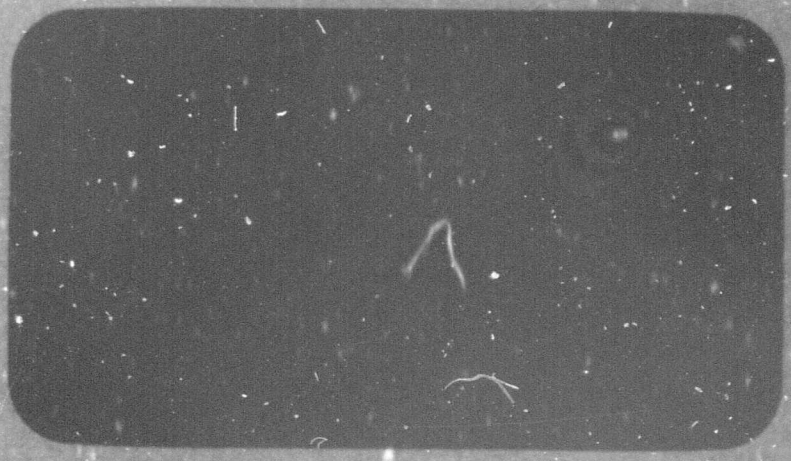
IITRI



Report No. IITRI-L6021-3
(Quarterly Progress Report)

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Headquarters
U.S. Army Medical Research and
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IITRI Project L6021
Contract No. DA-49-193-MD-2281
May 1 to July 30, 1965

I. INTRODUCTION

During this report period further studies were carried out on the electronic recording method for the detection of mosquito biting activity described in Quarterly Progress Report IITRI-L6021-2, and new applications for this apparatus were conceived and tested. New experimental approaches to the development of a systemically effective insect repellent were also conceived, and preliminary tests of the feasibility of these approaches were begun by utilizing the electronic method. A rescreening program of some known mosquito repellents with the electronic apparatus was also initiated.

II. THE ELECTRONIC RECORDING METHOD: FURTHER STUDIES

A. Recording Equipment

During the initial development of the electronic recording method, the question arose as to whether the Sanborn model 320 recorder was the best instrument to use in this application. We had the opportunity to evaluate another recording instrument of higher sensitivity than the model 320, the Sandborn model 7701A

direct-writing oscillographic recorder. This instrument has 1000-fold greater sensitivity than model 320 (0.5 microvolts/mm for model 7701A compared with 0.5 millivolts/mm for model 320).

It was found that at the maximum sensitivity level for model 7701A, the external noise almost completely obliterated the patterns of the mosquito biting activities. Only at sensitivity levels of 200 microvolts/mm (which approach the highest sensitivity of 0.5 millivolts/mm for model 320) could noise be adequately shielded out and clear biting activity patterns obtained. The patterns (Figure 1) are very similiar to those obtained with the model 320, although a paper speed of 50 mm/sec is the maximum speed attainable with model 7701A, compared to 100 mm/sec with model 320.

Figure 1 shows engorgement, salivation, and withdrawal patterns as recorded with the model 7701A recorder. The lack of detail showing valve activity of the mosquito's mouthparts in the patterns recorded with model 7701A can probably be explained by the slower maximum paper speed and slower response time of the writing stylus of this recorder. It is interesting to note in Figure 1 that at the sensitivity of 200 microvolts/mm (0.2 millivolts/mm) although only one mosquito is biting the mouse, the displacement from the base line is 3- to 5-fold greater (30- to 50 mm) than it was when recorded on model 320 (10 mm) at a sensitivity of 0.5 millivolts/mm. This greater displacement is approximately proportional to the sensitivity difference; model 7701A is about 2.5 times more sensitive than model 320 at these settings.

