A LARVICIDAL PRODUCT OF SUCROSE FEEDING BY ADULT MOSQUITOES

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ABSTRACT. During the course of regular laboratory maintenance of colonies of *Aedes aegypti* and *Culex quinquefasciatus,* it was discovered that sugar cotton removed from cages of adults was lethal to all instars of larvae of both species as were aqueous extracts of the cotton. This same finding applied to field-collected material and was unrelated to blood feedings. The activity of the toxic compound was of such magnitude that 2 female mosquitoes interacting with the sucrose cotton produced a larvicide which killed 100% of the specimens. The major peak obtained from high pressure liquid chromatography (HPLC) of the toxic extract was isolated, bioassayed, and its structure partially characterized from its ultraviolet, infrared and mass spectra. It is hypothesized that the adult female mosquitoes included in this study deposit a chemical while feeding on sucrose-soaked cotton, which interacts with it to form a potent larvicidal compound which is not species specific.

Our prior investigations designed to develop new vector control agents have centered around rather standard approaches, i.e., the modification of known molecular structures to maximize biological activity against mosquitoes (Micks, et al. 1967, 1972; Micks and Chambers 1974). This work resulted in the development of a new, commercially available control agent (Micks, et al. 1968, 1969). However, in our search for chemical compounds with insecticidal activity, there was never any indication that products or by-products of adult mosquitoes might be lethal to mosquito larvae, a phenomenon which we first observed approximately two years ago.

The purpose of the present study was to elucidate the relationship of this toxic factor to various mosquito species in terms of its larvicidal activity, and to determine its molecular structure.

MATERIALS AND METHODS

Colonies of *Aedes aegypti* (L.) and *Culex quinquefasciatus* Say have been maintained continuously in our laboratory for more than two decades under temperature and humidity-controlled conditions (approximately 80°F (27°C) and 80% RH). The adults, which are kept in cages measuring about 2 sq ft (0.6 sq. m), have continuous access to 5% sucrose (prepared with deionized water) and are provided weekly blood feedings. The sugar-soaked cotton rolls, which are 1.5 in (3.8 cm) long and ⅜ in (1 cm) diam. (dental cotton), are changed three times weekly. Since the larvae are reared through from the eggs in pans of water kept separate from the cages, there is no opportunity for the larvae to contact the sucrose. However, on one occasion when caged *Ae. aegypti* females deposited eggs on the sugar cotton, in addition to using the wet paper towelling which is the regular oviposition site, the cotton was flooded with water in a separate pan in order to obtain the maximum number of larvae. The resulting hatch was approximately 100% and the 1st instar larvae normal, however, they were all dead the following day. Since we had never had occasion to observe this phenomenon before, we conducted several preliminary experiments which demonstrated that this finding was reproducible.

The majority of tests were done with eggs, larvae, pupae and adults obtained from the two laboratory colonies. However, the adults of field-collected *Aedes sollicitans* (Walker), *Ae. triseriatus* (Say) and *Cx. quinquefasciatus* were also used in some experiments. The larvae were reared in metal pans approximately 25 × 30 cm containing about 400 ml of water (depth - 1 cm), and fed with laboratory chow. The immature stages of both *Ae. aegypti* and *Cx. quinquefasciatus,* which always served as the test subjects, were exposed to the cotton removed from the colony cages containing approximately 1,500–2,000 adult female and male mosquitoes. The initial series of tests conducted in triplicate used 8 cotton rolls and 150 specimens per pan (400 ml water) for each of the larval instars and pupae. Each test included a control which was identical in every respect except that the sucrose cotton had not been exposed to adult mosquitoes. All subsequent experiments were done with depression plates with 12 wells per plate. The maximum capacity of each well was approximately 1 ml. Later experiments using known numbers of adults were initiated in micro-cages consisting of 10 oz plastic tumblers in which was placed a 100 ml beaker containing a specific number of pupae. The plastic glass was covered with netting held in place with a rubber band. A small hole (0.5 in (1.3 cm) diam) was cut in the netting and closed by a cork stopper to facilitate access to the cot-

ton roll wedged between the top of the beaker and the plastic container. Because of the need to better quantitate the test material, a single roll of sucrose cotton was made available to specific numbers of adults ranging from a single specimen to as many as 100, for a specified number of days. At the end of the exposure period, approximately 1 ml of deionized water was added to each cotton roll and the contents squeezed out and equally divided between three wells in a depression plate. Five to seven 4th instar larvae of either *Ae. aegypti* or *Cx. quinquefasciatus* were introduced into each well and the mortality always compared with sucrose controls in triplicate at the end of 24 hrs. Each of these sets of tests was repeated two or more times in separate series using corresponding sucrose cotton controls maintained with the same exposure except for the absence of mosquitoes.

