69-22,284

LAWSON, Donald Eugene, 1926-
THE EFFECTS OF METEPA ON GAMETOGENESIS AND EMBRYOGENESIS IN THE LARGE MILKWEED BUG, ONCOPELTUS FASCIATUS (DALLAS).

The University of Nebraska, Ph.D., 1969
Entomology

University Microfilms, Inc., Ann Arbor, Michigan

THE EFFECTS OF METEPA ON GAMETOGENESIS AND EMBRYOGENESIS IN THE LARGE MILKWEED BUG, *Oncopeltus fasciatus* (Dallas).

by

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A THESIS

Presented to the Faculty of

The Graduate College in the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Department of Entomology

Under the Supervision of Professor Harold J. Ball

Lincoln, Nebraska

June, 1969

TITLE

The Effects of Metepa on Gametogenesis and Embryogenesis in the Large Milkweed Bug, Oncopeltus fasciatus (Dallas)

BY

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GRAD 52002 300 5-66(T)

AKNOWLEDGMENTS

I owe my deepest appreciation to Professor Harold J. Ball who provided me with attentive guidance throughout the time this work was being conducted. I wish to thank Professor Roscoe E. Hill for his continued support and encouragement during the years required to complete my work.

I am also greatly appreciative to Carla Tollefson for her valuable assistance in many facets of this study.

A very special thanks must go to my wife, Lorraine, whose patience and understanding have been miraculous. I am very grateful and thankful for her staunch support.

TABLE OF CONTENTS

INTRODUCTION

Considerable research is currently being directed toward discovering means of controlling insects other than by applying insecticides. There are several promising concepts of control which minimize the use of insecticides and one of these is the use of sterilizing techniques. Basically, such techniques involve the use of an agent to sterilize the male insect prior to release into the environment or the use of agents in baits or attractants to be contacted by the wild population already in the environment. The efficacy of such measures depends on the degree to which sterile males compete with normal males both in numbers and mating vigor.

The most notable successes have been achieved by sterilizing large numbers of male insects with gamma irradiation and releasing them in quarantined or isolated situations to compete with wild males. Promising results have also been attained by exposing wild insect populations to chemosterilants where it was possible to present them in a bait form which was highly attractive to the subject insects.

The sterile-male release method has been the most effective, but its success depends on rather specific circumstances of isolation and quarantine which are difficult to attain with most insects. The method requires the rearing of very large numbers of male insects, a difficult undertaking for most economically important species especially

when conducted on a large scale.

In view of these and other considerations, some emphasis is currently being placed on the development of chemosterilant techniques. Many of the studies have been concerned with the screening of potentially active chemicals, whereas other research has dealt with the efficacy of field application. The more basic work has been accomplished through the participation of several disciplines.

The purpose of this study was to characterize the effects and mode of action of a particular chemosterilant, metepa (tris [1-(2 methyl) aziridinyl] phosphine oxide) on the large milkweed bug, Oncopeltus fasciatus (Dallas). Attention was given to determination of the locus of attack and the relationship of dosage to effects on tissue from both embryonic and reproductive sources. The effects of dosage level on egg viability and female fecundity were also studied.

LITERATURE REVIEW

The development of sterilizing agents which inhibit or prevent normal reproduction in insects parallels closely the development of cancer therapeutic agents. The requirements for a mode of action are similar in both cases. The cells of neoplastic tissue are similar to germinative cells of the reproductive organs, in that the rate of cell division is greater than that ordinarily found in somatic tissue. The criterion for judging the efficiency of a compound for either purpose would be based on the degree of interference as contrasted to or with proper cellular division. The most successful groups of compounds for both purposes are the alkylating agents and antimetabolites.

The earliest reference to the unique biological properties of materials subsequently used as anti-cancer agents was made by Paul Ehrlich (1898) who reported the action of ethylenimine and ethylene oxide on animal tissue. These two compounds are closely related alkylating agents and possess biological activity similar to other chemicals of this group.

