IITRI



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(Annual Report)

# DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Headquarters
U.S. Army Medical Research and
Development Command
Office of the Surgeon General
Washington, D.C.

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# DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

November 1, 1964, through October 31, 1965

Contract No. DA-49-193-MD-2281
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## FOREWORD

This is Report No. IITRI-L6021-4 (Annual Report) on IITRI Project L6021, Contract No. DA-49-193-MD-2281, entitled "Development of an Orally Effective Insect Repellent." Previous work on the contract was conducted at IIT Research Institute from May 1, 1962 through October 31, 1964 under IITRI Project C222. The period covered by this report is November 1, 1964 through October 31, 1965. This investigation was supported by the U.S. Army Medical Research and Development Command, Office of the Surgeon General.

The project was directed by Dr. E. J. Hawrylewicz, Assistant Director of Life Sciences Research. The author wishes to acknowledge the various contributions of Dr. Harbans Lal, Mr. Harold G. Wakeley, Mr. Robert Fosler, Mr. Hugh J. O'Neill, and Mr. Robert S. Levi during the course of this work. Mr. Merl L. Kardatzke performed the statistical analyses of the electronically recorded data.

All data are recorded in Logbooks C14999, C15429, C15492, and C13755 and in the form of actual electronic chart recordings, motion picture films, and 35-mm slides.

Respectfully submitted,

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## ABSTRACT

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

The objective of this program is the development of insect repellents that can be administered systemically, preferably orally. In view of the results of experiments in which a new assay procedure was used, previous estimates of the potency of some repellents should be revised.

In order to study chemically induced feeding behavior of mosquitoes, chemicals that induce engorgement of mosquitoes were investigated. Although adenosine-5'-monophosphate stimulates engorgement to some extent, no nucleotide or other compound tested equals the engorgement-stimulating properties of adenosine-5'-triphosphate.

Skin permeability to various chemicals was investigated, and although these studies are incomplete, efforts in this direction were suspended temporarily.

A new assay procedure was developed in which an electronic device records the probing, penetration, salivation, engorgement, and withdrawal of a mosquito in relation to its host. Each act is distinct and can be separately recognized in the recording of a single mosquito bite. The chart recordings were correlated with the physiological events occurring within the mosquito during engorgement and salivation.

Statistical methods were applied to information derived from the electronic recordings, and statistically valid criteria for assessing the repellent-attractant phenomena of chemical compounds were investigated.

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# DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

## I. INTRODUCTION

The objective of this program is the development of an insect repellent that is effective when administered systemically, preferably orally. Such a repellent should afford more uniform and longer-lasting protection from insect bites than conventional surface repellents, and it should significantly reduce the spread of arthropod-borne diseases as well as the discomfort and annoyance caused by the bite of an insect.

Because the much needed information on the physiological basis of insect attraction and repulsion is largely unavailable, the development of a systemic repellent cannot be approached in a direct way. The host specificity demonstrated by most species of mosquitoes and the occurrence of some humans who appear to be naturally unattractive to mosquitoes indicate that the physico-chemical factors underlying insect attraction may be altered within physiological limits. Thus the ultimate objective of this program appears to be within the scope of realization.

This year's investigation was divided into two phases. The first phase concerned further characterization of substances that stimulate engorgement of mosquitoes. This information was to be applied to the design of chemical compounds or to the utilization of other biological means by which receptor stimulation may be antagonized and engorgement inhibited.

Also, initial attempts were made to measure the skin permeability of potential systemic insect repellents.

The second phase involved the development of a more accurate and reliable method to assay the responses of mosquitoes to potential repellents. Since insect salivation in the host is the mechanism by which arthropod-borne diseases are transmitted, the ability to discern all of an insect's responses to a potential repellent, independent of whether engorgement ensues, could be fundamental to the success of this program.

## II. CHEMICALLY INDUCED FEEDING BEHAVIOR OF MOSQUITOES AND SKIN PERMEABILITY OF POTENTIAL INSECT REPELLENTS (PHASE I)

### A. Chemically Induced Feeding of Mosquitoes

In vitro methods for testing chemically induced feeding behavior have been described in reports on Project C222.

Since adenosine-5'-triphosphate has been found to stimulate mosquito engorgement (ref. 1), investigations were initiated to define other chemical structures that may stimulate chemoreceptors of mosquitoes.

Table 1 gives data on feeding of mosquitoes as induced by adenine nucleotides and outdated whole human blood. Whereas nearly all the mosquitoes fed on blood, adenine nucleotides induced feeding in only half the mosquitoes. The amount of fluid ingested by the mosquitoes was nearly the same in all cases.

Table 2 shows the effect of time of exposure on total feeding induced by adenosine monophosphate. Exposure of mosquitoes to the feeding cylinder for 1 hr gave optimum

Table 1

EFFECT OF ADENINE NUCLEOTIDES AND BLOOD ON FEEDING OF MOSQUITOES THROUGH ARTIFICIAL MEMBRANE

| Agent[a] | Conc. | % of 50 Mosquitoes Feeding | | Solution Ingested per Mosquito, µl | |
|---|---|---|---|---|---|
| | | Mean ± SE | Number[b] of Expts. | Mean ± SE | Number[b] Of Expts. |
| Blood | Whole | 88.2 ± 6.02 | 13 | 3.95 ± 0.005 | 13 |
| Adenosine-5'-triphosphate | $1 \times 10^{-2}$ | 12.0 ± 2.71 | 4 | 5.10 ± 1.80 | 4 |
| | $1 \times 10^{-3}$ | 63.0 ± 1.74 | 27 | 3.63 ± 0.15 | 17 |
| | $1 \times 10^{-4}$ | 40.0 ± 8.50 | 8 | 3.28 ± 0.15 | 18 |
| Adenosine-5' monophosphate | $1 \times 10^{-1}$ | 23.0 | 2 | 2.95 | 2 |
| | $1 \times 10^{-2}$ | 53.0 + 1.12 | 13 | 2.78 ± 0.18 | 13 |
| | $4 \times 10^{-2}$ | 43.0 | 3 | 4.0 | 3 |
| | $1 \times 10^{-3}$ | 29.0 ± 4.76 | 8 | 3.16 ± 0.24 | 8 |
| | $1 \times 10^{-4}$ | 8.0 | 2 | 4.60 | 2 |

[a]Dissolved in 0.15 M sodium chloride at pH 7.2.

[b]50 mosquitoes were exposed in each experiment.

results. Exposure for longer than 1 hr did not increase the number of mosquitoes fed. Therefore 1 hr was selected as the time of exposure for all experiments.

Table 2

EFFECT OF TIME OF EXPOSURE ON AMP[a]-INDUCED FEEDING RESPONSE OF MOSQUITOES

| Time of Exposure, min | % of Mosquitoes Feeding | Solution Ingested per Engorged Mosquito, µl |
|---|---|---|
| 30 | 37.9 | 2.1 |
| | 39.6 | 2.4 |
| 60 | 56.1 | 2.0 |
| | 52.7 | 2.3 |
| 90 | 52.8 | 2.2 |
| | 56.9 | 2.5 |
| 120 | 53.8 | 2.2 |
| | 52.7 | 2.3 |
| 180 | 55.6 | 2.9 |
| | 51.7 | 2.1 |

[a]10 µmoles of adenosine monophosphate in 0.15 M sodium chloride.

Mosquitoes once fed on blood do not feed again until at least 72 hr. It was of interest to know the interval after which the mosquitoes fed on adenine nucleotides are susceptible to feeding again. Therefore mosquitoes were fed on a $10^{-3}$ M adenosine triphosphate solution. The engorged mosquitoes were separated and exposed to anesthetized mice after various time intervals. The data in Table 3 show that mosquitoes fed adenosine triphosphate exhibited normal feeding 18 hr after first feeding. Nearly all the mosquitoes fed adenosine triphosphate bit the mice 18 and 24 hr after the first feeding.

Table 3

SUBSEQUENT FEEDING ON MICE BY ATP[a]-FED MOSQUITOES

| Time after Feeding | Number Engorged per Number Exposed | % Engorged |
|---|---|---|
| 15 min | 2/25 | 8.0 |
| | 3/24 | 12.5 |
| 2 hr | 14/34 | 41.2 |
| | 7/17 | 41.2 |
| 4 hr | 15/35 | 42.9 |
| | 16/37 | 43.2 |
| 18 hr | 29/33 | 87.9 |
| | 25/25 | 100.0 |
| 24 hr | 30/34 | 88.2 |
| | 29/35 | 82.9 |

[a]1 μmole of adenosine triphosphate in 0.15 M sodium chloride.

Efforts were made to determine whether other chemical structures induce feeding in mosquitoes. Table 4 lists the chemical agents that failed to induce any feeding in adult mosquitoes. The water-insoluble compounds were suspended with the aid of Tween 80 in trace quantity. Tween 80 in control experiments did not noticeably induce feeding in mosquitoes.

Table 4

CHEMICAL COMPOUNDS THAT FAILED TO INDUCE FEEDING IN MOSQUITOES

| Compound | Conc., M |
|---|---|
| Acetycholine (in presence of 0.1 mg/cc physostigmine) | $10^{-3}$ |
| Metrazol | $10^{-2}$ |
| | $10^{-3}$ |
| | $10^{-4}$ |
| Creatinine phosphate | $10^{-2}$ |
| | $10^{-3}$ |
| | $10^{-4}$ |
| Adenosine-3', 5-cyclic phosphate | $10^{-2}$ |
| | $10^{-3}$ |
| | $10^{-4}$ |
| Trimethyl phosphate | $10^{-2}$ |
| | $10^{-3}$ |
| | $10^{-4}$ |
| Methyl eugenol[a] | $10^{-2}$ |
| | $10^{-3}$ |
| | $10^{-4}$ |
| Trimedlure[b] | $10^{-2}$ |
| | $10^{-3}$ |
| | $10^{-4}$ |
| Adenosine | $10^{-2}$ |

[a] Oriental fruit fly attractant.

[b] 3-Chloro-6-methyl cyclohexane-1-carboxylic acid, tertiary butyl ester (Mediterranean fruit fly attractant).

## B. Skin Permeability of Potential Insect Repellents

Preliminary experiments were conducted to devise a procedure suitable for studying the skin penetration of potential systemic repellents. The apparatus consists of a glass cylinder (2-cm ID) open at both ends. Shaved abdominal skin from a freshly killed mouse was dissected from the mouse, stretched over one of the open ends of the cylinder, and secured with a rubber band. The cylinder was placed in an external glass chamber containing Ringer's solution. The external chamber rested on a magnetic stirrer in a constant-temperature water bath. The internal and external chambers contained 10 and 40 ml of Ringer's solution, respectively. The internal solution was oxygenated, while the external solution was stirred magnetically throughout the experiments. To prevent hydrostatic pressure from influencing the diffusion, the levels of internal and external solutions were kept the same.

The Ringer's solution and the skin were allowed to equilibrate for 15 min. Thiourea-$C^{14}$ (0.1 ml, containing not more than 0.05 mg of thiourea/ml) was added in the internal cylinder in some experiments and to the external chamber in others. The external surface of the skin always faced the external chamber. Small aliquots (0.1 to 0.2 ml) were taken from both chambers at several time intervals and radioassayed.

