EFFECTS OF CHEMOSTERILANTS ON THE DEVELOPMENT OF MALARIAL PARASITES IN MOSQUITOES [1, 2]

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INTRODUCTION. In recent years resistance to chlorinated hydrocarbon insecticides in insects of medical importance has become more widespread and reports of failure to maintain control with the newer organophosphorus compounds have become more prevalent. New insecticides are continually being developed but resistance often follows after extended agricultural usage. Alternate methods of control other than insecticides acting in the conventional manner have been proposed. These include the use of pathogens or parasites as a means of biological control of a specific insect, the genetic manipulation of populations, and the use of ionizing radiation and chemicals to sterilize the insect sexually. (World Health Organization, 1963).

The eradication of the screw-worm fly, *Callitroga hominivorax* (Cqrl.), from the southeastern United States by the release of males sexually sterilized by gamma radiation demonstrated that an insect population could be controlled by radiation techniques (Knipling, 1960). For this method to be successful, large numbers of the insect had to be reared, made sterile without affecting their viability and released in the normal population in such numbers that the released sterile males could successfully compete with the normal males. Laboratory experiments with *Anopheles quadrimaculatus* Say indicated that this procedure might be an effective method of mosquito control in areas with small mosquito populations. However, when irradiated male mosquitoes were released in the field, no demonstrable reduction occurred in natural populations (Weidhaas, *et al.*, 1962). It was believed that the dose of radiation necessary to render a male sterile altered its behavior or viability in such a manner that it was unable to compete sexually with the normal males present in the area.

A number of compounds are known that will affect the metabolism of the arthropod or its reproductive tissues in such a manner that sexual sterility can be achieved without altering its behavior or longevity. These chemosterilants have the additional advantage of ease of application to the insect either in its food or as an insecticide, and they eliminate the need for expensive radiation facilities. Since these compounds appear to affect only rapidly proliferating cells and tissues at the appropriate dosage level, they may likewise modify or prevent the multiplication of pathogenic agents within their arthropod vectors.

It is possible that a single compound applied as insecticide could act as a sexual sterilant to a mosquito and prevent the transmission of disease by the insect. The potentialities of this dual approach to malarial control were first emphasized by Lindquist (1961).

The ideal chemosterilant for a malaria control program should be an agent that will prevent the transmission of viable sporozoites either by inhibiting or interfering with the sporogonous cycle of the parasite in its mosquito host. It should sexually sterilize the insect without affecting its viability, should be nontoxic to humans and should be applicable as a residual insecticide.

Three groups of chemosterilants—alkyl-

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ating agents, antimetabolites and the nonalkylating agents — have been extensively evaluated by the U. S. Department of Agriculture as sexual sterilants. Of these, the alkylating agents have exhibited the most activity against mosquitoes.

The alkylating agents or radiomimetic substances produce many of the biological end-effects observed after treatment with ionizing radiations. At low concentrations they act upon the genetic material of the cell producing chromosomal breakage and gene mutation. With higher dosages carcinogenic or growth-inhibitory effects are evident, usually accompanied by the death of both dividing and non-dividing cell.

The most effective alkylating agents are all derivatives of aziridine (Chang, *et al.,* 1964). These include tepa, metepa, tretamine and apholate. Tepa has been used medically for the treatment of malignant lymphomas. Apholate and related compounds are commercially used in the textile industry as polymerizing agents to make fabrics crease resistant. These agents are considered hazardous to man if not appropriately handled. For example, tepa is very irritant to mucous membranes, particularly the eyes; nausea, vomiting or diarrhea may occur after overexposure; extravasation into subcutaneous tissues may cause pain, swelling and occasional sloughing. In rats they are known to produce bone marrow depression. Barnes (1964) and Hayes (1964) have recently reviewed the toxicology of alkylating agents.

The nonalkylating agents such as hempa are structurally similar to tepa but have very low mammalian toxicity and lack alkylating properties. They have been extensively evaluated in houseflies (Chang, *et al.,* 1964).

The first studies on the concurrent effect of a chemosterilant on the mosquito host and its malarial parasite were made by Altman (1963). He demonstrated that exposure of *Aedes aegypti* to a tepa residue of 10 mg./ft.^{2} on a glass surface prior to or just after infection with the chicken malarial parasite (*Plasmodium gallinaceum,* 8-A strain) produced a marked reduction in malarial parasite development, decrease in mosquito transmission rate and non-viable mosquito eggs.

