A GENETIC STUDY OF APHOLATE-RESISTANCE
IN *Aedes aegypti* (L.)

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Abstract of Dissertation Presented to the Graduate Council
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A GENETIC STUDY OF APHOLATE RESISTANCE
IN Aedes aegypti (L.)

By

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An investigation was undertaken to study the mode of inheritance of apholate resistance in Aedes aegypti (L). Sterility-concentration curves were determined for each sex of the resistant, susceptible, and F_1 hybrid genotypes. The SC_{50} values were 48.30 for the resistant male, 2.26 for the susceptible male, 4.14 for the F_1 male (resistant female x susceptible male), and 19.50 for the F_1 male (susceptible female x resistant male); and 31.61 for the resistant female, 3.22 for the susceptible female, 8.94 for the F_1 female (resistant female x susceptible male), and 16.87 for the F_1 female (susceptible female x resistant male). The results of genetic crosses indicated that apholate resistance in A. aegypti was a quantitative trait with a marked paternal influence on the resistance of F_2 and backcross progeny.

There was no marked cross-resistance to tepa and metepa in the apholate-resistant strain, but increased tolerances to both chemicals were found. There was no loss of resistance through 9 generations after

selection pressure was removed from a sub-colony of the apholate-resistant strain.

The examinations of chromosome squashes were negative for major chromosomal aberrations in the apholate-resistant strain.

A GENETIC STUDY OF APHOLATE RESISTANCE
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INTRODUCTION

Since the introduction of large-scale use of insecticides for control of insect pests, the most difficult problem in achieving control has been the marked capacity of insects to develop resistance to insecticides. The problems of dealing with a perpetual search for newer and more efficient insecticides, as well as undesirability of persistent chemical residues, required of entomologists the imagination to devise new control measures. One of the most promising of recently advanced control measures was the sterile-insect technique. The utilization of this technique would involve releasing sterile insects into a normal population or sterilizing the natural population. The value of this control measure was demonstrated by the eradication of the primary screwworm fly, Cochliomyia hominivorax, from the southeastern United States in 1957 by releasing millions of adult screwworm flies sterilized by exposure to gamma-radiation in the pupal stage (Baumhover, 1966).

After the success of the screwworm eradication program, considerable interest developed in the possibility of sterilizing field populations of insects, and, also, the possibility of reducing the cost of sterilization. Chemicals that would induce sexual sterility in insects have received much attention, because it would in all probability be less expensive to use chemicals, and the mobility of the sterilization equipment would be greater than with radiation equipment.

Also, the necessity for the mass rearing of insects for release could be avoided if a chemical were applied directly to a natural field population.

During the past decade, thousands of chemicals have been evaluated for sterilant activity, and probably the most effective compounds have been alkylating agents, particularly the aziridines.

Following the discovery of these chemosterilants, many researchers speculated that insect populations would develop resistance to the sterilizing effects of chemicals by Darwinian selection as in insecticide resistance. Since entomologists had seen the development of insecticide resistance to almost every class of chemical toxicant, there was reason to expect resistance to chemosterilants. Indeed, following experimental selection with alkylating agents, chemosterilant resistance was subsequently developed in laboratory colonies of Aedes aegypti (L.) (Hazard et al., 1964) and Musca domestica (L.) (Abasa and Hansens, 1969).

Investigators at the Insects Affecting Man and Animals Research Laboratory, United States Department of Agriculture, Gainesville, Florida, induced resistance to the sterilizing effects of apholate, in Aedes aegypti within a few generations after selection pressure was initiated. (Hazard et al., 1964). This strain was made available, and the investigation reported herein was undertaken to elucidate the inheritance of this apholate resistance and to describe the resistant strain in terms of the level of resistance and other aspects of interest that existed in the strain.

REVIEW OF LITERATURE

Resistance to Chemosterilants

There have been only a few reports on the development of chemosterilant resistance in insects, and all these have involved the induction of resistance in laboratory strains under artificial selection conditions. An excellent review on the use of chemicals to induce sterility in field populations is presented by LaBrecque (1968). However, since the chemosterilants have been used in the field as experimental compounds only, there has been little opportunity for field populations of insects to develop resistance.

The earliest report on chemosterilant resistance was made by Hazard et al. (1964). These researchers reported a 4-to-5-fold tolerance to apholate in A. aegypti following selection for 11 generations. The fourth larval instar was the stadium treated with apholate. This apholate-selected strain is the one used during the course of the investigation described herein. Patterson et al. (1967) stated that this strain, since the report of Hazard et al. (1964), had been maintained under selection pressure of 25 ppm of apholate and had increased the level of resistance in the larval stage to 20-fold. These researchers found only a 6-to-7-fold level of resistance in the adult mosquitoes which were fed apholate in sugar water solutions. They further demonstrated that no cross-resistance to tepa was present in the strain, and only a 3-to-4-fold tolerance was found when larvae were treated with metepa.

There have been two reports of metepa resistance induced in two insect species. Sacca et al. (1966) reported an increased tolerance to metepa which was applied topically to house flies, M. domestica. However, they also noted that no resistance was observed when adult house flies were treated topically with tepa and hempa. Klassen and Matsumura (1966) found that metepa resistance was induced in A. aegypti by applying selection pressure to fourth-instar larvae. There appeared to be an improvement in the fertility of the selected strain when compared to the parental strain. The parental strain produced a preponderance of non-embryonated eggs, as the hatching rate was usually about 40%. They suggested that the selected strain laid only viable eggs, which hardly seems reasonable and leads the author to consider other possible mechanisms, i.e. increasing the vigor of the strains by Darwinian selection via the treatment with metepa. These workers further demonstrated that tissue homogenates of the metepa-resistant strain detoxified 3 times as much metepa during incubation in the presence of metepa for 30 minutes. The metepa determinations were assessed by micro-chemical analysis for phosphorous.

Morgan et al. (1967) failed to develop resistance to apholate in house flies when they subjected the adults continuously to concentrations of apholate that produced less than 100% sterility. These workers observed that a strain under selection with .01% of apholate showed an increase in sterility from less than 10% in the F₅ to 70% in the F₃₀, with a subsequent decrease in sterility to 20% in the F₅₅ generation. The strain has maintained this level of sterility in a more or less static manner to the F₁₀₀ generation. They postulated

that the initial buildup of sterility was due to an accumulation of genetic damage and presented cytological evidence that substantiated this idea. Chromosome squash preparations yielded evidence of chromosome stickiness, fragmentation, deletions, and the absence of an autosomal homologue. In this same experiment, a colony of house flies was selected with metepa in the adult food; because of reductions in fecundity and fertility the strain died out in the F_{10} generation.

Observations similar to those of Morgan et al. (1967) were made by George and Brown (1967); when they applied selection pressure of hempa on larvae of A. aegypti, sterility increased until the strain was lost in the F_6 generation. They attributed the increased sterility to inheritable recessive genetic defects which accumulated in the strain due to inbreeding.

Abasa and Hansens (1969) reported a 5-fold increase in tolerance to apholate in the house fly. They fed an apholate-sugar bait to adults for 35 generations and first observed a tolerance of apholate in the F_{15} generation. The selected strain showed a slight cross-tolerance to metepa, but not to methotrexate.

The net analysis of laboratory chemosterilant selection applied on A. aegypti and M. domestica indicates that the only development of chemosterilant resistance of any magnitude was the apholate-resistant strain of A. aegypti reported first by Hazard et al. (1964) and later by Patterson et al. (1967). This was the only report of a species of insect that could tolerate relatively high concentrations of a chemosterilant and apparently maintain a reasonable level of fecundity and fertility. The strain of A. aegypti reported as resistant to metepa by Klassen and Matsumura (1966) had only a 3-to-4-fold level of

resistance, and most of the other chemosterilant selections have resulted in a strain of insects more susceptible to the chemosterilant than the parent strain. This apparent accumulation of genetic damage was expected by many researchers and has been one of the basic considerations in the argument that field populations of insects would not develop resistance when treated with chemosterilants. There have been very few large scale field experiments with chemosterilants against native populations of insects, but Meifert et al. (1967) made the observation that after two years of treating a population of house flies on Grand Turk Island in the Bahamas, the fertility of the flies on the island declined slightly, and no resistance developed.

Apholate as a Chemosterilant

The initial report on the use of apholate as a chemosterilant was published by LaBrecque (1961). He found that apholate and two other aziridine compounds induced sterility in both sexes of house flies.