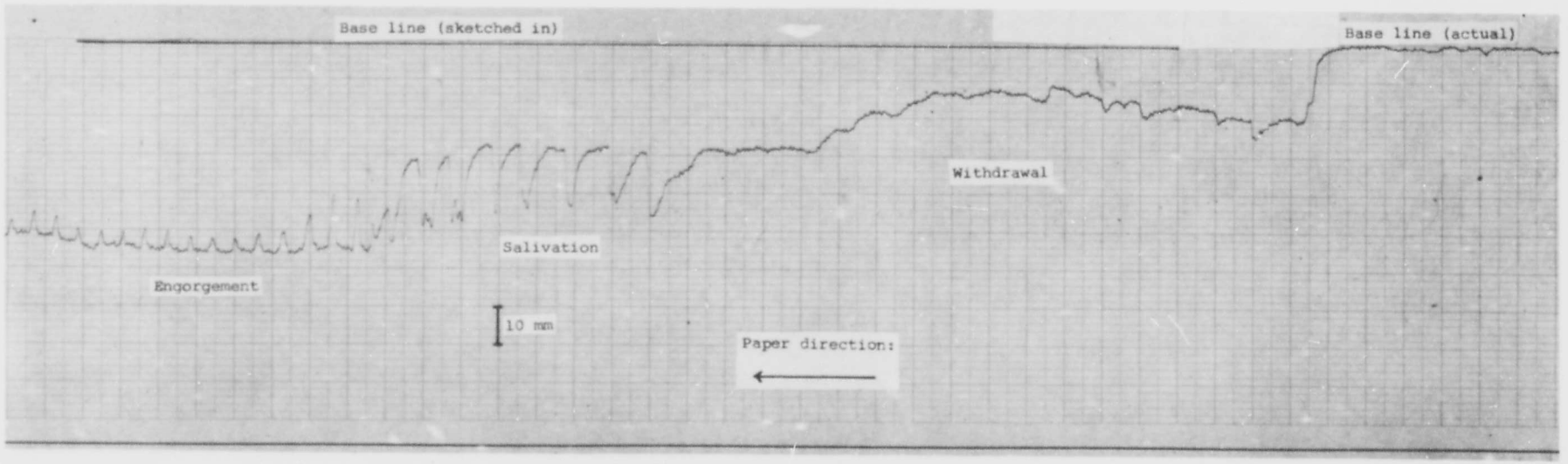

Figure 1. Engorgement, salivation, and withdrawal patterns of single mosquito bite recorded with Sanborn Model 7701A recorder. Paper speed: 50 mm/sec.

An attempt was also made to view these patterns on an oscilloscope. To date we have not succeeded in reproducing the patterns either on a conventional oscilloscope or on a storage oscilloscope, in which the image is retained for about 30 seconds after it has swept across the screen.

Our conclusion at this point is that the Sanborn model 320 recorder is indeed the best instrument to use for our purposes.

Further experiments were also performed with the wire screens. A bronze screen between the mouse and the mosquitoes seemed to give somewhat better results than a copper screen. To test the assumption that a coating of tin on the screens would not only obliterate the differences between the copper and the bronze but also form a continuous conducting layer of metal on the surface of the screen and thus better contacts between the crosshatch wires, a number of copper and bronze screens were electroplated with tin.

Electronic recordings made with these tin-plated screens were decidedly inferior to those made with the uncoated metal screens. The displacement from the base line with a single mosquito biting was only about 3 to 5 mm, indicating greater electrical resistance of the screen, and the bite characteristics could barely be distinguished. Apparently the tin microcrystals deposited by the electroplating process considerably decreased the electrical conductivity of the screens.

The tin-plated screens were then passed quickly back and forth over the flame of a bunsen burner in order to melt the deposited tin and break down the crystal structure. A test of

the screens after this treatment showed that their electrical properties were restored and that differences between the copper and bronze screens were indeed obliterated. The tin-melt-coated screens did not seem to show any particular advantage over the uncoated bronze screens, though the coated screens could conceivably have more uniform electrical properties.

In order to complete the description of the electronic recording apparatus presented in the previous report, Figure 2 shows the wiring diagram of the resistor-battery box used in this work.

B. Feeding Behavior: Pool Feeding

In the previous Quarterly Report, two types of feeding behavior, originally described by Gordon and Lumsden,[1] were interpreted from the chart recordings of the electronic system: capillary feeding and pool feeding. In order to substantiate the interpretation proposed for pool feeding in the chart recordings, a model system was devised that could approximate the conditions under which a mosquito exhibits pool feeding.

The unshaven abdominal skin of a freshly killed mouse was streched over one end of a glass cylinder (2-cm internal diameter), hair side out, and secured with a rubber band. About 10 cc of outdated citrated human blood at 37°C was added to the glass cylinder, with the mouse skin at the bottom of the tube. This arrangement is similiar to the in vitro testing system described

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[1]Gordon, R. M. and Lumsden, W. H. R., Ann. Trop. Med., 33, 259, 1939.