Bertram, *et al.,* (1964) studied the effects of a closely related compound, thiotepa, in the same host-parasite system used by Altman except the 8-B parasite strain was utilized. The same general observations on malarial oocyst reduction were made, but Bertram noted that dosage levels for thiotepa were approximately four times greater than that needed by Altman to produce a comparable effect. In fact, the dose of thiotepa necessary to suppress almost completely oocyst development or sporozoite infectivity approached the dosage lethal to the vector.

The present studies were initiated to develop a standard laboratory test procedure for the evaluation of chemosterilants in malaria control, to study certain factors responsible for variations in response observed in different laboratories and to compare the relative efficacy of tepa, metepa, apholate and hempa in a standardized system.

MATERIALS AND METHODS. Unless otherwise indicated, a Bangkok strain of *Aedes aegypti* (Linn.) was the mosquito host. One experiment utilized *Anopheles stephensi* Liston (Delhi, India strain). All mosquitoes were reared in the departmental insectary at approximately 27° C. Mosquitoes used for any one experiment were approximately 5 to 10 days from date of pupation, had mated at random prior to the test date and were maintained at 27° C. in "Precision" Low Temperature Cabinets at 75–85 percent relative humidity after receiving an infective blood meal and chemosterilant exposure.

The 8-A strain of *Plasmodium gallinaceum* Brumpt was used in all but two experiments with *Aedes aegypti* which utilized the 8-B strain. It was maintained by sporozoite passage in White Leghorn chickens and most mosquito infections were made on chicks with parasitemia ranging between 5–10 percent. The simian parasite, *P. cynomolgi bastianelli* Garn-

ham, maintained in *Macacca mulatta,* was used for the *Anopheles stephensi* experiment.

The degree of malaria parasite development was assessed by quantitative observations made on the malarial oocysts on the seventh day after the infective meal. The procedures for these counts and measurements have been previously described (Ward, *et al.,* 1960). Mosquito mortality was recorded on day seven and a sample of 300 eggs was checked for hatching from each treated and control group.

All chemosterilants studied were fresh samples provided by A. B. Borkovec of the Entomology Research Division, U. S. Department of Agriculture. The following compounds were supplied:

(1) Tepa (ENT-24915Xw) is tris-(1)-aziridinyl)-phosphine oxide.

(2) Metepa (ENT-50003e) is tris-(2-methyl-1-aziridinyl)-phosphine oxide.

(3) Apholate (ENT-26316) is 2,2,4,4,6,6-hexahydro-2,2,4,4,6,6-hexakis-(1-aziridinyl)-1,3,5,2,4,6-triazatriphosphorine.

(4) Hempa (ENT-50882a) is hexamethyl-phosphoric triamide.

All chemical dilutions were made in methanol no earlier than one day prior to conducting an experiment. Initially 1-pint glass jars with glass Petri dish covers were used as treatment chambers (Altman, 1963). Later, the World Health Organization Adult Mosquito Test kit (World Health Organization, 1960) served as the test chamber with chemicals applied to treated filter paper (Bertram, 1964), bond paper or glass liners. Treated papers or surfaces were allowed to dry 4 to 24 hours prior to introducing insects. A methyl alcohol control was used in each experiment. At the conclusion of each experiment, all chemosterilant-treated surfaces were inactivated by a 12-hour soaking in 5 percent acetic acid.

To determine the effect of a chemosterilant upon an engorged mosquito, females were transferred to treatment cages 2 hours subsequent to an infective blood meal. They were kept in the treatment cage for 3 hours, except for the first experiment, and then transferred to a holding cage. During the treatment period, observations were made to determine if any repellent effect was evident and the cage was agitated at 30-minute intervals to assure an even distribution of mosquitoes. Aside from the preparation of test papers or glass liners the tests adhered to the World Health Organization procedure for the evaluation of insecticides.

RESULTS. Apholate and *Plasmodium cynomolgi* in *Anopheles stephensi.* The inside surfaces of pint glass jars and Petri dish covers were treated with apholate applied at concentrations of 10 and 20 mg./square foot. Four hours later, 50 infected anophelines were placed in each treatment chamber for two hours.

