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Report No. IITRI-L6021-16

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Annual Progress Report

Philip Kashin

November 1968

Supported by

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

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

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IIT RESEARCH INSTITUTE
10 West 35th Street
Chicago, Illinois 60616

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ABSTRACT

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

The dual objectives of this program are to develop insect repellents for topical application that are superior to and longer lasting than those available today and to develop insect repellents that are effective when administered orally.

During this year the evaluation of compounds for mosquito repellency by the electronic recording method was continued, and the results were statistically analyzed in a digital computer program. A new electronic "bitometer" that facilitates the compilation of laboratory repellency data was utilized in these tests. Certain modifications were incorporated into the instrument to make the test data more precise.

Work was continued on a hypothesis -- the GABA hypothesis -- developed during the course of this work that could explain the physicochemical basis of a mosquito's attraction to warm-blooded hosts. Gamma-aminobutyric acid (GABA), a substance known to cause inhibition in the transmission of nervous impulses across certain synaptic structures, was found in mosquitoes. It was hypothesized that the interactions of GABA with carbon dioxide, heat, and water vapor form the basis of mosquitoes attraction to their hosts. Evidence supporting the hypothesis was obtained from chemical studies of the interactions of GABA with carbon dioxide, correct predictions of chemical structures that on the basis of the hypothesis should repel mosquitoes, and direct in vivo physiological investigations.

FOREWORD

This is Report No. IITRI-L6021-16 (Annual Progress Report) on IITRI Project L6021, entitled "Development of an Orally Effective Insect Repellent." The report covers the period from November 1, 1967 through October 31, 1968.

This project is being sponsored by the U.S. Army Medical Research and Development Command, Office of the Surgeon General, Washington, D.C. 20315, under Contract No. DA-49-193-MD-2281 - and is being conducted by IIT Research Institute, Technology Center, Chicago, Illinois 60616. Previous work under this contract was conducted by IIT Research Institute from May 1, 1962 through October 31, 1967.

The project leader for this program is Mr. Philip Kashin, under the administrative supervision of Dr. E. J. Hawrylewicz. The electronic circuitry of the new mosquito "bitometer-timer" was designed by Mr. Blayne Arneson. The statistical analyses of the electronically recorded repellency test data were performed by Mr. Merl L. Kardatzke, who also devised the computer program for determining the repellency index and the statistical confidence limits for the test compounds. Helpful suggestions and discussions for the physiological phases of the work were contributed by Dr. William F. Danforth, Biology Department, Illinois Institute of Technology.

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

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

All repellency test data are recorded in IITRI Logbooks C17599 and C18467. The computer output sheets also form part of our permanent records.

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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

I. INTRODUCTION

The objectives of this program are to develop better topical insect repellents than those currently available, and if possible, to develop an insect repellent that is effective when administered orally. Such repellents should afford more uniform and longer-lasting protection from bites of predacious insects. Success in this undertaking could result in a significant reduction of human suffering from disease and discomfort caused by insect bites.

The experimental rationale and approaches followed in the work during this report period were largely established in previous years (ref. 1 to 3). The research effort this year was essentially a continuation and consolidation of these approaches.

Further experimental observations were made to test the validity of the gamma-aminobutyric acid (GABA)-carbon dioxide (CO_2) hypothesis described in previous reports (ref. 2 to 3). This hypothesis could explain the physiological mechanisms governing a mosquito's attraction to warm-blooded hosts. Recently, preliminary in vivo physiological confirmation of this hypothesis was obtained.

To obtain further information on the chemical structures that are repellent to mosquitoes, repellents that are GABA analogues were tested. The rationale previously presented (ref. 3) is constructed on a physiological basis. This work is directed toward elucidating the general electronic configurations of compounds that are repellent to mosquitoes.

An instrument -- "bitometer-timer" -- was previously described (ref. 3) that makes the electronic screening of potential mosquito repellents more efficient and accurate. The instrument essentially consists of 2 digital timers and a meter relay. One timer runs continuously throughout the test period, while the other is actuated only when an insect bites a host. A direct and immediate measure of the percentage of biting time thus becomes available for insertion into a computer program that yields statistical confidence limits for repellency. The circuitry of this instrument has been slightly modified from that previously reported (ref. 3). The modification, described herein, makes the "bitometer-timer" more sensitive and accurate.

