments with rats it was found that glycogen reserves dis-
appeared completely two to six hours after administration.

Buck and Keister (1949) and Buck et al. (1952) studied the physio-
logical effects of DDT on Phormia larvae and found that the respiratory
quotient, in case of DDT poisoning, rose from 0.74 to 0.84. This shift
would correspond to 11 times the normal carbohydrate oxidation, assuming
that all the oxygen taken up is involved in this process. The oxygen
uptake of poisoned larvae was markedly decreased by ligating the larvae
behind the brain, indicating that the high uptake characteristic of DDT
poisoning was due to neuromuscular activity.

Considerable research has been done on the effects of DDT on various
enzyme systems. Hurst (1947) reported that DDT and analogous compounds
act by indirect blocking of cytochrome oxidase and succinic dehydroge-
nase which may be accomplished by uptake and storage of the insecticides
in the phospholipids of the cell membranes. Johnston (1951) reported
that DDT inhibited succinoxidase and cytochrome oxidase from rat heart
in vitro, but not succinic dehydrogenase.

Sacktor (1950) compared the cytochrome oxidase activity of two strains of house flies. The DDT resistant strain had a greater oxidase activity than did the normal (susceptible) strain. He suggested that one of the explanations for the resistance of house flies to DDT may be the greater cytochrome oxidase activity. Anderson et al. (1954) reported that DDT, DDE or 1,1-dichloro-2,2-bis(p-chlorophenyl) ethane and related compounds inhibited cytochrome oxidase and succinoxidase systems of house flies in vitro, and to a lesser extent succinic dehydrogenase. Perry and Sacktor (1955) compared 10 strains of susceptible and DDT-resistant house flies for their ability to absorb DDT and convert DDT to DDE, and their cytochrome oxidase activity. There was no direct relationship between cytochrome oxidase activity and the ability to change DDT to DDE. It became apparent thus, that no single factor was involved in the mode of action and resistance toward this insecticide.

Morrison and Brown (1954) studied the in vitro effects of 26 insecticidal compounds upon cytochrome oxidase obtained from the coxal muscle of the American cockroach. All the chlorinated hydrocarbons completely inhibited cytochrome oxidase at $10^{-3}$ M. The organic phosphorus compounds were all stimulatory at $10^{-5}$ M. TEPP was stimulatory at $10^{-3}$ M concentration also. The organic phosphorus insecticides parathion or O,O-diethyl O-p-nitrophenyl phosphorothioate and malathion or S-[1,2-bis(ethoxycarbonyl)ethyl] O,O-dimethyl phosphorothioate were completely inhibitory at $10^{-3}$ M. Nicotine was stimulatory at concentrations of $10^{-3}$ and $10^{-5}$ M and rotenone at $10^{-5}$ only. Pyrethrins and allethrin completely inhibited cytochrome oxidase at $10^{-3}$ M; at this same concentration phenothiazine
and thiocyanates markedly inhibited cytochrome oxidase.

Brown and Brown (1956) injected adult, male American cockroaches with lethal doses of DDT, methoxychlor, parathion, malathion, and butoxy thiocyanodiethyl ether in corn oil. Cytochrome oxidase activity in the coxal muscles of the poisoned insects decreased slightly in 24 hours, but only with butoxy thiocyanodiethyl ether was the reduction markedly significant. It was concluded that DDT did not affect the cytochrome oxidase activity in vivo and the in vitro inhibitions reported by other workers were due to non-specific adsorption of the insecticide on the enzyme surface.

Dixon and Needham (1946) and Lewis (1948) have shown that methyl bromide inhibited the sulfhydryl-dependent enzymes like urease, succinic dehydrogenase and yeast hexokinase in vitro.

Mode of action studies of organic phosphorus insecticides have also been numerous in recent years (Fukuto 1957). It is generally agreed that the toxicity of organic phosphorus insecticides to mammals is associated with the inhibition of cholinesterases, although other enzymes such as liver esterase, chymotrypsin and trypsin have also been inhibited by these compounds (Kilby and Youatt 1954). Metcalf and March (1953), Augustinsson and Grahn (1954), Chefurka and Smallman (1955) and Metcalf et al. (1955) have shown that the toxicity of organic phosphorus insecticides to insects was clearly associated with the cholinesterase enzyme system. Aldridge and Davison (1952) have studied the mechanism of inhibition of cholinesterase by para-oxon or diethyl p-nitrophenyl phosphate and have concluded that the inhibition results from irreversible phosphorylation at some active site.
O'Brien (1956) showed that malathion depressed cholinesterase activity sharply, immediately after treatment of cockroaches. The activity of the enzyme rose slowly until death. The succinoxidase system was also inhibited but was not believed to be the cause of death. It has been shown by Richards and Cutkomp (1945) and Tobias et al. (1946) that DDT does not have any effect on cholinesterase.

Torda and Wolff (1949) reported inhibition of mammalian carbonic anhydrase by DDT. Anderson and March (1956), however, failed to detect any inhibition of carbonic anhydrase in Periplaneta americana and concluded that inhibition of this enzyme was not a factor in the toxicity of DDT.

Judah and Williams-Ashman (1951), using rabbit kidney preparations, showed that the toxic action of dinitrophenols was fundamentally due to their ability to block oxidative phosphorylation, thus preventing the utilization of energy derived from the oxidation of carbohydrates. The acceleration of respiration and glycolysis of intact cells, produced by intoxication due to these chemicals apparently resulted from the increased availability of orthophosphate and adenylic acceptors which normally limit carbohydrate metabolism.

Dahm and Kearns (1951) did not attribute differences in levels of phosphoarginine and pyruvic acid in adult house flies to the effects of DDT poisoning.

Winteringham et al. (1953, 1955) devised a technique for incorporating P\textsuperscript{32} into various metabolic intermediates of the house fly. These intermediates were extracted and separated by paper chromatography. Since phosphorylated intermediates are involved in a most vital manner in the
function of muscle, any disturbances in the normal distribution of these intermediates caused by a foreign compound would be of great significance in explaining the mode of action of the compound. Winteringham et al. (1954) also showed that methyl bromide caused considerable depletion of adenosine triphosphate (ATP) in house fly thoracic tissue. Winteringham and Hellyer (1954) compared the effects of methyl bromide, ethylene dibromide and ethylene dichloride on the distribution of P\(^{32}\)-labeled intermediates extracted from thoracic muscle tissue of house flies. The slow depletion of phosphoglycerate caused by the first two chemicals suggested inhibition of triose phosphate dehydrogenase. Depletion of ATP and arginine phosphoric acid by methyl bromide indicated a rapid blocking of the phosphorylation of nucleotides. Winteringham (1956) reported that levels of ATP in the tissues of house flies fall drastically 10 hours after topical application of DDT.

Rosedale (1948) found that adenylic acid was absent in termites, *Trinervitermes havilandi*, killed with DDT.

Slade (1945) suggested that lindane, the gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane, may be isosteric with the B-vitamin, meso-inositol, and thus block some vital process. There is evidence, however, which points to the failure of the above hypothesis (Kearns 1956). No specific biochemical evidence to explain the mode of action of lindane has yet been given. Mullins (1954, 1955) has advanced a physical basis for the insecticidal action of the isomers of 1,2,3,4,5,6-hexachlorocyclohexane. He proposed a model membrane composed of a lattice of cylindrical lipoprotein molecules oriented in such a way that passage was provided for ions and small molecules from one side of the membrane to another.
A foreign molecule entering the interspace without causing distortion in the three surrounding lipoprotein molecules would have a narcotic effect relative to its thermodynamic activity. He used as a model, a membrane lattice composed of lipoprotein molecules spaced 2 Å apart and having 40 Å diameter so that one could inscribe a circle 8.5 Å in diameter in the resulting interspace. DDT and lindane molecules constructed from Stuart models and imposed in the interspace drawn on a comparable scale, showed that only DDT molecules in the end-on position and lindane, of all the isomers of BHC, could enter the membrane interspace at plane and end-on orientations. The attractive forces of the halogens in these orientations were also in the most favorable position to act on the surrounding lipoprotein molecules.

Thus it has been pointed out that several studies have been done in the past on the mode of action of insecticides. Conflicting results have been obtained as regards the inhibition of cytochrome oxidase and succinic dehydrogenase. There have been differences in the methods of application of the insecticides. The more recent work points towards the desirability of in vivo studies.
MATERIALS AND METHODS

General

Rearing of the house flies

Adult, female house flies, Musca domestica L.\(^1\) were used as the test insects in these studies, because the thorax of the house fly is a good source of muscle tissue and the insects are easily reared in the laboratory. A convenient rearing method, outlined by the Chemical Specialities Manufacturers Association, is available for this insect (Anonymous 1956). The fly larval medium\(^2\) was mixed with yeast and non-diastatic diamalt according to the directions outlined in the rearing method. Glazed clay jars, about one gallon in capacity, were used to rear the larvae. When the larvae were ready to pupate, a layer of sand about two inches deep was put on top of the medium. Most of the larvae pupated in the sand and were separated easily by means of a sieve. The pupae were placed in wire screen cages where the adults emerged and were fed diluted condensed milk. In all the experiments, unless specified differently, three- to five- day old females were used.

Insecticides and fumigants

The following insecticides and fumigants were used:

\(^{1}\)This insecticide-susceptible strain was obtained from Dr. T. P. Sun, Shell Chemical Corp., Modesto, Calif.

