Report No. IITRI-L6021-8
(Annual Report)

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

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

IIT RESEARCH INSTITUTE

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November 1, 1965, through October 31, 1966

Contract No. DA-49-193-MD-2281
IITRI Project L6021

Prepared by

Philip Kashin

of

IIT RESEARCH INSTITUTE
Technology Center
Chicago, Illinois 60616

for

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

Copy No.____ November 11, 1966

FOREWORD

This is Report No. IITRI-L6021-8 (Annual Report) on IITRI Project L6021, Contract No. DA-49-193-MD-2281, entitled "Development of an Orally Effective Insect Repellent." The period covered by this report is November 1, 1965, through October 31, 1966. Previous work on this contract was conducted at IIT Research Institute from May 1, 1962, through October 31, 1964, under IITRI Project C222, and from November 1, 1964, through October 31, 1965, under IITRI Project L6021. This investigation was supported by the U.S. Army Medical Research and Development Command, Office of the Surgeon General.

The project was directed by Dr. E. J. Hawrylewicz, Assistant Director of Life Sciences Research. Technical assistance was provided by Mr. Robert Fosler and Miss Eileen M. Gross during various phases of the work. Dr. Andrew Dravnieks performed the vibrating capacitor-potential measurements of GABA and the interreactions of gamma-aminobutyric acids with carbon dioxide and water vapor in this system. Dr. George Sumyk and Mr. Anthony M. Gross performed the amino acid analyses of the dialyzable mosquito extracts. Dr. Sidney Katz was consulted in the preparation of the crayfish abdominal stretch receptor and in the techniques of obtaining electrophysiological recordings from the preparations.

Helpful advice and suggestions were offered by Dr. William F. Danforth of the Biology Department of the Illinois Institute of Technology. Mr. Merl L. Kardatzke performed the statistical analyses of the electronically recorded data and devised the computer program for determining the repellency and index and confidence limits of the test compounds.

All data are recorded in Logbooks C13755, C16400, C16417, C16586, C16230, C16667, C16939, and C17088. The computer output sheets and electronic chart recordings are also retained as part of our permanent records.

Respectfully submitted,
IIT RESEARCH INSTITUTE

Philip Kashin

Philip Kashin
Associate Biochemist
Life Sciences Research

Approved by:

E. J. Hawrylewicz

E. J. Hawrylewicz
Assistant Director
Life Sciences Research

PK/cg

ABSTRACT

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

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

During this year compounds were tested for mosquito repellency by the electronic recording method, and the results were statistically analyzed. A computer program was devised that yields a repellency index and confidence limits in comparison with controls for each test compound on the basis of data obtained from the electronic recordings.

A hypothesis was developed that could explain the mechanism of attraction of mosquitoes to warm-blooded animals. The hypothesis states that gamma-aminobutyric acid (GABA), a substance which is known to inhibit transmission of nerve impulses across certain synaptic junctions in some animal species, may also play an inhibitory role in the nervous system of mosquitoes. GABA was shown to reversibly combine with carbon dioxide (CO_2), depending upon temperature and CO_2 tension, and it was proposed that the GABA-CO_2 complex formed no longer possesses the synaptic inhibitory power of GABA alone. The interreactions of GABA with CO_2 and heat were hypothesized to form the basis of mosquitoes' attraction to hosts.

Although ultimate proof of the hypothersis was not achieved, evidence for the validity of the theory was obtained from correct predictions of chemical structures that repel mosquitoes and from chemical work on GABA-CO_2 complexes. This work is still in progress.

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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

I. INTRODUCTION

The objective of this program is the development of an orally effective insect repellent. Such a repellent could be more easily used and afford more uniform and long-lasting protection than conventional surface repellents. It could also significantly reduce human suffering from the diseases and discomfort caused by the bites of insects.

Since much needed information on the physiological basis of insect attraction and repulsion is largely unavailable, a major part of this year's effort was devoted to the elucidation of the neurophysiological events that can culminate in an insect finding and landing upon its host. A hypothesis was developed that could explain the mechanism of attraction of mosquitoes to warm-blood animals. Although ultimate proof has not yet been achieved, experimental results to date are entirely consistent with the hypothesis.

Predictions of chemical structures that should repel mosquitoes on the basis of the hypothesis were demonstrated experimentally to be correct and highly efficient as repellents. Preliminary studies of the structural specificities of these compounds indicated, in confirmation of the hypothesis, that certain requirements exist beyond which repellency is either reduced or completely lost. Further, this new family of

effective compounds exhibits considerable water solubility and thus offers the possibility of being excreted in the sweat when administered systemically. Since the presumed mechanism of action of these compounds is based upon certain neurophysiological processes that may commonly occur among insects affecting man, these compounds may exhibit a wide spectrum of repellent activity.

The electronic recording method for the testing of repellency (ref. 1-3) was refined, and a computer program was devised for determining the repellency index of test compounds. Day-to-day variations of the biting activity of mosquitoes was taken into account by the program in the determination of the repellency index, thus affording a more realistic appraisal of the repellent efficacy of test compounds. A considerable amount of the hand work necessary for the computations was thus obviated, and a screening protocol on a large scale has become feasible. The assays of the last groups of compounds tested during this report period reflect the computerized methods (Table 7).

For clarity, this report is divided into three parts. Section I is introductory; section II deals with the neurophysiological approach and experimental work undertaken toward the possible elucidation of the mechanism of insect-host interactions, and Section III deals with further applications and refinements of the electronic recording system, i.e., the statistical methods for analyzing the repellency of test compounds, as well as the tests themselves.

II. NEUROPHYSIOLOGY OF MOSQUITO-HOST INTERACTIONS

A. Trapping and Testing of Animal Effluents for Mosquito Attractancy

The literature is replete with methods of testing insects for specific attractancy to various odors and effluents. In many cases specific attractants have been identified and chemically characterized. However, chemical substances that emanate from mammalian hosts and specifically attract mosquitoes have not been unequivocally established.

Three definitely established components necessary for host-finding by mosquitoes are CO_2, moisture, and warmth. That CO_2 has an activating effect upon mosquitoes has been generally recognized and amply confirmed since the work of Rudolphs (ref. 4). Christophers (ref. 5) states, "Not only is there very strong evidence that warmth attracts *Aedes aegypti* in a very pronounced way in the urge to feed, but there has not been described in the literature any other attractive influence so active and characteristic in its results." We nevertheless tested effluents of animals and humans for mosquito attractancy by using methods of collection that had not been attempted previously for this purpose.

To obtain samples for the attractancy test, 20 anesthetized mice were used. The mice were placed into a glass tube 30 in. in length and 2 in. in inside diameter. Dry CO_2-free air was

admitted into one end of the tube at a flow rate of 5 liters/min. The effluent air was passed into a Y-shaped tube, which was immersed in a mixture of dry ice and acetone in order to condense and trap any volatiles emanating from the mice. After a 30-min collection period, the Y-shaped tube was removed, and the open ends were immediately sealed with Teflon stoppers. A control sample was collected from the 30-in. tube before the mice were placed into it. The experimental and control samples were kept frozen until used.

Part of each sample was injected into an Aerograph model 204 two-channel detection system for separation and detection of the collected effluents. This device uses a flame-ionization detector and an electron-capture detector (electrons originate from a radioactive source) to analyze the effluents. The device traps gases on an oil-impregnated inert carrier, which is slowly heated to drive off the trapped gases at different temperatures. The gases then pass through the two detectors, and a chart recording shows the positions of gas evolution. Many components were found in the effluents trapped from the mice.

The attractancy test was performed in a 30 x 12 x 12 in. glass tank covered with cheesecloth mesh. Approximately 500 female Aedes aegypti mosquitoes were anesthetized by cooling and placed into the glass tank. When the mosquitoes began to revive, the two previously collected samples (control and mouse

effluents) were placed into the glass tank, opened, and dropped onto two watchglasses at opposite ends of the tank. The revived mosquitoes were carefully observed for 1 hr to determine whether the samples had any orienting influences upon their flight.

The sample containing the mouse effluents showed no difference in attractance from the control sample, and in fact, hardly a single mosquito landed upon or near either watchglass.

The experiment was repeated with nonanesthetized mice, and a trap containing liquid oxygen was inserted in parallel with the dry ice-acetone trap. But even at the much lower temperature of liquid oxygen, no effluent attractive to mosquitoes was detected. Similarly, human effluents, which were collected at 0°C, were not attractive to mosquitoes.

Although the results are not entirely conclusive, they are also not surprising or unexpected. They confirm the results of other such investigations utilizing different methods of collection of airborne vapors. The attractive properties of certain amino acids, especially lysine, have been described (ref. 6). However, it was later recognized that the attractiveness of lysine and other basic amino acids is largely due to trapped CO_2, either absorbed or bound in carbaminoyl complexes with the amino groups. The attractancy was in direct proportion to the amount of CO_2 bound (ref. 7), and these amino

acids were no longer attractive when bound CO_2 was removed. However, it was also indicated that certain amino acids, such as tyrosine, cystine, and the prolines, may attract mosquitoes, even though bound CO_2 was apparently removed (ref. 8).

On the fairly well-documented assumption that heat and CO_2, and moisture are the most important, if not the only factors involved in attracting mosquitoes to their warm-blooded hosts, we evolved a hypothesis to explain how the interactions of heat, moisture, and CO_2 operate in directing the mosquito to its host and to explain the neurological events guiding this activity within the mosquito. If subsequent work proves this hypothesis correct, a rational physiological and biochemical basis for interfering with host attraction can be established.

B. A Possible Mechanism of Host-Finding by Mosquitoes

For many years, GABA has been known to inhibit the transmission of afferent impulses across synaptic junctions of crustaceans, such as crayfish (ref. 9). The evolutionary relationship between crustacea and insects suggests that perhaps GABA or some GABA-like substance may also inhibit the transmission of impulses across the synaptic junction of insects, with special reference to mosquitoes. Indeed, GABA has been shown to be present in the nervous tissue of many mammalian and nonmammalian species. If GABA or a GABA-like substance (henceforth designated as GABA for convenience) does

inhibit the transmission of impulses in the synapses of mosquitoes, how can CO_2 interact with this substance to cause host attraction in mosquitoes?

The work of Lipsitz and Brown (ref. 7) describes the high affinity that CO_2 has for lysine and the carbamino complex formed between CO_2 and this amino acid. It is possible that GABA can also form a carbaminoyl derivative with CO_2 just as lysine does. Indeed, alpha-aminobutyric acid is one amino acid that shows a high attractance ratio for mosquitoes and significantly absorbs CO_2 (ref. 8).

If GABA does inhibit the transmission of impulses across synaptic junctions, perhaps when GABA is complexed with CO_2 it can no longer function as an inhibitor and thus permits a much greater number of impulses to pass across the junction. In this case, CO_2 would act as an activator since it would deactivate the inhibitor. CO_2 may thus indirectly act as an irritant to the mosquito in the sense that there may be an increase in the number of afferent impulses arriving in the mosquito's central nervous system in the presence of CO_2.

In the presence of CO_2, mosquitoes become activated and take to the wing. Initially, flight may be directionless and merely reflect an "attempt" by the mosquito to escape the "irritant." If, however, the CO_2 level in the mosquito's environment persists, the mosquito continues to fly, probably randomly. If the mosquito should happen to fly into a warm

updraft of air, the heat may serve to uncouple the CO_2 from the GABA, and the mosquito may become somewhat less irritated and activated, and more "comfortable." That CO_2 can be driven from its complex with lysine by heating has been shown by Lipsitz and Brown (ref. 7).

As the mosquito approaches the source of heat more closely, the GABA-CO_2 complex is increasingly cleaved, and the insect becomes less irritated. If the heat source, however, originates from a warm-blooded mammal, then, as the mosquito approaches the heat more closely, the CO_2 content of the air also increases. Therefore, although the GABA-CO_2 complex uncouples at a faster rate, it also forms at a faster rate. A quickly reversing interplay of activation and inhibition now drives the mosquito directly to its host and eventuates in a landing. The warmth and the high CO_2 content at the surface of the host skin greatly stimulate the activity of the mosquito, and probing movements are one of the expressions of this heightened activity.

The effect of warmth is evident also in the need for warmth in the blood or in other fluids as a stimulus to feed. We have found as have others (ref. 10) that when we feed Aedes aegypti, through a membrane, the fluids behind the membrane must be warm for feeding to be effective. Probing is an expression of a generalized increase of activity due to CO_2. The tapping of a supply of warm blood and the mosquito's avidity for the source of the warmth may be an expression of the mosquito's

attempt to decrease the irritant effects of CO_2 by bringing more warmth into its body. Blood, however, also contains CO_2, so the chain of events leading to engorgement is only further stimulated, and the mosquito continues feeding to repletion. Chemical stimuli on the taste receptors of the labrum also are undoubtedly involved at this point.

Another mechanism can come into play when engorgement is complete. When the mosquito is fully distended with blood, the posterior pharyngeal valve, which is involved in "swallowing," can no longer open to receive more blood because of the back pressure of blood from the mosquito's abdomen. The valve is tightly shut, and an outward pressure is exerted upon it. This outward pressure can cause the stimulation of other nerves that are pressure receptors. The stimuli from these receptors can act antidromically or otherwise inhibit afferent impulses originating at the synapse, which contains the GABA bound with CO_2. This new set of impulses can effectively inhibit, or "switch off" the afferent impulses which originally activated the mosquito, so that the activator-irritant (CO_2) becomes ineffective. Therefore, the mosquito withdraws its mouthparts and settles down to digest its meal. The difficulty of activating mosquitoes after full distention with blood has long been recognized. This postulated chain of events is diagrammed in Figure 1.