The apholate treatment produced a marked decrease in malarial oocyst number which was significant at the 1 percent level (Table 1). No significant differences were present between the 10 and 20 mg. levels. Mortality was doubled with exposure at either level. Mature, motile sporozoites were observed in oocysts from treated mosquitoes.

TABLE 1.—Effect of apholate on development of *P. cynomolgi* in *Anopheles stephensi.* Two-hour mosquito exposure in treated jar.

Treatment	Malarial oocysts ♀♀ dissected	Mean no. ±S.E.	♀♀ mortality
Control	10	260.4±66.0	26%
10 mg. apholate/ft.²	6	17.7±13.3	62%
20 mg. apholate/ft.²	8	32.0±21.0	64%

Chemosterilants and *Plasmodium gallinaceum* in *Aedes aegypti.* The pint jar was found to be an impractical treatment chamber. It is difficult to apply a uniform deposit of chemosterilant on the inner surfaces due to the irregular shape of the jars. In addition, a fumigant effect was present due to variations in volatility of different chemosterilants.

The World Health Organization adult

mosquito test kit as used by Bertram (1963) proved to be the best method for evaluating these compounds. Treatment and holding tubes are screened at one end and have a plastic slide at the other end for mosquito transfer. This design overcomes the main difficulties experienced with the pint jars and the transfer of mosquitoes can be accomplished much faster. These tubes were designed for use with insecticide-impregnated paper linings. Initial experiments replaced these papers with filter and bond paper impregnated with chemosterilants. Later, a treated glass liner was used.

In accordance with the procedure outlined by Bertram, Whatman No. 1 filter papers were treated with various concentrations of apholate and placed in the test cages four hours after treatment. Observations on the mosquitoes (Table 2) indicated that with increasing dosage of chemosterilant, there was a slight increase in female mortality, a marked decrease in hatch rate but no change in malarial oocyst development in terms of number or size.

During the preparation of the above filter papers it was observed that the methanol solution of chemosterilant was absorbed almost instantaneously by the relatively coarse paper, apparently leaving little chemosterilant residue on the surface. It was decided that a better coating of the paper might be effected by using a relatively smooth, non-porous bond paper.[3]

[3] Erasable Prudential Bond Typewriter Paper, #110-E, Prudential Paper Products, Elmhurst, N. Y.

The next experiment was designed to compare the effects of tepa, metepa and hempa applied to bond paper on mosquito and parasite development under the same conditions as in the preceding apholate experiment (Table 3). Unexpectedly, no reduction in oocyst number was observed in the tepa group as previously was noted (Altman, 1963 and Bertram, *et al.*, 1964). Mortality was normal for the tepa and metepa treatments except for an anomaly at the 20 mg./ft.^{2} level of tepa. The lower mean oocyst count for 100 mg./ft.^{2} of hempa is not significantly lower than the other values. The hempa produced an excessive mortality of 80 percent. At the end of 24 hours half the *A. aegypti* at this level were dead. An experiment was conducted with hempa applied at 500 mg./ft.^{2}. In one hour, 24/25 females were knocked down and all were dead within 24 hours. All oocysts from these tepa, metepa and hempa treatments were nor-

TABLE 3.—Effect of tepa, metepa and hempa on development of *P. gallinaceum* (8-A) in *Aedes aegypti* (Bangkok). Three-hour mosquito exposure on treated bond paper in WHO kit.

Treatment	Malarial oocysts ♀♀ dissected	Malarial oocysts Mean no. ± S.E.	♀♀ mortality
Control	15	47.3±16.0	6%
20 mg. tepa/ft.^{2}	15	41.9±13.0	28.6%
40 mg. tepa/ft.^{2}	23	43.4± 8.4	4.2%
20 mg. metepa/ft.^{2}	21	42.2±13.5	4.6%
40 mg. metepa/ft.^{2}	22	42.0±11.7	4.4%
100 mg. hempa/ft.^{2}	5	31.6±16.4	80.0%

TABLE 2. Effect of apholate on development of *P. gallinaceum* (8-A) in *Aedes aegypti* (Bangkok). Three-hour mosquito exposure on treated filter paper in WHO kit.