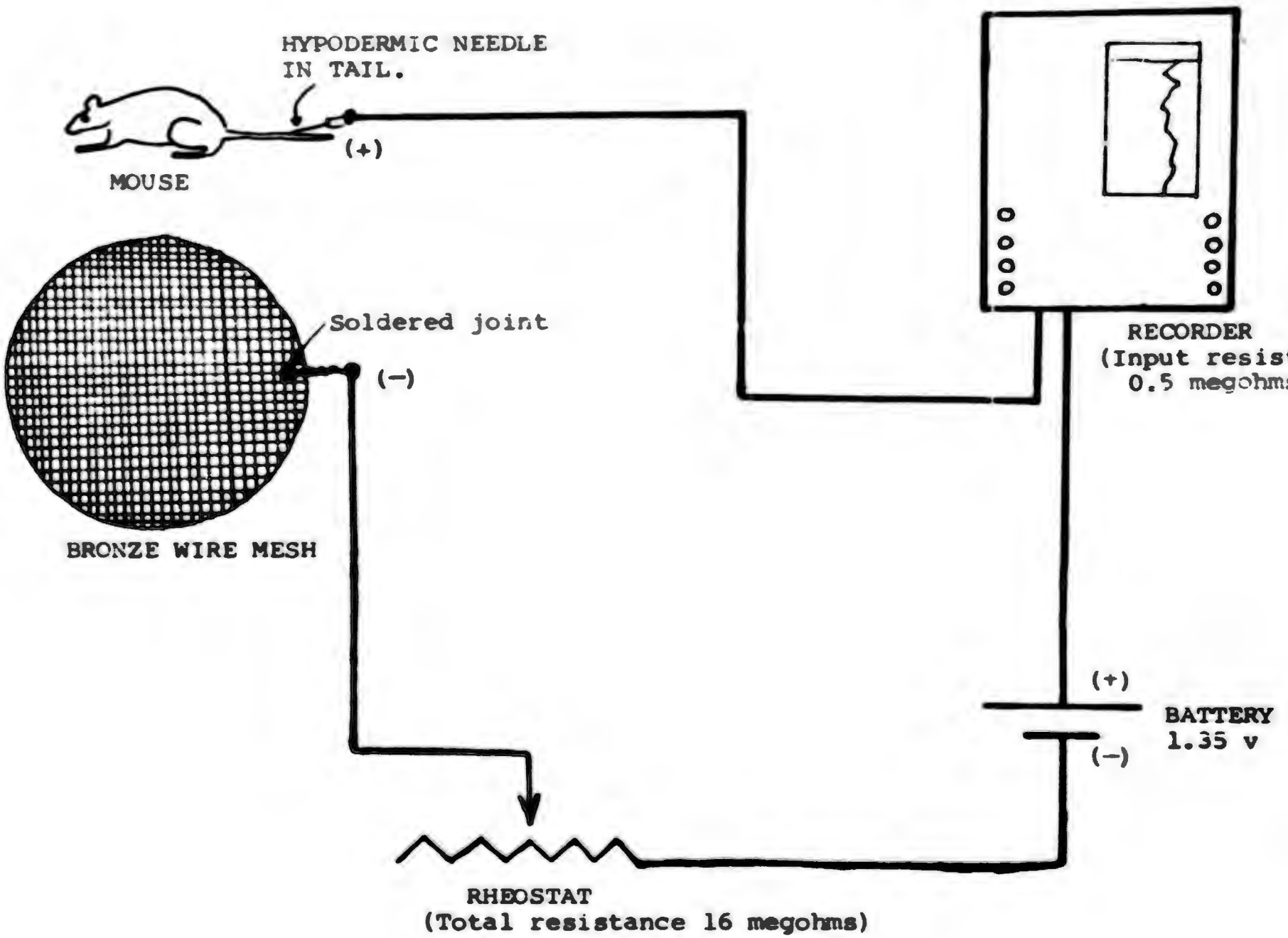




**Figure 1**

CIRCUIT DIAGRAM OF ELECTRONIC RECORDING APPARATUS

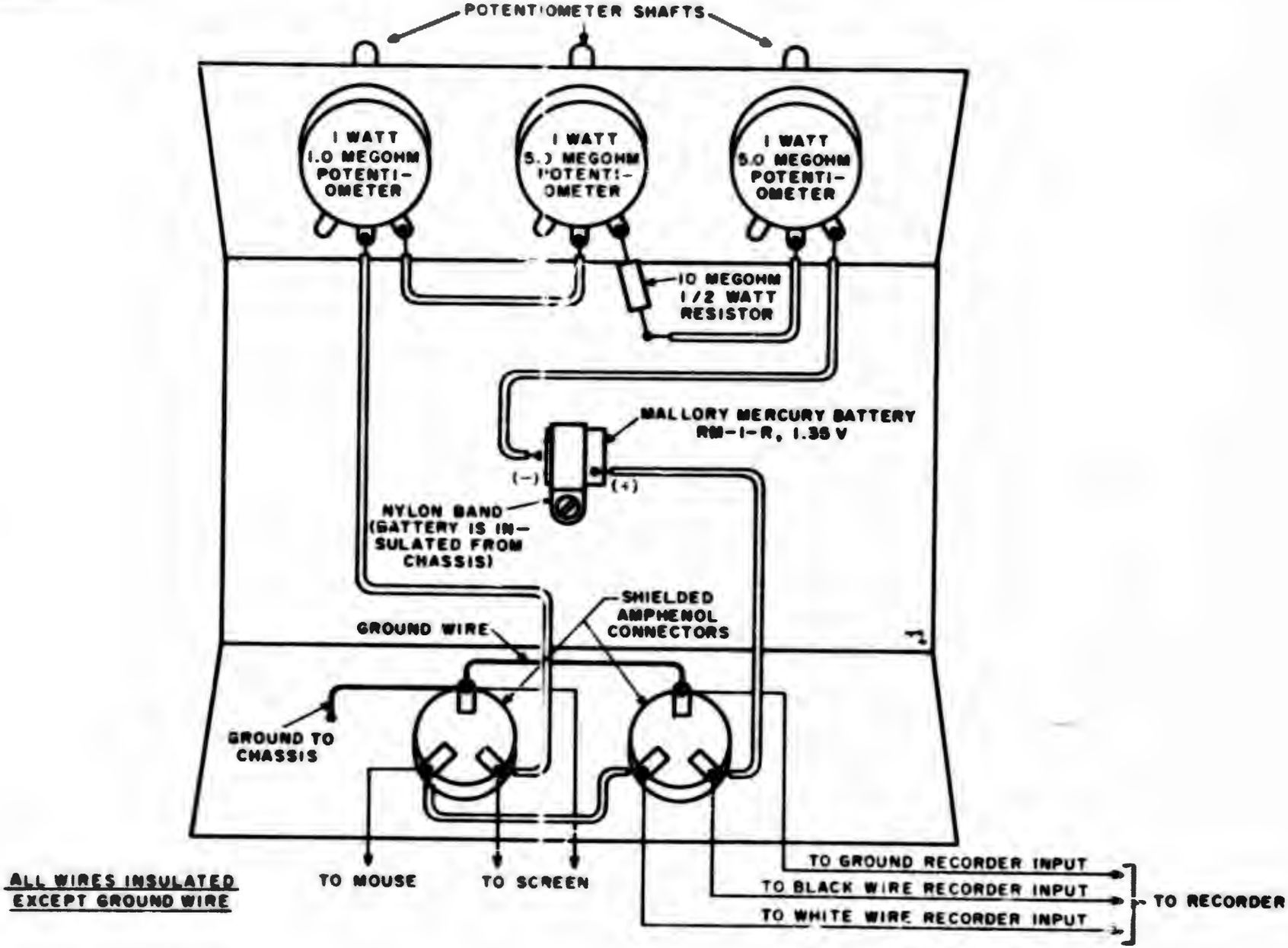


Figure 2

WIRING DIAGRAM OF RESISTOR-BATTERY BOX

as the mosquito is completing the circuit, and there is a displacement from the base line (point of no current flow) on the recorder chart paper for the entire time the mosquito is biting. When the proboscis is withdrawn, the circuit is broken. The writing stylus immediately returns to the base line, indicating that virtually infinite resistance has been reestablished between the mouse and the bronze mesh.

## B. Discussion of Method

The question of whether the flow of an electric current could change a mosquito's behavior or create artifacts in the system was considered. By considering the total resistance of the circuit and the total voltage available, it was calculated that a maximum of $8.2 \times 10^{-8}$ amp is available in the circuit, if there is no resistance between the mouse and the mesh. When a single mosquito bites a mouse through the screen, the average displacement of the stylus is 10 mm from the base line. In calibrating the recorder (which was always set at its highest sensitivity level of 0.5 mv/mm and at maximum gain), it was observed that a displacement of 3.4 mm from the base line is equivalent to 1 mv. A displacement of 10 mm therefore indicates a voltage drop of 2.94 mv across the recorder.

From the known input resistance of the recorder (0.5 megohms) and the measured voltage drop across the recorder when a mosquito bites, it can be shown that approximately $6.0 \times 10^{-9}$ amp flows

through a mosquito at the time of the bite. This value is probably an overestimate, since the resistance of the mosquito is not taken into account. This quantity of electricity is extremely small and probably no more than the static electrical charge that the insect attains during free flight. Therefore we concluded that artifacts that might affect a mosquito's activities are not introduced by this flow of electrical current. We could not detect changes in a mosquito's behavior even when all resistances were removed from the circuit. A resistance of 16 megohms between the battery and the recorder seemed to give the most uniform recordings.

The question of whether biting was the only activity that could cause a departure from the base line in the recording was also considered. When male Aedes aegypti L. mosquitoes or female mosquitoes whose proboscises were amputated were placed in the mesh-covered container, no displacement from the base line was observed at any time. We concluded that only the biting female mosquito causes displacement.

Further experiments were performed with the wire screens. A bronze screen between the mouse and the mosquitoes seemed to give somewhat more uniform 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 create better contacts between the crosshatch wires, a number of copper and bronze screens were electroplated with tin.

radioactive counts was divided by the number that had engorged, and the total average intake per mosquito was calculated in terms of microliters of test substance (ref. 3). Repellency was judged to be inversely proportional to engorgement. Thus, if a test substance inhibits engorgement but not penetration or salivation, the repellent properties of this substance would be assessed erroneously. Since the success of this program may well depend upon our ability to define all of a mosquito's responses to a repellent or attractant substance, the development of a more dependable method of assay was considered indispensible. Our efforts in this direction are described below.

## III. THE ELECTRONIC RECORDING OF THE MOSQUITO BITE (PHASE II)

### A. Principle and Methods

We reasoned that our objective could be accomplished if the contact between a mosquito and its host were an integral part of an electrical circuit connected in series. The mosquito's bite and withdrawal from the host could be used as the "making" and "breaking" switch in the circuit.

Since it is impossible to connect an electric wire directly to a mosquito, a 50- or 100-mesh bronze screen is used to separate the mosquito from its host. The screen covers a 1-pint cylindrical ice cream carton (or glass container if simultaneous visual observation is desired), and female Aedes aegypti L.

mosquitoes are placed into the container. A wire is connected from the screen through a series of resistances totaling 16 megohms to the negative pole of a 1.35-v standard mercury cell (Mallory Mercury Battery RM-1-R or RM-12-R). The circuit is then carried from the positive pole of the mercury cell to the input of a Sanborn model 320 two-channel general-purpose recorder (The Sanborn Company, Waltham, Mass.). Another lead then continues the circuit from the recorder to the tail of an anesthetized mouse, where it is imbedded via a hypodermic needle soldered onto the lead. The mouse is placed on top of the bronze screen and contact of the mouse's paws with the screen is carefully avoided. The hair on the mouse's body is sufficient to insulate the mouse from the screen, and no current flows. The entire circuit is thus connected in series (Figure 1*). The screen is negative with respect to the mouse. Figure 2* shows the wiring diagram of the resistor-battery box used in the circuit.

When the mosquito bites the mouse, it holds on to the wire screen with its legs and passes its proboscis through the spaces of the mesh and into the mouse. When the skin of the mouse is penetrated, the circuit between the mouse and the screen is completed through the mosquito, and the resulting current flow is registered by a displacement of the writing stylus of the recording instrument. The current flows continuously as long

*These figures have been corrected. In previous reports, the negative and positive poles were reversed.

Table 5

RADIOACTIVITY IN INTERNAL AND EXTERNAL CHAMBERS AFTER ADDITION OF THIOUREA-$C^{14}$ TO THE DIFFUSION SETUP

| Time after Thiourea Addition, min | Radioactivity,[a] counts/min | |
|---|---|---|
| | External Chamber (0.2-ml aliquot) | Internal Chamber (0.1-ml aliquot) |
| **Thiourea-$C^{14}$ Added to Internal Chamber** | | |
| 30 | 19.5 | 782.5 |
| 60 | 15.4 | 745.2 |
| 90 | 27.3 | 769.2 |
| 150 | 17.0 | 754.1 |
| 210 | 17.2 | 755.3 |
| 240 | 16.0 | 805.1 |
| 270 | 24.7 | 769.1 |
| 300 | 17.3 | 836.1 |
| **Thiourea-$C^{14}$ Added to External Chamber** | | |
| 15 | 1315.8 | 15.6 |
| 30 | 1572.3 | 16.4 |
| 45 | 1388.9 | 18.2 |
| 60 | 1362.4 | 14.7 |
| 90 | 1408.5 | 17.9 |
| 120 | 1543.2 | 16.0 |
| 150 | 1453.5 | 16.7 |
| 180 | 1381.2 | 18.4 |
| 210 | 1529.1 | 17.6 |
| 240 | 1453.5 | 19.5 |
| 320 | 1519.8 | 22.5 |

[a]Background radioactivity was nearly 18.

Results from two typical experiments are given in Table 5. These data show that the experimental setup did not detect any measurable inward or outward diffusion of thiourea-$C^{14}$ through the excised skin. The reason for this failure to detect penetration through skin is not clear, since other investigators using similar techniques (ref. 2) succeeded in detecting penetration of various compounds through skin.