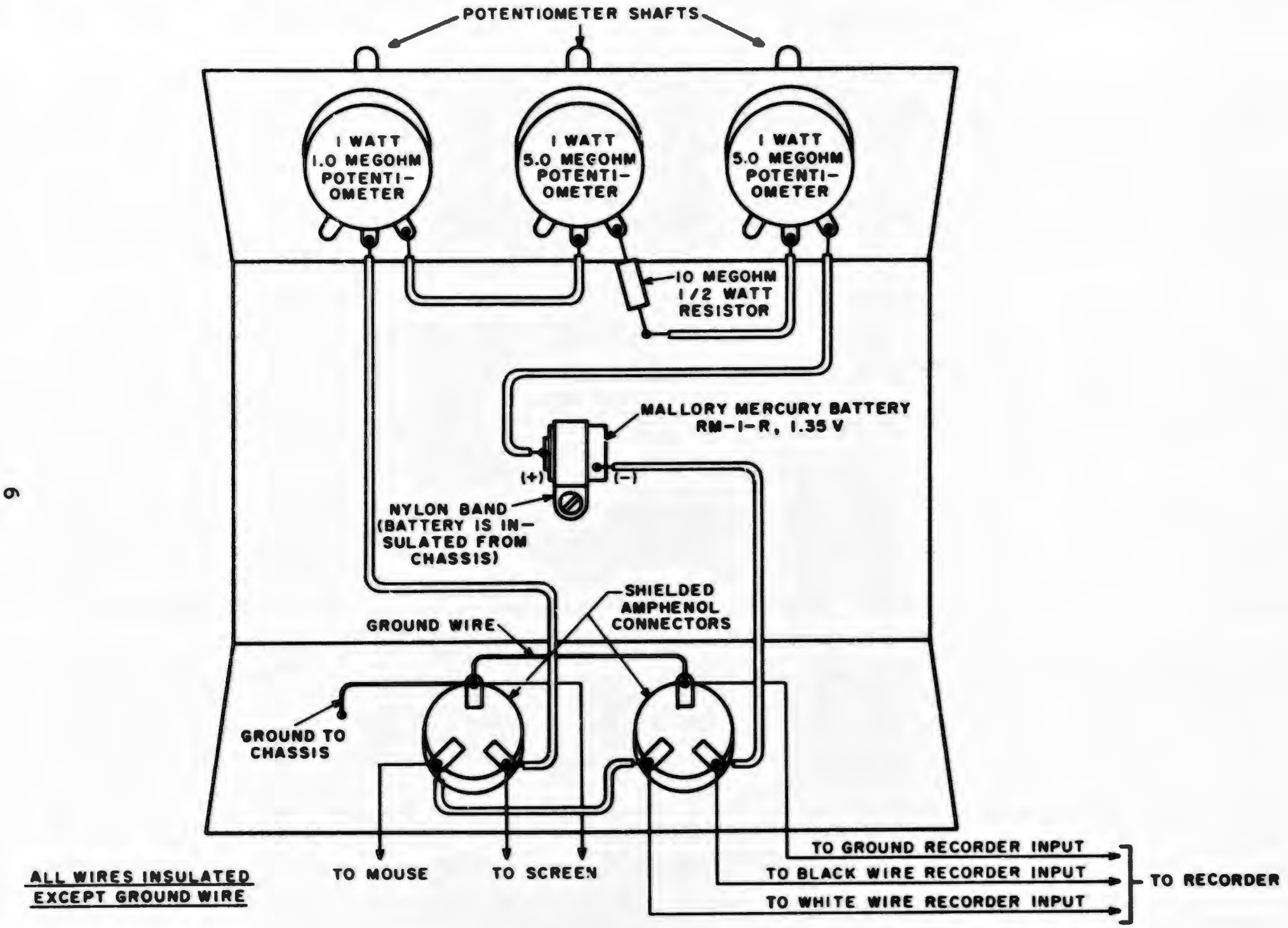

Figure 2. WIRING DIAGRAM OF RESISTOR-BATTERY BOX

in previous reports, except that the unshaven mouse skin was used instead of the Baudruche membrane to cover the bottom of the tube. The skin on the bottom of the tube was placed in contact with a 50-mesh bronze screen covering a glass vessel containing female mosquitoes (Aedes aegypti L.).

The screen was connected to the electronic recording system, and the electrode usually placed in the mouse's tail was allowed to dip into the blood solution. The hair on the skin of the mouse was sufficient to insulate the blood from the screen. No current flowed until the mosquito, holding on to the screen, penetrated the skin with its mouth parts. In a quite exaggerated fashion, the mosquito was thus presented with a pool of blood upon which to feed, and electronic recordings of feeding activity were made. If the interpretations of the pattern representing pool feeding are correct, then a similiar pattern should be recorded.

Figure 3 shows an engorgement pattern made during this experiment. The general shape of the peak is similiar to what was previously seen for engorgement during pool feeding, but the peak amplitude is much greater than that previously seen. This system is an exaggerated model of pool feeding, and the mosquito has responded in an equally exaggerated fashion. The time to complete engorgement was also quite prolonged, lasting more than 5 min. The low frequency of peaking (5/sec), the height of the peaks, and the prolonged engorgement times correspond in every way to the attributes previously interpreted as characterizing pool feeding. We may therefore have a greater degree of confidence

