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Toxicity of azobenzene and certain related compounds to insects

Silas S. Sharp
Iowa State College

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TOXICITY OF AZOBENZENE AND CERTAIN RELATED COMPOUNDS TO INSECTS

by

Silas S. Sharp

A Thesis Submitted to the Graduate Faculty for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Entomology

Iowa State College
1947
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INTRODUCTION

The insecticidal action of azobenzene has been known for a decade, but it was only with the relatively recent (Blauvelt 1945a) discovery of its efficacy for the control of the red spider mite in greenhouses that the compound achieved any extensive application. Although azobenzene fits peculiarly well this small niche in the wide field of pest control, the restricted use of the material as compared with the newer synthetic organic insecticides has resulted in a limited literature. Few detailed quantitative studies of the comparative action of azobenzene and related compounds have been reported. Information concerning the mode of action of azo compounds on insects has been almost entirely lacking.

This investigation was undertaken for the purpose of making a comparative quantitative study of the insecticidal efficiency of azobenzene and certain selected related chemical agents, and with the hope of contributing to our knowledge of the action of insecticides by observing the method of action of azobenzene on insects and tracing the fate of the toxicant in the insect body.
REVIEW OF LITERATURE

Chemistry

The azo compounds are characterized by containing the --N=N-- grouping within the molecule. The formula of azobenzene, sometimes called benzeneazobenzene, which is a typical member of this class of compounds, is given below.

![Diagram of azobenzene structure]

Azobenzene was first prepared by Mitscherlich (1834) by treating an alcoholic solution of nitrobenzene with potassium hydroxide and distilling the product. It is an orange-red crystalline compound with a melting point of 68° and a boiling point of 293° (Heilbron and Bunbury 1943). Azobenzene exhibits to a remarkable degree the phenomenon of metastability (Friedlaender and Tammann 1897), a property which may contribute to its efficiency when used as a fumigant (Sharp 1946). Azobenzene is insoluble in water but soluble in most organic solvents with a red color. Weak reducing agents convert azobenzene into hydrazobenzene but benzidine is obtained when stronger reducing agents are used (Thorpe 1921).
Insecticidal Use of Azobenzene

Over a century elapsed between the discovery of azobenzene in 1934 and the first recorded use of the compound as an insecticide. Fink and Vivian (1936) stated that the toxic action of azobenzene had been observed in 1934. They found that azobenzene at a concentration of five parts per million was toxic to mosquito larvae when the substance was dissolved in acetone and the resultant solution dispersed in water. These findings were corroborated in subsequent investigations by Fink et al (1938), who ascertained that azobenzene at five parts per million caused 88% mortality to fourth instar Culex larvae in 16 hours.

The work of Bushland (1940) indicated that azobenzene was as toxic as rotenone to young screwworm (Cochliomyia americana) larvae, both compounds having a minimum lethal concentration of 0.05-.08%.

In field tests for the control of the European corn borer (Pyrausta nubilalis), Questel et al (1941) found that azobenzene applied in a spray at the rate of 1 pound per 100 gallons caused 59.1% reduction in borers. A 74.9% reduction in borer population was secured when the rate of application was 3 pounds per 100 gallons of spray.

Swingle and coworkers (1944) investigated the toxicity
of azobenzene to a number of insects when applied as an undiluted dust. They found it toxic after 48-hour treatment to the American cockroach and to the larvae of the following insects: cabbage looper, Colorado potato beetle, cross-striped cabbage worm, diamondback moth, Hawaiian beet webworm, imported cabbage worm, melon worm, southern armyworm, southern beet webworm, termites, and yellow woody bear. Azobenzene was non-toxic to the rice weevil and to the larvae of the fall webworm and the greenhouse leaf tier.

Haring (1946) obtained satisfactory control of the Colorado potato beetle, Mexican bean beetle, eastern tent caterpillar, European corn borer, and red spider mite with azobenzene dusts. An ovicidal action was claimed on the red spider mite. Azobenzene was found to have little effect on the squash bug, several species of aphids, and the greenhouse leaf tier.

Although the investigations cited indicated some insecticidal action for azobenzene, they also demonstrated that the compound did not possess sufficient potency to compete with more efficient insecticides on the market. Only the discovery and development by Blauvelt (1945a, 1945b) of the use of azobenzene as a greenhouse fumigant for the control of the red spider mite, a
pest not readily controlled by the standard agents, kept
the compound from being relegated to the growing number
of chemicals tried as potential insecticides and found
wanting. Blauvelt (1946b) stated that over 100,000
pounds of azobenzene fumigant were used in American green-
houses during the period from September, 1945 to November,
1946.

Blauvelt (1945a) reported 90-99.75% kill of all
stages of the red spider mite on roses and other green-
house plants. Azobenzene is in many respects an ideal
compound for the control of this pest, combining high
toxicity for the mite with low phytotoxicity and very low
toxicity to man.

Blauvelt's original (1945a) method of azobenzene fumi-
gation consisted of applying a proprietary paste contain-
ing 70% azobenzene to the greenhouse steam pipes. The
steam was then turned on to heat the pipes and cause vola-
tilization of the material. An alternative method (Blau-
velt 1946a) consists of vaporizing azobenzene crystals by
the use of lamps or hot plates. There has recently been
developed commercially a pressure fumigator in which the
azobenzene is mixed with a combustible compound. Igni-
tion of the latter causes the release of azobenzene vapor.
Effect of Azobenzene on Mammals

The observation by Baumann and Herter (1877) of the voiding of bloody urine by a dog following administration of azobenzene is apparently the earliest published record of the effect of this compound on warm-blooded animals. Blauvelt (1946b) cites a report by Saarbach in 1881 that azobenzene caused hemoglobinuria and methemoglobinemia in dogs and rabbits. These symptoms have not been confirmed by subsequent investigators, and Blauvelt (1946b) points out that there is room for suspicion that the effects in these cases may have been due to nitrobenzene present as an impurity in the compound used.

Bodansky (1924), in researches on the physiological effect of compounds related to hydrazine, found that mild anemias were produced in rabbits when azobenzene in olive oil emulsion was injected subcutaneously.

Allen and Page (1930) reported that azobenzene had an erythrolytic action in dogs when given orally. The effects disappeared when administration of the chemical was stopped.

The studies of Maruya (1938) on the renal changes induced in albino rats by the oral administration of azo compounds indicated that azobenzene caused cloudy swelling
and hyaline degeneration in the epithelium of the convoluted urinary tubules.

