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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Annual Progress Report

November 1967

Philip Kashin
Life Sciences Research Division

U.S. Army Medical Research and Development Command
Office of The Surgeon General
Washington, D.C. 20315

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ABSTRACT

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

The objective of this program is to develop insect repellents that can be administered systemically, preferably orally.

During this year the evaluation of compounds for mosquito repellency by the electronic recording method continued, and the results were statistically analyzed for significant differences from control values in a specialized digital computer program. A new electronic "bitometer" that facilitates the compilation of laboratory repellency data was developed.

Work continued on a hypothesis developed during the course of this work that could explain the physiochemical basis of a mosquito's attraction to warm-blooded hosts. Gamma-aminobutyric acid (GABA) was found in aqueous extracts of mosquito heads and bodies, and it was hypothesized that the interactions of GABA with with carbon dioxide, heat, and water vapor form the basis of mosquitoes' attraction to hosts. Evidence supporting the validity of the hypothesis was obtained from chemical studies of the interactions of GABA with carbon dioxide, correct predictions of chemical structures that should repel mosquitoes, and direct in vivo physiological investigations.

FOREWORD

This is Report No. IITRI-L6021-12 (Annual Progress Report) on IITRI Project L6021, entitled "Development of an Orally Effective Insect Repellent." The report covers the period from November 1, 1966, through October 31, 1967.

This project is being sponsored by the U.S. Army Medical Research and Development Command, Office of The Surgeon General, Washington, D.C. 20315, under Contract No. DA-49-193-MD-2281 and is being conducted by IIT Research Institute, Technology Center, Chicago, Illinois 60616. Previous work under this contract was conducted by IIT Research Institute from May 1, 1962, through October 31, 1966.

The project leader for this program is Mr. Philip Kashin, and the work is directed by Dr. E. J. Hawrylewicz. Technical assistance during this report period was provided by Mr. Clarence E. Boyle. The electronic circuitry of the new mosquito bitometer-timer described was designed by Mr. Blayne Arneson. The amino acid analysis on the mosquito extracts was performed by Mr. Anthony M. Gross. The compounds described in Appendix A were synthesized by Mr. Karl Rosemann. The statistical analyses of the electronically recorded repellency test data were performed by Mr. Merl L. Kardatzke, who also devised the computer program for determining the repellency index and the statistical confidence limits for the test compounds. Helpful suggestions and discussions for the physiological phases of the work were contributed by Dr. William F. Danforth, Biology Department, Illinois Institute of Technology.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

The citation of any trade names in this report does not constitute an official indorsement or approval of the use of such commercial hardware or software.

All data are recorded in IITRI Logbooks C17088, C17259, C17189, C17495, and C17599. The electronic chart recordings and the computer-output sheets also form part of our permanent records.

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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

I. INTRODUCTION

The objective of this program is to develop an orally effective insect repellent. Such a repellent could be more easily used and could afford more uniform and long-lasting protection than conventional topically applied repellents. Success in this undertaking could result in a significant reduction of human suffering from diseases and discomfort caused by the bites of insects.

The experimental rationale and approaches followed in the work during this report period were largely established in previous years (ref. 1,2). The research effort this year was essentially a continuation, consolidation, and, in some cases, modifications of these approaches.

Further experimental observations were made to test the validity of the gamma-aminobutyric acid (GABA)-carbon dioxide (CO_2) hypothesis outlined in previous reports (ref. 2). We recently obtained preliminary in vivo physiological confirmation of this hypothesis, as discussed in Section II.

We continued to test repellents that are GABA analogues to obtain further information on the chemical structures that are repellent to mosquitoes. The rationale presented in Section III is constructed on a physiological basis and could unify our views on the general molecular configurations required for compounds that seem to be generally repellent to mosquitoes.

We modified our computer program so that the confidence limits for repellency levels of test compounds are more easily compared in terms of efficacy ranking. The modifications are described in Section IV.

We designed, constructed, and made pilot studies on a new electronic recording instrument that is considerably more economical and efficient to use for purposes of repellency testing than the Sanborn model 320 recorder presently employed in those studies. The new instrument, also described in Section IV, has the sensitivity required to detect mosquito biting, can be easily fabricated, and can make the electronic recording method for repellency testing more readily accessible to other laboratories. Three of these instruments have been constructed in our laboratories.

Section V outlines a future-work program that should be pursued to logically extend our findings to achieve the ultimate goal of developing an orally effective insect repellent.

II. GABA HYPOTHESIS

A. Amino Acid Analyses of Mosquito Extracts

An amino acid analysis of water-soluble, dialyzable, whole-body extracts of mosquitoes (A. aegypti) was reported in our previous Annual Progress Report (ref. 2). This analysis showed that a high level of GABA is present in these extracts.

Further amino acid analyses were performed on water-soluble dialyzable mosquito extracts to confirm the presence of GABA as well as to obtain a rough estimate of its distribution between the heads and the bodies of the insects. The results, shown below, were calculated on the basis of 500 heads and bodies.

Gaba Content, μM	
Heads	Bodies
0.068	0.168
0.016	0.256 } Same extract
	0.280 }

It is apparent that the GABA content of the extracts is variable, although the existence of GABA in both the head and the body of A. aegypti is confirmed. Since the parameter of feeding was not controlled for these insects, the variability may have been due to the nutritional state of the mosquitoes when the extracts were made. Although none had access to a blood meal, some had ad libidum access to a 10% sucrose solution until the time of extraction. Auclair (ref. 3) found that the free amino acids extractible from the haemolymph of the German cockroach was influenced by diet, and Villeneuve (ref. 4) showed that the titers of haemolymph free amino acids of Agria (Pseudosarcophaga) affinis raised on pork liver were significantly higher than those of the free amino acids of the haemolymph of larvae raised on an artifical, synthetic diet.

If a starved mosquito has less free GABA associated with its nervous structure (this assumption is reasonable, since the free amino acid pool during starvation is expected to be depleted), the amount of GABA that would have to be bound by CO_2 to activate the insect would be less, since less inhibitor would be available, and very little further depletion of the inhibitor could cause activation. This response is obviously advantageous to the starved insect, since the insect would be much more sensitive to the presence of a potential host and thus more "avid" in terms of host-seeking behavior. Therefore if the ratio of CO_2-bound GABA to free GABA must exceed a certain proportion before the insect is activated, this ratio is reached or exceeded more quickly in a starved insect than in one that is in a good nutritional state, i.e., has recently had a blood meal. This reasoning corresponds with what is known to be true of mosquito behavior in terms of responsiveness to CO_2 before and after a blood meal. It is difficult to activate fully gorged mosquitoes. Furthermore, for purposes of repellency testing, mosquitoes that have been starved for at least 24 to 48 hr prior to testing are always more sensitive indicators of repellency.

The above reasoning leads to the possibility that an optimum balance that would result in the most efficient orienting and host-finding movements may exist between the heat, the water vapor, and the CO_2 emanating from a natural host. Therefore the relative gradients of CO_2, water vapor, and heat may be an important determinant of host preference by mosquitoes, and the effective ratios of CO_2-bound GABA and free GABA that cause inhibition and activation may differ from one mosquito species to another. Further work should shed light on these interrelationships.

The finding that GABA is present in the body as well as in the head is of interest. The main site of the exchange of respiratory gases in an insect is the trachea, which open to the air via the spiracles. These openings permit free passive diffusion of gases in and out of the insect's body and thus permit gas exchange to take place in the open circulatory system of the insect. The trachea obviously has an intimate association with atmospheric conditions, and an activating mechanism such as the one proposed would be advantageously situated in these structures, as well as in the sensory organs of the head.

B. Binding of CO_2 by Amines

The GABA hypothesis as originally presented (ref. 5) stated that GABA combines with CO_2 and that the GABA-CO_2 complex does not possess the synaptic inhibitory power of GABA alone. It was further stated that GABA, which is known to intermediate synaptic inhibition in nervous structures of animals in which it is found, may also intermediate synaptic inhibition in a mosquito. It was hypothesized that the formation of a GABA-CO_2 complex alternatively inhibits and activates mosquitoes, depending upon the temperature and the CO_2 tension of the environment. The interactions of GABA with CO_2, heat, and water vapor were theorized to form the basis of a mosquito's attraction to a host. The hypothesis was strengthened on the basis of later evidence that showed that the GABA-CO_2 complex is highly reversible and extremely sensitive to temperatures in the mammalian temperature range and that water vapor is essential for the initial complex formation (ref. 2).

An essential point in our hypothesis was that the binding of CO_2 by GABA must be easily reversible and sensitive to temperature. We have shown (ref. 2) that at a constant temperature, the complex spontaneously dissociates and that after 15 min only about one-fifth of the carbamino compound remains as compared to a solution that stands 1 min before assay.

It is of interest to explore the literature to determine whether the results of other investigators correspond to our findings in terms of CO_2 binding by amines and to obtain a clearer understanding of the binding of CO_2 by amino groups in physiochemical terms.

The association of CO_2 with amino groups in solution to form carbamates has been long and intensively studied because of its importance in the transport of CO_2 in the blood. Certain facts, which have been revealed by these studies, are summarized by Edsall and Wyman (ref. 6) essentially as follows:

(1) CO_2 reacts with ammonia, amines, amino acids, and proteins. The following equation represents the reaction of CO_2 with amino acids:

$$H_2N\text{-}R\text{-}COO^- + CO_2 \rightleftarrows {}^-OOC\text{-}HN\text{-}R\text{-}COO^- + H^+$$

CO_2 reacts only with uncharged amino groups. An amino acid must be in the anionic form for the reaction to occur.*

(2) The reactive species is molecular CO_2, not carbonic acid or any of its ionization products.

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*It is of interest to note in this context that Edwards and Kuffler (ref. 42), have shown that the amino and the carboxyl end groups are essential for inhibitory activity. The combining of CO_2 with the amino group can thus modify the chemical characteristics of the GABA molecule in such a way that it can no longer function as a neuroinhibitor.

(3) The net reaction is exothermic. A rise in temperature therefore causes the carbamino compound to dissociate into the free amine and CO_2.

(4) Carbamino compounds are decomposed in acid solutions but are stable in basic solutions.

(5) Calcium and barium salts of carbamino compounds are soluble in water. Therefore the free carbonate can be precipitated by the addition of a barium or calcium salt and the carbamino compound remains in solution.

Our assays of CO_2-bound GABA were based on the last fact (ref. 2).

A number of Scandinavian workers recently active in the study of carbamino formation have described the reversibility of these complexes with time at a constant temperature. The reversibility of a carbamino complex with amino acids at a constant temperature was shown in detailed studies of Jensen and Faurholt (ref. 7). Olsen et al (ref. 8) and Jensen et al (ref. 9) studied the carbamates of the alkylamines, and Jensen et al (ref. 10) studied carbamate formation in amino alcohols. In all cases, reversibility was a characteristic of the reaction and the complexes were practically entirely destroyed with time. The carbamino compounds were gradually replaced by a stable equilibrium in which bicarbonate and carbonate ions were produced. Therefore the fact of spontaneous reversibility appears to be firmly established.

We then explored the question of the physiochemical aspects of the effect of temperature in the binding of CO_2 to amines. It is known that the only form of an amino acid that reacts with CO_2 is the anion $H_2N\,R\,COO^-$, or, more simply R^- (ref. 6,11).

Therefore:

$$H_2N\text{-}R\text{-}COO^- + CO_2 \rightleftarrows {}^-OOC\text{-}HN\text{-}R\text{-}COO^- + H^+ \qquad (1)$$

Or:

$$R^- + CO_2 \rightleftarrows AM^{--} + H^+ \qquad (2)$$

where Am^{--} represents the carbamino compound.

The equilibrium constant is:

$$K_{Am} = \frac{(Am^{--})(H^+)}{(R^-)(CO_2)}$$

Pinsent et al (ref. 12) studied the total heat evolved in the following successive reactions:

$$NH_3 + CO_2 \rightarrow H_2H\text{-}COO^- + H^+ \quad (3)$$

$$H^+ + NH_3 \rightarrow NH_4^+ \quad (4)$$

They found that ΔH of the overall reaction is -9.0 kcal/M at 0°C, -12.7 kcal/M at 20°C, and -15.6 kcal/M at 40°C. For Reaction 4, ΔH = 12.5 kcal/M ($\Delta Cp = 0$) at 25°C. Substracting the ΔH of Reaction 4 from that of the overall Reaction results in a ΔH for Reaction 2 of approximately +3.5 kcal/M at 0°C, 0 kcal/M at 20°C, and -3 kcal/M at 40°C (ref. 13). The ΔH in the overall Reaction in the formation of the carbamino compound is therefore very low and extremely sensitive to temperature changes. On physiochemical grounds, the postulated interactions of temperature with CO_2 and GABA are therefore quite realistic.

Reactions 3 and 4 were performed in aqueous solutions. In the tissue, the H^+ released from GABA when GABA forms a complex with CO_2 is probably taken up by tissue proteins, as well as by other GABA molecules.

In terms of mosquito behavior, if the released H^+ combines with an amino group in the tissue to form $-NH_3^+$, the above relationships probably hold, as for NH_4^+ formation. If the temperature increases as the mosquito approaches a host, the ΔH of the isolated carbamino reaction decreases. It reaches zero at about 20°C and becomes slightly negative thereafter.

On the other hand, if the released H^+ combines with a $-COO^-$ group in the tissue, a relatively large positive entropy change occurs. In carboxylic acid ionizations, heat effects are negligible and entropy changes are the most important contributions to free energy. Therefore both entropy changes and heat exchanges may occur. The entropy terms should therefore probably also be considered in carbamino complex formation and destruction, however it is likely to be negligible at physiological pH.

The following constants for GABA at 25°C are also of interest (ref. 14):

Constant	Values for GABA	
	pK_1	pK_2
pK_A	4.031	10.556
ΔF°, cal/M	4,400	14,400
ΔH°, cal/M	405	12,070
ΔS°, cal/deg/M	-17.1	-7.8
ΔC_p, cal/deg/M	-34	-5

The ΔH of formation of GABA $(NH_3)^+$ is not very different from that of GABA $(NH_4)^+$, and similar thermodynamic relationships probably exist. We could not find any references to the ΔH of formation of the carbamates of amino acids and therefore cannot carry out the calculation for GABA. Although the net reaction is exothermic, the actual formation of the carbamate appears to be endothermic at low temperatures and exothermic at high temperatures. Therefore the ΔH of formation and decomposition of a GABA-CO_2 complex is such that the equilibrium is extremely responsive to small temperature changes within the physiological range. We have experimentally shown that this is indeed the case (ref. 2). The GABA-carbamino complex is precipitously decomposed between 20 and 40 C.

Pinsent (ref. 12) also determined the kinetics of Reaction 3. He found that the velocity constant $(k') = -d(CO_2)/dt\,[(CO_2)(NH_3)] = 74\ M^{-1}\ sec^{-1}$ at 0°C and $1130\ M^{-1}\ sec^{-1}$ at 40°C. Therefore decomposition is very rapid as temperature increases.

It is also of interest to note that at 18°C, CO_2 reacts with alpha-alanine $(k' = 10^{4.82})$ approximately 1.5 times slower than with beta-alanine $(k' = 10^{5.04})$ (ref. 7). On electrostatic grounds this fact is not surprising. A new negatively charged COO^- group is formed by the reaction of CO_2 with the amino group. Electrostatic work must be done against the repulsion of the negatively charged carboxyl group already present. These similarly charged groups are much closer together in alpha-alanine than in beta-alanine; therefore the reaction would be expected to be slower with the alpha amino acid than with the beta form. It can be safely presumed on these grounds that CO_2 reacts very rapidly with GABA, since the distance between the carboxyl and the amino groups is comparatively great.

Stadie and O'Brien (ref. 15) also showed that the velocity of carbamate formation depends on the concentration of free CO_2. Therefore in a relatively high CO_2 environment, the GABA-CO_2 complex would probably form very rapidly.

C. Modification of GABA Hypothesis

Further considerations of the GABA hypothesis in the light of other investigations have led us to believe that a modification of the originally stated hypothesis may be in order.

Various investigators (ref. 16-18) have shown that certain neuromuscular preparations of crayfish respond to applications of glutamate with large depolarizations caused by activation of the excitatory synaptic membrane. Glutamate was also shown to have excitatory effects in neuromuscular preparations of insects. Kerkut et al (ref. 19) have shown that a profused cockroach leg preparation gives an increased contraction after the addition of acetylcholine, L-glutamic acid, D glutamic acid, and L-aspartic acid. It was further shown that GABA causes a marked inhibition

of the contraction; the inhibitory effect is quickly reversible with washing. In the cockroach preparation, GABA has little inhibitory effect on contractions induced by acetylcholine but easily inhibits contractions caused by glutamic acid.

Kerkut and Walker (ref. 20) studied the effects of L-glutamate, acetylcholine, and GABA on the miniature endplate potentials (mepps) and the contractures of the coxal muscles of the cockroach. It was found that glutamate increases the amplitude and the frequency of both mepps and contractures and that GABA decreases the amplitude and the frequency. The threshold concentration of L-glutamate for the increase was exactly equal to the threshold concentration of GABA for the decrease (10^{-6} g/ml). Therefore on a mole-for-mole basis, the mutually antagonistic effects of GABA and glutamic acid were nearly equivalent (the ratio is approximately 1.4 moles of GABA to 1.0 moles of glutamate). In this preparation, acetylcholine had no effect on the mepps and the contractures. Since during stimulation of the cockroach nerve glutamate was previously shown to be released in quantities related to the degree of stimulation (ref. 21), Kerkut and Walker suggested that glutamate is the excitatory transmitter in certain synaptic junctions of the cockroach.

The facts that GABA inhibited the mepps caused by glutamate and that acetylcholine had no effect on mepps in the coxal muscle in the cockroach preparation may indicate that a specific neuroinhibitory-neuroexcitatory system involving glutamate and GABA exists in certain insect synapses. In terms of our GABA hypothesis, if one examines the structure of a GABA–CO_2 complex with the use of molecular models, it can be observed that the molecular conformation of the complex is not very different from that of glutamic acid. It is therefore quite possible that the GABA–CO_2 complex is not only noninhibitory, but actually stimulatory. Therefore the combining of CO_2 with GABA may not only remove a molecule of inhibitor, but also add a molecule of stimulator. This view appears to lend even further support to the GABA hypothesis, in that activation may result directly from the complexing of CO_2 with GABA instead of indirectly through the qualitative decrease of the inhibitory power of GABA through association with CO_2.

D. Physiological Studies of GABA Hypothesis

The GABA–CO_2 complex has been shown to be easily reversible, depending upon the temperature and the CO_2 tension in the immediate environment of the GABA molecule (ref. 2,22). Previous attempts made to show that GABA–CO_2 complexes do not have the neuro-inhibitory powers of GABA alone have not been successful. The design of the experiments, however, was considered inadequate to either prove or disprove the hypothesis (ref. 2). The assay system in which crayfish intestine was utilized (ref. 23) had

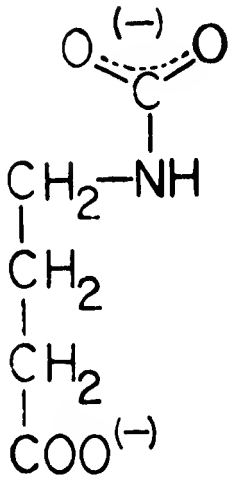
to be continuously aerated, and aeration may have caused the depletion of the GABA-CO_2 complex. Also, the intestinal tissue itself may not have been suitable for the purposes of this assay.

During this year we performed other experiments with the crayfish intestine preparation. Since the GABA-CO_2 complex is quite labile and since it is difficult to assess whether it is actually present in the experimental situation, we synthesized N-acetyl GABA (Appendix A). This compound is not subject to the types of instability to which GABA-CO_2 is subject. GABA was acetylated at the amino group, where CO_2 is bound. The structures of GABA-CO_2 (ref. 24), N-acetyl GABA, and glutamic acid are compared in Figure 1.

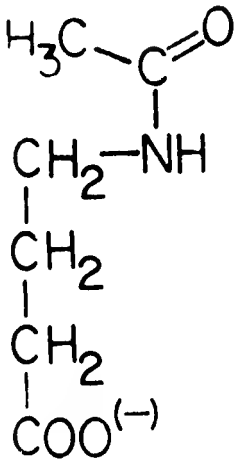
It can be seen that there is a certain degree of analogy among the three structures. We reasoned that if the GABA-CO_2 complex does not inhibit impulse transmission, N-acetyl GABA-CO_2 may also not inhibit impulse transmission. We tested the effect of N-acetyl GABA on acetylcholine-stimulated contractions of the crayfish intestine. The results are shown in Figure 2. It can be seen in Figure 2a that N-acetyl GABA did not inhibit intestinal contractions when acetylcholine was added and, in fact, appeared to have some stimulatory properties of its own. When GABA was added to the medium instead of N-acetyl GABA, no contractions could be observed upon the addition of acetylcholine (Figure 2b). GABA was also capable of causing cessation of the contractions stimulated by N-acetyl GABA (Figure 2c).

Although these findings would be expected if the GABA hypothesis were correct, they do not confirm the hypothesis. The molecular conformation of N-acetyl GABA is not the same as that which results from a GABA-CO_2 complex, and the charge distribution in the two molecules is different.

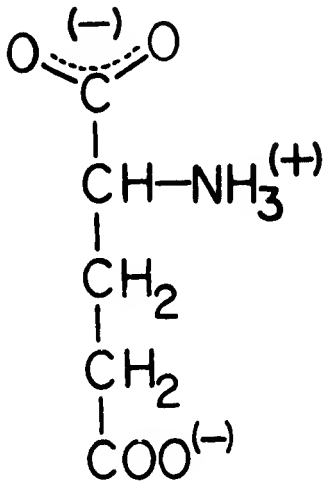
We also tried to observe a release from GABA inhibition when air containing 5% and 1% CO_2 was bubbled through the bathing medium instead of ordinary air. We thought that the CO_2-enriched gas would help preserve any GABA-CO_2 complex formed. We found that the air containing 5% CO_2 was detrimental to the crayfish intestine preparation, which did not survive long. The 1% CO_2-air mixture, however, appeared to be well tolerated by the intestine. We observed two phenomena in this series of experiments that seemed encouraging. First, there was a considerably more rapid recovery by the intestine after GABA inhibition when aerated with the 1% gas mixture. Whereas with ordinary air at least three or four saline washes were required after GABA inhibition before responsiveness to acetylcholine returned, only one saline wash was required after aeration by the 1% CO_2 mixture. Second, there seemed to be a slow return of the intestinal contraction after 1- or 2-min 1% CO_2 aeration of the solution containing GABA and acetylcholine. Although we also occasionally observed a resumption of contractions during aeration with air, it took two or three times as long as during 1% CO_2 aeration.