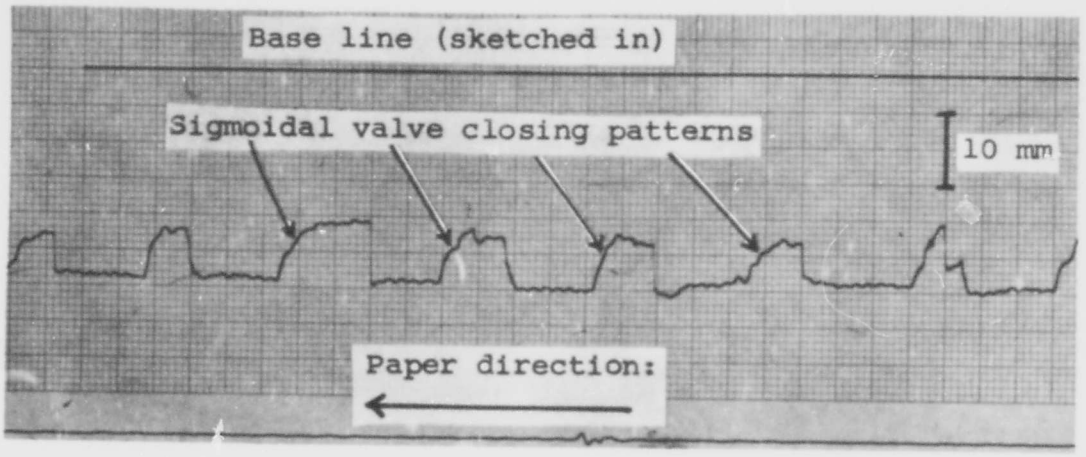

Figure 3. Mosquito engorgement in vitro. Note amplitude of peak and sigmoidal shape of upward sweep in valve closing patterns. Paper speed: 100 mm/sec.

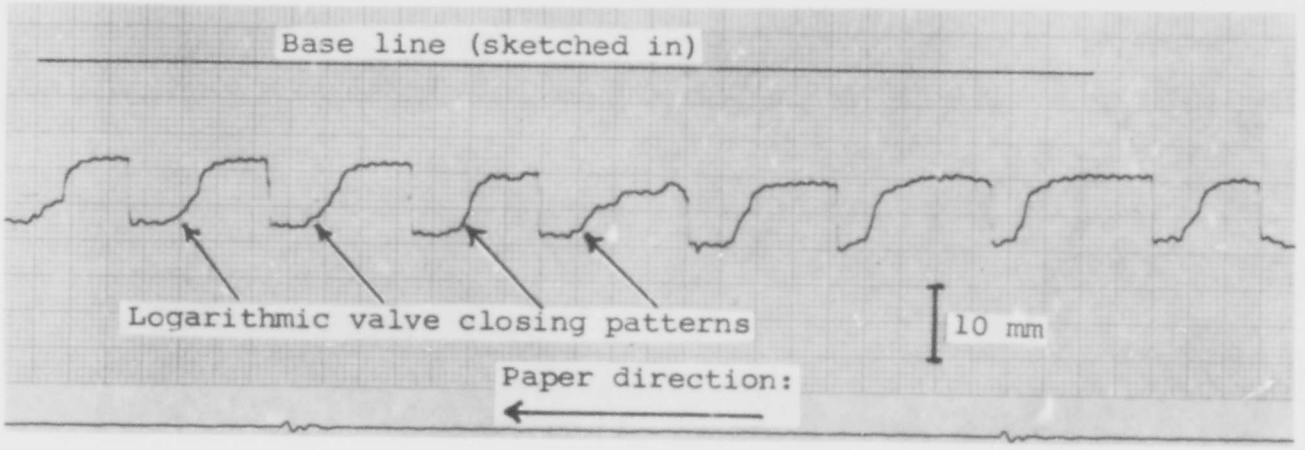

Figure 4. Mosquito salivation in vitro. Note logarithmic shape of upward sweep in valve closing patterns.
Paper speed: 100 mm/sec.

in our assessments of these recorded patterns.

It is interesting to note the considerably greater displacement from the base line during the engorgement of a single mosquito in the in vitro blood system. Since the current does not pass through the high resistance of a mouse and since blood itself is a good conductor of electricity, we may assume that considerably more current passes through the apparatus when the mosquito bites in the in vitro than in the in vivo system. Consequently, the change in resistance when the mosquito bites in vitro will be considerably greater than when it bites the mouse. This apparently appears in the recording as a greatly increased displacement from the base line.

In the recordings made with the more sensitive recorder (Sanborn model 7701A) there is also a greater displacement from the base line with a single mosquito bite (Figure 1). The response of an instrument of high sensitivity to low current flow is apparently equivalent to the response of an instrument of lower sensitivity to high current flow.

Figure 4 shows a salivation pattern recorded in the in vitro system at a paper speed of 100 mm/sec. Here it can be clearly seen that the salivation pattern is the exact reversal of the engorgement pattern. There are two factors that distinguish the salivation from the engorgement patterns. The first is that the lower portion of the engorgement pattern, representing the free flow of blood through the food canal and past the open anterior pharyngeal valve, lasts for a longer time than the lower portion of the salivation pattern, which represents the

free flow of saliva from the insect at the moment of saliva deposition. The second factor is that the closing of the anterior pharyngeal valve (which is involved in engorgement) appears to be much faster than that of the salivary valve. These differences are reflected in the closing patterns of these valves; i.e., the upward slopes in the two recordings.