Smith et al. (1943) investigated the influence of dietary protein on the toxic effect of azobenzene in rats. The drug was administered orally by dissolving it in olive oil and incorporating the solution in the food mixture. Most animals died in a few days on a diet containing 0.1% of azobenzene. At lower concentrations, azobenzene fed to rats on a low protein diet produced a centrallobular hyaline degeneration of liver cells, a more or less marked erythropoietic reaction, and a hemosiderosis of spleen, liver, and kidneys. The effects were not permanent, for when azobenzene was removed from the diet the hepatic lesions disappeared without trace and the splenic and renal symptoms were greatly reduced.

Elson and Warren (1944) administered azobenzene dissolved in arachis oil to rats by peritoneal injection. The urine of the treated animals was collected and analyzed in an effort to determine the metabolic changes undergone by the chemical in the body. They believed that the azobenzene was largely reduced in the body to hydrazobenzene, and the latter then rapidly excreted as a water-soluble derivative formed possibly by conjugation with sulphuric or glucuronic acid.

There is ample evidence in the literature (Kinoshi
1937, Cook et al 1940, Law 1941, Smith et al 1943) that certain azo compounds, especially p-dimethylaminoazo- benzene (Kinoshita 1937, Smith et al 1943), have a cancerogenic action within the body but no such effect is produced by azobenzene (Smith et al 1943, Elson and Warren 1944).

Very little information is extant concerning the toxicological action of azobenzene on man. Blauvelt (1946b) reported that he had exposed himself to the fumigation concentrations of azobenzene vapor for one to several hours at a time for a total of nearly 100 hours, as well as having had considerable contact with the material in preparation. He could detect no ill effects and two complete analyses of his blood and urine failed to reveal any symptoms of injury.

Non-insecticidal Uses of Azobenzene

Azobenzene is the parent substance of a large class of compounds, some of which are important dyes. Conant and Tischler (1939) state that almost none of the latter are prepared from the parent compound but according to Gregory (1944) it does serve as a starting point in making certain dyestuffs. Gregory also asserts that azobenzene is used in firefighting, as an ingredient of
carbon tetrachloride fire extinguishers; in mining, as a flotation agent for sulphide ores of copper, zinc, and lead; and in paper and soap manufacture, as an antioxidant for rosin soaps.

Mechanism of Action on Insects

There have been no direct statements in the literature concerning the mechanism of the action of azobenzene on insects. When used against those arthropods with haustellate mouthparts, it must obviously function by penetration of the integument or the tracheal system. The compound has been tested chiefly on insects with chewing mouthparts, and in this case the principal method of ingress into the insect body is unsettled. Swingle et al (1944) found that some mandibulate insects were killed by azobenzene when their food was dusted, although they consumed but a trace of it. This strongly indicates a contact action.

Azobenzene is vaporized when used for the control of the red spider mite in greenhouses. Sharp (1946) suggests that the action in this case may be due to very fine metastable droplets of azobenzene in contact with the mite, rather than by the penetration of the compound as a gas.
PART ONE. COMPARATIVE TOXICITY OF AZOBENZENE AND NINE RELATED COMPOUNDS TO HOUSEFLY LARVAE

Materials

Biological

In order to compare quantitatively the toxicity of several compounds, it was desirable to have a test insect that could be reared in large numbers in a manner that would assure a minimum of variation in the biological material. The housefly, which has a short life cycle and which is relatively easy to rear in quantity under controlled conditions, fits these specifications admirably and was adopted as the insect to be used in this study.

The houseflies were reared on a larval food mixture modified somewhat from that of Richardson (1932). The formula actually developed and used by the writer is given below.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rolled oats</td>
<td>1 5/8 lb.</td>
</tr>
<tr>
<td>Alfalfa meal</td>
<td>7/8 lb.</td>
</tr>
<tr>
<td>Fleischmann's yeast</td>
<td>1/2 cake</td>
</tr>
<tr>
<td>Malt extract</td>
<td>30 ml.</td>
</tr>
<tr>
<td>Tap water</td>
<td>1875 ml.</td>
</tr>
</tbody>
</table>
Female flies oviposited readily in small jars of the larval food mixture placed in the breeding cages. A measured volume of eggs was then transferred to enameled rearing pans 3½ inches deep and 10 inches in diameter containing half the amount of food mixture given in the above formula.

When adult flies were desired, pupae were removed from the rearing pans to shallow containers and placed for emergence in screen breeding cages 24 x 14 x 14½ inches in dimension. One side of such a cage was grooved to receive a glass plate which could be removed to facilitate cleaning. One end of the cage was closed with cheesecloth, so attached as to form a sleeve through which food and oviposition receptacles could be inserted and removed without the escape of flies.

The adult flies were fed on a mixture consisting of half tap water and half homogenized milk. The liquid was placed in a vial inverted on a disc of filter paper in half a petri plate. This arrangement served as a "self-feeder" and as the fluid was imbibed by the flies or evaporated, more flowed down to keep the filter paper moist. The food supply was renewed twice daily.

The fly culture was kept in an insulated room maintained by a thermostat at a temperature of approximately 27°C.
Chemical

The chemicals used in these tests were mostly procured from commercial sources and were of the highest grade readily available. The azo and related compounds utilized in screening tests with fly larvae are listed with their formulae and sources in Table I.

Methods

Preparation of materials for testing

The toxicity of a compound was determined by mixing measured amounts of it with the larval food. Approximately 50 grams of this material were placed in a glass Stender dish 30 mm deep by 55 mm in diameter, a method of treatment described by Du Chanois (1947).

The food mixture for a test was prepared according to the following formula:

- Rolled oats 24 grams
- Alfalfa meal 14 grams
- Water-malt-yeast suspension 62 grams
- Toxicant as measured

