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Report No. IITRI-L6021-24

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Final Report

Philip Kashin, Ph.D.

October 1970

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Washington, D.C. 20315

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

IIT Research Institute
10 West 35th Street
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ABSTRACT

This report summarizes experimental studies conducted for the U.S. Army Medical Research and Development Command. The objectives of the program were to develop improved topical insect repellents and if possible to develop a repellent that is effective upon oral administration.

An insect "bitometer" was developed that detects and records the probing, penetration, salivation, engorgement and withdrawal of a mosquito from the skin of the host. The device was employed for assay of a number of potential mosquito repellents. To provide statistical tests of significance the repellency index for test compounds was contrasted with that for control compounds by means of a specialized digital computer program.

In order to establish a basis upon which to search for new and more efficient repellents, studies were conducted to explore possible mechanisms of attraction of mosquitoes to an animal host. A hypothesis was developed to explain how the interactions of moisture, temperature, and carbon dioxide may operate in host attraction. Gamma-aminobutyric acid (GABA) was found in mosquitoes, where it may play a role in mediating synaptic inhibition in the nervous system. It was postulated that when GABA combines with carbon dioxide, the resulting carbamino-GABA compound is no longer neuroinhibitory, and furthermore that the reaction of GABA with carbon dioxide underlies the mechanism by which mosquitoes are activated by carbon dioxide. Experimental studies supported the concept that carbamino-GABA is a neurostimulatory compound. On the basis of this work, numerous GABA-like volatile compounds, as well as N-substituted GABA-like compounds were screened for mosquito repellency. In most instances these compounds were found to be significantly repellent in low concentrations.

During the last quarter of the program a comparison was made between the "bitometer" repellency assay method and with the standard method used by the U.S. Department of Agriculture. The results indicated that the two assay methods corresponded well.

FOREWORD

This project was 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 was conducted by IIT Research Institute, Technology Center, Chicago, Illinois 60616 during the period of May 1, 1962 through October 31, 1970. This is Report No. IITRI-L6021-24 (Final Report) on IITRI Project L6021, entitled "Development of an Orally Effective Insect Repellent." The report covers the period from November 1, 1964 through October 31, 1970.

During the period covered by this report Dr. Philip Kashin, Research Biochemist, served as the project leader, under the administrative supervision of Dr. E. J. Hawrylewicz and more recently Mr. A. M. Shefner, Assistant Director, Life Sciences Research. During the period of May 1962 to October 1964 the project leader was Dr. H. Lal, Research Pharmacologist. The principal laboratory assistants during the program were Mr. S. Ginocchio, Technical Assistant, R. Foster, Technical Assistant, and A. M. Gross, Associate Biochemist. The statistical analyses were performed by Mr. Merl L. Kardatzke, who also developed the computer program for determining the repellency index. Valuable 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 trade names in this report does not constitute their official endorsement or approval.

The experimental data are recorded in IITRI Logbooks C16400, C16586, C16939, C17088, C17259, C17495, C17599, C18467, and C19669.

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I. INTRODUCTION

The objectives of this program were to develop topical insect repellents superior to those currently available. Furthermore, if possible, to develop an insect repellent that is effective when administered orally. In order to achieve these goals, three separate but related lines of research were followed:

a. A rapid and accurate method was developed for laboratory assay of candidate repellents.

b. Studies were conducted to elucidate the basic physiological mechanisms of attraction of hematophagous insects to their warm-blooded hosts. Understanding of this mechanism would allow a premeditated selection or synthesis of compounds that interfere with the responses of an insect to the host.

c. Compounds were tested for repellency throughout the program. Many of these compounds were selected on the basis that certain chemical structures, or electronic configurations, similar to that of gamma-aminobutyric acid (GABA) alter the mosquito's responses to the host.

This report briefly summarizes experimental results obtained since the initiation of the project. Details of these experiments were provided in past reports. In addition the report presents the findings of investigations conducted during the last contract year.

II SUMMARY OF PAST WORK

A. The Insect "Bitometer"

In order to make laboratory evaluation of repellents rapid and accurate, considerable effort was devoted to the development of a repellency assay method in which the bite of a mosquito can be detected and recorded electronically (ref. 1, 2).

This instrument "Bitometer", has been described in detail in a past report and publication (ref. 3). It consists essentially of two digital timers and a meter relay. One timer runs continuously throughout the period of the test, while the other is actuated only when a mosquito bites the host mouse. A direct and immediate measure of the percentage of time of biting thus becomes available for insertion into the computer program. All the pertinent electronically monitored data are thus obtained in a simple and convenient way and the necessity to manually measure these parameters from a chart recording is obviated. Three "Bitometer-timers" were constructed and they have proven to be useful and efficient tools in repellency screening.

A specialized digital computer program was written that statistically compares biting data between repellent-treated and untreated control mice. The percentage of mosquitoes engorged (E) is added to the percentage of biting time (P), to yield a repellency index for each trial. To provide statistical tests of significance, the repellency index for test compounds is contrasted with that for controls. The statistical methods upon which the computer program is based were presented in past reports and a publication (ref. 4).