GABA-CO_2



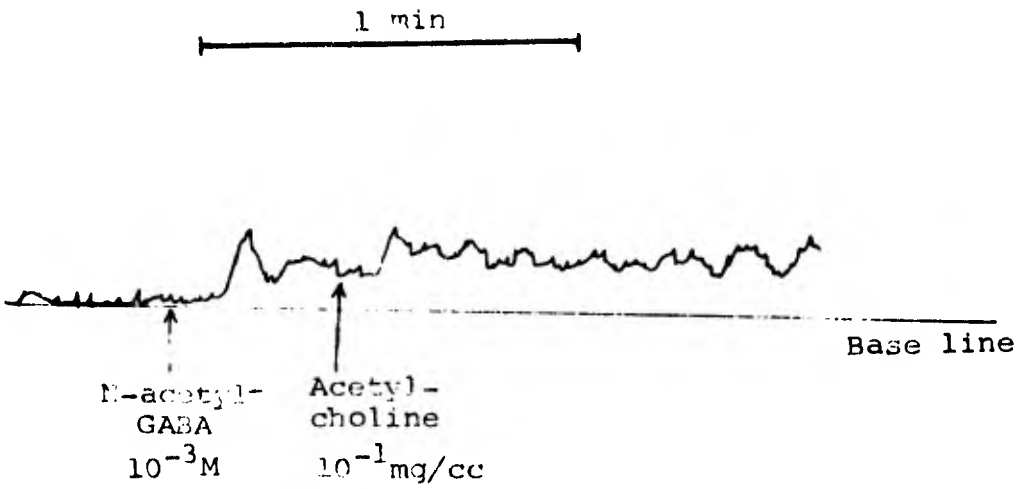
N-Acetyl GABA



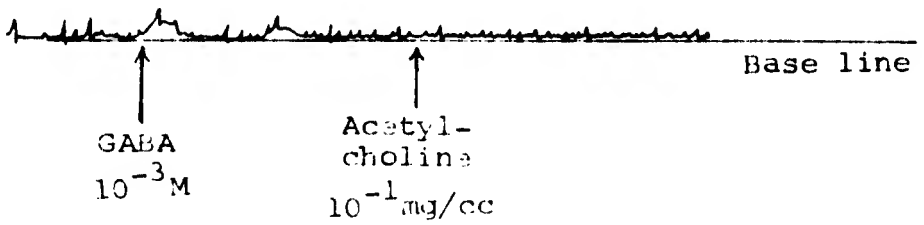
Glutamic Acid

Figure 1

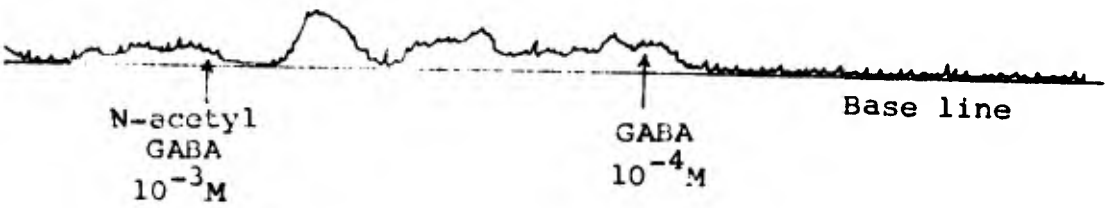
CONFIGURATION OF GABA-CO_2, N-ACETYL GABA, AND GLUTAMIC ACID (AT PHYSIOLOGICAL pH)



(a) N-ACETYL GABA FOLLOWED BY ACETYLCHOLINE



(b) GABA FOLLEWED BY ACETYLCHOLINE



(c) N-ACETYL GABA FOLLOWED BY GABA

Figure 2

EFFECTS OF N-ACETYL GABA AND GABA
ON ACETYLCHOLINE-STIMULATED CONTRACTIONS OF THE CRAYFISH INTESTINE

Though these results were of interest, they were still not conclusive or clear cut. Other considerations also confused our results. Since a GABA-CO_2 complex appears to be structurally similar to glutamic acid, it would be reasonable to assume that a tissue responsive to glutamic acid in terms of synaptic impulse transmission would be more likely to show responsiveness to a GABA-CO_2 complex (if responsiveness to this complex exists) than a tissue not responsive to glutamic acid. Although the crayfish intestine derived from Astacus astacus L is reported to contract with the application of L-glutamate (ref. 25), the intestines derived from Orconectes or Cambarus (used in this work) were inhibited by glutamate after one initial quick contraction; inhibition by glutamic acid was also reported by Florey (ref. 23) for preparations of intestines derived from these genera. We previously reported preliminary findings (ref. 22) that indicated that glutamate did cause the intestine to contract, but these results could not be duplicated or confirmed in subsequent work. Therefore we could not explain the apparent contractions caused by N-acetyl GABA, if the analogy to glutamic acid is followed.

The work of Kerkut et al (ref. 19) showed that glutamic acid profused through the leg of the cockroach Periplaneta americana caused the leg to contract, while GABA abolished the contractions. Kerkut and Walker (ref. 20) found that applications of glutamic acid increased the miniature end-plate potentials for the coxal muscles of this cockroach, while GABA decreased them. These results suggested that Periplaneta americana would be an ideal animal in which to study the physiological effects of the interactions of GABA and CO_2. Dr. Carroll N. Smith of the U.S.D.A. Laboratories in Gainesville, Florida, kindly provided us with a colony of Periplaneta americana for the studies described below.

An adult cockroach was isolated from the colony and cooled for about 5 min to deactivate it somewhat for ease in handling. The cockroach, with its ventral side up, was pinned to a cork block, and the ventral sternites over the third thoracic segments were removed to expose the thoracic ganglia and the nerves supplying the legs. Then a small portion of the chitin was removed from the coxa of the third thoracic leg. Stimulating electrodes were placed on the thoracic ganglia, and a stimulator (Grass model S4) delivered shocks to the electrodes at the rate of one shock every 2 sec. The strength of the stimulus was adjusted so that the leg gave a small twitch to every stimulus. The stimulus strength varied from one preparation to the next.

When a 10^{-4} M solution of GABA in crayfish saline (ref. 23) was placed on the exposed muscle of the coxa from which the chitin was removed, the leg slowly stopped twitching because of the inhibitory effect of GABA. A fine stream of CO_2 was then directed at the exposed muscle, and the twitching resumed, weakly at first, then more strongly. When the stream of CO_2 was removed, the twitches of the leg became weaker and weaker, and finally stopped.

This reestablishment of inhibition was probably due to the slow dissociation of CO_2 from GABA. The heat of the illuminating lights on the preparation probably also contributed to the dissociation. This finding was in exact conformity with our predictions and expectations in terms of the reactions of GABA with CO_2 (ref. 5). When a stream of air was directed at the exposed muscle, the inhibition persisted, few or no contractions were observed. Another stream of CO_2 again caused the leg to twitch. Simply breathing on the preparation was also sufficient to release the inhibition and cause the leg to twitch.

These experiments were performed very recently, and all our observations of the responses of the cockroach leg were visual. In the near future, we will make chart recordings of our results with a sensitive electrical transducer. Two experiments have been done to date, and both gave similar responses. If these results can be further substantiated in future work, the GABA-CO_2 hypothesis will probably have been proven in a physiological system. The implications of these results may be rather far-reaching, not only in terms of mosquito behavior, but also in terms of other organisms in which GABA is naturally found to play a role in neuroinhibition.

III. REPELLENTS DESIGN BASED ON GABA HYPOTHESIS

On the basis of the theory of insect attraction and the repellency data already presented (ref. 2) and on the basis of the repellency data presented in this report, it may be possible to develop a rationale upon which we can base the design of insect repellents.

We have tested many substances that we considered volatile structural analogues of GABA. We assumed that these substances would interfere with the host recognition mechanism of mosquitoes by increasing neuroinhibition and would thus upset the "inhibition-activation balance" as the insect approaches a host. The GABA analogues tested to date have largely borne out this prediction and have shown considerable repellency in low concentrations. These tests were performed only to obtain evidence of whether this approach is of value. We were not yet concerned with other practical and important considerations such as deleterious effects on systemic or topical application or length of time of action. If an approach to the basic design of repellents could be achieved in terms of "intrinsic repellency" of compounds, other attributes essential for effective repellency could be considered, and eventually a molecule could be built that has the desired properties for potent and long-lasting repellency.

In the following discussion, all data referred to can be found in Quarterly Progress Report No. IITRI-L6021-9 (ref. 26). From these data and previously reported data (ref. 2) it appears that compounds with general structures that contain an amino group on one end of the molecule and a hydroxyl function on the other end are significantly repellent at the 97.5% confidence level at a concentration of 1.0 mg per square inch of skin. In the progression from a two-carbon amino alcohol (2-aminoethanol) to a six-carbon amino alcohol (6-amino-1-hexanol), there does not seem to be an optimal number of carbons with which repellency is greatest. The repellency of 4-aminobutanol was shown to be significantly repellent at 1.0 mg/in.^{2}, but not at 0.1 mg/in.^{2}. It is of interest that although 6-amino-1-hexanol is the only compound in the progression that is a solid (mp, 52 to 54°C), its repellency is of the same order of magnitude as the smaller-chain liquid amino alcohols.

N,N-dimethylethanolamine is also repellent at 1.0 mg/in.^{2} but just misses being significantly repellent at 0.1 mg/in.^{2} if 97.5% is taken as the level of acceptance. This fact is of interest in terms of the electron-inducing properties of the methyl group. The dimethylamine configuration may be more nucleophilic (electron dense) than the amino group alone and may thus effect a greater difference in terms of electronic configuration between the two functional groups of the molecule.

Double bonds (pi bonds) in organic compounds are known to be structures that are relatively nucleophilic. If our approach is correct, compounds containing double bonds in lieu of amino groups should be repellent when incorporated into a carbonyl or a hydroxyl compound. We therefore tested 3-butene-2-ol and found it to be repellent at a highly significant level of confidence at 0.1 mg/in.^{2}. Structures of this type have also been cited by Dethier (ref. 27) as being repellent.

We tested ethanolpropanolamine to observe the effect of the insertion of an amine into an otherwise symmetrical molecule. We previously discussed the fact that 1,6-hexanediol is not an effective repellent (ref. 26,28) and attributed this nonrepellency to the symmetrical configuration of the molecule and to the equal electron distribution within the molecule. Replacement of a CH_2 group with an amine group in this structure places a nucleophilic center between the two relatively electrophilic hydroxyl radicals. This change in the electronic configuration of the molecule is apparently sufficient to produce significant repellency at a concentration of 0.1 mg/in.^{2}.

We previously reported (ref. 2) that the mild hydrolysis product of 4-aminobutyraldehyde diethylacetal was very repellent at low levels of application and seemed to be the most repellent compound of the amino-carbonyl family tested. The presumed

product, free aminobutyraldehyde,* is a very close analogue of GABA. The diethylacetal of the corresponding 4-aminobutyraldehyde was hydrolyzed in acetone overnight in the cold with two or three drops of 0.1 N hydrochloric acid (HCl). We tried to repeat these results but used one drop of concentrated HCl instead of 0.1 N HCl. After this product stood in the refrigerator overnight, it was not significantly repellent, even at 1.0 mg/in.^{2}.

Since the concentrated HCl may have caused the amino group to become ionized and may thus have changed its electronic characteristics as well as its repellency, we added a little sodium bicarbonate to neutralize the acidity. The material then became repellent at 1.0 mg/in.^{2} but not at 0.1 mg/in.^{2}. When we tried the solution of hydrolyzed acetal made some months previously with dilute HCl, it still had the very high level of repellency originally observed and showed significant repellency at a level of 0.001 mg/in.^{2}. Apparently either the concentrated acid had caused some decomposition in our present sample or we had not successfully neutralized the acid with the sodium bicarbonate. Nevertheless, we pursued this question further.

When we had attempted to hydrolyze 4-aminobutyraldehyde diethylacetal with reflux in aqueous acidified solution, the product was nonrepellent. It also showed a negative Schiff reaction (specific reaction for free aldehyde). Since we had kept this solution in the refrigerator, we attempted to neutralize it by adding solid sodium bicarbonate. Neutralization was complete when the pH reached about 6.8. We tested the resulting solution in the Schiff test and found that it was intensely Schiff positive. We then tested the neutralized product for repellency. The material was significantly repellent at a concentration of approximately 0.01 mg/in^2. Although there may have been some destruction of the product during the reflux procedure and the rather long intervening time at a low pH, the product was still considerably repellent. Its repellency was probably previously masked by the low pH of the solution when first tested. The low pH also accounted for the negative Schiff test originally observed. This compound is apparently a much more effective repellent at near physiological pH ranges. These findings are very encouraging, since we succeeded in confirming our previous results and learned that the repellency properties of this type of compound are best expressed at a neutral pH. This class of repellents may therefore be compatible with physiological systems in terms of pH.

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*The reaction mixture containing the presumed aminoaldehyde that we produced in our laboratory is as yet ill-defined. Although highly repellent, this mixture must be fractionated, and the active components must be identified.

We also tested diethylaminoacetaldehyde diethylacetal and found it to be not significantly repellent at 1.0 mg/in.^{2} but repellent at 10.0 mg/in.^{2}. Upon hydrolysis in acetone with the addition of one drop of concentrated HCl and standing overnight in the refrigerator, this compound became significantly repellent at 1.0 mg/in.^{2} but not at 0.1 mg/in.^{2}. The increase in repellency in this case may also be due to hydrolysis to the free aldehyde. Neutralization or the use of milder hydrolysis conditions may increase the repellency even further.

In the tests discussed below, the 95% confidence level was accepted as repellent instead of the 97.5% level, because we changed our statistical analysis to a two-tailed test of significance instead of a one-tailed test. This change necessitated lowering the confidence level so that this would be comparable to that previously used. The reason for this change as well as a full discussion of our computational methods are taken up in Section IVB.

Appendix B shows the results of the repellency testing of certain compounds submitted by Dr. Ronald P. Quintana of the University of Tennessee, as well selected compounds in the continuation of tests of the validity hypothesis (that analogues of GABA are repellent to mosquitoes) previously presented (ref. 2). In Appendix B the results are not reported directly in terms of confidence levels, but in terms of "weighted percent of controls" and "upper bound." An upper bound of 100 is equivalent to the 95% level of confidence. Numbers below 100 denote a higher significance level, and numbers above 100 denote a lower significance level. The number just above that labeled "upper bound" is the percent reduction of biting due to the test compound as compared to the biting of untreated controls. This method of reporting results will also be discussed in Section IV B.

We observed an occasional reversal of repellency, in which a compound at a higher concentration is not significantly repellent at the 95% level, while the same compound at a lower concentration is significantly repellent. The reason for these reversals cannot be directly accounted for or easily explained. The possibility of such occurrences are implicitly stated in any statistical confidence limit. These occurrences may be, though need not necessarily be, ascribed to experimental error. Some of these reversals are subsequently discussed; in some cases a more comprehensive evaluation in terms of repeat tests may be indicated.

In the series of compounds we assayed for the University of Tennessee, significant repellency was noted for compound A036 at a level of 5.0 mg/in.^{2}. A035 was repellent at 10.0 mg/in.^{2}, but lost repellency at 5.0 mg/sq in. A032 was significantly repellent at 25 mg/in.^{2}, but not at 10 mg/sq in. A033 was repellent at 50 mg/in.^{2}, but was not significantly repellent at 30 mg/in.^{2} (A033 was assayed only once at these levels however, and should be assayed again to achieve greater statistical stability).

Compound A029 was significantly repellent at all levels tested thus far (down to 30 mg/in.^{2}) and has to be tested further to observe at which point it is no longer repellent. A028 was significantly repellent at 50 mg/in.^{2}, but did not reach the 95% confidence limit at 30 mg/in.^{2}. A016 was significantly repellent at all levels tested (50 and 30 mg/in.^{2}). A014 was significantly repellent at 50 and 30 mg/in.^{2}, but not at 10 mg/in.^{2}. A013 was significantly repellent at all levels tested, down to 5 mg/in.^{2}. Compound Y006 was significantly repellent at 0.1 mg/in.^{2}. Compounds Y007 and Y008 were both significantly repellent at 1.0 mg/in.^{2}, but not at the 0.1 mg/in.^{2} level of application.

The compound 3-amino-1-propanol was significantly repellent at 1.0 mg/in.^{2} but not repellent at 0.1 mg/in.^{2}. On the other hand, the diethylamino derivative of this compound was repellent at 0.1 mg/in.^{2}. Therefore an electron-releasing substituent on the amino group that makes this group more nucleophilic enhances the repellency of the compound. The compound however loses repellency at 0.01 mg/in.^{2}.

If the hydroxyl group is right next to the diethylamino functional group, as in 1-diethylamino-2-propanol, repellency is also retained at 0.1 mg/in.^{2} but lost with further dilutions. If the hydroxyl group is two carbon atoms away from the diethylamino functional group, as in 4-diethylamino-1-butanol, repellency is still retained at 0.1 mg/in.^{2}, but not retained at lower levels. Therefore the distance between functional groups does not appear to significantly influence repellency. This is reminiscent of the situation with the amino alcohols previously discussed.

If methyl groups instead of ethyl groups are the substituents on the amino group, as in 3-dimethylamino-1-propanol, repellency is not evident at the 95% level of confidence even at a concentration of 1.0 mg/in.^{2}. One of the aforementioned reversals, however, is evident. The 3-dimethylamino-1-propanol unexpectedly shows significant repellency at 0.01 mg/in.^{2}, although not at any other level tested. The 1-in-20 statistical chance for such a occurrence possibly evidences itself here. In view of the consistent nonrepellency at the other test levels, reassessing this compound appears to be of little value. These results are consistent with the fact that methyl groups are less electron inducing than ethyl groups. The compound 1-dimethylamino-2-propanol is also not repellent at 1.0 mg/in.^{2}, a result consistent with the above line of reasoning. It is of interest to note that a reversal is again seen in the assay of this compound. The 1-dimethylamino-2-propanol shows repellency only at 0.01 mg/in.^{2}. This was also the only level at which 3-dimethylamino-1-propanol showed significant repellency. The chemical similarity of the two compounds is apparent, and the consistent reversals seen in the assays of these two compounds at the same treatment levels may conceivably

have some physiological significance outside of mere statistical discontinuity.

In 4-dimethylamino-1-butanol the functional groups are separated by two carbon atoms. Significant repellency is retained even at 0.001 mg/in.^{2}, and we have not yet reached the nonrepellent dilution. The electron-withdrawing properties of the hydroxyl group mainly affect carbon atom two, while carbon atom three is free to contribute its electrons to the dimethylamino group and enhance the nucleopilic properties of that group. Therefore a greater charge differential can be maintained with the butanol derivative than with the propanol derivatives. In this respect it is interesting to note that in amino acids, the pK, or the basicity of the amino group, increases as it is separated from the carboxyl group.

The compound 1,1,3,3-tetramethyl urea was of interest because it is a symmetrical compound containing substituted amino groups on each side of a carbonyl moiety. In the tests of this compound, a reversal was again seen. The 0.1-mg/in.^{2} treatment was significantly repellent, while the 1.0-mg/in.^{2} treatment or any other level of treatment was not significantly repellent. Again, we have no explanation for these results. In general, however, this compound does not appear to be outstanding as a repellent. We have already suggested the possibility that if the oxygen-containing moiety is located in the middle of a molecule or is surrounded by alkyl groups, the molecules loses its effectiveness as a repellent (ref. 26). This compound appears to offer further evidence of the validity of the hypothesis. The electron-withdrawing properties of the oxygen-containing group are neutralized by the inductive properties of the surrounding electron-releasing groups.

Further support for our line of reasoning was obtained from the results of the assay of diethylaminoacetone. This compound is not repellent at 1.0 mg/in.^{2} or at 0.1 mg/in.^{2}. The carbonyl moiety is again bonded on both sides to electron-inducing groups.

To test the effects of the presence of two oxygen atoms with an amino group in the same molecule, we synthesized GABA ethyl ester. (The synthetic method is described in Appendix A.) Although the oxygens in this compound are also surrounded by alkyl moieties, the two oxygens are juxtaposed, and the total electron-withdrawing properties of the two oxygens in close proximity should be greater than when only one oxygen is present between alkyl groups. This compound showed significant repellency at 10 mg/in.^{2} and at 1 mg/in.^{2}. We could not test further, since we had used up all that we had available. Our tests with this compound will continue when more can be obtained. It is interesting, however, that GABA ethyl ester is significantly repellent at 1.0 mg/in.^{2}, in contrast to the results with 1,1,3,3,-tetramethylurea and diethylaminoacetone.

When we tested the repellency of 2-(2-aminoethyoxy)-ethanol, a compound that contains an amino group separated by two carbons from a midchain oxygen at one end of the molecule and a hydroxyl group separated by two carbons from the same midchain oxygen at the other end of the molecule, we found that the compound was no more repellent than any of the omega-amino hydroxy compounds tested (ref. 26). It was significantly repellent at 1.0 mg/in.^{2} but not at 0.1 mg/in.^{2}. Apparently, an oxygen atom surrounded by alkyl groups was again shown to contribute little to the electronic disproportionation of the molecule and thus has little influence on overall repellency.

We previously reported (ref. 26) that 3-butene-2-ol was repellent at a concentration of 0.1 mg/in.^{2}. We continued testing this compound during this report period and found that significant repellency was retained at 0.01 mg/in.^{2} but lost at 0.001 mg/in.^{2}. Therefore a compound in which the nucleophilic structure is in the form of a pi bond exerts as high a level of repellency as a compound in which this structure is in the form of a free or substituted amine. The electronic properties rather than the actual substituents in the molecule are therefore again proven to be the factors that determine potent mosquito repellent efficacy.

We retested the solution of 4-aminobutyraldehyde diethylacetal that had been hydrolyzed in water with reflux in the presence of HCl and subsequently neutralized with sodium bicarbonate. We had previously used (ref. 28) this solution, and we were interested in assessing the stability of the compound with time. It continued to show significant repellency, even at concentrations of 0.001 mg/in.^{2}. It lost significant repellency at 0.0001 mg/in.^{2}. This substance therefore appears to be a very powerful mosquito repellent.

We then studied the effects of a benzene ring on the repellency of aminocarbonyl compounds. The compound 2-aminobenzaldehyde, a light-yellow solid, is sufficiently stable that it can be obtained commercially as the free aldehyde. This compound was significantly repellent at 0.001 mg/in.^{2} but lost significant repellency below this concentration. Therefore this benzenoid aminoaldehyde is considerably repellent even though it is a solid. These results are of great interest when compared to previously reported repellency tests of m-aminodiethylbenzamide (ref. 1). This amino analogue of m-diethyltoluamide (DEET), which is also a solid, was shown to have no repellency, even at 10 mg/in.^{2}. These results seem to indicate again that neither the precise chemical constitution of a compound nor the distance between the functional groups is as important for repellency as the actual electronic configuration of the molecule. The oxygen-atom in the 2-aminobenzaldehyde is at an end position and has no electronic input other than that from the ring. It thus retains its essential electrophilic (electron-poor) properties. On the other hand, m-aminodiethylbenzamide has an oxygen moiety that not only

receives electrons from the ring (because of the inductive effects of amino substitution for the methyl group) but also from the diethylamino group on its other side.