In the recording of engorgement, the valve closure pattern shows an abrupt ascent to its maximum peak, with possibly a slight slowing down as complete closure is approached. This results in the sigmoidal appearance in the recorded pattern of valve closure. The salivation valve, on the other hand, is apparently slow in closing at the beginning, but speeds up as complete closure is approached. This results in the logarithmic-type closure pattern recorded for the salivary valve (compare Figures 3 and 4).

The longer straight portion in the upper part of the salivation pattern (Figure 4) probably represents a period during which the salivary reservoir is being replenished. The sudden expulsion of saliva is represented by the abrupt downward sweep in the recorded pattern. At slower paper speed engorgement or salivation can be determined by the amount of time the writing stylus spends in the various positions. Thus engorgement at a paper speed of 5 mm/sec is seen as a series of rapid ascents from a base position (Figure 5), while salivation at a paper speed of 1 mm/sec (Figure 6) is seen as a series of rapid descents from a base position.

C. Activity Measurements

Since the electronic system is sufficiently sensitive to respond to the fine intricacies of valve action of mosquito mouthparts

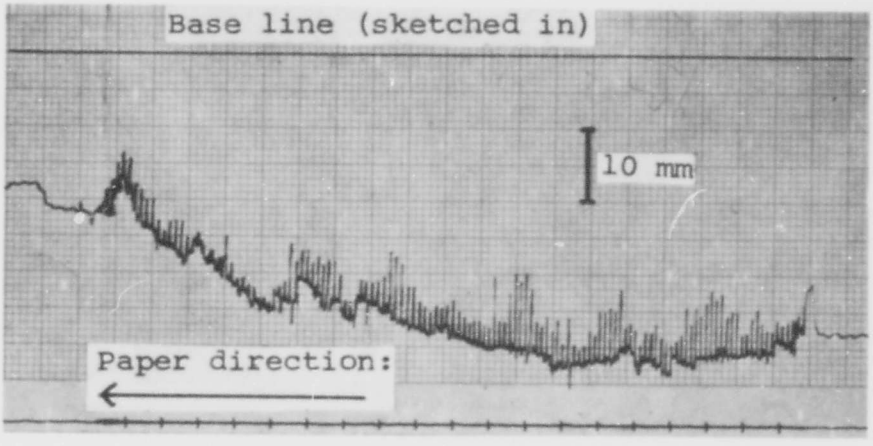

Figure 5. Engorgement in vitro.
Paper speed: 5 mm/sec.

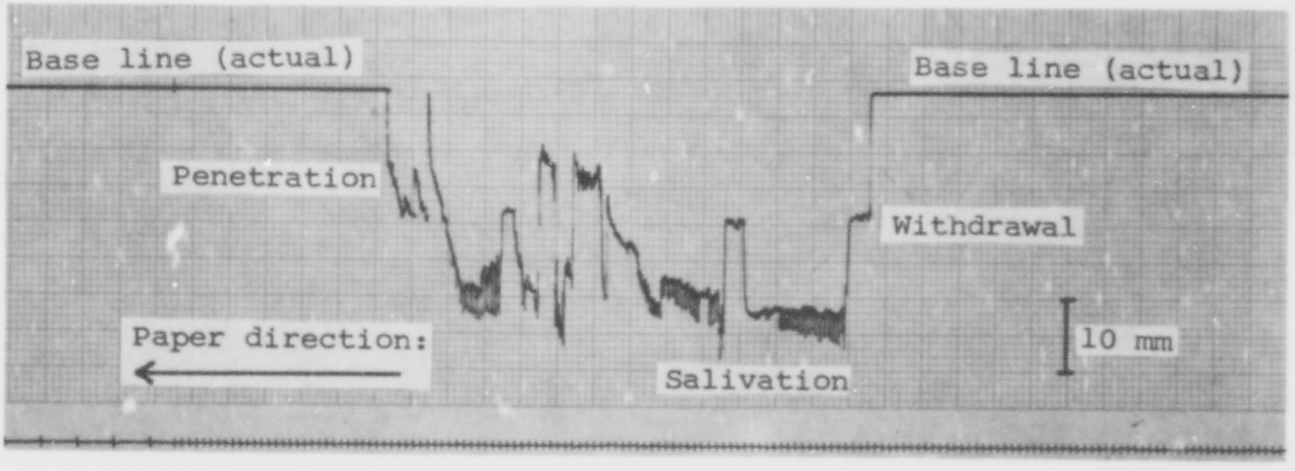

Figure 6. Penetration, salivation, and withdrawal in vitro.
Paper speed: 1 mm/sec.

during engorgement and salivation, we attempted to determine whether it would be possible, with a suitable electrical arrangement, to detect and record walking activities of a mosquito. In order to accomplish this, a square plastic frame was wound on four sides with fine-gage copper wire and enclosed on the remaining two sides with clear Plexiglass. One of the Plexiglass sides had a hole drilled into it so that the mosquitoes could be placed inside, and the hole was closed with a cork.