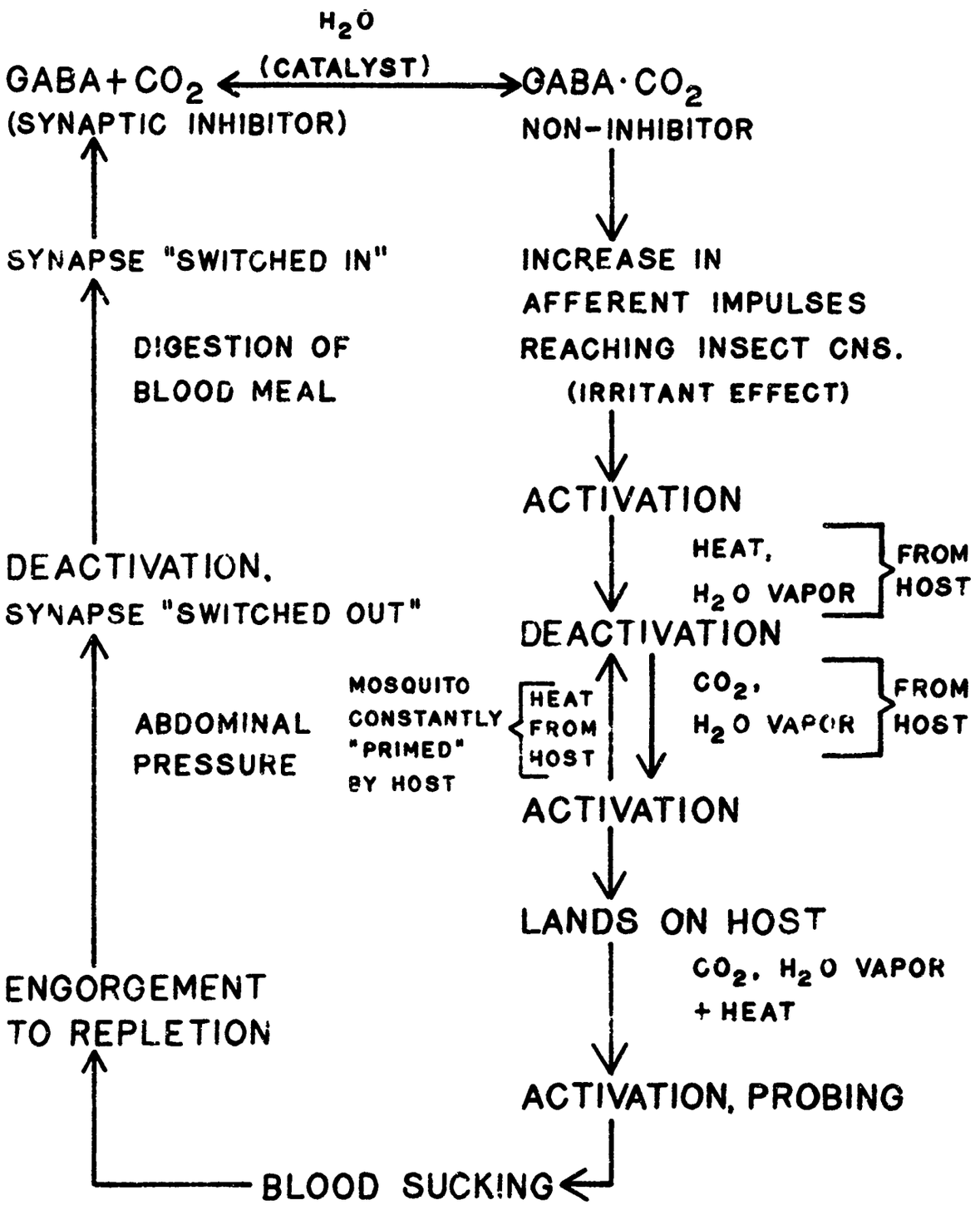


Figure 1

HYPOTHETICAL NEUROCHEMICAL REACTIONS CULMINATING
IN MOSQUITO ATTRACTION AND ENGORGEMENT

Although purely speculative, these assumptions, if proven correct, would pave the way for a totally new approach to the development of mosquito repellents and contribute to our understanding of insect host-seeking behavior in neurophysiological terms.

Before further experimental work was carried out, a literature review was conducted to determine whether our hypothesis was consistent with the findings of other investigators in terms of the possible role of GABA-CO_2 complexes at synaptic junctions. We found that many of the results of others could be successfully interpreted in terms of our hypothesis.

C. Literature Review

Before any functional significance for GABA was realized, its presence was discovered in such widely diverse material as bacteria, yeast, mold, fungus, chlorella, and higher plant tissues. Mammalian brains were also found to contain large amounts of GABA. The chemical identity of GABA from brain extracts was subsequently proven by Udenfriend (ref. 11). Bacteria, plants, and brain tissue extracts and particles contain specific decarboxylases that catalyze the production of GABA from glutamic acid. Brain tissue has the most active decarboxylase activity.

In 1953, Florey (ref. 12) observed that extracts from mammalian central nervous tissue exert inhibitory effects upon discharge of the crustacean stretch receptor, an organ that sends impulses into its associated neuron when stretched. The inhibitor was named Factor I. A rapid and accurate bioassay that could differentiate variances of Factor I activity of 10% or less was devised utilizing the stretch receptor preparation. The tissue to be assayed is heated, suspended in suitable crayfish saline, diluted as required, and applied to the test preparation. When the identity of Factor I was tentatively established as GABA, reference standards that cause the same degree of inhibition were used to estimate the Factor I activity of unknown extracts.

By using a similar assay procedure, Elliott and van Gelder (ref. 13) sought to establish whether GABA is stored in an inactive condition and released when its activity is called for and whether the free, active form could be disposed of when no longer required. Their estimation of the presence of GABA was based solely upon inhibition of the crustacean stretch receptor, not upon chemical analysis of GABA actually present in the test solutions. Their procedures and results were as follows.

When unheated brain suspensions were incubated with known amounts of GABA aerobically with glucose in the medium, their GABA content increased, as shown by the release of "occluded" GABA from the homogenates of the brain slices when heated. "Occluded" GABA was defined as that fraction in the brain that was released only by heating and that caused inhibition in the stretch receptor preparation. When brain slices were incubated with known amounts of GABA aerobiocally without glucose in the medium, their GABA content decreased; i.e., GABA uptake from the medium was less marked than when glucose medium was used. When brain slices were incubated with known amounts of GABA anaerobically with or without glucose, they lost still more occluded GABA, and no GABA had been taken up from the medium. No evidence of rapid GABA destruction was found.

This study shows that apparently no mechanism rapidly lowers the concentration of GABA. Although these experiments indicate that metabolizing brain slices can absorb GABA, the mechanism of occlusion is not understood.

The results of Elliott and van Gelder may possibly be explained on the basis of a GABA-CO_2 complex. If the only substrate of brain metabolism (glucose) is removed or if respiration is inhibited by anaerobic conditions, no CO_2 would be evolved by the brain slices, and thus GABA could not be "occluded." Heating releases the "occluded" GABA and would also be capable of dissociating a GABA-CO_2 complex.

Under anaerobic conditions or in the absence of glucose under aerobic conditions, no occluded GABA could be detected.

Edwards and Kuffler (ref. 14) studied the blocking effect of GABA and related compounds on single nerve cells in the crustacean stretch receptor. They showed that amino and carboxyl end groups are essential for blocking and that blocking was optimal if 3 carbon atoms separated the amino group from the carboxyl group. Some guanido acids, such as guanidoacetic acid, beta-guanidopropionic acid, beta-guanidobutyric acid, and gamma-guanidobutyric acid, were almost as effective as GABA in blocking sensory discharges.

"Adaptation" to the inhibitor substance was noted in most preparations after several minutes. The discharge frequency of the receptor cell gradually increased with time; this increase showed that inhibition was becoming less marked. Stirring the solutions after adaptation occurred often restored inhibition. This occurred especially with the GABA solution. Stirring caused a restoration of inhibition equivalent to that of adding fresh GABA solution. The effect of stirring was not always as great with the other compounds. The order of effectiveness of restoration of inhibition with stirring was as follows: GABA > beta-guanidopropionic acid > guanidoacetic acid > beta-guanidobutyric acid. Stirring was ineffective with beta-alanine, delta-aminopentanoic acid, and gamma-guanidobutyric acid.

To test the hypothesis that GABA can be inactivated by deamination in the synapse, amine oxidase inhibitors, such as isopropyl isonicotinic hydrazide and isonicotinic hydrazide, were added to the blocking concentration of GABA. No change was seen in the synaptic inhibitory potential. It was concluded that the inhibitory transmitter was not broken down by an amine oxidase. An alternative proposed was that the inactivation of GABA can occur through an uptake of GABA by the tissue. The work of Elliott and van Gelder discussed above was quoted in this regard. This possibility, however, was not studied.

Kravitz et al (ref. 15) studied the substrates of GABA metabolism in lobster excitatory and inhibitory axons. They found that the principal difference between the two axons was the activity of glutamic decaboxylase; decarboxylase activity in the inhibitory axon was 11 times greater than that in the excitatory axon. The inhibitory axon was capable of synthesizing more GABA than it could destroy. The decarboxylase in the inhibitory axon was inhibited at high GABA concentrations, but even at one-third of the inhibitory axon's normal activity, synthesis of GABA balanced destruction.

It appears unlikely that rapid chemical removal of GABA at the site of inhibition would explain the stirring effect described by Edwards and Kuffler. This effect could be explained on the basis of our hypothesis. The CO_2 diffusing out of metabolizing cells at the synapse could bind GABA and thus inactivate

its inhibiting properties. Stirring the solution would obviously bring fresh GABA to the synaptic site, and inhibition would once again be evident and continue until more CO_2 diffused out to bind the GABA again. The "adaptation" described for other compounds for which stirring was not effective in restoring inhibition is not intended to be explained by this hypothesis.

It has been amply established that GABA is a depressant in the central nervous system and other nervous tissue of vertebrates (ref. 16,17). This amino acid has also been shown to play a role in the normal physiology of the insect. Price (ref. 18) obtained extracts from the head of a fly, Musca domestica, and found that GABA is in a higher concentration in this region than in any other region of the animal. Ray (ref. 19) showed that GABA exists in the central nervous system of the cockroach, Periplaneta americana.

Since GABA is found associated only with nervous tissue in animals, GABA in insects also is probably involved in the physiology of nervous tissue. The physiological effects of GABA have been studied in a few insect preparations. Vereshtchagin et al (ref. 20) showed that high concentrations of GABA and beta-alanine had a depressing effect on the nerve chain of a caterpillar, Dendrolinus pini. Suga and Katsuki (ref. 21) showed that GABA had an inhibitory effect on the locust prothoracic ganglion. Gahery and Boistel (ref. 22) found that

GABA had a complete inhibitory effect on response at the level of the ascending giant fibers in the cockroach, Periplaneta americana. Since conduction along the giant fibers was not modified by GABA, these authors concluded that GABA has a selective action on synaptic transmission.

Curtis and Phillis (ref. 23) postulated that GABA acts either on postsynaptic membranes or intraneuronic processes that control membrane properties. Amino acids with a chemical structure similar to that of GABA had a similar effect (ref. 22). Recent investigations of Kerkut et al (ref. 24) showed that GABA causes marked inhibition of contraction of a profused cockroach leg. The inhibitory concentration of GABA at the threshold was 5×10^{-6} g/ml, about the same order of magnitude noted in the crustacean stretch receptor.

The purpose of our investigation is to establish whether GABA or some closely related compound is present in mosquito nervous tissue and plays a central role in the mosquito-host interaction. The evidence presented above is mainly indirect. To our knowledge, no investigations have been conducted on the effect of CO_2 in synaptic junctions, on the interaction of CO_2 with GABA or on the existence of GABA in mosquitoes. Available information does not rule out our hypothesis, which can, in fact, explain some puzzling phenomena associated with GABA activity described by various investigators. This literature review was not intended to be comprehensive, but rather a

brief survey of pertinent information and a reinterpretation of experimental findings on the basis of our hypothesis.

D. Tests of the Hypothesis: Experimental Results

1. GABA in Mosquitoes

A central point in the hypothesis is whether GABA or some other CO_2-binding component involved in the transmission or the inhibition of impulses in the insect nervous system can be found in mosquitoes.

To determine whether GABA is present in mosquitoes, a group of female *Aedes aegypti* mosquitoes was starved for 2 days and then frozen. About 0.8 g of the frozen mosquitoes was ground in a glass homogenizer with about 5 cc of water as the extractant, since GABA is very soluble in water. The insoluble residue was separated by centrifugation and discarded. The opalescent supernatant was heated for 10 min in boiling water, and much material precipitated from the solution. This material was separated by centrifugation and also discarded. The clarified supernatant (water extract) was placed in a dialysis bag and dialyzed with stirring at 4°C overnight against distilled water. The dialyzand (water external to the dialysis casing) was freeze-dried, and the residue was redissolved in 1 cc of 0.1 N hydrochloric acid. An aliquot of this solution was taken for amino acid analysis, which was performed on a Technicon Auto-Analyzer. The results are presented in Table 1.

Table 1

AMINO ACID ANALYSIS OF WATER-SOLUBLE, DIALYZABLE MOSQUITO EXTRACT

Amino Acid[a]	Micromoles
Unidentified peak	----
Aspartic acid	.041
Threonine	.048
Serine	.111
Glutamic acid	.160
Proline	.209
Glycine	.084
Alanine	.822
Valine	.043
Cystine	Trace
Methionine	.013
Isoleucine	.022
Leucine	.052
Tyrosine	.034
Phenylalanine	.028
Unidentified peak	----
GABA	.168
Ammonia[b]	.163
Ornithine	.015
Lysine	.066
Histidine	.125
Tryptophan	.023
Arginine	.136

[a]In order of elution from column.

[b]Ammonia absorption from air makes this determination inaccurate.

GABA was eluted from the column just before ammonia; this result indicates the basicity of this amino acid. Basicity gives further evidence of a capability for binding CO_2. GABA is not, however, as basic as lysine or arginine, and therefore its binding of CO_2 is probably not as strong as that of lysine or arginine. GABA-CO_2 binding, therefore, should be fairly reversible and quickly responsive to CO_2 tension in the air. GABA must possess all these properties to play the postulated role in the nervous transmission of mosquitoes. GABA can be visualized as behaving somewhat like the hemoglobin of blood. Although hemoglobin reversibly binds and quickly responds to relative CO_2 and oxygen concentrations in the environment, GABA can reversibly bind and quickly respond relative atmosphere CO_2 concentrations.