In order to determine the applicability of the "Bitometer" method to the evaluation of repellents, a series of statistically designed tests were performed. The basic assumptions tested were as follows:

a. Factors that affect the rate of biting by mosquitoes of untreated control mice also affect the biting rate of mice treated with repellent.

b. There are no differences of practical significance between the data output from the three meters used in the tests.

c. The utilization of the electronic biting data, i.e. the amount of actual biting time as electronically recorded, makes an independent and significant contribution to total assay of repellency regardless of percent mosquito engorgement.

Details of the experimental and statistical procedures used in making these evaluations have been previously described. The three assumptions were proven to be correct, thus the validity of the electronic repellency assay method was further documented.

Recently, a direct comparison of repellency assays were made of a number of repellents provided by the U.S. Department of Agriculture, Gainesville, Florida. The repellency of these compounds was initially determined at the USDA by the standard procedure and then tested by the "Bitometer" method in our laboratories. A high degree of correspondence between the two methods was established. Results of these comparisons are discussed in detail in a later section of the report.

B. The GABA Hypothesis

A hypothesis was proposed to explain how the interactions of moisture, temperature, and carbon dioxide (CO_2) may operate to attract a mosquito to a warm-blooded host. Gamma-aminobutyric acid (GABA) was found in mosquitoes (A. aegypti), where it may play a role in mediating synaptic inhibition within the nervous system. The GABA combines with CO_2 either in aqueous solution or as a dry deposit in the presence of water vapor. The resulting carbamino-GABA compound was shown to be easily decomposed in the temperature range of warm-blooded hosts. It was proposed that the carbamino-GABA compound does not possess the synaptic inhibitory power of uncombined GABA, and that the interactions of GABA with the higher than normal moisture, atmospheric CO_2 concentration, and temperature in the vicinity of a host underlie the insects host-seeking behavior.

Studies designed to test this hypothesis were reported in detail in past reports. The hypothesis was tested in a physiological system using the change in the spontaneous discharge rate of a portion of the isolated central nervous system of a cockroach as a function of various treatments.

Carbon dioxide markedly increased the spontaneous discharge rate of this preparation, and GABA decreased it. Increasing concentrations of GABA in CO_2-treated saline solutions caused a steady potentiation of the neurostimulatory effect of CO_2. This potentiation was apparent up to and including a GABA concentration of approximately 10^{-3} M, which was neuroinhibitory in the absence of CO_2. The neuroinhibitory effects of GABA predominated in the CO_2-treated solutions at higher GABA concentrations.

Amino acids are known to form carbamates in the presence of CO_2, and it was proposed that carbamino-GABA could be the actual neurostimulatory agent in the CO_2-treated solutions. Gamma-hydroxybutyric acid (GHB) is a GABA analogue, but it contains a hydroxyl group in place of the amino group of GABA. This substance was about two orders of magnitude less neuroinhibitory than GABA. Since GHB has no amino group, it cannot form a carbamino compound. The presence of GHB in CO_2-treated saline solutions caused a steady decline in the stimulatory action of CO_2 as a direct function of GHB concentration. The stimulatory effects of CO_2 were also suppressed in GABA solutions containing GHB. These data supported the suggested role of carbamino-GABA as a neurostimulator. The fact that the neurostimulatory action of CO_2 was abolished in the presence of GHB concentration that in themselves were not neuroinhibitory suggested the presence of an excitatory site on the synaptic membrane that is different from the inhibitory site.

When tested on the cockroach preparation beta-hydroxy-gamma-aminobutyric acid (BHGA), another GABA analogue, was about equal to GABA in its neuroinhibitory potency. Similarly to GABA, BHGA has an amino group, but it also contains an electronegative hydroxyl group proximal to the amino group. Nearby electronegative groups reduce the tendency of amines to form carbamates, and CO_2 was less stimulatory in the presence of BHGA than in the presence of GABA. These results further suggested that the neurostimulatory action of CO_2 was directly related to carbamino formation.

N-acetyl-GABA (NAG), a chemically stable analogue of carbamino-GABA was shown to possess neurostimulatory properties similar to those of L-glutamate, which may be a functional excitatory transmitter in insect neuro-muscular synapses. The structural similarities between L-glutamate, carbamino-GABA, and NAG were demonstrated, and it was proposed that the similarities could account for their corresponding effects on nervous tissue. The fact that NAG, L-glutamate, carbamino-GABA, and GHB have terminal electronegative groups was adduced as further evidence that these compounds probably act principally at the same exitatory synaptic site. Therefore, CO_2

not only abolishes the neuroinhibitory action of GABA, but in forming a carbamino compound causes GABA to become neurostimulatory.

The reaction of GABA with CO_2 is extremely rapid in both directions. Thus formation of the carbamino compound is very responsive to CO_2 concentration and the diffusion rate of CO_2 in the tissue is probably the only rate-limiting factor in the reaction. Results of these studies were reported in the last annual report and in a paper to be submitted for publication (ref. 5).

C. Tests of Selected Repellents

Based on the GABA hypothesis, it was reasoned that volatile analogues of the non-volatile GABA, may repel mosquitoes by neutralizing the stimulatory effects of CO_2 emanating from a host, i.e., by increasing neuroinhibition and causing loss of host recognition.