This area of investigation was temporarily abandoned, and new efforts were directed toward the development of an accurate and reliable method for assaying mosquito biting, regardless of whether engorgement occurs. In all previous experiments the assay of repellency was based solely upon engorgement, and there was no way to determine whether the mosquito had penetrated the skin of the host and not engorged. This method of assay is seriously limited, because salivation, not engorgement, is the vector by which mosquito-borne diseases are disseminated.

The previous method used to determine the attractant or repellent properties of a compound involved the intravenous injection of radioiodinated serum albumin (RISA) together with a fluorescent dye (Blancophore) or the addition of these compounds to an in vitro test system. Engorgement was determined by measuring the radioactivity of the total group of mosquitoes exposed to the test system and counting (under ultraviolet light) the number that had taken up the fluorescent dye. The number of

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 the 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, although the coated screens could conceivably have more uniform electrical properties. Bronze screens have been used in all work to date.

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 Sanborn model 7701A direct-writing oscillographic recorder. This instrument has 1000-fold greater sensitivity than model 320 (0.5 $\mu$v/mm for model 7701A compared with 0.5 mv/mm for model 320).

It was found that at the maximum sensitivity level for model 7701A, external noise almost completely obliterated the patterns of the mosquito biting activities. Only at sensitivity levels of 200 μv/mm (which approach the highest sensitivity of 0.5 mv/mm for model 320) could noise be adequately shielded out and clear biting activity patterns obtained. The patterns recorded were very similar to those obtained with the model 320. Since a paper speed of 50 mm/sec is the maximum speed attainable with model 7701A, compared with 100 mm/sec with model 320, details of the recordings were not as distinct.

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 sec after it has swept across the screen.

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

## C. Results and Interpretations

A visual observation and an electronic recording were made simultaneously during the course of a single mosquito bite. The pattern recorded at a paper speed of 1 mm/sec during the observed probing activities of the mosquito were generally irregular (Figure 3).*

*In all figures, a displacement of 10 mm is equivalent to a voltage drop of 2.95 mv.

When the withdrawal of the mosquito's proboscis was observed visually, there was invariably an immediate return to the base line in the chart recording.

When the mosquito was observed to be still and in the biting attitude, the chart recording became much more regular (Figure 4). Rapid downward peaks occurred, although no engorgement was observed. While the mosquito was still maintaining the biting position, the recorder pattern then changed in character (Figure 5). As the recording progressed, the peaking reversed from a downward to an upward direction (Figure 5, right side). The position of the mosquito did not change.

While this pattern continued, the mosquito was suddenly observed to be filling with blood; engorgement had begun. The engorgement pattern continued (Figure 6, left side) for a period of about 4.5 min, after which time the mosquito was fully distended with blood. The proboscis was then withdrawn, and the recording immediately returned to the base line (Figure 6, right side). At the time of withdrawal, there was again an indication of pattern reversal, and the peaks seemed to change from the upward to a downward direction.

A pattern was recorded at a higher paper speed, 100 mm/sec, while the mosquito was engorging (Figure 7), because the pattern can be analyzed more carefully at this higher paper speed. Note that a rise toward the base line indicates increasing resistance

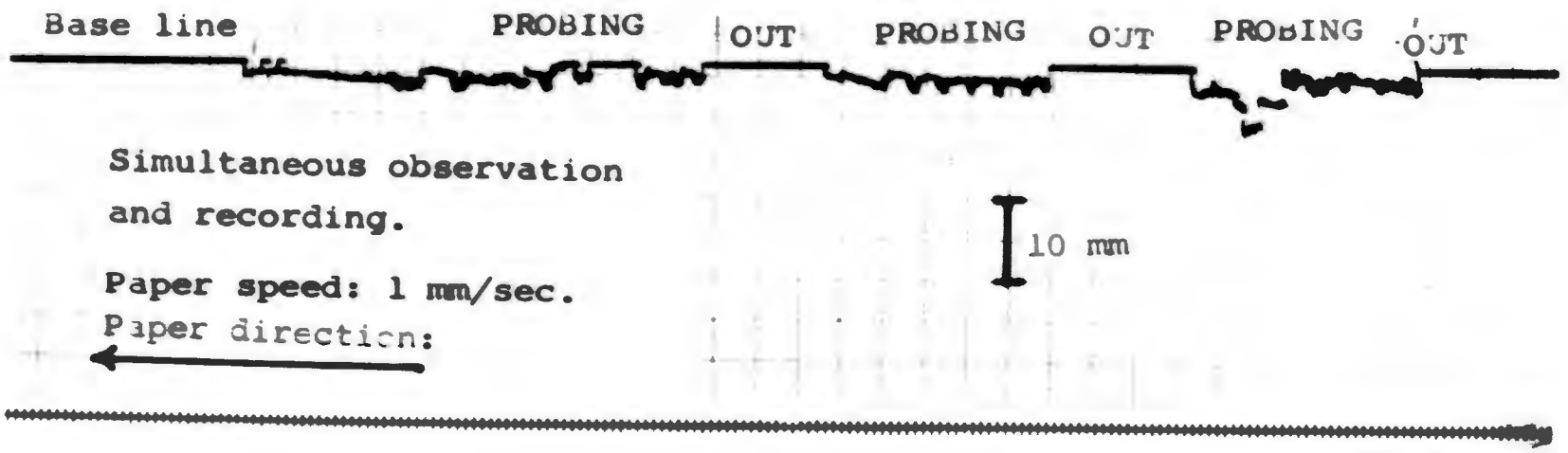


**Figure 3**

**CHART RECORDING OF A SINGLE MOSQUITO PROBING INTO AN ANESTHETIZED MOUSE**

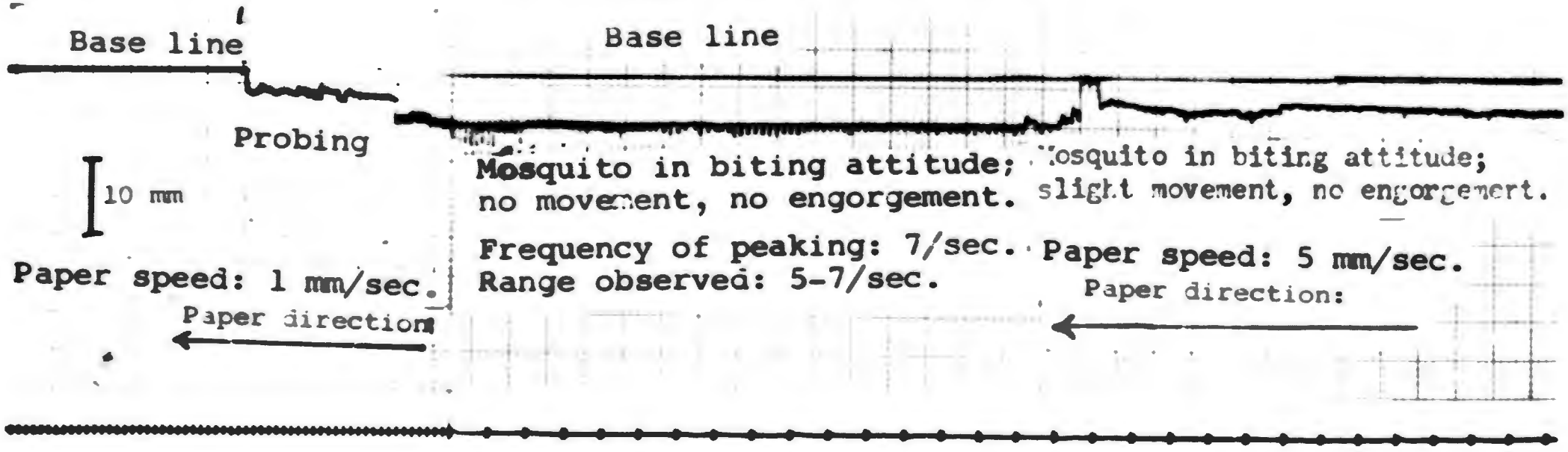


**Figure 4**

**CHART RECORDING OF A SINGLE MOSQUITO PROBING INTO AN ANESTHETIZED MOUSE FOLLOWED BY APPARENT BITING (Note Downward Peaks)**

pattern is envisaged as representing the reopening of the anterior pharyngeal valve. The electrolyte pathway is reestablished as the cibarial and pharyngeal pumps again expand to receive more blood, and the process is repeated.

Although the engorgement pattern can be identified by actually observing the mosquito swelling with blood, the act of salivation was not observed, and therefore the interpretation of its pattern is somewhat tenuous. Figure 8 is a 100 mm/sec recording of the pattern in Figure 4, which showed downward peaking at a paper speed of 5 mm/sec. This pattern was interpreted to be representative of salivation because it is the exact reversal of the engorgement pattern. Here, the valve that controls salivation remains closed until sufficient saliva accumulates. When the valve is closed, there is a higher resistance in the circuit because of the severing of the electrolyte pathway. This is indicated in the recording by the closer proximity of the upper straight line portion of the tracing to the base line. The salivary pump then contracts, the saliva valve opens, and saliva spurts out. The sudden decrease in resistance at the moment of saliva deposition (electrolyte flow) causes the abrupt downward peaking in the recording. (The small sinesoidal waves in the recordings at high paper speed represent a slight 60-cycle A.C. ripple from the recorder and should be ignored).

Figure 9 is another 100 mm/sec recording of a single mosquito bite and shows a mosquito that apparently went from engorgement to salivation and back to engorgement in about 1 sec. In microscopic observations of a mosquito feeding on a mouse's ear, Griffiths and Gordon (ref. 5) described the repeated observation of a "...discharge of fluid from the tip of the labrum when feeding was in progress on the mouse's ear." They further state, "There seems no reason to doubt that this fluid was salivary secretion discharged from the hypopharynx." Apparently, we have now observed this phenomenon electronically.

We have observed salivation occurring in the initial penetration (Figure 4), during engorgement (Figure 9), and during final withdrawal of the mouth parts (Figure 6, right side). Gordon and Lumsden (ref. 6) and Griffiths and Gordon (ref. 5) have also noted salivation during initial penetration and during engorgement in their microscopic observations.

Recordings of the engorgement phase show that in some cases the upward peaking takes rather large excursions (Figure 6, left side), while in other cases the excursions are very small and at times hardly visible (Figure 10). The total feeding time was 4 to 6 min in recordings with large excursions, and only 1 to 2 min in those with smaller excursions. During a single mosquito bite the excursions ranged from very small to fairly large within a short period of time (Figure 11). We therefore conclude that the sizes of the excursions are not individual characteristics of different mosquitoes but that every mosquito is capable of showing

in the system and that descent from the base line indicates decreasing resistance. Correlation of the recorded patterns with the proposed movements of the cibarial and pharyngeal pumps during feeding (ref. 4) leads to the following interpretations. The lower straight-line portion of this pattern is envisaged in terms of blood flowing from the mouse through the food canal of the mosquito's labrum and into its cibarial and pharyngeal pumps. The electrolyte (blood) pathway established causes lower resistance between the mouse, the mosquito, and the mesh, and the departure from the base line is the greatest. As the pattern rises toward the base line, the recorder is responding to an increase of electrical resistance in the system. This phase is therefore interpreted as representing the closing of the anterior pharyngeal valve, thus severing the electrolyte pathway and causing the increased resistance.