The amino acid analysis also showed peaks that we could not immediately identify. Taurine and beta-alanine have been found in mosquito extracts (ref. 25), and the unidentified peaks may have been these amino acids.

It should be noted that this amino acid analysis gives only relative values, not absolute concentrations of free amino acids in mosquitoes.

In general, however, the standard methods of amino acids analysis do not appear to be sufficiently sensitive for our purpose. Although GABA definitely appears to be present in our aqueous extracts, the quantitative results were not consistent. The question of how much free amino acid cannot be extracted by our methods

because of nonspecific binding to the discarded insoluble tissue cannot be answered. In enzymatic fluorometric methods that are available, 1×10^{-11} mole GABA can be detected (ref. 26). This is approximately 1000-fold more sensitive than amino acid analysis methods and may make it possible to determine the GABA content of a single mosquito. Furthermore, investigators using enzymatic methods have obtained 95 to 100% recoveries of GABA added to brain homogenates (ref. 27).

It may also be possible to histologically localize the site of synthesis of GABA in the mosquito. A histochemical method for the demonstration of GABA metabolism in tissue sections of nervous tissue has recently become available (ref. 28). We plan to investigate the GABA content of mosquitoes by enzymatic and histological means in order to gain a clearer insight into nervous process governing mosquito behavior.

2. CO_2 Binding by GABA

Our first approach to testing our hypothesis was qualitative. Whether GABA can indeed bind CO_2 is basic to the hypothesis. In order to ascertain whether CO_2 is bound by GABA, a 0.5 M solution of GABA was prepared, and CO_2 was bubbled through it for 10 min at room temperature. The excess CO_2 absorbed in the water was precipitated by the addition of excess saturated barium hydroxide solution. The precipitate was centrifuged off, and the supernatant

was treated with additional saturated barium hydroxide solution. No further precipitate was observed. Upon placing the test tube containing the supernatant in hot water, more white flocculent precipitate formed.

When the experiment was performed with distilled water, no further precipitate was noted when the tube was placed under hot water. Therefore, GABA appeared to have bound CO_2 and made it unavailable for precipitation with barium hydroxide until heat was applied.

These preliminary results were interpreted to mean that GABA does bind CO_2 and that heat breaks the GABA-CO_2 complex. The binding site is at the amino group of GABA in the form of carbamino complex (ref. 29,30). In order to study quantitatively the binding of CO_2 by GABA in relation to temperature, the following experiments were carried out.

A 0.1 M solution of GABA was divided into 5 aliquots of 6 cc each. Each aliquot was allowed to equilibrate at the following temperatures; 3, 10, 28, 37, and 57°C. After equilibration, CO_2 was bubbled through each solution at a moderate rate for 5 min. The solutions were maintained at temperature during the CO_2 treatment. Paraffin oil was then layered on each solution to a thickness of about 1/4 in. and 4 cc of a saturated solution of barium hydroxide was added to each GABA-CO_2 solution. Immediately, a white precipitate formed, which represented the excess CO_2 (unbound CO_2) in the GABA solutions.

The solutions were allowed to stand at temperature 15 min after the addition of the barium hydroxide and then centrifuged for 15 min at 2000 rpm in the cold. Cold temperatures are known to preserve carbamino compounds (ref. 30), and the paraffin oil prevented further absorption of CO_2 from the atmosphere. Water blanks were run as controls at each temperature. The precipitate of barium carbonate from the first centrifugation was discarded, and each GABA solution from which excess CO_2 had been removed was placed in boiling water for 10 min. After boiling, another precipitate was visible in the GABA solutions, which represented the CO_2 that was bound to the GABA and that was removed by the higher temperature. The excess barium hydroxide still present in the solution reacted with the released CO_2 to form the second precipitate. Little or no second precipitate was noted in the blank solutions containing only water.

In order to estimate the amount of the carbamino compound that was formed at the various temperatures, the second precipitate was centrifuged as before, and the supernatant was discarded. The precipitate was washed once with about 10 cc of distilled water, washings were discarded, and 10 cc of 0.1 N sulfuric acid was added to the precipitate. The precipitates were heated with sulfuric acid in boiling water for 30 min. During this time an exchange reaction occurred in which barium sulfate was formed from the barium carbonate originally present. The amount of sulfuric acid unexchanged was determined by titration with 0.1 N sodium hydroxide using phenolphthalein as an

indicator and was subtracted from the total sulfuric acid originally added. The difference obviously represents the amount of sulfate that was exchanged to form barium sulfate, and is directly proportional to the amount of barium carbonate in the second precipitate. This method is essentially that described in Roessler and Brown (ref. 31) for the estimation of carbamino lysine.

The results of this experiment are shown in Figure 2. It is apparent that the CO_2 binding by GABA is low at 3°C, rises to a maximum at about 18°C, and rapidly descends to practically zero binding at 57°C. The amount of CO_2 bound to GABA at physiological temperature (37°C) is only about one-third of that bound at 18°C. A very important assumption in the hypothesis can thus be substantiated. As the mosquito approaches a warm-blooded animal, the GABA-CO_2 complex is dissociated at an increasingly rapid rate.

Figure 2 also shows that there is less binding of CO_2 at temperatures below 18°C. This result may at first seem unexpected since low temperatures should preserve the carbamino compound. However, these results can be explained in terms of pH. The rate of breakdown of the carbamino compound depends not only on temperature, but also on pH (ref. 30) and varies directly with hydrogen ion concentration over a wide range.

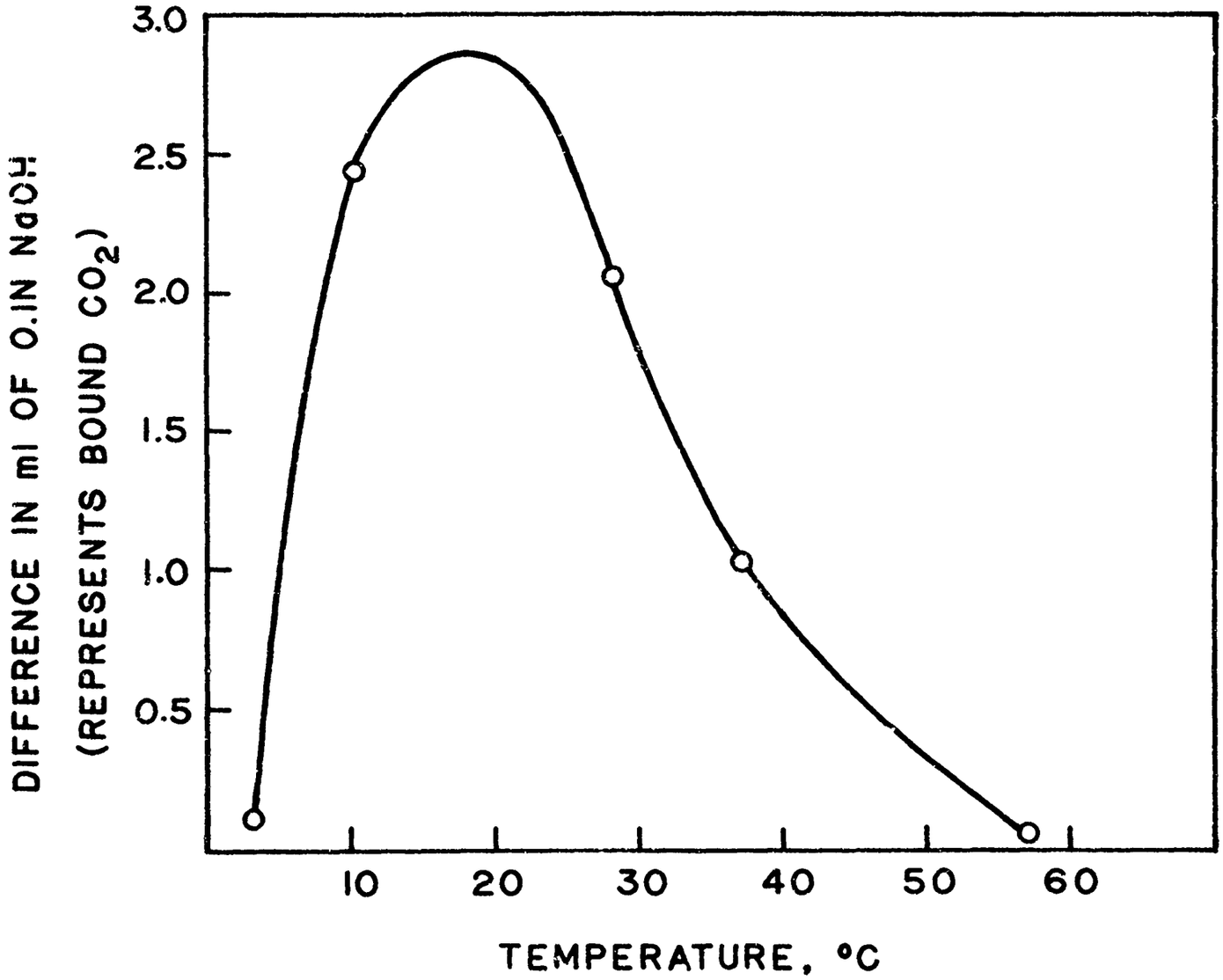


FIG. 2 EFFECT OF TEMPERATURE ON THE FORMATION OF A GABA-CO_2 COMPLEX.

The ionized carbamino compound (—NHCOO$^-$) is stable, but the molecular form (—NHCOOH) dissociates rapidly to CO_2 and free amine. Only the anionic form of a simple amino acid is reactive. Thus CO_2 reacts with $-NH_2$ groups but not with $-NH_3^+$ groups (ref. 30,32). It is well known that gases are more soluble in liquids at low temperature than at high temperature. At 0°C, 0.3346 g of CO_2 is soluble in 100 g of water at 1 atm pressure, and at 18°C, 0.1789 g of CO_2 is soluble in water under the same conditions (ref. 33). Almost twice as much CO_2 is soluble in water at 0°C than at 18°C. Although the hydration reaction of CO_2 to the ionic species HCO_3- and CO_3- is very slow in the absence of catalysts such as carbonic anahydrase (ref. 32), there is almost twice as much hydrogen ion present at 0°C as at 18°C. The increased solubility of CO_2 at low temperature and the resulting lowered pH of the solution explains the decreasing fraction of the carbamino GABA formed at low temperature during the bubbling of CO_2.

In experiments designed to verify this point, we found that in bubbling CO_2 through a 0.1 M solution of GABA at 15°C, the pH at equilibrium was about 5.4, and the pH of a 0.1 M solution of GABA at 0°C after equilibration with CO_2 was about 5.2. Apparently the difference of 0.2 pH units was sufficient to cause the pronounced dissociation of the carbamino compound observed at low temperatures (Figure 2).

The influence of the transport of CO_2 by blood provided the impetus for extensive research in the mechanism of binding of CO_2 by blood and serum proteins. These studies have shown that molecular CO_2 is itself the species that reacts with amino groups, not H_2CO_3, HCO_3^-, or $CO_3^=$ (ref. 30,32). A mosquito is known to respond instantly to various atmospheric levels of CO_2. Since the preliminary slow hydration step for CO_2 is not necessary for carbamino formation, our concept of a quickly reversible carbamino-GABA hypothesis gains support.

The addition of moisture to the CO_2-containing atmosphere increases the kinetic effects of CO_2 in an additive way (ref. 34). We envision moisture as playing the role of a catalyst for the reaction of amino groups with CO_2. It may be reasonably assumed that the nervous structure involved in the detection of CO_2 is not dry, but is enveloped in a water film (ref. 35). Thus, the GABA in the receptor is probably in solution.

The differences between the kinetic effects of "dry" and "wet" CO_2 may lie in whether the CO_2 must first be dissolved in the moisture film at the receptor site (as in the case of "dry" CO_2), or is already in solution when it reaches the receptor site (as in the case of "wet" CO_2), thus facilitating entry. The additivity of the kinetic effects of moisture and CO_2 can be understood in these terms.

Another factor that may contribute significantly to the additive effects of moisture and CO_2 is the comparatively great solubility of CO_2 in water. Of the three main constituents of air, the water solubility of CO_2 is about 50 times greater than that of oxygen, and 100 times greater than that of nitrogen (ref. 33). It is conceivable that the concentration of CO_2 in the water vapor micelle is considerably higher than that in the dry air. Water vapor reaching the target neural tissue of a mosquito may thus carry proportionately higher concentrations of CO_2 into these structures. Under such circumstances, additivity would be expected.

Moisture alone is also known to be an attractant to mosquitoes (ref. 34). If moisture catalyzes the combination of CO_2 and free amino groups, it probably also catalyzes the reverse reaction, i.e., dissociation of the carbamino complex. This is diagramed in Figure 1. In this respect, moisture may resemble heat, since both agents contribute to the distruction of carbamino complexes. The attractivity of moisture can then be understood in the same terms as the attractivity of heat. It is interesting to note that moisture becomes unattractive when the ambient air is cooler than 60°F, or 15.5°C (ref. 34). This temperature corresponds to the temperature in Figure 2 where the greatest association of GABA with CO_2 occurs; in this region the complex is least affected by changes in temperature, and probably also therefore by changes in relative humidity.

Further evidence for factors influencing the binding of CO_2 by GABA was obtained in quite another way. The gradient of an electrical field in the air gap between two conducting plates depends on the existence of dipole layers between the plates and the electric charges on or between the plates. The electrical field gradient in the gap is evidenced by the generation of an AC signal in the connecting circuit when one plate is vibrated with respect to the other. Essentially, this apparatus is a vibrating capacitor and is used for the detection of surface effects between different materials (ref. 36).

A polar vapor between the capacitor plates can change the electrical topology of the plate in many ways; the change is reflected by a change in the sensor electrical signal. Diagrammatic representations of the apparatus are shown in Figures 3 and 4.

The apparatus was used by coating the sensor plate of the capacitor with 0.05 ml of a 1.0% GABA solution, which was allowed to dry on the plate. The plate was equilibrated with dry nitrogen, and then 0.5% CO_2 was added, and the mixture was passed between the vibrating-capacitor plates. If CO_2 is bound by GABA, changes in the electrical properties of the capacitor will occur. Essentially no change occurred in the electrical field between the capacitor plates. Figure 5 shows the results.