To test this assumption, the repellency of a number of commercially available compounds was studied by the electronic method. The first indication that this line of reasoning was valid was provided by the finding that 4-aminobutanol, an alcohol analogue of GABA, was significantly repellent on the skin of a mouse at a concentration of 1.0 mg/in^2. All other omega-amino alcohols tested, from 2 to 6 carbon atoms, also showed the same level of repellency. This is about one order of magnitude less repellent that Deet, which exhibited significant repellency at 0.1 mg/in^2 of skin. Neither n-butyl alcohol nor n-butylamine alone showed significant repellency at 1.0 mg/in^2. Apparently for the repellency to be expressed both the amino group and the hydroxyl group must be present on the same molecule.

Based on the fact that certain well-known mosquito repellents, such as Deet and Rutgers 612, bear no obvious relationship to the structure of GABA this approach was subsequently broadened. Within their molecules these compounds were shown to contain certain relationships of nucleophilic and electrophilic moieties. The electronic configurations of these substances could be correlated with the electronic configuration of GABA. Thus it was proposed that the electronic configuration with a molecule (rather than its actual chemical constitution) coupled with its ability to be volatilized, determines its repellent properties. Investigations undertaken to test this proposition demonstrated that double bonds (pi-bonds) in relatively nucleophilic organic compounds can act as the nucleophilic electron donor in place of the amino group in a carbonyl or hydroxyl compound.

A substance such as 1,6-hexanediol, which is a symmetrical molecule, has an electron distribution that is equal in all parts of the molecule and is not an effective repellent (ref. 6). However, replacement of a CH_2 group with an amine group in this structure, as in ethanolpropanolamine, places a nucleophilic center between two relatively electrophilic hydroxyl radicals. This change in the electron configuration is sufficient to produce significant repellency at 0.1 mg/in^2. On the other hand, the two active repellents Rutgers 612 and 2,2,4-trimethylpentane-1,3-diol (TMPD), possess one hydroxyl group each on carbon 1 and carbon 3. There is no amine group or obviously nucleophilic group in either compound, yet they are active repellents. In the two compounds the hydroxyl group on carbon 3 is surrounded by alkyl moieties on both sides, while the hydroxyl group on carbon 1 has an alkyl group on only one side. Therefore, more electrons can be induced into the electronegative hydroxyl group on carbon 3 than on carbon 1 resulting in a less acidic inner group than the end hydroxyl group. The middle hydroxyl group is relatively nucleophilic, and the end one is relatively electropholic. Thus, the presence of an electrophilic and a nucleophilic moiety in these repellents is demonstrated and the electronic distribution of GABA-like compounds in Rutgers 612-like compounds are preserved.

On the other hand, if in a Rutgers 612-like compound the hydroxyl group in carbon 1 is removed to carbon 2, and that in carbon 3 to carbon 4, the resulting family of hexanediols shows a diminished repellency (ref 6). This is consistent with our expectations, since there are less differences in the electronic properties of the two hydroxyls when one is removed from the terminal position of the molecule. When the two hydroxyls are exactly equivalent, as in 1,6-hexandiol or 1,3-propanediol, repellency is even further diminished (ref. 6).

The repellency of m-diethyl toluamide (Deet) was also interpreted in these terms. In Deet the diethylamide group is located in the meta position, a position of relatively low electron density. An oxygen is double-bonded to a carbon atom, which in turn is bonded in the meta-position to the toluene ring on one side and to a diethylamine moiety on the other side. The diethylamine moiety has a relatively nucleophilic environment, while the carbonyl moiety is a relatively electrophilic. Since the meta position is not much activated in the toluene moiety the dearth of electrons of the carbonyl moiety is offset very little by the electrons in the ring. Although the electrons in the benzene ring itself are relatively abundant, they are not available to the carbonyl region and participate mainly in the resonance stabilization of the pi-electron structure of the benzene ring. Therefore the condition of a

relatively electrophilic-moiety juxtaposed with a relatively nucleophilic moiety continues to be maintained. The carbonyl group is sandwiched between two nucleophilic moieties, the benzene ring and the diethylamine. If this reasoning is correct, then diethylbenzamide should be as good a repellent as Deet, since there is not even a methyl group present (as in toluene) to activate the ring in any way. This was indeed the case. Alternatively, highly activating groups as substituents in the benzene ring should decrease the repellency of the benzamide moiety. Again, we have reported that m-aminodiethylbenzamide is useless as a repellent.

III. CURRENT WORK

During the last project year additional compounds were tested to study the relationship between molecular structure and mosquito repellency. Experiments were conducted to further evaluate the validity of the GABA hypothesis. In addition studies were conducted to determine the effects of L-lactic acid in the presence and absence of CO_2 on the central nervous system of the cockroach. This was done to explain the reported attraction of mosquitoes to lactic acid. A comparison was made between the "Bitometer" method used at IIT Research Institute and the standard method used by the U.S. Department of Agriculture for evaluating compounds for mosquito repellency.

A. Tests of New Compounds for Repellency

A number of commercially available compounds were used in studies of relationships between molecular structure and repellency. These compounds were chosen on the basis of the presence of nucleophilic and electrophilic moieties in a single molecule. Of special interest was the repellency assay of 3-hydroxybutyric acid which was significantly repellent at 1.0 and 0.1 mg/in^2 of skin.