The valve-closing pattern seems to have a bimodal character; first it shows a high positive slope and then it suddenly changes to an almost zero slope. The zero slope portion could represent the contraction of the pharyngeal pump, which forces blood back past the open posterior pharyngeal valve and into the gut, while the anterior pharyngeal valve remains closed. The process of swallowing is represented by the zero slope portion of the pattern, while valve closure is represented by the positive slope portion of the pattern. The sudden subsequent drop in resistance in the

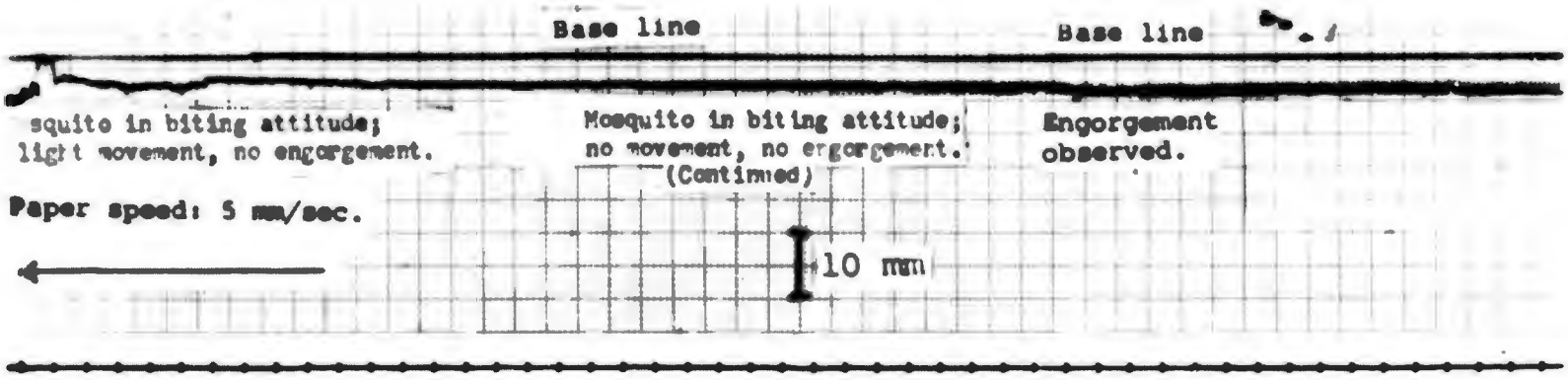


Figure 5

CHART RECORDING OF A SINGLE MOSQUITO PROBING INTO AN ANESTHETIZED MOUSE DURING VISUAL OBSERVATION OF ENGORGEMENT
(Note Reversal of Peaking and Upward Peaks)

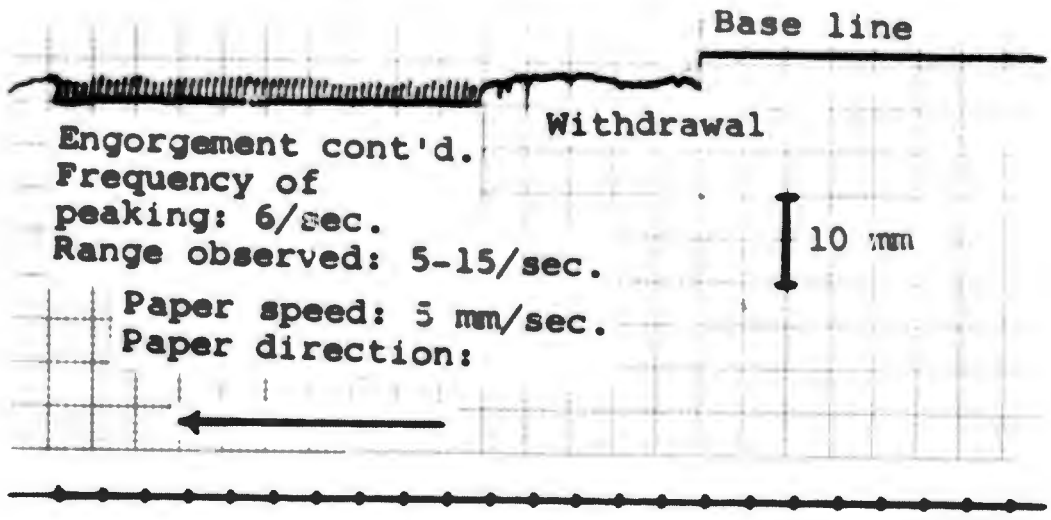


Figure 6

CHART RECORDING OF ENGORGEMENT AND PATTERN REVERSAL JUST BEFORE WITHDRAWAL OF PROBOSCIS
(Total Feeding Time: 4.5 min)

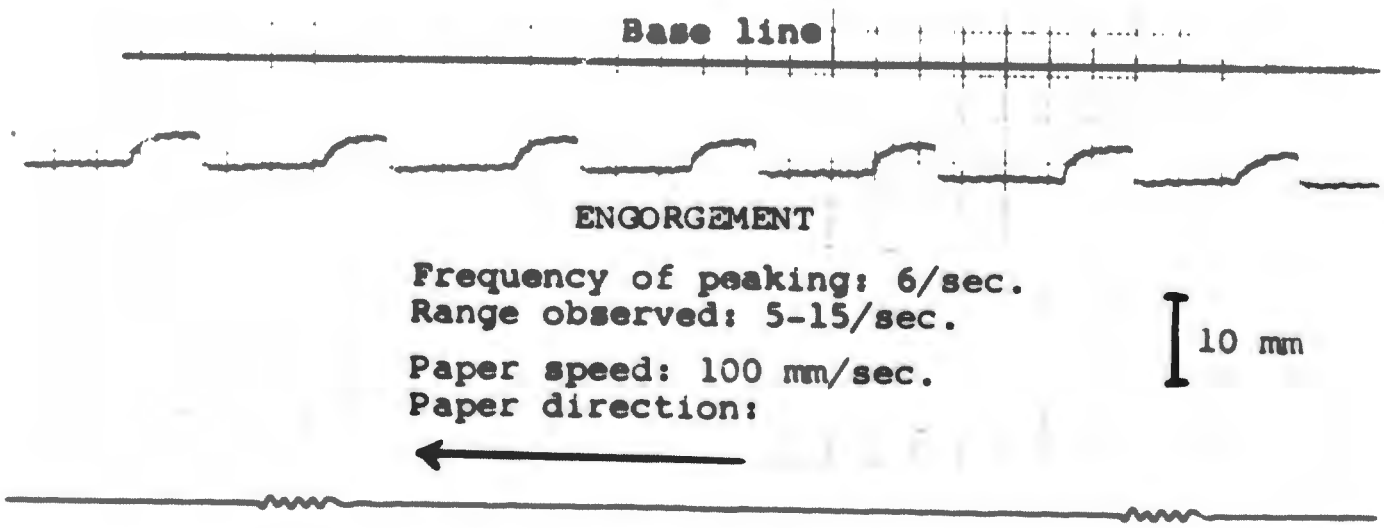


Figure 7

CHART RECORDING OF ENGORGEMENT AT A HIGH PAPER SPEED

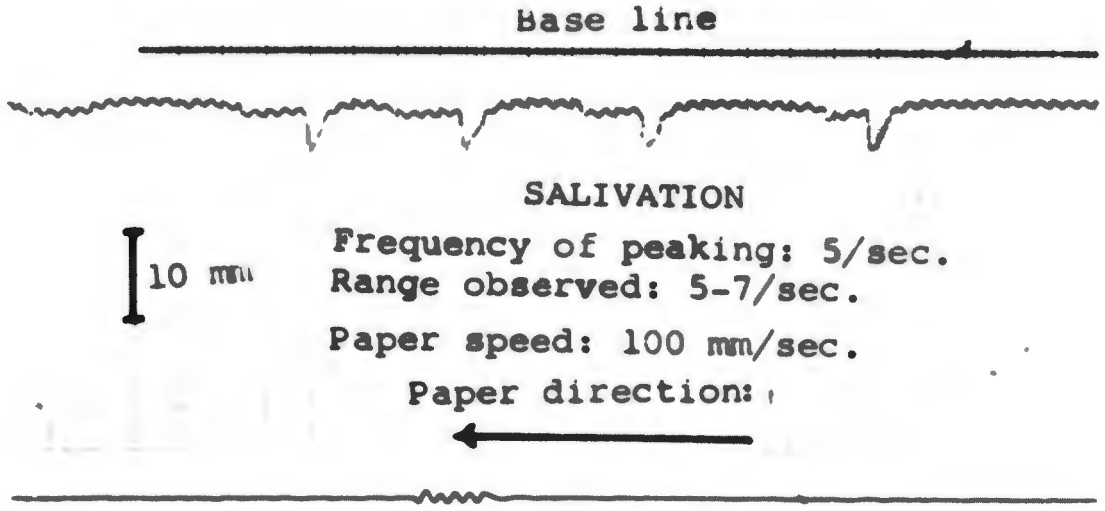


**Figure 8**

**CHART RECORDING OF SALIVATION AT A HIGH PAPER SPEED**

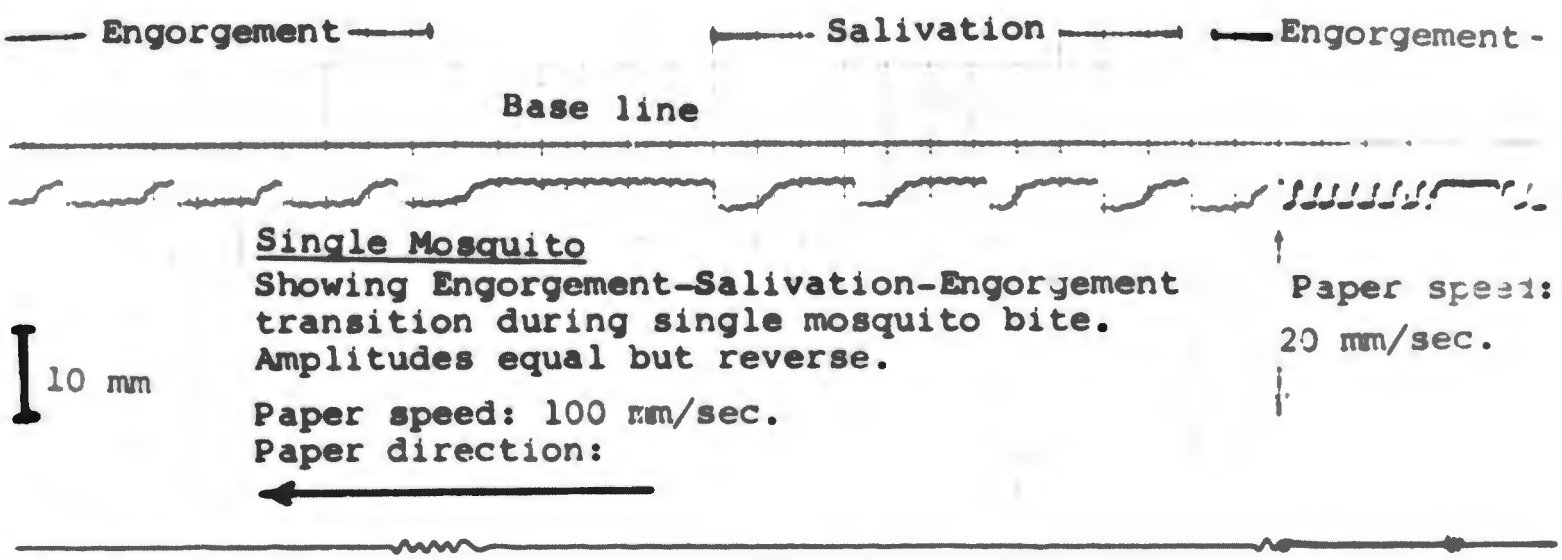


**Figure 9**

**CHART RECORDING OF ENGORGEMENT-SALIVATION-ENGORGEMENT TRANSITION AT A HIGH PAPER SPEED**

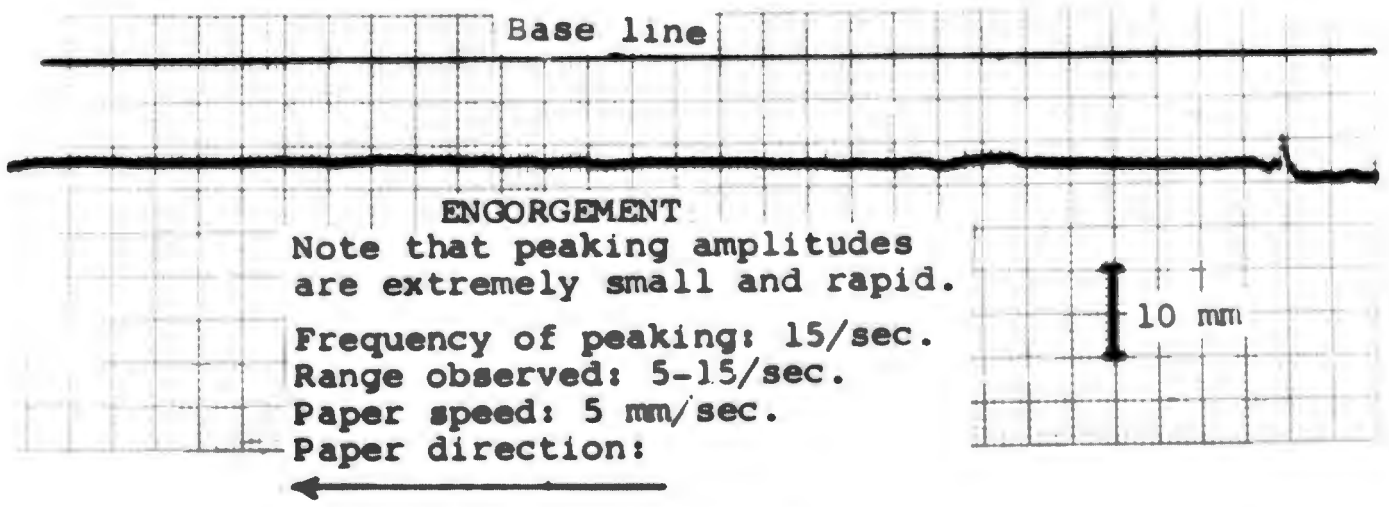


**Figure 10**

**CHART RECORDING OF ENGORGEMENT WITH SMALL EXCURSIONS OF PEAKS (Total Feeding Time: 1.5 min)**

the full range of engorgement patterns. The particular pattern shown by different mosquitoes is therefore a function of some factor outside the mosquito per se.