A measure of the spread of the legs of a mosquito when standing still showed the average span to be between 4 and 6 mm. The fine-gage copper wire was therefore wound at a distance of about 1 mm between strands, with each three strands (3 mm) electrically insulated from the next three strands. The alternating groups of strands were then independently connected to the negative and positive poles of a 1.35-volt mercury cell in an alternating fashion. With this arrangement, a mosquito standing or walking on the wire strands would be very likely to contact at least two strands of opposite electrical charge.

The same resistor-battery box as was used in the electronic recordings was used as the power and resistance source, and the leads that formerly went to the mouse and the screen were now connected to the two groups of wire strands in such a way as to have each vicinal group of three strands oppositely charged. The leads were then connected through the recorder exactly as for the electronic recording of biting activity, and mosquitoes were placed into the activity box.

The results of this experiment showed that when the mosquito moved or landed on the wire strands and bridged two oppositely charged strands with his legs, the mosquito closed the series circuit, and the recorder responded by showing a departure from the base line. When the mosquito moved up or down the wire strands, the recorder responded with erratic and abrupt departures from the base line. If the mosquito flew or just stood still, the recorded line became straight.

The mosquitoes were not affected by the small flow of electrical current, since they were observed to land on the wire strands at least as frequently as on the Plexiglass and not to depart from the strands with any greater frequency than from the sides. The experiment indicates the feasibility of devising an electronic apparatus to measure and record insect activity without the necessity of visual observation. Such a device could be used in automatically assessing a mosquito's response to a repellent or attractant substance and the duration of activity of such a substance. The Sanborn model 320 recorder is probably not the best instrument to use for this purpose. Wire grids are available with the crosshatch points insulated from each other, and recorders are available that can automatically monitor the position and activity of each insect by scanning the X and Y axes of the grid at any desired time intervals. This information can be stored and reproduced at any future time.

III. DEVELOPMENT OF A SYSTEMICALLY EFFECTIVE INSECT REPELLENT

It has generally been stated that in order for a compound to exhibit repellent activity, it must be volatile. This view seems to be substantiated by the findings in our laboratory and by others that compounds having a low vapor pressures or high molecular weights are generally poor repellents. In previous reports it was shown that even when good repellents, such as diethyl toluamide (DEET) or allethrin, are injected intradermally, intraperitoneally, or intravenously into a mouse, mosquito engorgement was not completely prevented, although it was curtailed. Whether this lack of repellent activity was due to non volatility of the repellent substance by its inability to pass through the skin or to a metabolic alteration of the repellent molecule, which rendered it non repellent, could not be ascertained. Since the test mice were exposed to the mosquitoes for about 1 hr, metabolic alteration during this time may well have played a role in the failure of these compounds to repel. Previous reports describing radioisotope tracer studies of C^{14}-labeled DEET showed that the radioactivity was soon localized in the liver, kidneys, and other excretory organs.

In order to determine why the injected repellent loses its activity, the electronic method was utilized. A group of mosqutoes was placed in a pint cardboard ice-crean container covered with a bronze mesh. A mouse was anesthetized and placed on top of the mesh. The mesh and the mouse were connected to the recording apparatus as previously described. When it was observed that

many of the mosquitoes in the group were simultaneously biting the anesthetized mouse and the recorder was responding characteristically, the mouse in one experiment was injected with 0.05 ml of pure DEET and in another experiment with 0.05 ml of 94% allethrin. The recorder was observed to determine whether there was immediate withdrawal of the mosquitoes from the mouse before there was any possibility of metabolism of the high concentration of these compounds.

The recorder patterns showed that there was no withdrawal of the mosquitoes from the mouse immediately following the injections in either experiment nor for the 30-min time period that the observations continued. In both cases, however, prolonged salivation patterns appeared in the recordings immediately after the injections. In both cases about 10% of the mosquitoes had engorged, but it was not determined whether this engorgement took place before or after the injection of the compounds. In future work we will inject radioiodinated serum albumin (RISA) simultaneously with the repellent substance to determine when this engorgement took place.

The mouse injected with allethrin died during the test, but the recorder patterns clearly showed that probing continued after the death of the mouse. It was shown early in the development of the electronic system that mosquitoes will probe into a dead mouse but not engorge. This is illustrated in Table 1. In this experiment a mouse injected intravenously with RISA before death was used as bait. Essentially no radioactive RISA was ingested by the mosquitoes, though the recordings showed active probing.

Table 1

Mosquitoes Feeding on Dead Mouse

	Counts per min
25 γ of mouse's blood	244.5
Radioactivity in 50 mosquitoes after attack on dead mouse for approximately ½ hour. (Mouse used 5 min post mortem)	18.7
Background Count	14.4

These experiments give preliminary indications that a repellent substance will not be active unless it is volatilized and that therefore a systemically effective repellent must be capable of being excreted through the skin. Salivation appeared to be considerably stimulated by the injected repellents. The interesting possibility emerging from these studies is that if a repellent substance is rendered nontoxic, prevented from being metabolized, and preserved in the circulation for a reasonable time period, mosquitoes may be prevented from engorging. If individuals who are already infected with an arthropod-borne disease could be treated with this substance, the spread of these diseases may be significantly reduced.