We have previously reported that 4-hydroxybutyric acid is not only neuroinhibitory to the central nervous system of the cockroach but also completely desensitizes this nerve tissue to the stimulatory effects of CO_2. We assumed that this desensitization was due to the fact that a carbamino compound could not be formed by GHB and that it competes with endogenous GABA or carbamino-GABA for the receptor site. Since GHB is a solid, no attempt was made to test its repellency.

However, a closely related compound, 3-hydroxybutyrate, is a liquid at room temperature, and was tested as a repellent. The results of the assay showed that 3-hydroxybutyric acid is strongly repellent at both 1.0 and 0.1 mg/in^2. Therefore it appears that a compound chemically related to one that has an effect on the nervous system of the cockroach, also influences mosquito biting. It is possible that 3-hydroxybutyrate exerts repellency by desensitizing the receptor structures of the mosquito to CO_2 emanating from the host in the same way as the 4-hydroxy compound does in the cockroach. Thus the "inhibition-activation balance" (ref. 7) is upset, and the mosquito loses the cues for host recognition.

Acree et al (ref. 8) and Smith et al (ref. 9) reported that lactic acid is an attractant only in the presence of CO_2. These investigators also found that mosquitoes are attracted to humans in proportion to the amount of lactic acid that could be isolated from their skin. The structure of 3-hydroxybutyric acid is similar to that of lactic acid, differing from it by one methylene group. The structures of the two compounds are as follows:

3-Hydroxybutyric acid Lactic acid

If the mechanism of repellency of 3-hydroxybutyrate is to desensitize the mosquitoes neural receptor structure to CO_2 (by analogy with the effect of GHB on the cockroach central nervous system), then lactic acid may also have such an effect, but to a lesser degree. Lactic acid in conjunction with CO_2 exposure, could play a role similar to that which we hypothesized heat plays in the attraction of mosquitoes to hosts, i.e., the partial restoration of neuroinhibition in the presence of CO_2 (ref. 7). The same end result is thus accomplished by both heat and lactic acid, but by different mechanisms. Heat restores neuroinhibition by the decomposition of carbamino-GABA, while lactic acid may restore neuroinhibition by partially desensitizing the receptor structures to CO_2. Thus, in the presence of CO_2, both heat and lactic acid can act as attractants, since both affect carbamino-GABA. The former destroys it, while the latter competes with it at the receptor site. In both cases, the activating and irritating effects on the insect of the formation of carbamino-GABA are ameliorated.

On the other hand, however, Skinner et al (ref. 10) reported that lactic acid is repellent to mosquitoes. The apparently conflicting observations could possibly be resolved by stoichiometric considerations. The repellent or attractant properties of lactic acid can be expressed as a function of the relative proportions of lactic acid (as measured by its vapor pressure) and CO_2 in the environment. A substance such as 3-hydroxybutyric acid, which has a relatively high vapor pressure, is apparently predominantly repellent. Lactic acid, which has a much lower vapor pressure exerts repellency or attractancy depending upon the amount of CO_2 in the air, and the ambient temperature which effects its vapor pressure. Thus a small change in the inhibition-activation balance can grossly influence the judgement as to whether a substance is a repellent or an attractant.

Pursuant to this line of reasoning, we undertook to determine the effect of L-lactic acid on the spontaneous firing rate of the cockroach central nervous system, and on the CO_2 sensitivity of the preparation. The results of these experiments showed that the stimulatory effects of CO_2 were considerably modified in the presence of lactic acid, and almost totally absent at lactate concentrations of $>10^{-4}$ M. However, independent of the presence of CO_2 lactate in concentrations of $>10^{-2}$ M was found stimulatory to the cockroach central nervous system in two separate experiments.

If this is indeed the case, then lactate can be attractant or repellent depending upon concentration. If the lactate concentration is not too high, then the activating effects of CO_2 are abolished in its presence, and in an atmosphere containing CO_2 it is attractive. Higher atmospheric lactate concentration in the absence of CO_2 repel due to its neurostimulatory action. This could possibly explain the conflicting reports of attractancy (ref. 8, 9) and repellency (ref. 10) of lactic acid to mosquitoes.

B. Tests of the GABA Hypothesis

Experiments were continued to test the validity of the GABA-CO_2 hypothesis (ref. 7). In view of the previously described results where it was shown that gamma-hydroxybutyric acid desensitizes the cockroach ventral nerve cord to the neurostimulatory action of CO_2, it was of interest to determine the responses to a solution containing a mixture of GHB, GABA, and CO_2. The presence of GHB in a solution containing GABA and CO_2 could modify the responses of the preparation to the stimulatory effects of GABA with CO_2, and also provide some insight into identity of the sites of action of GABA, GHB, and carbamino-GABA.

Two GABA concentrations were used that were previously shown to be highly effective in potentiating the effects of CO_2 (10^{-4} M and 5 x 10^{-4} M). The GHB (10^{-4} M) concentration used was that which did not completely abolish the CO_2 effect, but which considerably modified it. The results showed that 10^{-4} M GHB in a solution containing 10^{-4} or 5 x 10^{-4} M GABA and 10% CO_2 considerably modified and dampened the stimulatory carbamino-GABA effect. These results were interpreted as providing further evidence that there are two sites on the synaptic membrane, one inhibitory and one excitatory. Both GHB and GABA can bind at both sites, but more GHB is required at the inhibitory site to block spontaneous bioelectric activity than at the excitatory site to block the action of carbamino-GABA.