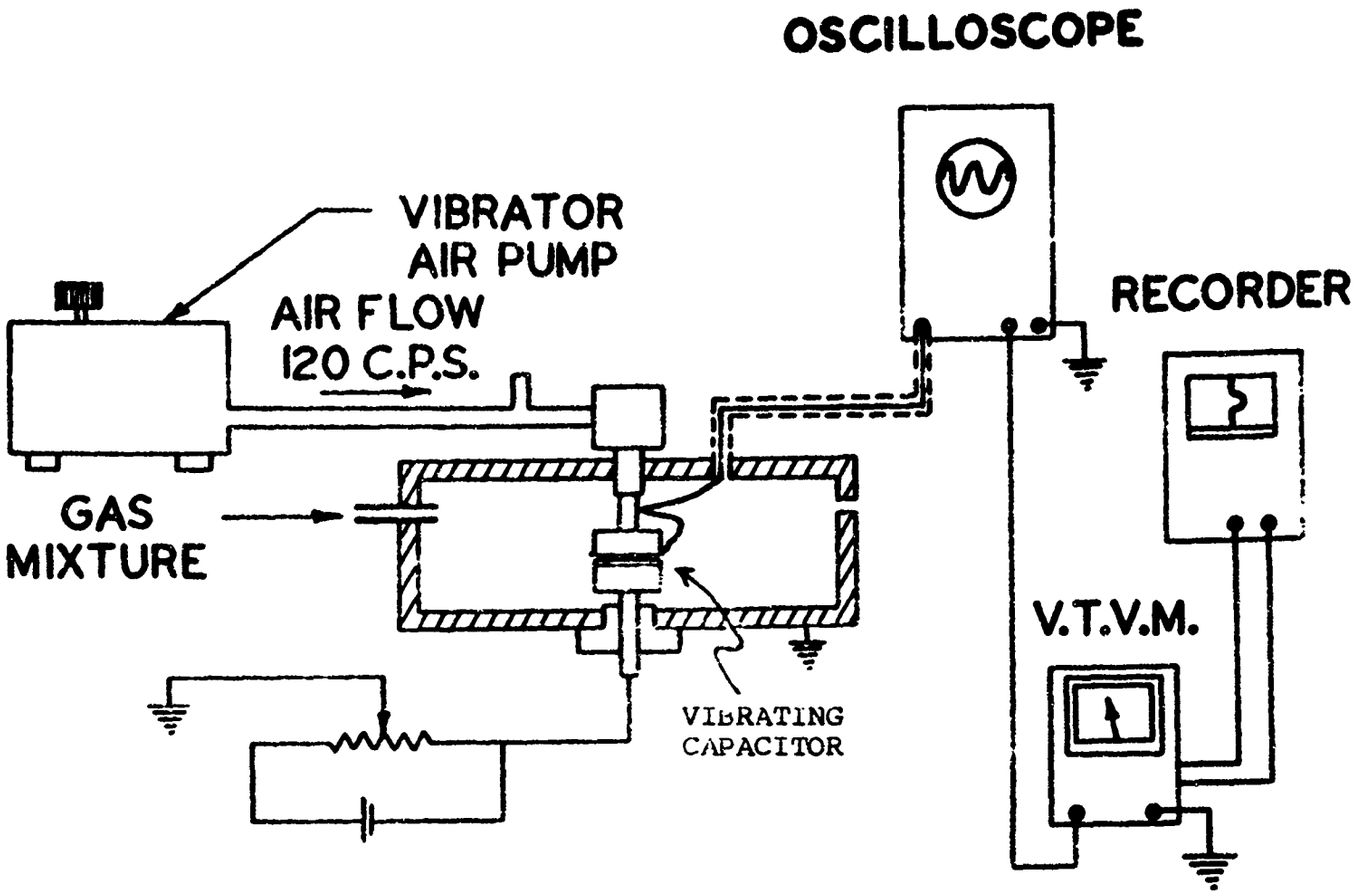


Figure 3

DIAGRAM OF VIBRATING CAPACITOR APPARATUS

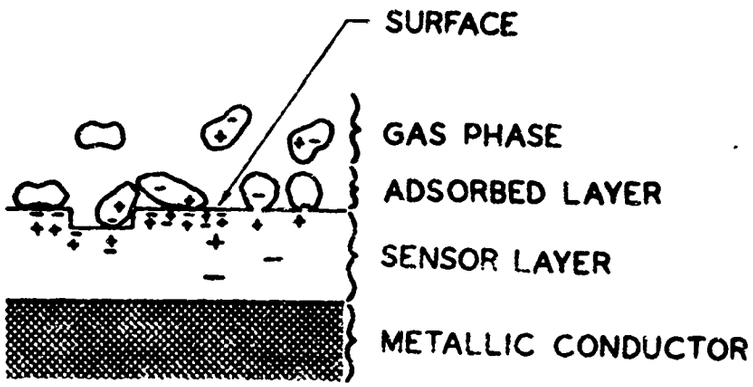


Figure 4

HYPOTHETICAL RELATIONSHIPS BETWEEN
GAS PHASE AND GABA COATED SENSOR
PLATE IN VIBRATING-CAPACITOR APPARATUS

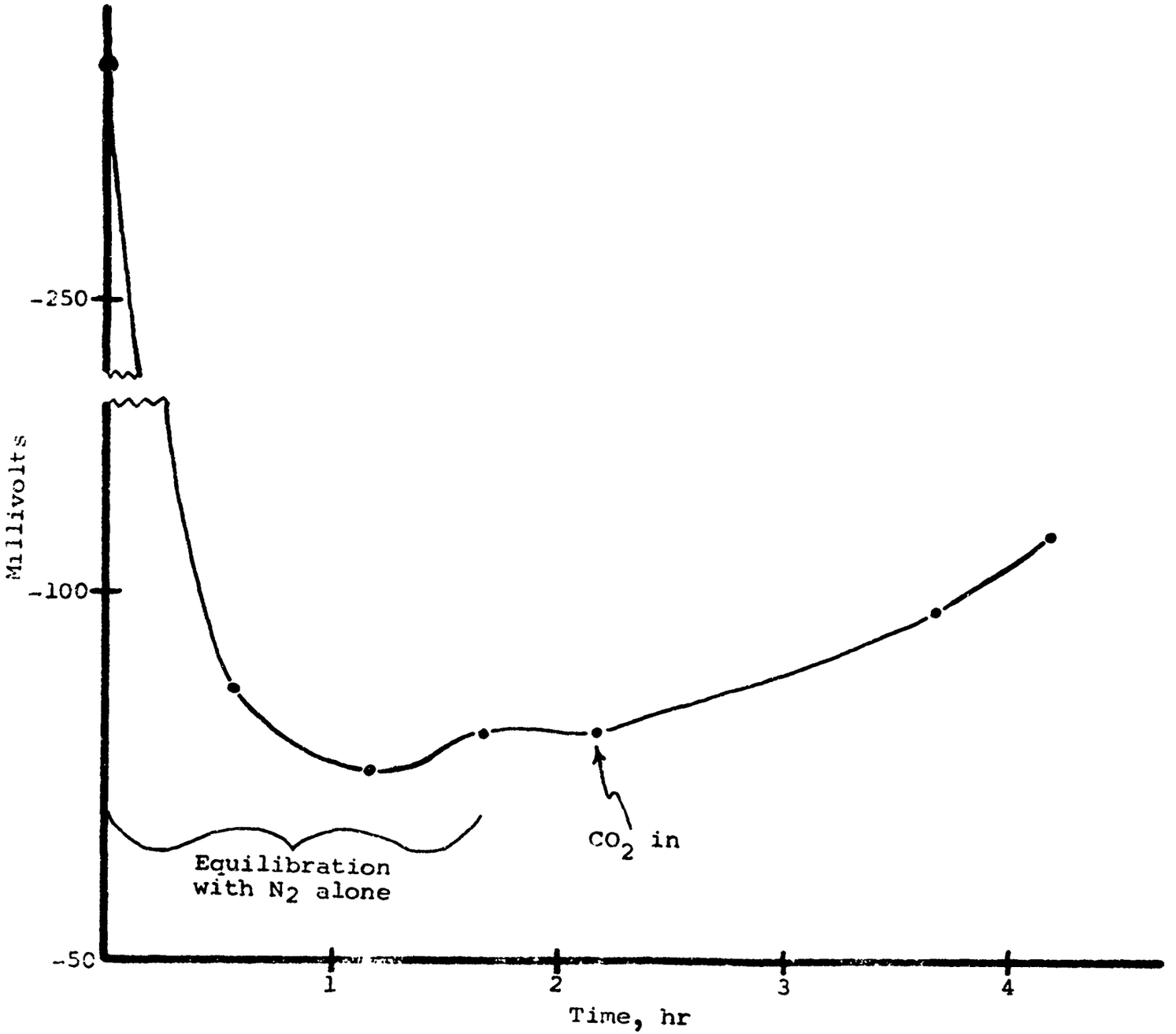


Figure 5

EFFECT OF DRY CO_2 ON GABA

When the plate was equilibrated with nitrogen containing water vapor (20% relative humidity) and then 0.5% CO_2 was added to the gas mixture between the vibrating-capacitor plates, an immediate and pronounced change occurred in the electrical properties of the apparatus upon the addition of CO_2. Figure 6 shows these changes. The potential drifted toward more negative values when water vapor and nitrogen without CO_2 were passed between the capacitor plates. When CO_2 was added, the potential precipitously changed to more positive values. After about 1 hr, a drift toward a more negative direction in the electrical field was observed as the CO_2-nitrogen-water mixture continued to pass over the GABA. We cannot explain this phenomenon at the present time, though a gradual reorientation of the molecules coating the sensor plate may be suggested.

The electrical model showed that CO_2 did not combine with GABA unless moisture was present. The water vapor-GABA-CO_2 model system therefore conforms to what we know about mosquito behavior, and the hypothesis obtains further support.

We then tested the reversibility of the binding of CO_2 by GABA at a given temperature and the effect of CO_2 concentration on the amount of GABA bound in the carbamino complex. In order to accomplish this, the amount of CO_2 bound to the GABA solution was determined after different times of reaction with the saturated barium hydroxide solution at a constant

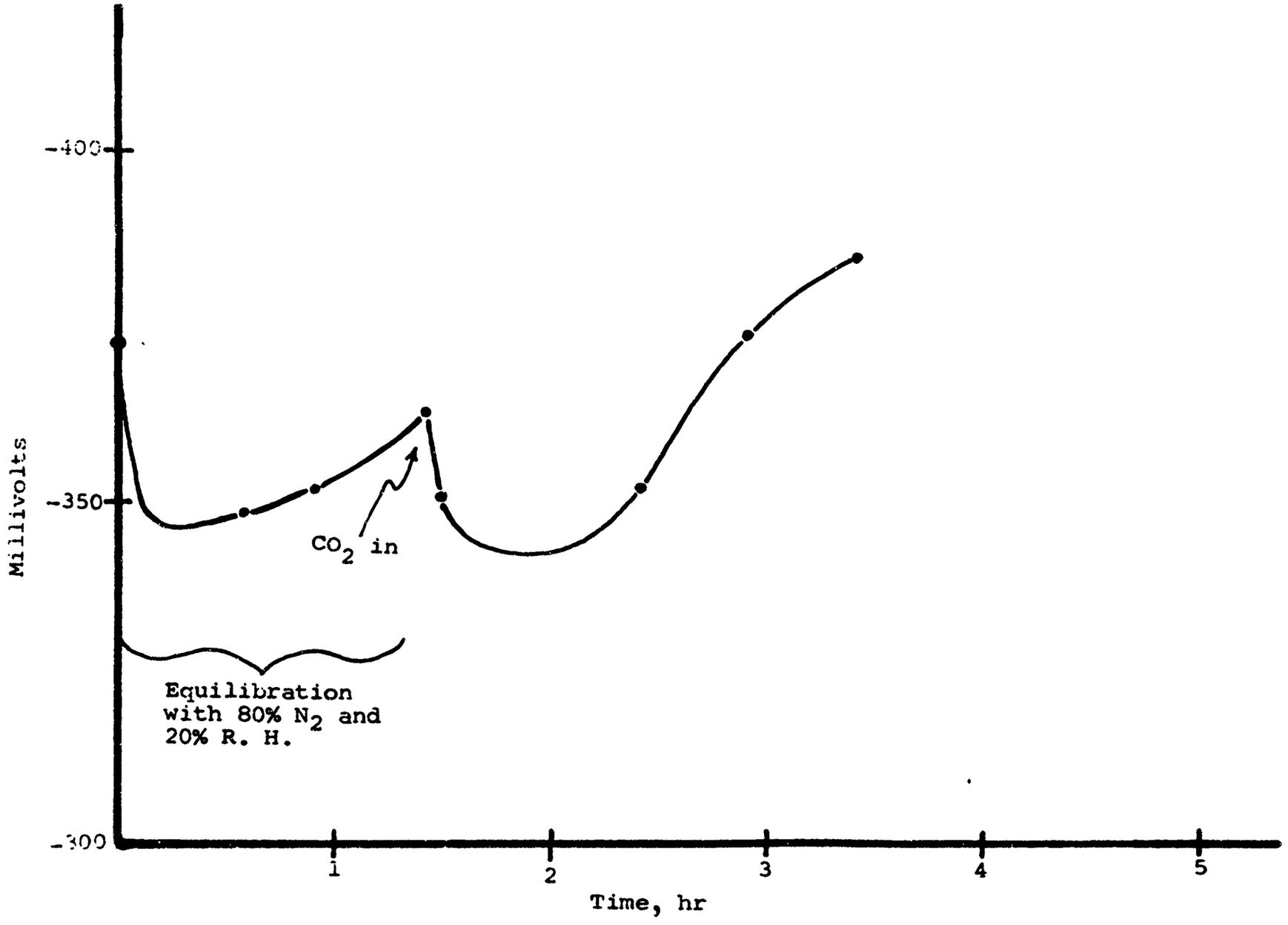


Figure 6

EFFECT OF CO_2 AND 20% RELATIVE HUMIDITY ON GABA

temperature. The experiment was carried out at 25°C with a 0.5 M solution of GABA. CO_2 was bubbled into each of two 0.5 M GABA solutions for 5 min and into each of two distilled water blanks. Then saturated barium hydroxide was added to all four solutions. The precipitate of barium carbonate that formed immediately was removed within 1 min from one of the blanks and from one of the GABA solutions by rapid filtration under pressure. This first precipitate represented the excess CO_2, as before. The other GABA solution and blank were allowed to stand 15 min before filtration. Each solution was then boiled for 30 min to precipitate the GABA-bound CO_2 and titrated after treatment with sulfuric acid, as previously described.