This amount of material was sufficient to fill two of the Stender dishes.
Table I. Compounds tested for toxicity to housefly larvae.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Formula</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-amino-5-azoanisole</td>
<td>4-NH₂C₆H₅-3(OCH₃)N:NC₆H₄-2-OCH₃</td>
<td>E</td>
</tr>
<tr>
<td>p-aminoazobenzene</td>
<td>NH₂C₆H₄N:NC₆H₅</td>
<td>E</td>
</tr>
<tr>
<td>2-amino-5-azotoluene</td>
<td>NH₂(CH₃)C₆H₃N:NC₆H₄CH₃</td>
<td>E</td>
</tr>
<tr>
<td>aminoazoxylene hydrochloride (Techn.)</td>
<td>NH₂(CH₃)C₆H₂N:NC₆H₃(CH₃)₂·HCl</td>
<td>E</td>
</tr>
<tr>
<td>4, 4'-azotoluene</td>
<td>CH₃C₆H₄N:NC₆H₄CH₃</td>
<td>*</td>
</tr>
<tr>
<td>azobenzene</td>
<td>C₆H₅N:NC₆H₅</td>
<td>E</td>
</tr>
<tr>
<td>azoxybenzene</td>
<td>C₆H₅N:N(O)C₆H₅</td>
<td>E</td>
</tr>
<tr>
<td>benzenearzodiphenylamine</td>
<td>4-C₆H₅N:N C₆H₄NHC₆H₅</td>
<td>E</td>
</tr>
<tr>
<td>benzeneazoresorcinol</td>
<td>4-C₆H₅N:NC₆H₃·1,3-(OH)₂</td>
<td>E</td>
</tr>
<tr>
<td>benzidine</td>
<td>NH₂C₆H₄C₆H₄·NH₂</td>
<td>E</td>
</tr>
<tr>
<td>Chrysoidin R</td>
<td>2-CH₃C₆H₅N:NC₆H₂-5-CH₃-2,4-(NH₂)₂·HCl</td>
<td>N</td>
</tr>
<tr>
<td>Congo red</td>
<td>(1-NH₂C₁₀H₆-4-SO₂Na-2-N:N-1-C₆H₄-4-)₂</td>
<td>N</td>
</tr>
<tr>
<td>diazoaminobenzene</td>
<td>C₆H₅N:NNH₂C₆H₅</td>
<td>E</td>
</tr>
<tr>
<td>p-dimethylaminoazobenzene hydrochloride</td>
<td>(CH₃)₂NC₆H₄N:NC₆H₅·HCl</td>
<td>C</td>
</tr>
<tr>
<td>p-dimethylaminoazophenylarsonic acid</td>
<td>4-(CH₃)₂NC₆H₄N:NC₆H₄-4-AsO₃H₂</td>
<td>E</td>
</tr>
<tr>
<td>Compound</td>
<td>Formula</td>
<td>Source</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>--------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>4, 4-dinitroazoxybenzene (Techn.)</td>
<td>NO₂C₆H₄N:N(O)C₆H₄NO₂</td>
<td>E</td>
</tr>
<tr>
<td>Hydrazine hydrate</td>
<td>NH₂NH₂·H₂O</td>
<td>F</td>
</tr>
<tr>
<td>Hydrazobenzene (Pract.)</td>
<td>C₆H₅NNHNHC₆H₅</td>
<td>E</td>
</tr>
<tr>
<td>p-hydroxyazobenzene</td>
<td>C₆H₅N:NC₆H₄·OH</td>
<td>E</td>
</tr>
<tr>
<td>Orange II</td>
<td>4-NaOSO₂C₆H₄N:N-1-C₁₀H₆-2-OH</td>
<td>**</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>C₆H₅NNNH₂</td>
<td>F</td>
</tr>
<tr>
<td>Phenylhydrazine hydrochloride</td>
<td>C₆H₅NNNH₂·HCl</td>
<td>E</td>
</tr>
<tr>
<td>Semicarbazide hydrochloride</td>
<td>NH₂CONH₂·HCl</td>
<td>F</td>
</tr>
<tr>
<td>Sudan III</td>
<td>C₆H₅N:NC₆H₄·4-N:N-1-C₁₀H₆-2-OH</td>
<td>C</td>
</tr>
</tbody>
</table>

**Legend:**

C - Coleman and Bell Co., Cincinnati, Ohio.
F - Fisher Scientific Co., St. Louis, Mo.
* - Prepared by the writer after the method of Perkin (1890).
** - Prepared by the writer after the method of Adkins et al (1940).
Water-soluble toxicants were mixed with the food by dissolving them in the water-malt-yeast suspension prior to the addition of the latter to the dry ingredients.

The homogeneous dispersion of water-insoluble toxicants presented a more difficult problem. In the preliminary investigations designed to establish a uniform procedure for testing, such a material was ground as finely as possible in an agate mortar, then mixed thoroughly with the dry alfalfa meal before preparation of the food. Even with continued grinding, it was not possible to reduce the substance to the desired degree of fineness, and erratic results were obtained.

A new method of adding the water-insoluble compounds was evolved, based on the fact that they were soluble in organic solvents. The substance was dissolved in the minimum amount (7 ml) of acetone required to moisten the 14 grams of alfalfa meal used in the formula. The container of treated alfalfa meal was then allowed to stand uncovered overnight in the laboratory while the acetone evaporated completely. Evaporation of the solvent left the toxicant impregnated in and on the particles of alfalfa meal in a very fine state of subdivision. The treated alfalfa meal was then mixed with the other food ingredients as above described.
Method of testing

General procedure. Forty 3-day old fly larvae were selected at random from a rearing pan and placed on the treated food mixture in a Stender dish. The dish was covered with a square of finely-woven muslin held in place by a stout rubber band. Inasmuch as the action of azo compounds is relatively slow, the tests were allowed to run for 72 hours, at the end of which period the container was opened and a mortality count made. Two milliliters of tap water were added through the muslin cover daily during the test period to compensate in part for evaporation. During the 3-day testing period the Stender dishes were kept in trays in a room maintained at a temperature near 27°C.

As a rule, the 100 grams of treated food was divided between two Stender dishes, resulting in a sample size of 80 insects.

Screening tests. All of the compounds were initially subjected to screening tests in order to select those with greatest toxicity for further detailed comparative study. Each of the 24 compounds was tested at a concentration of 2 grams per 100 grams of food mixture on at least two replicates of 80 insects each. Compounds exhibiting any
toxicity were retested at lower concentrations.

**Detailed tests.** For each of the ten compounds chosen for quantitative comparison, six to nine concentrations spanning the toxic range on a logarithmic scale were selected. For each concentration 400 insects, subdivided into six replicates of 80 insects each, were used.

**Checks.** An untreated check was run every day that a test was set up. The number of check insects totaled 2600 during the course of the experiments.

**Mortality counts**

Mortality counts were made by spreading the contents of a Stender dish on a sheet of paper toweling and separating the living and dead larvae from the food mixture.

**Results and Discussion**

**Method of mixing toxicant with food**

It was mentioned previously that in preliminary experiments water-insoluble toxicants were ground in a mortar as finely as possible prior to mixing with the food. Erratic results were obtained and were attributed to coarse or irregular particle size causing a lack of homogeneous distribution of the insecticide in the food. When the
substances were dispersed as described in acetone solution, greater toxicity and consistent results were secured. This is not surprising, as the surface area of a given mass of material increases enormously with repeated subdivision, and more molecules of toxicant at any one time would be in contact with those surfaces of the insect through which penetration occurs.

It was believed that the solvent might possess some toxic action but numerous tests in which acetone alone was applied to the food showed that this was not the case.

**Screening tests**

As a result of screening tests, the following compounds were found to be non-toxic to housefly larvae at a concentration of two grams per 100 grams of food mixture and were not investigated further: 2-amino-5-azoanisole, benzeneazoresorcinol, benzidine, Chrysoidin R, Congo red, p-dimethylaminoazobenzene hydrochloride, p-dimethylaminoazophenylarsonic acid, 4,4'-dinitroazoxybenzene, p-hydroxyazobenzene, orange II, and Sudan III.