If carbamino-GABA is the physiologically active form of CO_2 then it could be expected that BHGA would be less effective in potentiating the effects of GABA in the presence of CO_2. This is due to the fact that the electronegative hydroxyl group next to the amino group would repress carbamino formation due to mutual repulsion between the carboxylate group of the carbamino compound and the hydroxyl group on carbon 3. Although BHGA can form a carbamino compound, it probably would not do so as avidly as GABA. Thus, BHGA provided the opportunity to study the neurostimulatory action of CO_2 in the presence of a compound that is intermediate between GABA and GHB in terms of carbamino formation. It was found that BHGA was not as stimulatory in the presence of CO_2 which agreed with the expectations if it is assumed that carbamino compounds are of physiological importance.

The data of these experiments support the hypothesis that carbamino-GABA may be the physiologically active form of CO_2 in the insect nervous system. The findings can be summarized as follows:

a. GABA potentiates the effect of CO_2, and the potentiation is maximal in different preparations at about 10^{-4} M or 5 x 10^{-4} M GABA. The potentiation also appears to persist even at the inhibitory concentration of 10^{-3} M GABA.

b. GHB reduces the sensitivity of the cockroach ganglia preparation to the neurostimulatory effects of CO_2 as a direct function of GHB concentration. This is presumably due to its inability to form a carbamino compound with CO_2, and blocking of the carbamino-GABA receptors.

c. GHB interferes with the neurostimulatory action of CO_2 even in the presence of GABA.

d. BHGA is less effective in potentiating the neurostimulatory action of CO_2, presumably due to its decreased ability to form carbamino compounds.

C. Comparison of Repellency Assay Methods

Comparative repellency tests of compounds provided by the U.S. Department of Agriculture for blind testing by the "Bitometer" method were completed. The results were submitted to Dr. Weidhaas, who subsequently provided the names of the compounds, as well as the results of the standard USDA tests. The analysis of the "Bitometer" repellency tests of these compounds is shown in Appendix A.

All control mice were in these tests were treated with acetone alone, while all test mice were treated with the stated amount of repellent in an acetone solution. Table 1 shows the upper bounds for each of the treatment levels, and Table 2 the decreasing order of efficacy of each compound at each test level. The results where the upper bound indicated more significant repellency with decreasing concentration were ignored, and the judgement of repellent efficacy was made at the higher concentration. On basis of the "Bitometer" test, the repellency of the compounds was ranked as follows:

Compound	Relative Rating
A, B, D	Good
E, I, J	Intermediate
F, H	Poor

Table 1

UPPER BOUNDS FOR EACH TREATMENT LEVEL (mg/sq in)

Compound	1.0	0.1	0.01
USDA - A	35.5	71.2	120.1
USDA - B	37.7	64.6	104.1
USDA - D	35.3	35.7	121.1
USDA - E	35.5	79.5	187.0
USDA - F	110.1	115.5	144.1
USDA - H	137.7	77.7	95.5
USDA - I	58.1	59.2	89.4
USDA - J	72.8	59.1	102.3

Table 2

DECREASING ORDER OF EFFICACY AT EACH TREATMENT LEVEL

1.0	0.1	0.01
USDA - D	USDA - D	USDA - I
USDA - A	USDA - J	USDA - H
USDA - E	USDA - I	USDA - J
USDA - B	USDA - B	USDA - B
USDA - I	USDA - A	USDA - A
USDA - J	USDA - H	USDA - D
USDA - F	USDA - E	USDA - F
USDA - H	USDA - F	USDA - E

Compound E was placed in the "intermediate" category because repellency decreased very rapidly as concentration decreased to 0.1 and 0.01 mg/in^2 of skin. Compound D appeared to be the best repellent in the group. Upon completion of the evaluation Dr. Weidhaas sent us the following identification of the compounds.

A = M-2020	A mixture of dimethyl phthalate, 40%; dimethyl carbate, 30%; and ethyl hexanediol, 30%.
B = ENT-375	Ethyl hexanediol
D = ENT-22542	Deet
E = ENT-6217	Isopropyl-3-phenylhydracrylate
F = ENT-4262	Isobornyl 4-morpholineacetate
H = ENT-5103	2-Ethylbutyraldehyde glyceryl acetal
I = ENT-6484	Methyl N,N-diisoprophyladipamate
J = ENT-12142	5-Ethyl-2,4-heptanediol

The relative rating of the chemicals by the U.S.D.A. was based on the ratio of the protection time of the chemical to that of M-2020 against A. aegypti. Ethyl hexanediol was placed in the "good" category, although a relative rating was not assigned to it. Table 3 shows the comparison of the relative ranking.