Gordon and Lumsden (ref. 6) and Griffiths and Gordon (ref. 5) described two types of feeding behavior of mosquitoes. In one type, called capillary feeding, the mosquito's fascicle was inserted in a capillary, which pulsated rapidly during feeding; the feeding time was about . min. In the other type, called pool feeding, blood was extravasated into the tissues when the fascicle pierced a capillary during probing, causing a small hemorrhage. The mosquito then sucked up the blood as fast as it flowed into the tissues until it became completely engorged. Feeding by this method took as long as 10 min.

We therefore suggest that the large excursions in the recordings with prolonged feeding times represent pool feeding and that the smaller excursions with shorter feeding times represent capillary feeding. The relative availability and high pressures of blood in capillary feeding would permit much faster operation of the cibarial and pharyngeal pumps. A greater frequency of peaking in capillary feeding would be expected, and this indeed seems to be the case in the recordings (compare Figure 10 with Figure 6). The smaller peaking excursions during capillary feeding are possibly due to incomplete closing of the anterior pharyngeal valve (during contraction of the pharyngeal pump) as a result of

the pressure of inrushing blood from the capillary and the more rapid activities of the sucking mechanism. The electrolyte pathway is thus not as completely severed by the action of the anterior pharyngeal valve in capillary feeding as it is when there is a less abundant supply of blood and lower pressures in pool feeding.

The electrical resistance change during valve closure in capillary feeding is thus not as great as in pool feeding since valve closure is not as efficient. In pool feeding the cibarium and pharynx fill more slowly. Slower and more efficient valve closure results in greater electrical resistance changes during valve activity. The lower frequency of peaking, greater excursions of the peaks, and prolonged time of engorgement are characteristic of pool feeding, while higher frequency of peaking, smaller excursions of the peaks, and comparatively shorter feeding times are characteristic of capillary feeding in these recordings.

In order to further substantiate these interpretations, a model system was devised that could approximate the conditions under which a mosquito may exhibit pool feeding. The unshaven abdominal skin of a freshly killed mouse was stretched over one end of a glass cylinder (2 cm ID), 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. The skin on the bottom of the tube was placed in contact with a 100-mesh bronze screen covering a vessel containing female Aedes aegypti L. mosquitoes. 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 mouthparts. 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, a similar pattern should now be recorded.

Figure 12 shows a 100 mm/sec recording of an engorgement pattern made during this experiment. The general shape of the peak is similar to that 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 factors previously interpreted as characterizing pool feeding.

It is interesting to note the considerably greater displacement from the base line during 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. In the recording this appears as a greatly increased displacement from the base line.

Figure 13 shows a salivation pattern recorded in the in vitro system at a paper speed of 100 mm/sec. Here the salivation pattern is the exact reversal of the engorgement pattern. Two factors distinguish the salivation from the engorgement patterns. First, 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. Second, the closing of the anterior pharyngeal valve (which is invclved 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.

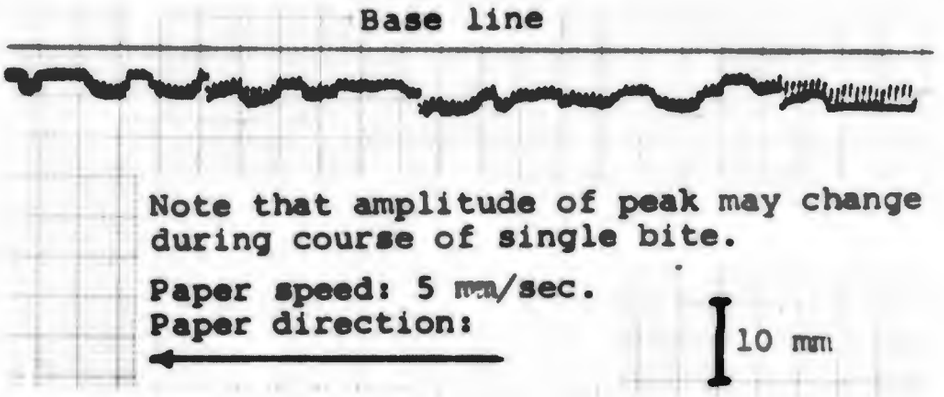


**Figure 11**

**CHART RECORDING OF A SINGLE MOSQUITO BITE WITH DIFFERENT SIZES OF EXCURSIONS**

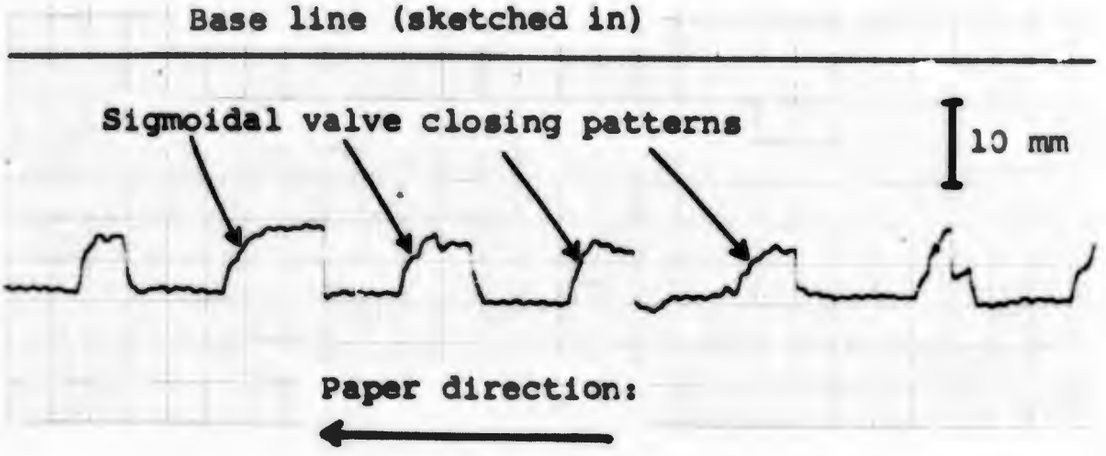


**Figure 12**

**CHART RECORDING OF MOSQUITO ENGORGEMENT IN VITRO AT A HIGH PAPER SPEED (Note amplitude of peak and sigmoidal shape of upward sweep in valve-closing patterns.)**

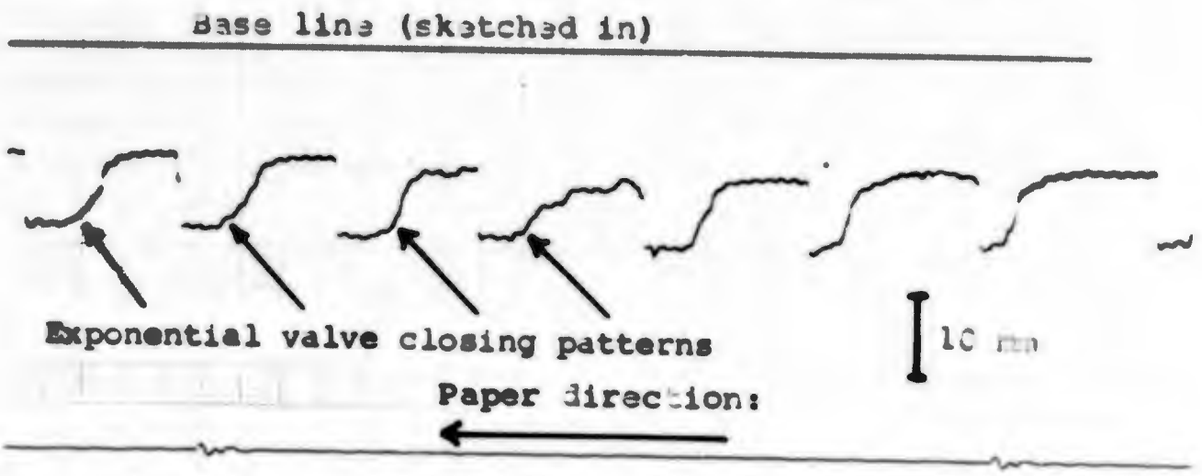


**Figure 13**

**CHART RECORDING OF MOSQUITO SALIVATION IN VITRO AT A HIGH PAPER SPEED (Note exponential shape of upward sweep in valve-closing patterns.**

In the recording of engorgement, the valve closure pattern shows an abrupt ascent to its maximum peak. This results in the sigmoidal-type appearance of 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 exponential-type closure pattern recorded for the salivary valve (compare Figures 12, 13, and 9).

The longer straight portion in the upper part of the salivation pattern (Figure 8) probably represents the period during which the salivary reservoir is being replenished while the salivary valve remains closed. The sudden explusion of saliva is represented by the abrupt downward sweep in the recorded pattern. The longer straight line portion in the lower part of the engorgement pattern (Figure 7) represents the period during which blood is flowing from the mouse through the mosquito's food canal and into the ciburium. The closing of the anterior pharyngeal valve servers the electrolyte (blood) pathway when the pharynx is full; this is represented by the abrupt upward sweep in the recorded pattern.

At a slower paper speed engorgement or salivation can be determined by the amount of time the writing stylus spends in the various positions. Thus the in vitro engorgement pattern at a paper speed of 5 mm/sec is seen as a series of rapid ascents from a base position (Figure 14), while salivation at a paper speed of

1 mm/sec (Figure 15) is seen as a series of rapid descents from a base position (compare also Figure 4, middle, and Figure 6, left side). Although the general form of these recorded patterns has been observed repeatedly, variations in the details of the tracings have occasionally appeared.

When a group of about 50 mosquitoes was placed in the container and permitted to bite simultaneously, the recorded pattern was very irregular, and there was a general obscuring of the characteristics of the individual bites (Figure 16). Also, the departure from the base line was greater than that seen in the single mosquito bite. This greater departure is expected, since the resistance between the mouse and the mesh during the biting of a group of mosquitoes is less than during the biting of a single mosquito.

However, the departure is not proportional to the number of mosquitoes biting. The first mosquito that bites changes the resistance of the system from an infinite to a finite value. Subsequent bites do not cause as large a change, and proportionality should therefore not be expected. In effect, when a group of mosquitoes bites, a number of miniature parallel circuits are set up between the mouse and the mesh. If the resistances of all the mosquitoes are equal, each biting mosquito should draw the same quantity of current. In terms of amperage then, proportionality and additivity might be expected as the number of parallel units (biting mosquitoes) in the circuit increases. We have not yet