The explanation for the lack of toxicity of these chemicals while other closely related compounds were toxic is beyond the scope of this study but it is a problem that merits inquiry. In general there appears to
be a loss of toxicity with increased substitution in the molecule.

Benzeneazodiphenylamine, 4,4'-azotoluene, and 2- amino-5-azotoluene exhibited some toxic action but were not used for detailed tests because of the limited availability of the first two and the failure of the third to give consistent results.

Detailed tests

Azobenzene and the following nine compounds were chosen for a detailed analytical comparative study: p- aminoazobenzene, aminoazoxylene hydrochloride, azoxybenzene, diazoaminobenzene, hydrazine, hydrazobenzene, phenylhydrazine, phenylhydrazine hydrochloride, and semicarbazide hydrochloride.

The data secured in these experiments were analyzed statistically by the method of Bliss (1935). The data obtained for azobenzene and the statistical methods used in computing its dosage-mortality curve are outlined in Table II. A summary of the data and the statistical values for each of the other compounds will be found in Tables III to XI.

The mortality in the 2600 control insects observed during the course of the tests was 0.3%. This value was
assumed to represent the normal mortality of the insect; correction for the normal mortality was made by the use of Abbott's (1925) formula to arrive at the figures for net percent mortality.

The dosage-mortality curves of the ten compounds are delineated in Figures 1-10. Comparative statistics of the dosage-mortality lines are given in Table XII.

An inspection of Table XII reveals that the compounds in order of toxicity to third instar housefly larvae are: phenylhydrazine > diazoaminobenzene > semicarbazide hydrochloride > phenylhydrazine hydrochloride > hydrazo-benzene > azoxybenzene > azobenzene > hydrazine > p-amino-azobenzene > aminoazoxyylene hydrochloride.

In view of the relative toxicity of these different compounds to housefly larvae, it is suggested that study is needed concerning the effect of those higher in the series than azobenzene upon those pests, mainly the red spider mite, now controlled by the latter substance.
Table II. Calculation of dosage-mortality curve of azo-benzene for third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage g./100</th>
<th>Sample Size</th>
<th>Mortality %</th>
<th>Log dosage x 100</th>
<th>Probit Kill</th>
<th>Empiric-weight</th>
<th>Sample weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>480</td>
<td>0.5</td>
<td>1.000</td>
<td>2.424</td>
<td>0.081</td>
<td>1</td>
</tr>
<tr>
<td>0.13</td>
<td>480</td>
<td>4.9</td>
<td>1.114</td>
<td>3.345</td>
<td>0.194</td>
<td>1</td>
</tr>
<tr>
<td>0.175</td>
<td>480</td>
<td>12.3</td>
<td>1.243</td>
<td>3.840</td>
<td>0.384</td>
<td>1</td>
</tr>
<tr>
<td>0.23</td>
<td>480</td>
<td>26.4</td>
<td>1.362</td>
<td>4.369</td>
<td>0.558</td>
<td>1</td>
</tr>
<tr>
<td>0.30</td>
<td>480</td>
<td>46.0</td>
<td>1.477</td>
<td>4.900</td>
<td>0.635</td>
<td>1</td>
</tr>
<tr>
<td>0.40</td>
<td>480</td>
<td>78.5</td>
<td>1.602</td>
<td>5.789</td>
<td>0.579</td>
<td>1</td>
</tr>
<tr>
<td>0.525</td>
<td>480</td>
<td>85.7</td>
<td>1.720</td>
<td>6.087</td>
<td>0.422</td>
<td>1</td>
</tr>
<tr>
<td>0.69</td>
<td>480</td>
<td>91.6</td>
<td>1.839</td>
<td>6.379</td>
<td>0.248</td>
<td>1</td>
</tr>
<tr>
<td>0.92</td>
<td>480</td>
<td>98.5</td>
<td>1.964</td>
<td>7.170</td>
<td>0.098</td>
<td>1</td>
</tr>
</tbody>
</table>

\[
S(w) = 3.199
\]

\[
S(wx) = 4.7743
\]

\[
S(wy) = 16.0680
\]

\[
S(wx^2) = 7.2867
\]

\[
S(wxy) = 24.7311
\]

\[
S(w) = 3.199
\]

\[
\bar{x} = \frac{S(wx)}{S(w)} = 1.4924
\]

\[
\bar{y} = \frac{S(wy)}{S(w)} = 5.022
\]

\[
A = S(wx^2) - \bar{x} S(wx) = 0.1615
\]

\[
S(wxy) = 24.7311
\]

Slope of dosage-mortality line \( b = \frac{S(wxy) - x S(wy)}{A} = 4.670 \)

Regression equation: \( Y = 5.0222 - 4.670(X - 1.4924) \)

Computation of LD<sub>50</sub>: \( X = \bar{x} \frac{1}{b(Y - \bar{y})} = 1.4876 \)

\[
\text{Antilog} \frac{1.4876}{100} = 0.3073
\]
Fig. 1. Toxicity of azobenzene to third instar larvae of *Musca domestica.*
Table III. Toxicity of p-aminoazobenzene to third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample Size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.38</td>
<td>480</td>
<td>9.7</td>
</tr>
<tr>
<td>0.50</td>
<td>480</td>
<td>25.5</td>
</tr>
<tr>
<td>0.66</td>
<td>480</td>
<td>39.7</td>
</tr>
<tr>
<td>0.87</td>
<td>480</td>
<td>57.8</td>
</tr>
<tr>
<td>1.15</td>
<td>480</td>
<td>72.8</td>
</tr>
<tr>
<td>1.50</td>
<td>480</td>
<td>75.7</td>
</tr>
<tr>
<td>2.00</td>
<td>480</td>
<td>84.2</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[ \bar{x} = 1.9302 \]
\[ \bar{y} = 5.0409 \]
\[ S_{wx} = 7.2363 \]
\[ S_{wy} = 18.8985 \]
\[ S_{wx}^2 = 14.1547 \]
\[ S_{wxy} = 37.0616 \]

\[ A = 0.1872 \]

Slope of dosage-mortality line = 3.1181

Equation for regression line: \[ Y = 5.0409 - 3.1181(X - 1.9302) \]