Since the discovery that apholate induced sterility in house flies, 34 other species of insects and mites have been sterilized in laboratory tests with apholate (LaBrecque, 1968). A review of recent literature revealed an additional 7 species of insects which have been sterilized with apholate. These species were Heliothis zea (Boddie) and Heliothis virescens (F.) (Soto and Graves, 1967), Oulema melanopus (L.) (Ezueh and Hoopingarner, 1967), Ostinia nubilalis (Hubner) (Harding, 1967), Hypera postica (Gyllenhal) (McLaughlin and Simpson, 1968), Bracon hebetor (Say) (Valcovic and Grosch, 1968), and Epilachna varivestris Mulsant (Webb and Smith, 1968).

Apholate is an alkylating agent and a radiomimetic compound. The five chief types of cytotoxic alkylating agents are mustards, ethyleneimines, sulfonic esters, epoxides, and certain N-alkyl-N-nitroso compounds. Apholate, by virtue of the six ethyleneimine rings on the molecule, is in the second of the above general groups. Alkylation is defined as the replacement of a hydrogen atom of a molecule by an alkyl group, and alkylating agents have a markedly varied structure and composition. The only chemical property that distinguishes this heterogeneous group of compounds is the propensity to alkylate electron-rich centers (Turner, 1968). Turner further delineated the type reaction which is thought to occur during a biological alkylation. The general scheme of the reaction is listed below:

In the above reaction scheme, the rate depends upon the concentration of the alkylating agent and the target molecule as follows:

$$\text{rate} = [RY] [HX]$$

The ionization of the alkylating agent (RY) occurs at the same time as the bond with the nucleophile (HX). Since alkylating agents are electrophiles (electron loving), the nucleophiles with which they react are polar compounds that donate an electron pair to the carbon in the alkylating agent.

Therefore, the chief nucleophilic centers that might be a target for alkylation are organic and inorganic anions, amino groups, and sulfide groups. The range of possible nucleophile targets creates a very complex problem as pertains to the elucidation of the critical

alkylations that could result in sterility. Since elucidation of the mode of action of a chemosterilant would require not only the determination of which compound (s) is alkylated, but the site of alkylation, the wide range of nucleophile targets would make an investigation of mode of action an insurmountable task.

Although a great deal of work has been done concerning the cytological and cytogenetic aspects of chemosterilization (LaChance et al., 1968), the author could find no literature pertaining to the use of apholate and related aziridine chemosterilants for induction of visible morphological mutants in insects. However, although ethyleneimine demonstrated no sterilant activity in house flies (Borkovec et al., 1964), it has been used as a mutagen (Lim and Snyder, 1968 and Alexander and Glanges, 1965). These two reports dealt with induction of translocations and recessive lethals, but also contained information concerning the production of morphological mutants using ethyleneimine for treatment of Drosophila melanogaster.

No reports were found on the absorption and distribution of apholate in insects. However, studies using the related aziridine compounds, metepa and tepa, have been reported. Plapp et al. (1962) found that larvae and adults of Culex tarsalis degraded metepa completely within 48 hours after treatment. Also, they found that house flies injected with metepa degraded nearly 50% of the chemical within 2 hours. In another investigation, Chamberlain and Hamilton (1964) found a rapid rate of degradation of metepa in the screwworm fly, Cochliomyia hominivorax (Coquerel), and in the stable fly, Stomoxys calcitrans (L.), and identified the principal metabolite as phosphoric

acid. Chang et al. (1966) noted that 50% of the tepa injected into house flies had been degraded in 2 hours. Based on these studies on degradation of aziridine chemosterilants, it is not unreasonable to assume that a similar rapid degradation of apholate would occur in insects.

Based on an investigation by Ristich et al. (1965), there appeared to be a positive correlation between the per cent ethyleneimine in apholate analogs and per cent sterility induced in treated house flies. A series of apholate-analogs substituted with chlorine and dimethylamino groups on the aziridine rings and between the rings were evaluated for sterilitant activity. This work agreed with a later paper by Crystal (1966), which showed that the presence of substituents on the aziridine-ring carbon atoms reduced the sterilitant activity of the compound. These investigations indicated that the per cent aziridine-ring in a compound was important for chemosterilization; however, Glancey (1965) and George and Brown (1967) demonstrated that A. aegypti (L.) was sterilized by treatment of the larvae with hempa, a phosphoramidate that is structurally and sterically related to tepa but is not an alkylating agent. Therefore, in spite of the assumption based on earlier work that the alkylating property was necessary for sterilitant activity, it apparently is not.

Kilgore and Painter (1964) reported that normal house fly eggs had a low concentration of DNA initially after oviposition, but this concentration increased rapidly prior to hatching. Sterile eggs from females treated with apholate demonstrated no increase in DNA content. They also found that lactate dehydrogenase activity increased rapidly

in developing eggs, but the activity in sterile eggs was not increased. The authors suggested that ovarian damage, hormonal imbalance, or a number of other metabolic abnormalities could have caused the effect on DNA synthesis; however, based on their investigation no determination on direct or indirect effects was implied.

Mendoza and Peters (1968) found a significant reduction in a DNA specific alkaline phosphatase in the ovaries of southern corn rootworms following injection of apholate. They attributed the decrease in activity to a direct inhibition of phosphatase by apholate; however, it seems probable that other metabolic pathways were affected and indirect effects could have caused this inhibition.

Sharma and Rai (1969) observed a "deterioration" of brain tissue and midgut epithelium in A. aegypti females which were treated with apholate in the larval stage. Both tissues were severely affected, and a lack of golgi bodies was noted in the midgut epithelium. Golgi complexes are most prevalent and well developed in secretory cells, and some evidence points to a role as a secretor of macromolecules for these complexes. Also, golgi vacuoles develop into zymogen granules, which contain and store the proteins produced by the ribosomes (Mahler and Cordes, 1966). It appears, therefore, that a reduction in enzymatic activity would probably occur in the midgut epithelium. However, Rai and Sharma (in press) have demonstrated that normal ovaries transplanted into the abdomens of chemosterilized females developed normally. This indicated that either a considerable repair of damaged cells occurred or those damaged cells continued to function, possibly at a suboptimal level.

Turner (personal communication) has completed some preliminary experiments concerning uptake and metabolism of apholate by larvae of the apholate-resistant strain compared to the apholate-susceptible strain. The susceptible strain showed an uptake of apholate almost 3 times greater than the resistant, and the rate of disappearance was equal in both strains. The data collected by Turner indicated that simple uptake is apparently a very important factor in apholate resistance; however, the resistance can not be attributed to simple accumulation alone. He used a concentration of 100 ppm of apholate as a treatment for the larvae. This concentration is nearly 50 times greater than the SC_{50} for the susceptible strain reported by Patterson et al. (1967), and based on the author's work exceeds the SC_{90} for the resistant strain. Therefore, it is probable that the high concentration used for treatment changed any difference that existed between the two strains.

Maheswary (1968) found that ovarian alkaline phosphatase activity was suppressed in both the apholate-resistant and susceptible strains of A. aegypti following exposure of the larvae to apholate. However, gross protein content of ovaries from the resistant strain was not affected by apholate treatment. In contrast, protein content of ovaries from the susceptible strain was severely reduced.

Formal Genetics of Aedes aegypti

A complete review of the status of the formal genetics of A. aegypti was recently presented by Craig and Hickey (1967). They recorded a total of 87 mutants that have been isolated; many of these which involve color patterns were found from examinations of strains

of diverse geographical origin and others which involve structural aberrations were obtained by inbreeding. Of the 87 mutants reported, 34 have been assigned to 3 linkage groups. All the mutants, except those that represent polymorphisms from diverse areas, have arisen spontaneously during inbreeding. A. aegypti demonstrates considerable color variation in strains from diverse geographical origin and apparently carries a great deal of concealed variability, even in laboratory strains. Van deHey (1964) found a mutation load of 1.32 visible mutants per mosquito during inbreeding experiments with 4 strains of A. aegypti. He observed a higher rate of spontaneous mutants during inbreeding than was observed following irradiation with inbreeding.

Linkage data thus far collected indicate there is reason to suspect the definitive linkage map in A. aegypti will be fairly short. The total map distance of the three linkage groups at present is 110 units ($43 + 37 + 30$), and this led Craig and Hickey to speculate that the maximum length may be approximately 150 units. Certainly, additional linkage studies will lengthen the map. However, since Akstein (1962) and Mescher and Rai (1966) observed a maximum of 2 chiasmata per bivalent, this was further evidence to support a short map.

Since crossing over occurs in both sexes, and there has been no standardized program of experimentation, linkage data from various laboratories have been variable. Variations in map distances from different laboratories remain unexplained; however, some progress has been made in finding the reasons for these variations. According to Craig and Hickey, age and sex have a marked influence on crossing

over; other sources of variation are the larval diet and the temperature of the larval medium. It appears that a uniform program of experimentation needs to be adopted before many of the problems in the variations of linkage data will be overcome.