\(^{2}\)Chemical Specialities Manufacturers Association (CSMA) standard fly larval medium. The Ralston Purina Co., St. Louis, Mo.
Hydrocyanic acid\(^3\), ethylene dibromide or 1,2-dibromoethane\(^4\), ethylene dichloride or 1,2-dichloroethane\(^4\), carbon disulfide\(^5\), pyrethrum extract containing pyrethrins and cinerins (see formula I)\(^6\), DDT or 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane (II)\(^7\), and Dipterex or O,O-dimethyl 2,2,2-trichloro-1-hydroxyethylphosphonate (III)\(^8\).

Application of the insecticides and fumigants

Considerable time was spent in selecting low dosages which would allow an exposure period of from four to six hours to increase the physiological effects of toxicants, except for pyrethrum extract and hydrocyanic acid where the exposure periods used were one to three hours. In the work with cytochrome oxidase the dosages of the fumigants were higher than in the rest of the work; the onset of paralytic symptoms at these higher dosages was very rapid, hence the exposure periods were kept short. In all cases, however, dosages and exposure periods were regulated carefully so that the insects were alive at the time of tissue extraction.

\(^3\)Cyanogas, active ingredient - calcium cyanide 42%. Obtained from the American Cyanamid Co., 30 Rockefeller Plaza, New York 20, N. Y.

\(^4\)Obtained from the Douglas Chemical Co., 620 E. 16th Avenue, North Kansas City 16, Mo.

\(^5\)Baker Analyzed Reagent grade, obtained from the J. T. Baker Chemical Co., Phillipsburg, N. J.

\(^6\)Pyrethrum extract 20% obtained from John Powell and Co., Inc., New York 16, N. Y.

\(^7\)p,p' - DDT, obtained from Geigy Agricultural Chemicals, Saw Mill River Road, Ardsley, N. Y.

\(^8\)Obtained from Farbenfabriken, Bayer Laboratory, Blue Point, L. I., N. Y.
Esters

Chrysanthemic esters

Pyrethrin I
Cinerin I

Pyrethric esters

Pyrethrin II
Cinerin II

Structures

<table>
<thead>
<tr>
<th>Esters</th>
<th>R</th>
<th>R'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chrysanthemic esters</td>
<td>R</td>
<td>R'</td>
</tr>
<tr>
<td>Pyrethrin I</td>
<td>-CH₃</td>
<td>-CH₂CH=CHCH=CH₂</td>
</tr>
<tr>
<td>Cinerin I</td>
<td>-CH₃</td>
<td>-CH₂CH=CHCH₃</td>
</tr>
<tr>
<td>Pyrethrin II</td>
<td>-C(O)OCH₃</td>
<td>-CH₂CH=CHCH=CH₂</td>
</tr>
<tr>
<td>Cinerin II</td>
<td>-C(O)OCH₃</td>
<td>-CH₂CH=CHCH₃</td>
</tr>
</tbody>
</table>

(I) Pyrethroids

(II) DDT

(III) Dipterex

Two round-bottom flasks, with volumes of 5400 and 5500 ml. respectively, were used for application of the fumigants. A measured volume of each liquid fumigant was applied with a micro-pipette to a cotton swab attached to the rubber stopper of the flask. In the case of hydrocyanic acid, calcium cyanide was placed on a small aluminum foil boat and the latter attached to the stopper. Reaction of atmospheric water vapor with the calcium cyanide produced the hydrocyanic acid. The other insecticides either were applied topically as acetone solutions on the mesonotum of the flies with a one-μl. pipette or the flies were exposed to a film of the insecticide on a glass plate. During the exposure periods, the
flies were confined either in the flasks or in a carton containing the glass plate. The topically treated flies were confined in plain cartons. The exposure periods are given under the Results and Discussions.

Preparation of homogenates

The treated and untreated flies were immobilized temporarily by refrigeration and transferred to a watch glass resting on a mixture of sodium chloride and ice. The heads, abdomens, wings and legs were removed quickly and the thoraces dropped into a Potter-Elvehjem tissue homogenizer\(^9\) containing 0.01 M phosphate buffer solution (pH 7.4). The buffer solution consisted of a solution of potassium dihydrogen phosphate and dipotassium hydrogen phosphate in glass-distilled water. The homogenizer containing buffer solution and fly thoraces was submerged in a salt–ice mixture contained in a 25-ml Erlenmeyer flask. The tissue was dispersed by attaching the pestle to an electric stirrer\(^10\). Unless otherwise stated, the tissue was dispersed for a one-minute period and the homogenate kept immersed in the salt–ice bath. Immediately before use the homogenate was sieved through a double layer of cheese-cloth to remove the particles of cuticle.

Cytochrome Oxidase

Cytochrome oxidase is the terminal enzyme in the aerobic electron transport scheme (Figure 1). The activity of cytochrome oxidase in the


house fly muscle homogenate was determined by measuring spectrophotometrically the amount of reduced cytochrome c, \( \text{Cy}[\text{Fe}^{++}] \), present at a definite time interval (Sacktor 1950 and Cooperstein and Lazarow 1951). This method is based upon the rate of oxidation of reduced cytochrome c which has a sharp absorption maximum at 550 mp. (Keilin 1933). As the reduced cytochrome c is oxidized, the optical density decreases; the rate of decrease is a measure of the oxidase activity. The optical density observed at a particular time gives the sum of the densities of reduced and oxidized cytochrome c, \( \text{Cy}[\text{Fe}^{++}] \). Thus for a mixture of oxidized and reduced cytochrome c the relationship between light absorption and their concentrations is expressed by the formula:

\[
\log \left( \frac{I_o}{I} \right)_t = \alpha_o \left( \text{Cy}[\text{Fe}^{++}] \right)_L + \alpha_r \left( \text{Cy}[\text{Fe}^{+}] \right)_L
\]

(1)

where:

- \( I_o \) = intensity of incident light,
- \( I \) = intensity of transmitted light,
- \( t \) = time interval after the addition of enzyme preparation,
- \( \alpha_o \) = extinction coefficient of oxidized cytochrome c,
- \( \alpha_r \) = extinction coefficient of reduced cytochrome c,
- \( \text{Cy}[\text{Fe}^{++}] \) = oxidized cytochrome c,
- \( \text{Cy}[\text{Fe}^{+}] \) = reduced cytochrome c, and
- \( L \) = length of the absorption cell.

After all the cytochrome c has been oxidized,

\[
\log \left( \frac{I_o}{I} \right)_{\text{inf}} = \alpha_o \left[ \text{Cy} \right]_L
\]

(2)
where:

\[ \text{Inf} = \text{time when all the reduced cytochrome c has been oxidized, and} \]

\[ [\text{Cyt}] = \text{sum of the concentrations of reduced and oxidized cytochrome c. In this case and in expressing the concentration of } [\text{Cy}^{\text{Fe}^{++}}] \text{below the terms are placed in parenthesis to indicate the concept of concentration.} \]

By combining formulae (1) and (2), the concentration of reduced cytochrome c, \( [\text{Cy}^{\text{Fe}^{++}}] \), at any time \( t \), can be expressed as follows:

\[
[\text{Cy}^{\text{Fe}^{++}}] = \frac{\log \frac{L_0^{1/T}}{T} - \log \frac{L_0^{1/T}}{T}}{\alpha - \alpha_0} \quad (3)
\]

Therefore,

\[
\log([\text{Cy}^{\text{Fe}^{++}}]) = \log \frac{OD_t - OD_{\text{Inf}}}{(\alpha_T - \alpha_0)}
\]

\[
= \log(OD_t - OD_{\text{Inf}}) - k \quad (4)
\]

where:

\[ OD_t = \text{optical density of the solution at time } "t", \]

\[ OD_{\text{Inf}} = \text{optical density of the solution after complete oxidation of reduced cytochrome c, and} \]

\[ k = \text{constant.} \]

Thus by obtaining the logarithm of \( (OD_t - OD_{\text{Inf}}) \) the term \( \log([\text{Cy}^{\text{Fe}^{++}}]) \) + \( k \) at any time was obtained. By subtracting this value from the one obtained at zero time, the change in the concentration of reduced cytochrome c with time, \( \Delta \log([\text{Cy}^{\text{Fe}^{++}}]) \), was calculated. In order to compare the activities of cytochrome oxidase in homogenates from the control and treated flies the relationship, \( \Delta \log([\text{Cy}^{\text{Fe}^{++}}]) \) per minute, was
obtained by dividing $\Delta \log(Cy[Fe^{++}])$ by the time intervals, one through five minutes after adding the enzyme preparation. The rate of change in the concentration of reduced cytochrome c, $\Delta \log(Cy[Fe^{++}])$ per minute was averaged and expressed as the change in concentration of reduced cytochrome c per minute.

To determine the purity of the cytochrome c\textsuperscript{11} sample, an aqueous solution of cytochrome c (approximately 0.17 mg. per ml.) was reduced by sodium hydrosulfite ($Na_2S_2O_4 \cdot 2H_2O$), and the optical density curve obtained with a Beckman, model DU, spectrophotometer, using one-cm. cells.

In order to see the effect of homogenate concentration on enzyme activity, 20 thoraces were homogenized in five ml. of the cooled phosphate buffer solution as described previously to give an approximately three per cent (by weight) concentration of tissue. This homogenate was filtered through cheese-cloth and diluted as follows:

- Conc. 1 - 0.5 ml. in 50 ml. phosphate buffer solution (pH 7.4).
- Conc. 2 - 0.5 ml. in 25 ml. phosphate buffer solution (pH 7.4).
- Conc. 4 - 1.0 ml. in 25 ml. phosphate buffer solution (pH 7.4).