Table 3

RELATIVE RANKING OF REPELLENTS

Rank	IITRI	U.S.D.A.	
Good	A = M-2020	A = M-2020	1.00
	B = ENT-375	B = ENT-375	
	D = Deet	D = Deet	2.28
Intermediate	E = ENT-6217	H = ENT-5103	1.17
	I = ENT-6484	I = ENT-6484	1.32
	J = ENT-12142	J = ENT-12142	1.64
Poor	F = ENT-4262	F = ENT-4262	0.18
	H = ENT-5103	E = ENT-6217	.30

In his letter of August 7, 1970 Dr. Weidhaas stated.... "We can see why ENT-5103 could be placed in the poor category. Therefore, ENT-6217 would be the only chemical placed in a different category from our results..."

Although it would be desirable to test additional compounds for comparative purposes, the results substantially increase our confidence in the validity of the electronic recording method as a screen for mosquito repellents.

IV. CONCLUSIONS

The investigations summarized in this report have included three main areas of research:

a. Development of an accurate statistically-based method to evaluate mosquito repellency of test compounds.

b. Testing a hypothesis that could explain the basic physiological mechanisms that drive mosquitoes to warm-blooded hosts.

c. Testing the mosquito repellency of certain chemical structures that are related to the structure of GABA and exploring the nature of a repellent and the mechanism of repellency.

The repellency test method developed in our laboratories as well as the assumptions upon which it was based have been thoroughly documented. A "blind" comparison between this method and the standard U.S.D.A. repellency test method showed a high degree of correspondence between the two methods. Thus it can be concluded that the electronic method provides a rapid and accurate appraisal of a candidate repellent.

Experimental evidence was obtained which supports the hypothesis that when GABA combines with CO_2, the resulting carbamino-GABA compound is a neurostimulator. This finding may have important implications that underlie the mechanism by which CO_2 triggers host-seeking behavior of mosquitoes. The suggestion that volatile chemical analogues of GABA may exert repellency was supported by the evaluation of selected compounds for mosquito repellency by the electronic recording method. The theoretical and methodological approaches developed during the program may contribute to the elucidation of the physiological mechanisms governing the interactions of hematophagous insects and their warm-blooded hosts.

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APPENDIX A

REPELLENCY ASSAY OF SELECTED COMPOUNDS

CONTROL VALUES

REPELLENC OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

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
	-0.00000	38.46	57.50	95.96			1	1
	-0.00000	42.31	59.77	102.07			1	2
	-0.00000	28.85	61.90	90.75			2	3
	-0.00000	46.30	85.76	132.05			2	4
	-0.00000	85.19	98.00	183.19			2	5
	-0.00000	65.45	82.40	147.85			3	6
	-0.00000	30.19	81.32	111.51			3	7
	-0.00000	50.00	81.47	131.47			3	8
	-0.00000	7.55	52.71	60.26			4	9
	-0.00000	41.07	66.67	107.74			4	10
	-0.00000	38.46	72.63	111.09			5	11
	-0.00000	16.00	40.51	56.51			5	12
	-0.00000	75.51	89.46	164.97			6	13
	-0.00000	60.38	85.90	146.28			6	14
	-0.00000	25.00	59.97	84.97			7	15
	-0.00000	56.25	91.77	148.02			7	16
	-0.00000	56.00	68.77	124.77			7	17
	-0.00000	32.65	52.10	84.75			8	18
	-0.00000	21.28	39.50	60.78			8	19
	-0.00000	69.81	40.87	110.68			9	20
	-0.00000	72.55	75.58	148.13			9	21
	-0.00000	27.66	76.17	103.83			10	22
	-0.00000	36.00	67.80	103.80			10	23
	-0.00000	43.48	52.83	96.31			11	24
	-0.00000	17.07	45.95	63.02			12	25
	-0.00000	6.82	11.69	18.51			12	26
	-0.00000	23.64	66.27	89.91			13	27
	-0.00000	26.92	98.14	125.06			13	28
	-0.00000	43.40	87.10	130.50			14	29
	-0.00000	79.63	92.30	171.93			14	30
	-0.00000	32.56	79.23	111.79			14	31
	-0.00000	21.28	43.53	64.81			15	32
	-0.00000	48.08	79.67	127.74			15	33
	-0.00000	48.08	87.43	135.51			15	34
	-0.00000	37.25	72.60	109.85			16	35
	-0.00000	19.61	39.07	58.67			17	36
	-0.00000	36.00	68.33	104.33			18	37
	-0.00000	81.25	54.17	135.42			18	38
	-0.00000	27.66	48.10	75.76			19	39
	-0.00000	63.04	72.22	135.26			19	40
	-0.00000	80.77	75.07	155.84			20	41
CONTROL	-0.00000	42.91	67.37	110.28	100.0	-0.0000=CONTRAST		
		21.19	19.26	35.64	(112.7)UPPER BOUND	6.7383=STANDARD ERROR		

