UPTAKE AND METABOLISM OF APHOLATE IN LARVAE OF APHOLATE-RESISTANT AND SUSCEPTIBLE STRAINS OF *AEDES AEGYPTI* (L.)[1]

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ABSTRACT. Uptake and metabolism of apholate were observed in 4th-stage larvae of apholate-resistant (R) and susceptible (S) strains of *Aedes aegypti* (L.). Uptake of apholate was significantly greater in the S strain, but the metabolism of apholate was equal in the 2 strains. Although uptake was implicated as partially contributing to the resistance mechanism, it did not completely explain it.

The selection of an apholate-resistant strain of *Aedes aegypti* (L.) was described by Hazard et al. (1964) and later in a separate report by Patterson et al. (1967). This strain was selected with apholate by treating early 4th-stage larvae until pupation. We have maintained selection pressure of 25 ppm of apholate in rearing water on this strain through the F_{83} generation with no further increase in resistance. The present paper reports a study of the nature of the resistance mechanism. We used gas chromatography to determine the uptake of apholate by 4th-stage larvae from aqueous solutions and the persistence of apholate after the termination of treatment. Our intention was to determine whether the resistance is associated with reduced uptake or increased metabolic capacity and, if so, whether these factors could explain the 10X and 20X levels of resistance observed in females and males, respectively, by Seawright (1972).

METHODS AND MATERIALS. *Aedes aegypti* were reared by setting ca. 2000 larvae in plastic trays containing 4 liters of water that was gently aerated with an aquarium pump and maintained at $30 \pm .5°$ C. Larvae were fed a mixture of 2 parts liver powder and 1 part brewer's yeast at a rate such that a surplus of food was continuously present. All larvae used for testing were set and harvested on the same time schedule in an effort to achieve uniform size and weight.

Uptake of apholate by the resistant (R) and susceptible (S) strains was studied by treating groups of 4th-stage larvae for 4 hours with apholate solutions of 16, 32, and 48 ppm, and for 1, 2, and 4 hours with 64 ppm. Persistence of apholate was ob-

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[1] Mention of a pesticide or a proprietary product in this paper does not constitute a recommendation or an endorsement of this product by the USDA.

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served at 0, 1, 2, 4, and 8 hours in larvae treated for 4 hours in a solution of 64 ppm of apholate. Treatments were replicated 3 to 6 times, with 250 4th-stage larvae per treatment, in a water bath maintained at 30° C. After treatment, larvae were washed on a screen with distilled water, and in the uptake tests, they were held in distilled water for 5 minutes to wash unabsorbed apholate from the cuticle. Our preliminary work showed that the washing and soaking steps were necessary to achieve repeatability for the gas chromatographic (GC) analysis.

Larvae in each sample were killed in 5 ml of chloroform, and the samples were stored in a freezer for further processing. Each sample was extracted 4 times by grinding the pupae with 5 ml portions of chloroform in the presence of 5 g of sodium sulfate. The combined extracts from a sample were poured onto a silica gel (J. T. Baker 3405) column, that retained the apholate, but cleaned the sample of chloroform-soluble components that interfered with the GC analysis. The apholate was then eluted with methanol, and this solution was concentrated to an appropriate volume with vacuum and a 50° C water bath.

A Hewlett-Packard Model 5750 instrument equipped with a Malpar flame photometric detector was operated in the phosphorous mode. The 50-cm glass column (44 mm ID) contained 5% OV–101 (w/w) on 80–100 mesh Gas Chrom Q preconditioned overnight at 250° C and was operated isothermally at 210° C. Temperatures of the injection port, the detector, and the line connecting the oven to the detector were 220°, 230°, and 220° C, respectively. Flow rates of the gases in ml/minute were: nitrogen (carrier) 160, hydrogen 200, and oxygen 40.