A $1.3 \times 10^{-5} \text{M}$ solution of cytochrome c was prepared in 0.01 M phosphate buffer (pH 7.4). To 10 ml. of this cytochrome c solution was added 0.03 ml. of a freshly prepared 1.2 M solution of sodium hydrosulfite. The solution was shaken vigorously for two minutes and 2.5 ml. of this solution were transferred to a one-cm. spectrophotometer cuvette. Next, 0.5 ml. of the diluted homogenate was added to the cuvette containing reduced cytochrome c. The actual amounts of original three per cent

\textsuperscript{11}Obtained from General Biochemicals Inc., Chagrin Falls, Ohio
homogenate added to each cuvette were 0.005, 0.010, and 0.020 ml., respectively. The cuvette was shaken to mix the contents thoroughly and optical density values read at one-minute intervals for a period of five minutes. At the end of this period 0.1 ml. of $10^{-3} \text{M}$ potassium ferricyanide solution was added to oxidize the cytochrome c. The values of $\Delta \log ([\text{Cy}^{\text{Fe}^{+}}])$ per minute were then calculated and comparisons made between the control and the treated samples.

Since there were slight day to day variations in the enzyme activity and the weights of the thoraces, 40 female flies of the same age and from the same breeding cage were used to make two cytochrome oxidase determinations (20 fly thoraces were used in each determination). One group of 20 flies was used as control and the other group was subjected to treatment. For all these determinations one ml. of the original three per cent homogenate was diluted in 25 ml. of buffer solution.

Succinic Dehydrogenase

Succinic dehydrogenase is the enzyme involved in the oxidation of succinate to fumarate (Figure 1). The activity of succinic dehydrogenase was determined manometrically (Bonner 1955). It has been shown by Slater (1949) that no simple method for estimating succinic dehydrogenase has been found, hence, any assay for this enzyme has to be carried out in the presence of some or all the components of the succinic dehydrogenase-cytochrome oxidase system. The expression "succinic dehydrogenase system" which is used below refers to the enzymes which catalyze the anaerobic oxidation of succinate, including cytochrome b and succinic dehydrogenase.

The manometric assay of the succinic dehydrogenase system depends
on the measurement of oxygen consumption during succinate oxidation in the presence of cyanide and methylene blue. The reactions this system undergoes are as follows:

\[
\begin{align*}
\text{Succinate} + \text{Methylene blue} & \rightarrow \text{Leucomethylene blue} + \text{Fumarate} \\
\text{Leucomethylene blue} + O_2 & \rightarrow \text{Methylene blue} + H_2O_2
\end{align*}
\]

\[
\text{Succinate} + O_2 \rightarrow \text{Fumarate} + H_2O_2
\]

Thus the final reaction in this assay is with leucomethylene blue and the product of the oxidation is hydrogen peroxide. The observed oxygen uptake must be halved because in the living system cytochromes are present and the over-all reaction is as follows:

\[
\text{Succinate} + \frac{1}{2}O_2 \rightarrow \text{Fumarate} + H_2O.
\]

In the present work, this correction was effected by taking manometer readings for 30 minutes and assuming that this was the oxygen consumption for a one-hour period.

Reagents

- 0.01 M methylene blue
- 0.10 M NaCN (adjusted to pH 7.0).
- 0.40 M Na succinate
- Phosphate buffer to give a final concentration of 0.10 M phosphate at pH 7.4.

A Warburg apparatus\textsuperscript{12} was used to measure the rate of oxygen consumption. Reaction vessels with one side arm and capacities varying

\textsuperscript{12}Gilson Medical Electronics Circular Refrigerated Model EWB-3, Gilson Medical Electronics, Route 1, Middleton, Wis.
from 16 to 19 ml. were used. All the experiments were carried out at 37° C.

Forty female house flies were obtained from a rearing cage and two separate homogenates prepared by dispersing 20 thoraces in 2.5 ml. of the phosphate buffer solution (pH 7.4) described above under the topic "preparation of homogenate" giving an approximately six per cent (by weight) homogenate. Three such control experiments were conducted. In the rest of the experiments 20 flies were used for each of the insecticide treatments and another 20 from the same rearing cage as controls. The following reagents were added to the main compartment of each reaction flask:

<table>
<thead>
<tr>
<th>Addition</th>
<th>ml. per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 M NaCN (adjusted to pH 7.0)</td>
<td>0.30</td>
</tr>
<tr>
<td>0.01 M methylene blue</td>
<td>0.30</td>
</tr>
<tr>
<td>0.40 M Na-succinate</td>
<td>0.20</td>
</tr>
<tr>
<td>0.176 M phosphate buffer (pH 7.4)</td>
<td>1.70</td>
</tr>
<tr>
<td>Total volume</td>
<td>2.50</td>
</tr>
</tbody>
</table>

Into the side arm was added 0.5 ml. of the homogenate. Thus each flask contained a total volume of three ml. The reaction flask was attached to the manometer, and after an equilibration period of five minutes, the contents of the side arm were tipped into the main compartment. Manometer readings were taken for a period of 30 minutes at five-minute intervals. A comparison of oxygen consumption values of the homogenates was made to determine the effect of insecticides on enzyme activity. Each homogenate preparation was run in either triplicate or quadruplicate series.
Over-all Glycolysis

Over-all glycolysis can be summarized as the production of two molecules of lactic acid from one glucose molecule (Figure 1). The over-all glycolytic activity of house fly thoracic muscle tissue was measured manometrically under anaerobic conditions (LePage 1948, Potter 1957). Over-all glycolysis is accomplished by a complex series of reactions which can be divided into four stages. First, glucose is esterified and transformed to fructose-1,6-diphosphate. Adenosine triphosphate (ATP) acts as the source of phosphorus. In the next stage, fructose-1,6-diphosphate is split into two triose phosphates, which are oxidized to phosphoglyceric acid. This oxidative step is coupled with the phosphorylation of adenosine diphosphate (ADP) to ATP. In the third stage, phosphoglyceric acid is transformed to phosphopyruvic acid, which transfers an energy-rich phosphate to ADP. In the fourth stage, pyruvic acid is reduced to lactic acid. Under experimental conditions, when the tissue is ground, there is a diminution in coenzyme concentration, due to dilution and partial destruction. Hence, in an assay for over-all glycolytic activity it is necessary to add various coenzymes and cofactors. The following mixture (Potter 1957) was used in these experiments:

<table>
<thead>
<tr>
<th>Addition</th>
<th>ml. per flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (glass-distilled)</td>
<td>0.75</td>
</tr>
<tr>
<td>0.024 M KH₂PO₄-K₂HPO₄ buffer (pH 7.4)</td>
<td>0.30</td>
</tr>
<tr>
<td>0.50 M KHCO₃</td>
<td>0.15</td>
</tr>
<tr>
<td>Addition</td>
<td>ml. per flask</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>0.40 M Nicotinamide(^{13})</td>
<td>0.30</td>
</tr>
<tr>
<td>0.01 M K-ATP(^{14})</td>
<td>0.10</td>
</tr>
<tr>
<td>0.20 per cent DPN(^{15})</td>
<td>0.20</td>
</tr>
<tr>
<td>0.04 M K-HDP(^{16})</td>
<td>0.15</td>
</tr>
<tr>
<td>0.10 M MgCl(_2)</td>
<td>0.20</td>
</tr>
<tr>
<td>0.10 M Glucose</td>
<td>0.30</td>
</tr>
<tr>
<td>0.15 M Na-pyruvate(^{13})</td>
<td>0.10</td>
</tr>
<tr>
<td>0.20 M KF</td>
<td>0.15</td>
</tr>
<tr>
<td>Homogenate</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Total volume 3.00

For each day's work 20 flies were used as control and another 20 were treated with an insecticide or fumigant. Twenty thoraces were homogenized in 2.5 ml. of phosphate buffer solution.

The Warburg flasks used in these determinations were equipped with a side arm and venting tube and had capacities varying from 16 to 19 ml. Before preparing the tissue homogenates the components of the mixture listed above were added to the main compartment of each flask (which

\(^{13}\) Obtained from Sigma Chemicals, 4648 Eastern Avenue, St. Louis 13, Mo.

\(^{14}\) Adenosine triphosphate. Obtained from Pabst Laboratories, 221 No. LaSalle Street, Chicago, Ill.

\(^{15}\) Diphosphopyridine nucleotide, Coenzyme 1. Obtained from Schwarz Laboratories, Inc., 230 Washington Street, Mt. Vernon, N. Y.

\(^{16}\) Hexose diphosphate. Obtained from Sigma Chemicals as the barium salt and converted to the potassium salt by the method of Dounce and Beyer (1948).
was then kept partly immersed in chopped ice), and 0.25 ml. of 65 per cent trichloroacetic acid solution was added to the side arm of each flask. The tissue homogenate was added to the main compartment of each flask and the flask attached immediately to a manometer. The manometers were transferred to the water bath (at 37° C.) and connected to a gassing manifold; the flasks were then flushed with a mixture of 95 per cent nitrogen and five per cent carbon dioxide\(^\text{17}\) for a period of 15 minutes. During this period the Brodie solution in the manometer arms was moved up and down several times to remove air from the system. At the end of 15 minutes, gassing was stopped and the Brodie solution in the manometer arms brought to the zero point. Manometer readings were taken at 10-minute intervals over an incubation period of approximately 40 minutes. The trichloroacetic acid in the side arm was then tipped into the main compartment. This gave a final concentration of five per cent trichloroacetic acid, which, according to LePage (1948) was sufficient to precipitate the proteins. Carbon dioxide evolution and production of lactic acid in the reaction mixture were used to compare the glycolytic activity of the control and the treated flies.