Larval toxicity studies with the isolated toxic fraction from both *Ae. aegypti* and *Cx. quinquefasciatus* cage cotton also used depression plates. The residue was dissolved in sufficient water to make a series of concentrations ranging from 10 mg/ml to 10 μg/ml. Tests were done in triplicate and repeated several times, using 4–5 larvae/well except for the 1st instars of which 10–15 specimens were employed, using 0.5 ml quantities of the toxic fraction per well. Some tests used mixtures with sucrose. The aqueous fraction obtained with ethyl acetate extraction had already been tested and found to be non-toxic to mosquito larvae. Mortality was recorded after 24 hrs.

ISOLATION OF THE LARVICIDAL PRODUCT. Sucrose cotton was removed from the *Cx. quinquefasciatus* and *Ae. aegypti* colony cages every 2–3 days and stored separately in closed containers in the refrigerator until a sufficient quantity accumulated for extraction. It was first soaked in water and filtered (approximately 200 cotton rolls/200 ml water). The toxic material was isolated from this aqueous solution by extracting 3 times with 100 ml of ethyl acetate. The combined ethyl acetate layers were evaporated to dryness under vacuum. The residue was suspended in methanol (5 ml) and filtered with a 1.2 μM thickness Gelman Acrodisc filter. A control extract was also prepared by extracting with ethyl acetate approximately the same amount of cotton soaked in 5% sucrose.

HPLC analyses of the extracts were carried out by injecting 20 μl of the extract and eluting with methanol-water (70:30 and 50:50) solvent mixtures. Methanol solutions of uric acid and sucrose were also run to obtain retention times. Uric acid was included since it is known to be the main constituent of mosquito excreta (Irreverre and Terzian 1959). The HPLC profiles from this isolated toxic material showed one major peak in addition to several minor peaks and the retention time of the major peak did not match with either uric acid or sucrose. The toxic material represented by this major peak was then isolated by repeated injection of the material onto the column and the collected fractions were pooled and evaporated to dryness under vacuum and a portion of the residue dissolved in ethyl acetate for further spectral analyses.

CHEMICAL SPECTRA OF THE LARVICIDAL COMPOUND. The ultraviolet spectrum of the larvicidal compound dissolved in methanol was obtained using a Perkin-Elmer Lamda-3 double-beam spectrophotometer. The infrared (IR) spectrum was obtained in Nujol (mixture of long-chain aliphatic hydrocarbons, a commonly used IR solvent) using a Perkin-Elmer Model 710-B spectrophotometer.

The electron impact and fast atom bombardment (FAB/MS/MS) mass spectra were obtained using Finnigan-4000 EI/CI and Kratos MS50-TA (Midwest Center for Mass Spectrometry, University of Nebraska, Lincoln) mass spectrometers, respectively.

RESULTS AND DISCUSSION

Exposure of each of the instars of *Cx. quinquefasciatus* larvae to cotton from the *Cx. quinquefasciatus* colony cage resulted in virtually complete mortality except for the 4th instar larvae, which were slightly less susceptible (Table 1). The 1st instars were dead within 12 hrs and the 4th instar larvae generally died within 24 hrs. When such larvae did succeed in pupating, some of the pupae died. When egg rafts of *Cx. quinquefasciatus* were exposed to sucrose cotton, the larvae died before the hatching process was completed. Pupae exposed to the cage cotton were unaffected. Essentially the same results were obtained when *Ae. aegypti* stages were exposed to *Ae. aegypti* cage cotton.

Reciprocal experiments were conducted with *Ae. aegypti* larvae exposed to *Cx. quinquefasciatus* cage cotton and vice versa. In both instances,

Table 1. Effects of sucrose cotton from the *Culex quinquefasciatus* colony cage on immature stages of *Cx. quinquefasciatus*.