A monograph published in 1958 by the New York Academy of Sciences presents a comprehensive picture of research accomplished up to that time on the clinical and biological effects of alkylating agents. Schmidt (1958) observed that about 45 years had elapsed before interest was shown in the biological properties of alkylating agents as first described

by Ehrlich. Following the onset of World War II, considerable effort was placed on studying the effects of nitrogen mustard derivatives on biological systems. These efforts were directed primarily towards possible usage in chemical warfare, although other aspects of these compounds were also investigated.

In 1942, Goodman and Gilman noted the effects of alkylating chemicals on lymphoid tissue and rapidly dividing cells as subsequently reported by Gilman and Philips (1946). These latter authors pointed out that a large family of related chemicals remained to be evaluated for their effects on proliferative cells. Subsequently such evaluations were made, and many new compounds were synthesized for this purpose.

The phenomena of induced insect sterilization was first reported by Runner (1916) who observed that cigarette beetles, Lasioderma serricorne (Fabricius) produced infertile eggs after exposure to roentgen rays. Later, Muller (1927) noted that mutations could be induced in Drosophila melanogaster through exposure to radiation.

In 1938, Knipling proposed the idea of introducing sterile males into a natural population of screw-worm flies to achieve control. After several years of preliminary research, such a program was initiated by Baumhover (1954) on the island of Curacao. The program was remarkably successful and resulted in complete eradication of the screw-worm fly on the island. The details of this venture have been

well described by Lindquist (1959) and Knipling (1960).

The success of this method on Curacao and later in a number of our southern states gave rise to interest in the use of chemicals to induce insect sterility. In 1959, Knipling advanced a theoretical model involving the use of chemical sterilizing agents in a natural population. At that time, investigations were being carried out to determine what chemicals could be used for sterilization.

In 1960 and 1961, La Brecque, et al. reported that some alkylating agents (aziridinyl derivatives) induced sterility in house flies. The damage done by these chemicals to the affected tissue was of a type similar to that caused by radiation, thus giving rise to the designation of the aziridinyls as "radiomimetic" compounds. Other types of compounds were also investigated by La Brecque and all were grouped under the generic term "chemosterilant".

After structure-activity relationships became known, many compounds were assayed to determine their value as chemosterilants. Hundreds were found to have some degree of sterilizing activity, although relatively few were really promising. Chemosterilants can be grouped into three different categories according to their mode of action; antimetabolites, miscellaneous compounds, and biological alkylating agents.

The antimetabolites are primarily female sterilants although some will also sterilize males. Antimetabolites act

by blocking synthesis or activity of nucleic acids.

The miscellaneous compounds form an uncertain group which includes several very promising chemicals. The most important seem to be a series of structurally related amides. The amides possess good sterilizing properties but are not alkylating chemicals. Bŏrkovec (1964) reported that these compounds apparently did not have mutagenic properties. However, subsequent investigation by Palmquist and LaChance (1965) showed that they do possess mutagenic qualities and thus must be considered as hazardous as the alkylating agents insofar as handling is concerned. Also, they have longer residual properties which could present problems in field dissemination.

The term "alkylating agent" denotes a compound that is capable of replacing a hydrogen atom with an alkyl radical; the first agent thoroughly studied for its alkylating effects was nitrogen mustard. The activity of nitrogen mustard depends on the splitting off of a chlorine atom in solution, leaving a positively reacting intermediate. The action of the other mustards is the same and theoretically all react readily with carboxyl groups of proteins and phosphate groups of nucleic acids. The general scheme for the reaction is shown in Figure 1.

Many other chemicals having alkylating properties were tested and all were found to be similarly reactive. Since these chemicals were alike in no other respects, their action