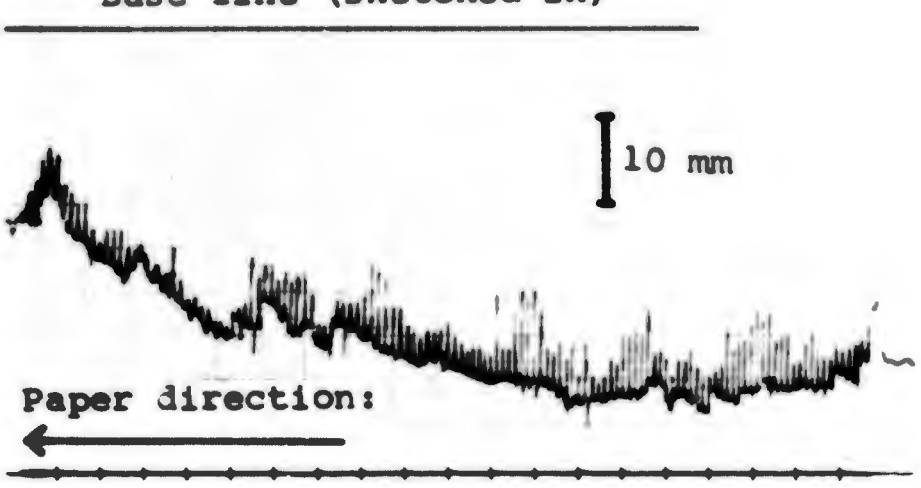


Figure 14

CHART RECORDING OF ENGORGEMENT IN VITRO
AT A PAPER SPEED OF 5 MM/SEC

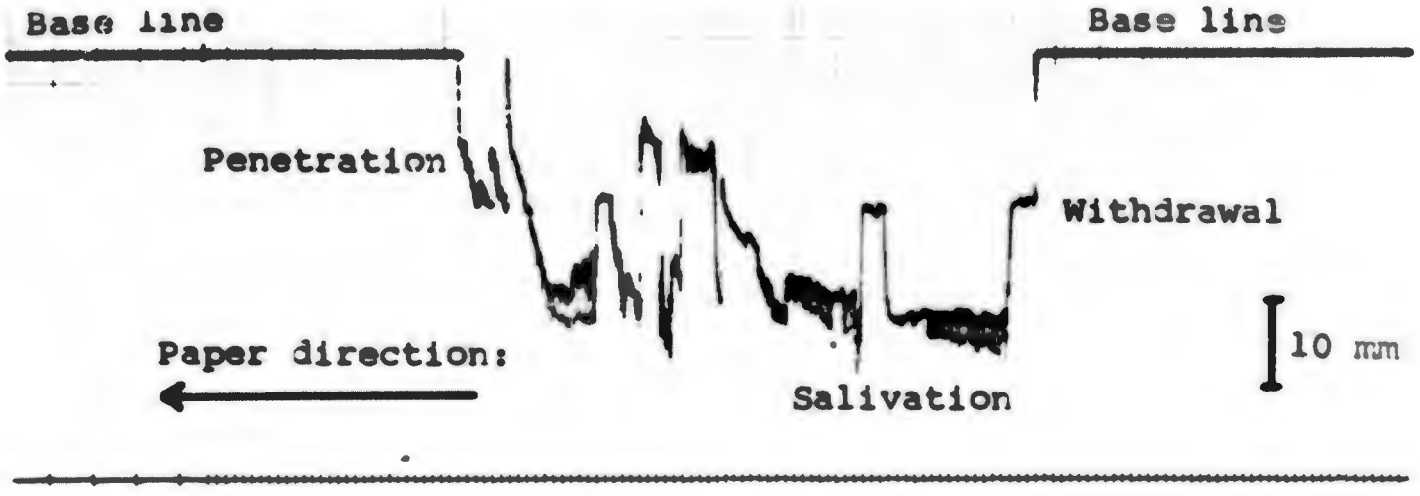


Figure 15

CHART RECORDING OF PENETRATION, SALIVATION,
AND WITHDRAWAL IN VITRO AT A PAPER SPEED OF 1 MM/SEC

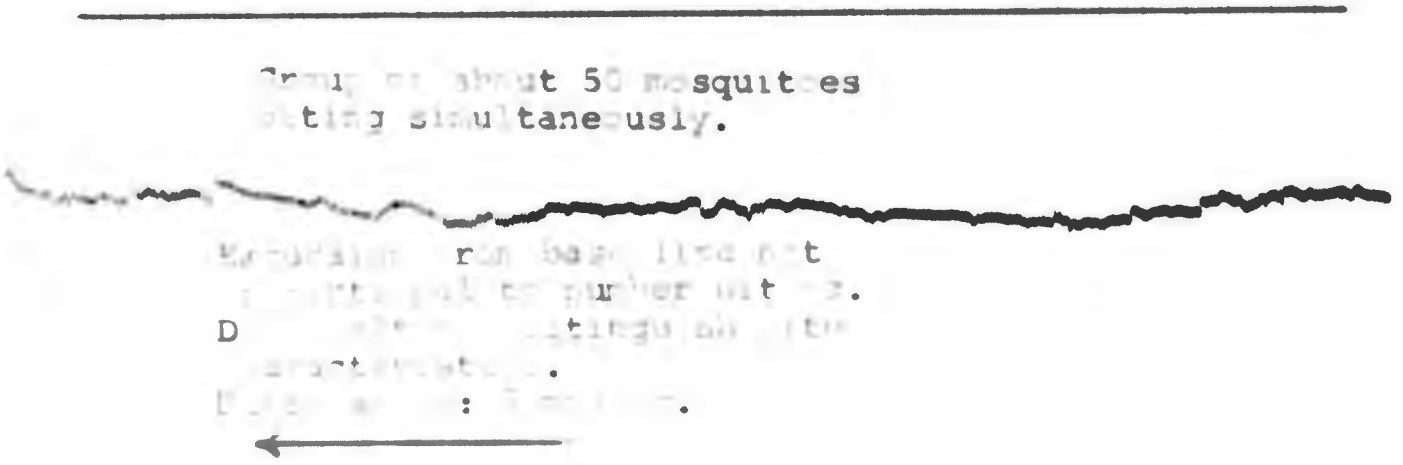


Figure 16

CHART RECORDING OF GROUP OF MOSQUITOES BITING SIMULTANEOUSLY

succeeded in quantifying the apparatus in terms of number of mosquitoes simultaneously biting, although certain parameters that have been utilized are probably valid criteria of repellency. These parameters are discussed later.

D. Activity Measurements

As a corollary to the electronic recording method for detecting mosquito biting, we attempted to determine whether it is possible, with a suitable electrical arrangement, to detect and record the 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-v 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 resistor-battery box 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 such that each vicinal group of three strands was oppositely charged. The leads were then connected through the recorder, as before, and mosquitoes were placed into the activity box.

As a result, when the mosquito moved or landed on the wire strands and bridged two oppositely charged strands with its legs, it closed the series circuit. 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 landed on the wire strands at least as frequently as on the Plexiglass sides, and they did not depart from the strands with any greater frequency than from the sides. This experiment indicates the feasibility of devising an electronic apparatus to measure and record insect activity without visual observation. Such a device could be used in automatically assessing mosquitoes' movements in response to a repellent or actractant substance and the duration of activity of such a substance. The Sanborn model 320 recorder is probably not the best instrument 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 time interval. This information can be stored and reproduced at any future time.

## E. The Development of a Systemically Effective Insect Repellent

### 1. Intravenous Injection of Repellents and Simultaneous Electronic Recording

In general, in order for a compound to exhibit repellent activity, it must be volatile. This view seems to be substantiated by our experiments and by those of others in which compounds with a low vapor pressure or a high molecular weight were found to be generally poor repellents. In previous reports on IITRI Project C222, 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 decreased. Whether this lack of repellent activity was due to the inability of repellent vapors to pass through the skin or to a metabolic alteration of the repellent molecule, rendering it nonrepellent, 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 on Project C222 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 mosquitoes was placed in a pint cardboard ice-cream container and 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 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. When radioiodinated serum albumin (RISA) was injected simultaneously with the repellent compounds, some mosquitoes were found to be

radioactive. Therefore not only probing but also engorgement took place after injection of the repellents. The mouse injected with allethrin died during the test, but the recorder patterns clearly showed that probing continued after the death of the mouse.

2. Diazotization of Repellents to Red Blood Cells

In another approach to the development of a systematically effective repellent, we attempted to chemically bond repellent molecules to circulating red blood cells. Precedent for such bonding by diazotizing various chemicals and protein antigens to red blood cells has been well established in the field of immunology (ref. 7-9). The red blood cell retains its stability and the antigen molecule retains its antigenic specificity after this treatment.

If a repellent molecule could be diazotized to a red blood cell, the large size of the red blood cell, compared with the size of the repellent molecule, may protect the molecule from enzymatic 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. It was not possible to predict what effect the chemical treatment of the red blood cells

would have upon repellency, and this work was undertaken to determine whether this approach would be fruitful.

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 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 Appendix I.

A study of the repellent activity of the compound is given in Table 6. 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. However, this result was not necessarily indicative of how diazotization or bonding of the amine to red blood cells would influence the repellent or engorgement-inhibiting properties of the compound. The method used for diazotizing the m-amino-N,N-diethylbenzamide and bonding it to sheep red blood cells is given in Appendix II.

Table 6

REPELLENT PROPERTIES OF m-AMINO-N,N-DIETHYLBENZAMIDE AS DETERMINED BY FEEDING ON MICE

| Concentration Applied to Mouse Belly, mg | Number Fed per Total | Average Percentage Fed |
|---|---|---|
| 0.1 | 30/52<br>36/53<br>18/55 | 52.5 |
| 1.0 | 5/45<br>18/51<br>20/52<br>36/53 | 39.3 |
| 10.0 | 6/52 | 11.5 |

A volume of sheep red blood cells equivalent to about one quarter of the total red blood cells present in a mouse (0.2 cc of packed sheep red blood cells) was reacted with m-diazo-N,N-diethylbenzamide and suspended in isotonic saline in a total volume of 0.5 cc. The suspension was injected intravenously into the tail vein of a mouse. After about 2 min, to allow for adequate circulation and distribution, the mouse was anesthetized, placed on a 50-mesh bronze screen covering a vessel containing 50 mosquitoes, and electronic recordings were made.

The recordings showed that the attack upon the mouse by the mosquitoes was almost immediate. Patterns of mosquito probings, salivation, and engorgement were evident throughout the entire recording period of 30 min, and 20% of the mosquitoes were found to have gorged to repletion in this time.

The results of these experiments show that neither high concentrations of circulating protein-bound repellents nor circulating free repellents are capable of preventing mosquitoes from biting. Vaporization thus indeed appears to be essential for repellency.

## F. Assay of Repellents with the Electronic System: Statistical Analysis

In view of the above experiments, the successful development of a systemic insect repellent will probably be largely dependent upon the discovery of a substance whose vapors are effective in repelling mosquitoes in extremely low concentrations. This substance should be reasonably stable to metabolic degradation, and either have an affinity for skin in itself, or be alterable by chemical manipulation to increase skin affinity. In the search for such a substance. the importance of defining all of a mosquito's responses to the potential repellent cannot be overestimated. The electronic method offers this capability.

The application of the electronic system to the screening of 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 include: Does the concentration of a repellent that prevents engorgement also prevent probing and salivation? If not, what concentration of repellent is necessary to prevent

probing and salivation? The time periods in which these compounds retain their repellent activity also can be monitored automatically.

Although the base line in the electronic recordings becomes more displaced as the number of mosquitoes biting simultaneously increases, the total displacement is not related in a simple way to the number of mosquitoes biting. A method was therefore developed to reduce the data obtained from the chart recordings into meaningful and realistic numerical terms, and to make them amenable to statistical analysis.

The compound to be tested for repellency was applied to the skin of anesthetized test mice, as described in previous reports on Project C222, and electronic recordings of mosquitoes' responses to the test compounds were made. The mosquitoes were exposed to the test mouse for 30 min.

In order to analyze the chart recordings made during that period, two parameters were utilized: the percentage of time that the recording was displaced from the base line during the 30-min period, and a measure of the displacement (in cm) from the base line taken every 10 cm in the chart recordings. A chart speed of 1 mm/sec was used for all of these recordings, and 18 displacement measurements were obtained for each test. Since the increment of displacement successively decreases as more mosquitoes bite, a more valid measure of this displacement in terms of the number of mosquitoes biting would be given by

Table 8 (cont.)

| Compound | Conc. on Mouse, mg | % of Time Displaced (P) | Standard Displacement Distance $\sqrt{D^2/n}$ (D) | % of Mosquitoes Engorged (E) | Significance at the 95% Confidence Level[a] |
|---|---|---|---|---|---|
| 1,2-Cyclopentane-acetic acid, N-sec-butyl- | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| 2-Naphthol-1,2,3,4-tetrahydro- | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | C | 0 | 0 | S |
| | 0.1 | 0 | 0 | 0 | |
| m-Dioxane, 5-ethyl-5-nitro-2-propyl[b] | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 54 | 0.47 | 1.9 | NS |
| | 0.1 | 85 | 0.48 | 4.0 | |
| Vanillic acid diethylamide | 1.0 | 43 | 2.4 | 18 | NS |
| | 1.0 | 54 | 2.1 | 26 | |
| N,N-Diethyl-2,6-dimethyl benzamide | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| Cyclopentaneacetic acid, -1-hydroxy-cyclohexyl ester | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 0 | 0 | 0 | S |
| | 0.1 | 0 | 0 | 0 | |

(D) for the control group was 1.1, while the mean percent engorgement (E) was 30%.

Table 8 shows the results obtained when these parameters were used to measure the mosquito repellency of various test compounds with the electronic method.

Discriminant function analysis was applied to these data by using a standard computational program (ref. 15). This analysis yielded a linear combination that maximized separation between the control group and the group of typical low-level treatment cases (0.1 mg/cc). The percent displacement (P) and the percent engorgement (E) could be used without the variable for standard displacement distance (D) as a simplified discrimination function, as illustrated in Figure 17. The line of best separation for the discriminant function is shown and represents those values of the discriminant function that give the least number of misclassifications.