The results showed that after standing 15 min only one fifth of carbamino compound remained compared to the solution which stood 1 min before filtration. The water blanks showed no difference in these time intervals. We interpret these results as evidence of the reversibility of the carbamino complex.

If CO_2 spontaneously dissociated from the complex with the amino group, it becomes available for immediate precipitation with the excess barium hydroxide in the solution and is thus removed from the possibility of further reaction with an amino group. The amount of carbamino-bound CO_2 will thus be a function of the amount of CO_2 available, and of the time that it is exposed to the barium hydroxide. Since Figure 2 was constructed after a reaction time of 15 min at each temperature, the amount

initially bound at each temperature was probably higher. The relative values, however, should be accurate. Figure 2 is therefore still an adequate representation of relative binding at different temperatures.

We can conclude that the binding of CO_2 by amino groups is a reversible reaction at any given temperature and that the amount of carbamino complex is a function of the concentration of CO_2. These results support the hypothesis that the degree of CO_2 binding is a function of both CO_2 concentration and temperature. In the vicinity of the host, the nervous tissue of the mosquito can bind enough of the CO_2 emanating from the host to keep the insect "primed," in spite of the higher temperatures.

This concept leads to the possibility that any insect attractant must contain activating as well as deactivating principles in orienting the insect toward its host. On the one hand, the activator functions as an irritant to the insect, and on the other hand, the deactivator offers the promise of relief. Thus, the insect is continuously "primed" and "unprimed" as it approaches the host, and a delicate balance between activation and inhibition leads to the finding of and the attack upon the host.

A source exhuding only CO_2 and no heat has been shown to have mainly activating, but little orienting, effects on mosquitoes. We can therefore ask what are the effects of heat alone (in the complete absence of CO_2)? Under these conditions,

if the above speculations are correct, we should observe no orientation to the heat source. To our knowledge, no such experiments have been performed with mosquitoes in a totally CO_2-free atmosphere. In future work, we intend to study the effects of heat on mosquitoes in the complete absence of CO_2.

As a corollary to this concept, there should be an optimum balance between the heat, water vapor, and CO_2 emanating from a host that would result in the most efficient orienting and host-finding movements. We will attempt to study the behavior of mosquitoes in an olfactometer, in which the CO_2 content, water vapor, and temperature of an airstream can be independently controlled. The relative gradients of CO_2, water vapor, and heat emanating from a host may be an important determinant of host preference by mosquitoes. The effective ratios of CO_2-bound and free GABA which cause inhibition and activation may also differ from one mosquito species to another.

3. Bioassay of GABA and GABA-CO_2 Complexes

Florey (ref. 37) described the use of isolated hind gut of crayfish in the assay of GABA. This preparation is considerably simpler than the crayfish stretch receptor for determining GABA activity. We therefore used it in our preliminary studies.

The intestine was removed from the cryafish and suspended by threads between a support in the bottom of a test tube and the writing arm of a kymograph. The intestine was bathed in a saline medium described by Florey (ref. 37), kept at room

temperature, and continuously aerated. When a solution of 10^{-3} M acetylcholine was added to the solution, the intestine contracted. When a solution of 10^{-3} M GABA was added to the solution before 10^{-3} M acetylcholine, the intestine did not contract upon the addition of acetylcholine.

If our hypothesis is correct, the GABA solution that had CO_2 gas bubbled through it before addition to the medium should not inhibit intestinal contraction when acetylcholine is added. We found, however, that treating the GABA with gaseous CO_2 prior to addition did prevent contraction when acetylcholine was added.

We do not consider these result conclusive, since a certain amount of the GABA-CO_2 complex may have reverted back to GABA to cause inhibition because of the continuous aeration of the solution, as proscribed in the procedure. Even when the air supply to Florey's crayfish intestine preparation was occluded and the solution was not mixed, no adaptation was noted during a 5-min observation period (ref. 38). The crayfish intestine therefore may not be a suitable tissue for the purposes of this assay.

Although there are other premutations of the test system that we will try before abandoning the gut preparation, it does not appear that this preparation is adequate for either proving or disproving our hypothesis. Smooth muscle, is a relatively undefined tissue, and may not be sensitive to GABA-CO_2 complexes. Continuous aeration of the preparation may dissociate the GABA-CO_2 complex which was added.

Edwards and Kuffler (ref. 14) did not stir the CABA solution around the crayfish stretch receptor when release from inhibition was noted, and they obtained inhibition of impulse transmission when the solution was stirred. Therefore the crayfish stretch receptor may be the preparation of choice in answering the question posed by the hypothesis. This preparation is considerably more difficult than that of the intestine. We therefore obtained the consulting services of Dr. Sidney Katz, a neurophysiologist, in the preparation of the crayfish abdominal stretch receptor. This work is presently in progress.

E. Predictions of Repellent Chemical Structures on the Basis of the GABA Hypothesis

Although we have not yet obtained ultimate proof of the hypothesis, it is difficult to resist speculating on how the hypothesis, if true, would affect our approach to the design of mosquito repellents. There are two possible alternatives: one is to alter the emanations of the host; the other is to increase, by some means, the amount of free GABA in the mosquito's nervous system. The first alternative is patently not feasible, since interference with the emanations of CO_2, water vapor, and heat from the host would imply interference with the basic life processes of mammals. The second alternative

is therefore the one we explored. Although GABA itself is non-repellent, probably due to little or no vapor pressure, volatile structural analogues of GABA that we have investigated exhibited considerable repellency toward mosquitoes. The repellency of these analogues compared favorably and in some cases were in preliminary trials superior to the best repellents currently available. These results lend further support to the hypothesis, in that we accurately predicted repellent structure on the basis of our assumptions. Presumably, these GABA analogues were capable of penetrating the mosquito's nervous and sensory structures, causing deactivation and cessation of host-seeking activities. These results are further discussed in Section III.

III. ASSAY OF REPELLENTS WITH THE ELECTRONIC RECORDING SYSTEM AND STATISTICAL ANALYSES

A. Statistical Analyses

Statistical methods for assay of the repellency of compounds by the electronic recording method were first presented in our last annual report (ref. 1). Table 2, which was prepared in a manner similar to that described in the last annual report, shows the sums of the averages of the percent displacement, P, and percent engorgement, E. This is the disciminant function, $\bar{P} + \bar{E}$, as developed in that report. P and E are averaged over the number of trials of a single compound at a single concentration. The standard displacement distance, D, is also given in Table 2, though this value was not used in our computations, and was found statistically to contribute little to the measure of repellency.

The test for significant differences is developed as follows. The set of controls upon which the analyses (ref. 1) were based contained $n_c = 14$ observations. The mean for the variable $\bar{P} + \bar{E}$ was $\bar{X}_c$, or 113.6, and the estimated variance, S_c^2, was equal to 1585.5. By using a t test for significant differences of the means, the relationship can be stated as follows:

$$\frac{\bar{X}_c - \bar{X}_i}{\sqrt{S_c^2\left(\frac{1}{n_c} + \frac{1}{n_i}\right)}} \geqq t_{0.95}(n_c - 1) \qquad (1)$$

Table 2

ANALYSIS OF MOSQUITO REPELLENCY OF VARIOUS COMPOUNDS BY ELECTRONIC RECORDING METHOD

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Standard Displacement Distance (D), $\sqrt{\Sigma D^2/n}$	Mosquitoes Engorged (E), %	$\bar{P} + \bar{E}$, %	Significance at 95% Confidence Level[a]
N-Amyl succinimide	0.1	0	0	0		
	0.1	0	0	0		
	0.1	7.3	0.047	0	4.1	S
	0.1	5.3	0.118	3.8		
	0.01	35.6	0.326	4.0	39.6	S
2-Cyclohexyl-cyclohexanol	0.1	83	0.567	30.9	113.9	NS
N,N-Diethyl-m-isopropyl-benzamide	0.1	26.6	0.403	6.0		
	0.1	24.1	0.268	1.9	29.2	S
Cyclohexanol-2-phenyl	0.1	19.0	0.307	5.7		
	0.1	51.5	0.769	11.1	43.7	S
Methyl anthranilate	0.1	21.4	0.205	1.9		
	0.1	0	0	0		
	0.1	87.0	0.799	25.0	56.2	S
	0.1	75.6	0.329	15.0		
4-Cyclohexene-1,2-dicarboxy-imide, N-propyl	0.1	0	0	0		
	0.1	0	0	0		
	0.1	36.8	0.486	5.6	15.6	S
	0.1	17.8	0.273	1.8		

Table 2 (cont.)

Table 2 (cont.)

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Standard Displacement Distance (D), $\sqrt{\Sigma D^2/n}$	Mosquitoes Engorged (E), %	$\bar{P} + \bar{E}$, %	Significance at 95% Confidence Level[a]
N,N-Diethyl-2,6-dimethyl benzamide	0.1	0	0	0	22.9	S
	0.1	0	0	0		
	0.1	24.2	0.328	6.2		
	0.1	50.8	0.450	10.3		
Succinamic acid, N,N-diethyl-sec-butyl ester	0.1	1.27	0.606	0	2.53	S
	0.1	0	0	0		
	0.1	24.0	0.328	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0		
	0.01	51.0	0.450	2.3	37.6	S
	0.01	0	0	0		
	0.01	27.5	0.710	0		
	0.01	60.8	0.729	8.0		
2-Naphthol-1,2,3,4-tetrahydro	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	32.9	0.208	0	32.5	S
	0.01	28.2	0.654	3.8		
Propionanilide-N-butyl	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	61.2	0.721	16.4	60.2	NS
	0.01	37.2	0.449	5.6		

Table 2 (cont.)

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Standard Displacement Distance (D), $\sqrt{\Sigma D^2/n}$	Mosquitoes Engorged (E), %	$\bar{P} + \bar{E}$, %	Significance at 95% Confidence Level[a]
1,2-Cyclohexane-dicarboximide-N-sec-butyl	0.1	0	0	0		
	0.1	0	0	0	0	S
	0.01	57.3	0.755	15.0		
	0.01	28.9	0.423	9.0	55.1	S
N,N-Diethyl benzamide	0.1	0	0	0		
	0.1	0	0	0		
	0.1	0	0	0	0	S
	0.1	0	0	0		
	0.01	50.1	0.331	5.4		
	0.01	0	0	0	27.8	S
Succinamic acid, N,N-dipropyl-sec-butyl ester	0.1	0	0	0		
	0.1	0	0	0	0	S
	0.01	34.6	0.506	3.1		
	0.01	57.4	0.644	14.6	54.9	S
Controls	-	100	1.082	41.8		
	-	97.1	0.534	17.3	128.2	-

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

where $\bar{X}_i$ is the mean of n_i observations for a test group. If Equation 1 is true, the treated group is significantly different from the control group at a 95% confidence level. By using these statistics for the control group and $t_{0.95}$ (13) = 1.771, Equation 1 is simplified:

$$\frac{113.6 - \bar{X}_i}{\sqrt{1585.5\,\frac{1}{14} + \frac{1}{n_i}}} \geqq 1.771 \qquad (2)$$

Equation 2 gives:

$$113.6 - 70.5\sqrt{\frac{1}{14} + \frac{1}{n_i}} \geqq \bar{X}_i \qquad (3)$$

for a threshold condition with a significant repellent effect. Thus, if $n_i = 2$, the mean for the sum $\bar{P} + \bar{E}$ must be less than or equal to 60.3%.

For the new data, in which $n_c = 2$ and $\bar{X}_c = 128.1$, the previous estimate of variance, $S_c^2 = 1585.5$, is used. The threshold condition becomes:

$$\bar{X}_i \leqq 128.1 - 70.5\sqrt{\frac{1}{2} + \frac{1}{n_i}} \qquad (4)$$

For $n_i = 2$, the threshold is:

$$128.1 - 70.5\sqrt{\frac{1}{2} + \frac{1}{2}} = 57.6\% \text{ for the control group.}$$

For the three sets of variables measured, based on discriminant function analysis, the percent of time displaced (P) is the best single measure of repellency; the percent of mosquitoes engorged (E) is the next best measure; and the standard displacement distance (D) is the least reliable measure. The best variables for measuring repellency are $\overline{P}$ and $\overline{E}$. These variables are the only ones required in these calculations.

Table 3 lists the threshold values for various numbers of tests (n_i) on a compound at a specific concentration. These values were derived from Equation 4. If these values are exceeded, the experimental compound is not significantly different from the control.

Table 3

THRESHOLD VALUES FOR n_i

Number of Trials (n_i)	Threshold Value for Significant Difference
1	41.8
2	57.6
3	63.7
4	67.0
6	70.5
10	73.5

The results obtained with the electronic recording method show that we have a very useful instrument for screening potential mosquito repellents. Compounds that show repellency significantly above that of the controls can be applied in decreasing concentrations to test the concentration limit for repellency.

The slightest degree of penetration of the skin by the insect is recorded, and contributes to our judgement of repellent efficacy. A mosquito landing or probing on a host may not be easily distinguished visually from an actual bite with penetration. The effectiveness of a repellent, especially in very low concentrations may be erroneously assessed if this distinction is not accurately made. Our method eliminates any possibility of error in this judgement. A concentration of repellent that is just sufficient to prevent penetration of the host's skin is all that is necessary, regardless of whether a landing occurs. Such penetrations can be sensitively determined with the electronic recording method. We have microscopically observed the penetration of mosquito mouthparts into a transilluminated mouse's ear while simultaneous electronic recordings were made. The recorder responded only when the mosquito's fascicle actually penetrated the skin of the ear. If the proboscis merely touched the ear or quickly moved from one part of the ear to another in an apparent "searching" movement without penetration, the recorder did not respond. There is no doubt that when the base line in the recording is displaced, the insect has actually penetrated the host's tissue.