In an effort to find such an ideal substance, it occurred to us that if a repellent molecule could be chemically bonded to a certain proportion of circulating red blood cells, this objective might be achieved. It is well established that antigen molecules can be diazotized to red blood cells,[2,3] and that the red blood cell retains its stability and the antigen molecule retains its antigenic specificity after this chemical treatment.

If a repellent molecule could be diazotized to a red blood cell, the large size of the red blood cell compared to the size of the repellent molecule may protect the molecule from enzymatic

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[2]Landsteiner, K., "The Specificity of Serological Reactions," Dover Publications Inc., New York Chapter V.

[3]Kabat, E. A. and Mayer, M. M., "Experimental Immunochemistry," Charles C. Thomas Co., Springfield, Ill. pp. 122, 798.

attack by sterically hindering the approach or proper orientation of catabolic enzymes. The half-life of a red blood cell is between 2 and 3 months, and thus the activity could be retained in the circulation for a considerable period of time. Since the molecules are bound to red blood cells, the repellent probably would not be toxic in vivo. If the molecule does not lose its repellent activity after diazotization, it is possible that this system may approach the requirements for the ideal engorgement-inhibiting substance.

In order to diazotize a compound, an amino group coupled to a benzene ring must be available. If the methyl group of DEET were substituted by an amine, this compound could be diazotized and coupled to a red blood cell. A search of the literature revealed that neither the synthesis of m-amino-N, N-diethylbenzamide, nor the physical constants for this compound has been reported and that the compound is unavailable commercially. We therefore synthesized the compound in our laboratories. The synthetic procedure is described in the Appendix.

We have not yet attempted the diazotization of this compound to red blood cells, but a study of the repellent activity of the compound is given in Table 2. It is apparent that the substitution of the amino group for the methyl group of DEET has reduced the effectiveness of this compound as a mosquito repellent, but it cannot yet be predicted how diazotization of the amine will influence the repellent or engorgement-inhibiting properties of the compound.

Table 2

Repellent Properties of m-amino-N,N-diethylbenzamide as Determined by Feeding on Mice

Concertration Applied Mouse Belly mg	Number Fed/ Total	Average Percentage Fed
0.1	30/52	
0.1	36/53	52.5
0.1	18/55	
1.0	5/45	
1.0	18/51	
1.0	20/52	
1.0	36/53	39.3
10.0	6/52	11.5

IV. RESCREENING OF REPELLENTS WITH THE ELECTRONIC SYSTEM

The application of the electronic system in screening mosquito repellents will probably be most useful at the limits of concentration for maximum repellent activity exhibited by potential repellents. The questions that the electronic method could answer are whether the concentration of a repellent that prevents engorgement also prevents probing and salivation. If not, then what concentration of repellent is necessary to prevent probing and salivation? The time periods in which these compounds retain their repellent activity can also be automatically monitored with the electronic method.

Work in this area has just begun. Preliminary results demonstrating the applicability of this method are shown in Table 3. No conclusion can be drawn at this point because of the insufficiency of accumulated experimental data.

V. SUMMARY AND FUTURE WORK

Different recording instruments and wire screens were tested. It has been concluded that the Sanborn model 320 recorder and 50- or 100-mesh bronze screens are best suited for use in the electronic recording method.

A new application whereby mosquito activity could be measured electronically was successfully tested, and suggestions for the further improvement of an apparatus to measure mosquito activity were offered.

An in vitro model system to duplicate in vivo pool feeding was devised, and the recorded patterns for this system confirmed

Table 3

Repellent Properties of Some Known Repellents
as Determined by Feeding and Electronic Recording

Compound	Concentration Applied to Mouse Belly (mg)	Number Fed/Total (30 min feeding time)	Electronic Recordings
Allethrin	1.0	0/54	No biting seen
"	0.1	0/54	19 separate instances of biting
"	0.01	5/53	At least 24 separate instances of biting
"	0.001	0/51	49 separate instances of biting
"	0.001	0/56	1 bite at start of test
"	0.001	1/50	At least 2 separate instances of biting
"	0.001	1/53	At least 4 separate instances of biting
DEET	1.0	2/61	Considerable biting activity seen in all cases. Exact number of bites could not be determined.
"	0.1	2/56	
"	.01	25/45	
"	.001	27/53	

the interpretations and predictions inferred from in vivo pool feeding.

A possible approach to the development of a systemic means of preventing mosquito engorgement was presented based on findings obtained with the electronic method, and work has been initiated in this direction.

A program to rescreen known mosquito repellents utilizing the electronic method has begun, and preliminary results demonstrating the applicability of this method were presented.

Future work will be devoted to pursuing the experimental approaches outlined above.