LD$_{50}$ = 0.826
Fig. 2. Toxicity of p-aminoazobenzene to third instar larvae of Musca domestica.
Table IV. Toxicity of aminoazoxylene hydrochloride to third instar larvae of Musca domestica.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>480</td>
<td>4.3</td>
</tr>
<tr>
<td>1.25</td>
<td>480</td>
<td>9.3</td>
</tr>
<tr>
<td>1.60</td>
<td>480</td>
<td>16.0</td>
</tr>
<tr>
<td>2.00</td>
<td>480</td>
<td>32.6</td>
</tr>
<tr>
<td>2.50</td>
<td>480</td>
<td>43.4</td>
</tr>
<tr>
<td>3.20</td>
<td>480</td>
<td>64.3</td>
</tr>
<tr>
<td>4.00</td>
<td>480</td>
<td>96.4</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[ \bar{x} = 2.3522 \]

\[ \bar{y} = 4.8326 \]

\[ S_{wx} = 6.9578 \]

\[ S_{wy} = 14.2948 \]

\[ S_{wx}^2 = 16.4459 \]

\[ S_{wxy} = 34.0537 \]

\[ A = 0.0798 \]

Slope of dosage-mortality line = 4.9035

Equation for regression line:

\[ Y = 4.8326 - 4.9035(X - 2.3522). \]

\[ LD_{50} = 2.434. \]
Fig. 3. Toxicity of aminoazoxylene hydrochloride to third instar larvae of *Musca domestica*.
Table V. Toxicity of azoxybenzene to third instar larvae of Musca domestica.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.17</td>
<td>480</td>
<td>5.3</td>
</tr>
<tr>
<td>.20</td>
<td>480</td>
<td>16.8</td>
</tr>
<tr>
<td>.235</td>
<td>480</td>
<td>25.9</td>
</tr>
<tr>
<td>.275</td>
<td>480</td>
<td>44.3</td>
</tr>
<tr>
<td>.32</td>
<td>480</td>
<td>60.7</td>
</tr>
<tr>
<td>.38</td>
<td>480</td>
<td>87.6</td>
</tr>
<tr>
<td>.45</td>
<td>480</td>
<td>99.3</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[
\bar{x} = 1.4393 \\
\bar{y} = 4.9687 \\
S_{wx} = 4.1078 \\
S_{wy} = 14.1808 \\
3_{wx^2} = 5.9474 \\
S_{wxy} = 20.7030 \\
A = .0350
\]

Slope of dosage-mortality line = 8.36

Equation for regression line:

\[
y = 4.9687 - 8.36(x - 1.4393).
\]

LD$_{50}$ = 0.277
Fig. 4. Toxicity of azoxybenzene to third instar larvae of *Musca domestica*.
Table VI. Toxicity of diazoaminobenzene to third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.030</td>
<td>480</td>
<td>0.7</td>
</tr>
<tr>
<td>0.044</td>
<td>480</td>
<td>12.0</td>
</tr>
<tr>
<td>0.063</td>
<td>480</td>
<td>50.7</td>
</tr>
<tr>
<td>0.091</td>
<td>480</td>
<td>80.3</td>
</tr>
<tr>
<td>0.132</td>
<td>480</td>
<td>94.5</td>
</tr>
<tr>
<td>0.191</td>
<td>480</td>
<td>98.7</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[
\bar{X} = 0.8336 \\
\bar{Y} = 5.0569 \\
S_{wx} = 1.6586 \\
S_{wy} = 10.0561 \\
S_{wx^2} = 1.4561 \\
S_{wxy} = 8.8072 \\
A = 0.0735
\]

Slope of dosage-mortality line = 5.7524

Equation for regression line:

\[
Y = 5.0569 - 5.7524(X - 0.8336)
\]

\[LD_{50} = 0.066\]
Fig. 5. Toxicity of diazoaminobenzene to third instar larvae of *Musca domestica*.
Table VII. Toxicity of hydrazine to third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>480</td>
<td>1.4</td>
</tr>
<tr>
<td>.16</td>
<td>480</td>
<td>19.3</td>
</tr>
<tr>
<td>.25</td>
<td>480</td>
<td>39.3</td>
</tr>
<tr>
<td>.40</td>
<td>480</td>
<td>51.8</td>
</tr>
<tr>
<td>.63</td>
<td>480</td>
<td>85.5</td>
</tr>
<tr>
<td>1.00</td>
<td>480</td>
<td>99.5</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[
x = 1.5057 \\
y = 4.9908 \\
Swx = 3.6678 \\
Swy = 12.1578 \\
Swx^2 = 5.7050 \\
Swxy = 18.9905 \\
A = .1824 \\
\]

Slope of dosage-mortality line = 3.7544

Equation for regression line:

\[
Y = 4.9908 - 3.7544(X - 1.5057). \\
LD_{50} = 0.322
\]
Fig. 6. Toxicity of hydrazine to third instar larvae of *Musca domestica*.
Table VIII. Toxicity of hydrazobenzene to third instar larvae of Musca domestica.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10</td>
<td>480</td>
<td>0.9</td>
</tr>
<tr>
<td>0.13</td>
<td>480</td>
<td>5.1</td>
</tr>
<tr>
<td>0.16</td>
<td>480</td>
<td>12.6</td>
</tr>
<tr>
<td>0.20</td>
<td>480</td>
<td>21.1</td>
</tr>
<tr>
<td>0.25</td>
<td>480</td>
<td>45.3</td>
</tr>
<tr>
<td>0.32</td>
<td>480</td>
<td>77.0</td>
</tr>
<tr>
<td>0.40</td>
<td>480</td>
<td>93.6</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[ \bar{x} = 1.3747 \]
\[ \bar{y} = 4.8409 \]
\[ Swx = 3.5454 \]
\[ Swy = 12.4847 \]
\[ Swx^2 = 4.9294 \]
\[ Swxy = 17.5275 \]
\[ A = 0.0555 \]

Slope of dosage-mortality line = 6.573

Equation for regression line:

\[ Y = 4.8409 - 6.573(X - 1.3747) \]

LP_{50} = 0.250
Fig. 7. Toxicity of hydrazobenzene to third instar larvae of *Musca domestica*. 

**CONCENTRATION IN LOGARITHMS**

**MORTALITY IN PROBITS**
Table IX. Toxicity of phenylhydrazine to third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.020</td>
<td>480</td>
<td>3.4</td>
</tr>
<tr>
<td>0.032</td>
<td>480</td>
<td>18.2</td>
</tr>
<tr>
<td>0.050</td>
<td>480</td>
<td>38.7</td>
</tr>
<tr>
<td>0.080</td>
<td>480</td>
<td>57.8</td>
</tr>
<tr>
<td>0.125</td>
<td>480</td>
<td>97.8</td>
</tr>
<tr>
<td>0.200</td>
<td>480</td>
<td>93.2</td>
</tr>
<tr>
<td>0.32</td>
<td>480</td>
<td>99.5</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[ \bar{x} = 0.8177 \]

\[ \bar{y} = 5.0561 \]

\[ s_{wx} = 2.1146 \]

\[ s_{wy} = 13.0750 \]

\[ s_{wx}^2 = 1.9851 \]

\[ s_{wxy} = 11.4554 \]

\[ A = 0.226 \]