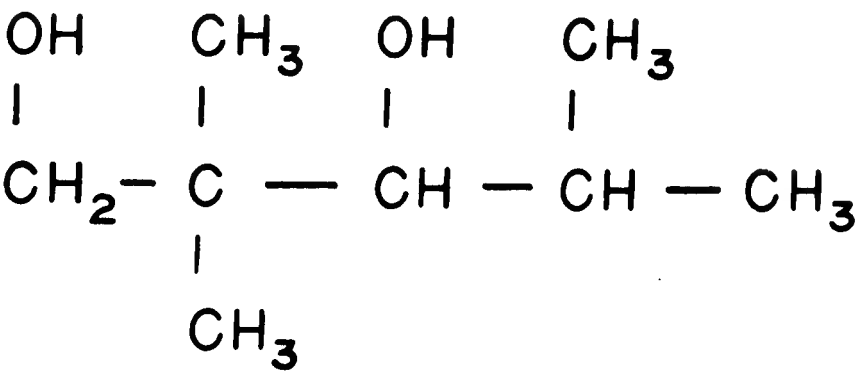
Repellency tests of N,N-diphenylformamide showed that this compound is significantly repellent at all levels tested thus far, down to 0.01 mg/in.^{2}, and have not yet reached the repellency limits in terms of concentration. In this molecule, a free aldehyde is bonded directly to a diphenylamino group. The relationships of a nucleophilic moiety (diphenylamine) and an electrophilic moiety (aldehyde) are retained in the molecule, and a high degree of repellency is evidenced.

For purposes of comparison, Appendix B also shows an assay of DEET. DEET was significantly repellent at 0.1 mg/cc, but not at 0.01 mg/cc. Three tests were performed at each concentration level, but no tests at the same concentration level were performed on the same day. This method is the most statistically efficient way to perform these repellency assays, as it minimizes the day effect. All repellency tests to be henceforth reported will be performed in this way, with corresponding controls.

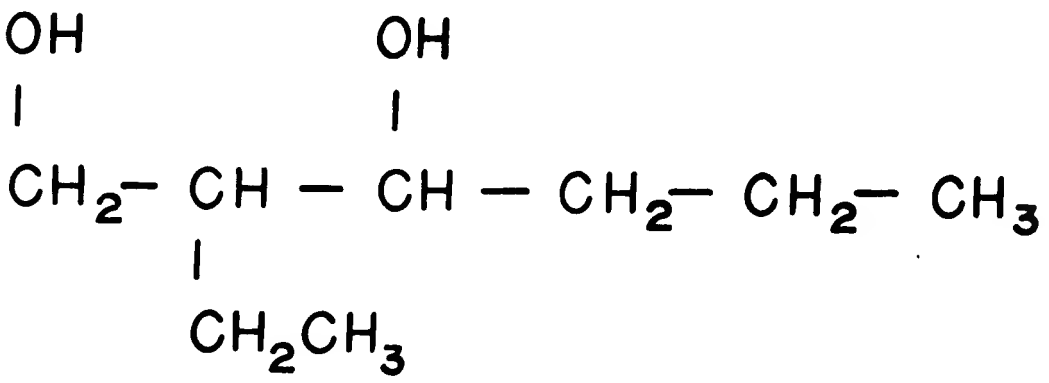
Since the rationale upon which we based these investigations is apparently largely correct, it is of interest to consider what consistencies, if any, there may be between these GABA analogues and known, established repellents that have no obvious relationship to the structure of GABA.

Figure 3 shows the structures of Rutgers 612 and TMPD (2,2,4-trimethylpentane-1,3-diol). These compounds have very similar structures, and they are both good repellents. There are two hydroxyl groups in each compound, one at the first carbon atom and one at the third carbon atom. The hydroxyl group on carbon three is surrounded by alkyl moieties on both sides in both compounds, while the hydroxyl group on carbon 1 has an alkyl chain on only one side in both compounds. Therefore more electrons can flow into the hydroxyl group on carbon 3 than into the hydroxyl group on carbon 1. The result is that the inner hydroxyl group is, in general, a less acidic (i.e. more basic) hydroxyl group (i.e., less able to lose a proton) than the end hydroxyl group. In other words, the inner hydroxyl group has a denser electron input from the surrounding hydrocarbon chains than the end hydroxyl group. Therefore the two hydroxyl groups are not equivalent. The middle one is relatively nucleophilic, and the end one is relatively electrophilic.

In the case of effective GABA analogues reported here, the two functional groups are also not equivalent. One is a relatively nucleophilic amino group, and one is a relatively electrophilic hydroxyl or carbonyl group. Therefore the electronic properties of both Rutgers 612-like compounds and GABA-like compounds are essentially preserved. It is therefore possible



2, 2, 4 TRIMETHYLPENTANE-1, 3-DIOL
(TMPD)



2-ETHYL-1-3-HEXANEDIOL
(RUTGERS 612)

Figure 3

THE STRUCTURE OF TWO REPELLENTS CONTAINING 1,3-DIOL GROUPS

that these two substances operate via the same basic mechanism, i.e., neuroinhibition. If in the 612-like compounds the hydroxyl group on carbon 1 is removed to carbon 2 and that on carbon 3 is removed to carbon 4, the resulting family of hexanediols has diminished repellency (ref. 28). This result is consistent with our expectations, since there are less differences in the electronic properties of the two hydroxyls when one is removed from the end position. When the two hydroxyls are exactly equivalent, as in 1,6-hexanediol or 1,3-propanediol, repellency is even further diminished (ref. 29).

Interpreting the repellency of DEET in these terms is also possible. The ability of substituents on the benzene ring to activate the ring toward further substitution is in the order shown in Figure 4. A methyl (CH_3) group is the least-activating group (in terms of electrophilic aromatic substitution) of any of the family of substituents shown in Figure 4. Whatever ring-activating ability it possesses is oriented to the ortho and the para positions and very little to the meta position. Therefore the meta position is the least activated; i.e., the electron induction from the methyl group into the benzene ring is least effective in the meta position.

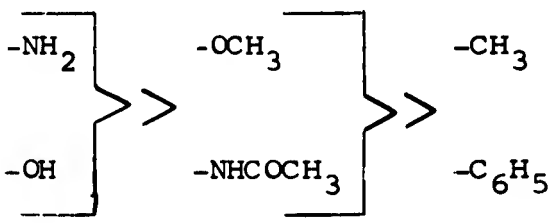


Figure 4

ELECTRON-RELEASING ABILITY
OF GROUPS ACTIVATING BENZENE RING

In DEET, the diethylamide group is located in the meta position, a position of relatively low electron density. A double-bonded oxygen atom is present on a carbon atom, which is in turn bound to the ring on one side and to a diethylamine moiety on the other side. The diethylamine moiety has a relatively nucleophilic environment, while the carbonyl ($-\overset{O}{\overset{\|}{C}}-$) moiety is relatively electrophilic because of the double-bonded oxygen. This dearth of electrons is offset very little by the electrons of the ring, since the meta position is not activated

much in the toluene moiety. Although the electrons in the ring itself are relatively abundant, they are not available to the carbonyl region and mainly continue to participate in the resonance stabilization of the pi-electron structure of the benzene ring.

Therefore in DEET the condition of a relatively electrophilic moiety juxtaposed with a relatively nucleophilic moiety continues to be maintained. The carbonyl group is actually sandwiched between two nucleophilic moieties, the benzene ring and the diethylamine. Therefore diethylbenzamide should be as good a repellent as (if not a better repellent than) DEET, since there is not even a methyl group present (as in toluene) to activate the ring in any way. We have found that this is indeed the case (ref. 1).

Alternatively, highly activating groups as substituents on the benzene ring should decrease the repellency of the benzamide moiety. We have mentioned previously that m-aminodiethylbenzamide is useless as a repellent (ref. 1).

Therefore the predictions of repellent structures derived from the hypothesis appear to be largely borne out, and new classes of repellents that may be more effective than those presently used may become available. Future work should shed more light on the interrelationships between repellents and neuroinhibitors, and their applications to the development of orally effective insect repellents.

IV. REPELLENCY ASSAY

A. Method

Before discussion of our statistical treatment of repellency assay data, a brief review of our repellency testing method (ref. 1,30,31) is presented.

The basic method consists essentially of the completing of a series electrical circuit by a mosquito when it bites an anesthetized host animal. A bronze screen, which covers a container of *Aedes aegypti* mosquitoes, is connected to a 1.35-v battery in series with resistances totaling about 15 megohms, and then to a recorder (Sanborn model 320, two-channel general-purpose recorder, Sanborn Co., Waltham, Mass.). The circuit from the recorder continues to the tail of an anesthetized host mouse, where the wire is imbedded via a hypodermic needle soldered to the lead. The mouse lies on top of the screen and is insulated from the screen by the hair on its body.

When a mosquito bites, it holds onto the wire mesh with its legs and passes its proboscis through the mesh into the mouse. When the proboscis penetrates the skin of the mouse, the series electrical circuit is completed by the insect and the recorder responds with a displacement of the recording stylus from the baseline. The stylus does not move unless the mosquito proboscis actually penetrates the mouse skin, and baseline displacement is a direct reflection of the biting activity of the mosquitoes.

In repellency assay, a known weight of the test substance in acetone solution is spread on a 1-in.^{2} area of the belly of the anesthetized mouse. When the acetone dries, the treated area is masked from the untreated areas, and the mouse is placed on top of the wire screen to explose it to the previously starved mosquitoes in the container. If repellency is good, there is little or no displacement of the recording stylus from the baseline, and very few or none of the mosquitoes are found to have taken blood.

We occasionally observe displacement without engorgement, a result that means that the mosquitoes merely probed, possibly salivated, and withdrew without actually sucking blood. However, we have never observed engorgement without displacement, a result that is important for the ultimate validity of our method. The detection of probing with or without engorgement is an essential component in the total measurement of repellency, since an infected mosquito need not draw blood to spread disease or cause discomfort but need merely probe and salivate in the host's skin.

B. Statistical Treatment

In our method of repellency testing, about 50 mosquitoes are exposed for 30 min to an anesthetized mouse treated with the test repellent. Three parameters are available for measurement when the test is completed. These are:

(1) The percentage of time that the stylus is displaced from the baseline during the 30-min period

(2) The distance that the stylus is displaced from the baseline as measured at periodic intervals on the chart recording (This measure is roughly proportional to the number of mosquitoes biting simultaneously.)

(3) The percentage of mosquitoes that take blood during the test period.

To decide which of these parameters or combinations of parameters would give efficient separation of untreated controls from low-level (borderline) repellent-treated cases, a discriminant function analysis was applied. This analysis, which employs a standard computational program (ref. 32), yielded a linear combination that maximized separation between these two groups. It was found from this analysis that the percentage of time that the stylus is displaced (P) and the percentage of mosquitoes that engorge (E) could be used without the variable for displaced distance as a simplified discriminant function (ref. 2). The sum P + E, which we defined as the "repellency index," approximated the discriminant function that gave the least number of misclassifications and permitted the evaluation of test compounds for statistical significance with respect to the distribution of the values of the function P + E for the control groups. An analysis of variance was used to estimate the statistical variation of the control groups, and in this way a means of testing statistically significant differences from the controls was provided.

In our last Annual Progress Report (ref. 2) we presented an analysis of variance of the repellency index for control groups and showed that the day effect was definitely significant (i.e., mosquito biting in the control group varied significantly from day to day). This effect was therefore taken into account in assessing the confidence level for the test groups. The number of mosquitoes exposed in the control tests was, however, not firmly established as being significant and was therefore not taken into account in the statistical treatment. We have since obtained sufficient new control data (Table 1) to further test the effect of number of mosquitoes as well as age of the mosquitoes used in the control groups.

The day effect was again proven to be significant in these control groups, while age and number effects did not prove significant. We therefore confirmed our findings with these new control groups and also determined that the age of the insects does not significantly affect our test results. The ages ranged from about 4 days to 25 days, and the number of mosquitoes exposed ranged from about 16 to 57. In the majority of cases, however, the age was 8 days and the number of mosquitoes was near 50. Our past treatment of the data therefore remains valid.

The following is a summary of our approaches and objectives in the statistical analysis of the repellency data.

The repellency of each compound is characterized by the percent of mosquitoes engorged out of approximately 50 that are used in each test and the percent displacement in the electronic recording (i.e., biting activity) during a 30-min test period as compared to parallel control tests.

Table 1

ANALYSIS OF VARIANCE OF CONTROL VALUES OF REPELLENCY INDEX

Effect	Sum of Squares	Degree of Freedom	Mean Square	F	Significance Level
Day	40046.5	18	2224.6	1.962	5%
Number	3733.2	1	2733.2	2.410	not significant
Age	270.2	1	270.2	0.238	not significant
Error	32882.1	29	1133.9		
	75932.0	49			

Each compound at each dose level tested yields a mean repellency index, which is the sum P + E, and the significance of differences of this index from the control values is tested by taking day-to-day variation into account. A compound can then be judged by the maximum level of significance at which it tests significantly different from the control tests. A compound that has received more testing is more likely to test significantly different from the control. This situation also occurs if the number of control tests is not the same for the days on which the compounds are tested.

Day-to-day variation, which proved significant in every analysis of variance performed on control groups, is taken into account in such a way that days with lower average control values would be less likely to test significantly different if the test values are the same. In most cases, reduced biting for controls can be expected to correlate with reduced biting for the test compounds. Such a response, however, is impossible for test results that are zero or near zero. We know of no suitable transformation to avoid this problem. The result is that the level of significance is of value primarily for testing the absolute difference from the control tests. Comparative ranking of test compounds can be made by testing these absolute differences at different concentrations of the compounds or by uniformly multiplying all control values by a desired fraction. To perform the latter operation, we incorporated into our computer program the ability to take any desired fraction of the total control values for each day of testing (ref. 22.26). Therefore we were able to compare biting to 0.9, 0.75, or any other fraction of the control value.

This operation provides proof of repellency by at least some percent below the control activity. The test of significance has therefore been adapted to test for significant deviation below any desired fraction of the control values. Therefore we can state that a test compound has proved to be significantly repellent at 10% (or 50%) below the control values with some level of confidence.

We recently reviewed the confidence levels stated for tests of significance of each compound. Since in testing our compounds we had been essentially interested only in the reduction of biting activity, we had used a one-tailed test of significance (t-test). However, in view of the possibility of a slight skewing of the distribution of the repellency index, we decided to take the slightly more conservative approach of using a two-tailed test of significance. This change does not affect the program, only the significance levels that are an input to the program. The change causes the significance levels to be shifted downward by one step in all cases. Whereas we previously chose the 97.5% level as our accepted significance level, we now accept the 95% confidence level as significant. Therefore if in a previous test a compound was significantly repellent at the 97.5% confidence level, it is now significant at the 95% confidence level.

Three other major modifications were also made in the computer program during this report period. In the first modification, the mean values for the controls for each day are computed automatically when the control data are put in before the test data; the need to separately calculate and put in these means at the beginning of the program is thus eliminated (ref. 22). The second modification eliminates the need for a separate analysis of variance (ANOVA) for each new group of tests. The variance for each new set of controls is now calculated, and an analysis-of-variance table is printed by the computer. The ANOVA appears immediately after the controls, with an F-test for significance of the day effect. The computations for the sum of squares (SS), the degree of freedom (DF), and the mean square (MS) are also printed. The error mean square (error MS) is the control group variance actually used in the subsequent tests of significance (ref. 22). The third modification involves a change in the way the confidence levels are printed out, so that more information can be obtained in a single computer run. As stated above, in order to estimate how different control values were from test values, the control values were multiplied by some fraction in order to ascertain the point at which repellency did not differ significantly from the chosen fractional value of the controls at the 95% confidence level. In this way, the compounds could be ranked according to their repellency at given treatment levels, even when two compounds are repellent at the 95% confidence level. In other words, we were able to state that one compound reduced biting more than another, even though both were significantly repellent. This method of handling the data, however, became cumbersome. The fractions chosen for multiplying the control values were discrete, and a continuous comparison in terms of differences from controls could not be obtained. This method is also expensive in terms of computer time, since the data had to be computed separately for each fraction of the control values.

Therefore a new computer program was developed that gives a continuous ranking of compounds in terms of control values. We can now determine at a glance the effectiveness of a repellent, i.e., how much biting is decreased as compared to the optimally weighted (ref. 2) controls. Appendix B shows the data that were referred to in Section III and that were computed with the new program.

The major change in the computer printout can be seen in the column that was formerly labeled "confidence level (PCT);" this column now reads "weighted percent of controls." Two numbers for each test compound are entered in this column. The first, unlabeled number is the percentage of decrease in biting from the controls for a given series of tests. The second number, labeled "upper bound," replaces the confidence limit; any value below 100 indicates that this compound is significantly repellent at the 95% confidence limit. The magnitude of the number indicates

how close to the 95% confidence limit the compound is repellent. A very low number indicates that the compound is more repellent than a higher number even though both may fall within the 95% confidence limit, if both numbers are less than 100. A value of 100 or greater for the upper bound indicates that the compound is not significantly repellent as compared with parallel controls, and that it does not fall within the 95% confidence limit. Comparison of the entires in this report with those in a previous report (ref. 22) illustrates these points.

Therefore with the new computer program, the repellency of various compounds can be ranked according to their differences from controls at similar levels of treatment, and sound judgements of relative repellency are based on firm statistical grounds.

The following discussion presents the formulas used to define the percent of control and the upper bound, that indicates statistical significance. It also includes a brief description of the application of these indicators.

The program for the contrast analysis was modified so that the weighted mean of the repellency index is given as a percent of the control response and statistical significance is indicated by the 95% confidence level upper bound. The 95% confidence level, represented by a value of 100 for this statistic, replaces the indication of confidence level and permits a comparative ranking of compounds by their significance level. Uncertainty concerning the extremes of the distribution are ameliorated by this approach. The 95% limits are relatively stable with respect to variations from normality (i.e., a robust statistical estimate) as compared with higher levels of confidence.

To give a complete formulation of these statistics, it is necessary to repeat the formulation of the t-test for significant difference (ref. 2).

The following formulas take into account the fact that the repellency index for untreated controls usually shows significant day-to-day variation. This day effect is removed in the contrast analysis, which includes an efficient statistical test of significance of variations from the mean of the control observations as follows.

$$W_i = \frac{1}{\frac{1}{M_i} + \frac{1}{N_i}} \qquad (1)$$

$$\overline{X(C)} = \frac{\sum_i W_i X_i(C)}{\sum_i W_i} \tag{2}$$

$$\overline{X(T)} = \frac{\sum_i W_i X_i(T)}{\sum_i W_i} \tag{3}$$

$$K(T) = \overline{X(C)} - \overline{X(T)} \tag{4}$$

where

$X_i(C)$ is the mean of control observarions on day i

$X_i(T)$ is the mean of test observations on day i

M_i is the number of control observarions on day i

N_i is the number of test observations on day i

W_i is the weight for the contrast on day i

K(T) is the weighted average of the contrast for the test group.

Equation 4 is the optimum contrast weighted by the number of test and control observations on each day.

Let S^2 represent the mean square error and let f be the number of degrees of freedom for the error in the analysis of variance for the day-to-day variation in control observations. Then the variance for K(T) is:

$$\text{Var } K(T) = \frac{S^2}{\sum_i W_i} \tag{5}$$

The two-tailed test of significance, which allows equal risk of variations above and below the control mean, is used at the 95% confidence limit. If Equation 6 applies, the test group, T, is significantly different from the control group at the 95% confidence level.

$$\frac{K(T)}{\sqrt{\text{Var } K(T)}} \geq t_{0.975}^{f} \tag{6}$$

For comparison it is convenient to represent the repellency index of the tests as a percent of the repellency index of the controls:

$$\text{Percent of controls} = \frac{\overline{X(T)}}{\overline{X(C)}} \tag{7}$$

The upper limit for this percent of controls is a convenient indicator of significance as well as of the level of established merit. The upper limit is developed by multiplying by $\sqrt{\text{Var } K(T)}$ and adding $\overline{X(T)}$ to both sides of the inequality of Equation 6:

$$\overline{X(T)} + K(T) > \overline{X(T)} + t_{0.975}f \sqrt{\text{Var } K(T)} \tag{8}$$

$K(T)$, as developed in a previous report (ref. 2), is as shown in Equation 9, or the difference between the means of the control observations and the test observations on day i.

$$K(T) = \overline{X(C)} - \overline{X(T)} \tag{9}$$

Substituting in the left side of the inequality of Equation 8 gives:

$$\overline{X(T)} + K(T) = \overline{X(C)} \tag{10}$$

And dividing by the left side of 2 gives:

$$1 > \frac{\overline{X(T)} + t_{0.975}f \sqrt{\text{Var } K(T)}}{\overline{X(C)}} \tag{11}$$

Equation 11 is our test of significance. The right side of Equation 11 is the upper bound for the percent of control repellency index. If the upper bound is equal to or greater than 1, the repellency index of the test compound is not significantly different from that of the untreated controls. The number 1 is given in terms of percent, and is equivalent to 100 in the upper bound computation of the computer output. This index is useful for indicating the level of reduction of biting that can be assured at a given point in testing.

If few tests have been run, a compound occasionally shows a low percent of control but has a high upper bound. These results may seem contradictory, but they simply indicate that further testing may be required to establish the efficacy of the test compound.

The index automatically takes into account control variation and the number of test and control observations for all days of the test. Therefore tests can be scheduled for convenience and maximum efficiency.

During this year we performed another discriminant function analysis to retest the validity of our repellency index. In using this type of analysis, we previously found (ref. 1) that the sum of the percent of the total number of exposed mosquitoes engorged (E) plus the percent of the total time of displacement from the baseline (P) in the electronic recording method was sufficient to adequately separate controls from the low-level treatment (borderline) cases. The results of our recent reanalysis with new data showed that this method of distinguishing control from test groups is still valid, and no statistical basis for changing our approach was shown. The efficiency of the repellency index will continue to be reevaluated from time to time.

We also tested an innovation of our repellency testing method. We "conditioned" our mosquitoes by placing the wire mesh on top of the container 24 hr before the group of mosquitoes was actually used for the test. Previously, the mesh had been placed on top of the mosquito container immediately before the actual test. The new procedure resulted in a smaller variance, but the repellency index of the controls was reduced; i.e., we obtained a more uniform response, but the overall response level was lower. A statistical discriminant function analysis of the controls showed, however, that this procedure does not significantly improve or in any way change the results of the tests, and we no longer use this "conditioning" procedure.

It should be stated in conclusion that our underlying objective in using these methods is to detect small changes that result in significant differences from control values at a high level of confidence. While most other test methods are oriented toward yielding an assured level of protection, that is, toward observing the point at which a predetermined desired level of reduced biting is obtained, our method is oriented toward the screening of possible candidate repellents with maximum sensitivity for testing statistically significant differences from controls. Therefore a certain amount of biting and engorging is permitted before the test compound is rejected, and missing possible good repellents because of individual mosquito variation or other uncontrolled or uncontrollable circumstances is extremely unlikely.