**Determination of lactic acid**

Lactic acid produced in the Warburg flasks was determined by the colorimetric method of Barker and Summerson (1941). According to these authors glucose and other interfering substances were removed by treatment of the sample with copper sulfate and calcium hydroxide. An aliquot of the resulting solution was heated with concentrated sulfuric acid to con-

\(^{17}\)Obtained from Matheson Co., Inc., Joliet, Ill.
vert lactic acid to acetaldehyde, which was then determined colorimetrically by reaction with \( p \)-hydroxydiphenyl in the presence of copper ions.

**Reagents**

- \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O}, 20\% \)
- \( \text{CuSO}_4 \cdot 5\text{H}_2\text{O}, 4\% \)
- \( \text{Ca(OH)}_2, \) powdered
- \( \text{H}_2\text{SO}_4, \) concentrated, sp. gr. 1.84
- Solution of \( p \)-hydroxydiphenyl, 1.5\%, in 0.5\% NaOH

To clear the filtrate further, the contents of the reaction flask were filtered very carefully after the addition of trichloroacetic acid, and the filtrate centrifuged. A one or two ml. aliquot of the supernatant solution was added to one ml. of 20 per cent copper sulfate solution. The volume was made to 10 ml. with distilled water. Approximately one gram of powdered calcium hydroxide was added to the solution and the mixture shaken vigorously. The mixture was allowed to stand at room temperature for half an hour with occasional shaking and then centrifuged at a relative centrifugal force of 200 \( \times \) gravity for 10 minutes. A one ml. aliquot of the clear supernatant was used for final color development. Every care was taken not to include any solid material when transferring the supernatant liquid to a clean test tube. The test tube and contents were immersed in an ice bath and six ml. of pre-cooled, concentrated sulfuric acid were added. The test tubes were shaken during the addition of the acid to prevent excessive heating. After all the acid had been added, the tubes were placed in a boiling water bath for five minutes and then cooled to 30° C. or slightly below. One drop each of the four per
cent copper sulfate and \( p \)-hydroxydiphenyl reagents were added. The tubes were shaken and incubated at 28-30° C. in a beaker of water for 30 minutes. The tubes were then heated in boiling water for 90 seconds, cooled, and the samples transferred to colorimeter tubes.

The solutions were read against a reagent blank using a filter with maximum absorbance at 560 mu. in a Klett-Summerson photoelectric colorimeter. As recommended by Summerson (1939) and Barker and Summerson (1941), the results were calculated in terms of a simultaneously prepared standard, rather than by reference to a previously measured one. This assured similar treatment of the test sample and the standard. The concentration of lactic acid in the test sample was calculated as follows:

\[
\text{Concentration of lactic acid in test sample} = \frac{\text{Concentration of lactic acid in the standard} \times \text{Reading of test sample}}{\text{Reading of standard}}
\]

Distribution of \( P^{32} \)-Labeled Intermediates

Incorporation of \( P^{32} \) into tissues of the house fly

Newly emerged female house flies were kept in the rearing room overnight without food or water. The flies were immobilized by brief refrigeration and 25 females transferred to an all-glass metabolism chamber (Figure 2) patterned after a chamber used by Winteringham et al. (1955). The cylindrical chamber was approximately 400 ml. in volume. The floor was connected to the chamber with a ground-glass joint and contained a feeding well in the center with a capacity of approximately one ml. The feeding well was filled with absorbent Cellucotton. The top of the
Figure 2. Metabolism chamber used to incorporate $^{32}$P into the tissues of house flies
chamber consisted of a reservoir unit with a long dropper extending to just above the feeding well at the bottom of the chamber; the flow of liquid from the reservoir to the feeding well was controlled by a glass stopcock. The chamber had two openings on the side for circulating dry air. Two lots of carrier-free P$^{32}$ obtained as a phosphate in weak hydrochloric acid solution,$^{18}$ were used in these experiments. One lot of the radioisotope had a specific activity of 3.88$\pm$ 10 per cent milli­curies and the other, 0.284$\pm$ 10 per cent millicurie per milliliter (mc./ml.). For each experiment an aliquot of the radioactive solution containing 50 microcuries (mc.) of P$^{32}$ was diluted to 2.5 ml. with distilled water; 150 mg. of glucose were added to the solution and it was neutralized to pH 7.0 with 1 N potassium hydroxide. The neutralized radioactive solution was then transferred to the reservoir above the metabolism chamber and the solution added dropwise into the feeding well. The flies were allowed to feed on the radioactive solution for 24 hours, during which time almost all the solution was either consumed or evaporated. At the end of this period the flies were transferred to a one-quart, cylindrical, cardboard carton covered on both ends with wire-screen. The flies were kept in these cartons for another 24 hours in order to allow the P$^{32}$ to become incorporated into the house fly tissues and metabolic intermediates. The flies were fed diluted, condensed milk during this latter 24-hour period.

Extraction of the thoracic muscle tissue

The radioactive flies were taken out of the carton and were sub-

---

$^{18}$ Obtained from Oak Ridge National Laboratory, Oak Ridge, Tenn.
jected to either insecticidal or fumigant treatment as described above or were used as untreated or control flies. Ten flies from the above treatments were then confined in a small wire-screen cage (approximately one-cm. cube) and plunged into liquid nitrogen. The frozen flies were transferred to a watch glass kept cold on a dry-ice:acetone mixture. The heads, abdomens, wings and legs were quickly removed; the remaining thoraces were returned to the small wire-screen cage and immersed in a cold (-15°C) 50 per cent ethanol solution containing five per cent (v/v) orthophosphoric acid which was added to remove external contamination of the thoraces with P32-phosphate. The cage and thoraces were then rinsed successively in 50 per cent aqueous ethanol and glass-distilled acetone, both maintained at -15°C. The washed thoraces were then dropped into 1.8 ml. of 1 N formic acid in 50 per cent ethanol (at -10°C to -15°C) contained in a tissue homogenizer which was cooled by immersing it in an acetone-ice mixture. The thoraces were homogenized for 10 minutes and then centrifuged at a relative centrifugal force of 250xgravity for 10 minutes. The mixture was kept cold during centrifugation by placing the 12-ml. centrifuge tubes used in this process in 250-ml. centrifuge tubes containing chopped ice. The supernatant liquid was decanted and stored at a temperature of -10°C. The sediment was dispersed twice with ethanolic formic acid as described above and all the supernatant liquid from centrifugation was pooled and concentrated to approximately one ml. under high vacuum, using liquid nitrogen to trap the solvent. The concentrated extract was finally centrifuged at room temperature for 10 minutes at a relative centrifugal force of
SOOxgravity. The resulting supernatant liquid was used for paper chromatographic separation of the P\textsuperscript{32}-labeled metabolic intermediates as described below.

**Chromatographic separation of the metabolic intermediates**

The intermediary metabolites extracted in the above manner were separated by uni-dimensional, descending paper chromatography. The solvent used was a mixture of formic acid, acetone, and water (14:60:26 parts by volume) as recommended by Burrows *et al.* (1952) and Winteringham *et al.* (1955). The latter investigators used three solvent systems for further separation of ATP, ADP, arginine phosphate, adenosine monophosphate, and glucose-1-phosphate. However, further separation of these compounds by the solvents recommended by Winteringham *et al.* (1955) was not very satisfactory and therefore further separation of these compounds was not undertaken in the present study. The ratio of ATP + ADP to inorganic phosphate could be computed from the movements of these compounds during the initial chromatographic separation (using the formic acid:acetone:water solvent mixture) and this ratio was used as an indication of total phosphorus utilized for the production of high energy bonds.

Whatman No. 1 filter paper, in one-inch wide strips, was used for the chromatographic separation. The paper was soaked in 0.1 N hydrochloric acid overnight, rinsed six times in glass-distilled water, dried, and cut into strips 60 cm. long which were folded to provide a distance of 50 cm. for solvent travel. A cylindrical glass jar, 30 cm. in diameter and 60 cm. in height, was used as the chromatography chamber.
The chamber was maintained at 3° to 4° C. (in a refrigerator) and contained about 300 ml. of the solvent mixture at the bottom and a filter-paper lining on the walls to facilitate equilibration of the solvent vapors within the chamber. Twelve to 16 µl. of the concentrated extract were applied with a micropipette on the strip so that the diameter of the application spot was approximately 0.5 cm. The strip was then placed inside the chamber. After an equilibration period of about three hours, 80 ml. of the solvent mixture were added to the trough at the top of the chamber and the chromatograms allowed to develop. The solvent front advanced about 45 cm. from the point of application (Rf 0) in about nine hours. Usually two to four strips were processed at one time. The strips were taken out of the chamber, air-dried, and scanned for radioactivity with an ultra-thin window Geiger-Mueller tube. This equipment has been described by Schmidt and Dahm (1956). The following non-radioactive compounds were used to locate Rf values (ratio of distance traveled by compounds to distance traveled by the solvent front) of known compounds and to help identify the radioactive fractions separated by the same method of chromatography: ATP19, glucose-1-phosphate19, 3-phosphoglyceric acid20, and dipotassium hydrogen phosphate. All the above compounds were converted to the hydrogen-form by adding Dowex 50, cation exchange resin to solutions of above salts in distilled water and

19 Obtained as the dipotassium salt from Dr. D. E. Metzler, Dept. of Chemistry, Iowa State College, Ames, Iowa.

20 Obtained as the barium salt from Dr. S. Aronoff, Dept. of Botany, Iowa State College, Ames, Iowa.
stirring the solution. The locations of the unlabeled compounds on paper strips were detected colorimetrically using the spray reagent recommended by Bandursky and Axelrod (1951). After the chromatograms were sprayed they were dried at 80° C. for about two minutes and exposed to ultraviolet radiation at a distance of about 15 cm. from a Champion 15-watt, ultra-violet germicidal lamp. The location of the three organic phosphate compounds was indicated by the appearance of blue spots on the chromatograms; the location of the inorganic phosphate was indicated by a yellow-green spot. The \( R_f \) values were determined by measuring the distances of the colored spot and the solvent front from the origin and computing their ratio.