At the stated conditions, 5 μliters of concentrated extract were injected for analysis. The retention time for apholate was 1.25 minutes. The requirements for conditioning the column for apholate analysis were the same as those reported previously by Bowman and Beroza (1966). Once the column was conditioned, the minimum detectable amount of apholate was 0.05 ng/5 μliters injected. Another peak present in all samples had a retention time of 0.80 minutes; however, it was resolved from the apholate peak and did not interfere. Quantification was based on peak height. Recoveries from insect tissue spiked with apholate were 95% or better.

RESULTS. Differences were observed in uptake of apholate in the 4-hour treatments at all concentrations (Tables 1 and 2). However, there was no difference in uptake between the 2 strains when the larvae were treated for only 1 or 2 hours in 64 ppm. Regression analyses (AOV) showed that the relationship between uptake and duration of treatment or concentration was linear (Table 3). However, the fit ($r^2=0.52$) of the R strain data for the relationship of uptake over time was insufficient to define uptake as directly proportional to duration of treatment. The small accumulation of apholate between 2 and 4 hours in the R larvae suggested the presence of an uptake limit. The GC analysis was specific for apholate, and no information was obtained concerning metabolites; thus, any reference to metabolism in this discussion relates solely to the

Table 1. Uptake and persistence of apholate in 4th-stage larvae of *A. aegypti* treated in an aqueous solution of 64 ppm apholate.

Duration of exposure or postexposure period (hr)	Average residue (ng/mg) $\pm S_{\bar{x}}$[a]	
	Resistant strain	Susceptible strain
	Exposure	
1	2.67±0.23	2.58±0.42
2	4.31± .30	4.45± .37
4	4.85± .36	7.65± .25
	Post-exposure[b]	
1	3.62±0.21	6.74±0.48
2	2.58± .15	5.87± .38
4	1.50± .22	4.43± .45
8	.55± .08	2.07± .22

[a] Mean of 6 replications; mg given as wet weight.
[b] After treatment in 64 ppm apholate for 4 hr.

Table 2. Uptake of apholate in 4th-stage larvae of *A. aegypti* exposed to various concentrations of aqueous solutions of apholate for 4 hr.

Concentration (ppm)	% Sterility[a] in R males	Average residue (ng/mg) $\pm S_{\bar{x}}$[b] Resistant strain	Susceptible strain
16	2	0.58±0.02	1.03±0.01
32	20	1.39± .07	2.38± .29
48	50	2.11± .13	4.79± .12
64	70	3.43± .34	5.75± .79

[a] Data taken from Seawright (1972); sterility in S males 100 percent at all concentrations.
[b] Average of 3 replications; mg given as wet weight.

Table 3. Linear regression for uptake and metabolism of apholate in *A. aegypti* larvae.

Measurement	Slope	Correlation (r)
Resistant		
Uptake (time)	0.64	0.72
Uptake (concentration)	.05	.96
Metabolism	—.49	—.88
Susceptible		
Uptake (time)	1.68	0.93
Uptake (concentration)	.10	0.94
Metabolism	—.69	—.91

persistence of apholate in larvae. A regression analysis of the persistence data revealed a linear relationship between apholate residues and time after exposure with negative slopes of 0.69 for S and 0.49 for R. Further analysis showed no significant difference between these slopes; thus, there was no apparent difference in metabolic capacity for apholate between the 2 strains.

DISCUSSION. Resistance to the sterilizing effects of apholate can be attributable to several possible mechanisms including reduced uptake, increased degradation, differential distribution of the alkylating agent in the larval tissues, or presence of protective molecules. However, before evaluation of the available data pertaining to the resistant strain, a few brief comments on apholate and alkylation reactions are appropriate. Apholate, like other aziridinyl compounds, is an alkylating agent and is extremely active in biological systems, causing sterility by alkylation of organic and inorganic anions, amino groups, and sulfide groups. Thereby, it disrupts and inhibits cellular processes. Most importantly, an aziridine causes changes in DNA which result in dominant lethality in gametes produced by a chemosterilized insect. Probably the alkylation of nucleophiles occurs at random in the body of an insect, thus producing damage throughout. Although it must be recognized that tissues would be sensitive to a varying extent, related to their developmental maturity, severe genetic damage could prevent the proper function of cells in proliferating tissue. Our experience (unpublished data) has been that apholate induces chromosome damage in brain tissue as well as gonadal tissue of larvae of *A. aegypti.* Therefore, apholate usually causes genetic damage throughout a mosquito larva, and this suggests a resistance mechanism(s) would then either protect the entire larva or would simply block the effect of apholate in the gonadal tissue, which is the critical site of action.