Appendixes C and D show the actual computer program listing (in Fortran IV) that permits the statistical manipulations to be automatically executed by the IBM 7094 computer. Appendix C shows the program, used previously (ref. 22), that yields the confidence level in terms of percent, and appendix D shows the program that yields the weighted percent of controls, with upper

bounds. The program in Appendix D is the one we have used here and will use from now on. Section I of Appendix E shows the computer program listing that yields a complete tabulation of the raw input data, which permits checks to be made to ascertain that all input data are accurate prior to statistical analysis. This program also calculates the repellency index. Section II of Appendix E shows an example of the output of raw data that this program yields.

C. New Bitometer Timer

1. Description

In view of the finding from our discriminant function analysis that only the variables P (percent displacement) and E (percent engorgement) are needed to achieve a sensitive separation of test groups from control groups in our assay of mosquito repellency, we developed a new apparatus that can give a direct readout for the parameter bite time. The number of engorged mosquitoes, however, must still be determined by hand count.

The apparatus consists essentially of two electronic digital timers and a meter relay, all of which are actuated by solid-state circuitry. One timing meter is activated only when the mosquitoes bite, while the other timing meter runs continuously during the test. All the pertinent electronic data are thus obtained in a very simple way.

While the Sanborn instrument requires the bite time to be measured directly from the chart recording, a task that is time consuming and possibly error prone, the new instrument permits the bite time to be read directly from one timing meter and the total time of the test from the other. After the data are recorded, a reset button is pushed and the apparatus is ready for the next test. Also, the cost of constructing this instrument, approximately $125.00, is considerably less than the cost of the Sanborn recorder.

The instrument also has a built-in safety device. For instance, if the paw of a test mouse accidentally touches the screen during a test or if the mouse urinates, events that essentially cause a short circuit or an overload, the instrument automatically shuts itself off and thus invalidates the test.

The new apparatus is shown in Figure 5 as it is set up during repellency testing. (The untreated portions of the mouse were not masked in this Figure in order to show the wire mesh.)

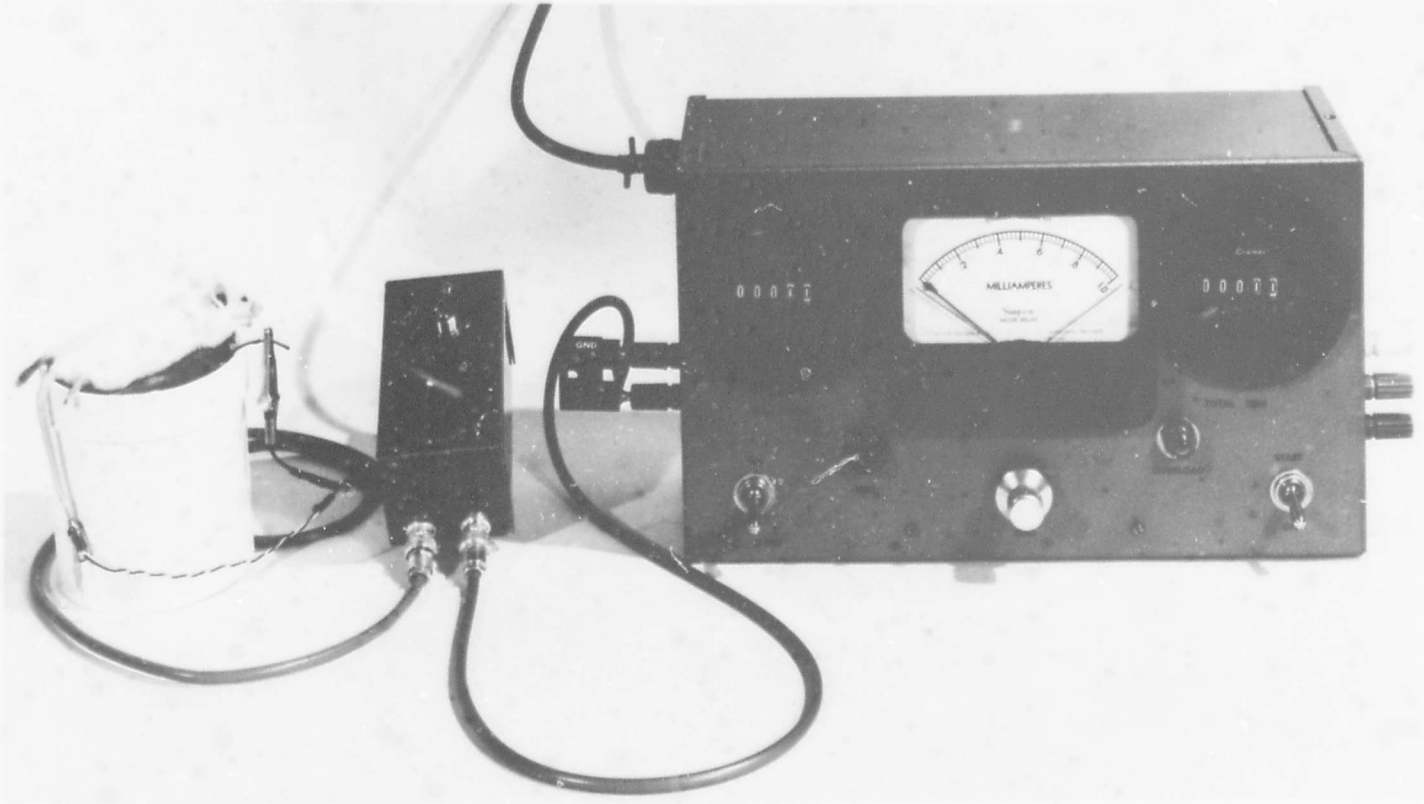


Figure 5

NEW BITOMETER-TIMER

2. Circuitry

a. Field-Effect Voltmeter Circuit

A schematic diagram of the sensitive field-effect transistor voltmeter circuit, which controls the timers, is shown in Figure 6. The input signal is applied to the gate of Q1, the input resistance of which is nominally in excess of 10^{15} ohms. To maintain a constant input resistance, the input signal is shunted by a fixed resistance of 10^{10} ohms (R11). Extreme caution should be excercised in wiring the input circuitry of the field effect transitor (FET) to minimize the possibility of leakage paths. The gate terminal and the high side of the 10^{10}-ohm resistor should be floated on a Teflon or ceramic standoff insulator. Both the standoff insulator and the 10^{10}-ohm resistor should be washed with a chemically pure solvent such as Freon TF after assembly. Also, the FET should be washed, particularly around the leads. Layout of the remaining circuitry is not critical.

The FET load resistor must be selected to match the characteristics of the particular FET used. This is accomplished by setting the zero adjust at its midpoint, temporarily connecting a 10,000-ohm potentiometer in place of R2, and adjusting for zero deflection on M-1. This adjustment is independent of the settings of R6 and R8. The potentiometer is then removed and measured to determine the final value for R2. At balance, the voltages at points A and B are equal, and hence no current flows through the meter.

The three diodes CR-1, CR-2, and CR-3 comprise the main regulator for the power supply (Figure 7) and establishes reference points for the relay control logic. Point B is approximately 1 v more positive than point C. This voltage appears across the 500-ohm potentiometer (R8) whose setting adjusts the potential at terminal 2 of the μA710C integrated circuit module. This integrated circuit is a differential comparator whose output at terminal 7 depends upon the state of the two inputs, terminals 2 and 3. When terminal 2 is positive with respect to 3, the output is 1.0 v negative relative to terminal 1. Since Q3 has its emitter also common to pin 1 on the μA710C, this transistor is reverse biased and no collector current flows. Since the collector current of Q3 supplies the base drive for Q4, this transistor is also nonconducting and the relay, RY-2, is deenergized.

When a positive voltage is applied to the input, both Q1 and Q2 conduct more heavily, and an increased voltage drop across R4 results. Point A drops to a lower potential. Since point A supplies the second input to the comparater, the difference between the two inputs decreases. When terminal 3 becomes more negative than 2 (by approximately 1 mv), the output (pin 7) switches abruptly to a 3 v positive, again with respect to pin 1. This causes both Q3 and Q4 to conduct, and the relay is energized.

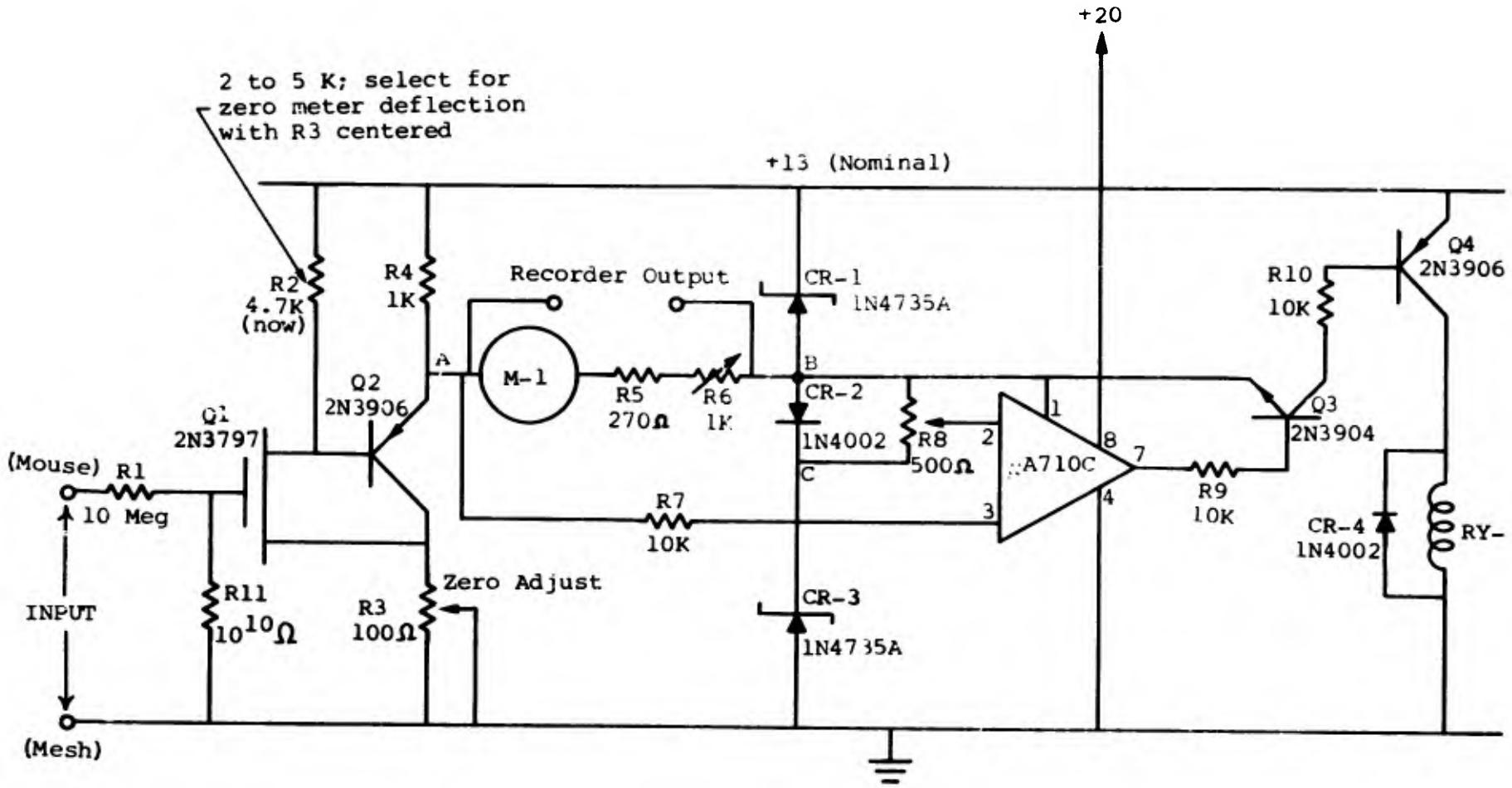


Figure 6.

FIELD EFFECT VOLTMETER CIRCUIT

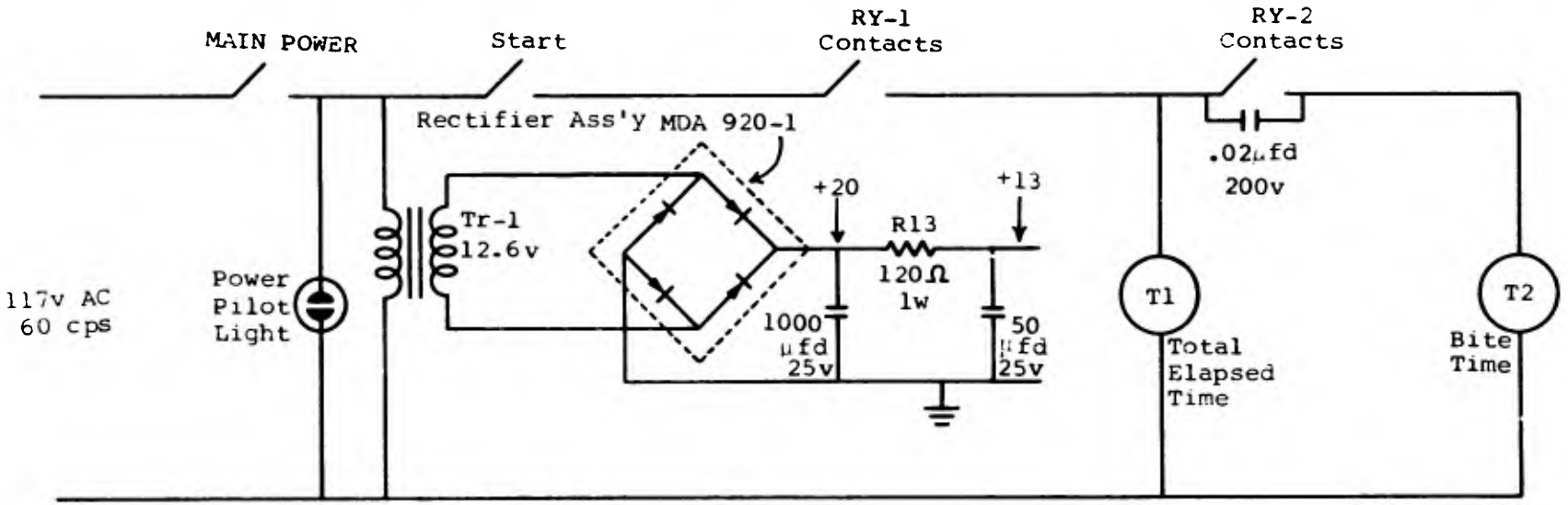


Figure 7.

POWER SUPPLY AND TIMER CIRCUIT

There are two calibration adjustments in this circuit. Full-scale meter sensitivity is set by R6 and the threshold level at which the relay trips is set by R8. R6 should be set before R8.

The meter used in this system has an adjustable contact that can be manually set to energize an external circuit at any desired deflection. In this case, it used to control the overload relay. The overload circuit is shown in Figure 8. At low currents, the meter contact is normally open. The relay control transistor, Q5, is conducting, since base bias is provided through the overload lamp. In the event of lamp failure, this transistor does not conduct and the timers do not start. The base current of the transistor is not sufficient to light the lamp in normal operation.

As the current increases to the point at which the meter contact closes, 13 v is applied to the lamp and Q5 turns off; the relay RY-1 is thus deenergized. As is shown below, this event stops both timers.

b. Power Supply and Timer Circuit

The power supply for the low-voltage circuits (Figure 7) consists of a standard 12.6-v filament transformer (Tr-1) followed by a full-wave bridge rectifier. This supplies a nominal 20 v DC at the bridge output, which is then regulated to 13 v by the diode string in the voltmeter circuit (Figure 6). Each IN4735A is a 6.2-v zener diode, while IN4002 is a forward-biased standard silicon diode. No further regulation is required.

The main power switch applies power to the low-voltage power supply and to the start switch. Therefore the meter can be adjusted before an experiment is started.

When all adjustments have been completed and the timers reset, the start switch may be placed in the on position. This procedure applies power to T1 to record the total elapsed time, unless an overload condition exists. (Recall that RY-1 is normally energized except for an overload condition.) If an overload exists, the instrument should be turned off and the cause of the overload corrected.

Relay RY-2 becomes energized, when a mosquito (or mosquitoes) complete the circuit and timer T2 starts recording the total bite time. Upon the completion of the experiment, the start switch may be turned off, and the ratio T2/T1 represents percentage of bite time. This ratio is correct even if the experiment is terminated by an overload. A .02 μfd capacitor across the contacts of RY-2 reduces the break hysteresis in the relay contacts at threshold values.

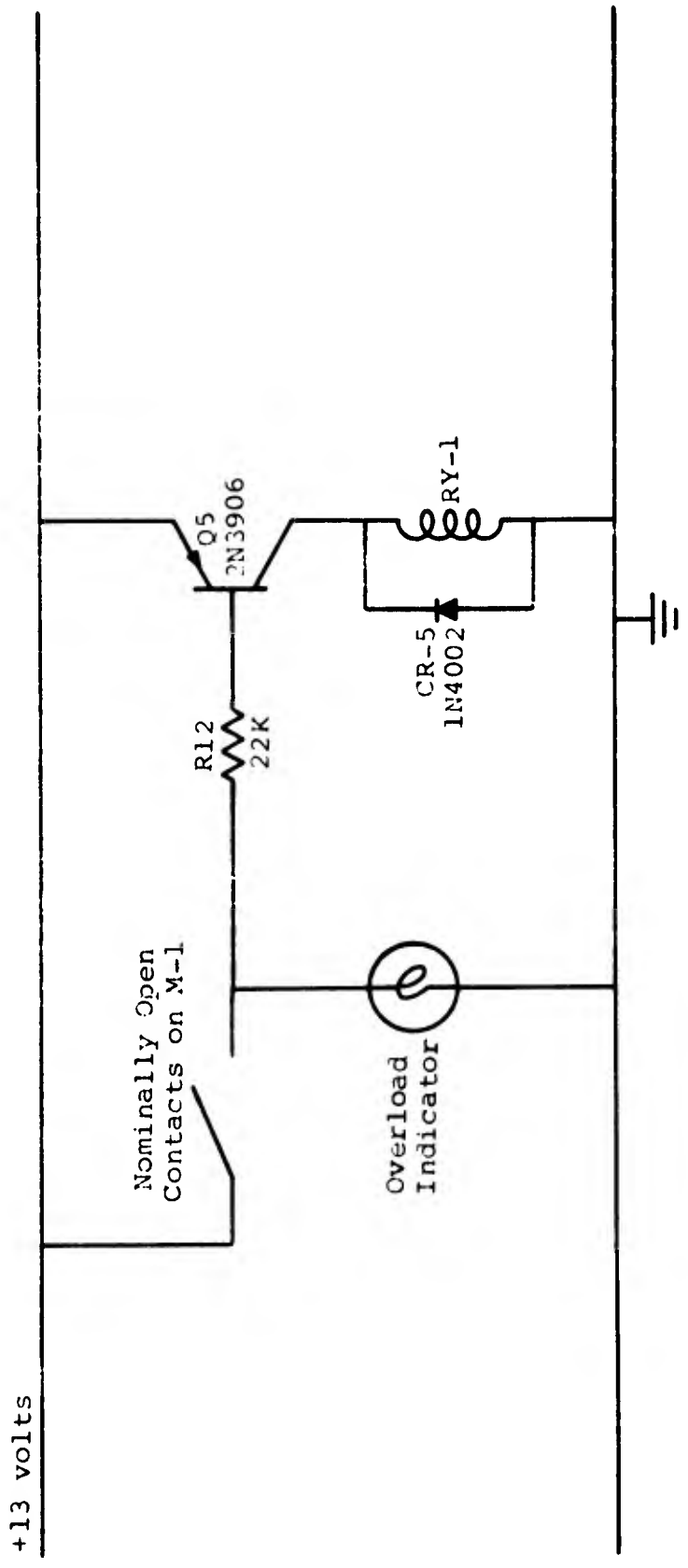


Figure 8.

OVERLOAD CIRCUIT

c. Potential Box

Since the electronic circuitry associated with the meter and the relay control is basically a voltage-sensing system, a voltage source must be included in the input circuit. The potential box is illustrated in Figure 9. The source is a 1.35 v mercury cell with the terminal voltage adjusted by means of a 1-megohm potentiometer. The 10^8-ohm resistor (R15) is the terminating resistor of the potential box. It is connected across the output terminals to lower the time constant (recovery rate) of the field-effect voltmeter circuit when the mosquitoes stop biting, so that a zero reading on M-1 is quickly achieved, and T2 instantly stops. If a high resistance path (Rx) such as a mosquito bite connects the input terminal, the maximum voltage at the output is:

$$V_0 = 1.35 \frac{10^8}{R_x + 10^8}$$

In actual operation, however, the voltage is less, depending upon the setting of the potentiometer connected across the cell.

The output of the potential box is connected to the timer unit, while the input is wired into the experiment by using the special input cable shown in Figure 10. The function of the 10-megohm resistor (R16) built into the cable together with the 0.0047-μf capacitor in the potential box is to filter out any AC component that may be present. Cable lengths are not critical.

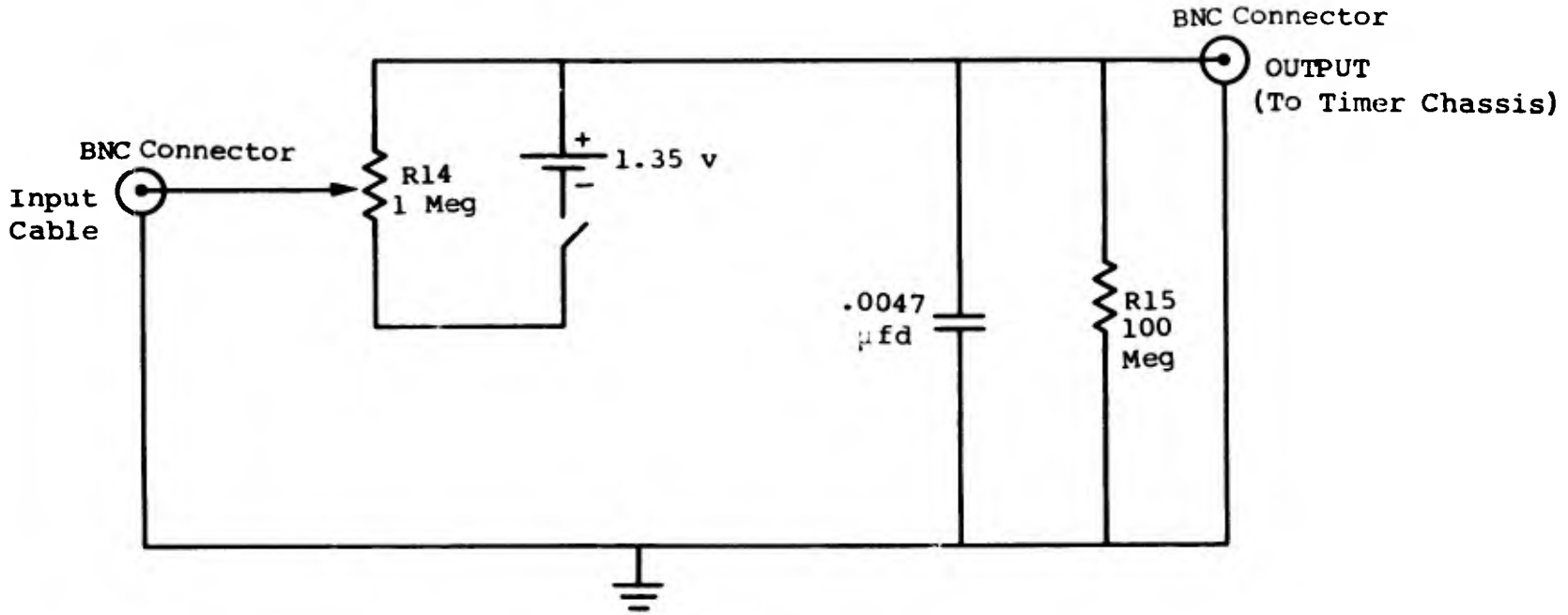


Figure 9.