In the case of the chromatograms containing the labeled intermediary metabolites the area under each peak on the recording of the chromatogram was measured with an optical planimeter\(^2\). The area under each peak was then taken as an index of the total amount of the labeled compound present.

\(^2\)Optical planimeter with adjustable tracing arm. Planimeter type 236A. Filotecnica, Milano, Italy. Loaned by Dr. W. E. Scholtes, Dept. of Agronomy, Iowa State College, Ames, Iowa
RESULTS AND DISCUSSION

Cytochrome Oxidase

The optical density curve for reduced cytochrome c is shown in Figure 3; a sharp peak at 550 nm confirmed the purity of the material (Keilin 1933). The effect of different homogenate concentrations on the activity of cytochrome oxidase is shown in Figures 4 and 5. The rate of change (logarithmically) of reduced cytochrome c per minute, $\Delta \log \text{Cy}[Fe^{++}]$ per minute plotted in Figure 4 shows that over the range of the three concentrations used the cytochrome oxidase activity was directly proportional to the homogenate concentration. When the activity of three homogenate concentrations, expressed as $\Delta \log \text{Cy}[Fe^{++}]$, was plotted against time (Figure 5), the activity of the two lower concentrations, containing 0.005 ml. and 0.010 ml. homogenate per cuvette, was very low. Therefore it was decided to use a higher concentration, containing 0.020 ml. homogenate per cuvette, for the experiments with insecticides because this concentration provided greater sensitivity to detect any inhibition of the enzyme by the toxicants used.

Effects of the insecticides and fumigants on the cytochrome oxidase activity of house fly thoracic muscle tissue

The methods of application, dosages, exposure periods, and effects of the insecticides and fumigants on cytochrome oxidase activity in the homogenates from fly muscle tissue are shown in Table 1.

Hydrocyanic acid, carbon disulfide and Dipterex inhibited cytochrome oxidase activity. Keilin (1929) reported that cyanide and hydrogen sulfide
Figure 3. Optical density curve for reduced cytochrome c; the concentration of cytochrome c was approximately 0.17 mg. per ml. The curve was prepared using a one-cm. cell in a Beckman model DU spectrophotometer.
Figure 4. The change in the activity of cytochrome oxidase (as estimated by measuring the change in the concentration of reduced cytochrome c) by varying the concentration of the homogenate obtained from house fly thoracic muscle tissue.
Figure 5. Cytochrome oxidase activity (as determined by the rate of change of concentration of reduced cytochrome c) of three homogenate concentrations prepared from female house fly thoracic muscle tissue.
Table 1. Effect of insecticides and fumigants on the cytochrome oxidase activity of female house fly thoracic muscle homogenates as determined by measuring spectrophotometrically the rate of change of concentration of reduced cytochrome c (single assays)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Method of application</th>
<th>Dosage</th>
<th>Exposure period (minutes)</th>
<th>Cytochrome oxidase activity*</th>
<th>Inhibition (%)</th>
<th>Stimulation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocyanic acid</td>
<td>Gas</td>
<td>1 mg. Ca(CN)$_2$/liter (l.)</td>
<td>10</td>
<td>49</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td>Ethylene dibromide</td>
<td>Gas</td>
<td>3.7 μl./l.</td>
<td>60</td>
<td>-</td>
<td>46</td>
<td>15</td>
</tr>
<tr>
<td>Ethylene dichloride</td>
<td>Gas</td>
<td>37 μl./l.</td>
<td>120</td>
<td>-</td>
<td>28</td>
<td>42</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>Gas</td>
<td>18 μl./l.</td>
<td>120</td>
<td>22</td>
<td>23</td>
<td>-</td>
</tr>
<tr>
<td>Pyrethrum extract (20%)</td>
<td>Film</td>
<td>685 μl./ft.$^2$</td>
<td>25</td>
<td>-</td>
<td>16</td>
<td>33</td>
</tr>
<tr>
<td>DDT</td>
<td>Film Topical</td>
<td>100 mg./ft.$^2$</td>
<td>25</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dipterex</td>
<td>Film Topical</td>
<td>200 mg./ft.$^2$</td>
<td>60</td>
<td>19</td>
<td>12</td>
<td>-</td>
</tr>
</tbody>
</table>

*Per cent inhibition and stimulation calculated by measuring $\Delta \log \left(\frac{Cy}{Fe^{+4}}\right)$ per minute in the homogenates prepared from treated and control flies.
inhibited cytochrome oxidase activity. Carbon disulfide seems to have the same mode of action. Morrison and Brown (1954) showed that malathion and parathion, which, like Dipterex, are both organic phosphorus insecticides, inhibited cytochrome oxidase (*in vitro*) obtained from the leg muscles of the American cockroach. Brown and Brown (1956), however, reported no significant *in vivo* inhibition of cytochrome oxidase obtained from the American cockroach. Experiments reported here, however, show that Dipterex, which is an organic phosphorus insecticide like malathion and parathion, inhibited cytochrome oxidase from the house fly thoracic muscle tissue.

When the female house flies were exposed to ethylene dibromide, ethylene dichloride, and pyrethrum extract, a stimulatory effect on the cytochrome oxidase activity of thoracic muscle tissue homogenate was produced (Table 1). The precise biochemical reason for this effect is not known. The secondary stimulations produced by these toxicants may be similar to the observations of Judah and Williams-Ashman (1951) who reported that dinitrophenols which blocked oxidative phosphorylation increased the rate of carbohydrate metabolism. This increased activity was thought by these authors to be due to the availability of orthophosphate and adenylic acid in excess of normal amounts. The stimulatory effect on cytochrome oxidase by these toxicants can also be explained in the light of the hypothesis put forward by Monod and Cohn (1952). These authors proposed that the synthesis of an enzyme is linked to its activity and that the substrates act as effective inducers of synthesis. Thus the increased activity of cytochrome oxidase may be due to the inhibition of some other enzyme with subsequent accumulation of
Exposure of flies to DDT produced no detectable effects on the cytochrome oxidase activity of thoracic muscle tissue homogenates. Sacktor (1950), Anderson et al. (1954), Morrison and Brown (1954), and Ludwig et al. (1955) have reported that DDT inhibited cytochrome oxidase in vitro. Sacklin et al. (1955) reported that the effects of DDT cannot be explained on the basis of inhibition of cytochrome oxidase. However, Brown and Brown (1956), using in vivo techniques, concluded that DDT did not inhibit cytochrome oxidase and any inhibition detected by using in vitro methods was due to non-specific adsorption of DDT on the enzyme surface. These results indicate that the methods of insecticide application and enzyme assay have considerable effect on the results.

Succinic Dehydrogenase

The activity of two succinic dehydrogenase preparations from the same population of normal, untreated flies is shown in Figure 6. The mean value for oxygen uptake of these homogenate preparations was 44 ml. (range 32.7-52.4 ml) per mg. of dry weight of tissue per hour. There was very little variation between homogenate preparations from the same population of flies.

Effects of the insecticides and fumigants on the succinic dehydrogenase activity of house fly thoracic muscle tissue

Table 2 shows the methods of application, dosages, exposure periods, and effects of insecticides and fumigants on the activity of succinic dehydrogenase. The dosages of fumigants in these experiments were
Figure 6. The succinic dehydrogenase activity of two homogenate preparations from normal, untreated female house fly thoracic muscle tissue measured manometrically in the presence of methylene blue and cyanide. One set of values is shown as circles and dots and the other as solid dots.
Table 2. Effect of insecticides and fumigants on the succinic dehydrogenase activity of female house fly thoracic muscle homogenates (triplicate or quadruplicate assays)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Method of application</th>
<th>Dosage</th>
<th>Exposure period (minutes)</th>
<th>Succinic dehydrogenase activity&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td>Hydrocyanic acid</td>
<td>Gas</td>
<td>1 mg</td>
<td>150</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca(CN)&lt;sub&gt;2&lt;/sub&gt;/l.</td>
<td>180</td>
<td>-</td>
</tr>
<tr>
<td>Ethylene Gas</td>
<td>Gas</td>
<td>0.9 µl./l.</td>
<td>240</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8 µl./l.</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6 µl./l.</td>
<td>240</td>
<td>12</td>
</tr>
<tr>
<td>Ethylene Gas</td>
<td>Gas</td>
<td>1.8 µl./l.</td>
<td>240</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.6 µl./l.</td>
<td>300</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.0 µl./l.</td>
<td>300</td>
<td>15</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>Gas</td>
<td>4.5 µl./l.</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.0 µl./l.</td>
<td>300</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.0 µl./l.</td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>Pyrethrum extract (20%)</td>
<td>Film</td>
<td>685 µl./ft.&lt;sup&gt;2&lt;/sup&gt;</td>
<td>60</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>685 µl./ft.&lt;sup&gt;2&lt;/sup&gt;</td>
<td>60</td>
<td>16</td>
</tr>
<tr>
<td>DDT</td>
<td>Film</td>
<td>100 mg./ft.&lt;sup&gt;2&lt;/sup&gt;</td>
<td>240</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Topical</td>
<td>0.15 µg./fly</td>
<td>240</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.30 µg./fly</td>
<td>240</td>
<td>13</td>
</tr>
<tr>
<td>Dipterex</td>
<td>Film</td>
<td>200 mg./ft.&lt;sup&gt;2&lt;/sup&gt;</td>
<td>240</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Per cent inhibition and stimulation calculated by comparing the oxygen uptake by homogenates prepared from treated and untreated control flies
considerably lower and the exposure periods were longer in all the cases as compared to the experiments with cytochrome oxidase.