The distribution of the discriminant function for the control group makes possible a test of significance of the mean of several independent observations with the same test compound.

The formula for the discriminant function is $114 - 79/\sqrt{n}$, where n is the number of observations. This formula can be applied for any number of observations of a test compound. In this case, two observations at the minimum dose were made for all test compounds used in plotting the points in Figure 17. The 95% confidence limit shown applies for the mean of the discrimination

Table 8 (cont.)

| Compound | Conc. on Mouse, mg | % of Time Displaced (P) | Standard Displacement Distance $\sqrt{D^2/n}$ (D) | % of Mosquitoes Engorged (E) | Significance at the 95% Confidence Level[a] |
|---|---|---|---|---|---|
| N,N,-Diethyl-p-isopropylbenzamide | 1.0 | 27 | 0.77 | 6.2 | S |
| | 1.0 | 13 | 0.74 | 5.6 | |
| Succinamide-N-amyl | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| Succinamic acid, N, N-diisopropyl-propyl ester[b] | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 98 | 0.82 | 34 | NS |
| | 0.1 | 80 | 1.03 | 12 | |
| Benzquinamide | 1.0 | 50 | 0.51 | 26 | S |
| | 1.0 | 24 | 0.54 | 11 | |
| 1,2-Cyclohexane-dicarboximide, N-isobutyl | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 27 | 0.31 | 0 | S |
| | 0.1 | 0 | 0 | 0 | |
| Methyl anthranilate | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| N,N-Diethyl benz-amide | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |

Table 8 (cont.)

| Compound | Conc. on Mouse, mg | % of Time Displaced (P) | Standard Displacement Distance $D^2/n$ (D) | % of Mosquitoes Engorged (E) | Significance at the 95% Confidence Level[a] |
|---|---|---|---|---|---|
| Allethrin | 1.0 | 0 | 0 | 0 | S |
| | 0.1 | 0.33 | 0 | 0 | |
| | 0.1 | 2.9 | 0 | 0 | |
| | 0.1 | 1.6 | 0 | 0 | S |
| | 0.1 | 23 | 0.21 | 0 | |
| | 0.1 | 0 | 0 | 0 | |
| | 0.1 | 0 | 0 | 0 | |
| | 0.01 | 52 | 0.70 | 9.5 | NS |
| | 0.001 | 38 | 0.68 | 0 | NS |

[a]The judgement of significance is based upon an average of the various determinations at the same concentration level. S denotes a significant difference from the control at a 95% level of confidence. NS denotes that the difference is not significant at the 95% level of confidence.

[b]These determinations were used in constructing Figure 17.

the square of the distances, thus weighing large displacements more than small ones. In order to minimize random variations in measurement and to obtain a more unified picture of repellent efficacy, the squares of the distances were added ($\Sigma D^2$), then divided by the number of observations, and a square root of the result taken. The resulting value, $\sqrt{\Sigma D^2/n}$, is related to a standard deviation, and is called the standard displacement distance (D) in this analysis.

It is possible that when repellent efficacy is high and when there are only sporadic and short-lived departures from the base line, the arbitrarily chosen measurement interval of 10 cm may not necessarily coincide with these departures. At such times, the value of $\sqrt{\Sigma D^2/n}$ would be zero. The percent deviation, however, would have a finite value, indicating that there was indeed some biting of the test mouse by the mosquitoes. Both these parameters, together with the known repellent concentration on the test animal, should yield a valid repellency profile for any test substance.

In order to test these methods, the data derived from the electronic recordings of a number of untreated control mice (used as controls during the repellent testing) are presented in Table 7. The control recordings show that in most cases the base line is displaced over 90% of the time during the period in which mosquito biting was monitored, with a mean percent displacement time (P) of 84% for the group. The mean standard displacement distance

Figure 17

SCATTER DIAGRAM SHOWING SEPARATION
BETWEEN CONTROLS AND LOW LEVEL TREATMENT CASES
(2 Observations)

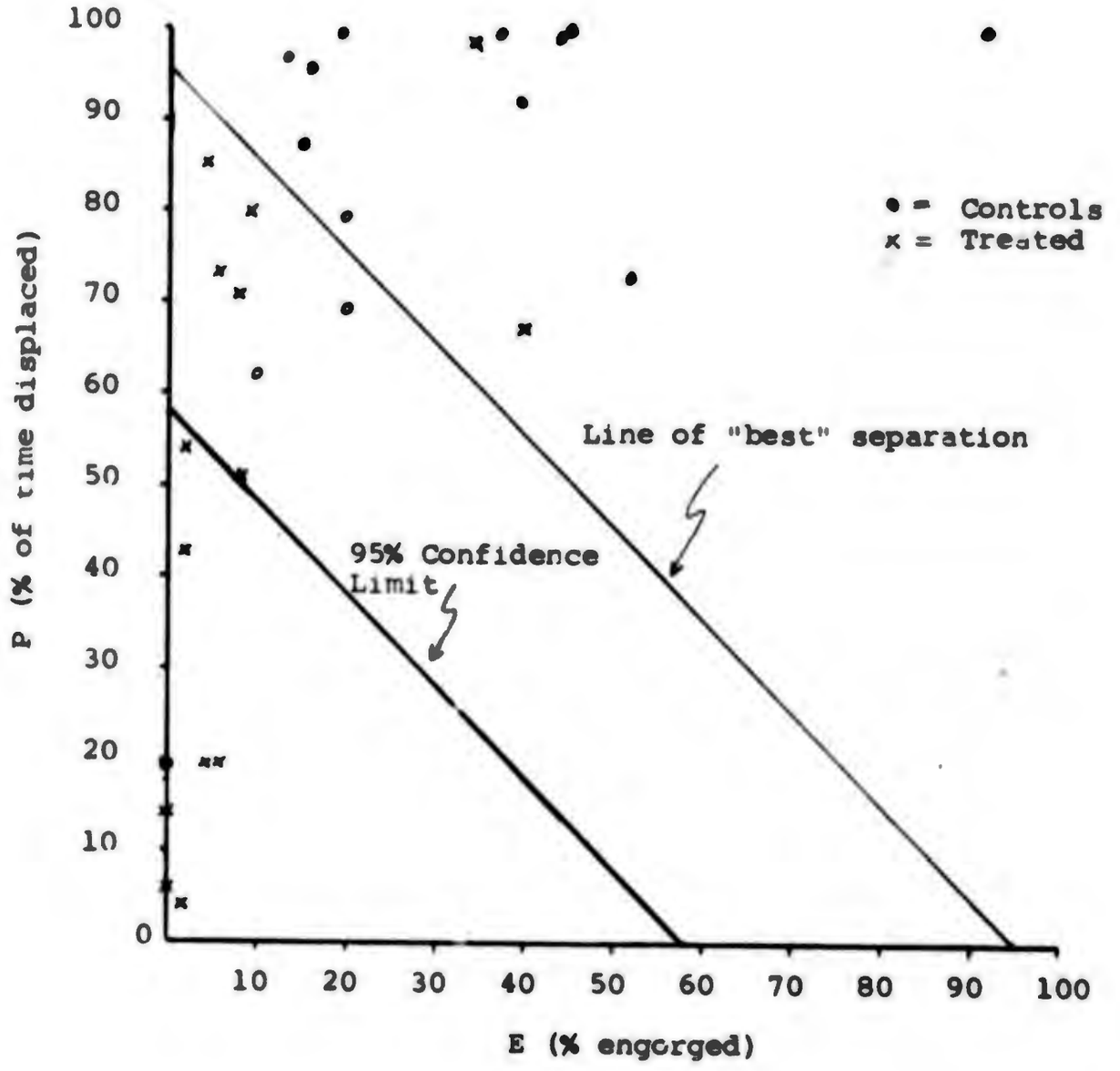

Table 8

ANALYSIS OF MOSQUITO REPELLENCY OF VARIOUS COMPOUNDS
BY THE ELECTRONIC RECORDING METHOD

| Compound | Conc. on Mouse, mg | % of Time Displaced (P) | Standard Displacement Distance $\sqrt{\Sigma D^2/n}$ (D) | % of Mosquitoes Engorged (E) | Significance at the 95% Confidence Level[a] |
|---|---|---|---|---|---|
| Benzyl benzoate | 1.0 | 80 | 1.3 | 39 | NS |
| | 1.0 | 36 | 0.4 | 10 | |
| Succinamic acid, N,N-dipropyl-, sec-butyl ester | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 64 | 0.59 | 16.3 | S |
| | 0.1 | 0 | 0 | 0 | |
| 2-Cyclohexylcyclohexanol | 1.0 | 16 | 0.090 | 0 | S |
| | 1.0 | 29 | 0.19 | 0 | |
| 4-Cyclohexene-1,2-dicarboximide, N-propyl | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| Succinamic acid, N,N-diisopropyl isopropyl ester | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 51 | 0.91 | 0 | NS |
| | 0.1 | 82 | 0.76 | 0 | |

Table 7

ANALYSIS OF ELECTRONIC RECORDINGS OF UNTREATED CONTROL MICE

| Control No. | % of Time Displaced (P) | Standard Displacement Distance $\sqrt{\Sigma D^2/n}$ (D) | % of Mosquitoes Engorged (E) |
|---|---|---|---|
| 1 | 92 | 0.82 | 39 |
| 2 | 100 | 1.7 | 45 |
| 3 | 95 | 1.6 | 16 |
| 4 | 87 | 1.6 | 15 |
| 5 | 99 | 1.2 | 44 |
| 6 | 96 | 1.5 | 13 |
| 7 | 100 | 1.4 | 92 |
| 8 | 19 | 0.17 | 0 |
| 9 | 69 | 1.2 | 20 |
| 10 | 73 | 0.95 | 52 |
| 11 | 62 | 0.87 | 10 |
| 12 | 99 | 1.4 | 19 |
| 13 | 99 | 0.44 | 37 |
| 14 | 79 | 0.52 | 20 |

Table 8 (cont.)

| Compound | Conc. on Mouse, mg | % of Time Displaced (P) | Standard Displacement Distance $D^2/n$ (D) | % of Mosquitoes Engorged (E) | Significance at the 95% Confidence Level[a] |
|---|---|---|---|---|---|
| N,N-Diethyl-m-isopropyl benzamide | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| Acetamide, N-amyl-alpha-butoxy[b] | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 73 | 1.3 | 6.2 | NS |
| | 0.1 | 43 | 0.63 | 2.0 | |
| 1,2-Cyclohexane-dicarboximide, N-sec-butyl[b] | 0.1 | 14 | 0.42 | 0 | S |
| | 0.1 | 5.5 | 0.11 | 0 | |
| Diethyltoluamide (DEET) | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 28 | 0.57 | 3.3 | S |
| | 0.1 | 0 | 0 | 0 | S |
| | 0.1 | 56 | 0.36 | 3.5 | |
| | 0.1 | 3.1 | 0 | 0 | |
| | 0.1 | 7.7 | 0.059 | 0 | |
| | 0.1 | 18 | 0.40 | 2.2 | |
| | 0.1 | 16 | 0 | 0 | |
| | 0.01 | 98 | 1.3 | 56 | NS |
| | 0.001 | 98 | 2.04 | 51 | NS |

Table 8 (cont.)

| Compound | Conc. on Mouse, mg | % of Time Displaced (P) | Standard Displacement Distance $\sqrt{\Sigma D^2/n}$ (D) | % of Mosquitoes Engorged (E) | Significance at the 95% Confidence Level[a] |
|---|---|---|---|---|---|
| 4-Cyclohexene-1,2-dicarboximide, N-isobutyl[b] | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 71 | 0.87 | 7.7 | S |
| | 0.1 | 3.7 | 0.072 | 2.0 | |
| Cyclohexanol-2-phenyl | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| Propionanilide, N-butyl | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 72 | 0.035 | 0 | S |
| | 0.1 | 0 | 0 | 0 | |
| Bicyclo(2'2'1)-5-heptene-2,3-dicarboximide, N-amyl-[b] | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 19 | 0.59 | 5.8 | S |
| | 0.1 | 19 | 0.57 | 4.3 | |
| Emivan | 1.0 | 82 | 0.96 | 26 | NS |
| | 1.0 | 97 | 0.96 | 47 | |
| N,N-Diethyl-m,n-propylbenzamide[b] | 1.0 | 0 | 0 | 0 | S |
| | 1.0 | 0 | 0 | 0 | |
| | 0.1 | 67 | 1.09 | 40 | NS |
| | 0.1 | 51 | 0.45 | 8.7 | |

function for these groups. If a larger number of observations are considered, as was the case for allethrin and DEET (Table 8). the discriminant functior changes as the square root of n changes in the above formula.