The mechanism of sex determination in A. aegypti was determined by McClelland (1962). He found that sex was determined either by a small block of a chromosome or a single gene, with the male being heterogametic. Gilchrist and Haldane (1947) postulated the same mechanism for sex determination in Culex pipiens. There has been speculation that the situation in Aedes and Culex mosquitoes is analogous to that in some species of Chironomus where sex is determined by an inversion that occurs only in the males (Beerman, 1955; Acton, 1957). A small-inversion mechanism would give results similar to the M/m system, but unfortunately examination of the polytene chromosomes in Aedes species has not been accomplished.

Cytogenetics of Aedes aegypti

In the past few years, marked progress has been made in the cytogenetics of A. aegypti. Paralleling the development of the formal genetics of A. aegypti, considerable interest has been shown in mitotic and meiotic karyotype descriptions and the effects of radiation and chemosterilants at the cellular level.

The first report concerning the mitotic karyotype of A. aegypti was made by Carter (1918), who listed the chromosome number as $2n = 4$, based on examination of sectioned gonads. Sutton (1942) used salivary gland preparations and indicated a diploid number of six. Then Rai

and Craig (1961) and Breland and Gassner (1961) gave a more detailed description of the mitotic chromosomes based on observations of squash preparations of larval brain tissue.

Based on size and location of centromere, Rai (1963a) described the normal karyotype of A. aegypti. The karyotype is composed of 3 pairs of chromosomes designated I, II, and III. Based on measurements on 10 mitotic, metaphase figures, the chromosomes averaged 5.4, 6.9, and 7.6 microns, respectively, in the brain tissue of larvae.

Mescher and Rai (1966) described spermatogenesis in A. aegypti. They observed that primary spermatocytes were present from the beginning of pupal life onward. Further observations were that usually in pupae 2 to 4 hours old, most of the cells seen were in pachytene, and that pachytene was of long duration and was seen in all preparations of pupal testes. The double nature of each homologue was clearly visible in diplotene, with the strands held together, presumably, only at points of chiasmata. Diakinesis was of short duration, during which the chromosomes were in a highly contracted state. At metaphase I, the number of chiasmata per bivalent ranged from 1-2, and the chromosomes moved away from their homologues in a non-synchronous fashion. This manner of movement was not observed in anaphase II, where all the chromosomes were observed to move together. The authors noted that the greatest impediment to clear observation was chromosome stickiness, particularly in metaphase I and anaphase I. Spermatids were observed after the first third of pupal life, and mobile sperm were seen by the time the males emerged. No reports pertaining to oögenesis in A. aegypti were found.

Because of difficulties involving chromosome stickiness, it has not been possible to map salivary chromosomes (Aldighieri, 1961, Mescher, 1963). In these attempts to prepare the salivary polytene chromosomes for observation, chromosomal stickiness resulted in chromatic masses.

Rai (1964) treated second-instar larvae of A. aegypti with 15 ppm of apholate and examined squashes of brain tissue for the effects of this treatment on somatic chromosomes. He observed that apholate induced numerous aberrations, including stickiness, deletions, ring chromosomes, dicentric chromosomes, and anaphase bridges. Although he made no observations on gonadal tissue, similar damage in developing gametes could be expected to result in dominant lethality. In adult females, which emerged from the apholate-treated larvae, oogonial maturation was almost completely inhibited. The ovarioles remained underdeveloped and eventually degenerated. Only rarely did eggs develop and these usually failed to hatch.

Aberrations similar to those observed by Rai were reported by George and Brown (1967), when they treated fourth-instar larvae of A. aegypti with hempa.

The aberrations induced in somatic chromosomes of A. aegypti by apholate and hempa were basically the same as those induced by x-irradiation (Rai, 1963b). Rai described the chromosomal aberrations induced in brain cells by x-irradiation as similar to those induced by apholate, and since he did not score the number of each class of aberration observed, it was assumed that the x-irradiation and apholate produced similar effects.

Statistical Analysis of Resistance Data

Resistance of insects to chemical toxicants is an invisible character, and the most familiar means for investigating the inheritance of resistance is an interpretation of toxicological data. Dosage-mortality curves give insight into the mode of inheritance. Tsukamoto (1963) presented an excellent description of the use of the log dosage-probit mortality curve in genetic investigations of insecticide resistance. In a later paper Tsukamoto (1964) presented a form of analysis of variance to determine the contribution of each chromosome in inheritance studies of resistance. In a third paper (1965), he described a statistical method for estimation of recombination values for insecticide resistance by elimination of errors in analysis stemming from both differential viability and partial manifestation of the resistant genotype. This third paper by Tsukamoto concentrated on a practical method to estimate overlapping phenotypes.

METHODS AND MATERIALS

The chemosterilants used in this investigation were apholate [2, 2, 4, 4, 6, 6 - hexakis (1 - aziridiny1) - 2, 2, 4, 4, 6, 6 - hexahydro - 1, 3, 5, 2, 4, 6 - triazatriphosphorine], tepa [tris (1 - aziridiny1) phosphine oxide], and metepa [tris (2 - methyl - 1 - aziridiny1) phosphine oxide]. Technical information pertaining to chemical and physical properties of these three chemicals were taken from Olin Mathieson Chemical Corporation (1964) and Kenaga (1966). This information is listed in the appendix, pages 77-79.

Concentration-Sterility Curves With Apholate

In order to study the mode of inheritance of chemical resistance, it is necessary to determine a concentration of chemical that will distinguish the susceptible and resistant phenotypes. A preliminary experiment was conducted to construct a concentration-sterility curve for the susceptible mosquitoes. The apholate-susceptible strain was used in these preliminary tests, because it was thought there would probably be less variation in results obtained.

Approximately 150 first-instar larvae of the apholate-susceptible strain of *A. aegypti* were placed in one liter of glass-distilled water. A mixture of 2 parts liver powder:¹ 1 part yeast extract v/v

1. Obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

was provided as food and was added each day as needed. The larvae required approximately one day to complete an instar and were usually in the fourth instar early on the fifth day. The rearing room for larvae and adults was maintained at $82 \pm 2^{\circ}$ F and 70-80% relative humidity. The technique for treatment in this experiment was a modification of that described by Dame et al. (1964). Approximately 100 fourth-instar larvae of the apholate susceptible strain were exposed in apholate solutions of 5, 10, 15, and 20 ppm until pupation. After the first 6 hours of treatment, liver and yeast mixture was added for food as described before. The pupae were usually collected on the second and third days after treatment. The pupae were sexed on the basis of size, the males being smaller than the females, and placed in cages 12 x 12 x 6 inches for eclosion. Three days after eclosion 25 treated males and 25 treated females were held in cages with untreated mosquitoes of the opposite sex 3 days for mating. Sugar cubes and water on cotton pads served as food during the period for mating. On the sixth day after eclosion the treated females and females mated to treated males were bled on a guinea pig. Two days following the blood meal, eggs were collected on wet filter paper, which had been placed inside a jar, 3 1/2 inches in diameter x 3 1/2 inches high, half-filled with water. The eggs were allowed 5 days for maturation before hatching was initiated by flooding the eggs with water, followed by placing the eggs in a vacuum for 30 minutes.

In the groups with treated males, egg samples were counted to determine the per cent sterility and net per cent sterility was

calculated by Abbot's formula as follows:

$$\text{NET \% Sterility} = \frac{\text{Sterility in Treatment} - \frac{\text{Sterility in Control}}{\text{Sterility in Control}}}{100}$$

A total count of the eggs laid by the treated females was made in order to estimate the loss of fecundity induced by the sterilant. Net per cent sterility was calculated by the following formula:

$$\text{NET \% Sterility} = 100(1-hf)$$

Where: h = the decimal frequency of hatch.

f = the decimal frequency of fecundity.

Since this technique gave reproducible results, it was adopted for establishing concentration-sterility curves for the resistant strain and F_1 hybrids obtained from crossing the resistant and susceptible strains.

Following the establishment of a treatment technique, experiments were conducted on the resistant strain and the F_1 hybrids to determine a range of concentrations of apholate that would yield an estimate of the SC_{50} of these genotypes. This was not necessary for the susceptible strain, since a range had been determined in the preliminary experiments.

One replication was conducted with each genotype at concentrations of 5, 10, 15, and 20 ppm of apholate. The treatment and handling schedule was consistent with the preliminary experiment. Induced sterility was assessed in each sex by mating with untreated mosquitoes from the susceptible strain.