Among the repellents tested in Table 2, some seem to be even more potent than diethyltoluamide (DEET) in this system. In this respect, succinamic acid, N,N-diethyl-sec-butyl ester; 2-naphthol-1,2,3,4-tetrahydro; N,N-diethyl benzimide; and possibly N-amyl succinimide and N-butyl propionanilide appear to be outstanding.

There is, however, a certain difficulty with this assay method. We compare our test groups with a group of controls which were pooled over a period of many days. We do not know whether the biting of the controls on the day of the test was equivalent to the biting of the controls of the pooled group. If the biting of the controls on the test date was for some reason different from the pooled group, our test of significance would not be accurate, since the biting of the mosquitoes may have been influenced by some random unknown factor. We have now devised a computer program designed to overcome this difficulty and give a more realistic evaluation based upon day-to-day comparisons. This program and further statistical evaluations are discussed in Section C.

B. Mosquito Repellency of GABA and GABA Analogues

If our hypothesis of the mechanism of GABA action is correct, when GABA or its chemical analogues are volatilized in the vicinity of a mosquito, the mosquito's biting behavior should be altered. To test this approach, gamma-amino-n-butanol and gamma-aminobutyraldehyde diethylacetal were tested

as mosquito repellents. The electronic recording method was used for assay, and statistical analyses were performed as described previously. During the course of these tests, 23 control experiments were carried out, and the control groups were pooled. The mean for the controls was $\bar{P} + \bar{E} = 87.63$. This is lower than that for the previously reported control group and indicates somewhat less biting. The variance (S_c^2) was 1807.6, which is larger than the previous variance.

Because of these differences, the threshold values for n_i, i.e., the values of $\bar{P} + \bar{E}$ that cannot be exceeded if repellency of the test compound is significantly different from control values at the 95% confidence interval are lower than for the previous controls. This actually tends to make the test of significance somewhat more demanding. Some of these threshold values are calculated in Table 4.

Table 4

THRESHOLD VALUES FOR n_i

Number of Trials (n_i)	Threshold Value for Significant Difference (95% bound)
1	13.02
2	33.79
3	42.81
4	48.06
10	59.95

GABA itself was found to have no significant repellency, even at a concentration of 10 mg/sq in. of skin. This was probably due to the fact that GABA is a salt and, as such, probably has a very low vapor pressure.

Gamma-amino-n-butanol (GABOH), however, is a liquid and at a concentration of 1 mg/sq in. of skin was considerably repellent. The degree of repellency was about the same order of magnitude as that of DEET. At a concentration of 0.1 mg/sq in. of skin, repellency was no longer evident. The repellency effects of GABOH were not long-lasting and disappeared after about 5 hr (Table 5). This may be due to a high rate of evaporation of this compound.

Table 5

REPELLENCY OF GABA AND GABOH

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Mosquitoes Engorged (E), %[a]	$\bar{P} + \bar{E}$, %	Significant at 95% Confidence Level
GABA	10.0	44.5	38.9		
	10.0	100.0	29.6	106.5	No
	1.0	33.3	56.9		
	1.0	100.0	63.0	126.5	No
Gamma-amino-n-butanol	1.0	0	0		
	1.0	0	0		
	1.0	0	0		
	1.0	0	0	0	Yes
	1.0	13.6	0		
	0.1	92.9	37.7		
	0.1	92.3	15.9	119.4	No
5-hr retest	1.0	14.4	16.7		
	1.0	87.5	64.3	111.4	No
	1.0	71.0	80.5		
24-hr retest	1.0	79.3	27.1	106.4	No

[a]30 to 50 mosquitoes were exposed in each test.

C. The Computer Program

1. Analysis of Variance for Controls

During the course of this work we realized that there were definite variations in control values from test to test. There are at least four possible sources of this variation:

(a) Day-to-day variations in biting. This variable may also be a function of the time of day the test was performed.

(b) The number of mosquitoes exposed to the test animal. This number is not always uniform because of the death of some of the insects prior to the test.

(c) The age of the mosquitoes at the time of testing.

(d) Natural attractancy or repellency of the test mouse.

The first three of these factors are amenable to statistical analysis; the fourth is uneconomical to assess. For our purposes, we assumed that the natural attractancy or repellency of the test mice is uniform. In any case, repeated trials on promising repellents would diminish the significance of this variable. We do not yet have enough data to determine the effect of age, since until now we had not recorded the age of the mosquitoes used in the tests. We now record the mosquito age and will perform an analysis of variance when enough data are accumulated.

We have named the sum, $\overline{P} + \overline{E}$, the repellency index. This repellency index was determined for a number of control experiments (untreated mice), and an analysis of variance of the results of the untreated control tests for the repellency index was performed. Separate components for day effects (day-to-day variations) were computed, and the effect of the covariate for the number of mosquitoes present was also determined. Table 6 shows the sum of squares that can be attributed to each of these effects.

Table 6

ANALYSIS OF VARIANCE OF REPELLENCY INDEX FOR CONTROLS

Effect	Degrees of Freedom	Sums of Squares	Mean Square	Variance Ratio, F	Approximate Significance Level, %
Number	1	3171	3171	3.990	94
Day	15	34833	2322	2.922	99
Error	24	19072	794.66		
Total	40	57076			

The day effect was definitely shown to be significant in the analysis of variance. The effect of the number of mosquitoes was not firmly established. The implications are as follows:

(a) The day-to-day variation in the control group should be taken into account in tests of significance of repellency.

(b) The number of mosquitoes actually present can have some effect on the repellency index, but the exact nature of this effect must yet be determined and will result in a minor adjustment of the repellency index.

(c) The control variance is 794.66, which is less than that in previous tests because of the removal of the component of variance associated with day-to-day variation.

2. Optimum Number of Controls

If we wish to test for significant differences from the untreated control group, we can assume equal variance for the control and experimental test observations. It is then possible to apply the following result, which minimizes the variance of comparisons between the control mean and each test group mean for a given expenditure of effort.

The total number of experimental units, k, is equal to the sum of the number of units allocated to the control group, u, plus the product of n, the number of test groups, times v, the number of tests for each test group:

$$k = u + nv \tag{5}$$

Hence:

$$v = \frac{k - u}{n} \tag{6}$$

If σ^2 is the variance of observations in the control group, then variance, V, of the contrast of the mean of the control group versus the mean of each test group is:

$$\frac{\sigma^2}{u} + \frac{\sigma^2}{v} \tag{7}$$

Substituting for v from Equation 6:

$$V = \left(\frac{1}{u} + \frac{n}{k - u}\right) \sigma^2 \tag{8}$$

We now differentiate V with respect to u, and equate to zero in order to determine the value of u that minimizes V:

$$\frac{dV}{du} = \frac{-1}{u^2} + \frac{n}{(k - u)^2} = 0 \tag{9}$$

Since u = 0 or u = k will leave the test group or the control group, respectively, unobserved, these can be ruled out as not optimum. Multiplying dV/du by $u^2(k - u)^2$ gives:

$$-(k - u)^2 + nu^2 = 0 \tag{10}$$

or

$$(n - 1)u^2 + 2ku - k^2 = 0 \tag{10a}$$

If n = 1, then:

$$2ku - k^2 = 0 \tag{11}$$

Hence:

$$u = \frac{k}{2} \tag{12}$$

If $n > 1$,

$$u = k \frac{-1 \pm \sqrt{n}}{n - 1} \tag{13}$$

But, since u must be positive, the negative root is rejected and we are left with:

$$u = k \frac{-1 + \sqrt{n}}{n - 1} = \frac{k}{\sqrt{n} + 1} \tag{14}$$

Substituting in Equation 6:

$$v = \frac{k - u}{n} = \frac{k(\sqrt{n} + 1) - k}{n(\sqrt{n} + 1)} = \frac{k\sqrt{n}}{n(\sqrt{n} + 1)} = \frac{k}{\sqrt{n}\,(\sqrt{n} + 1)} \tag{15}$$

Let $n = m^2$, then from Equation 14:

$$u = \frac{k}{m + 1} \tag{16}$$

and, from Equation 15:

$$v = \frac{k}{m(m + 1)} \tag{17}$$

Since integer numbers of tests are of interest we make a convenient choice of k to give us such solutions.

With $n = m^2$, let $k = m(m + 1)$, then:

$$v = 1 \text{ and } u = m \tag{18}$$

We may generate optimum solutions for m equal to any positive integer. All optimum solutions are of this form unless multiplying by an integer constant. Thus we can present Table 7, which shows the optimum allocation between test and control units.

Table 7

OPTIMUM ALLOCATION BETWEEN TEST AND CONTROL UNITS

Number of Tests Groups (n)	Number of Test Units (v)	Number of Control Units (u)	Total Number of Units (k)	Variance of the Contrast in σ^2 Units	Relative Efficiency
1	1	1	2	2.000	0.250
1	2	2	4	1.000	0.250
1	3	3	6	0.667	0.250
4	1	2	6	1.500	0.444
4	2	4	12	0.750	0.444
4	3	6	18	0.500	0.444
9	1	3	12	1.333	0.562
9	2	6	24	0.667	0.562
9	3	9	36	0.444	0.562
16	1	4	20	1.250	0.640
25	1	5	30	1.200	0.694
36	1	6	42	1.167	0.735
49	1	7	56	1.143	0.766
64	1	8	72	1.125	0.790
81	1	9	90	1.111	0.810
100	1	10	110	1.100	0.826
2	2	3	7[a]	0.833	0.343
3	3	5	14[a]	0.533	0.402
5	4	9	29[a]	0.361	0.478
6	2	5	17[a]	0.700	0.504
7	3	8	29[a]	0.458	0.528
8	6	17	65[a]	0.225	0.547

[a]Approximate optimization.

Some approximate optimizations are also given. Relative efficiency is computed by the formula:

$$\text{Relative efficiency} = \frac{n}{k} \cdot \frac{1}{\text{variance}},$$

where the variance is in σ^2 units.

Due to the significance of the day effect in the analysis of variance, our immediate objective is to minimize the variance of the basic unit of contrast between test observations and control observations on a given day. Hence if we expect to run 12 tests per day, we can test 9 compounds with one test each and use 3 control groups. Replication tests of these compounds could be performed on another day. If only six tests could be run, 2 should be control units.

3. Practical Considerations

In applying these values, one question remains to be answered. What is the experimental block over which we compile our test group? It does make a difference concerning the proportion of controls that is optimum, as shown by comparing 4 groups a day for 25 days with 100 groups for the same period (Table 7).

This decision can be made objectively by an analysis of variance to test for a day effect in the controls. If the day effect is significant, which we have found to be the case, then the number of groups each day determines the optimum ratio of controls to test units. Otherwise the logical experimental design that is analyzed as a unit would be considered as a whole. The need to test for day-to-day variation would indicate

a minimum of two controls per day of the experiment to ensure an adequate number of degrees of freedom in the estimate of residual variance. Until experience proves that day-to-day variance can be consistently neglected, it is safest to plan on a daily basis.

The proportion of control units increases with the number of test groups per day for optimum results. Also, it is advantageous to spread the testing of a given compound (or other variable of the test group) over several days to ensure the reproducibility of results and to guard against the effects of spurious experimental conditions.

Since the day-to-day variation is significant, the mean for each day must be calculated and used in the way indicated in the following discussion, which is the basis of a computer program devised for performing the comparisons between test and control groups.

4. Description of Computer Program and Analysis

The variance and the mean of the repellency index should be derived from the control data for each logical experimental unit. Day-to-day variation in the control should be taken into account, since it is statistically significant.

Taking this day-to-day variation into account is easily handled by a computer routine. The computer coding form serves also as a laboratory worksheet. Cards coded from this form serve as input to a program and provide routine documentation of all tests together with calculation of the percent of

mosquitoes engorged and of the percent of the time of deflection of the "bitometer" (ref. 2).

Analysis of variance of the control data is required to test for significance of day effect. The data input cards are also suitable for use as input to existing general-purpose analysis-of-covariance programs. This allows analysis of the effects of the total number of mosquitoes in the test and of mosquito age.

Reevaluation of the index of repellency will be done periodically in order to check our previously derived index. This will allow us to make use of a larger, more representative data base and will ensure optimum use of future experimental data.

The general advantages of this procedure are such that an insect repellent resulting in a 50% decrease in activity for two independent trials will establish a significant effect at a 95% confidence level. Since each trial would be performed with one mouse and approximately 50 mosquitoes, the index derived utilizing the insect "bitometer," provides an extremely economical screening tool.

The program was written for the IBM 7095 digital computer in the Fortran IV computer language. This program evaluates the weighted average of the contrast for each test group. Tests of significance are made and tables of the observations and the

results are created in a form suitable for printing on reproduction masters directly from the computer output magnetic tape. This eliminates time-consuming, error-prone table preparation. Calculation of the repellency index is also provided.

A simple ancillary program provides tables containing original input numbers as a convenience in checking for input errors. Tests for each compound must be sorted together to be suitable for final tabulation. The control observations are input first. The statistical formulae upon which the computer program is based is shown in the Appendix.

Tables 8 and 9 show the results of the first application of our new computerized program to repellency testing. Table 8 shows the control data, and Table 9 shows the repellency data. The computerized procedure will be used for analyzing all future repellency data that we obtain.