Slope of dosage-mortality line = 3.3805

Equation for regression line:

\[ y = 5.0561 - 3.3805(x - .8177). \]

\[ LD_{50} = 0.063 \]
Fig. 8. Toxicity of phenylhydrazine to third instar larvae of *Musca domestica*. 
Table X. Toxicity of phenylhydrazine hydrochloride to third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050</td>
<td>480</td>
<td>4.9</td>
</tr>
<tr>
<td>0.069</td>
<td>480</td>
<td>20.1</td>
</tr>
<tr>
<td>0.098</td>
<td>480</td>
<td>31.6</td>
</tr>
<tr>
<td>0.135</td>
<td>480</td>
<td>50.0</td>
</tr>
<tr>
<td>0.190</td>
<td>480</td>
<td>69.3</td>
</tr>
<tr>
<td>0.270</td>
<td>480</td>
<td>93.8</td>
</tr>
<tr>
<td>0.390</td>
<td>480</td>
<td>99.7</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[
\bar{x} = 1.1040 \\
\bar{y} = 5.0309 \\
S_{wx} = 3.1156 \\
S_{wy} = 14.1972 \\
S_{wx}^2 = 3.5903 \\
S_{wxy} = 16.2974 \\
A = 0.1507 \\
\]

Slope of dosage-mortality line = 4.1387

Equation for regression line:

\[
y = 5.0309 - 4.1387(x - 1.104) \\
LD_{50} = 0.125
\]
Fig. 9. Toxicity of phenylhydrazine hydrochloride to third instar larvae of *Musca domestica*.
Table XI. Toxicity of semicarbazide hydrochloride to third instar larvae of *Musca domestica*.

<table>
<thead>
<tr>
<th>Dosage gms per 100 gms</th>
<th>Sample size</th>
<th>Net % Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.028</td>
<td>480</td>
<td>0.7</td>
</tr>
<tr>
<td>0.050</td>
<td>480</td>
<td>7.5</td>
</tr>
<tr>
<td>0.090</td>
<td>480</td>
<td>47.3</td>
</tr>
<tr>
<td>0.16</td>
<td>480</td>
<td>76.4</td>
</tr>
<tr>
<td>0.28</td>
<td>480</td>
<td>85.1</td>
</tr>
<tr>
<td>0.50</td>
<td>480</td>
<td>92.8</td>
</tr>
</tbody>
</table>

Summary of statistical treatment:

\[
\bar{x} = 1.0704 \\
\bar{y} = 5.0342 \\
S_{wx} = 2.3323 \\
S_{wy} = 10.9696 \\
S_{wx}^2 = 2.7160 \\
S_{wxy} = 12.4548 \\
A = 0.2195 \\
\text{Slope of dosage-mortality line} = 3.2478 \\
\text{Equation for regression line:} \\
Y = 5.0342 - 3.2478(X - 1.0704). \\
LD_{50} = 0.115
Fig. 10. Toxicity of semicarbazide hydrochloride to third instar larvae of *Musca domestica*.
Table XII. Comparative statistics of the dosage-mortality lines of the compounds studied.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Slope of Dosage-Mortality Line</th>
<th>( \text{LD}_{50} ) Gms per 100 gms of Food</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylhydrazine</td>
<td>3.3805</td>
<td>0.063</td>
</tr>
<tr>
<td>Diazosaminobenzene</td>
<td>5.7525</td>
<td>0.066</td>
</tr>
<tr>
<td>Semicarbazide hydrochloride</td>
<td>3.2478</td>
<td>0.115</td>
</tr>
<tr>
<td>Phenylhydrazine hydrochloride</td>
<td>4.1387</td>
<td>0.125</td>
</tr>
<tr>
<td>Hydrazobenzene</td>
<td>6.5730</td>
<td>0.250</td>
</tr>
<tr>
<td>Azoxybenzene</td>
<td>8.3600</td>
<td>0.277</td>
</tr>
<tr>
<td>Azobenzene</td>
<td>4.6700</td>
<td>0.307</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>3.7544</td>
<td>0.322</td>
</tr>
<tr>
<td>p-aminoazobenzene</td>
<td>3.1181</td>
<td>0.826</td>
</tr>
<tr>
<td>Aminoazoxylene hydrochloride</td>
<td>4.9035</td>
<td>2.434</td>
</tr>
</tbody>
</table>
PART TWO. TOXIC EFFECTS OF AZOBENZENE ON HOUSEFLY
LARVAE AND ON COCKROACHES

Materials

Three day old larvae of the housefly (*Musca domestica* Linn.) and last instar nymphs and adults of the American cockroach (*Periplaneta americana* Linn.) were used as the test animals in observing the toxic effects of azobenzene on insects. The cockroaches were from a culture that had been maintained in the Iowa State College Insect Physiology Laboratory for a period of thirteen years. The colony was kept in a rectangular glass aquarium and fed on a diet of moistened whole wheat bread.

Methods

Administration of the toxicant

*Fly larvae.* Five hundred to 1500 three-day old fly larvae were taken from a rearing pan and transferred to another pan containing food mixture treated in the manner previously described with azobenzene at a concentration of 0.175 grams per 100 grams of food. The larvae were
allowed to remain in this medium for three days prior to removal for study.

**Cockroaches.** Azobenzene was administered to cockroaches by the method described by Swingle et al (1944). The undiluted, finely powdered toxicant was dusted evenly over the bottom of a battery jar six inches in diameter. No food nor water was provided and the roaches were allowed to remain in the jars until death ensued. Several control experiments were set up in order to ascertain that death was not due to starvation or other causes.

In testing the contact action of azobenzene on cockroaches, it was necessary to prevent any ingestion of the material by the insect. This was assured by sealing the mouthparts with cellulose acetate as described by Griffiths and Tauber (1943).

In experiments designed to study recovery from azobenzene poisoning, roaches were placed in contact with the insecticide in a battery jar for a period of two hours, then removed, washed in a stream of running water to remove adhering particles of azobenzene, and placed in a clean cage with food and water.

**Examination of the treated insects**

**Dissection.** Both fly larvae and cockroaches were
dissected following azobenzene poisoning in order to study the appearance of the internal structures of the insects. A dissecting stage was made by filling partially a petri dish with molten beeswax and allowing the wax to solidify. The beeswax provided a smooth surface to which an insect could be affixed and one that could be cleaned easily. When the surface became pitted with pin holes the wax was remelted; upon solidification a smooth surface was again presented.

For use in the dissection of maggots, tiny scalpels were constructed by fastening bits of the edge of a safety razor blade to matchsticks by means of a plastic cement. A larva was secured to the beeswax surface by fine insect pins and slit down either the mid-dorsal or mid-ventral line. The specimen was then covered with a drop of water to prevent drying. Slender pins were used as probing needles while inspecting the internal organs.