Treatment	Malarial oocysts ♀♀ dissected	Malarial oocysts Mean no. ± S.E.	♀♀ mortality	Egg hatch
Control	23	17.8±6.4	4.2%	92.0%
10 mg. apholate/ft.^{2}	24	14.5±3.7	14.3%	63.3%
20 mg. apholate/ft.^{2}	19	13.1±5.6	13.6%	64.8%
80 mg. apholate/ft.^{2}	20	13.0±5.6	20.0%	45.3%

mally developed at day seven so it was concluded that the bond paper was not an appropriate treatment surface despite its slower absorbency.

To overcome these problems of absorption, a non-porous material was considered to be the best surface for treatment. Plastic liners were initially considered but were later rejected due to possible interaction between the chemosterilant and the material. Finally, standard glass tubing, 40 mm in diameter, cut to 125 mm lengths proved to be an ideal lining material for the WHO test kit tube. It is easily obtained, fits smoothly in the treatment tube, can be easily coated with test materials and is reusable after cleansing in 5 percent acetic acid solution. Care should be taken that the tubes are exactly 125 mm long so that there is perfect contact with the screened top of the holder. Since it requires a period greater than 4 hours to dry the treated tubes thoroughly, all experimental tubes were coated with test materials late during the afternoon preceding the test date. In general, approximately 21 hours elapsed between the coating of the glass liners and introduction of infected mosquitoes.

The first experiment with the coated tubes involved a comparison of the effects of tepa, metepa, apholate and hempa on the 8-B strain of *P. gallinaceum* in the Bangkok strain of *A. aegypti* (Table 4). A significant reduction in oocyst number was observed in both the tepa and metepa treatment groups. Only one mosquito in the metepa group developed malarial oocysts while 80 percent of the controls had oocysts. The mean oocyst count for the 20 mg. apholate group was not significantly different from that of the 10 mg. level for this material. The tepa and metepa groups demonstrated a marked retardation in oocyst growth rate as determined by measurements made on day seven. At the tested levels, apholate and hempa did not affect the growth pattern of the sporogonous cycle of the parasite. Little mosquito mortality occurred during the test period except for a slight rise in the metepa group. No eggs from tepa or metepa treated females hatched. Significantly fewer eggs hatched from apholate and hempa treated females than from the controls. An examination of female genital tracts from tepa- and metepa-exposed mosquitoes showed almost complete destruction of the ovaries. Generally, the ovaries were an amorphous mass with the follicles almost completely fused together. If eggs were present, they were invariably sterile.

The final experiment (Table 5) verified the observations of the preceding experiment in another strain of *A. aegypti*. Both tepa and metepa produced significant reductions in oocyst number. The sample examined from the 10 mg./ft.^{2} tepa level had no oocysts on the midgut. At the 5 mg./ft.^{2} level, metepa-treated females showed no reduction in oocyst size but did at the 10 mg./ft.^{2} level as previously observed. Transmission experiments to

TABLE 4.—Effect of tepa, metepa, apholate and hempa on development of *P. gallinaceum* (8-B) in *Aedes aegypti* (Bangkok). Three-hour mosquito exposure on treated glass liners in WHO kit.

Treatment	Malarial oocysts ♀♀ dissected	Malarial oocysts Mean no. ± S.E.	Malarial oocyst size No. measured	Malarial oocyst size Mean diameter ± S.E.	♀♀ mortality	Egg hatch
Control	25	65.2±22.1	48	35.0±1.0 μ	0%	95.0%
5 mg.tepa/ft.^{2}	24	12.8± 7.4	48	24.9±0.6 μ	4.0%	0%
10 mg. metepa/ft.^{2}	21	0.3± 0.1	6	24.4±0.8 μ	12.5%	0%
10 mg. apholate/ft.^{2}	25	51.6±19.8	48	36.3±0.8 μ	3.8%	..
20 mg. apholate/ft.^{2}	21	39.2±18.0	46	35.6±0.6 μ	0%	66.7%
50 mg. hempa/ft.^{2}	25	51.5±20.4	48	37.7±0.8 μ	0%	26.2%

TABLE 5.—Effect of tepa and metepa on development of *P. gallinaceum* (8-B) in *Aedes aegypti* (Dietrich). Three-hour mosquito exposure on treated glass liners in WHO kit.