The code words used in the program and their meanings are as follows:

Control: untreated control animals

AO and YO series: compounds submitted for repellency testing by the University of Tennessee

Amylamine: $CH_3(CH_2)_4NH_2$

3BUNH2: n-tributylamine, $(C_4H_9)_3N$

sec-Butylamine: $CH_3CH_2(CH_3)CHNH_2$

CH3(3)PENT(OH)2: 2,2,4-trimethylpenatane-1,3-diol, $CH_2(OH)C(CH_3)_2CH(OH)CH(CH_3)CH_3$ (TMPD)

DEET: m-diethyltoluamide

GABA: gamma-aminobutyric acid

Hexylamine: $CH_3(CH_2)_5NH_2$

4NH2BUCHODEA: 4-aminobutyraldehyde diethylacetal, $NH_2(CH_2)_3CH(OC_2H_5)_2$ (Schiff-negative)

4NH2BUCHODYDWATER: 4-aminobutyraldehyde diethylacetal hydrolyzed with reflux in water (Schiff-negative)

4NHBUCHO(HYDRAC): 4-aminobutyraldehyde diethylacetal hydrolyzed in the cold in acetone (Schiff-positive)

4NH2BUCHO(HYDRACR): 5-hr retest of 4-aminobutyraldehyde diethylacetal hydrolyzed in the cold in acetone

4NH2BU(OH): 4-amino-1-butanol, $NH_2(CH_2)_4(OH)$

2NH2-1-BU(OH): 2-amino-1-butanol, $CH_2(OH)C(H)(NH_2)CH_2CH_3$

NH4(OH): ammonium hydroxide

In the computer program, the compound name is entered twice: once at the beginning of the tests of a compound at a stated concentration, and once at the end. The entry at the beginning introduces the compound, and the entry at the end indicates that the averages of the percent engorged ($\bar{E}$) and the percent time displaced ($\bar{P}$) are being added to give the repellency index ($\bar{P} + \bar{E}$).

Table 8

CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
CONTROL					
	-0.00000	18.42	98.12	116.55	
	-0.00000	63.89	98.33	162.22	
	-0.00000	20.00	68.22	88.22	
	-0.00000	11.11	78.17	89.28	
	-0.00000	24.00	89.33	113.33	
	-0.00000	8.33	62.39	70.72	
	-0.00000	16.13	46.61	62.74	
	-0.00000	8.57	35.67	44.24	
	-0.00000	37.50	73.39	110.89	
	-0.00000	26.83	64.22	91.05	
	-0.00000	39.53	93.78	133.31	
	-0.00000	18.42	51.61	70.03	
	-0.00000	22.58	51.11	73.69	
	-0.00000	50.00	97.17	147.17	
	-0.00000	69.23	75.06	144.29	
	-0.00000	41.18	89.33	130.51	
	-0.00000	26.47	35.50	61.97	
	-0.00000	75.00	91.67	166.67	
	-0.00000	82.14	66.56	148.70	
	-0.00000	81.58	82.22	163.80	
	-0.00000	57.14	88.28	145.42	
	-0.00000	59.09	52.22	111.31	
	-0.00000	12.77	37.50	50.27	
	-0.00000	29.17	92.83	122.00	
	-0.00000	14.58	61.67	76.25	
	-0.00000	9.30	76.94	86.25	
	-0.00000	11.90	52.67	64.57	
	-0.00000	40.43	98.33	138.76	
	-0.00000	13.04	27.67	40.71	
	-0.00000	23.68	78.61	102.30	
	-0.00000	32.61	87.53	120.14	
	-0.00000	70.21	96.54	166.76	
	-0.00000	63.83	98.33	162.16	
	-0.00000	50.00	70.56	120.56	
	-0.00000	56.82	39.44	96.26	
	-0.00000	40.00	93.33	133.33	
	-0.00000	24.53	83.33	107.86	
	-0.00000	11.90	22.22	34.13	
	-0.00000	37.50	71.11	108.61	
	-0.00000	41.30	95.00	136.30	
	-0.00000	17.39	62.22	79.61	
CONTROL	-0.00000	35.56	71.58	107.14	N.S.
		22.05	22.21	37.70	

Table 9

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
A003	75.00000	2.17	21.33	23.51	
A003	75.00000	2.17 -0.00	21.33 -0.00	23.51 -0.00	95
A013	5.00000	20.69	62.72	83.41	
A013	5.00000	20.69 -0.00	62.72 -0.00	83.41 -0.00	70
A013	10.00000	14.29	46.94	61.23	
A013	10.00000	14.29 -0.00	46.94 -0.00	61.23 -0.00	90
A013	50.00000	2.33	10.56	12.88	
	50.00000	16.67	21.17	37.83	
	50.00000	24.14	51.11	75.25	
	50.00000	0.00	0.00	0.00	
	50.00000	0.00	0.00	0.00	
A013	50.00000	8.63 11.11	16.57 21.21	25.19 31.96	99.9
A013(3.5HR RETST)	50.00000	9.30	52.94	62.25	
A013(3.5HR RETST)	50.00000	9.30 -0.00	52.94 -0.00	62.25 -0.00	97.5

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
A013(5.5HRRETEST)					
	50.00000	67.65	88.72	156.37	
A013(5.5HRRETEST)	50.00000	67.65	88.72	156.37	N.S.
		-0.00	-0.00	-0.00	
A013 (6HR RETEST)					
	50.00000	54.05	91.39	145.44	
A013 (6HR RETEST)	50.00000	54.05	91.39	145.44	N.S.
		-0.00	-0.00	-0.00	
A014					
	72.00000	17.50	31.18	48.68	
A014	72.00000	17.50	31.18	48.68	95
		-0.00	-0.00	-0.00	
A014					
	75.00000	2.70	10.11	12.81	
	75.00000	5.71	20.50	26.21	
	75.00000	47.37	94.06	141.42	
	75.00000	45.00	94.67	139.67	
	75.00000	2.00	7.89	9.89	
A014	75.00000	20.56	45.44	66.00	99.9
		23.45	44.91	68.33	
A016					
	30.00000	12.50	96.50	109.00	
A016	30.00000	12.50	96.50	109.00	N.S.
		-0.00	-0.00	-0.00	

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
A016					
	75.00000	0.00	0.00	0.00	
	75.00000	0.00	0.00	0.00	
A016	75.00000	0.00	0.00	0.00	99.9
		-0.00	-0.00	-0.00	
A016(5 HR RETEST)					
	75.00000	25.71	39.72	65.44	
	75.00000	17.07	71.06	88.13	
A016(5 HR RETEST)	75.00000	21.39	55.39	76.78	70
		6.11	22.16	16.05	
A018					
	75.00000	17.14	52.72	69.87	
	75.00000	0.00	1.44	1.44	
A018	75.00000	8.57	27.08	35.65	99
		12.12	36.26	48.38	
A028					
	75.00000	3.33	7.78	11.11	
	75.00000	41.18	96.67	137.84	
A028	75.00000	22.25	52.22	74.48	99
		26.76	62.85	89.61	
A028(2 HR RETEST)					
	75.00000	16.67	78.28	94.94	
A028(2 HR RETEST)	75.00000	16.67	78.28	94.94	90
		-0.00	-0.00	-0.00	

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
A029					
	75.00000	6.25	61.96	68.21	
	75.00000	20.00	59.57	79.57	
A029	75.00000	13.12	60.76	73.89	99
		9.72	1.69	8.03	
Y001					
	75.00000	11.11	22.78	33.89	
	75.00000	57.45	89.80	147.25	
Y001	75.00000	34.28	56.29	90.57	95
		32.76	47.39	80.16	
AMYLAMINE					
	1.00000	9.76	57.22	66.98	
AMYLAMINE	1.00000	9.76	57.22	66.98	70
		-0.00	-0.00	-0.00	
3BUNH2					
	1.00000	2.00	17.78	19.78	
	1.00000	0.00	0.00	0.00	
	1.00000	0.00	7.22	7.22	
3BUNH2	1.00000	0.67	8.33	9.00	99.9
		1.15	8.94	10.01	
SEC-BUTYLAMINE					
	1.00000	4.17	32.22	36.39	
SEC-BUTYLAMINE	1.00000	4.17	32.22	36.39	95
		-0.00	-0.00	-0.00	

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
CH3(3)PENT(OH)2					
	1.00000	0.00	1.33	1.33	
CH3(3)PENT(OH)2	1.00000	0.00	1.33	1.33	99.9
		-0.00	-0.00	-0.00	
CH3(3)PENT(OH)2					
	0.50000	0.00	0.00	0.00	
	0.50000	46.34	88.61	134.95	
CH3(3)PENT(OH)2	0.50000	23.17	44.31	67.48	99.9
		32.77	62.66	95.43	
CH3(3)PENT(OH)2					
	0.10000	9.30	49.06	58.36	
	0.10000	34.88	77.61	112.49	
CH3(3)PENT(OH)2	0.10000	22.09	63.33	85.43	99.5
		18.09	20.19	38.28	
DEET					
	1.00000	2.50	13.33	15.83	
DEET	1.00000	2.50	13.33	15.83	99.9
		-0.00	-0.00	-0.00	
DEET(5 HR RETEST)					
	1.00000	67.50	96.43	163.93	
DEET(5 HR RETEST)	1.00000	67.50	96.43	163.93	N.S.
		-0.00	-0.00	-0.00	

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
GABA					
	10.00000	29.63	100.00	129.63	
GABA	10.00000	29.63	100.00	129.63	N.S.
		-0.00	-0.00	-0.00	
GABA					
	1.00000	62.86	100.00	162.86	
GABA	1.00000	62.86	100.00	162.86	N.S.
		-0.00	-0.00	-0.00	
HEXYLAMINE					
	1.00000	7.69	79.46	87.15	
HEXYLAMINE	1.00000	7.69	79.46	87.15	N.S.
		-0.00	-0.00	-0.00	
4NH2BUCHODEA					
	0.10000	39.47	87.94	127.42	
4NH2BUCHODEA	0.10000	39.47	87.94	127.42	N.S.
		-0.00	-0.00	-0.00	
4NH2BUCHODEA					
	1.00000	12.50	51.94	64.44	
	1.00000	0.00	0.00	0.00	
	1.00000	22.00	90.83	112.83	
	1.00000	2.08	13.33	15.42	
	1.00000	0.00	0.00	0.00	
4NH2BUCHODEA	1.00000	7.32	31.22	38.54	99.5
		9.71	39.54	49.23	

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)	
4NH2BUCHOUEA						4
	10.00000	0.00	0.00	0.00		
	10.00000	0.00	0.00	0.00		
	10.00000	0.00	0.00	0.00		4
	10.00000	0.00	0.00	0.00		
4NH2BUCHOUEA	10.00000	0.00	0.00	0.00	99.5	
		-0.00	-0.00	-0.00		4
4NH2BUCHOHYDWATER						4
	0.01000	6.16	33.33	41.50		
4NH2BUCHOHYDWATER	0.01000	8.16	33.33	41.50	70	
		-0.00	-0.00	-0.00		4
4NH2BUCHOHYDWATER						4
	0.10000	6.00	35.83	41.83		
4NH2BUCHOHYDWATER	0.10000	6.00	35.83	41.83	70	
		-0.00	-0.00	-0.00		4
4NH2BUCHOHYDWATER						4
	1.00000	0.00	0.00	0.00		
	1.00000	27.50	58.67	86.17		
4NH2BUCHOHYDWATER	1.00000	13.75	29.33	43.08	70	4
		19.45	41.46	60.93		
4NH2BUCHO(HYDRAC)						
	1.00000	0.00	0.00	0.00		
	1.00000	0.00	0.00	0.00		4
4NH2BUCHO(HYDRAC)	1.00000	0.00	0.00	0.00	99.9	
		-0.00	-0.00	-0.00		

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/50.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
4NH2BUCHO(HYDRAC)	0.10000	0.00	0.00	0.00	
4NH2BUCHO(HYDRAC)	0.10000	0.00 -0.00	0.00 -0.00	0.00 -0.00	99.5
4NH2BUCHO(HYDRAC)	0.01000	4.26	30.28	34.53	
4NH2BUCHO(HYDRAC)	0.01000	4.26 -0.00	30.28 -0.00	34.53 -0.00	97.5
4NH2BUCHO(HYDRAC)	0.00100	0.00	0.00	0.00	
4NH2BUCHO(HYDRAC)	0.00100	0.00 -0.00	0.00 -0.00	0.00 -0.00	99.5
4NH2BUCHO(HYDRAC)	1.00000	42.11	91.29	133.39	
4NH2BUCHO(HYDRAC)	1.00000	42.11 -0.00	91.29 -0.00	133.39 -0.00	N.S.
4NH2BU(OH)	0.10000	14.00	63.89	77.89	
	0.10000	22.45	92.78	115.23	
	0.10000	45.00	86.11	131.11	
	0.10000	26.09	69.44	95.53	
4NH2BU(OH)	0.10000	26.88 13.10	78.06 13.62	104.94 23.17	70

Table 9 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	CONFIDENCE LEVEL (PCT)
4NH2BU(OH)					
	1.00000	22.92	69.33	92.25	
	1.00000	0.00	0.00	0.00	
	1.00000	0.00	0.00	0.00	
	1.00000	4.08	17.78	21.86	
	1.00000	0.00	0.00	0.00	
	1.00000	44.90	99.44	144.34	
4NH2BU(OH)	1.00000	11.98	31.09	43.08	99.9
		18.42	42.92	61.14	
2NH2-1-BU(OH)					
	1.00000	1.96	8.89	10.85	
	1.00000	9.09	61.67	70.76	
	1.00000	0.00	0.00	0.00	
	1.00000	0.00	0.00	0.00	
2NH2-1-BU(OH)	1.00000	2.76	17.64	20.40	99.9
		4.32	29.65	33.96	
NH4(OH)					
	1.00000	38.78	78.18	116.96	
NH4(OH)	1.00000	38.78	78.18	116.96	N.S.
		-0.00	-0.00	-0.00	

Directly beneath the averages is another set of numbers. These numbers are the sample standard deviations for the test group. This gives an estimate of the spread or variation of the individual measurements. In some of our cases, we have only one measurement at a given level. In these cases the standard deviation is of course zero.

The confidence level in the extreme right column of Table 7 tells us how certain we can be that the test compound is different from the controls. The confidence level is derived by the computer by contrasting the test group with the control group; this was done previously by hand. There is one important difference, however. The program is set up so that only the controls performed on the day that the compound was tested are used in the comparisons. Previously we had used a whole block of controls from different days in the derivation of the level of confidence, since it would have been extremely laborious to do day-to-day comparisons by hand. With our computer program, however, the job is done in about 30 sec.