POTENTIAL BOX

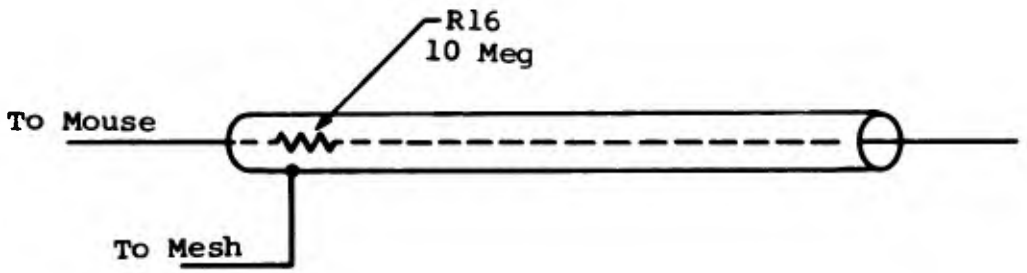


Figure 10.

INPUT CABLE

3. Calibration and Operation

The following procedure is followed to initially calibrate the instrument. Connect to 117 v AC outlet, turn AC power switch on. then:

(1) Turn off the battery switch on the battery box, short the leads to the mouse and the mesh, and adjust for zero on meter M-1 with the zero-adjust potiometer (R3).

(2) Set the 1-megohm potentiometer on the potential box (R14) to 1 on the dial, and switch the battery on. Then increase R14 to 3 on the dial.

(3) Adjust the 1 K potentiometer (R6) for full-scale deflection on M-1.

(4) Return the battery-box potentiometer (R14) to 1 on the dial; then increase R14 until the meter reads 0.1.

(5) Adjust the threshold of the 500-ohm potiometer (R8) until the bite-time timer (T2) just starts.

(6) Return the battery-box potentiometer (R14) to 1 on the dial to make sure that the bite-time timer (T2) stops.

(7) Unshort the mouse and the mesh leads, and connect the leads to the mouse and the mesh, respectively. Place the mouse on top of the mesh.

(8) Set the potential box potentiometer (R14) to between 5 and 7.5 on the dial; then immediately reset zero with the zero-adjust potiometer (R3) while no bites are occurring. Set the bite-time timer (T2) to zero and the elapsed-time timer (T1) to zero. The repellency test may now proceed. Turn start switch on.

Only step 8 need be followed in experiments after the initial calibration.

A components list for the construction of this instrument is given in Appendix F. The component layout of the finished bitometer-timer and battery box are shown in Figure 11. Figure 12 shows the wiring underneath the vector board, which is obscured in Figure 11.

We constructed three of these bitometer-timers for use in our repellency assays, and they should be of considerable aid in our future repellency testing.

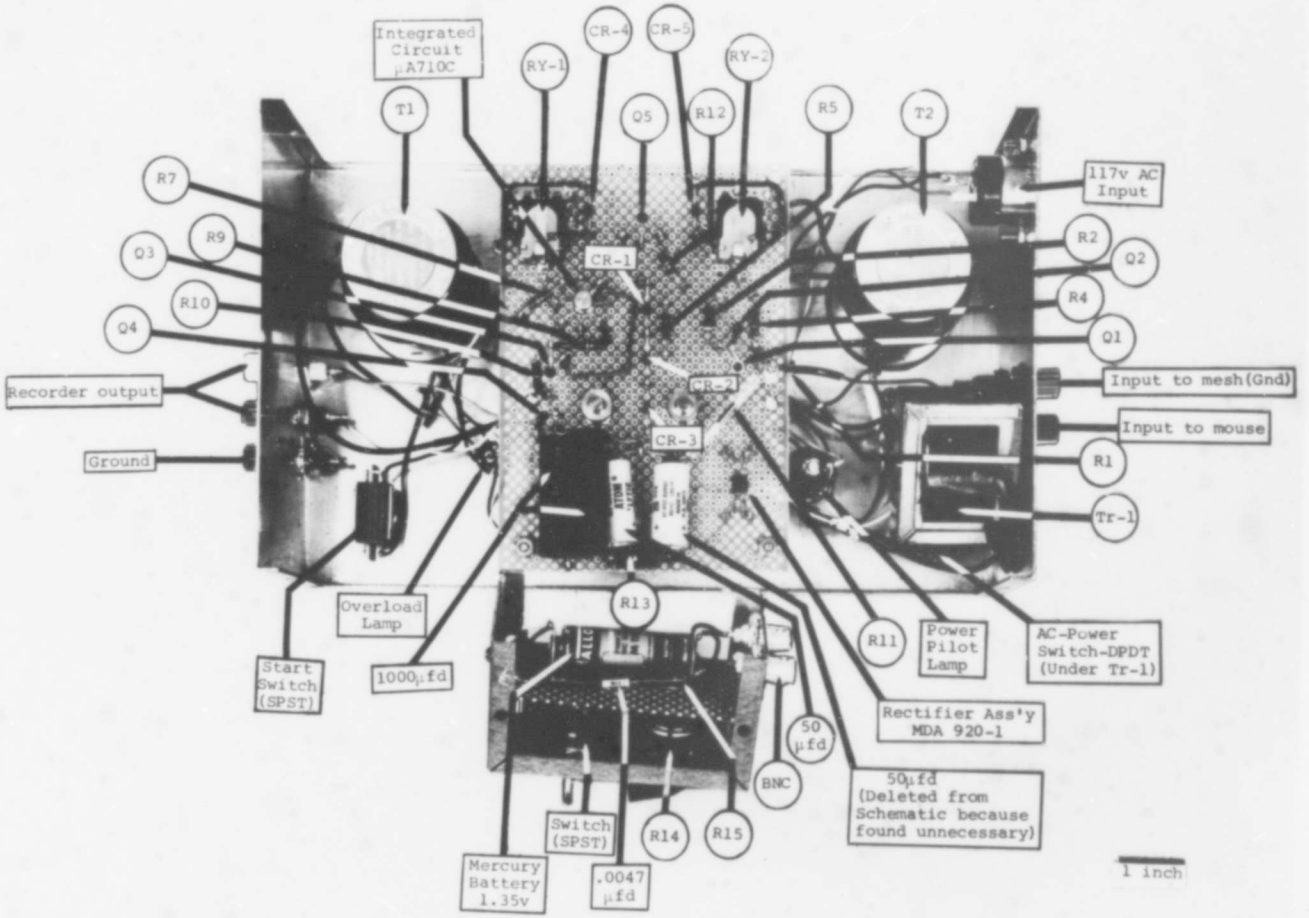


Figure 11.

COMPONENT LAYOUT OF FINISHED BITOMETER-TIMER AND BATTERY BOX

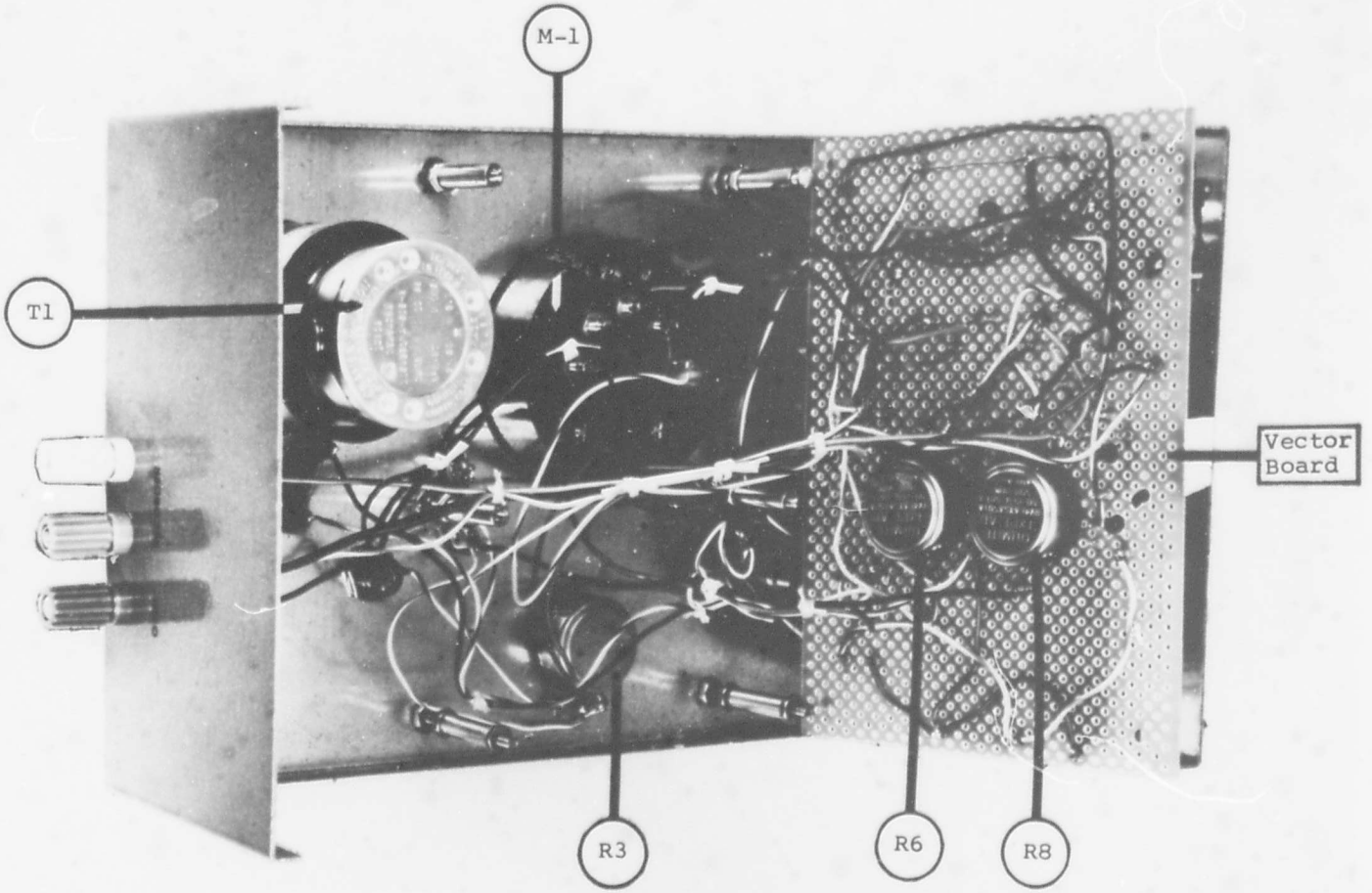


Figure 12.

WIRING UNDERNEATH VECTOR BOARD

V. SUMMARY AND DISCUSSION

A. GABA Hypothesis

We have presented (ref. 2) detailed expositions on the GABA hypothesis. Briefly, the hypothesis states that GABA, a substance known to inhibit the transmission of impulses across crustacean synapses and found exclusively in association with the nervous tissue of many mammalian and nonmammalian species, may also intermediate synaptic inhibition in a mosquito. It was proposed that GABA combines with CO_2 and that the resulting GABA-CO_2 complex does not possess the synaptic inhibitory power of GABA alone. Therefore in the vicinity of a potential host, where the CO_2 content is increased above the normal atmospheric CO_2 content, the mosquito is activated. This activation is hypothesized to underlie the host-seeking behavior of mosquitoes. GABA is therefore proposed as the actual CO_2 receptor substance in nervous tissues of mosquitoes.

Evidence to support this hypothesis was obtained when it was observed that GABA exists in aqueous dialyzable extracts of mosquitoes, that GABA does bind CO_2, and that the binding is easily destroyed by heat. The complex is rapidly dissociated as the temperature approaches that of a warm-blooded animal. We recently obtained actual physiological evidence that a GABA-CO_2 complex does not inhibit nervous transmission.

B. Repellents Design

On the basis of the GABA hypothesis, it was reasoned that volatile analogues of GABA may repel mosquitoes by neutralizing the stimulatory effects of CO_2 emanating from a host, i.e., by causing neuroinhibition and loss of host recognition. Our approach was subsequently broadened on the basis of the fact that certain well-known mosquito repellents such as DEET and Rutgers 612 bear no obvious relationship to the structure of GABA. Within their molecules, these compounds were shown to contain certain relationships of nucleophilic and electrophilic moieties. The electronic configurations of these substances were correlated with the electronic configurations of GABA, and it was proposed that the electronic configuration within a molecule (rather than its actual chemical constitution) coupled with its ability to be volatilized under ordinary conditions determines its repellent properties.

Investigations were subsequently undertaken to test this proposition, and the experimental evidence accumulated largely supported these predictions. Substances such as the presumed gamma-aminobutyraldehyde, o-aminobenzaldehyde, many substituted aminoaldehydes, amino-alcohols, and unsaturated alcohols (e.g., 3-butene-2-ol) that contain at least one nucleophilic and one electrophilic center within one molecule were essentially repellent to mosquitoes. Therefore a rational basis in the search for potent, long-lasting repellents has been proposed and thus far supported by our experimental findings.

In future work it may be possible to "tailor-make" repellents for topical or internal administration, that incorporate all the properties necessary for potent repellency and low toxicity. It may be possible, for instance, to construct a triglyceride for oral administration that has an amino or a substituted amino group on the last carbon atom of one or more of its fatty acid chains. During the normal sequential biochemical degradation of this triglyceride, amino alcohols, aldehydes, and unsaturated amino hydrocarbons will be formed. Fatty acids are usually found in sweat, and the degradation products of these synthetic triglycerides may therefore also be exuded in sweat. If the repellency of this class of substances parallels in actual use what we have found in our laboratory investigations, a very small amount of this material on the skin surface may be highly repellent to mosquitoes. We may thus be able to recruit the body's own normal metabolic processes to manufacture an orally effective insect repellent from ingested raw materials.

The actual existence of free fatty aldehydes as components of mammalian lipids has been suggested. Acylated alkenyl alpha-glycerol ethers are naturally occurring aldehydogenic lipids in mammalian tissues (ref. 33,34). An enzyme system capable of hydrolyzing 1-alkenyl glycerol 3-phosphorylcholine to yield a fatty aldehyde and a glycerol phosphorylcholine in rat liver has been described (ref. 35). During the biosynthesis of sphingosine by brain tissue, palmitoyl-CoA is reduced by NADPH* to palmitaldehyde, which is then incorporated into dihydrosphinogosine (ref. 36). A similar observation has been made in experiments with human subjects (ref. 37). The natural occurrence of free fatty aldehydes has also been demonstrated in mammalian heart muscle (ref. 38).

Amino aldehydes have also been implicated as precursors of collagen biosynthesis in rat skin (ref. 39), and several basic and neutral amino aldehydes were found to be present in tropocollagen fractions extracted from carp swim bladders (ref. 40).

Therefore our approach to the development of an orally effective insect repellent through the mediation of a metabolically produced free or substituted amino aldehyde appears to have a biochemical basis and reality in naturally occurring biosynthetic and degradative pathways.

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*Reduced nicotine adenine dinucleatide phosphate.

C. Repellency Assay

The electronic method developed in our laboratories that detects and records the bite of a mosquito and permits the visualization of every phase of the mosquito bite has been fully described in past reports (ref. 1) and publications (ref. 30,31). In applying this method to repellent bioassay, a statistical discriminant function analysis showed that only two parameters are necessary to achieve a good separation of control (untreated) from test (repellent-treated) mice. The parameters are (1) the percentage of time that the recording stylus is displaced in the electronic recording during the 30-min test period (P) and (2) the percentage of mosquitoes that engorge blood out of the approximately 50 mosquitoes that are employed in each test (E).

A specialized digital computer program was written that compares test cases to paralled control cases and ultimately yields confidence limits (upper bounds) for the tests in terms of the controls. These comparisons are made on a daily basis, since analysis of variance usually showed that the day on which a given test is performed contributes significantly to variations in the controls and therefore presumably also to variations in the tests. The computer program compares controls run on the same day as the test animals to derive statistical tests of significance.

Compounds can be comparatively ranked for repellent efficacy, depending upon how well significant repellency is retained as the concentration is diminished and upon the value of the upper bound. Therefore we have developed an accurate, sensitive, and efficient means for the assay of repellents by utilizing an electronic recording method.

A new apparatus recently designed and built in our laboratories gives a direct readout for the bite time, which is the only parameter needed from the electronic recorder in our bioassay method. The apparatus consists essentially of two electronic digital timers and a meter relay, all of which are actuated by solid-state circuitry. One timing meter is actuated only when the mosquitoes bite, and the other timing meter runs continuously during the test. All the pertinent electronic data are thus obtained in a very simple way. This new instrument permits the total bite time to be read directly from one timing meter and the total time of the test from the other. After the data are recorded, a reset button is pushed and the apparatus is ready for the next test.

VII. FUTURE INVESTIGATIONS

The approaches described above and detailed in previous reports will be continued in future work. We anticipate that our future-work program may encompass the following areas of investigation.

A. Search for New Repellents

The search for new repellents is no longer purely empirical, but has been narrowed to very specific demands in terms of molecular and electronic configurations of candidate repellents. Commercially available materials will be obtained for testing whenever possible, if they appear to fit our structural requirements. If certain promising structures are unavailable commercially, we will consult our organic chemists in the synthesis and the purification of these compounds.

B. Synthesis of Amino Triglycerides

The synthesis of amino triglycerides appears to be very promising in terms of the development of an orally effective insect repellent. We will require the active participation and cooperation of our organic chemists in this effort. Pending the availability of funds for this work, we will attempt to esterify into a triglyceride molecule the omega-amino acids that upon metabolic degradation may give rise to the classes of highly potent repellent substances discovered during this work. In general, these substances appear to be amino or substituted amino aldehydes. It should also be considered that ethers may be more aldehydogenic than esters (ref. 33,34), and the synthesis of various glycerol ethers will also be attempted. When available, these substances will be fed to small mammals (mice or rats) at various dose levels, and these animals will be tested for repellency. Later, we anticipate that primates, animals having sweat glands, may be fed these substances and subsequently tested for repellency.

C. Purification of Hydrolysis Products of 4-Aminobutyraldehyde Diethylacetal

Our experiments have shown that when the diethylacetal of 4-aminobutyraldehyde is hydrolyzed with acid and neutralized, the resultant product is an exceptionally potent mosquito repellent. We presumed that the active substance in the hydrolysis mixture is 4-aminobutyraldehyde. At present we have no evidence to support this assumption. One of our goals will be to fractionate, purify, and characterize the active repellent substance(s) in this mixture. The fractionation will be followed with bioassay for repellency at each step to observe the partitioning of the repellent activity.

D. Further Verification of GABA Hypothesis

We will continue to gather evidence to further establish the validity of the GABA hypothesis in physiological systems. The encouraging positive results thus far obtained with the cockroach (Periplaneta americana) preparation described will be further substantiated. The effect of repellent vapors on this preparation will also be studied.

We will also further investigate the distribution of GABA in mosquitoes. By a method recently available (ref. 41) it is possible to histologically localize the actual site(s) of GABA synthesis in tissue sections of nervous tissue. The method makes use of the fact that in the course of the metabolic pathway by which GABA is converted to succinic acid, a tetrazolium salt is reduced to insoluble formazan. To apply this histochemical method to the determination of the sites of GABA metabolism in mosquitoes, cold sectioning of mosquitoes will be performed, and the histochemical method will be applied. This work should add further support to the GABA hypothesis.

When we localize the neural tissue in the mosquito in which GABA is synthesized, we may be able to insert microelectrodes to study the action potentials of the nerve fibers in the absence and the presence of mosquito-repellent vapors and CO_2. It may thus be possible to conclusively demonstrate whether repellents cause neuroinhibition (as we have postulated) whether CO_2 causes activation, and the general validity of these investigations in terms of the mosquito itself.

E. Determination of Repellents Toxicity

Since the classes of compounds tested as repellents are new in terms of human application, we envision that toxicity investigations of the more potent substances will eventually have to be undertaken to establish their merit for human use. These tests should include toxicity studies for internal and topical administration, according to standards and procedures established by the U.S. Food and Drug Administration. IIT Research Institute is well equipped to carry out such a toxicity-testing program and has had substantial experience with toxicity-testing procedures. The toxicology studies will be initiated if funds are available.

F. Maintenance of Mosquito Colony

Our colony of Aedes aegypti (L) mosquitoes will continue to be bred and maintained for purposes of repellency testing throughout the course of this work.

VIII. CONCLUSION

We believe that the approaches delineated above have established a sound foundation for ultimate success in the development of an orally effective insect repellent. The basic theoretical approaches and methodological innovations developed during the course of this program appear to be yielding fruitful results. These concerted efforts should be sustained and even more intensified to hasten the solution of a very important problem.

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APPENDIX A

SYNTHESIS PROCEDURES

SYNTHESIS PROCEDURES

I. N-ACETYL GABA

The N-acetyl derivative of GABA was prepared by the method described by Mori,[1] with the amino acid and the acetic anhydride. After drying in vacuo over potassium hydroxide for 18 hr, the crude product (mp, 123 to 128°C) was recrystallized from ethyl acetate-ethanol to a melting point of 128.5 to 130.0°C (lit. mp, 126 to 127°C,[1] 129°C[2]).

II. ETHYL ESTER OF 4-AMINOBUTYRATE

Ethyl-4-aminobutyrate was prepared by the Fisher esterification procedure employing absolute ethanol, hydrogen chloride gas, and 4-aminobutyric acid. (The 4-aminobutyric acid was purchased from Sigma Chemical Company; found mp, 199.5 to 200°C; softens at 198°C; used without further purification.) The ethanol was saturated with hydrogen chloride, the acid was added; and the solution was allowed to stand at room temperature for 1 week. The solvent was removed, and the residue was dissolved in a small amount of water and neutralized with sodium carbonate. The basicified solution was continuously extracted with ether for 21 hr. Then the ethereal extract was dried, and the ether was removed at atmospheric pressure. Low vacuum distillation of the residue yielded a colorless distillate (bp at 24 mm Hg, 94.5 to 95.0°C; n_D^{20}, 1.4332). Proton magnetic resonance spectral data showed the distillate to be ethyl 4-aminobutyrate (lit.[3] bp at 12 mm Hg, 75 to 77°C).