Samples of hemolymph were procured for study by inactivating the maggots in acetic acid vapor in a small chamber of the type used by Viado (1941), puncturing the integument anywhere on the dorsal surface, and collecting the escaping hemolymph in a glass capillary tube.

Before dissection of a cockroach taken from the battery jar treatment chamber it was necessary to remove
all adhering particles of azobenzene in order to prevent contamination of the internal structures, some of which were to be examined for the presence of the toxicant. This was accomplished by holding the insect head upward under a forceful jet of water until all visible material was dislodged. The roach was then examined under a binocular microscope and any red crystals still clinging to the exoskeleton were removed by a fine camel's hair brush.

The roaches were usually taken for dissection when moribund from the effects of the poison, but before death had occurred. In the majority of such insects, one to many drops of hemolymph could be secured by amputating one of the antennae. The severed anten
al stub was inserted in a capillary tube and the hemolymph as extruded would push along the capillary to form an unbroken column of fluid. The collected hemolymph was expelled into a vial containing two to three milliliters of insect Ringer's solution for preservation.

After collecting the available hemolymph from a roach, the wings and legs were snipped off, and the insect affixed back down to the beeswax dissecting stage by pins thrust through the free edges of the pronotum. The tip of the abdomen was clipped off, then the
abdominal and thoracic sterna loosened by cutting carefully along the pleural region of either side with a pair of small dissecting scissors. The ventral body wall was removed by lifting up the rear tip with a pair of forceps and cutting or pulling away the underlying tissue.

Following a general inspection of the internal appearance, as much of the fat body as possible was removed and placed in a vial for subsequent examination. The alimentary canal was stretched out to one side and severed just posterior to the proventriculus and again near the anus. The freed portion of the digestive tube was removed to a glass slide and masses of attached fat teased away with insect pins. After removal of the fat, a cut was made just anterior to the point of debouchment of the Malpighian tubules, and the hindgut with attached tubules and the midgut transferred to separate vials. The body cavity of the roach was cleaned of any remaining fat and discarded.

**Extraction.** The fat bodies, midguts, and hindguts of treated cockroaches were subjected to extraction with various solvents in order to determine the amount and nature of the toxicant or products derived from it which might be present in those tissues.

In the case of fly larvae, no individual organs or tissues were collected for analysis. After being freed
of adhering food particles by washing in water, several hundred maggots at a time were ground in a glass mortar and the macerated mass subjected to extraction.

Acetone seemed to be the most efficacious of the several liquids tested as extracting agents but it is a very general solvent and so was not employed extensively, inasmuch as separate extraction of the water-soluble and fat-soluble fractions was desired.

Ether and hot water (85-95⁰C) were chosen as the two extracting media to be used. A given sample of cockroach tissues or macerated fly larvae was equally divided and one half extracted with hot water, the other half with ether. The material was left in ether overnight or longer, the supernatant liquid decanted off, and the residue washed with two changes of ether. The remaining solid material was then subjected to hot water extraction.

The half of the sample extracted with hot water was treated for about 20 minutes in each of two changes of the extractant. The residue was allowed to dry, then extracted with ether.

After cooling, the hot water extract was washed with ether in a separatory funnel, and the ether extract was washed similarly with water. The combined ether extracts and the combined water extracts were set aside
for chemical tests.

The hemolymph collected from roaches and preserved in physiological salt solution was extracted by shaking with a few milliliters of ether in a vial.

Chemical tests. Azobenzene is not a dye, but its solutions in organic liquids are strongly colored, passing from red through orange to yellow with increasing dilution. The yellow color is readily apparent in acetone solution at a concentration of 1 to 100,000. It should be stressed that this is not an acceptable method of identifying azobenzene since many compounds exhibit a similar coloration.

The solubility of azobenzene in hemolymph was tested by inserting a few fine crystals of the substance along the bore of a capillary tube and allowing hemolymph obtained by severance of the antenna of a normal roach to flow into the tube and over the crystals.

Hydrazobenzene and substituted hydrazobenzenes undergo the benzidine rearrangement when heated with mineral acids (Lucas 1935). The hot water extract was treated with one third its volume of concentrated hydrochloric acid and heated on the steam bath for one hour (Elson and Warren 1944) to convert any water-soluble hydrazo compound in the extract into benzidine for identification. The solution was neutralized with sodium hydroxide and
extracted with ether to remove any benzidine present. The ether was evaporated and the residue taken up with water. This aqueous solution was tested for the presence of benzidine by the Claus-Risler (1881) reaction which consists in the formation of a typical color sequence upon the addition of highly diluted bromine water to a benzidine solution. It was confirmed by the writer that this extraordinarily sensitive test would readily detect benzidine at a concentration of 1 to 500,000.
The Julius (1884) test, which depends on the formation of a dark blue precipitate when a saturated potassium dichromate solution is added to a benzidine solution, was also employed but is much less sensitive than the Claus-Risler reaction.

Results and Discussion

Observations on the treated insects

External. Fly larvae which had been in azobenzene treated food for a day or longer became lemon yellow or orange yellow in color. Larvae receiving a lethal dose of the poison gradually became inactive and usually died in an extended position. Most larvae turned brown after death.
Cockroaches maintained in contact with azobenzene ordinarily exhibited no apparent effect for 12-18 hours. After that time the majority of the insects became quiescent, typically falling over on their backs to succumb to the poison in 24-40 hours. Before losing the power of locomotion, a few roaches exhibited a hyper-irritability when disturbed.

It was observed that the cockroach nymphs were notably more resistant than the adults to azobenzene poisoning. Most of the adults used in the tests died in less than 36 hours, while none of the nymphs tested under identical conditions died in less than 48 hours. A suggested explanation for the lower nymphal susceptibility is presented in the next section.

**Internal.** Some, if not all, of the metabolic decomposition products of azobenzene are colored deep yellow, a fact seized upon for locating them in the insect body. Dissection of treated fly larvae revealed that their bright coloration was due to pigmented substances present primarily in the fat body and to a lesser extent in the hemolymph and alimentary canal. Presence or absence of extraneous pigment in the Malpighian tubules was impossible to ascertain, as these organs are normally yellowish. There was no evidence
whatever of yellow coloring in the body wall nor in other tissues not listed above.

Internal examination of poisoned cockroaches disclosed that the yellow breakdown compounds were restricted to the hemolymph, parts of the alimentary canal, the fat body, and, in the female imago, the developing eggs. In cockroaches exposed to more than a minimum amount of azobenzene, the fat body especially was always stained a deep lemon yellow. It is difficult to state with certainty from visual inspection that additional pigmentation was present in the Malpighian tubules because these organs are normally yellow. Other tests presented strong evidence, however, that the metabolic products derived from azobenzene do pass through the Malpighian tubules. The ventral nerve cord of every dissected roach was examined but no yellow coloration was ever noted. Likewise, the musculature, hypodermal cells, reproductive organs, and other tissues were not colored.