It is not surprising if the two methods show differences in repellency estimates. Some tests previously judged repellent at the 95% confidence level may now be judged non-repellent, and vice verse. The cause of this could be attributed to the fact that on the day these compounds were tested, the controls were for some reason biting more or less than usual. Our day-to-day contrasts remove these

differences, and the control and test groups are optimally weighed for day-to-day (minimal) variation. This is done by using the mean of the control group for each day in contrast with tests performed on that day.

For the data in Table 7, we choose 97.5% as the confidence level from which to differentiate control from test values. This seems reasonable, since testing by the daily comparison method with the computer program appears to demand a more critical decision in differentiating from controls, and the 97.5% level is well within the 95% level. Thus, borderline decisions are eliminated.

The computer program calculates 7 confidence levels: 99.9, 99.5, 97.5, 95.0, 90.0, 70.0, and NS (not significant). Any confidence level below 70.0 is denoted as NS. However, if we choose a confidence level of 99.5 as our point of rejection, a computer level of 99.0 would, for our purposes, be not significant (NS).

The confidence level calculation is a function of both the difference from control values and the number of tests in a group. Thus, if in some of our tests one test was performed on the compound and the repellency index was zero, the confidence level would be lower than if two or more tests were made on the same compound at the same level. Also, if the controls did not bite as well on that day as on another day,

the confidence level would be lower on the day that the controls did not bite as well, in spite of the fact that the test results may have been exactly the same (i.e., no biting) on both days. This is certainly a reasonable consideration from an intuitive standpoint, and statistically sound.

Table 9 shows that gamma-aminobutyraldehyde diethylacetal, a liquid, was quite repellent at a concentration of 10 mg/sq in. of skin. It also exhibited a significant repellency at a concentration of 1 mg/sq in. of skin. It was ineffective as a repellent at a concentration of 0.1 mg/sq in. of skin.

When the diethylacetal was hydrolyzed at 4°C overnight in an acetone solution containing a few drops of 0.1 N hydrochloric acid, the resulting compound, a light-yellow solution, which presumably contained free gamma-aminobutyraldehyde, was significantly repellent in preliminary trials at concentrations of 1.0, 0.1, 0.01, and 0.001 mg/sq in. of skin. The butyraldehyde is obviously a closer analogue of GABA than the acetal, since the carbonyl function is free. It is a considerably better repellent than the acetal and also exceeds the repellency of GABOH. The aldehyde is a closer analogue of GABA than GABOH, since it is a higher oxidation state.

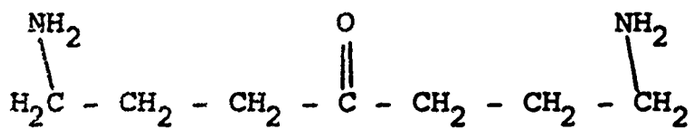
Since the actual degree of hydrolysis of the diethylacetal was uncertain under the conditions used, we attempted to hydrolyze the diethylacetal with reflex for about 3 min in water acidified with hydrochloric acid. This is the recommended method for the hydrolysis of acetals. The product of this

hydrolysis, which was dark amber, was tested for repellency and found to be totally ineffective as a mosquito repellent. The product of the hydrolysis in the acetone solution and the product obtained by reflux were then tested with Schiff reagent, which is a well-known reagent for the detection of free aldehydes. The product obtained by reflux was Schiff-negative, indicating that no free aldehyde was present.

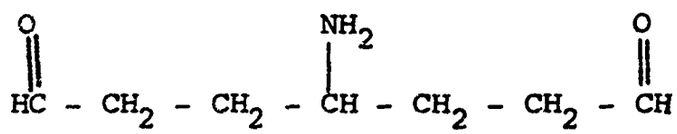
The product obtained by mild hydrolysis in acetone in the cold showed a definite Schiff-positive reaction, indicating that the free aldehyde was present in this solution. The aldehyde had therefore obviously decomposed during the reflux treatment, and the resulting product exhibited no repellency. This shows that the gamma-amino butyraldehyde is relatively unstable. Beilstein's Handbook (ref. 39) showed that the free amino-butyraldehyde is indeed an unstable substance. Since we do not know the actual degree of hydrolysis or decomposition of the acetal in the acetone solution, the repellent concentrations shown in Table 9 should be taken as high estimates. This substance may be repellent in lower concentrations than stated.

It is probably correct to conclude that GABA analogues considerably affect mosquito-biting behavior as predicted by our hypothesis. It should also be noted that the amino alcohols and amino aldehydes discussed here are soluble in water. This fact speaks for the possibility that these compounds or prototypes of them may be excreted with sweat subsequent to systemic administration.

Thus the possibility for the development of a completely new approach to the design of insect repellents is possible. For example, compounds such as



or



which are "double-edged" GABA analogues, as well as many other compounds based on the GABA structure may be designed that have the necessary physical and chemical properties to be potent and long-lasting mosquito repellents. Such compounds may also have a wide spectrum of activity toward other insects that are parasitic on warm-blooded hosts, since GABA has been found to be present in many insect species (ref. 18,19) and to play a role in the normal physiology of these insects.

It is interesting to observe the structural relationships of GABA and its analogues to repellency. GABA, as previously mentioned, is nonrepellent at the levels tested, probably because of its lack of appreciable vapor pressure. Analogues of GABA that are liquids, and have measurable vapor pressures, are definitely repellent. Thus, 4-amino-1-butanol is repellent at 1.0 mg/sq in. of skin, but loses repellency at 0.1 mg/sq in.

of skin. Table 9 shows that neither hexylamine nor sec-butylamine was repellent at a confidence level of 97.5. This indicates that an amino group alone is insufficient for repellency to be exhibited.

We next examined the effects of distance between the amino and the hydroxyl groups. Table 9 shows that 2-amino-1-butanol is repellent. We can conclude that repellency is retained, regardless of the distances between the amino and the hydroxyl groups up to at least 4 carbons. We have not yet investigated the effects of longer chain lengths between amino and hydroxyl groups, but will do so in future work.

These preliminary results are very encouraging in terms of our hypothesis. If the CO_2-bound GABA is causing activation and host recognition in the mosquito the volatilization of GABA-like substances in the vicinity of a host may cause deactivation, and the insect may no longer recognize its potential host. Table 9 also shows some assays of DEET and TMPD, Ch3(3)PENT(OH)2. TMPD has recently been found to exhibit repellency on the order of magnitude approaching that of DEET (ref. 40), and in our tests it indeed seems to show significant repellency at the levels tested.

We are justified in asking at this point: what structural similarities can be found among all these repellents? Although we can see the similarities between the structures of GABA and the amino-alcohols and aldehydes, there are no obvious analogies between the structures of DEET, TMPD, and GABA.

We are exploring these questions and have reason to believe that there are indeed certain similarities among all of these substances. Further discussion of these points will be resumed in our next quarterly report.

During our repellency testing, we also assayed a series of compounds submitted by the University of Tennessee. The results are shown in Table 9. In the AO and YO series of compounds, AO13 was found to be effective as a repellent at 50 mg/sq in. of skin and it retained its repellency for 3.5 hr after application. After 5.5 and 6 hr, the repellency of AO13 was diminished to a nonsignificant level. AO14 was repellent at 75 mg/sq in. of skin, as was AO16, but AO16 was not repellent at 30 mg/sq in. of skin. AO16 also lost its repellency 5 hr after application at the 75 mg/sq in. level.

AO18 was significantly repellent at 75 mg/sq in. of skin, as was AO28, but AO28 lost its repellency at this level after 2 hr.

AO29 was repellent at a concentration of 75 mg/sq in. of skin, and YO01 was not repellent at the 75 mg/sq in. of skin concentration at the 97.5% confidence level. YO01 is pure griseofulvin. The chemical constitution of the other compounds will be supplied by the University of Tennessee.

IV. DISCUSSION AND FUTURE WORK

On the basis of the hypothesis proposed in Section II, it was predicted that certain chemicals analogues of GABA would be repellent toward mosquitoes. These predictions have thus far proven correct, and a rational basis for the design of tailor-made repellents can thus be established. Although some of these substances in their present form may not be suitable for use as repellents because of a lack of chemical stability or possible tissue-irritating properties, they can serve as prototypes. Certain modifications of the basic design can be made so that undesirable properties will be removed and desirable properties retained. This involves a synthetic program that is essential for defining optimal structures. We believe at this point that we can be very specific in our demands upon the chemical constitution of a candidate repellent and that we have achieved a degree of specificity that allows for testing of compounds on a rational chemical and physiological basis.

In terms of the development of an orally effective insect repellent, we may be able to mobilize the resources of our body to synthesize the desired products. For example, if we feed an individual a compound that has an amino group or substituted amino group on the last carbon atom of an odd-numbered fatty acid, the well-known beta oxidation of fatty acids will stop

at the 3-carbon amino-carboxyl moiety. In the course of the degradation, amino alcohols, ketones, and aldehydes will be formed. All the amino alcohols and aldehydes that we have tested have considerable repellency and water solubility. The possibility arises therefore that some of the intermediate products of the degradation may arrive at the skin and be excreted with sweat. During the degradation there will be a number of points at which active compounds may be excreted, depending upon the length of the carbon chain.

Another possibility is the incorporation of omega-amino acids into glycerol esters. These should be analogous to fatty-acid triglycerids, which are often deposited in subdermal layers for insulation and storage. The possibility exists that the slow breakdown of these triglycerids may afford protection against mosquito bites.

There are many avenues of exploration, and many approaches that are now available. In future work, we intend to pursue these approaches and continue along the general lines of investigation described in this report.

V. SUMMARY

A hypothesis that could explain the mechanism of attraction of mosquitoes to mammals was proposed. The hypothesis involves the reversible binding of CO_2 to GABA, a substance that mediates synaptic inhibition in many animal species. Although GABA is a known inhibitor of nerve transmission, it is proposed that a GABA-CO_2 complex is not.

A literature survey was made, and the experimental results of other investigators were reinterpreted in terms of our hypothesis. No inconsistencies have been noted yet.

Experiments showed that GABA does indeed bind CO_2 and that the binding is reversed by heating. The reversal of binding was shown to occur in a physiological temperature range. Water vapor was shown to play an important role in the binding of CO_2 to the amino group of GABA.

Preliminary assays for GABA and GABA-CO_2 complexes in the crayfish intestine proved negative, but the intestine preparation may not have been adequate to show the effects of GABA-CO_2 complexes.

Amino acid analysis showed that GABA was present in aqueous extracts of mosquitoes. Tests of repellents based on our hypothesis have confirmed our predictions concerning the repellent efficacy of certain structures. A specific approach to the design of repellent chemicals with desired properties has been proposed.

A repellency assay system was devised utilizing the electronic method. An index of mosquito repellency, which is the sum of percent of mosquitoes engorged plus percent of biting time during a 30-min exposure period, was derived by using discriminant function analysis. These multiple measurements for tests using minimally treated host mice and untreated controls were used to derive a function to give best separation between the two groups. Hence a simple index consisting of the sum of these two variables is proposed as an indicator of repellency. The resulting variance of the index is calculated, and the confidence interval for significant variations from the control group is given as a function of the number of independent trials. Day-to-day variations in mosquito biting were found to be significant, and a day adjustment that compares controls and test data on a day-to-day basis has been incorporated into a computer program.

PUBLICATIONS RESULTING FROM CONTRACT

November 1, 1965, through October 31, 1966

1. Kashin, P. "The Electronic Recording of the Mosquito Bite," oral presentation, Annual Meeting of the American Mosquito Control Association, Atlanta, Ga., March 6-9, 1966.

2. Kashin, P., "Electronic Recording of the Mosquito Bite," J. Insect Physiol. 12, 281, 1966.

Others presently in preparation.

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APPENDIX

STATISTICAL FORMULAE

If day-to-day variation were not significant, the estimate of the mean of the control for each day could be the overall mean on the pooled control data. The first set of data were computed under this assumption. Since our analysis of variance has shown that day-to-day variation is significant, it must be taken into account. The following formulae are required.

To calculate the mean and variance for any test group taking into account day effects:

Xij (T) is the jth test observation on the ith day.

Xij (C) is the jth control observation on the ith day.

Ni is the number of test on day i.

Mi is the number of controls on day i.

The means for day i are:

$$\sum_{j=1}^{Ni} Xij\ (T)/Ni = \bar{X}i\cdot\ (T) \text{ for the test group,}$$

and

$$\sum_{j=1}^{Mi} Xij\ (C)/Mi = \bar{X}i\cdot\ (C) \text{ for the control group.}$$

The contrast for day i is:

$$\sum_{j=1}^{Mi} Xjk\ (C)/Mi - \sum_{j=1}^{Ni} Xjk\ (T)/Ni = \bar{X}i\ (C) - \bar{X}i\ (T).$$

The variance of the contrast for day i $= \sigma^2 \frac{1}{Mi} + \frac{1}{Ni}$.

Weights may be chosen to minimize variance in the weighted average.

The weight for the contrast for day i $= Wi = 1/\ \frac{1}{Mi} + \frac{1}{Ni}$.

The weighted average of the contrast for the test group, K(T) is:

$$K(T) = \frac{\sum_i Wi\ \left[Xi(C) - Xi(T)\right]}{\sum_i Wi} = \frac{\sum_i Wi\ Xi(C)}{\sum_i Wi} - \frac{\sum_i Wi\ Xi(T)}{\sum_i Wi}$$

The variance of the contrast for the test group, K(T) is:

$$\text{var } K(T) = \frac{\sigma^2}{\sum_{i=1} Wi}$$

If we substitute S^2, the estimate of variance from the analysis of variance on the control data for σ^2, we can test the statistical significance of the contrast K(T). The number of degrees of freedom in the analysis of variance is f.

The confidence level is 1 - P, where P is the probability level.

The t test with f degrees of freedom is t_P (f), and 1 - P confidence limits.

If $\frac{K(T)}{\sqrt{\text{Var } K(T)}} > t_p\ (f)$, then the test group T is significantly different from the control group.