The ranges of concentrations used to establish the SC_{50} values

for each sex of the resistant, susceptible, and F_1 hybrid genotypes ranged from 0.5 ppm to 80 ppm. Each concentration was replicated 3 - 5 times with 20 mosquitoes (selected at random from 100 adults treated as larvae) per replicate. The basic schedule for rearing, treating with apholate, and handling of the mosquitoes is listed in Table 1. The females were placed in individual oviposition cups, a four ounce squat cup lined with filter paper and half-filled with water. Also, the treated males were mated to a single female in the same size cup used for oviposition. These cups were covered by plastic screen secured by a rubber band. In the early concentration-sterility tests, an accurate account of the mortality and fecundity of females mated to treated males was recorded, and was used as a guideline for an approximation of the number of mosquitoes to be used at each concentration in order to insure there would be enough mated females to constitute a replication. Individual matings were not done with the treated females, since the source of fertile sperm was thought to be of little consequence. The females were mated en masse in cages 12 x 12 x 6 inches.

The data collected from the concentration-sterility experiments were analyzed by probit analysis as outlined in Finney (1952). Concentrations used were transformed to logarithms and net per cent sterilities were transformed to probits. A linear regression was performed on the transformed data, and estimates of SC_{50} values, slopes and fiducial limits of the SC_{50} values were calculated for the genotypes tested.

Table 1. Basic schedule for rearing and handling Aedes aegypti larvae, pupae, and adults during concentration-sterility experiments.

Day	Operation
0	Eggs were hatched and 100 first-instar larvae were placed in 1 liter of glass-distilled water with liver and yeast mixture as food.
1	Larvae in second instar. Food added.
2	Larvae in third instar. Food added.
3	Larvae in late third instar. Food added.
4	Fourth-instar larvae placed in apholate solutions. Food added 6 hours after treatment initiated.
5	Food added.
6	Pupae (mostly males) collected.
7	Pupae collected..
8	Pupae collected. Adult males emerging.
9	Adults emerging.
10	Adult emergence complete.
11	Treated adults mated to untreated mosquitoes of susceptible strain.
14	Females blooded on guinea pig.
16	Females placed in oviposition cups.
21	Eggs hatched in a vacuum and per cent sterility recorded.

Loss of Resistance

The loss of insecticide resistance by both field and laboratory populations of insects has been documented by many researchers. Usually the loss in resistance occurred after several generations during which the insects were not subjected to selection pressure by exposure to the chemical, and the population demonstrated susceptibility to concentrations of chemical to which it once was completely refractory.

An experiment was conducted through 10 generations to determine if any reduction in apholate resistance would occur in the resistant strain. A sub-colony was established and reared without apholate selection. Two groups of 100 larvae from each generation were treated with 25 ppm of apholate in the manner described previously. In addition to the two treated groups, a resistant control from the generation being assayed was reared and the sterility assessed. Also, two groups of 100 larvae from the susceptible strain were treated with 25 ppm of apholate, and a third group from the susceptible group was reared and the natural sterility was assessed. In each group, sterility was assessed by mating to virgin mosquitoes of the susceptible strain. Only the male sterility was recorded to use as an indication of any loss of resistance. Eggs were collected from the females en masse on filter paper in a jar half-filled with water, and samples of not less than 100 eggs were used for sterility counts.

Concentration-Sterility Curves For Tapa and Metepa

Other experiments were conducted to determine if cross-resistance to two closely related aziridine chemosterilants was manifest in the apholate-resistant colony. This experiment paralleled the work reported by Patterson et al. (1967), with the exception that the experiment described below assayed the effect of tapa and metepa on each sex.

The rearing schedule in the preceding section was used in these experiments. Approximately 100 fourth-instar larvae of the apholate-resistant and apholate-susceptible strains were treated at each concentration. Food was added 6 hours after treatment had begun. Both strains were exposed to 10, 20, 40, and 80 ppm of metepa and 2.5, 5.0, 10.0 and 20.0 ppm of tapa, with 3 replicates at each concentration. Since these two compounds deteriorate rapidly at a pH below 6.5, the solutions were buffered with 1 ml/liter of 1 M K_2HPO_4 . As in the experiments with apholate, treatment was continued until pupation. The pupae were rinsed and transferred to glass-distilled water. Mortality counts of larvae, pupae, and adults were recorded.

The adults, both males and females, were mated en masse to virgin untreated mosquitoes of the susceptible strain. The females were allowed to feed on a guinea pig and eggs were collected en masse on filter paper lining a glass jar, which was half-filled with water. Samples of not less than 200 eggs were hatched to assess sterility. For purposes of comparing the sterility induced in the resistant and susceptible strains, the results from the males

were plotted on log-concentration probit-sterility graphs, and SC_{50} values were estimated for the resistant and susceptible genotypes.

Cytogenetic Studies of Brain and Testes Squashes

Since the initiation of selection for apholate resistance, the resistant colony has been selected for over seventy generations with 25 ppm of apholate. In view of this rigid selection with a mutagenic chemical, it was suspected genetic damage could probably be detected by examination of chromosome squashes. Preparation of chromosome squashes for mosquito larval brain was described by Rai (1963a) and Breland (1961), and squash preparation of mosquito pupal testes was described by Mescher and Rai (1966). The techniques used in this investigation were a combination of desirable procedures selected from the methods of those authors above.

Rapid cell divisions in the brain of a mosquito occur intermittently, and the selection of the stage for studying mitosis in the life cycle was important. Usually the brain undergoes considerable growth preceding a larval moult, and the prepupal stage of the last larval instar was chosen for the present study, because the brain is much larger and more easily dissected than at earlier stages. The prepupa can be recognized by the presence of the developing pupal trumpets in the thoracic region.

With the aid of a low-power (about 20X) dissecting microscope, the head was removed from the body and placed in a hypotonic solution of 1% sodium citrate for 30 minutes. As reported by Morgan and

La Brecque (1964), this caused the cells to swell and produced more uniform squashes. The head was then placed in Carnoy's fixative (1 part 95% ethanol: 1 part glacial acetic acid) for 1 minute. The fixative caused the brain to change from a colorless, highly refractive body to an opaque white body which facilitated the dissection of brain tissue from the digestive tract.

After fixation, the head was placed in a drop of Belar's saline on a slide, and the brain was dissected out of the head capsule. The head capsule was teased open with minuten pins, which are ordinarily used for mounting small insects. One needle was placed in the mouth to hold the head in place. The second needle was inserted into the mouth and pushed anteriorly to cause the head capsule to split. The brain lies transversely between the eyes across the dorsal side of the head capsule. After the capsule was split, it was necessary to sever the nerves that connect to each eye and the circumcentric commissures that pass ventrally around the digestive tract.

The brain was transferred to a drop of aceto-orcein stain for ten minutes. Then the stain was washed off the slide with 45% acetic acid. A cover slip was placed over the brain, and the slide was squashed with the aid of a mechanism developed by Linkfield et al. (1967). Good, uniform preparations were obtained using 225 pounds of pressure per square inch. Slides were sealed with finger-nail polish and remained usable for approximately one month.

Testes were dissected from pupae, which were nearly ready to eclose, and from adults less than 24 hours old, and squashes were made for examination of meiosis.

The pupae selected were dark and easily distinguished, because the formation of adult scales was easily seen through the cuticle. The abdominal section was severed from the body and placed in hypotonic sodium citrate for 15 minutes. Then the whole abdomen was fixed in Carnoy's fixative for 1 minute. The sausage-shaped testes, which lie in the dorsolateral region of the sixth abdominal segment, were dissected from the abdomen in Belar's saline, and stained for 10 minutes in aceto-orcein stain. The stain was washed off the slide with 45% acetic acid. A coverslip was placed over the testes and the slide was squashed as described previously with 200 pounds of pressure per square inch.

Testes from adults were prepared as in the manner described above. The testes were dissected from the body by grasping the terminal segment of the male with a pair of fine forceps and applying a gentle, smooth pull. The mosquito was held in place by a needle held behind the thorax. The testes were left attached to the terminal segment, which served as a handle, during transfers from slide to slide.

Squashes were made of the brain and testes of mosquitoes from the susceptible strain, the resistant strain, and the F_1 hybrids from crosses of those two strains. Preparations were also made of the brain of larvae of the susceptible and resistant strains treated with 25 ppm of apholate.

The preparations were examined with a phase-contrast microscope, and photomicrographs were taken with a $3\frac{1}{4} \times 4\frac{1}{4}$ inch Polaroid[®] camera and a 35 mm camera. The Polaroid[®] film used was

Type 107/ASA 3000 black and white, and 35 mm films used were Panatomic-X (ASA 32) and Agfa Isopan (ASA 25). The Polaroid® prints were used immediately for comparing the chromosomes from different cells, and the 35 mm exposures were retained for a permanent record.