Ethylene dibromide, ethylene dichloride, pyrethrum extract, DDT, and Dipterex produced inhibitory effects on the activity of succinic dehydrogenase.

Lewis (1948) has shown that methyl bromide inhibited the sulfhydryl-group-dependent enzymes, including succinic dehydrogenase. Winteringham and Hellyer (1954) have, however, shown that the biochemical effects produced by ethylene dibromide and methyl bromide were fundamentally different. Since the latter authors have not reported the dosages used in their experiments there is no basis for comparing their results with those reported in this study. Methyl bromide caused a reduction in the tissue ATP level and ethylene dibromide did not have any such effect. In the present study, observations on the effect of ethylene dibromide on the ATP level in house fly thoracic tissue corroborate the findings of Winteringham and Hellyer (see Distribution of P$^{32}$-Labeled Intermediates below). Non-depletion of ATP in the tissue alone does not, however, rule out the possibility that ethylene dibromide inhibited succinic dehydrogenase.

Heppel et al. (1947) have shown that compounds possessing sulfhydryl groups and their precursors provided considerable protective action against ethylene dichloride poisoning in rats. This is indirect evidence for the action of ethylene dichloride on sulfhydryl-dependent enzymes in mammals. Thus it seems that inhibition of succinic dehydrogenase by ethylene dichloride was a primary factor in its mode of action.
No account of the effect of pyrethrum extract on succinic dehydrogenase activity was available in the literature; observations recorded here indicate that succinic dehydrogenase activity was inhibited by pyrethrum extract. Hurst (1945) stated that the susceptibility of insects to pyrethrins was dependent fundamentally on the effects of pyrethrins on the oxidative enzyme systems associated with the insect tissue. The inhibition of succinic dehydrogenase by pyrethrum extract as observed in this study could have been brought about by this mechanism.

Although in these studies DDT was found to exert an inhibitory effect on succinic dehydrogenase, Anderson et al. (1954) have reported that both DDT and its essentially non-toxic, dehydrochlorination product, DDE produced in vitro inhibition of succinic dehydrogenase. On this basis, these authors concluded that the inhibition of succinic dehydrogenase was not a primary factor in the mode of action of DDT.

Inhibition of succinic dehydrogenase seems to be the primary factor in the mode of action of Dipterex. O'Brien (1956) reported in vivo inhibition of succinic dehydrogenase by malathion, which, like Dipterex, is also an organic phosphorus insecticide.

Carbon disulfide produced a stimulatory effect on the activity of succinic dehydrogenase. This may be due to secondary effects of the toxicant. It may be recalled that carbon disulfide inhibited cytochrome oxidase activity. It also inhibited over-all glycolysis (see under Over-all Glycolysis below). The secondary stimulatory effects produced by toxicants have been discussed above under the effects of insecticides and fumigants on cytochrome oxidase. In this connection, Slater (1955)
has shown that the path of electron transfer in succinate oxidation was as follows:

\[
\text{Succinate} \rightarrow \text{Succinic dehydrogenase} \rightarrow \text{Cytochrome b} \rightarrow \text{Cytochrome c} \rightarrow \text{O}_2 \rightarrow \text{Cytochrome a}
\]

Thus it will be seen that the dehydrogenase system is very closely linked with the oxidase system and the secondary stimulatory effects on succinic dehydrogenase by carbon disulfide may be due to the primary effects on cytochrome oxidase.

Over-all Glycolysis

The accurate localization of inhibition or stimulation of over-all glycolysis in the whole chain of metabolic reactions is not easily determined in crude cell preparations. In the homogenates from normal, untreated house flies, an average of 48 \(\mu\)l of carbon dioxide (range 34.0 to 74.0 \(\mu\)l) and 57 \(\mu\)g of lactic acid (range 29.4 to 96.5 \(\mu\)g.) per mg. dry weight of tissue were produced. There were wide variations in the carbon dioxide and lactic acid production between the flies that had emerged on different dates. Table 3 shows the methods of application, dosages, exposure periods, and the effects of the insecticides and fumigants on over-all glycolytic activity as determined by assaying for both carbon dioxide and lactic acid production. It is apparent that there was very poor correlation between the amounts of carbon dioxide evolved and the lactic acid produced. Newburg (1956)\(^{22}\) has also observed a similar

\(^{22}\text{Newburg, R. W., Oregon State College, Corvallis, Ore. Private Communication, 1956.}\)
Table 3. Effect of insecticides and fumigants on the over-all glycolytic activity of female house fly thoracic muscle homogenates (duplicate assays)

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Method of application</th>
<th>Dosage</th>
<th>Exposure period (minutes)</th>
<th>Over-all glycolytic activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CO₂ production</th>
<th>Lactic acid production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocyanic acid</td>
<td>Gas</td>
<td>1 mg.</td>
<td>180</td>
<td>-</td>
<td>4.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ca(N)₂/l.</td>
<td>180</td>
<td>-</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Ethylene Gas</td>
<td></td>
<td>0.9 ml./l.</td>
<td>300</td>
<td>22</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.8 ml./l.</td>
<td>300</td>
<td>20</td>
<td>-</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6 ml./l.</td>
<td>300</td>
<td>35</td>
<td>-</td>
<td>25</td>
</tr>
<tr>
<td>Ethylene Gas</td>
<td></td>
<td>4.6 ml./l.</td>
<td>300</td>
<td>&lt;1</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.0 ml./l.</td>
<td>300</td>
<td>12</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>Carbon Gas</td>
<td></td>
<td>9.0 ml./l.</td>
<td>300</td>
<td>21</td>
<td>-</td>
<td>36</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.0 ml./l.</td>
<td>120</td>
<td>4</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Pyrethrum extract (20%)</td>
<td>Film</td>
<td>685 ml./ft.²</td>
<td>60</td>
<td>-</td>
<td>3</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
<td>4</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Film</td>
<td>100 mg./ft.²</td>
<td>300</td>
<td>11</td>
<td>-</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Topical</td>
<td>0.15 µg./fly</td>
<td>300</td>
<td>3</td>
<td>-</td>
<td>8</td>
</tr>
<tr>
<td>Dipterex</td>
<td>Film</td>
<td>200 mg./ft.²</td>
<td>240</td>
<td>16</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>32</td>
<td>-</td>
<td>26</td>
</tr>
</tbody>
</table>

<sup>a</sup>Per cent inhibition or stimulation in the over-all glycolytic activity was computed by measuring the CO₂ evolution and lactic acid production by the homogenates prepared from treated and untreated, control flies.
lack of correlation in studies involving insect tissue. The reportedly low level of lactic dehydrogenase in insect thoracic tissue may offer a possible explanation for this lack of correlation (Zebe 1956\textsuperscript{23} and Zebe and McShan 1957). Carbon dioxide evolution from the bicarbonate buffer used in the manometric technique is a function of changes in acid equivalents. Changes in acid equivalents occur at several places in the series of reactions comprising over-all glycolysis. Therefore the data from measurements of lactic acid production have been taken as the most important criteria for interpreting the effects of the insecticides and fumigants on over-all glycolytic activity in these experiments.

Effects of the insecticides and fumigants on over-all glycolysis in house fly thoracic muscle tissue

Ethylene dibromide, carbon disulfide, pyrethrum extract and Dipterex inhibited the over-all glycolytic activity (Table 3) of house fly thoracic muscle tissue. Ethylene dibromide has been shown to inhibit sulphydryl-group-dependent enzymes (Winteringham and Hellyer 1954). The inhibition of over-all glycolytic activity by ethylene dibromide may be due to its effects on these enzymes and especially on phosphoglyceraldehyde dehydrogenase. The location of specific reactions within the over-all glycolytic series of reactions that are inhibited by carbon disulfide, pyrethrum extract and Dipterex is not known. Pyrethrum extract has been shown to affect the enzymes associated with oxidative metabolism (Hurst

\textsuperscript{23}Zebe, E., University of Wisconsin, Madison, Wisconsin. Private Communication. 1956.
Hydrocyanic acid, ethylene dichloride and DDT produced some stimulation in glycolytic activity as judged by lactic acid production. Graham (1946) showed, by measurement of respiratory quotients, that acceleration of glycolytic activity in cyanide-poisoned insect tissue provided a compensatory mechanism for obtaining energy. Lauger et al. (1946) reported that the amounts of blood sugar and lactic acid increased when the rabbits were fed DDT at the rate of one and two grams per kilogram of body weight.

Distribution of $^{32}$P-Labeled Intermediates

The $R_f$ values of known, non-radioactive, metabolic intermediates, separated by paper chromatography and identified colorimetrically, as described under Materials and Methods above were as follows:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>$R_f$ values</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>Glucose-1-phosphate</td>
<td>0.42 ± 0.04</td>
</tr>
<tr>
<td>3-phosphoglyceric acid</td>
<td>0.60 ± 0.06</td>
</tr>
<tr>
<td>Inorganic phosphate (orthophosphate)</td>
<td>0.71 ± 0.04</td>
</tr>
</tbody>
</table>

These $R_f$ values were based upon 11 determinations.