Reduced uptake will be considered first as a possible resistance mechanism. In this context exclusion of apholate from the body represents the most direct method of resistance. In the present study, S larvae accumulated 1.7 times more apholate than R larvae; however, this value does not correspond to the levels of resistance (10-fold in R females and 20-fold in R males) and the linear relationship between log-concentration of apholate and probit-sterility reported previously (Seawright, 1972). In addition, males of the S strain were completely sterilized by treatment in 16 ppm apholate, but this treatment had no effect on the fertility of R males. The lack of a close correspondence between the uptake levels (1.7) and the resistance levels suggested that differential uptake was not responsible for resistance, but we were not sure that a close correspondence was necessary. To clarify this point, we

calculated a correlation coefficient for sterility and uptake observed at the various concentrations in the R strain. A significant correlation coefficient ($r=0.96$) was obtained that indicated a close association between uptake and sterility. It must be recognized that this correlation is based on exposures of ¼ (or less) of the normal treatment time used for sterilization, which was usually a minimum of 24 hours. Patterson et al. (1967) showed a 7-fold level of resistance in the R strain when adults of the R and S strains were fed *ad libitum* on sugar water solutions of apholate. This demonstration of a resistance mechanism in the R adults precludes a small difference in apholate uptake as the principal mechanisms of resistance.

A second type of resistance mechanism involves the metabolism of apholate. This is a tenuous subject because in the process of apholate break-down it is most probable that a nucleophile is alkylated, so that even though the apholate molecule per se would be eliminated, it would be at the expense of the organism. Since the rate of metabolism of apholate after the exposure period was the same in R and S larvae, the presence of different modes of detoxication, i.e., enzymatic pathways, are not likely. If different specific pathways were involved, the rate of metabolism would probably be faster in the R strain. Otherwise, the apholate would alkylate critical cellular constituents and cause a measurable sterility. However, as mentioned previously, a concentration that completely sterilized S larvae had no effect on R larvae.

Turner (1968) calculated ratios of LD_{50}/ED_{50} for the R and S larvae and obtained values of 1.6 and 7.7, respectively. He interpreted these ratios to mean a higher degree of selectivity of apholate for gonadal tissue in the S larvae. However, these ratios could indicate a protective effect of the genetic material in the whole body of the R larvae and not necessarily just the gonads. Turner also found the content and intracellular distribution of nucleic acids and proteins were the same in both strains. Presumably these measurements were made with untreated larvae, since there was no mention of apholate treatment. Apholate therefore does not completely disrupt the cellular processes, and the changes that lead to sterility are most likely subtle ones.

The production of a surplus of nonessential (or even essential) metabolites could provide a protective mechanism to shield nucleic acids from alkylation. This type of mechanism could work by simple chemical masking. Ferrer et al. (1968) found that injection of compounds with "active" SH groups reduced the sterilizing effects of apholate in house flies, *Musca domestica* L. Also, the presence of abnormal quantities of sulfhydryl-containing compounds was observed in radioresistant, tissue-culture HeLa cells by Revesz and Bergstrand (1963) and Morita (1973). In addition, Revesz and Modig (1965) found that a cysteamine-induced increase in glutathione levels occurred in a radioresistant HeLa tissue line and hypothesized this as the resistance mechanism. Morita (1973) also showed a noticeable tolerance to nitrogen mustard in a radioresistant cell line, though no clear parallelism in the degree of resistance to the two agents was observed. Protection from radiation in the cell lines and from apholate in the house fly (Ferrer et al., 1968) presumably results from the scavenger role of the increased cellular sulfhydryl content.