Penetration of the toxicant

Cockroaches having the mouthparts blocked with cellulose acetate and placed in contact with azobenzene died, demonstrating that the poison is capable of penetrating the integument of the insect. Moreover, the
time elapsing until the death of such insects did not differ appreciably from that in roaches with unsealed mouthparts similarly treated with azobenzene and which consequently ingested quantities of it. This experiment indicates that the main action of azobenzene is as a contact insecticide and that any action as a true stomach poison is slight.

The cellulose acetate itself exhibited no deleterious effects on the roaches during the period of the tests.

In those treated insects with unsealed mouthparts, dissection always revealed abundant unchanged crystals of azobenzene in the crop, but none of the toxicant was ever visibly discernible in the midgut. The wall of the foregut is apparently permeable to the azobenzene or its breakdown products. Roaches were placed in contact with finely powdered azobenzene for two hours, then washed free of externally adhering particles, and removed to a clean cage. Over a period of days the ingested azobenzene particles clinging to the crop walls of these insects gradually changed to a yellowish substance, presumably a decomposition product of azobenzene. The hemolymph retained its coloration until the complete disappearance of this material from the crop. It is the writer's belief that any alimentary action of azobenzene is due to the passage of the material through the walls of the crop. If this be the case, the compound
is still acting in the strict sense, only as a contact poison, inasmuch as the wall of the arthropod foregut is similar in structure and permeability to that of the external body wall, albeit less heavily sclerotized (Yonge 1936).

**Fate of toxicant in the body**

The visible distribution in the tissues of poisoned insects of yellow substances derived from azobenzene has already been mentioned. Results of tissue extraction were relied on to give further information concerning the nature and location of these decomposition products.

Extraction of cockroach tissues disclosed that the yellow pigment of the fat bodies was not extractible with hot water, but was extractible with fat solvents. The ether extract of the midgut was lightly colored, but there was no coloration in the hot water extract. Extraction of the hindgut and Malpighian tubules revealed both a water-soluble and a fat-soluble yellow color present.

Treated fly larvae were found to contain both water-soluble and fat-soluble yellow substances. These were obtainable regardless of whether the mass of ground maggots was extracted first with ether or with water.
The hemolymph of both poisoned roaches and fly larvae is of a deep stramineous color, and the coloring agent is not extractible with ether. Azobenzene is insoluble in water and tests showed that it is likewise insoluble in hemolymph in vitro. Evidently the compound undergoes some modification during its transit through the body wall to the hemocoele.

Elson and Warren (1944) found that in rats azobenzene is largely changed in the body to a watersoluble hydrazo derivative which is eliminated as such in the urine. They converted the hydrazo compound to benzidine for identification. Repeated attempts by the writer to establish a similar pattern of detoxication in insects have met with failure. When tested as described in the preceding section, no benzidine was identified in the hot water extracts of samples of several hundred maggots. Considering the sensitiveness of the Claus-Risler test for benzidine, it seems questionable if the latter or parent hydrazo compounds were present.

A group of roaches was exposed to a sublethal dose of azobenzene and then dissected daily to study the elimination of the toxicant from the body. The roach dissected at the end of the first day differed in no way
in internal appearance from those insects having received lethal doses. Thereafter the yellowish color in the fat body faded until barely perceptible on the fourth day. For the first three days, only azobenzene crystals were present in the crop, but on the fourth day some bright yellowish material was noted. In the roach dissected on the sixth day all crystals had disappeared and only the bright yellow substance remained. There was no diminution in the color of the hemolymph until the seventh day. On the eighth day when the last insect of the series was opened, the yellow material was gone from the crop and the hemolymph color had returned to normal.

This experiment indicates that the fat body serves as a storage place for excess toxicant until its removal by way of the hemolymph and the Malpighian tubules. The greater resistance of nymphal roaches to azobenzene is attributed to the relatively greater size of the fat body in the immature insect. This would permit the absorption of a greater amount of toxicant before the toxic level is reached.

The ascription of an excretory function to the fat body is not new. A number of insects are known in which the fat body plays an excretory role in the normal metabolism (Wigglesworth 1939). Miall and Denny (1886) state
that the fat body of the cockroach serves in an excretory capacity throughout life.

The well-colored extracts of the hindgut and Malpighian tubules suggest that the detoxified material is leaving the insect body through these structures. In addition, a yellowish fluid not present in normal roaches was collected from the lumen of the hindgut of a number of the poisoned animals.

In summary, it is believed that a water-soluble derivative of azobenzene in the hemolymph is carried to the Malpighian tubules for slow excretion. During continued exposure to azobenzene the water-soluble derivative enters the hemolymph more rapidly than it can be excreted by the Malpighian tubules. When this level is reached the excess substance is absorbed by the fatty tissues of the insect and stored there pending excretion. Death apparently occurs when both the hemolymph and the fat cells have reached the saturation point for the toxicant or its metabolic decomposition products.
SUMMARY AND CONCLUSIONS

1. Twenty-four azo and related compounds were tested for toxicity to third instar larvae of the housefly (*Musca domestica*) by mixing the substances with the larval food. An effective method of obtaining homogeneous distribution in the food mixture of water-insoluble toxicants is described.

2. The following compounds were found to be non-toxic at a concentration of two grams per 100 grams of food mixture: 2-amino-5-azoanisole, benzeneazoresorcinol, benzidine, Chrysoidin R, Congo red, p-dimethylaminoazo-benzene hydrochloride, p-dimethylaminoazophenylarsonic acid, 4,4'-dinitroazoxybenzene, p-hydroxyazobenzene, orange II, and Sudan III.

3. Detailed comparative tests of the toxicity of ten compounds to third instar housefly larvae revealed the following order of toxicity: phenylhydrazine > diazoaminobenzene > semicarbazide hydrochloride > phenylhydrazine hydrochloride > hydrazobenzene > azoxybenzene > azobenzene > hydrazine > p-aminoazobenzene > aminoazoxylene hydrochloride.

4. The toxic effect of azobenzene on housefly larvae and on cockroaches (*Periplaneta americana*) was investigated.
5. The main insecticidal action of azobenzene is due to its penetration of the integument. Any alimentary action is relatively slight.

6. During passage through the integument, azobenzene is changed to a water-soluble derivative which exists in the hemolymph, causing the latter to be straw-colored.

7. The water-soluble decomposition product is carried to the Malpighian tubules for excretion.

8. Excess absorbed substances derived from the toxicant are absorbed by and stored in the fat tissues of the insect body. This bright yellow material is not extractible with hot water.

9. The metabolic decomposition products of azobenzene were not identified.
LITERATURE CITED


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