Marker Strains

A multiple mutant marker strain called RED EYE was received from Dr. G. B. Craig of Notre Dame University, Notre Dame, Indiana; however, this strain had low rates of fecundity and fertility and, therefore, was not satisfactory for a sterility investigation. The following is a description of this RED EYE strain and strains derived from outcrossing this strain followed by selection of desired genotypes in the F₂ progeny.

RED EYE: This strain was homozygous for red eye, spot, and black-tarsi.

Red eye (re) was reported by McClelland (1966) as a sex-linked, recessive mutant that was expressed as a deep blood red eye color. It can be detected in larvae, pupae, and adults.

Spot (sp) was described by Craig and Van deHey (1962) as an autosomal, recessive gene in linkage group II. This mutant displays a marked sexual dimorphism. In the male, it is expressed by an absence of the spot normally found on each lateral, abdominal tergite, but in the female, the spots are enlarged and extend to the postero-lateral corners of the tergite.

Black-tarsi (blt) was also isolated by Craig and Van deHey (1962). It is an autosomal, recessive gene in linkage group III and is

expressed in both sexes by a reduction of the band of white scales found on the basal portion of most of the tarsal segments.

From the RED EYE strain, the following strains were selected from the F_2 progeny of a cross of RED EYE males to wild type females.

sp-blt: Homozygous for spot and black-tarsi.

sp: Homozygous for spot.

blt: Homozygous for black-tarsi.

Due to a failure in the heating equipment, the sp-blt and sp strains were lost, but subsequently recovered by inbreeding the blt strain.

Genetic Crosses

The backcross and F_2 progeny from the crosses of the apholate-resistant (R) and apholate-susceptible (S) genotypes listed below were assayed for fertility with 20 ppm of apholate by the method developed during the course of the concentration-sterility experiments.

F_2 Crosses

Cross A: F_1 female (S sp blt female x R wild male) x

F_1 male (S sp blt female x R wild male)

Cross B: F_1 female (R wild female x S sp blt male) x

F_1 male (R wild female x S sp blt male)

Backcrosses

Cross C: F_1 male (S blt female x R wild male) x S blt female

Cross D: F_1 female (S blt female x R wild male) x S blt male

Cross E: F_1 female (R wild female x S blt male) x S blt male

Cross F: F_1 female (R female x S male) x R male

Cross G: F_1 male (S female x R male) x R female

RESULTS AND DISCUSSION

Apholate Concentration-Sterility Experiments

The sterility data collected during the preliminary concentration-sterility experiments are presented in Tables 2 and 3. The results listed in Table 2 were from treated male groups, and Table 3 contains the data from the treated female groups. From these experiments, it was apparent that differences existed between the response of the reciprocal F_1 hybrid males. Also, the highest concentration tested had only a small effect on the resistant males and females.

The differences between the F_1 hybrid male groups were surprising, and suggested that the resistance was dominant in the male and additive or recessive in the female. The data for the female groups did not appear to be linear, but this could have been attributed to the methods of collecting eggs from the females en masse. When a group of females laid their eggs in a common place, more eggs were deposited on the water and would not hatch.

The results of those experiments conducted to determine SC_{50} values for the resistant male, resistant female, susceptible male, and susceptible female are presented in Tables 4, 5, 6, and 7, respectively. The results of the experiments on the F_1 hybrid males and females are presented in Tables 8, 9, 10, and 11. The SC_{50} values, slopes of the lines, and fiducial limits for SC_{50} values are presented in Table 12. These data are presented graphically in the

form of calculated regression lines in Figures 1 and 2 for the male and female genotypes, respectively. It was of interest that the slopes of the lines for the resistant male and female genotypes were over twice as great as the slopes of the lines for the susceptible male and female genotypes. This would indicate that the resistance was at a stable level and most likely would not increase. Patterson (1969, personal communication) indicated that selection applied to the resistant strain at 50 ppm and 100 ppm of apholate for several generations failed to increase the resistance.

Examination of the data from the individual egg counts revealed that only a few individuals of the susceptible strain, particularly the females, were partially fertile at the higher concentrations tested. Also, this was found in the F_1 hybrids, particularly in the F_1 female (susceptible male x resistant female) genotype. Approximately 10% of the supposedly susceptible females demonstrated a degree of resistance comparable to the F_1 female (resistant male x susceptible female); however, none of the susceptible males tested showed a marked degree of resistance.

The fiducial limits of the susceptible female and F_1 female (susceptible male x resistant female) genotype barely overlapped, and the fiducial limits of the F_1 hybrid female genotypes overlapped considerably. Theoretically, the F_1 hybrid female genotypes should be identical, and the latter overlap was expected. The large differences in slope and SC_{50} values are unexplainable in terms of monofactorial inheritance; however, the SC_{50} values and position of the two curves (in relation to resistant and susceptible female genotypes) indicated

Figure 1. Calculated regression lines for the sterility-concentration experiments conducted with apholate on the male genotypes listed below:

- o apholate-resistant (R).
- ▲ apholate-susceptible (S).
- F_1 male (S female x R male).
- ▲ F_1 male (R female x S male).

Figure 2. Calculated regression curves for the concentration-sterility experiments conducted with apholate on the female genotypes listed below:

- apholate-resistant (R).
- ▲ apholate-susceptible (S).
- F_1 female (S female x R male).
- ▲ F_1 female (R female x S male).

an incomplete dominance (additive effect) of the resistance. There was approximately a 10-fold resistance to the effects of apholate in the resistant female genotype.

The sterility induced in the 4 male genotypes tested was not as complicated as the data from the female experiments. The susceptible male and F_1 male (susceptible male x resistant female), with SC_{50} values of 2.26 and 4.14, were virtually the same in terms of susceptibility to apholate. There was approximately a 24-fold level of resistance to apholate in the resistant male. The SC_{50} for the resistant male was 16.7 ppm greater than the resistant female, but any difference between these two genotypes could have been exaggerated due to the two different methods for calculating sterility. The total reduction in fecundity was taken into account in the resistant female, but no allowance was taken for sperm mortality or inactivation in calculating the net sterility in the resistant male. If it is assumed that in addition to dominant lethals in the form of deletions, chromosome breaks resulting in acentric fragments and dicentric chromosomes, and other aberrations, there also occurred damage of another nature that resulted in less motile sperm, then the data from the male genotypes and female genotypes are not comparable for differences. The greater tolerance of apholate exhibited by the susceptible female over the susceptible male was probably due to the 10% of the females mentioned previously that were partially refractory to apholate.

The SC_{50} value for the F_1 male (resistant male x susceptible female) was 19.5 ppm, which was about 10 times the SC_{50} of the

Table No. 2. Initial concentrations and resultant sterilities obtained with apholate on males of resistant, susceptible, and F₁ hybrid genotypes, treated from fourth-larval instar until pupation.

Strain	Concentration of Apholate (ppm)	No. Eggs in Sample*	No. Eggs Hatched	% Hatch	Net % Sterility
Resistant male	Check	1674	1585	94.7	--
	5	1578	1456	92.3	2.4
	10	1002	904	90.2	4.5
	15	1083	964	89.0	5.5
	20	1662	1251	75.3	20.3
Hybrid male (Susceptible male x resistant female)	Check	732	701	95.8	--
	5	1200	486	40.5	53.0
	10	1164	292	25.0	73.9
	15	1032	155	15.0	84.5
	20	1500	78	5.2	94.8

* Total of 3 replicates.

Table 2. (Cont.d)

Strain	Concentration of Apholate (ppm)	No. Eggs in Sample*	No. Eggs Hatched	% Hatch	Net % Sterility
Hybrid male (Resistant male x susceptible female)	Check	942	855	90.0	--
	5	912	776	85.2	6.3
	10	630	434	68.7	24.4
	15	774	369	47.6	47.6
	20	981	396	40.4	55.6
Prelim Test S male	Check	3000	2820	94.0	--
	5	3000	864	28.8	69.4
	10	3000	66	2.2	97.6
	15	3000	0	0.0	100.0
	20	3000	0	0.0	100.0

* Total of 3 replicates.

Table No. 3. Initial concentrations and resultant sterilities obtained with apholate on females of resistant, susceptible, and F₁ hybrid genotypes treated from fourth larval instar until pupation.