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APPENDIX B

ASSAY OF COMPOUNDS FOR REPELLENCY

APPENDIX B

ASSAY OF COMPOUNDS FOR REPELLENCY

The control values upon which the tests of repellency of the following compounds were based are shown in Appendix B. The abbreviated compound names of the compounds listed on the computer program are defined below.

Computer Listing	Compound Name, Formula and Treatment
AO- and YO- series	Supplied by Dr. R. P. Quintana of the University of Tennessee.
3-NH2-1-PROPANOL	3-Amino-1-propanol $CH_2(OH)CH_2CH_2(NH_2)$
3-DEA-1-PROPANOL	3-Diethylamino-1-propanol $(C_2H_5)_2NCH_2CH_2CH_2(OH)$
1-DEA-2-PROPANOL	1-Diethylamino-2-propanol $(C_2H_5)_2NCH_2CH(OH)CH_3$
4-DEA-1-BUTANOL	4-Diethylamino-1-butanol $(C_2H_5)_2NCH_2CH_2CH_2CH_2OH$
3-DMA-1-PROPANOL	3-Diethylamino-1-propanol $(CH_3)_2N(CH_2)_3OH$
1-DMA-2-PROPANOL	1-Dimethylamino-2-propanol $(CH_3)_2NCH_2CH(OH)CH_3$
4-DMA-1-BUTANOL	4-Dimethylamino-1-butanol $(CH_3)_2N(CH_2)_4OH$
1133TETRAMETHUREA	1,1,3,3-tetramethylurea $(CH_3)_2NCON(CH_3)_2$
DEA ACETONE	Diethylamino acetone $(C_2H_5)_2NCH_2COCH_3$
GABA-ETHYL-ESTER	Gamma-aminobutyrate ethyl ester $(NH_2)CH_2CH_2CH_2COOC_2H_5$
22AMETHOXYETHANOL	2-(2-Aminoethoxy)-ethanol $H_2NCH_2CH_2OCH_2CH_2OH$

Computer Listint	Compound Name, Formula and Treatment
3-BUTENE-2-OL	3-Butene-2-ol $CH_3CH(OH)CH{:}CH_2$
4 AMBUTALDDEAHWNB	4-Aminobutyraldehyde diethyl acetal Hydrolyzed in water with hydrochloric acid, neutralized with base
2AM-BENZALDEHYDE	2-Aminobenzaldehyde $o\text{-}H_2N(C_6H_4)CHO$
NNDIPENYLFORMIDE	N,N-Diphenylformamide $HCON(C_6H_5)_2$
DEAACETALD DEACET	Diethylaminoacetaldehyde diethyl acetal $(C_2H_5)_2NCH_2CH<^{O(C_2H_5)}_{O(C_2H_5)}$
DEET	N,N-Diethyl-m-toluamide $C_6H_4CH_3\text{-}m\text{-}CON(C_2H_5)_2$

Control Values

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS	Day of Test	Test Number
CONTROL	-0.00000	5.77	63.00	68.77		22	1
	-0.00000	5.77	45.72	51.49		22	2
	-0.00000	3.85	22.66	26.51		22	3
	-0.00000	47.17	83.50	130.67		22	4
	-0.00000	18.00	61.76	79.76		23	5
	-0.00000	7.69	49.20	56.89		23	6
	-0.00000	8.16	43.56	51.72		23	7
	-0.00000	8.00	57.58	65.58		23	8
	-0.00000	11.76	52.98	64.75		24	9
	-0.00000	5.88	44.76	50.64		24	10
	-0.00000	12.24	42.38	54.62		24	11
	-0.00000	8.33	65.39	73.72		25	12
	-0.00000	3.77	53.39	57.16		25	13
	-0.00000	52.08	95.84	147.93		25	14
	-0.00000	52.27	98.94	151.21		26	15
	-0.00000	17.65	67.48	85.13		26	16
	-0.00000	62.00	94.86	156.86		27	17
	-0.00000	31.25	91.54	122.79		27	18
	-0.00000	28.26	95.71	123.97		28	19
	-0.00000	52.50	93.09	145.59		28	20
	-0.00000	36.00	63.43	99.43		28	21
	-0.00000	9.09	52.54	61.63		29	22
	-0.00000	10.20	56.13	66.34		30	23
	-0.00000	30.23	88.76	118.99		30	24
	-0.00000	19.61	67.99	87.60		31	25
	-0.00000	11.76	62.38	74.15		31	26
	-0.00000	15.38	91.99	107.37		32	27
	-0.00000	6.00	55.05	61.05		32	28
	-0.00000	7.84	52.81	60.65		33	29
	-0.00000	61.11	92.40	153.52		33	30
	-0.00000	23.53	97.13	120.66		34	31
	-0.00000	42.00	90.35	132.35		34	32
	-0.00000	6.38	38.18	44.56		34	33
	-0.00000	20.75	73.80	94.56		34	34
	-0.00000	14.89	63.17	78.07		35	35
	-0.00000	30.00	59.66	89.66		35	36
	-0.00000	10.20	53.78	63.98		36	37
	-0.00000	22.00	66.08	88.08		36	38
	-0.00000	36.73	84.75	121.48		37	39
	-0.00000	24.49	69.11	93.60		37	40
	-0.00000	22.45	95.98	118.43		37	41
	-0.00000	16.67	78.62	95.29		37	42
	-0.00000	16.98	92.73	109.71		38	43
	-0.00000	10.00	74.15	84.15		38	44

Control Values (Cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS	Day of Test	Test Number
	-0.00000	7.27	65.56	72.83		39	45
	-0.00000	9.62	77.18	86.80		39	46
	-0.00000	14.81	67.01	81.83		40	47
	-0.00000	28.30	85.08	113.38		40	48
	-0.00000	46.15	98.31	144.46		41	49
	-0.00000	37.04	97.45	134.48		42	50
	-0.00000	46.00	95.48	141.48		42	51
	-0.00000	5.66	39.05	44.71		43	52
	-0.00000	65.38	98.40	163.78		44	53
	-0.00000	70.91	87.01	157.92		44	54
	-0.00000	26.42	85.30	111.71		45	55
	-0.00000	36.00	68.82	104.82		45	56
	-0.00000	42.31	97.07	139.38		45	57
	-0.00000	10.00	66.48	76.48		46	58
	-0.00000	32.65	84.65	117.30		46	59
	-0.00000	56.86	98.95	155.81		47	60
	-0.00000	66.67	74.86	141.53		47	61
	-0.00000	40.00	85.14	125.14		47	62
	-0.00000	32.65	96.27	128.92		47	63
	-0.00000	29.17	78.13	107.30		47	64
	-0.00000	33.33	78.55	111.89		47	65
	-0.00000	33.33	86.78	120.11		48	66
	-0.00000	27.45	84.09	111.54		48	67
	-0.00000	16.67	48.73	65.40		49	68
	-0.00000	16.00	74.74	90.74		49	69
	-0.00000	35.29	79.94	115.24		49	70
	-0.00000	14.00	58.90	72.90		49	71
	-0.00000	30.00	94.17	124.17		49	72
	-0.00000	46.15	84.19	130.34		50	73
	-0.00000	16.98	73.20	90.19		50	74
	-0.00000	3.70	14.81	18.51		50	75
	-0.00000	4.00	22.85	26.85		50	76
	-0.00000	3.85	48.56	52.40		51	77
	-0.00000	9.09	60.56	69.66		51	78
	-0.00000	21.57	57.84	79.41		52	79
	-0.00000	8.00	51.81	59.81		52	80
	-0.00000	7.55	33.30	40.84		52	81
	-0.00000	17.31	60.09	77.40		53	82
	-0.00000	26.92	69.27	96.20		53	83
	-0.00000	4.26	38.58	42.84		54	84
	-0.00000	5.77	38.67	44.43		54	85
	-0.00000	20.41	77.38	97.79		55	86
	-0.00000	26.42	93.45	119.87		55	87
	-0.00000	14.81	75.80	90.61		56	88

Control Values (Cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
	-0.00000	28.85	77.85	106.70			56	89
	-0.00000	17.65	79.83	97.48			57	90
	-0.00000	16.33	63.31	79.63			57	91
	-0.00000	7.41	60.99	68.39			57	92
	-0.00000	12.00	50.66	62.66			58	93
	-0.00000	30.19	99.46	129.64			58	94
	-0.00000	20.75	78.17	98.93			59	95
	-0.00000	24.53	89.38	113.90			59	96
	-0.00000	12.96	50.11	63.07			60	97
	-0.00000	27.78	49.72	77.50			60	98
	-0.00000	14.00	71.19	85.19			61	99
	-0.00000	22.64	83.31	105.96			61	100
	-0.00000	13.46	59.72	73.19			62	101
	-0.00000	7.69	37.62	45.31			62	102
	-0.00000	7.27	64.88	72.15			63	103
	-0.00000	19.61	55.37	74.98			63	104
	-0.00000	25.45	81.82	107.28			64	105
	-0.00000	35.19	83.09	118.28			64	106
	-0.00000	24.44	81.16	105.60			65	107
	-0.00000	22.64	82.49	105.13			65	108
	-0.00000	19.61	42.22	61.82			1	109
	-0.00000	7.55	85.51	93.06			1	110
	-0.00000	36.54	80.44	116.98			2	111
	-0.00000	22.45	51.10	73.55			2	112
	-0.00000	10.20	56.24	66.45			3	113
	-0.00000	30.00	73.72	103.72			3	114
	-0.00000	11.54	60.39	71.92			3	115
	-0.00000	7.41	52.84	60.24			4	116
	-0.00000	18.87	67.73	86.60			4	117
	-0.00000	5.77	25.08	30.85			5	118
	-0.00000	14.81	72.87	87.69			5	119
	-0.00000	9.26	56.30	65.56			5	120
	-0.00000	38.00	72.13	110.13			6	121
	-0.00000	26.42	84.21	110.63			7	122
	-0.00000	3.85	56.33	60.18			8	123
	-0.00000	41.51	84.30	125.81			8	124
	-0.00000	66.67	58.60	125.26			9	125
	-0.00000	21.28	54.27	75.55			9	126
	-0.00000	44.12	94.87	138.99			10	127
	-0.00000	28.57	55.43	84.01			10	128
	-0.00000	32.61	59.68	92.29			10	129
	-0.00000	8.51	74.49	83.00			10	130
	-0.00000	50.00	84.04	134.04			11	131
	-0.00000	69.81	81.36	151.18			11	132
	-0.00000	15.22	45.48	60.70			11	133
CONTROL	-0.00000	23.50	69.37	92.87	100.0	-0.0000=CONTRAST		
		16.40	19.23	32.51	(107.4)UPPER BOUND	3.4595=STANDARD ERROR		

ANALYSIS OF VARIANCE (of Controls)

EFFECT	S.S.	D.F.	M.S.	F
DAY	77398.094	54.	1433.298	1.801
ERROR	62078.906	78.	795.883	
TOTAL	139477.000	132.	1056.644	

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.IN-CH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A036	50.00000	0.00	0.00	0.00			34	1
	50.00000	0.00	3.21	3.21			34	2
A036	50.00000	0.00	1.60	1.60	1.6	96.4296=CONTRAST		
		-0.00	2.27	2.27	(51.4)UPPER BOUND	24.4318=STANDARD ERROR		
A036	30.00000	6.38	17.60	23.98			33	1
	30.00000	5.77	70.68	76.45			33	2
A036	30.00000	6.08	44.14	50.21	46.9	56.8694=CONTRAST		
		0.43	37.54	37.10	(99.5)UPPER BOUND	28.2114=STANDARD ERROR		
A036	10.00000	4.00	19.44	23.44			33	1
	10.00000	10.20	63.00	73.20			33	2
A036	10.00000	7.10	41.22	48.32	45.1	58.7636=CONTRAST		
		4.39	30.80	35.19	(97.7)UPPER BOUND	28.2114=STANDARD ERROR		
A036	5.00000	2.13	15.55	17.68			34	1
	5.00000	7.55	49.04	56.59			34	2
A036	5.00000	4.84	32.30	37.13	37.9	60.6984=CONTRAST		
		3.83	23.68	27.51	(87.6)UPPER BOUND	24.4318=STANDARD ERROR		
A035	30.00000	0.00	0.00	0.00			32	1
	30.00000	0.00	0.00	0.00			32	2
A035	30.00000	0.00	0.00	0.00	0.0	84.2143=CONTRAST		
		-0.00	-0.00	-0.00	(66.8)UPPER BOUND	28.2114=STANDARD ERROR		
A035								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS			Day of Test	Test Number
A035									
	10.00000	7.55	48.06	55.61				32	1
	10.00000	0.00	0.00	0.00				32	2
A035	10.00000	3.77	24.03	27.80	33.0		56.4097=CONTRAST		
		5.34	33.98	39.32	(99.8)	UPPER BOUND	28.2114=STANDARD ERROR		
A035									
	5.00000	16.98	81.08	98.06				34	1
	5.00000	3.92	33.92	37.84				34	2
A035	5.00000	10.45	57.50	67.95	69.3		30.0808=CONTRAST		
		9.23	33.35	42.58	(119.0)	UPPER BOUND	24.4318=STANDARD ERROR		
A032									
	75.00000	0.00	26.83	26.83				28	1
A032	75.00000	0.00	26.83	26.83	21.8		96.1627=CONTRAST		
		-0.00	-0.00	-0.00	(74.7)	UPPER BOUND	32.5757=STANDARD ERROR		
A032									
	50.00000	0.00	0.00	0.00				28	1
A032	50.00000	0.00	0.00	0.00	0.0		122.9960=CONTRAST		
		-0.00	-0.00	-0.00	(52.8)	UPPER BOUND	32.5757=STANDARD ERROR		
A032									
	25.00000	0.00	0.00	0.00				28	1
A032	25.00000	0.00	0.00	0.00	0.0		122.9960=CONTRAST		
		-0.00	-0.00	-0.00	(52.8)	UPPER BOUND	32.5757=STANDARD ERROR		
A032									

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/50.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A032								
	10.00000	15.69	68.05	83.74			29	1
	10.00000	1.85	34.56	36.42			34	2
A032	10.00000	8.77	51.31	60.08	65.0	29.4145=CONTRAST		
		9.78	23.68	33.46	(123.7)UPPER BOUND	24.7430=STANDARD ERROR		
A033								
	50.00000	0.00	0.00	0.00			31	1
A033	50.00000	0.00	0.00	0.00	0.0	80.8720=CONTRAST		
		-0.00	-0.00	-0.00	(85.2)UPPER BOUND	34.5518=STANDARD ERROR		
A033								
	30.00000	0.00	13.66	13.66			31	1
A033	30.00000	0.00	13.66	13.66	16.9	67.2084=CONTRAST		
		-0.00	-0.00	-0.00	(102.1)UPPER BOUND	34.5518=STANDARD ERROR		
A033								
	10.00000	2.27	16.58	18.86			30	1
	10.00000	22.45	70.23	92.68			30	2
A033	10.00000	12.36	43.41	55.77	60.2	36.8972=CONTRAST		
		14.27	37.93	52.20	(120.9)UPPER BOUND	28.2114=STANDARD ERROR		
A029								
	50.00000	0.00	5.76	5.76			40	1
	50.00000	1.92	13.90	15.82			40	2
A029	50.00000	0.96	9.83	10.79	11.1	86.8136=CONTRAST		
		1.36	5.75	7.11	(68.7)UPPER BOUND	28.2114=STANDARD ERROR		
A029								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS			Day of Test	Test Number
AO29									
	30.00000	0.00	0.00	0.00				40	1
	30.00000	0.00	3.77	3.77				40	2
AO29	30.00000	0.00	1.88	1.88	1.9		95.7219=CONTRAST		
		-0.00	2.67	2.67	(59.6)	UPPER BOUND	28.2114=STANDARD ERROR		
AO28									
	50.00000	0.00	0.00	0.00				39	1
	50.00000	0.00	0.00	0.00				39	2
AO28	50.00000	0.00	0.00	0.00	0.0		79.8145=CONTRAST		
		-0.00	-0.00	-0.00	(70.5)	UPPER BOUND	28.2114=STANDARD ERROR		
AO28									
	30.00000	3.77	31.61	35.39				39	1
	30.00000	3.64	16.63	20.27				39	2
AO28	30.00000	3.70	24.12	27.83	34.9		51.9876=CONTRAST		
		0.10	10.60	10.69	(105.4)	UPPER BOUND	28.2114=STANDARD ERROR		
AO28									
	10.00000	7.27	51.71	58.99				6	1
	10.00000	17.65	59.22	76.87				7	2
AO28	10.00000	12.46	55.47	67.93	61.5		42.4509=CONTRAST		
		7.34	5.31	12.65	(112.5)	UPPER BOUND	28.2114=STANDARD ERROR		
AO28									
	5.00000	9.26	59.83	69.09				6	1
	5.00000	23.08	83.21	106.29				7	2
AO28	5.00000	16.17	71.52	87.69	79.4		22.6874=CONTRAST		
		9.77	16.53	26.30	(130.4)	UPPER BOUND	28.2114=STANDARD ERROR		
AO16									

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS			Day of Test	Test Number
A016									
	50.00000	0.00	0.00	0.00				38	1
	50.00000	0.00	0.00	0.00				38	2
A016	50.00000	0.00	0.00	0.00	0.0		96.9294=CONTRAST		
		-0.00	-0.00	-0.00	(58.1)	UPPER BOUND	28.2114=STANDARD ERROR		
A016									
	30.00000	2.00	8.18	10.18				38	1
	30.00000	6.00	47.99	53.99				38	2
A016	30.00000	4.00	28.08	32.08	33.1		64.8471=CONTRAST		
		2.83	28.15	30.98	(91.2)	UPPER BOUND	28.2114=STANDARD ERROR		
A014									
	50.00000	1.92	9.89	11.82				41	1
	50.00000	9.26	42.32	51.58				37	2
A014	50.00000	5.59	26.11	31.70	29.9		85.2445=CONTRAST		
		5.19	22.93	28.12	(70.5)	UPPER BOUND	24.7430=STANDARD ERROR		
A014									
	30.00000	4.08	34.23	38.31				37	1
	30.00000	0.00	0.00	0.00				37	2
A014	30.00000	2.04	17.12	19.16	17.9		88.0442=CONTRAST		
		2.89	24.21	27.09	(63.3)	UPPER BOUND	24.4318=STANDARD ERROR		
A014									
	10.00000	24.00	91.34	115.34				37	1
	10.00000	2.08	8.37	10.46				37	2
A014	10.00000	13.04	49.86	62.90	58.7		44.3019=CONTRAST		
		15.50	58.67	74.16	(104.1)	UPPER BOUND	24.4318=STANDARD ERROR		
A013									

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS			Day of Test	Test Number
AC13									
	30.00000	0.00	0.00	0.00				36	1
	30.00000	0.00	0.00	0.00				36	2
AD13	30.00000	0.00	0.00	0.00	0.0		76.0296=CONTRAST		
		-0.00	-0.00	-0.00	(74.0)	UPPER BOUND	28.2114=STANDARD	ERROR	
AO13									
	10.00000	0.00	0.00	0.00				35	1
	10.00000	3.70	19.43	23.13				35	2
	10.00000	16.33	68.38	84.71				42	3
	10.00000	24.49	63.54	88.03				42	4
AO13	10.00000	11.13	37.84	48.97	44.1		61.9540=CONTRAST		
		11.32	33.49	44.23	(80.0)	UPPER BOUND	19.9485=STANDARD	ERROR	
AO13									
	5.00000	3.92	27.44	31.36				35	1
	5.00000	0.00	6.53	6.53				35	2
	5.00000	4.00	28.49	32.49				37	3
	5.00000	14.58	65.48	80.06				42	4
	5.00000	16.33	78.17	94.50				42	5
AO13	5.00000	7.77	41.22	48.99	43.0		62.6369=CONTRAST		
		7.23	29.62	36.82	(73.6)	UPPER BOUND	16.8595=STANDARD	ERROR	
YO06									
	1.00000	1.96	34.00	35.96				44	1
	1.00000	0.00	0.00	0.00				44	2
YO06	1.00000	0.98	17.00	17.98	11.2		142.8713=CONTRAST		
		1.39	24.04	25.43	(46.2)	UPPER BOUND	28.2114=STANDARD	ERROR	
YC06									