Figure 7 shows semi-diagrammatically the tracings of scanned radio-chromatograms obtained with the procedures outlined above under Materials and Methods. The average $R_f$ values for the distinctly different peaks or fractions of radioactivity in these chromatograms (and their designation by Roman numerals) were: 0 (I), 0.21 (II), 0.44 (III), 0.58 (IV), 0.68 (V).
Figure 7. Tracings of radiochromatograms, scanned with a Geiger-Mueller tube, showing the distribution of P$_{32}$-labeled intermediates obtained from thoracic tissue homogenates of female house flies treated with various insecticides and fumigants (semi-diagrammatic)
Fraction I (Figure 7, \( R_f = 0 \)) was the origin of the chromatogram and, according to Winteringham et al. (1955), consisted of insoluble phosphoproteins. This fraction has not been included in the estimation of total, soluble \( P^{32} \)-labeled intermediates. Fraction II (\( R_f = 0.21 \)) contained ATP; the \( R_f \) value for this fraction compared favorably with an \( R_f \) value of \( 0.20 \pm 0.03 \) obtained with a non-radioactive ATP standard. Winteringham et al. (1955) have shown that very little ADP accumulates in the thoracic tissues of the house fly and that the \( \alpha, \beta, \) and \( \gamma \) phosphorus atoms of ATP are uniformly labeled with \( P^{32} \). Therefore fraction II probably did not contain much ADP. Fraction III (\( R_f = 0.44 \)) contained glucose-1-phosphate (c.f. \( R_f \) value of \( 0.42 \pm 0.04 \) obtained with a non-radioactive standard.) Winteringham et al. (1955) have shown that besides sugar phosphates, this fraction also contained arginine phosphate, adenosine monophosphate and an unknown compound. Fraction IV (\( R_f = 0.58 \)) has been identified as 3-phosphoglyceric acid (c.f. \( R_f \) value of \( 0.60 \pm 0.06 \) obtained with a non-radioactive standard). Fraction V (\( R_f = 0.68 \)) was identified as orthophosphate (c.f. \( R_f \) value of \( 0.71 \pm 0.04 \) obtained with a non-radioactive standard).

The quantitative distribution of \( P^{32} \)-labeled intermediates is summarized in Table 4 and discussed below in terms of the treatments used in these experiments.

**Experiments with untreated, control flies**

Almost 50 per cent (range 41.6-58.3 per cent) of the total soluble \( P^{32} \) was present as fraction II (ATP + ADP). Winteringham et al. (1955) reported an average of 38.4 per cent (range 31.7-46.3 per cent) for
Table 4. Quantitative distribution of P<sup>32</sup>-labeled metabolic intermediates separated chromatographically from thoracic muscle tissue homogenates of female house flies under different experimental conditions

<table>
<thead>
<tr>
<th>Treatment (dosage and exposure)</th>
<th>Number of experiments</th>
<th>Number of chromatograms</th>
<th>Per cent radioactivity, mean values and range of fractions</th>
<th>Ratio of: Fraction II</th>
<th>Fraction V</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>III (ATP+ADP) (Sugar phosphates, arginine phosphate, and adenosine monophosphate) (IV (3-phosphoglyceric acid) (Orthophosphate)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(%)                                               (%)           (%)                                               (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated control</td>
<td>4</td>
<td>8</td>
<td>49.3 (41.6-58.3) 7.8 (5.7-10.7) -</td>
<td>42.9 (35.9-52.5)</td>
<td>1.15</td>
</tr>
<tr>
<td>Hydrocyanic acid</td>
<td>3</td>
<td>3</td>
<td>10.2 (11.1-19.3) 6.1 (3.1-10.6) 11.9 (5.6-16.9) 71.8 (57.0-89.7)</td>
<td>43.4 (41.8-45.5)</td>
<td>1.09</td>
</tr>
<tr>
<td>Ethylene dibromide</td>
<td>3</td>
<td>6</td>
<td>47.3 (45.8-48.1) 9.3 (7.0-11.5) -</td>
<td>43.4 (41.8-45.5)</td>
<td>1.09</td>
</tr>
<tr>
<td>Ethylene dichloride</td>
<td>2</td>
<td>4</td>
<td>47.4 (42.6-51.5) 13.1 (9.9-17.1) -</td>
<td>39.5 (31.3-47.5)</td>
<td>1.20</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>3</td>
<td>8</td>
<td>27.9 (18.0-35.2) 8.2 (5.2-11.5) -</td>
<td>63.9 (55.3-75.8)</td>
<td>0.44</td>
</tr>
<tr>
<td>Pyrethrum extract (film)</td>
<td>2</td>
<td>4</td>
<td>28.1 (21.9-32.7) 9.5 (7.4-15.2) -</td>
<td>62.4 (59.1-67.3)</td>
<td>0.45</td>
</tr>
<tr>
<td>Pyrethrum extract (topical)</td>
<td>2</td>
<td>4</td>
<td>44.2 (38.0-50.8) 10.7 (8.7-11.6) -</td>
<td>45.1 (40.5-50.9)</td>
<td>0.98</td>
</tr>
<tr>
<td>DDT</td>
<td>4</td>
<td>10</td>
<td>30.5 (14.6-41.8) 9.8 (5.9-17.4) -</td>
<td>59.7 (48.5-76.7)</td>
<td>0.51</td>
</tr>
</tbody>
</table>
fraction II. Although Winteringham et al. (1955) used cyclopropane anesthesia to immobilize the flies before extraction they have shown that brief exposures to cyclopropane did not cause any changes in the normal distribution of phosphorylated intermediates.

Fraction III, consisting of sugar phosphates, arginine phosphate and adenosine monophosphate, contained about 7.8 per cent (range 5.7-10.7 per cent) of the total soluble radioactivity. Winteringham et al. (1955) reported an average of 25.6 per cent (18.6-30.8 per cent) of total soluble radioactivity for the same fraction.

Fraction IV, consisting of 3-phosphoglyceric acid, was absent from the control fly extracts. It seems that under normal conditions of activity there is very little 3-phosphoglyceric acid in the thoracic tissue of the house fly. Winteringham et al. (1955) have shown that about 21 per cent of the total soluble radioactivity was present as 3-phosphoglyceric acid. Zebe and McShan (1957) have shown that there is a system in insects which accomplishes an immediate and direct breakdown of metabolites to supply the large amounts of energy needed during flight. Since over-all glycolysis is not a very efficient process for providing energy (as, for example, the Krebs cycle is) it is not surprising to find an intermediary metabolite like phosphoglyceric acid essentially absent.

Fraction V, consisting of inorganic phosphate, contained about 43 per cent (range 35.9-52.5 per cent) of the total, soluble phosphorylated compounds (Table 4). The amount of inorganic phosphate in the "phosphate pool" of the housefly tissue depends on the phosphate utilized in the formation of high energy bonds and that released during the hydrolysis of
these bonds. The ratio of the total amounts of ATP + ADP (fraction II) to inorganic phosphate (fraction V) in the normal, untreated house flies gives an indication of the over-all production of phosphorylated metabolites, especially ATP + ADP. This ratio (fraction II to fraction V) in the normal untreated house flies was found to be 1.15 and has been taken as an index of the over-all high energy bond formation in the thoracic muscle tissue. Winteringham et al. (1955) have shown that any depletion in the ATP + ADP fraction would give an increased amount of inorganic phosphate, thus reducing the numerical value of this ratio. Values lower than 1.15 would indicate a biochemical disturbance resulting in poor conservation of energy.

**Fumigant and insecticidal treatments**

From the values in Table 1, it appears that hydrocyanic acid, carbon disulfide, pyrethrum extract (film), DDT and Dipterex caused a reduction in the amounts of ATP + ADP (fraction II); the corresponding ratios of the mean values of fraction II to fraction V, ATP + ADP to inorganic phosphate were 0.14, 0.44, 0.45, 0.51, and 0.82 respectively. It has been shown earlier under Results and Discussion that hydrocyanic acid, carbon disulfide, and Dipterex inhibited cytochrome oxidase and that pyrethrum extract and DDT inhibited succinic dehydrogenase to some extent. A reduction in the amounts of ATP and ADP could be brought about by inhibition of these enzymes.

In the case of ethylene dibromide and ethylene dichloride, the ratios of ATP + ADP to inorganic phosphate were 1.09 and 1.20 respectively. This indicates that these fumigants caused very little depletion of ATP.
and ADP. It will be recalled that these fumigants stimulated cytochrome oxidase activity (Table 1) and inhibited succinic dehydrogenase activity (Table 2).

Fraction IV, or 3-phosphoglyceric acid, was present only in the thoracic muscle extracts from house flies treated with hydrocyanic acid and Dipterex (Table 4). The presence of 3-phosphoglyceric acid in these cases indicated the operation of some metabolic pathway involving this intermediate. Earlier in this presentation it was shown that both hydrocyanic acid and Dipterex inhibited cytochrome oxidase. Thus it seems that in the case of hydrocyanic acid the glycolytic cycle, which includes 3-phosphoglyceric acid, is stimulated (see also presentation of over-all glycolysis under Results and Discussion and data in Table 3). In the case of Dipterex it may be recalled that over-all glycolysis was inhibited (Table 3). This suggests that some other metabolic pathway which involves 3-phosphoglyceric acid was present in the thoracic muscle tissue of these house flies; this type of metabolism supplies energy even when cytochrome oxidase and over-all glycolysis were inhibited by Dipterex.

Since fraction III consisted of a mixture of metabolites which could not be separated easily, the changes in this fraction with experimental treatments have not been used for comparative purposes.
The inhibition of enzymes is one of the primary factors in the mode of action of insecticides. Therefore, an attempt was made in the present study to examine the in vivo effects of a series of insecticides, representing a wide spectrum of chemical structures, on cytochrome oxidase, succinic dehydrogenase, over-all glycolysis and distribution of $^{32}$P-labeled metabolites involved in biological energetics. The house fly, *Musca domestica* L., was used as the test insect. The thorax of this insect is rich in muscle tissue and was used as the source of the enzymes and the $^{32}$P-labeled metabolites. Dosages and exposure periods were adjusted for the insecticides and fumigants so that living, though poisoned, flies were used to prepare homogenates of the thoracic muscle tissue for the enzyme studies and as the source of $^{32}$P-labeled metabolites.