The discrim'nant function is 0.13281P + 0.13729E, where P is the percent of time displaced and E is the percent engorgement. If the mean of this function in the two observations is less than 0.078, there is a significant deviation from the control group at the 95% confidence level. This threshold level for the discriminant function can be simplified tc give $P + E \gtrsim 58\%$ for the 95% confidence limit. Table 8 lists the significances of the differences from the control at the 95% confidence limit. Table 8 also shows that at concentrations of 0.01 and 0.001 mg/ml the repellent power of allethrin is not significantly different from the control at the 95% confidence level. This information necessitates a revision of previous estimates of the repellency of this substance.

A larger number of observations on the same compound would cause the threshold for significance to rise. The methods of statistical experimental design will be used to determine the sample sizes required for the detection of significant differences at different confidence levels, or where greater precision for deviations from the control group are required. Further work remains to be done on the analysis of repellents and the statistical evaluations.

## IV. DISCUSSION AND FUTURE WORK

Results obtained with the electronic recording method have indicated that a repellent must vaporize from the treated surface in order to be effective. The most potent repellent encountered in these studies is allethrin, and even this compound, coursing through the blood vessels and superficial capillaries of skin in massive and lethal quantities, conferred no protection against mosquito bites. The successful development of a systemic insect repellent may be largely dependent upon the discovery of a substance whose vapors are effective in extremely low concentrations. This substance should either have an affinity for skin or be alterable by chemical manipulation to increase skin affinity. On the other hand, a substance that has affinity for skin would necessarily be retarded in its vaporization from skin. Thus one of the requirements for a successful systemic insect repellent is the very factor that may limit its usefulness. The search for a repellent that is sufficiently effective in small enough quantities so that these difficulties may be overcome should not be abandoned, since this approach represents one of the alternatives available.

The other approach that should be pursued is that of suppressing the emanation of natural attractants from the skin. When an established surface repellent such as diethyltoluamide (DEET) is used to cover a defined area of human skin, mosquitoes are not inhibited from landing and biting a few millimeters from the boundary of the DEET application. The question therefore arises whether

DEET or any repellent is actually actively repellent or simply renders the treated area nonattractive. Nonattractiveness could be conferred by masking attractive effluents or by lowering the partial pressure of skin effluents that are attractive to insects. In this connection, Gouck and Bowman (ref. 10) measured the difference in carbon dioxide and moisture evolution from repellent-treated and untreated arms of human subjects. These authors showed that retardation of carbon dioxide evolution has little relation to the action of repellents. Since carbon dioxide has long been thought to be an activator of mosquitoes and not in itself attractive (ref. 11), these results are not surprising. Gouck and Bowman did not study the effects of repellents on other skin effluents. Such a study may be basic to the success of this program.

This proposed line of investigation would involve a study of emanations from human and other mosquito hosts under carefully controlled experimental conditions, with a view toward defining and chemically characterizing those emanations that attract mosquitoes. Since differences of attractiveness within and among animal species have long been recognized, attractive effluents could probably be altered within physiological limits. A knowledge of the chemical properties of these attractants could lead to the synthesis of tailor-made retardants for attractive effluents.

The levels of retardant substances required systemically to reduce attractive emanations would probably be considerably lower than levels of systemic repellents required to exude from the skin in effective concentrations. Also, a study of the biochemical and metabolic events leading to the production of these emanations could eventually provide a basis for inhibiting their production.

A technique for studying human effluents presently being used at IIT Research Institute involves the use of a specially designed all-glass apparatus capable of holding normal size adults. The effluents given off by the subjects are collected in a planned trapping sequence and analyzed by multidetection gas-chromatographic techniques. Similar or identical techniques could become an important part of this program. Since the environmental atmosphere is carefully defined, the data can be evaluated accurately. The new electronic assay procedure will be an important tool in characterizing mosquitoes' responses to various fractions of human or animal effluents.

Further statistical analysis of the recorded data will be made to discover the possibility of relationships between biting and other parameters not considered here, such as duration of exposure, number of mosquitoes exposed in the test situation, etc. Many factors that influence mosquito behavior are now accessible to study in a more analytical and critical fashion.

Little information is available in the literature regarding the biochemical or physiological basis upon which disease-transmitting capability is dependent. Not only does species specificity exist in terms of disease vectors, but even within the same species some strains are more potent transmitters of disease than others (ref. 12,13). As an approach to this problem, the electronic method can be utilized to study the differences between the bite characteristics of vector and non-vector mosquitoes. The fact that an infection such as dengue has been found not to be conveyed, or to be conveyed only with great difficulty by an infected mosquito whose feeding was interrupted (ref. 14), implies that there may be some feeding characteristics of the mosquito that are significant in disease transmission. In studies of the electronic recordings, just before withdrawal from the host, considerable salivation has been noted repeatedly. Upon interrupting the feed, this terminal salivation was abolished.

A survey of the biting characteristics of various mosquito species may yield clues to determining vector requirements. If salivation is indeed the key to disease transmission, it may be possible to screen for mosquitoes showing little or no salivation during biting in the recorded patterns. Areas where mosquito-borne diseases are prevalent may then be populated with males and females of these selected strains. The resultant genetic heterogeneity may considerably decrease the number of insects

capable of being good vectors of disease. Since these selected strains should have no survival disadvantage, introduction of such heterogeneity would probably be permanent and irreversible.

Alternatively, since a practical method of detecting mosquito salivation has been developed, the possibility exists that a compound that inhibits salivation may be discovered. Future screenings of compounds will be conducted with a view toward determining possible salivation-inhibiting properties.

## V. SUMMARY

1. Chemicals that stimulate mosquito engorgement in vitro were investigated, but no unified picture of chemical structures that stimulate mosquito engorgement has yet emerged. Studies of permeability of skin to potential repellents were initiated.
2. An electronic method for detecting mosquito biting and salivating activities in the skin of a host has been devised. Electronic recordings were interpreted in terms of valve activity during salivation and blood sucking.
3. A statistical approach to the interpretations of chart recordings made during repellent testing has been devised. Confidence limits for repellent testing have been established, and all future tests of repellents will be reported in terms of these confidence

limits. Other relationships will also be sought in further statistical analyses.

4. Other approaches to the development of a systemically effective insect repellent as well as new approaches to the prevention of the dissemination of arthropod-borne disease, have been indicated.

The future experimental program will be concerned with several interrelated lines of investigation.

1. Screening of chemical compounds with the electronic recording method. Experiments will be conducted to establish repellent properties of these compounds, to seek common chemical or physical properties that appear to confer repellency, and to determine the effects of these compounds on engorgement and salivation.
2. The study of animal and human emanations to establish their attractive characteristics and chemical identity. On the basis of this work, likely chemical candidates that may be ingested or otherwise systemically administered and that may alter or inhibit these emanations will be investigated.
3. Characterization of the biting profiles of different species and strains of mosquitoes to determine relationships between biting characteristics and the ability to transmit disease.

The screening of additional compounds and the study of skin effluents will be undertaken with a view towards establishing a rationale and molecular mechanism for the stimulation and inhibition of the biting drive of insects. Though current information permits only an empirical approach to this critical problem, it is hoped that further studies will lead to findings of practical as well as theoretical value.

PUBLICATIONS RESULTING FROM CONTRACT
FROM NOVEMBER 1, 1964, THROUGH OCTOBER 31, 1965


1. Kashin, P. and Wakeley, H., "An Insect 'Bitometer'," Nature, 1965, in press.

2. Kashin, P., "Electronic Recording of the Mosquito Bite," J. Insect Physiol., 1965, in press.

3. Kashin, P., "The Electronic Recording of Mosquito Biting Activity," oral presentation given at University of Notre Dame, Department of Biology, South Bend, Ind., June 21, 1965.

4. Kashin, P. and Hawrylewicz, E. J., "Electronic Recording of Mosquito Biting," film and slide presentation given at Institute for Dermatologic Communication and Education, Pacific Dermatologic Association, Portland, Oregon, July 27, 1965.

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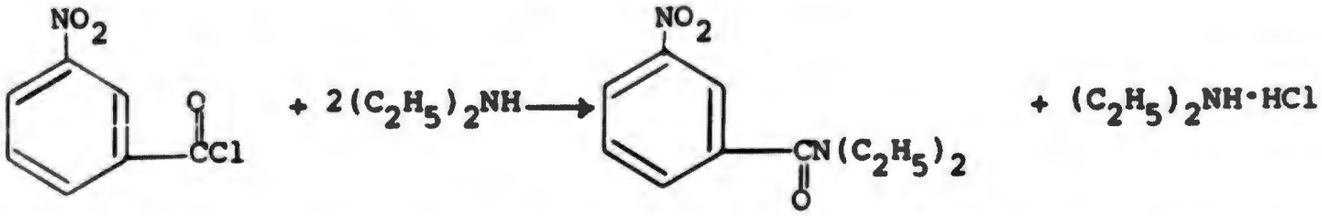
## APPENDIX I

## PREPARATION OF m-AMINO-N,N-DIETHYLBENZAMIDE

Preparation of N,N-Diethyl-m-nitrobenzamide

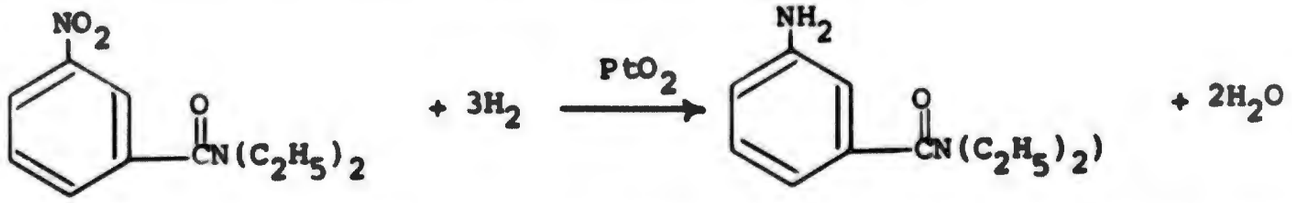


First, 18.56 g of m-nitrobenzoyl chloride (0.10 mole) dissolved in 200 ml of benzene was placed into a 500-ml 3-necked round-bottom flask fitted with a dropping funnel, mechanical stirrer, and reflux condenser. Then 15.4 g of diethylamine (0.21 mole) dissolved in 50 ml of benzene was added dropwise over 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 crude 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, 11.1 g of N,N-diethyl-m-nitrobenzamide (0.05 mole) was dissolved 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 66 to 88°C.

| Analysis: | Calculated for $C_{11}H_{16}N_2O$ | Found (1) | Found (2) |
|---|---|---|---|
| | 68.71 | 69.11 | 69.05 |
| | 8.39 | 8.49 | 8.63 |
| | 14.57 | 14.16 | 13.99 |

Infrared analysis showed this preparation to be essentially the desired product, although the CH out-of-plane bending bands of the aromatic ring indicate positional isomers other than the meta.

## APPENDIX II

## PREPARATION OF m-DIAZO-N,N-DIETHYLBENZAMIDE AND BONDING TO RED BLOOD CELLS

First, 1.0 g of m-amino-N,N-deithylbenzamide was dissolved in 100 ml of $H_2O$ containing 3.0 ml of 6 N HCl at 0°C. An aqueous solution of 0.35 g $NaNO_2$ was then added slowly with stirring. Addition of the $NaNO_2$ solution was stopped when starch-iodide test paper turned blue within 30 sec after the application of a drop of the reaction mixture. The reaction solution was yellow at this point. A little urea was added to destroy any excess $HNO_2$. The solution was quickly frozen in a dry ice-acetone bath and stored in the freezer until used. This solution contained m-diazo-N,N-diethylbenzamide.

Bonding of m-diazo-N,N-diethylbenzamide with washed sheep red blood cells proceeded as follows. To 7.0 cc of 0.11 M phosphate buffer, pH 7.38, was added 0.5 cc of the diazotized amine. Then 2.0 cc of this mixture was added to 0.2 cc of packed, washed sheep erythrocytes. The cell suspension was mixed gently and allowed to stand at room temperature for 10 min. After this time, the suspension was centrifuged in the cold at 1500 rpm for 10 min, and the supernatant was discarded. The cells were washed with centrifugation twice, 10 cc of saline was used in each washing. The cells were then suspended in saline to a total volume of 0.5 cc and taken up into a syringe.

A mouse was injected intravenously in the tail vein with the treated red blood cells, anesthetized after about 2 min, and tested for mosquito repellency.

In microscopic appearance the treated red blood cells were indistinguishable from normal sheep red blood cells.