Stage	% mortality (24 hrs)
Eggs	100
1st instar larvae	100
2nd instar larvae	100
3rd instar larvae	99
4th instar larvae	87
Pupae	0

mortality rates were essentially 100%. In some experiments in which larvae survived, their development was retarded, seldom advancing to the next instar. Occasionally, there was only partial shedding of the larval skins. Sugar cotton from blood-fed and non blood-fed cages of *Cx. quinquefasciatus* produced similar kills in exposed larvae, indicating that the toxic component was not associated with the blood meal. Sucrose cotton from cages of adult *Cx. quinquefasciatus, Ae. sollicitans* and *Ae. triseriatus* reared through from field collections of eggs or larvae was as lethal to larvae from both laboratory colonies as was the cotton from the colony cages.

Experiments conducted with filtered aqueous extracts of the cage cotton using 180 cotton rolls per liter of water, resulted in the death of all larval instars of both species in less than 24 hrs. The 1st instar larvae survived only 4–8 hrs.

Since the experiments with sucrose cotton obtained from the micro-cages of 100, 75, 50, 25, 15 and 10 adults invariably produced 100% mortality in the 4th instar larvae of both species, we reduced the number of specimens to which the cotton was exposed. Much to our surprise, a single cotton roll exposed to as few as 2 adult females resulted in 90–100% mortality in the test larvae (Table 2). The toxic factor reached its maximum activity following 2 days of exposure and further access of the mosquitoes to the sugar cotton did not accelerate mortality. Even one adult female interacted sufficiently with the cotton to produce some larval mortality. The capability to develop the larvicidal component seems to be confined to the females since batches of male pupae as large as 150 specimens allowed to hatch and contact sucrose cotton for as long as 5 days resulted in cotton which was non-toxic to larvae. These results suggest that the larvicidal compound formed is a highly active and potent one.

PARTIAL CHEMICAL CHARACTERIZATION. The HPLC retention times for the isolated toxic compound along with those of uric acid and sucrose are presented in Table 3. It can be seen that the retention times for these three compounds in both solvent systems are quite different, indicating that the toxic compound is neither sucrose nor uric acid. The control, which consisted of an ethyl acetate extract of cotton soaked in sucrose, did not show the toxic compound peak.

Table 2. Toxicity to *Culex quinquefasciatus* larvae (4th instar) of sucrose cotton exposed to two *Cx. quinquefasciatus* females for periods of 1 to 8 days.

No. days exposure	1	2	3	8	Control[1]
Mortality[2]	14/14	16/16	13/13	15/15	1/15
	17/17	19/19	15/16	15/15	0/17

[1] Sucrose cotton.
[2] Dead larvae/total.

Table 3. HPLC[1] retention times (minutes) for the isolated toxic compound, uric acid and sucrose.

	Methanol-water solvent (%v/v)	
Compound	70–30	50–50
Toxic compound	3.3	4.4
Uric acid	4.4	6.9
Sucrose	2.8	3.4

[1] Column: Altex ods reversed phase; flow rate: 1 ml/min; detector: uv 254 nm; sensitivity: 0.05 aufs.

The HPLC-isolated larvicidal compound was partially characterized by ultraviolet (UV) and infrared (IR) spectrophotometry and mass spectrometry. The UV spectrum shows an absorption maximum (λ_{max}) at 280nm (ϵ = 846). The IR spectrum has bands at (cm^{-1}): 3300–2500 (broad -OH region: absence of $-NH_2$); 1740, 1720, 1700 (three $-C=O$ groups); absence of aromatic peak around 800–600. This observation coupled with the low ultraviolet ϵ value indicate that the molecule is non-aromatic and has carbonyl ($-C=O$) group(s).

The direct probe electron impact mass spectrum (with Finnigan 4000 EI/CI Mass Spectrometer) shows mass (m/e) fragments at: 547 (MH^+), 435, 323, 305, 211, and 193; lower mass region indicates the molecule is non-aromatic. The difference of 112 mass units (a.m.u.) between 547, 435 and 323 could be due to three octyl groups and the difference of 18 a.m.u. between 435 and 417, and 211 and 193, shows that the compound could be polyhydroxylated. The mass spectrum obtained by the fast atom bombardment technique also confirmed the molecular weight of the compound to be 546.