Strain	Concentration of Apholate (ppm)	No. Eggs*	% Hatch (h)	Fecundity Frequency (f)	Net % Sterility**
Resistant female	Check	4620	79.5	1.00	--
	5	4755	76.9	1.00	0.0
	10	5052	81.1	1.00	0.0
	15	3237	77.1	0.70	32.2
	20	3396	73.3	0.73	32.7
Hybrid female (Resistant male x susceptible female)	Check	7473	95.9	1.00	--
	5	4974	86.5	0.66	40.4
	10	3879	67.9	0.52	63.2
	15	3009	67.4	0.40	71.9
	20	1752	50.3	0.23	88.0

* Total of 3 replicates.

** Net % sterility = 100 (1-fh).

Table 3. (Cont.d)

Strain	Concentration of Apholate (ppm)	No. Eggs*	% Hatch (h)	Fecundity Frequency (f)	Net % Sterility**
Hybrid female (Susceptible male x resistant female)	Check	6927	74.6	1.00	--
	5	6450	62.5	0.93	22.1
	10	4308	64.4	0.62	47.5
	15	3615	66.1	0.52	53.3
	20	1539	36.4	0.22	88.3
Prelim Test S female	Check	7035	94.0	1.00	--
	5	5628	84.0	0.80	24.25
	10	3345	67.3	0.47	63.80
	15	1338	47.7	0.19	89.70
	20	621	37.6	0.09	96.20

* Total of 3 replicates.

** Net % sterility = 100 (1-fh).

Table No. 4. Summary of concentration-sterility data used to calculate regression curve for the response of the resistant male to apholate.

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Sterility	% Net Sterility
Check	2034	1979	2.7	--
25	1566	1353	13.6	11.40
30	1189	922	22.5	20.30
35	1313	946	28.0	26.00
45	1431	904	36.8	35.00
50	1127	507	55.1	53.80
60	822	286	65.2	64.20
80	1174	132	88.8	88.40

* Total of 3 replicates.

Table No. 5. Summary of concentration-sterility data used to calculate regression curve for the response of the resistant female to apholate.

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Eggs Hatched	Fecundity Frequency	% Net Sterility
Check	1924	1839	95.6	1.00	--
20	1602	1392	86.9	.83	28.0
30	1445	1092	75.6	.75	43.3
35	1279	921	72.0	.66	52.5
45	961	536	55.8	.50	72.1
60	692	271	39.1	.36	86.0

* Total of 3 replicates.

Table No. 6. Summary of concentration-sterility data used to calculate regression curve for the response of the susceptible male to apholate.

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Sterility	% Net Sterility
Check	5952	5591	6.1	--
.5	3299	2713	17.8	12.5
1.0	5208	3888	25.4	20.5
2.0	4931	2502	49.3	46.0
4.0	5086	1778	65.1	62.8
6.0	4524	713	84.3	82.3
8.0	4633	452	90.3	89.3

* Total of 3 replicates.

Table No. 7. Summary of concentration-sterility data used to calculate regression curve for the response of the susceptible female to apholate.

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Eggs Hatched	Fecundity Frequency	% Net Sterility
Check	1934	1845	95.4	1.00	--
1	2142	1504	70.2	1.00	25.4
2	1730	1306	75.5	.89	29.6
4	1280	1001	80.7	.66	53.4
8	860	588	71.7	.44	68.1
12	586	252	45.1	.30	86.4
16	357	184	54.0	.18	90.1
20	264	72	28.6	.13	96.2

* Total of 3 replicates.

Table No. 8. Summary of concentration-sterility data used to calculate regression curve for the response of the F₁ male (susceptible female x resistant male) to apholate.

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Eggs Hatched	% Sterility	% Net Sterility
Check	1368	1264	92.4	7.6	--
4	1185	1007	84.9	15.1	8.1
8	2799	1974	70.5	29.5	23.7
12	1588	1026	64.6	35.4	30.1
16	2380	1162	51.2	48.8	44.6
24	1297	527	40.6	59.4	56.1

* Total of 3 replicates.

Table No. 9. Summary of concentration-sterility data used to calculate regression curve for the response of the F₁ male (resistant female x susceptible male) to apholate.

Concentrations of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Eggs Hatched	% Sterility	% Net Sterility
Check	2541	2436	95.8	4.2	--
2	2188	1638	74.8	25.2	21.9
4	3204	1683	52.5	47.5	45.2
8	3197	743	23.2	76.8	75.8
12	3402	602	17.7	82.3	81.5
16	1359	39	2.8	97.2	97.1

* Total of 3 replicates.

Table No. 10. Summary of concentration-sterility data used to calculate regression curve for the response of the F_1 female (resistant female x susceptible male) to apholate.

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Eggs Hatched	Fecundity Frequency	% Net Sterility
Check	1876	1674	89.2	1.00	--
4	1470	1325	90.1	.78	30.8
8	1292	1092	84.5	.68	42.6
12	1102	754	68.4	.58	60.4
16	970	646	66.6	.52	65.4

* Total of 3 replicates.

Table No. 11. Summary of concentration-sterility data used to calculate regression curve for the response of the F_1 female (susceptible female x resistant male) to apholate

Concentration of Apholate (ppm)	No. Eggs*	No. Eggs Hatched	% Eggs Hatched	Fecundity Frequency	% Net Sterility
Check	1310	1016	77.5	1.00	22.5
8	1134	944	83.2	.86	28.5
16	779	534	68.5	.59	59.6
24	591	222	37.5	.45	83.2
32	359	169	47.1	.27	87.3

* Total of 3 replicates.

Table 12. Summary of probit analyses of the data in Tables 4-11.

Genotype	SC ₅₀ (ppm)	b (Slope)	Fiducial Limits (P=.95)	
			Lower	Upper
F ₁ female (S female x R male)	16.87	4.10	14.5	19.5
F ₁ female (R female x S male)	8.94	1.55	4.1	19.3
S female	3.22	1.76	1.8	5.6
R female	31.61	3.47	27.4	36.4
F ₁ male (S female x R male)	19.50	2.01	9.5	39.9
F ₁ male (R female x S male)	4.14	3.02	3.2	5.4
S male	2.26	1.98	1.4	3.4
R male	48.30	4.47	45.2	51.6

Table 13. Summary of data from the experiment conducted to measure cross-resistance of the apholate-resistant male to metepa.

Strain	Concentration of Metepa (ppm)	No. Eggs*	No. Eggs Hatched	% Hatch	% net Sterility
Susceptible male	Check	600	588	98.0	--
	10	723	546	75.5	22.9
	20	684	354	51.7	47.2
	40	642	99	15.4	84.3
	80	600	15	2.5	97.4
Resistant male	Check	588	537	91.3	--
	10	693	618	89.2	2.3
	20	621	507	81.6	10.6
	40	627	470	74.9	18.0
	80	750	435	58.0	36.5

* Total of 3 replicates.

susceptible male. The fiducial limits did not overlap with any of the other 3 male genotypes, and the data showed a definite paternal effect. From these data it appeared the resistance was expressed as an incomplete dominant (additive effect) and was linked with the sex locus.

Based on the fiducial limits ($P = .95$) of the SC_{50} values and the log concentration-probit sterility curves (Figures 1 and 2), a concentration that would separate the resistant male from the susceptible male genotypes was selected at 20 ppm of apholate. The data from the female genotypes indicated that the selection of a concentration to separate the resistant from susceptible was impossible; therefore, it was decided to use the males for determining the mode of inheritance of the resistance.

Cross-Resistance Experiments

It was reasonable to expect that the apholate-resistant strain of A. aegypti would demonstrate resistance to other aziridine compounds. The data collected in the experiments to determine the sterility induced by tepa and metepa in the resistant and susceptible strains are presented in Tables 13, 14, 15 and 16. The net per cent sterilities from the treated male groups were graphed (Figure 3) and estimates of the SC_{50} values were taken from this graph. The data indicated a 5-fold tolerance of metepa and a 3-fold tolerance of tepa by the resistant males. The SC_{50} value for metepa on the resistant male might not be valid, because it was necessary to make a considerable extrapolation beyond the actual data.

The data from the female groups (Tables 14 and 16) indicated that the resistant female was more tolerant of metepa and tepa than

Figure 3. Regression lines visually fitted to data from the experiments designed to measure cross-resistance to tepa and metepa in the apholate-resistant (R) male.

- ▲ R male treated with tepa.
- △ S male treated with tepa.
- R male treated with metepa.
- S male treated with metepa.

Table No. 14. Summary of data from the experiment to measure cross-resistance of the apholate-resistant female to metepa.

Strain	Concentration of Metepa (ppm)	No. Eggs in Sample*	No. Eggs Hatched	% Hatch	% Net Sterility
Susceptible female	Check	600	588	98.0	--
	10	630	510	80.0	17.4
	20	678	513	75.6	22.8
	40	657	384	58.4	40.4
	80	360	171	47.5	51.5
Resistant female	Check	600	495	82.5	--
	10	600	417	69.5	16.9
	20	600	495	82.5	0.0
	40	648	333	51.4	37.7
	80	600	387	64.5	14.8

* Total of 3 replicates.