Techniques are described for the measurement of the activity of cytochrome oxidase (spectrophotometric), succinic dehydrogenase (manometric), over-all glycolysis (manometric and colorimetric), and the incorporation of $^{32}$P into the house fly tissue with subsequent extraction, chromatographic separation and radiometric scanning of the $^{32}$P-labeled metabolites.

Although detailed, quantitative summaries of the effects of the insecticides and fumigants are given under Results and Discussion, the following summary provides a condensed picture of the over-all effects. These effects are expressed in terms of stimulation (+), no significant effect (o), or inhibition (-). Where the data were inconclusive a question mark (?) is included along with the symbol expressing the effect.
In evaluating the effects of insecticides and fumigants on over-all glycolysis, the data for lactic acid production have been taken as the more important criteria than the data on carbon dioxide evolution. It will be seen that Dipterex inhibited all the enzyme systems studied and this may explain why only a few cases of insect resistance to organic phosphorous insecticides have been recorded.

The principal P³²-labeled metabolites separated chromatographically were identified as insoluble phosphoproteins (fraction I), ATP + ADP (fraction II), sugar phosphates + arginine phosphate + adenosine monophosphate (fraction III), 3-phosphoglyceric acid (fraction IV), and inorganic phosphate (fraction V). The ratio of ATP + ADP (fraction II) to inorganic phosphate (fraction V) has been taken as an index of the over-all high energy bond formation in the muscle tissue. In the untreated, control house flies this ratio was 1.15. The fraction containing

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Cytochrome oxidase</th>
<th>Succinic dehydrogenase</th>
<th>Over-all glycolysis CO₂ evolution</th>
<th>Lactic acid production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocyanic acid</td>
<td>-</td>
<td>o(?)</td>
<td>o(?)</td>
<td>+(?)</td>
</tr>
<tr>
<td>Ethylene dibromide</td>
<td>+</td>
<td>-(?)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethylene dichloride</td>
<td>+</td>
<td>-</td>
<td>-(?)</td>
<td>+</td>
</tr>
<tr>
<td>Carbon disulfide</td>
<td>-</td>
<td>+</td>
<td>-(?)</td>
<td>-</td>
</tr>
<tr>
<td>Pyrethrum extract</td>
<td>+</td>
<td>-</td>
<td>o(?)</td>
<td>-</td>
</tr>
<tr>
<td>DDT</td>
<td>o</td>
<td>-</td>
<td>-(?)</td>
<td>+(?)</td>
</tr>
<tr>
<td>Dipterex</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3-phosphoglyceric acid was absent in the control flies.

Hydrocyanic acid caused a depletion of the ATP + ADP fraction; the ratio of this fraction to inorganic phosphate was 0.15. The appearance of a 3-phosphoglyceric acid fraction confirmed the stimulatory effect of hydrocyanic acid on glycolytic activity. Ethylene dibromide and ethylene dichloride caused no abnormal effects on the distribution of the phosphorylated metabolites. Carbon disulfide, pyrethrum extract (exposure to a film), DDT and Dipterex also caused a reduction in the ATP + ADP fraction, the corresponding ratios of this fraction to inorganic phosphate being 0.44, 0.45, 0.51 and 0.82 respectively. In the case of house flies treated with Dipterex, it was found that 3-phosphoglyceric acid was present. Since Dipterex inhibited overall glycolysis, it was concluded that some other pathway involving 3-phosphoglyceric acid was present in the house fly intermediary metabolic scheme.
Albaum, H. C., and H. Kletzkin  

Albert, A.  

Aldridge, W. N., and A. N. Davison  

Anderson, A., and R. B. March  

Anderson, A., and R. L. Metcalf  

Anonymous  

Augustinsson, K., and M. Grahn  

Baldwin, E. E., and D. M. Needham  

Bandursky, R. S., and B. Axelrod  

Barker, S. E., and W. H. Summers  

Barron, E. S. G., and T. N. Tahmisian  
Blanchard, L., and G. Dinulescu
1932. Le metabolisme glucidique chez les larves de Gastrophiles au
Biol. 110:343-44.

Bodine, J. H.
1928. Insect metabolism. The anaerobic metabolism of an insect

Bonner, W. D.
eds. Methods in enzymology 1:722-29. New York, N. Y.,
Academic Press, Inc.

Brown, A. W. A.
1951. Insect control by chemicals. New York, N. Y., John Wiley
and Sons, Inc.

Brown, B. E., and A. W. A. Brown
1956. The effects of insecticidal poisoning on the level of cyto-
chrome oxidase in the American cockroach. Jour. Econ. Ent.
49:675-79.

Buck, J. B., and M. L. Keister
1949. Respiration and water loss in adult blowfly, Phormia regina
and their relation to physiological action of DDT. Biol.
Bull. 97:64-81.

, and I. Posner
Soc. America 45:369-84.

Burrows, S., F. S. M. Grylls, and J. S. Harrison
1952. Paper chromatography of phosphoric esters. Nature 170:800-
01.

Calaby, J. H.
Biophys. 31:294-99.

Chefurka, W.
Glycolysis in the house fly, Musca domestica L. Enzymologia
17:73-89.

1955. The occurrence of a direct oxidative pathway of carbohydrate
metabolism in the fly, Musca domestica L. Biochim. et
Oxidase metabolism of carbohydrates in insects II. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the house fly, Musca domestica L. Enzymologia 18:209-27.

and B. N. Smallman


Cooperstein, S. J., and A. Lazarow


Dahm, P. A.


Dahl, P. A., and G. W. Kearns


Davis, J. G., and W. K. Slater


Dixon, H., and D. Needham


Dounce, A. L., and J. Beyer


Ferguson, J.


Fink, D. E.


Fukuto, T. R.

Gilmour, D.


Graham, K.


Harvey, G. T., and S. D. Beck


Heppel, L. A., V. T. Porterfield, and N. E. Sharpless

Hurst, H.

Johnson, F. H., H. Eyring, and M. J. Polissar
1954. The kinetic basis of molecular biology. New York, N. Y.,
John Wiley and Sons, Inc.

Johnston, C.
1951. The in vitro effect of DDT and related compounds on the
31:375-82.

Judah, J., and H. Williams-Ashman
48:33-42.

Kears, C. W.

Keilin, D.
1925. On Cytochrome, a respiratory pigment, common to animals,
yeast, and higher plants. Proc. Roy. Soc. (Lond.) (B)

(B) 104:206-53.

1933. Cytochrome and intracellular respiratory enzymes. Ergeb.
Enzymforsch. 2:239-71.


1940. Succinic dehydrogenase-cytochrome system of cells — intra-
cellular respiratory system catalyzing aerobic oxidation of

Kilby, B. A., and G. Youatt
1954. The inhibition of trypsin and chymotrypsin by certain organic

Lüger, P., R. Pulver, C. Montigel, R. Wiesmann, and H. Wild
1946. Mechanism of intoxication of DDT insecticides in insects and
warm-blooded animals. Geigy Co., Inc., 89-91 Barclay Street,
New York 8, N. Y.

LePage, G. A.
176:1009-20.
Lewis, S. E.

______, and E. C. Slater

______, and ______

Lord, K. A.

______, and ______

Ludwig, D., M. C. Barsa, and C. T. Cali


Metcalf, R. L.

______, and ______

______, and R. B. March

______, and M. G. Maxon
Meyerhof, O.,

and K. Lohmann

and

Monod, J., and M. Cohn

Morrison, P. E., and A. W. A. Brown

Mullins, L. J.


O’Brien, R. D.

Parfentjev, J. A., and W. Devrient

Perry, A. S., and B. Sacktor

Potter, V. R.
Quastel, J. H.

Richards, A., and L. Cutkomp

Riemschneider, R., and H. Otto

Rockstein, M.

Rosedale, J. L.


Sacktor, B.


Schmidt, C. H., and P. A. Dahm

Sexton, W. A.

Shafer, G. D.

Shepard, H. H.
Slade, R. 
1945. The 8 isomer of hexachlorocyclohexane (Gammexane), an insecticide with outstanding properties. Chem. Ind. 314-19.

Slater, E. C. 


Slater, W. K. 

Spencer, E. Y., and R. D. O'Brien 

Summerson, W. H. 

Tischler, N. 

Tobias, J., J. Kollros, and J. Savit 

Torda, C., and H. G. Wolff 

Winteringham, F. P. W. 

and J. M. Barnes

P. M. Bridges, and G. C. Hellyer

and G. C. Hellyer

P. M. Bridges, and A. Weatherley

P. M. Loveday, and G. C. Hellyer

Wolsky, A.

Zebe, E. C., and W. H. McShan
ACKNOWLEDGMENTS

The writer wishes to express his sincere gratitude to Dr. Paul A. Dahm for suggesting this problem, guidance during the course of investigation and help in the preparation of the manuscript. Sincere thanks are also due to Dr. H. M. Harris for general supervision during the course of study. Thanks are due to Mrs. J. Croll and Mr. W. A. Brindley for technical assistance and to Dr. Adolf Voigt and Mr. Milo Voss of the Institute for Atomic Research for helping provide the radioactive phosphorus used in these investigations.

This work was made possible by financial assistance from the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Projects No. 1256 and 1257 and from the United States Atomic Energy Commission, Project No. 6, Contract AT(ll-1)-59.