LARVAL TOXICITY. The larval toxicity of the compound alone (prior to HPLC purification) and in combination with sucrose is shown in Table 4. The presence of sucrose did not materially enhance the mortality. It should be noted that these results represent 24-hr mortality readings whereas all specimens were dead in 48 hr. A series of additional experiments using the HPLC-isolated toxic compound was designed to determine its toxicity to all larval instars. Table 5 shows that the *Cx. quinquefasciatus* 1st instar larvae were the most susceptible and the 4th instars the least affected. High mortality of the 1st three instars occurred at the 50 μg/ml concentration level. Virtually identical results were

Table 4. Susceptibility of larvae to the toxic compound (with and without sucrose) prior to HPLC purification.

	% mortality (24 hrs)					
	Toxic compound			Toxic Compound + sucrose[1]		
Larval instar	0.1[2]	0.2	0.3	0.1	0.2	0.3
AA1[3]	40	85	100	75	99	100
AA4	100	100	100	100	100	100
CQ1	90	100	100	95	100	100
CQ4	15	50	100	33	50	100

[1] 0.2 ml sucrose (5%) added to each well. Total volume in all experiments brought up to 0.5 ml with the addition of water.

[2] Vol/ml of toxic compound.

[3] Letters denote mosquito species and the numbers indicate instar.

Table 5. Susceptibility of *Culex quinquefasciatus* larvae to the HPLC-isolated toxic compound extracted from *Cx. quinquefasciatus* cage cotton.

	% mortality (24 hrs) Toxic compound (μg/ml)				
Instar	20	30	40	50	100
1	0	90	100	100	100
2	0	0	50	95	100
3	0	0	0	87	100
4	0	0	0	50	100

obtained when *Ae. aegypti* larvae were exposed to the toxic compound isolated from *Ae. aegypti* cotton extracts. In all cases, there was no mortality in the controls. The compound is more toxic than the data indicate since water is unavoidably added to each well with the introduction of the larvae.

Earlier studies on prior generations of the same *Cx. quinquefasciatus* and *Ae. aegypti* colonies used for the present work showed that several bacterial species were present in the gut (Ferguson and Micks 1961, Micks et al. 1961), thus posing the possibility that bacteria could be the source of the toxic factor. However, our findings do not support this hypothesis. First of all, water-soaked cotton kept for several days in micro-cages with several hundred adults per cage in repeated tests with *Cx. quinquefasciatus* and *Ae. aegypti* produced no larval mortality when its contents were tested against larvae. Likewise, extracts of cotton to which 1 to 4 separate 1 ml applications of 5% sucrose were made were not toxic to larvae. Thirdly, if a bacterial toxin were elaborated, increasing the time of exposure of adult mosquitoes to the sugar cotton at the 80°F (27°C) test temperature would be expected to increase its amount and potency thus accelerating larval mortality. No such effect occurs. Finally, a small, wet piece of paper towelling (about 3 in (7.6 cm) square) is the regular surface on which hundreds of *Ae. aegypti* routinely lay thousands of eggs once or twice each week. Several of these egg papers are set in water every week after embryonation and drying. The larvae hatched normally and completed their development in spite of abundant bacterial growth. Thus it seems highly likely that the toxic component of the sugar cotton is formed from the interaction of the sucrose with products of the mosquito per se and the cotton. Although the saliva could conceivably be involved, excretory products are excreted in larger amounts and seem more likely to play a significant role. In fact, Irreverre and Terzian (1959) showed that uric acid, ammonia, urea, amino acids and protein were present in the excreta obtained over a 2-week period from females of *Ae. aegypti* and *Cx. pipiens* fed only on 4% sucrose. They found that excretion increased greatly in the 7-day period following the blood meal, with uric acid forming a high proportion of the nitrogenous matter, after which the level was low again (Terzian, et al. 1957). Since our findings cannot be explained on the basis of an effect of uric acid per se, we hypothesize that adult mosquitoes of the species included in this study deposit a chemical while feeding on sugar cotton which interacts with it to form a larvicidal compound. Furthermore, our evidence indicates that this toxic compound is not species specific. Chemical analysis of the compound, which will be presented in detail elsewhere, shows that it is a non-aromatic one with 3 carbonyl groups, not completely water-soluble, and with a molecular weight of 546. Additional studies are underway to complete the identification of its molecular structure.

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