No similar data are available to document abnormal quantities of normally occurring metabolites in the R strain, but Turner and Maheswary (1969) recorded data on the effect of apholate on ovarian protein content.

With ovaries from females treated with apholate as larvae, they found a decrease in specific activity of alkaline phosphatases in both strains, and a decrease in protein content in the S strain, but no protein reduction in the R strain. Indeed, the protein content of treated R females was actually higher than that of untreated R females. The significance of the apparent apholate-induced increase in protein con-

tent is unknown. More alkaline phosphatase activity was noted in untreated R females than in the untreated S females, but again there is no direct way to relate this observation to resistance. When Turner and Maheswary (1969) treated females of both strains with apholate, the decrease in alkaline phosphatase activity was more pronounced in the R strain. The fact that the enzyme activity was reduced indicates that apholate inhibited or interfered with the enzyme-catalyzed reaction or with enzyme synthesis. The former is the more probable, because interference with synthesis implies a direct effect on the nucleic acids, which would most likely cause permanent damage resulting in sterility. This could not have happened, because protein production increased, which would be unlikely if genetic damage was extensive. It is recognized that part of the protein produced in the presence of apholate could very well be dysfunctional because of alkylation. It is also clear that the nucleic acids (the genetic material) were not alkylated, even though the depression of alkaline phosphatase activity indicates the presence of apholate in the ovaries. Chromosome squashes we have observed of brain tissue of R larvae treated with apholate showed no chromosome damage typical of the type reported by Rai (1964), indicating the protective effect is also effective in shielding the genetic material of somatic cells as well.

Presumably all alkylating agents induce sterility in the same manner, but structural relationships will influence the activity of a compound so that some induce sterility at lower concentrations. The overall effect of alkylation, though, will be expressed as sterility. Therefore, there was reason to expect that an apholate-resistant strain would also be tolerant of other aziridines. This question was investigated by Patterson et al. (1967) when they treated the apholate-resistant strain with tepa and metepa. They observed a 4-fold tolerance to metepa but saw no evidence of cross-resistance to tepa. The resistance was therefore not specific for apholate, although it was not clear whether the same mechanism(s) was responsible. Klassen and Matsumura (1966) also reported a 2- to 3-fold tolerance to metepa in a laboratory metepa-selected strain of *A. aegypti*, determined that metepa breakdown *in vitro* was 3 times greater in the resistant strain, and attributed the metepa tolerance to this greater metabolic breakdown. However, their *in vitro* tests were conducted with 50 μg of metepa per mg larval homogenate, and much smaller quantities, ca. 110 ng/mg (Seawright et al. 1973) of aziridine were found in mosquitoes treated in a sterilizing concentration. The high substrate level casts some doubt on the validity of the significant difference in metabolic capacity because of the low aziridine level that is actually necessary to sterilize a mosquito.

Finally, we also considered the possibility that a repair mechanism(s) might confer resistance. However, it seems unlikely that repair would be efficient enough to correspond to the levels of resistance, partly because, when the R strain was compared to the S strain for radiosensitivity, the same sterility was induced in both strains (B. J. Smittle, personal communication). The chromosomal aberrations induced by a chemosterilant and by radiation are similar; hence, if repair were operative, there should have been some strain difference in radiosensitivity.

By extrapolation (with due regard for the pitfalls) of the uptake regression, we obtained an estimate of about 100 ng of apholate required to sterilize 1 S larva. This estimate compares favorably with data on the amounts of other aziridinyl compounds required to sterilize mosquitoes (Seawright et al. 1973) and house flies (Chang and Borkovec 1964). Since the average larva in our tests weighed 3 mg, this estimate of 100 ng/larva is about 0.003 percent of the body weight. Therefore, any attempt to determine the nature of the resistance mechanisms(s) would be exceedingly difficult because of the small quantity of apholate necessary to induce sterility and the nonspecificity of the alky-

lation reactions. In addition, apholate resistance was shown to be a quantitative trait (Seawright 1972), indicating that several of the factors discussed herein may be involved.

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