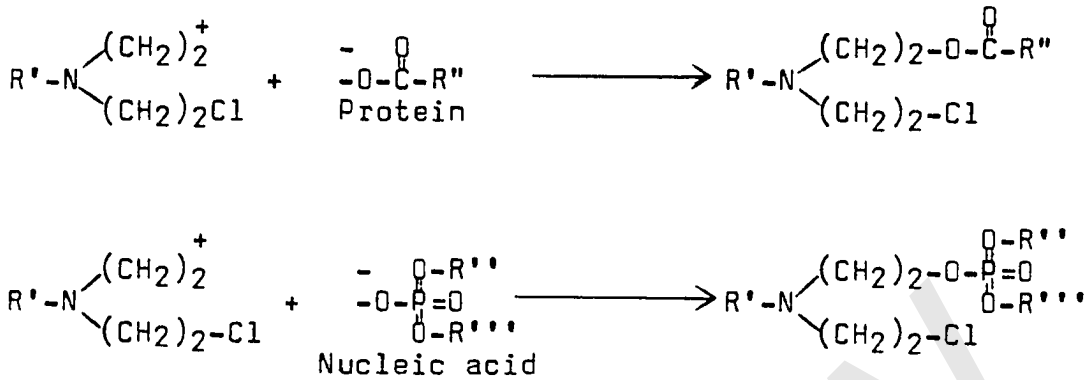

Figure 1. General reaction scheme of mustards with proteins and nucleic acids.

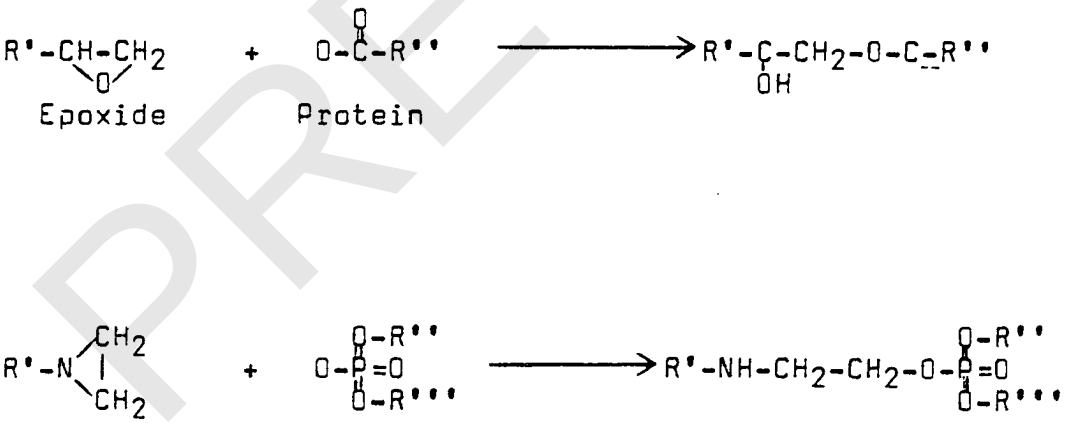

Figure 2. General reaction scheme of epoxides and ethylenimines with proteins or nucleic acids.

was concluded to be due to their alkylating abilities in biological systems. These substances include epoxides, ethylenimines and methane sulfonates.

As previously mentioned, the ethylenimines and epoxides had already been cited by Paul Ehrlich for their unusual properties. Subsequent investigations of these compounds and their many derivatives by Ross (1958), Stacey, et al. (1958), Alexander (1960), and others have shown them to be the most reactive of the known alkylating agents.

An important property of alkylating agents is the number of reactive groups contained in each molecule. The original materials tested (the mustards) have two reactive groups and such compounds proved to be much more active than compounds with only one reactive group. The alkylating agents therefore are described as mono-functional, bi-functional or poly-functional according to the number of reacting groups. The general alkylating scheme is shown in Figure 2 (Ross, 1958).

Other data by Ross, as presented in Figures 3, 4 and 5, indicates that the radiomimetic action of the aziridines might be explained by the reactions with the available groups of the nucleic acids. Since there are many reactive sites, it would seem possible that the attachment of an alkylating agent to a nucleic acid molecule would affect its ability to properly function as genetic material.