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
Y006								
	0.10000	20.00	50.86	70.86			44	1
	0.10000	26.53	80.10	106.63			44	2
Y006	0.10000	23.27	65.48	88.75	55.2	72.1042=CONTRAST		
		4.62	20.67	25.29	(90.2)UPPER BOUND	28.2114=STANDARD ERROR		
Y006								
	0.01000	0.00	0.00	0.00			4	1
	0.01000	16.67	74.22	90.89			5	2
Y006	0.01000	8.33	37.11	45.44	71.8	18.9225=CONTRAST		
		11.79	52.48	64.27	(142.3)UPPER BOUND	23.7023=STAND RD ERROR		
Y007								
	10.80000	0.00	0.00	0.00			45	1
Y007	10.80000	0.00	0.00	0.00	0.0	118.6375=CONTRAST		
		-0.00	-0.00	-0.00	(54.8)UPPER BOUND	32.5757=STANDARD ERROR		
Y007								
	1.00000	0.00	2.77	2.77			45	1
	1.00000	2.00	18.85	20.85			45	2
Y007	1.00000	1.00	10.81	11.81	10.0	106.8312=CONTRAST		
		1.41	11.37	12.79	(53.3)UPPER BOUND	25.7534=STANDARD ERROR		
Y007								
	0.10000	8.00	65.58	73.58			45	1
	0.10000	42.59	97.65	140.25			45	2
Y007	0.10000	25.30	81.61	106.91	90.1	11.7263=CONTRAST		
		24.46	22.68	47.14	(133.4)UPPER BOUND	25.7534=STANDARD ERROR		
Y008								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
Y008								
	1.00000	2.04	21.89	23.93			46	1
	1.00000	4.00	13.71	17.71			46	2
Y008	1.00000	3.02	17.80	20.82	21.5	76.0729=CONTRAST		
		1.39	5.78	4.40	(79.6)UPPER BOUND	28.2114=STANDARD ERROR		
Y008								
	0.10000	4.44	36.43	40.87			46	1
	0.10000	9.09	69.90	78.99			46	2
Y008	0.10000	6.77	53.16	59.93	61.9	36.9583=CONTRAST		
		3.29	23.67	26.95	(119.9)UPPER BOUND	26.2114=STANDARD ERROR		
3-NH2-1-PROPANOL								
	1.00000	0.00	0.00	0.00			23	1
	1.00000	0.00	0.00	0.00			23	2
3-NH2-1-PROPANOL	1.00000	0.00	0.00	0.00	0.0	63.4886=CONTRAST		
		-0.00	-0.00	-0.00	(76.8)UPPER BOUND	24.4318=STANDARD ERROR		
3-NH2-1-PROPANOL								
	0.10000	5.88	20.44	26.32			23	1
	0.10000	1.96	16.23	18.19			23	2
3-NH2-1-PROPANOL	0.10000	3.92	18.34	22.26	35.1	41.2290=CONTRAST		
		2.77	2.98	5.75	(111.8)UPPER BOUND	24.4318=STANDARD ERROR		
3-DEA-1-PROPANOL								
	1.00000	0.00	4.30	4.30			27	1
	1.00000	0.00	0.00	0.00			27	2
3-DEA-1-PROPANOL	1.00000	0.00	2.15	2.15	1.5	137.6743=CONTRAST		
		-0.00	3.04	3.04	(41.8)UPPER BOUND	28.2114=STANDARD ERROR		
3-DEA-1-PROPANOL								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
3-DEA-1-PROPANOL								
	0.10000	2.13	36.48	38.60			27	1
	0.10000	11.11	58.46	69.57			27	2
3-DEA-1-PROPANOL	0.10000	6.62	47.47	54.09	38.7	85.7395=CONTRAST		
		6.35	15.54	21.90	(78.9) UPPER BOUND	28.2114=STANDARD ERROR		
3-DEA-1-PROPANOL								
	0.01000	11.32	43.50	54.82			4	1
	0.01000	3.85	45.19	49.04			5	2
3-DEA-1-PROPANOL	0.01000	7.58	44.35	51.93	77.2	15.2801=CONTRAST		
		5.29	1.20	4.09	(147.7) UPPER BOUND	23.7023=STANDARD ERROR		
1-DEA-2-PROPANOL								
	1.00000	0.00	0.00	0.00			53	1
	1.00000	0.00	0.00	0.00			53	2
1-DEA-2-PROPANOL	1.00000	0.00	0.00	0.00	0.0	86.7964=CONTRAST		
		-0.00	-0.00	-0.00	(64.8) UPPER BOUND	28.2114=STANDARD ERROR		
1-DEA-2-PROPANOL								
	0.10000	2.00	21.67	23.67			53	1
	0.10000	5.88	31.37	37.26			53	2
1-DEA-2-PROPANOL	0.10000	3.94	26.52	30.46	35.1	56.3332=CONTRAST		
		2.75	6.66	9.61	(99.9) UPPER BOUND	28.2114=STANDARD ERROR		
1-DEA-2-PROPANOL								
	0.01000	4.44	31.36	35.80			57	1
	0.01000	8.16	46.12	54.28			57	2
1-DEA-2-PROPANOL	0.01000	6.30	38.74	45.04	55.0	36.7952=CONTRAST		
		2.63	10.44	13.07	(117.8) UPPER BOUND	25.7534=STANDARD ERROR		
1-DEA-2-PROPANOL								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
4-DEA-1-BUTANOL	1.00000	0.00	0.00	0.00			23	1
	1.00000	0.00	0.00	0.00			23	2
4-DEA-1-BUTANOL	1.00000	0.00	0.00	0.00	0.0	63.4886=CONTRAST		
		-0.00	-0.00	-0.00	(76.8)UPPER BOUND	24.4318=STANDARD ERROR		
4-DEA-1-BUTANOL	0.10000	0.00	0.00	0.00			23	1
	0.10000	0.00	0.00	0.00			23	2
4-DEA-1-BUTANOL	0.10000	0.00	0.00	0.00	0.0	63.4886=CONTRAST		
		-0.00	-0.00	-0.00	(76.8)UPPER BOUND	24.4318=STANDARD ERROR		
4-DEA-1-BUTANOL	0.01000	0.00	0.00	0.00			24	1
	0.01000	0.00	13.90	13.90			24	2
4-DEA-1-BUTANOL	0.01000	0.00	6.95	6.95	12.3	49.7183=CONTRAST		
		-0.00	9.83	9.83	(102.9)UPPER BOUND	25.7534=STANDARD ERROR		
4-DEA-1-BUTANOL	0.00100	0.00	0.00	0.00			24	1
	0.00100	4.08	24.88	28.96			24	2
4-DEA-1-BUTANOL	0.00100	2.04	12.44	14.48	25.5	42.1905=CONTRAST		
		2.89	17.59	20.48	(116.2)UPPER BOUND	25.7534=STANDARD ERROR		
3-DMA-1-PROPANOL								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
3-DMA-1-PROPANOL								
	1.00000	11.76	59.26	71.03			56	1
	1.00000	11.11	53.44	64.55			56	2
3-DMA-1-PROPANOL	1.00000	11.44	56.35	67.79	68.7	30.8689=CONTRAST		
		0.46	4.12	4.58	(125.8)UPPER BOUND	28.2114=STANDARD ERROR		
3-DMA-1-PROPANOL								
	0.10000	14.81	78.61	93.43			56	1
	0.10000	31.48	76.50	107.96			56	2
3-DMA-1-PROPANOL	0.10000	23.15	77.56	100.71	102.1	-2.0514=CONTRAST		
		11.79	1.49	10.29	(159.1)UPPER BOUND	28.2114=STANDARD ERROR		
3-DMA-1-PROPANOL								
	0.01000	0.00	0.00	0.00			60	1
	0.01000	0.00	6.67	6.67			60	2
3-DMA-1-PROPANOL	0.01000	0.00	3.33	3.33	4.7	66.9542=CONTRAST		
		-0.00	4.71	4.71	(84.8)UPPER BOUND	28.2114=STANDARD ERROR		
3-DMA-1-PROPANOL								
	0.00100	1.92	23.61	25.53			60	1
	0.00100	3.85	41.82	45.67			60	2
3-DMA-1-PROPANOL	0.00100	2.88	32.72	35.60	50.7	34.6857=CONTRAST		
		1.36	12.88	14.24	(130.7)UPPER BOUND	28.2114=STANDARD ERROR		
1-DMA-2-PROPANOL								
	1.00000	7.55	65.16	72.71			58	1
	1.00000	5.77	30.00	35.77			58	2
1-DMA-2-PROPANOL	1.00000	6.66	47.58	54.24	56.4	41.9109=CONTRAST		
		1.26	24.87	26.12	(114.9)UPPER BOUND	28.2114=STANDARD ERROR		
1-DMA-2-PROPANOL								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
1-DMA-2-PROPANOL								
	0.10000	3.85	31.66	35.50			58	1
	0.10000	12.50	59.95	72.45			58	2
1-DMA-2-PROPANOL	0.10000	8.17	45.80	53.97	56.1	42.1770=CONTRAST		
		6.12	20.00	26.12	(114.7)UPPER BOUND	28.2114=STANDARD ERROR		
1-DMA-2-PROPANOL								
	0.01000	4.26	38.56	42.81			59	1
	0.01000	1.96	24.64	26.60			59	2
1-DMA-2-PROPANOL	0.01000	3.11	31.60	34.71	32.6	71.7075=CONTRAST		
		1.62	9.84	11.46	(85.5)UPPER BOUND	28.2114=STANDARD ERROR		
1-DMA-2-PROPANOL								
	0.00100	5.56	77.73	83.29			59	1
	0.00100	13.21	69.16	82.37			59	2
1-DMA-2-PROPANOL	0.00100	9.38	73.45	82.83	77.6	23.5668=CONTRAST		
		5.41	6.06	0.65	(130.7)UPPER BOUND	28.2114=STANDARD ERROR		
4-DMA-1-BUTANOL								
	1.00000	0.00	0.00	0.00			25	1
	1.00000	0.00	0.00	0.00			25	2
4-DMA-1-BUTANOL	1.00000	0.00	0.00	0.00	0.0	92.9379=CONTRAST		
		-0.00	-0.00	-0.00	(55.3)UPPER BOUND	25.7534=STANDARD ERROR		
4-DMA-1-BUTANOL								
	0.10000	0.00	0.00	0.00			25	1
	0.10000	0.00	0.00	0.00			25	2
4-DMA-1-BUTANOL	0.10000	0.00	0.00	0.00	0.0	92.9379=CONTRAST		
		-0.00	-0.00	-0.00	(55.3)UPPER BOUND	25.7534=STANDARD ERROR		
4-DMA-1-BUTANOL								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/50.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
4-DMA-1-BUTANOL								
	0.01000	0.00	0.00	0.00			25	1
	0.01000	0.00	0.00	0.00			25	2
4-DMA-1-BUTANOL	0.01000	0.00	0.00	0.00	0.0	92.9379=CONTRAST		
		-0.00	-0.00	-0.00	(55.3)UPPER BOUND	25.7534=STANDARD ERROR		
4-DMA-1-BUTANOL								
	0.00100	0.00	0.00	0.00			25	1
	0.00100	5.77	29.47	35.24			25	2
4-DMA-1-BUTANOL	0.00100	2.88	14.74	17.62	19.0	75.3165=CONTRAST		
		4.08	20.84	24.92	(74.2)UPPER BOUND	25.7534=STANDARD ERROR		
1133TETRAMETHUREA								
	1.00000	4.08	47.07	51.15			48	1
	1.00000	18.00	55.72	73.72			48	2
1133TETRAMETHUREA	1.00000	11.04	51.40	62.44	53.9	53.3874=CONTRAST		
		9.84	6.12	15.96	(102.5)UPPER BOUND	28.2114=STANDARD ERROR		
1133TETRAMETHUREA								
	0.10000	0.00	0.00	0.00			48	1
	0.10000	4.08	27.40	31.48			48	2
	0.10000	14.29	57.57	71.85			49	3
1133TETRAMETHUREA	0.10000	6.12	28.32	34.45	39.0	64.5142=CONTRAST		
		7.36	28.80	36.02	(78.3)UPPER BOUND	20.8355=STANDARD ERROR		
1133TETRAMETHUREA								
	0.01000	1.92	17.91	19.83			62	1
	0.01000	0.00	2.49	2.49			62	2
1133TETRAMETHUREA	0.01000	0.96	10.20	11.16	18.8	48.0902=CONTRAST		
		1.36	10.90	12.26	(113.6)UPPER BOUND	28.2114=STANDARD ERROR		
1133TETRAMETHUREA								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
1133TETRAMETHUREA								
	0.00100	0.00	4.25	4.25			62	1
	0.00100	0.00	9.25	9.25			62	2
1133TETRAMETHUREA	0.00100	0.00	6.75	6.75	11.4	52.4949=CONTRAST		
		-0.00	3.53	3.53	(106.4)UPPER BOUND	28.2114=STANDARD ERROR		
DEA ACETONE								
	1.00000	14.29	66.93	81.21			49	1
	1.00000	10.00	45.91	55.91			49	2
DEA ACETONE	1.00000	12.14	56.42	68.56	73.2	25.1254=CONTRAST		
		3.03	14.86	17.89	(123.4)UPPER BOUND	23.6034=STANDARD ERROR		
DEA ACETONE								
	0.10000	12.50	69.94	82.44			49	1
	0.10000	4.65	44.48	49.13			49	2
DEA ACETONE	0.10000	8.58	57.21	65.79	70.2	27.9011=CONTRAST		
		5.55	18.01	23.56	(120.5)UPPER BOUND	23.6034=STANDARD ERROR		
GABA-ETHYL-ESTER								
	10.00000	11.11	67.51	78.62			47	1
	10.00000	4.08	22.95	27.04			47	2
GABA-ETHYL-ESTER	10.00000	7.60	45.23	52.83	41.1	75.6004=CONTRAST		
		4.97	31.51	36.48	(76.9)UPPER BOUND	23.0345=STANDARD ERROR		
GABA-ETHYL-ESTER								
	1.00000	20.00	72.31	92.31			47	1
	1.00000	3.85	36.19	40.03			47	2
GABA-ETHYL-ESTER	1.00000	11.92	54.25	66.17	51.5	62.2614=CONTRAST		
		11.42	25.54	36.96	(87.3)UPPER BOUND	23.0345=STANDARD ERROR		
22AMETHOXYETHANOL								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS			Day of Test	Test Number
22AMETHOXYETHANOL									
	1.00000	0.00	0.00	0.00					
	1.00000	16.98	44.74	61.72				65	1
								65	2
22AMETHOXYETHANOL	1.00000	8.49	22.37	30.86	29.3		74.5049=CONTRAST		
		12.01	31.63	43.64	(82.7)UPPER BOUND		28.2114=STANDARD ERROR		
22AMETHOXYETHANOL									
	0.10000	20.00	62.20	82.20					
	0.10000	17.31	37.40	54.71				65	1
								65	2
22AMETHOXYETHANOL	0.10000	18.65	49.80	68.46	65.0		36.9072=CONTRAST		
		1.90	17.54	19.44	(118.4)UPPER BOUND		28.2114=STANDARD ERROR		
3-BUTENE-2-OL									
	0.01000	5.66	24.72	30.38					
	0.01000	10.00	55.67	65.67				55	1
								55	2
3-BUTENE-2-OL	0.01000	7.83	40.20	48.03	44.1		60.8017=CONTRAST		
		3.07	21.89	24.95	(95.8)UPPER BOUND		28.2114=STANDARD ERROR		
3-BUTENE-2-OL									
	0.00100	20.00	76.97	96.97					
	0.00100	6.38	35.02	41.41				55	1
								55	2
3-BUTENE-2-OL	0.00100	13.19	56.00	69.19	63.6		39.6433=CONTRAST		
		9.63	29.66	39.29	(115.3)UPPER BOUND		28.2114=STANDARD ERROR		
4AMBUTALDDEAHWNB									
	1.00000	0.00	0.00	0.00					
	1.00000	0.00	0.00	0.00				24	1
								24	2
4AMBUTALDDEAHWNB	1.00000	0.00	0.00	0.00	0.0		56.6690=CONTRAST		
		-0.00	-0.00	-0.00	(90.7)UPPER BOUND		25.7534=STANDARD ERROR		
4AMBUTALDDEAHWNB									

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION OF MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
4AMBUTALDDEAHWNB								
	0.10000	0.00	0.00	0.00			24	1
	0.10000	0.00	0.00	0.00			24	2
	0.10000	0.00	24.12	26.12			50	3
	0.10000	8.00	33.70	41.70			50	4
4AMBUTALDDEAHWNB	0.10000	2.00	14.95	16.95	28.9	43.9618=CONTRAST		
		4.00	17.54	20.59	(86.1)UPPER BOUND	17.7247=STANDARD ERROR		
4AMBUTALDDEAHWNB								
	0.01000	14.00	61.16	75.16			26	1
	0.01000	1.96	5.96	7.92			26	2
	0.01000	5.56	35.75	41.30			50	3
	0.01000	0.00	15.97	15.97			50	4
4AMBUTALDDEAHWNB	0.01000	5.38	29.71	35.09	38.5	54.4627=CONTRAST		
		6.19	24.35	30.27	(80.1)UPPER BOUND	18.4687=STANDARD ERROR		
4AMBUTALDDEAHWNB								
	0.00100	6.52	53.35	59.87			26	1
	0.00100	4.00	25.00	29.00			26	2
4AMBUTALDDEAHWNB	0.00100	5.26	39.18	44.44	37.6	73.7339=CONTRAST		
		1.78	20.05	21.83	(85.2)UPPER BOUND	28.2114=STANDARD ERROR		
4AMBUTALDDEAHWNB								
	0.00010	11.11	55.17	66.28			4	1
	0.00010	9.43	55.75	65.18			5	2
4AMBUTALDDEAHWNB	0.00010	10.27	55.46	65.73	98.0	1.3437=CONTRAST		
		1.19	0.41	0.78	(168.5)UPPER BOUND	23.7023=STANDARD ERROR		
2AM-BENZALDEHYDE								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/50.1NCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
2AM-BENZALDEHYDE								
	1.00000	0.00	0.00	0.00			50	1
	1.00000	0.00	0.00	0.00			50	2
	1.00000	0.00	0.00	0.00			52	3
	1.00000	0.00	0.00	0.00			52	4
2AM-BENZALDEHYDE	1.00000	0.00	0.00	0.00	0.0	63.4157=CONTRAST		
		-0.00	-0.00	-0.00	(55.8)UPPER BOUND	17.7247=STANDARD ERROR		
2AM-BENZALDEHYDE								
	0.10000	0.00	0.00	0.00			50	1
	0.10000	0.00	0.00	0.00			50	2
	0.10000	0.00	0.00	0.00			52	3
	0.10000	0.00	0.00	0.00			52	4
2AM-BENZALDEHYDE	0.10000	0.00	0.00	0.00	0.0	63.4157=CONTRAST		
		-0.00	-0.00	-0.00	(55.8)UPPER BOUND	17.7247=STANDARD ERROR		
2AM-BENZALDEHYDE								
	0.01000	0.00	0.00	0.00			51	1
	0.01000	0.00	0.00	0.00			51	2
	0.01000	0.00	2.40	2.40			52	3
	0.01000	0.00	0.00	0.00			52	4
2AM-BENZALDEHYDE	0.01000	0.00	0.60	0.60	1.1	59.8258=CONTRAST		
		-0.00	1.20	1.20	(63.6)UPPER BOUND	19.0201=STANDARD ERROR		
2AM-BENZALDEHYDE								
	0.00100	0.00	0.00	0.00			51	1
	0.00100	3.77	50.00	53.77			51	2
	0.00100	3.85	51.25	55.10			52	3
	0.00100	1.89	19.36	21.25			52	4
	0.00100	2.04	17.95	19.99			61	5
	0.00100	0.00	0.00	0.00			61	6
	0.00100	5.77	27.76	33.53			1	7
	0.00100	5.66	25.23	30.89			1	8
2AM-BENZALDEHYDE	0.00100	2.87	23.94	26.82	37.5	45.5167=CONTRAST		
		2.27	19.44	21.06	(75.2)UPPER BOUND	13.7658=STANDARD ERROR		
2AM-BENZALDEHYDE								

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/50.1N.CM)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
2AM-BENZALDEHYDE	0.00010	4.26	13.41	17.67			54	1
	0.00010	6.25	16.96	23.21			54	2
	0.00010	16.28	78.78	95.06			61	3
	0.00010	0.00	0.00	0.00			61	4
	0.00010	38.89	68.54	107.43			1	5
	0.00010	5.56	34.71	40.27			1	6
2AM-BENZALDEHYDE	0.00010	11.87	35.40	47.27	65.5	24.9430=CONTRAST		
		14.28	31.80	43.91	(110.5)UPPER BOUND	16.2879=STANDARD ERROR		
2AM-BENZALDEHYDE	0.00001	6.00	17.79	23.79			54	1
	0.00001	4.17	24.94	29.11			54	2
2AM-BENZALDEHYDE	0.00001	5.08	21.37	26.45	60.6	17.1847=CONTRAST		
		1.30	5.06	3.76	(189.6)UPPER BOUND	28.2114=STANDARD ERROR		
NNDIPHENYLFORMIDE	1.00000	6.12	27.62	33.75			49	1
	1.00000	7.69	42.49	50.19			49	2
NNDIPHENYLF	1.00000	6.91	35.06	41.97	44.8	51.7206=CONTRAST		
		1.11	10.51	11.62	(95.1)UPPER BOUND	23.6034=STANDARD ERROR		
NNDIPHEN	.0000	3.85	50.36	54.21			49	1
	.10000	3.85	24.06	27.90			49	2
NNDIPHENYLFORMIDE	0.10000	3.85	37.21	41.05	43.8	52.6327=CONTRAST		
		-0.00	18.60	18.60	(94.1)UPPER BOUND	23.6034=STANDARD ERROR		
NNDIPENYLFORMIDE	0.01000	6.00	16.02	22.02			4	1
	0.01000	1.85	7.07	8.92			5	2
NNDIPENYLFORMIDE	0.01000	3.93	11.55	15.47	22.5	51.9525=CONTRAST		
		2.93	6.33	9.26	(93.0)UPPER BOUND	23.7023=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES (Cont.)

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
DEAACETALD DEACET	1.00000	7.84	38.67	46.51			2	1
	1.00000	22.45	75.47	97.92			2	2
DEAACETALD DEACET	1.00000	15.15 10.33	57.07 25.02	72.22 36.35	75.8 (134.9)UPPER BOUND	23.0518=CONTRAST 28.2114=STANDARD ERROR		
DEAACETALD DEACET	0.10000	12.24	50.77	63.02			2	1
	0.10000	5.88	47.24	53.12			2	2
DEAACETALD DEACET	0.10000	9.06 4.50	49.01 2.50	58.07 7.00	61.0 (120.0)UPPER BOUND	37.1987=CONTRAST 28.2114=STANDARD ERROR		
DEET	1.00000	0.00	0.00	0.00			8	1
	1.00000	0.00	0.00	0.00			10	2
	1.00000	0.00	0.00	0.00			9	3
DEET	1.00000	0.00 -0.00	0.00 -0.00	0.00 -0.00	0.0 (39.4)UPPER BOUND	97.7766=CONTRAST 19.3150=STANDARD ERROR		
DEET	0.10000	1.85	11.41	13.26			8	1
	0.10000	2.17	13.21	15.38			9	2
	0.10000	0.00	12.30	12.30			11	3
DEET	0.10000	1.34 1.17	12.31 0.90	13.65 1.58	13.1 (50.9)UPPER BOUND	89.8033=CONTRAST 19.5454=STANDARD ERROR		
DEET	0.01000	21.43	81.56	102.99			11	1
	0.01000	22.64	56.45	79.09			9	2
	0.01000	19.23	55.95	75.18			8	3
DEET	0.01000	21.10 1.73	64.65 14.65	85.75 15.06	83.6 (121.3)UPPER BOUND	16.9538=CONTRAST 19.5454=STANDARD ERROR		
DEET	0.00100	23.21	91.21	114.43			11	1
	0.00100	34.00	61.23	95.23			9	2
	0.00100	40.00	98.68	138.68			10	3
DEET	0.00100	32.40 8.51	83.71 19.82	116.11 21.78	111.7 (147.6)UPPER BOUND	-12.2616=CONTRAST 18.9485=STANDARD ERROR		