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
USDA-A	1.00000	0.00	0.30	0.30				1	1
	1.00000	0.00	0.50	0.50				2	2
	1.00000	0.00	0.10	0.10				3	3
USDA-A	1.00000	0.00	0.30	0.30	0.2		122.1074=CONTRAST		
		-0.00	0.20	0.20	(35.5)UPPER BOUND		20.7267=STANDARD ERROR		
USDA-A	0.10000	12.96	57.30	70.26				2	1
	0.10000	0.00	10.50	10.50				4	2
	0.10000	0.00	0.80	0.80				5	3
USDA-A	0.10000	4.32	22.86	27.19	28.2		73.5069=CONTRAST		
		7.48	30.21	37.69	(71.2)UPPER BOUND		21.1371=STANDARD ERROR		
USDA-A	0.01000	9.26	46.43	55.69				7	1
	0.01000	15.69	48.50	64.19				8	2
	0.01000	47.17	92.45	139.62				9	3
USDA-A	0.01000	24.04	62.46	86.50	79.2		22.3570=CONTRAST		
		20.29	25.99	46.28	(120.1)UPPER BOUND		21.1371=STANDARD ERROR		
USDA-B	1.00000	2.08	7.50	9.58				1	1
	1.00000	0.00	0.20	0.20				2	2
	1.00000	0.00	0.07	0.07				3	3
USDA-B	1.00000	0.69	2.59	3.28	2.5		119.3663=CONTRAST		
		1.20	4.25	5.46	(37.7)UPPER BOUND		20.7267=STANDARD ERROR		

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
USDA-A								
	0.10000	3.70	12.28	15.98			2	1
	0.10000	2.00	12.37	14.37			4	2
	0.10000	7.27	29.73	37.01			5	3
USDA-A	0.10000	4.33	18.13	22.45	21.7	90.2231=CONTRAST		
		2.69	10.05	12.63	(44.8)UPPER BOUND	21.1371=STANDARD ERROR		
USDA-B								
	0.01000	14.29	42.10	56.39				
	0.01000	16.67	48.50	65.17			7	1
	0.01000	17.31	66.98	84.28			8	2
USDA-B	0.01000	16.09	52.53	68.61	63.3	39.5013=CONTRAST	9	3
		1.59	12.92	14.26	(104.1)UPPER BOUND	21.1371=STANDARD ERROR		
USDA-D								
	1.00000	0.00	0.20	0.20				
	1.00000	0.00	0.17	0.17			1	1
	1.00000	0.00	0.10	0.10			2	2
USDA-D	1.00000	0.00	0.16	0.16	0.1	122.2535=CONTRAST	3	3
		-0.00	0.05	0.05	(35.3)UPPER BOUND	20.7267=STANDARD ERROR		
USDA-D								
	0.10000	0.00	0.27	0.27				
	0.10000	0.00	0.07	0.07			3	1
	0.10000	0.00	0.00	0.00			5	2
USDA-D	0.10000	0.00	0.11	0.11	0.1	123.3982=CONTRAST	6	3
		-0.00	0.14	0.14	(35.7)UPPER BOUND	21.1371=STANDARD ERROR		

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
USDA-D								
	0.01000	34.78	84.37	119.15			7	1
	0.01000	0.00	5.72	5.72			8	2
	0.01000	31.48	98.72	130.20			9	3
USDA-D	0.01000	22.09	62.94	85.02	60.3	21.2361=CONTRAST		
		19.20	50.07	69.90	(121.1)UPPER BOUND	21.1371=STANDARD ERROR		
USDA-E								
	1.00000	0.00	0.00	0.00			1	1
	1.00000	0.00	1.15	1.15			2	2
	1.00000	0.00	0.00	0.00			3	3
USDA-E	1.00000	0.00	0.38	0.38	0.3	122.0094=CONTRAST		
		-0.00	0.66	0.66	(35.5)UPPER BOUND	20.7267=STANDARD ERROR		
USDA-E								
	0.10000	2.13	14.60	16.73			3	1
	0.10000	15.38	73.13	88.52			5	2
	0.10000	5.56	56.73	62.29			6	3
USDA-E	0.10000	7.69	48.16	55.84	43.9	69.2354=CONTRAST		
		6.88	30.19	36.33	(79.5)UPPER BOUND	21.1371=STANDARD ERROR		
USDA-E								
	0.01000	71.11	91.50	162.61			7	1
	0.01000	57.69	98.81	156.50			8	2
	0.01000	57.41	94.81	152.21			9	3
USDA-E	0.01000	62.07	95.04	157.11	146.2	-49.7058=CONTRAST		
		7.83	3.66	5.22	(187.0)UPPER BOUND	21.1371=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