Treatment	Malarial oocysts		Malarial oocyst size		No. chicks infected/ No. chicks bitten
	♀♀ dissected	Mean no. ± S.E.	No. measured	Mean diameter ± S.E.	
Control	10	14.2±7.5	47	41.9±1.0 μ	4/10
5 mg. tepa/ft.^{2}	10	1.6±1.1	16	17.8±0.8 μ	0/6
10 mg. tepa/ft.^{2}	10	0	..	..	..
5 mg. metepa/ft.^{2}	8	3.8±1.2	28	41.3±1.3 μ	6/12
10 mg. metepa/ft.^{2}	9	1.6±1.6	12	23.7±1.6 μ	1/12

young chicks (75–100 gm.) were conducted with single control and treated mosquitoes. Infection rates were similar for control and 5 mg. metepa/ft.^{2} groups; very low for the 10 mg. metepa/ft.^{2} level, and apparently no transmission occurred by females exposed to 5 mg./ft.^{2} tepa. Transmissions were not attempted with the 10 mg./ft.^{2} tepa-treated females since no oocysts were observed. The prepatent period of the transmissions by the control group averaged 8.5 days while that of the 5 mg./ft.^{2} metepa was 10.1 days. This difference was apparently related to relative sporozoite dosage rather than an effect on the parasite by the chemosterilant. Examination of erythrocytic parasites from these transmission experiments revealed no differences in morphology as a consequence of chemosterilant treatment.

SUMMARY AND CONCLUSIONS. The adoption of glass liners for the World Health Organization adult mosquito insecticide test kit appears to be the most satisfactory method of evaluating the residual activity of chemosterilants against malaria-infected mosquitoes. This procedure is more efficient than the use of treated jars and overcomes the problem of absorption that is encountered when treated filter paper is used in the WHO test kit.

The discrepancies in response of *Aedes aegypti* and *Plasmodium gallinaceum* to varying dosage levels of tepa compounds as reported by Altman (1963) and Bertram, *et al.*, (1964) appear directly related to the surface to which the chemosterilant is applied rather than to differences in mosquito or parasite strains. In the present experiments no reduction in malarial oocyst development occurred with tepa and metepa applied at levels up to 40 mg./square foot on bond paper. On the other hand, marked activity was shown by application of 5 mg./square foot of these materials on glass liners.

Tepa and metepa proved to be the only tested materials which showed promise as residual chemosterilants in these laboratory tests. Apholate exhibited some activity against *P. cynomolgi* but none against *P. gallinaceum*. Since there was considerable mortality in *Anopheles stephensi* exposed to apholate at low levels, it probably does not hold much potential for future evaluation in this test system. Although hempadid reduce the egg hatch considerably, it had no effect on malarial parasites below the near-lethal level for the vector.

Although virtually complete suppression of the sporogonous cycle of the malarial parasite and full steriliity of the female vector can be achieved in a laboratory model, the applicability of this approach to the control of human malaria requires careful consideration. The problems of toxicity in handling these compounds are evident, and the genetic hazards of the alkylating agents to man do not warrant their dissemination in the form of a residual spray. There is a strong possibility that these agents may have a mutagenic effect on the malarial parasite which could alter its response to a given host system or its activity to antimalarial compounds. Future investigations should take these details into consideration.

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OVIPOSITION STUDIES WITH *AEDES VEXANS* IN THE LABORATORY

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INTRODUCTION. Females of *Aedes vexans* (Meigen) were engorged in the field to obtain fertile eggs for colonization attempts. This paper presents data on oviposition by these insects and results of laboratory experiments using several substrates to stimulate oviposition.

Mosquitoes were obtained while feeding on the collector in the field during the summers of 1961 and 1962 in Dane County, Wisconsin. Females were covered with vials only after blood became visible through the body wall. Feeding was rarely interrupted using this procedure. Mosquitoes were given no food other than this blood.

Oviposition vials were made by cutting pyrex glass tubing, 28 mm (OD), into 75 mm lengths. Cotton gauze was bound over the mouth of the vial with a rubber band. The base of the vial was filled with a plaster of paris layer approximately 6 mm thick. This plaster bottom was covered with an absorbent cotton disc as an oviposition substrate. Vials were placed in petri plates containing water. This procedure maintained the cotton disc at saturation and obviated the frequent addition of water.

DURATION OF THE PREOVIPOSITION PERIOD. Data are based on 107 females collected in each of the years 1961 and 1962. Oviposition occurred 2–8 days after engorgement in 1961 and 3–12 days afterward in 1962 (Table 1). Most of the ovi-

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