Table No. 15. Summary of data from the experiment conducted to measure cross-resistance of the apholate-resistant male to tepa.

Strain	Concentration of tepa (ppm)	No. Eggs*	No. Eggs Hatched	% Hatch	% Net Sterility
Susceptible male	Check	600	582	97.0	--
	2.5	561	216	38.5	60.3
	5.0	600	108	18.0	81.4
	10.0	600	60	10.0	89.7
	20.0	600	0	00.0	100.0
Resistant male	Check	600	516	86.0	--
	2.5	600	441	73.5	14.5
	5.0	609	252	41.4	51.8
	10.0	600	60	10.0	88.4
	20.0	600	0	00.0	100.0

* Total of 3 replicates.

Table No. 16. Summary of data from the experiment conducted to measure cross-resistance of the apholate-resistant female to tepa.

Strain	Concentration of Tepa (ppm)	No. Eggs*	No. Eggs Hatched	% Hatch	% Net Sterility
Susceptible female	Check	600	582	97.0	--
	2.5	615	477	77.6	20.0
	5.0	621	447	71.9	25.8
	10.0	612	339	55.4	42.8
	20.0	no eggs	--	--	100.0
Resistant female	Check	639	537	84.0	--
	2.5	600	474	79.0	5.9
	5.0	654	546	83.5	0.6
	10.0	600	360	60.0	28.6
	20.0	no eggs	--	--	100.0

* Total of 3 replicates.

Table No. 17. Summary of mortality in the apholate-resistant and susceptible strains after treatment with increasing concentrations of metepa.

Metepa Male Concentration of Metepa (ppm)	Susceptible Strain			Resistant Strain		
	No. Treated*	No. Dead	Net % Mortality	No. Treated	No. Dead	Net % Mortality
Check	300	0	--	300	1	.3
10	300	0	--	300	0	--
20	300	6	2.0	300	28	9.3
40	300	57	19.0	300	18	6.0
80	300	81	27.0	300	60	20.0

<u>Metepa Female</u>						
Check	300	0	--	300	0	--
10	300	11	3.6	300	13	4.3
20	300	0	--	300	11	3.7
40	300	6	2.0	300	67	22.3
80	300	5	1.7	300	65	21.6

* Total of 3 replicates.

Table No. 18. Summary of mortality in the apholate-resistant and susceptible strains after treatment with increasing concentrations of tepa.

Tepa Male Concentration of Tepa (ppm)	Susceptible Strain			Resistant Strain		
	No. Treated*	No. Dead	Net % Mortality	No. Treated	No. Dead	Net % Mortality
Check	300	10	3.3	300	29	9.7
2.5	300	28	9.3	300	17	5.7
5.0	300	48	16.0	300	30	10.0
10.0	300	67	22.3	300	150	50.0
20.0	300	262	87.3	300	243	80.9

<u>Tepa Female</u>						
Check	300	10	3.3	300	36	12.0
2.5	300	52	17.4	300	26	8.7
5.0	300	34	11.3	300	19	6.3
10.0	300	55	18.3	300	110	36.6
20.0	300	288	96.1	300	250	83.5

* Total of 3 replicates.

the susceptible female. A reduction in fecundity was noticed to correspond to increasing concentration in the females treated with both tepa and metepa; however, the reductions in the tepa treatments were more severe than the metepa treatments.

Mortality data collected during these experiments are presented in Tables 17 and 18 for metepa and tepa, respectively. Mortality as a result of the metepa treatments was low; however, a greater mortality was noticed in the resistant female compared to the susceptible female.

Mortality in the tepa treatments was quite severe, and no major differences existed between strains. At 20 ppm there were only a few individuals that survived to become adults. Most of the mortality was seen in the pupal stage and emerging adults. The dead adults were apparently very weak and never left the surface of the water. Larval mortality was negligible in all treatments, but at the highest concentrations of both compounds, some larvae required nearly a week to complete the fourth instar and pupate. These pupae invariably died.

Non-Selected Apholate-Resistant Strain

The expected loss of resistance after termination of apholate selection in the resistant strain was not observed through 9 generations. Figure 4 summarizes the data from this experiment in terms of per cent hatch observed in each generation. The level of resistance in the non-selected colony increased compared to the regular apholate-resistant colony and maintained a per cent hatch from a minimum of 15% to a maximum of 30% greater than the selected colony. Closer

Figure 4. Summary of data from the experiment to determine the permanence of apholate resistance in the absence of apholate selection pressure. Each point on the graph is the average of two replicates.

- non-selected apholate-resistant strain.
- assay groups from the non-selected strain treated with 25 ppm apholate.
- ▲ apholate-resistant strain treated each generation with 25 ppm apholate.

examination of the data indicated that this difference could have resulted from a loss of genetic damage. This statement is based on the data from the F_1 generation, which placed the assay group of resistant males almost half-way between the regular apholate-selected colony and the non-selected colony in the parent generation. In the succeeding 3 generations, there was an apparent increase in the resistance level, but then a decrease occurred in the F_5 . The lowest per cent hatch occurred in the F_6 generation, but this deviation from the other data could have possibly resulted from the temperature of the rearing room, which was 5° F lower than usual and delayed pupation by 1 day. This extra day of treatment probably increased the sterility induced.

Instead of losing apholate resistance, the non-selected colony became more tolerant of apholate and after 9 generations maintained a higher per cent hatch when treated with apholate than the regular apholate-resistant colony. The non-selected resistant strain also increased in per cent hatch above the 90% level and maintained that level for the duration of the experiment. Males of a susceptible colony maintained as a control were completely sterilized each generation by the apholate treatment.

Genetic Crosses

The results from the F_2 crosses and backcrosses are presented in Tables 19 and 20, respectively. These data did not fit any genetic models for 3 genes or less, and since there are only 3 linkage groups in A. aegypti, a quantitative type of inheritance was probably involved in apholate resistance. There was a definite paternal effect on the

Table No. 19. Summary of sterility in F_2 progeny, treated with 20 ppm of apholate, of Cross A: F_1 female (S sp blt female x R wild male) x F_1 male (S sp blt female x R wild male) and Cross B: F_1 female (R wild female x S sp blt male) x F_1 male (R wild female x S sp blt male).

Cross	Marker Phenotype	No. Sterile	No. Fertile	Total
A	+ +	61	178	239
	+ blt	20	57	51
	sp +	14	37	77
	sp blt	5	4	9
Total		100	276	376
B	+ +	89	0	89
	+ blt	22	2	24
	sp +	49	2	51
	sp blt	13	0	13
Total		173	4	177

Table No. 20. Summary of sterility in backcross progeny, treated with 20 ppm of apholate, of Cross C: F_1 male (S blt female x R wild male) x S blt female, Cross D: F_1 female (S blt female x R wild male) x S blt male, Cross E: F_1 female (R wild female x S blt male) x S blt male, Cross F: F_1 female (R female x S male) x R male, and Cross G: F_1 male (S female x R male) x R female.

Cross	Marker Phenotype	No. Sterile	No. Fertile	Total
C	+	66	72	138
	blt	69	72	141
Total		135	144	279
D	+	78	24	102
	blt	81	12	93
Total		159	36	195
E	+	147	12	159
	blt	126	6	132
Total		273	18	291
F	+	108	84	192
G	+	5	174	179

resistance of the males, and apparently the gene (s) on the sex chromosome had a greater effect when coupled to the male locus. This was easily seen when comparing the number of resistant males of Crosses A to B and C to D.

Although it was quite clear that resistance was manifest in more males that had a resistant male ancestor in the parent cross, as opposed to those with susceptible male ancestors, the mechanism for such a phenomenon was not clear. An expression of the resistance as a dominant when coupled with the male locus did not fit the data, as obviously the resistance was manifest in repulsion (Cross D) in some individuals and absent (Crosses A and C) in coupling in many individuals. The large number, about 50%, of sterile males in Cross C indicated that the resistance was certainly not due to a single dominant gene and linkage data with blt as a marker in Cross C and with sp and blt as markers in Cross A clearly showed that the resistant phenotype was not exclusively dependent on either of linkage groups 2 and 3. Therefore, a rather complicated mode of inheritance appeared to be involved. Perhaps differential expressivity of the resistant genotype was involved, but certainly this would have been expressed in the concentration-sterility curves established for the F_1 males, whereas it was not. Therefore, the expression of apholate resistance appears to be complicated and quantitative in nature with more than 3 genes involved. The sex chromosome derived from the male parent probably had a major role in the resistant phenotype, but the exact nature and mechanism of this role was not evident from the data.