The status of knowledge on the use of ionizing radiation

Group	pKa	Fraction of groups in reactive form
Proteins		
a-carboxyl	3.0-3.2	0.9999
Carboxyl (aspartyl)	3.0-4.7	0.9999-0.999
Carboxyl (glutamyl)	4.4	0.999
Phenolic hydroxyl (tyr)	10.4	0.001
Sulfhydryl (terminal cyst)	7.9-8.5	0.100-0.060
Sulfhydryl (non-term cyst)	10.8	5×10^{-4}
Imidazolium (histidine)	5.6-7.0	0.990-0.76
a-ammonium	7.6-8.4	0.440-0.110
e-ammonium (lysine)	9.4-10.6	$10^{-2} - 10^{-3}$
Guanidinium (arginine)	11.6-12.6	$10^{-4} - 10^{-5}$
Nucleic acids		
Primary phosphoryl	2.0	0.9999
Secondary phosphoryl	6.0	0.960
Aromatic hydroxyl (uracil, thymine)	10.2	0.002
Aromatic hydroxyl (guanine)	10.1	0.0025
Sugar hydroxyl	13.0	10^{-5}
Aromatic amino (guanine)	2.3	0.9999
Aromatic amino (adenine, cytosine)	3.7-4.2	0.999

Figure 3. pKa of reactive groups of proteins and nucleic acids at pH 7.5.