VI. RECORDS

The author wishes to acknowledge the technical assistance of Mr. Robert Fosler. Mr. Robert S. Levi carried out the chemical syntheses. All data and methods are recorded in Logbooks C13755 and C15492 and in the form of the actual chart recordings.

Respectfully submitted,

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APPENDIX

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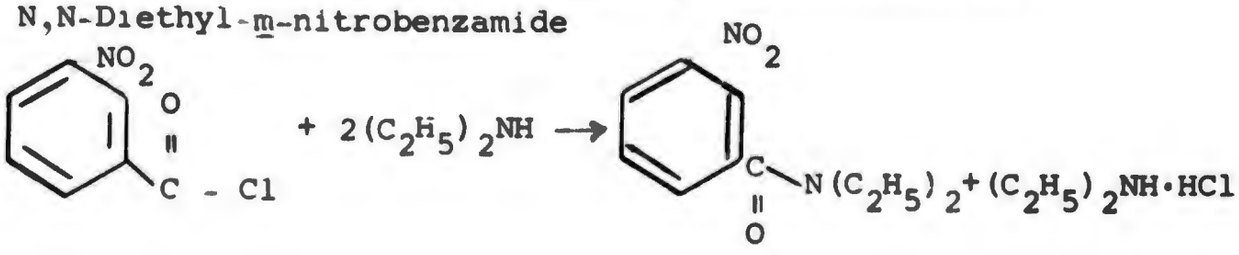
Preparation of m-Amino-N,N-diethylbenzamide

N,N-Diethyl-m-nitrobenzamide

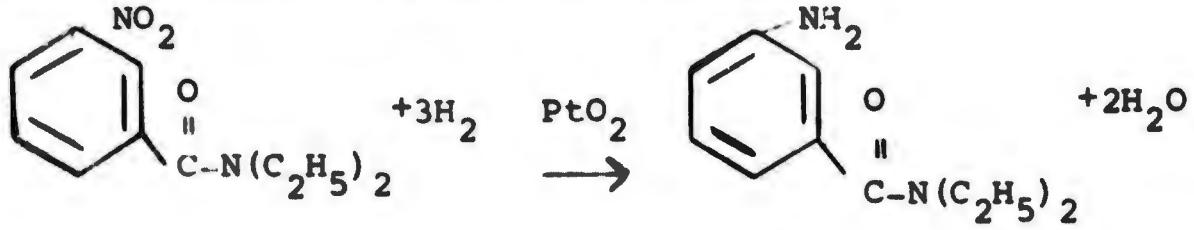


First 18.56 g of m-nitrobenzoyl chloride (0.10 mole) dissolved in 200 ml of benzene was placed into a 500-ml 3-necked round-bottom flask fitted with a dropping funnel, mechanical stirrer, and reflux condenser. Then 15.4 g of diethylamine (0.21 mole) dissolved in 50 ml of benezene was added dropwise, over a 10-min period to the m-nitrobenzoyl chloride solution with vigorous stirring. The temperature of the reaction mixture increased about 20 to 30°C during addition, but no cooling was necessary. Upon completion of addition, the reaction mixture was refluxed for 30 min. The white crystalline m-nitrobenzoyl diethylamine hydrochloride (10.6 g, 97%) was filtered by suction and washed with about 50 ml of benzene.

The combined filtrate was placed in a separatory funnel and washed twice with 50-ml portions of 2% Na_2CO_3, twice with 50-ml portions of 2% HCl, and finally washed to neutrality with distilled H_2O. The solvent was removed from the benzene layer by flash evaporation under aspirator pressure, yielding 21.9 g of crude product (m.p. 75 to 78°C).

The curde product was recrystallized from about 50 ml of 95% EtOH-H_2O mixed solvent. The resulting colorless plate crystals were dried at room temperature under vacuum, giving 19.29 g (87%) of the desired N,N-diethyl-m-nitrobenzamide (m.p. 76 to 78°C).

Preparation of m-Amino-N,N-diethylbenzamide



First 11.1g of N,N-diethyl-m-nitrobenzamide (0.05 mole) was disloved in 250 ml of 95% ethanol and placed in solution with 0.1 g PtO_2 (Adam's catalyst) in a glass jar set into a shaker hydrogenation apparatus. The system was evacuated and then hydrogenated under about 40 psi until hydrogen uptake was complete (30 min). Addition of fresh catalyst resulted in no further hydrogen uptake.

The catalyst was filtered by suction and rinsed with fresh solvent. The solvent was then evaporated by using a flash evaporator and aspirator at reduced pressure.

The yellow orange oil was transferred to a microdistillation apparatus and distilled at 170 to 172°C and 3.0 mm Hg pressure by using an oil bath for heating. The light yellow product (8.20 g; 85% yield), solidified upon storing in the freezer. The melting point was 86 to 88°C. Analysis:

	Calculated for $C_{11}H_{16}N_2O$	Found (1)	Found (2)
	68.71	69.11	69.05
	8.39	[illegible] 49	8.63
	14.57	14.16	13.99

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