Cytogenetic Experiment

Examination of chromosomes from the larval brain and adult testes divulged only one aberration in the resistant strain from the normal karyotype seen in the susceptible strain. The single aberration is shown in Figure 5c in the form of a deletion or absence of stainable nucleic acid in one arm of chromosome III (according to Rai, 1963a). This deletion, however, was observed only in one cell in this particular squash, with other cells on the slide normal. The number of brain squashes examined were 36, 27, 20, and 20 for the resistant, susceptible, F_1 (susceptible female x resistant male), and F_1 (resistant female x susceptible male) genotypes, respectively. Figures 5b and 5d are representative of the brain mitoses at metaphase examined in the apholate-resistant mosquitoes. Figures 6c, 5a, 6d, and 6a show mitotic prophase, metaphase, anaphase and telophase from the susceptible strain. The lack of observable aberrations corresponded to the data from the non-selected resistant strain. Those data indicated a loss of genetic damage by an increase in per cent hatch after selection was terminated. Major aberrations such as translocations would have persisted in the population for several generations (Curtis, 1968). Terminal breaks and relatively large deletions that produced no dominant effects would have also persisted for some time. However, the per cent hatch in the non-selected resistant strain increased to a value comparable to normal hatch in A. aegypti that were never exposed to apholate in one generation.

Figure 5. Mitotic chromosomes from brain tissue of Aedes aegypti.

- a. Apholate-susceptible strain.
- b. Apholate-resistant strain.
- c. Apholate-resistant strain; note apparent deletion.
- d. Apholate-resistant strain.

Figure 6. Mitotic chromosomes from brain tissue of apholate-susceptible strain of Aedes aegypti.

- a. Normal telophase.
- b. Metaphase from mosquito treated with 25 ppm apholate during fourth larval instar.
- c. Normal prophase.
- d. Normal anaphase.

Figures 6b and 7a-7d show the chromosomal damage caused by treatment of the susceptible strain with 20 ppm of apholate. Chromosomal stickiness and a loss of normal shape and form are seen in Figures 6b, 7a, and 7c. In Figure 7d, the chromosomes appeared to have constrictions which were not normal. Normal meiotic figures from adult squashes are shown in Figures 8a-9d. No more than 2 chiasmata per bivalent were observed, which agreed with Mescher and Rai (1966). Figures 9a and 9b demonstrate the difficulties encountered in working with the pupal testes for squashes. The chromosomes in metaphase I and anaphase I adhered to form a single chromatic mass. It was impossible to distinguish individual chromosomes, and at the first observation it appeared as though the mosquitoes had been accidentally exposed to apholate.

In accordance with Mescher and Rai (1966), the earliest stage at which chromosomes became clearly visible was pachytene. They attributed this lack of visible leptotene and zygotene stages to somatic pairing of chromosomes in the mitotic division preceding meiosis. Pachytene apparently is of long duration relative to other stages in A. aegypti and was present in all individuals examined in much greater numbers than the other stages.

Figure 7. Mitotic chromosomes from brain tissue of apholate-susceptible strain of Aedes aegypti treated with 25 ppm of apholate during fourth larval instar.

Figure 8. Meiotic chromosomes from adult testes of Aedes aegypti: a., b., and d., diplotene; c. diakinesis. Note maximum of two chiasmata per bivalent.

Figure 9. Meiotic chromosomes from testes of Aedes aegypti:

- a. and b., chromosomes prepared from pupal testes, which appear as a single chromatic mass.
- c. prophase II from adult testis.
- d. metaphase II from adult testis.

SUMMARY AND CONCLUSIONS

Chemosterilant resistance has been of concern to entomologists since the initial discoveries that certain compounds would effectively render insects sterile. Many researchers were familiar with the problems encountered in development of insecticide resistance by field populations of insects, and this prompted numerous groups of investigators to attempt to induce chemosterilant resistance in insects under experimental conditions in the laboratory. Resistance to apholate was subsequently developed in Aedes aegypti (L). This investigation was undertaken to elucidate the mode of inheritance of the apholate resistance in the species mentioned above, to determine whether any cross-resistance to tepa and metepa was evident in the apholate strain, and to study the permanence of the resistance through succeeding generations upon removal of selection pressure. In addition, a study was made on the chromosomes of the apholate-resistant strain to determine whether the mutagenic agent, apholate, had induced large deletions, terminal breaks, and translocations in the population, which had been exposed to apholate for more than 70 generations.

The mosquitoes were exposed to the chemosterilant in the larval stage. Sterility-concentration curves were established for each sex of the resistant, susceptible, and F_1 hybrid genotypes, and SC_{50} values were calculated by probit analysis for each genotype. The SC_{50} values were 48.30 for the resistant male, 2.26 for the susceptible

male, 4.14 for the F_1 male (resistant female x susceptible male), and 19.50 for the F_1 male (susceptible female x resistant male); and 31.61 for the resistant female, 3.22 for the susceptible female, 8.94 for the F_1 female (resistant female x susceptible male), and 16.87 for the F_1 female (susceptible female x resistant male).

From the concentration-sterility data, 20 ppm of apholate was selected as a concentration to discriminate the resistant male and F_1 male (susceptible female x resistant male) genotypes from the susceptible male and F_1 male (resistant female x susceptible male) genotypes. Progeny from genetic crosses were subjected to 20 ppm of apholate for bioassay in order to assess resistance. The results of the crosses indicated that apholate resistance in A. aegypti was a quantitative trait, with the additional complication of a marked paternal influence on the resistance of F_2 and backcross progeny.

There was no marked cross-resistance to tepa and metepa present in the apholate-resistant strain, but there were increased tolerances of 5-fold to metepa and 2.5-fold to tepa in the resistant males. Estimates of the tolerance levels of tepa and metepa were gained by examining probit sterility-log concentration graphs of the data collected from treated males of the resistant and susceptible strains.

There was no loss of resistance through 9 generations after selection pressure was removed from a sub-colony of the apholate-resistant strain. The resistance level, which was based on per cent hatch, actually increased during the first 4 generations, but then it decreased and remained fairly stable through the ninth generation.

The examination of chromosome squashes of the apholate-resistant mosquitoes for major chromosomal aberrations were negative, except for an apparent deletion found in a single cell of one individual. The lack of major aberrations substantiated the rate of disappearance of an inherent sterility of about 20% in 2 generations after apholate selection was terminated.

It is suggested that future investigations use an approach to the selection of genetic crosses to yield information concerning the heritability of the trait.

APPENDIX

APHOLATE

Chemical Name: 2,2,4,4,6,6-hexakis (1-arizidinyl) - 2,2,4,4,6,6-Hexahydro-1,3,5,2,4,6,-triazatriphosphorine.

Other Designations: SQ 8388

ENT 26316

OM 2174

Structural Formula:

Where R is:

Physical Properties:

White Powder

M. P. 150° C

Molecular formula $C_{12}H_{24}N_9P_3$

Molecular weight 387.4

Approximate solubility (% weight)

Water: 33%

Ethanol: 15%

Chloroform: 31%

APHOLATE (Cont.'d)

Approximate solubility (% weight) (Cont.'d)

Methanol: 14%

Acetone, xylene, mineral oil, hexane: 1%

Stability: Moisture, high temperature, low pH, proteinaceous materials and highly sorptive carriers cause polymerization.

METEPA

Chemical Name: tris (2-methyl-1-aziridinyl) phosphine oxide.

Other Designations: Metaphoxide

Methyl Aphoxide

ENT 50003

M APO

Structural Formula:

Where R is

Physical Properties:

Molecular formula: $\text{C}_9\text{H}_{18}\text{N}_3\text{OP}$

Molecular weight: 214.9

TEPA

Chemical Name: tris (1-aziridinyl) phosphine oxide.

Other Designations: Aphoxide

A P O

ENT 24915

TEPA (Cont.d)

Structural Formula:

Where R is

Physical Properties:

Molecular formula: $\text{C}_6\text{H}_{12}\text{N}_3\text{OP}$

Molecular weight: 172.9

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BIOGRAPHICAL SKETCH

Jack Arlyn Seawright was born September 9, 1941, at Ware Shoals, South Carolina. In 1959, he was graduated from Ware Shoals High School. He received the degree of Bachelor of Science with a major in Biology from Clemson University in January, 1964. In December, 1965, he received the degree of Master of Science with a major in Entomology from Clemson University. From August, 1965, he has been enrolled at the University of Florida in pursuit of the degree of Doctor of Philosophy with a major in Entomology.

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This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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