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
USDA-F								
	1.00000	0.00	22.50	22.50			10	1
	1.00000	15.69	42.04	57.72			11	2
	1.00000	7.32	36.80	44.12			12	3
USDA-F	1.00000	7.67	33.78	41.45	50.7	39.8743=CONTRAST		
		7.45	10.11	17.74	(110.1)UPPER BOUND	22.5323=STANDARD ERROR		
USDA-F								
	0.10000	22.00	71.44	93.44			14	1
	0.10000	16.67	79.87	96.53			15	2
	0.10000	30.00	61.03	91.03			16	3
USDA-F	0.10000	22.89	70.78	93.67	78.2	26.2495=CONTRAST		
		6.71	9.43	2.76	(115.5)UPPER BOUND	21.5730=STANDARD ERROR		
USDA-F								
	0.01000	49.06	92.04	141.10			15	1
	0.01000	50.00	98.43	148.43			18	2
	0.01000	9.62	47.74	57.36			19	3
USDA-F	0.01000	36.22	79.41	115.63	104.6	-5.1581=CONTRAST		
		23.05	27.61	50.60	(144.1)UPPER BOUND	21.1371=STANDARD ERROR		
USDA-H								
	1.00000	11.11	50.37	61.48			10	1
	1.00000	13.04	37.82	50.87			11	2
	1.00000	12.00	58.11	70.11			12	3
USDA-H	1.00000	12.05	48.77	60.82	78.3	17.1187=CONTRAST		
		0.97	10.24	9.64	(137.7)UPPER BOUND	22.5323=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON HOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
USDA-H								
	0.10000	0.00	0.00	0.00			14	1
	0.10000	45.10	68.73	113.83			15	2
	0.10000	2.70	20.97	23.67			16	3
USDA-H	0.10000	15.93	29.90	45.83	40.4	71.6444=CONTRAST		
		25.29	35.23	60.07	(77.7)UPPER BOUND	21.5730=STANDARD ERROR		
USDA-H								
	0.01000	26.04	96.22	122.30			15	1
	0.01000	2.08	7.57	9.65			18	2
	0.01000	5.77	42.19	47.96			19	3
USDA-H	0.01000	11.31	48.66	59.97	56.0	49.0282=CONTRAST		
		12.93	44.68	57.28	(95.5)UPPER BOUND	21.1371=STANDARD ERROR		
USDA-I								
	1.00000	0.00	0.45	0.45			13	1
	1.00000	2.13	8.58	10.71			12	2
	1.00000	0.00	0.77	0.77			10	3
USDA-I	1.00000	0.71	3.27	3.98	4.7	80.0461=CONTRAST		
		1.23	4.61	5.83	(58.1)UPPER BOUND	21.5730=STANDARD ERROR		
USDA-I								
	0.10000	0.00	0.00	0.00			14	1
	0.10000	6.38	3.33	9.72			15	2
	0.10000	9.80	50.67	60.47			17	3
USDA-I	0.10000	5.40	18.00	23.40	17.5	88.6925=CONTRAST		
		4.98	28.34	32.47	(59.2)UPPER BOUND	21.5730=STANDARD ERROR		

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
USDA-I								
	0.01000	8.00	29.77	37.77			14	1
	0.01000	21.15	82.29	103.44			19	2
	0.01000	23.08	57.03	80.11			20	3
USDA-I	0.01000	17.41	56.36	73.77	54.5	59.7232=CONTRAST		
		8.21	26.27	33.29	(89.4)UPPER BOUND	22.0370=STANDARD ERROR		
USDA-J								
	1.00000	0.00	0.00	0.00			13	1
	1.00000	0.00	3.94	3.94			12	2
	1.00000	4.17	40.87	45.03			10	3
USDA-J	1.00000	1.39	14.94	16.33	19.4	67.6957=CONTRAST		
		2.41	22.54	24.94	(72.3)UPPER BOUND	21.5730=STANDARD ERROR		
USDA-J								
	0.10000	2.04	1.03	3.07			14	1
	0.10000	5.26	13.80	19.06			17	2
	0.10000	4.17	29.77	33.93			15	3
USDA-J	0.10000	3.82	14.87	18.69	17.3	88.8111=CONTRAST		
		1.64	14.40	15.44	(59.1)UPPER BOUND	21.5730=STANDARD ERROR		
USDA-J								
	0.01000	27.45	60.33	87.78			14	1
	0.01000	24.44	84.75	109.19			19	2
	0.01000	9.43	52.57	62.00			20	3
USDA-J	0.01000	20.44	65.88	86.33	67.4	42.8769=CONTRAST		
		9.65	16.79	23.63	(102.3)UPPER BOUND	22.0370=STANDARD ERROR		

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13. ABSTRACT An insect "bitometer" was developed that detects and records the probing, penetration, salivation, engorgement and withdrawal of a mosquito from the skin of the host. The device was employed for assay of a number of potential mosquito repellents. To provide statistical tests of significance the repellency index for test compounds was contrasted with that for control compounds by means of a specialized digital computer program. Studies were conducted to explore possible mechanisms of attraction of mosquitoes to an animal host. A hypothesis was developed to explain how the interactions of moisture, temperature, and carbon dioxide may operate in host attraction. Gamma-aminobutyric acid (GABA) was found in mosquitoes, where it may play a role in mediating synaptic inhibition in the nervous system. It was postulated that when GABA combines with carbon dioxide, the resulting carbamino-GABA compound is no longer neuroinhibitory, and furthermore that the reaction of GABA with carbon dioxide underlies the mechanism by which mosquitoes are activated by carbon dioxide. Experimental studies supported the concept that carbamino-GABA is a neurostimulatory compound. On the basis of this work, numerous GABA-like volatile compounds, as well as N-substituted GABA-like compounds were screened for mosquito repellency. In most instances these compounds were found to be significantly repellent in low concentrations. A comparison was made between the "bitometer" repellency assay method and with the standard method used by the U.S. Department of Agriculture. The results indicated that the two assay methods corresponded well.

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	ROLE	WT	ROLE	WT	ROLE	WT
Mosquito						
Repellent						
Gamma-aminobutyric acid						
Carbon dioxide						
Neuroinhibitor						
Activation						
Electronic						
Bitometer-Timer						
Repellency index						
Computerized repellency assay						