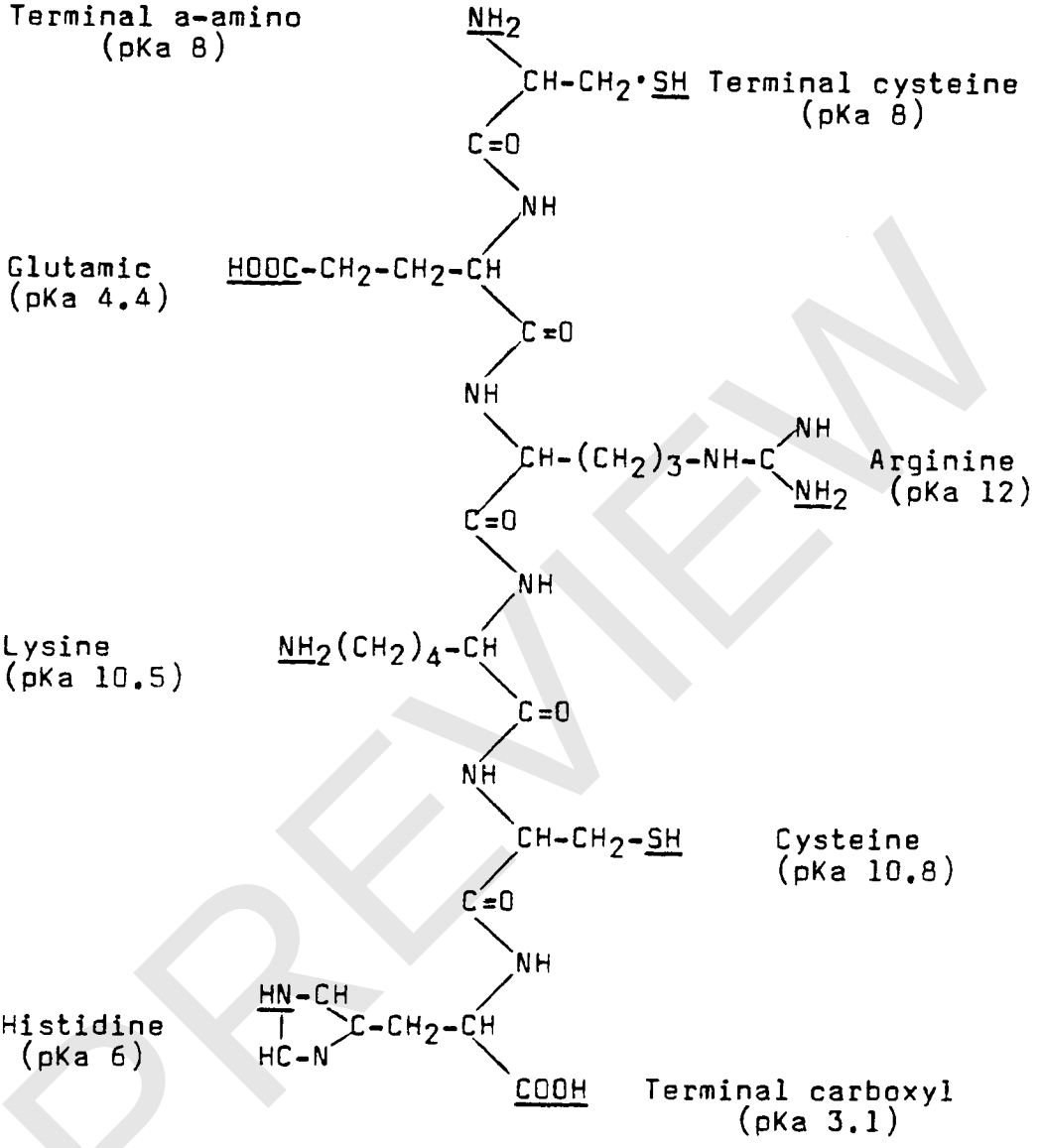

Figure 4. Hypothetical protein showing possible reactive groups (after Ross, 1958).

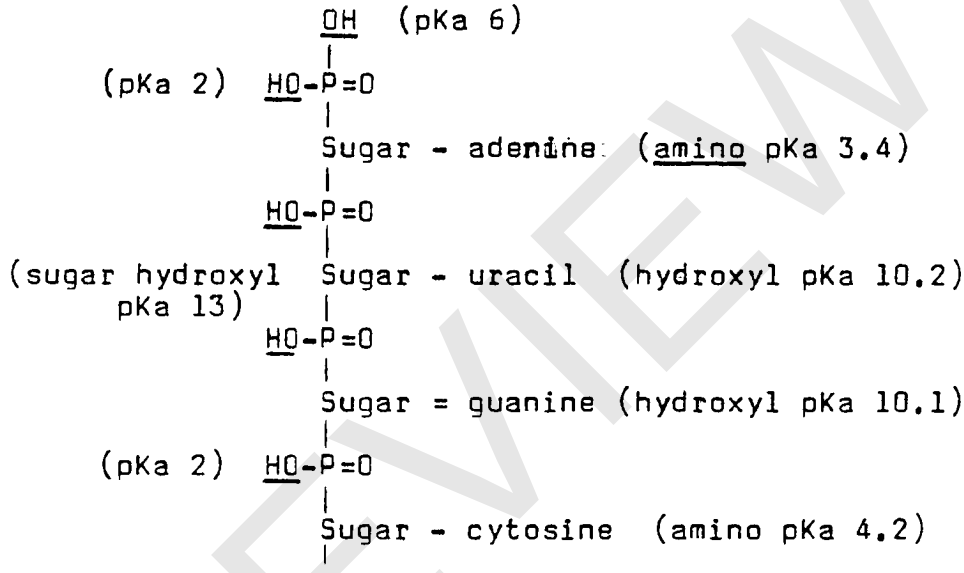

Figure 5. Section of a nucleic acid molecule showing possible reactive sites (after Ross, 1958).

and chemicals for the induction of dominant lethal mutations in insects was reviewed by LaChance (1967), who pointed out that sterility in an insect may occur for a variety of reasons. The pehnomena of aspermia and sperm inactivation is common in males; infecundity and inability to mate takes place in females, whereas dominant lethal mutations occur in both sexes. Any of these conditions can be induced by both ionizing radiation and chemicals, and probably a combination of both results under some treatment conditions.

On the basis of earlier work (LaChance and Riemann, 1964) it was thought that the most important causative factor for sterility in insects was the induction of dominant lethal mutations. Embryos of the screw-worm fly were studied following treatment of oocytes and sperm with ionizing radiation and tretamine [2,4,6-tris (1-aziridinyl)-s-triazine]. The effects of irradiation were observed in oocytes during the first two meiotic divisions in the form of chromosome bridges and fragments of chromosomes. The effects of tretamine were not obvious until the first cleavage divisions occurred or after the treated nuclei had undergone one replication of chromatin material.

The induction of dominant lethal mutations and the resulting death of the embryo as a consequence of chromosomal breakage and bridge formation has been generally accepted as the activity pattern for ionizing radiation. However, the

pathway for the induction of dominant lethal mutations produced by chemicals is a matter of some conjecture. As was pointed out earlier, some workers felt that crosslinking of DNA molecules did occur and that this phenomenon led to alteration of the genetic complement.