APPENDIX C

COMPUTER PROGRAM LISTING THAT YIELDS CONFIDENCE LEVEL IN TERMS OF PERCENT

Appendix C

Fortran IV Listing of Program Which Yields Confidence Levels in Percent.

```
      DIMENSION DATE(2),COMP(3),REMARK(4),RM(300)
      DIMENSIONCOMPCK(3),X(300),CN(300),W(300),T(16),TM(300),TITLE(84)        2
      DIMENSION FTAB(13),FTABLE(11)
      DATA (FTAB(I),I=1,13)/78HEFFECT S.S.   D.F. M.S.   F   ----------
     1-----------------   DAY ERROR TOTAL/
      READ(5,11)IDAYS,VARC,FRAC,T                                              1
      READ(5,92)TITLE                                                  3       4
      CA=0.
      IPAGE=0
      TDN=0.
C80   DAYEF= 0.
      DAYDF=-1.
    2 WRITE(6,91)TITLE                                                         7
      ILINE = 0
       IPAGE =1 +IPAGE
    1 READ (5,9) DATE , COMP , DOSE,ITEST,AGE,TN  ,ENG,TT  ,TD,REMARK        10
       PCTE=ENG/TN     *100.
      PCTD=TD /TT     *100.
      RDX =(PCTE+PCTD)
      ILINE= ILINE +01
      IF(TDN)25,25,22
   22 IF(COMP(1).NE.COMPCK(1)) GO TO 30
      IF(COMP(2).NE.COMPCK(2)) GO TO 30
      IF(COMP(3).NE.COMPCK(3)) GO TO 30
```

```
23 IF(DOSE    -DOSECK    )  30,24,30
24  SPCTE= SPCTE+PCTE
   SSRDX =SSRDX +RDX **2
    SRDX = SRDX +RDX
   SSPCTD=SSPCTD+PCTD**2
    SPCTD= SPCTD+PCTD
   SSPCTE=SSPCTE+PCTE**2
   TDN  =TDN+1.                                                    11
   X(ITEST) =  X(ITEST)+RDX                                        12
   TM(ITEST)= TM(ITEST)+1.
   NTDN=TDN                                                                33
   WRITE(6,8)    DOSE,PCTE,PCTD,RDX,ITEST,NTDN
   DOS =DOSE
   IF(ILINE-44) 1,2,2
30 IF(DOS   ) 47,47,29
47 DO 62 I=1,IDAYS
   CN(I) = TM(I)
   IF(CN(I))62,62,63
63 IF(VARC)46,65,46                                                        50
65 DAYEF=DAYEF+CN(I)*(X(I)/CN(I))**2.
   DAYDF=DAYDF+1.
46 RM(I) =(X(I)/CN(I))*FRAC
62 CONTINUE
   FTABLE(1)=DAYEF-(SRDX**2)/TDN
   FTABLE(9)=SSRDX-(SRDX**2)/TDN
   FTABLE(5)=FTABLE(9)-FTABLE(1)
   FTABLE(2)=DAYDF
   FTABLE(10)=TDN-1.
   FTABLE( 6)=TDN-1.-DAYDF
   DO64I=1,9,4
64 FTABLE(I+2)=FTABLE(I)/FTABLE(I+1)
   FTABLE(4)  =FTABLE(3)/FTABLE(7)
   VARC=FTABLE(7)                                                          70
29 SSPCTE=((SSPCTE-(SPCTE**2)/TDN)/(TDN-1.))**.5                           71
   SSPCTD=((SSPCTD-(SPCTD**2)/TDN)/(TDN-1.))**.5                           72
   SSRDX =((SSRDX -(SRDX **2)/TDN)/(TDN-1.))**.5
   SPCTE=SPCTE/TDN
   SPCTD=SPCTD/TDN
   SRDX=SRDX/TDN
```

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```
      DO7 I=1,IDAYS                                                                  31
      IF(TM(I)*CN(I)) 6,7,6                                                          32
    6 X(I) = X(I) / TM(I)   - RM(I)                                                  33
      W(I) = 1./ (1./TM(I) + 1./CN(I) )                                              34
      SX = SX - X(I)*W(I)                                                            35
      Sw = Sw +       W(I)                                                           36
    7 CONTINUE                                                                       37
      ILINE =ILINE + 6                                                               38
      SX = SX/Sw                                                                     39
       SDX =(VARC /Sw)**.5                                                           40        94
      TS = SX/SDX                                                                    41
      TOUT = T(1)                                                                    41
      DO 32 J= 4,16,2                                                                42
      IF(TS-T(J)   ) 31,31,32                                                        43
   31 TOUT = T(J-3)                                                                  44
      GO TO 33                                                                       45
   32 CONTINUE                                                                       46
      TOUT = T(15)                                                                   47
   33 WRITE(6,90)COMPCK,DOSECK,SPCTE,SPCTD,SRDX,TOUT,SX,  SSPCTE,SSPCTD,
     1SSRDX,SDX,COMP                                                                       106
      IF(DOS)51,66,51
   51 IF(ILINE-36)49,48,48
   66 WRITE(6,93)(FTAB(I),I=1,11),(FTABLE(J),J=1,4),FTAB(12),(FTABLE(J),
     1J=5,7),(FTAB(I),I=6,9),FTAB(13),(FTABLE(J),J=9,11)                                   112
                                                                                           144
   48 WRITE(6,91)TITLE,COMP
      ILINE=0
   49 DO 50 I=1,IDAYS                                                                50
      X(I)=0                                                                         51
   50 TM(I)=0                                                                        52
      SX = 0                                                                         53
      Sw = 0                                                                         54
       SPCTE=0
       SPCTD=0
       SRDX =0
      SSPCTE=0
      SSPCTD=0
      SSRDX =0
      TDN=0
```

```
 25 COMPCK(1)=COMP(1)
    COMPCK(2)=COMP(2)
    COMPCK(3)=COMP(3)
    DOSECK=DOSE
    GO TO 24
  8 FORMAT(1H ,17X,F11.5,3F12.2,40X2I5)
  9 FORMAT(A6,A2,2A6,A5,F5.5,I2,F3.0,4F5.0,5X,4A5)
 10 FORMAT(1H1,64X,50H  WEIGHTED CONTRAST ANALYSIS BY M.L.KARDATZKE,PA
   1GE  I5/98H0  DATE          COMPOUND    DOSE (MG)ITEST  AGE N(ENG)/N
   1(EXP)  DISPLACED/TIME   PCT(ENG) PCT(DSP) 16HR-INDEX   REMARK)
 11 FORMAT(I5,F7.0,F3.3,
   1                  6(A4,F6.3)/ 15X,2(A4,F6.3))
 90 FORMAT(1H0,2A6,A5,F11.5,3F12.2,5XA4,8XF12.4, 9H=CONTRAST
   1     /29X3F12.2,17XF12.4,15H=STANDARD ERROR///1H 2A6,A5)
 91 FORMAT(//6(13A6,A2/)/1H 2A6,A5)
 92 FORMAT(13A6,A2)
 93 FORMAT(28H1     ANALYSIS OF VARIANCE  ////
   1            5(6XA6)/1X5(6XA6)/7XA6,F12.3,F12.0,2F12.3//
   1                              7XA6,F12.3,F12.0, F12.3/1X,4(6XA6)/
   1                              7XA6,F12.3,F12.0, F12.3/1H1)
    END
```


Note: A card with the number "1" punched in column 29 should be inserted at the end of the data deck as a signal that the data is completed.

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APPENDIX D

COMPUTER PROGRAM THAT YIELDS WEIGHTED % OF CONTROLS, WITH UPPER BOUNDS

Appendix D

Fortran IV Listing of Program Which Yields Confidence Levels in Terms of Upper Bounds and Weighted Percent of Controls.

```
      DIMENSION DATE(2),COMP(3),REMARK(4),RN(300)
      DIMENSIONCOMPCK(3),X(300),CN(300),A(300),T(16),TM(300),TITLE(84)          2
      DIMENSION FTAB(13),FTABLE(11),TSTAT(100)
      DATA (FTAB(I),I=1,13)/78HEFFECT S.S.   D.F. M.S.   F   ----------
     1----------   DAY ERROR TOTAL/
      DATA                                    (TSTAT(I),I=1,100)/12.71,
     24.303,3.182,2.776,2.571,2.447,2.365,2.306,2.262,2.228,2.201,2.179,
     32.160,2.145,2.131,2.120,2.110,2.101,2.093,2.086,2.080,2.074,2.069,
     42.064,2.060,2.056,2.052,2.048,2.045,2.042,2.039,2.037,2.035,2.033,
     52.031,2.029,2.027,2.025,2.023,2.021,2.019,2.018,2.016,2.015,2.014,
     62.013,2.012,2.011,2.010,2.009,2.008,2.007,2.006,2.005,2.004,2.003,
     72.002,2.001,2.000,10*2.000 ,10*1.995 ,10*1.990 ,10*1.987 ,1.984/
      WRITE(6,999)(TSTAT(J),J=1,100)                                          1
  999 FORMAT(1X10F8.3)
      READ(5,11)IDAYS,VARC,FRAC                                               8
      READ(5,92)TITLE                                                3        10
      CA=0.
      IPAGE=0
      TDN=0.
      DAYEF= 0.
      DAYDF=-1.
```

```
 2 WRITE(6,91)TITLE                                                              13
   ILINE = 0
    IPAGE =1 +IPAGE
 1 READ (5,9) DATE , COMP , DOSE,ITEST,AGE,TN  ,ENG,TT  ,TD,REMARK              16
    PCTE=ENG/TN      *100.
   PCTD=TD /TT      *100.
   RDX =(PCTE+PCTD)
   ILINE= ILINE +01
   IF(TDN)25,25,22
22 IF(COMP(1).NE.COMPCK(1)) GO TO 30
   IF(COMP(2).NE.COMPCK(2)) GO TO 30
   IF(COMP(3).NE.COMPCK(3)) GO TO 30
23 IF(DOSE   -DOSECK    )  30,24,30
24  SPCTE= SPCTE+PCTE
   SSRDX =SSRDX +RDX **2
    SRDX = SRDX +RDX
   SSPCTD=SSPCTD+PCTD**2
    SPCTD= SPCTD+PCTD
   SSPCTE=SSPCTE+PCTE**2
   TDN  =TDN+1.
   X(ITEST) =  X(ITEST)+RDX                                          11
   TM(ITEST)= TM(ITEST)+1.                                           12
   NTDN=TDN
   WRITE(6,8)   DOSE,PCTE,PCTD,RDX,ITEST,NTDN                              39
   DOS =DOSE
   IF(ILINE-44) 1,2,2
30 IF(DOS  ) 47,47,29
47 DO 62 I=1,IDAYS
   CN(I) = TM(I)
   IF(CN(I))62,62,63
63 IF(VARC)46,65,46
65 DAYEF=DAYEF+CN(I)*(X(I)/CN(I))**2.                                      56
   DAYDF=DAYDF+1.
46 RM(I) =(X(I)/CN(I))*FRAC
```

```
62 CONTINUE
   FTABLE(1)=DAYEF-(SRDX**2)/TDN
   FTABLE(9)=SSRDX-(SRDX**2)/TDN
   FTABLE(5)=FTABLE(9)-FTABLE(1)
   FTABLE(2)=DAYDF
   FTABLE(10)=TDN-1.
   FTABLE( 6)=TDN-1.-DAYDF
   DO64I=1,9,4
64 FTABLE(I+2)=FTABLE(I)/FTABLE(I+1)
   FTABLE(4)  =FTABLE(3)/FTABLE(7)
   VARC=FTABLE(7)
29 SSPCTE=((SSPCTE-(SPCTE**2)/TDN)/(TDN-1.))**.5                    75
   SSPCTD=((SSPCTD-(SPCTD**2)/TDN)/(TDN-1.))**.5                    76
   SSRDX =((SSRDX -(SRDX **2)/TDN)/(TDN-1.))**.5                    77
   SPCTE=SPCTE/TDN
   SPCTD=SPCTD/TDN
   SRDX=SRDX/TDN
   DO7 I=1,IDAYS                                          31
   IF(TM(I)*CN(I)) 6,7,6                                  32
 6 XP=X(I)/TM(I)
   X(I) = X(I) / TM(I)  - RM(I)
   W(I) = 1./ (1./TM(I) + 1./CN(I) )                      34
   SXP=SXP+XP*W(I)
   SX = SX - X(I)*W(I)                                    35
   SW = SW +      W(I)                                    36
 7 CONTINUE                                               37
   ILINE =ILINE + 6                                       38
   SX = SX/SW                                             39
   SXP=SXP/SW
    SDX =(VARC /SW)**.5                                   40      102
   NDEGFR=FTABLE(6)
   IF(NDEGFR-100)72,72,70
72 T95=TSTAT(NDEGFR)
   GOTO 71
70 T95=1.960
71 T95=T95*SDX
   TS = SX/SDX                                            41
   PCTCNT=(SXP/(SXP+SX))*100.
   UPLIM=((SXP+T95)/(SXP+SX))*100.
```

```
      TOUT = T(1)                                                          41
      DO 32 J= 4,16,2                                                      42
      IF(TS-T(J)   ) 31,31,32                                              43
   31 TOUT = T(J-3)                                                        44
      GO TO 33                                                             45
   32 CONTINUE                                                             46
      TOUT = T(15)                                                         47
   33 WRITE(6,90)COMPCK,DOSECK,SPCTE,SPCTD,SRDX,PCTCNT,
     1SX,SSPCTE,SSPCTD,SSRDX,UPLIM,SDX,CCMP                                121
      IF(DOS)51,66,51
   51 IF(ILINE-36)49,48,48
   66 WRITE(6,93)(FTAB(I),I=1,11),(FTABLE(J),J=1,4),FTAB(12),(FTABLE(J),
     1J=5,7),(FTAB(I),I=6,9),FTAB(13),(FTABLE(J),J=9,11)                   127
   48 WRITE(6,91)TITLE,COMP                                                158
      ILINE=0
   49 DO 50 I=1,IDAYS                                                      50
      X(I)=0                                                               51
   50 TM(I)=0                                                              52
      SX = 0                                                               53
      SXP= 0.
      SW = 0                                                               54
       SPCTE=0
       SPCTD=0
       SRDX =0
      SSPCTE=0
      SSPCTD=0
      SSRDX =0
      TDN=0
   25 COMPCK(1)=COMP(1)
      COMPCK(2)=COMP(2)
      COMPCK(3)=COMP(3)
      DOSECK=DOSE
      GO TO 24
    8 FORMAT(1H ,17X,F11.5,3F12.2,40X2I5)
    9 FORMAT(A6,A2,2A6,A5,F5.5,I2,F3.0,4F5.0,5X,4A5)
   10 FORMAT(1H1,64X,50H  WEIGHTED CONTRAST ANALYSIS BY M.L.KARDATZKE,PA
     1GE  I5/98H0  DATE          COMPOUND    DOSE (MG)ITEST  AGE N(ENG)/N
     1(EXP)  DISPLACED/TIME   PCT(ENG) PCT(DSP) 16HR-INDEX   REMARK)
```

```
11 FORMAT(I5,F7.0,F3.3,
  1                   6(A4,F6.3)/ 15X,2(A4,F6.3))
90 FORMAT(1H0,2A6,A5,F11.5,3F12.2,2XF7.1,12XF12.4,9H=CONTRAST/29X3F12
  1.2,2X1H(F6.1,12H)UPPER BOUND
  1                        F12.4,15H=STANDARD ERROR///1H 2A6,A5)
91 FORMAT(//6(13A6,A2/)/1H 2A6,A5)
92 FORMAT(13A6,A2)
93 FORMAT(28H1      ANALYSIS OF VARIANCE  ////
  1            5(6XA6)/1X5(6XA6)/7XA6,F12.3,F12.0,2F12.3//
  1                                7XA6,F12.3,F12.0, F12.3/1x,4(6XA6)/
  1                                7XA6,F12.3,F12.0, F12.3/1H1)
   END
```

Note: A card with the number "1" punched in column 29 should be inserted at the end of the data deck as a signal that the data is completed.

APPENDIX E

COMPUTER PROGRAM LISTING THAT YIELDS COMPLETE TABULATION OF RAW INPUT DATA AND EXAMPLE OF OUTPUT OF RAW DATA

Appendix E

I. Fortran IV Listing of Program that Yields Tabulation of Raw Input Data

```
      DIMENSION DATE(2),COMP(3),REMARK(4)
      CA=0.
      IPAGE=0
    2 WRITE(6,10) IPAGE                                                    2
      ILINE = 0
       IPAGE =1 +IPAGE
    1 READ (5,9) DATE , COMP , DOSE,ITEST,AGE,TN  ,ENG,TT  ,TD,REMARK     4
       PCTE=ENG/TN     *100.
      PCTD=TD /TT     *100.
      RDX =(PCTE+PCTD)
      ILINE= ILINE +01
      WRITE(6,8) DATE , COMP , DOSE,ITEST,AGE,ENG,TN,TD,TT,PCTE,PCTD,RDX
     1,REMARK,ILINE                                                        9
      IF(ILINE-25) 1,2,2
    8 FORMAT(1HO,A6,A2,2X,2A6,A5,F9.5,I5,F5.0, F7.0,1H/,F7.0,F10.1,1H/,F
     17.0,F8.1,F9.1,F8.1,2X,4A5 ,I3)
    9 FORMAT(A6,A2,2A6,A5,F5.5,I2,F3.0,4F5.0,5X,4A5)
   10 FORMAT(1H1,64X,50HREPELLENCY INDEX OF P.KASHIN,BY M.L.KARDATZKE,PA
     1GE  I5/98HO  DATE          COMPOUND    DOSE (MG)ITEST  AGE N(ENG)/N
     1(EXP)  DISPLACED/TIME   PCT(ENG) PCT(DSP) 16HR-INDEX   REMARK)
      END
```

II. Example of Raw Data Output

REPELLENCY INDEX OF P.KASHINABY M.L.KARDATZKE.PAGE 0

DATE	COMPOUND	DOSE (MG)	ITEST	AGE	N(ENG)/N(EXP)	DISPLACED/TIME	PCT(ENG)	PCT(DSP)	R-INDEX	REMARK		
6/30/67	DEET	1.00000	8	10.	0./ 52.	0.0/ 194.	0.0	0.0	0.0		2	1
7/19/67	DEET	1.00000	10	21.	0./ 35.	0.0/ 184.	0.0	0.0	0.0		8	2
7/07/67	DEET	1.00000	9	17.	0./ 48.	0.0/ 213.	0.0	0.0	0.0	POSITIONED WRONGBEG	1	3
6/30/67	DEET	0.10000	8	10.	1./ 54.	21.0/ 184.	1.9	11.4	13.3		1	4
7/07/67	DEET	0.10000	9	10.	1./ 46.	24.1/ 183.	2.2	13.2	15.4	MOUSE DIED AT END	2	5
7/26/67	DEET	0.10000	11	15.	0./ 54.	23.0/ 187.	0.0	12.3	12.3		2	6
7/26/67	DEET	0.01000	11	15.	12./ 56.	151.3/ 186.	21.4	81.6	103.0		2	7
7/07/67	DEET	0.01000	9	10.	12./ 53.	102.4/ 181.	22.6	56.4	79.1		2	8
6/30/67	DEET	0.01000	8	10.	10./ 52.	107.7/ 193.	19.2	55.9	75.2		2	9
7/26/67	DEET	0.00100	11	15.	13./ 56.	171.3/ 188.	23.2	91.2	114.4		3	10
7/07/67	DEET	0.00100	9	10.	17./ 50.	113.7/ 186.	34.0	61.2	95.2		3	11
7/19/67	DEET	0.00100	10	22.	20./ 50.	179.6/ 182.	40.0	98.7	138.7		7	12

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APPENDIX F

PARTS LIST FOR BITOMETER-TIMER

PARTS LIST FOR BITOMETER-TIMER

Description	Number	Manufacturer	Part Number or Type
Capacitors			
500 mf, 25 WVDC	2	Cornell Dubilier	Type BR400-25
50 mf, 25 WVDC	1	Sprague	TVA-1206
0.0047 mf, low power	1	TRW	"Mylar" type 608
0.02 mf, 200 VDC	1	Aerovox	P8292ZN7
Transistors			
2N3904	1	Motorola	2N3904
2N3797 (FET)	1	Motorola	2N3797 (Field Effect)
2N3906	3	Motorola	2N3906
Resistors (All ±10% 0.5 watt unless otherwise stated)			
10,000 megohms (2%)	1	Victoreen	10,000-Megohm "glass"
100 megohms, 2 watts	1	Aerovox	CPX-2-2
4.7 K	1		
270 ohms	1		
120 ohms, 2 watts	1		
10 K	3		
1 K	1		
22 K	1		
Integrated circuit	1	Fairchild	μA710c (Commercial version)
Diodes			
Zener diode	2	Motorola	1N4735A
Crystal diode	3	Motorola	1N4002
Rectifier assembly	1	Motorola	MDA920-1
Transformer Filament-12.6 v CT. @ 1.5 A	1	Triad	F-25X
Potentiometers			
1 megohm	1	Ohmite	Type AB, CU-1052
100 Ohms	1	Ohmite	Type J
500 Ohms	1	Ohmite	Type AB CLU-5011
1000 Ohms	1	Ohmite	Type AB CLU-1021
BNC Connectors			
Bulkhead receptacle	2	Amphenol	UG-1094/U
Plug	2	Amphenol	UG-88/U

PARTS LIST FOR BITOMETER-TIMER

Description	Number	Manufacturer	Part Number or Type
Toggle switches			
DPDT; 3 A, 125 v	1	Arrow	
SPST; 3 A, 125 v	2	Arrow	
Dial plate, calibrated, 1-11	1	Mallory	381
Totalizer timers (T_1, T_2); time range, 999.99M; rating, 115/60	2	Cramer	636E; 636WK100-A0008A
Meter relay (M-1) 0-1 DC mamp	1	Simpson	Model 29XA, 7040 annular
Pilot light socket (power)	1	Dialight	95-0408-0931-241
Pilot light socket (overload)	1	General Electric	101-3830-0933-201
Pilot lamp (power)	1	General Electric	NE-51 T-3⅛ (neon)
Pilot lamp (overload)	2	General Electric	Type 344 T-1$\frac{3}{4}$ (filament)
Relays (RY-1, RY-2)	2	Potter & Brunfield	75-504 or 41 55 04
1.35 V. Mercury Cell	1	Mallory	RM-12R
Battery clip for 1.35-mercury cell	1	Keystone	139
Binding posts	5	Johnson	Various colors
Epoxy glass vector board	1	Vector	38F464
Vector pins	1 PKG	Vector	T28S1
Minibox (aluminum)			
12"L x 7" W x 4" H	1	Bud Radio	CU-2111-A
4"L x 2.25" W x 2⅛" H	1	Bud Radio	CU-2103-A
Twist-lock connectors			
Female; 10 A, 250 v	1	Hubbell	7427
Male base; 10 A, 250 v	1	Hubbell	7466

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Security Classification

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author)

IIT Research Institute
10 West 35th Street
Chicago, Illinois 60616

2a. REPORT SECURITY CLASSIFICATION

UNCLASSIFIED

2b. GROUP

N/A

3. REPORT TITLE

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Annual Progress Report - November 1, 1966, through October 31, 1967

5. AUTHOR(S) (First name, middle initial, last name)

Philip Kashin

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
November 1967	93	41

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
DA-49-193-MD-2281	IITRI-L6021-12
b. PROJECT NO.	
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

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11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	U.S. Army Medical Research and Development Command, Office of The Surgeon General, Washington, D.C.

13. ABSTRACT

The objective of this program is to develop insect repellents that can be administered systemically, preferably orally. During this year the testing of compounds for mosquito repellency by the electronic recording method continued, and the results were statistically analyzed for significant differences from control values in a specialized digital computer program. A new electronic "bitometer" that facilitates the compilation of laboratory repellency data was developed. Work was continued on a hypothesis developed during the course of this work that could explain the physiochemical basis of a mosquito's attraction to warm-blooded hosts. Gamma-aminobutyric acid (GABA) was found in aqueous extracts of mosquito heads and bodies, and it was hypothesized that the interactions of GABA with carbon dioxide, heat, and water vapor form the basis of mosquitoes' attraction to hosts. Evidence supporting the validity of the hypothesis was obtained from chemical studies of the interactions of GABA with carbon dioxide, correct predictions of chemical structures that should repel mosquitoes, and direct in vivo physiological investigations.

DD FORM 1 NOV 65 1473

UNCLASSIFIED

Security Classification

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
mosquito, repellent, gamma aminobutyric acid, neuroinhibition, carbon dioxide, electronic recording, repellency index, computer, oral, systemic, activation, inhibition, sweat, lipids.						