Hedin, et al. (1967) working with the boll weevil, observed cytological alterations in testicular tissue following treatment of males with tepa [tris (1-aziridinyl) phosphine oxide]. Clumping of chromatin, anaphase bridges, fragmentation and general tissue necrosis occurred in most cases. Abnormalities in the conformation of mitotic figures were commonly noted. Hedin's work compares strikingly with similar observations made by Riemann (1967) concerning the effects of ionizing radiation on the testicular tissue of the screw-worm fly. The descriptions of damage were similar to those of Hedin et al. (1967) with regard to the appearance of chromosome bridges and chromatin clumping. Hedin did not note chromosomal breaks or fragmentation as previously reported by Riemann (1967).

Recent work by Mendoza and Peters (1968) demonstrated some of the effects of an aziridinyl alkylating agent, apholate [2,2,4,4,6,6,-hexakis (1-aziridinyl)-2,2,4,4,6,6-hexa-hydro-1,3,5,2,4,6-triaza-triphosphorine] on the reproductive organs of the southern corn root worm (Diabrotica undecimpunctata howardii). The effects of apholate on alkaline phosphatase activity were investigated particularly because

of the relationship of alkaline phosphatase to DNA synthesis. Although apholate injections to adult insects caused a decrease in alkaline phosphatase, and at high levels sperm inactivation and limited mortality, no mention was made as to whether treatment with apholate resulted in sterility. However, the unpublished portion of Mendoza's work (Mendoza PhD thesis, 1964) indicated that a degree of sterility was attained even at treatment levels not causing sperm inactivation. Sterility was complete at higher levels.

Several authors have reported that atrophy of testicular and ovarian tissue occurred when relatively high treatment levels of chemosterilants were used (Riemann, 1967; Hedin, et al, 1967; Mendoza and Peters, 1968; Smittle, et al, 1966). Effects on other tissues were not reported but almost all workers reported increased mortality when insects were exposed to high chemosterilant levels.

The use of chemosterilants for insect control on a practical basis has been attempted for a number of insect species. Both the sterile-male release method and treatment of natural populations have been tested.

The first field control experiments with insect chemosterilants were carried out in 1961 by LaBrecque, et al. (1962) against the house fly. An isolated refuse dump in the Florida Keys was baited with corn meal treated with tepa. Several days later, measurements were made of the over-all fly population and per cent hatch of eggs laid.

Observations indicated that both the fly population and per cent of egg hatch were markedly reduced. During the following year LaBrecque, et al.(1963), carried out a similar experiment at a poultry farm. Granular corn meal, granulated sugar and vermiculite containing 0.5 per cent metepa were used as baits in this test. The corn meal bait reduced egg hatch to less than 10 per cent of normal, but the other baits were less satisfactory.

During 1962 and 1963, the chemosterilant-bait experiments were expanded and treatments were made on three islands in the West Indies to ascertain whether it would be possible to eradicate house flies in an isolated situation by the use of chemosterilant baits (Meifert, et al, 1967a). Eradication was not accomplished for several reasons, but a good rate of control, ranging from 50 to 90 per cent was achieved.

In 1962 and 1963 large scale releases of tepa-sterilized male Mexican fruit flies (Anastrepha ludens) were carried out along the Mexican border near Tijuana (Steiner, 1965) and in mango groves in the interior of Mexico (Shaw, 1965). These releases resulted in a fairly good degree of control especially in the border area where the treatments were credited with suppression of a potentially serious infestation.

Sterile-male release experiments with apholate-treated fruit flies (Drosophila melanogaster) were initiated in 1961 and 1962 in tomato plots by Mason, et al.(1968). Suppres-

sion of fly numbers was achieved in these plots during the period of time the releases were made. The success of these experiments led to further tests in tomato plots in 1963 and 1964 (Mason and Smith, 1968). Apholate-treated baits were used in this investigation in an attempt to induce sterility into the natural population. A maximum of about 63 per cent suppression was obtained as compared with a 93 per cent control obtained with Diazinon granules.

A small field experiment was conducted in 1962 by Davich for the purpose of controlling the boll weevil by the release of apholate-sterilized males. This venture produced promising results and similar experiments were subsequently carried out in 1964 (Davich, et al. 1967) on a much larger scale. Apholate-sterilized males were released in nine cotton fields and a definite reduction in boll weevil damage resulted. Eradication was not accomplished in any of the fields but some control was achieved. The effectiveness of this experiment was reduced because of mortality and reduced competitiveness among the chemosterilant-treated males.

In addition to the papers cited above, there have been a number of other investigations regarding the effects of chemical sterilizing agents on reproduction in insects. Most of this work has been concerned with determining the degree and form of sterilizing activity produced by various chemicals tested on several different insects. The results of these investigations has been adequately reviewed by Borkevec

(1966). More recently, additional work has been carried out on several aspects of the chemosterilization technique. Considerable effort has been placed on screening chemicals (Beroza and LaBrecque, 1967, Bhalla and Robinson, 1966, 1968, Crystal, 1966a and 1968a, Ezueh and Hoopingarner, 1967, Fye and LaBrecque, 1967, Harding, 1967, Harries and Wiles, 1966, Harris and Grazar, 1966, Haynes, et al, 1966, Kaloostian, 1968, Klassen, et al, 1968, Mason and Smith, 1967, Morgan, et al, 1967, Pershal and Naidu, 1966, Toppozada, et al, 1966, and Young and Snow, 1967).

The screening investigations were carried out in USDA facilities for the most part. Three species of flies were used for nearly all this work; house flies, Mexican fruit flies and screw-worm flies. About 2000 chemicals have been tested and the results of these efforts have helped formulate a more rational method of selecting candidate chemicals.

Some workers have placed more emphasis on the effects of chemosterilization on the insect and its reproductive activities (Crystal, 1968a, 1968c, Davis and Eddy, 1966, Hathaway, et al, 1966, Hedin, et al, 1967a, Henneberry, et al, 1966, Ladd, 1966 and Webb and Smith, 1968). Such work was concerned primarily with the gross effects on reproductive tissues and the subsequent manifestations of such effects in reductions in numbers of progeny. Various degrees of damage to reproductive and somatic tissues were reported in

several different insects.

Investigations concerning the physiology and chemistry of chemosterilants and their action on insect tissues have been reported by Chang, et al, 1966a, 1967, Chang and Borkovec 1966b, Hedin, et al, 1967b, Kido and Stafford, 1966 and North, 1967. The results of these studies have been of great value in establishing relationships between the chemical structure of the chemosterilants and their activity in biological systems.

Other work has been concerned with methods of field application and the importance of treatment variables on sterilizing effects in the insect (Cox, et al, 1967, Crystal, 1966b, 1967, George and Brown, 1967, Gilliland and Davich, 1966, Kissam, et al, 1967, McFadden and Rubio, 1966, Maitlen and McDonough, 1967, Meifert, et al, 1967 and Patterson, et al, 1967). For the most part, these investigations have extended the results of the chemical screening tests to additional species of insects. Considerable contribution has been made by these workers in helping to establish groupings of insects in relation to their response to certain types of chemosterilants.

MATERIALS AND METHODS

Culture Methods

The milkweed bugs (Oncopeltus fasciatus, Dallas) used in this study were from a culture which has been continuously maintained for over 16 years in the University of Nebraska Insectary. The insects, therefore, were quite homogenous in their growth characteristics, size and other attributes. All individual insects used in this series of experiments were reared under identical conditions so that variations in growth rate, nutrition or other factors would be minimized.

Stock cultures of insects were maintained in wide-mouth quart jars with the opening covered with 80 grade cheese cloth held in place with a rubber band. The cultures were maintained on a diet of milkweed seed which was provided in small packets made by stapling plastic screen around a thin layer of seeds. This method prevented waste of seed, since it kept the seed away from the bottom of the jar where it could become encrusted with fecal waste. Distilled water was provided in 125 ml. bottles containing a number 3, six-inch dental wick from which the insects could obtain water as necessary.

The stock cultures were kept in a rearing room at about 27^{o} C. Eggs were laid in clusters on the outside surface of the cheesecloth from where they were collected by simply