II. GABA HYPOTHESIS

A hypothesis was presented in previous reports (ref. 2,3) that attempted to explain the neurochemical mechanism by which the interaction of moisture, warmth, and CO_2 could operate to drive a mosquito to its warm-blooded host. We proposed that gamma-aminobutyric acid (GABA), a substance that is thought to mediate synaptic inhibition in the arthropod central nervous system (ref. 4), also exists in mosquitoes; that it could combine with CO_2 in the presence of water vapor, and that the resulting GABA-CO_2 complex does not possess the synaptic inhibitory power of GABA alone. The hypothesis was supported by showing that GABA exists in aqueous dialyzable extracts of the bodies (ref. 2) and the heads (ref. 3) of mosquitoes; that GABA does indeed combine with CO_2 only in the presence of water vapor (ref. 2); and that the GABA-CO_2 complex is extremely labile to heat, dissociating almost completely into GABA and CO_2 at mammalian temperatures.

We hypothesized that the interactions in the insect of GABA with higher than normal moistures, atmospheric CO_2 concentrations, and warmth when the insect is in the vicinity of a potential host could cause a quickly-reversing interplay of activation and inhibition that could underlie the insect's host-seeking behavior (ref. 2,3). The quick coupling and uncoupling of CO_2 and GABA as the host is approached could also prevent an adaptation of the mosquito to CO_2 as the host is approached, which would hinder its host-seeking behavior.

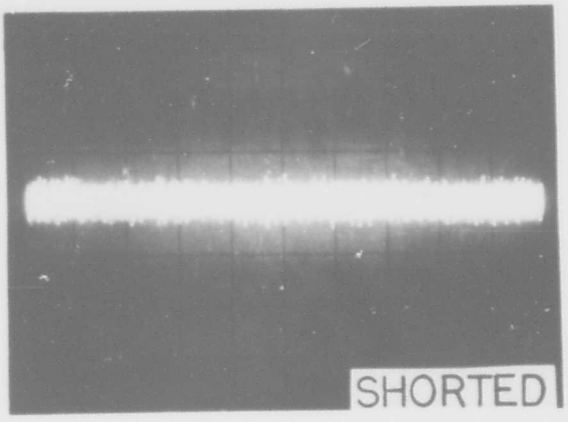
Investigations undertaken during this report period were directed toward testing the actual physiological effects of GABA and GABA-CO_2 complexes on insect nervous tissue. Extensive experimentation and research were undertaken to find a suitable physiological preparation to test these effects. Many of these preparations yielded doubtful, unclear results. Thus, the crayfish intestine was found unsuitable because it was not stimulated by glutamate (ref. 2), which as explained later is important to be able to demonstrate the effects of a GABA-CO_2 complex, and the cockroach leg-twitch experiment (ref. 3,5) was inadequate due to a lack of reproducibility and uncertainty in terms of the many variables that were unavoidably introduced from one preparation to another.

After further research and experimentation, a preparation that utilizes the isolated central nervous system of the cockroach, Periplaneta americana (L) was selected. The central nervous system of this cockroach spontaneously fires a continuous train of nervous discharges when isolated from the animal, without any external stimuli (ref. 6). The change in the system's spontaneous discharge rate under the influence of the test compounds is the parameter measured. An increase in this rate under the influence of a test compound indicates stimulation, while a decrease indicates inhibition. Both effects are largely reversed by washing the preparation with saline after respective treatments. Complications that arose in the use of this preparation were resolved, and the preparation is proving to be effective in the GABA-CO_2 study.

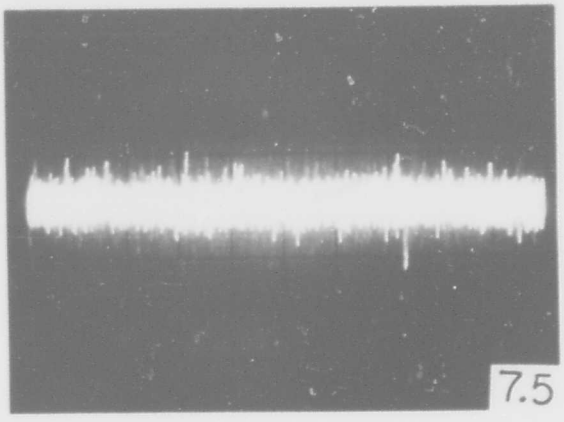
One of the difficulties encountered was the fact that lowered pH alone could cause an increased spontaneous firing rate in the preparation. Since the pH of a solution is lowered when CO_2 is bubbled into it, the effects of pH on the preparation was studied.

We found that between pH 8.5 and 5.75, the electrical discharge rate of the preparation was barely affected. However, when pH 5.5 was reached, the preparation's spontaneous firing rate increased without the presence of CO_2. This rate increased even further at lower pH. These results are shown in Figure 1a through 1e, which are photographs of the face of the cathode ray tube of the oscilloscope, showing the nerve discharges.

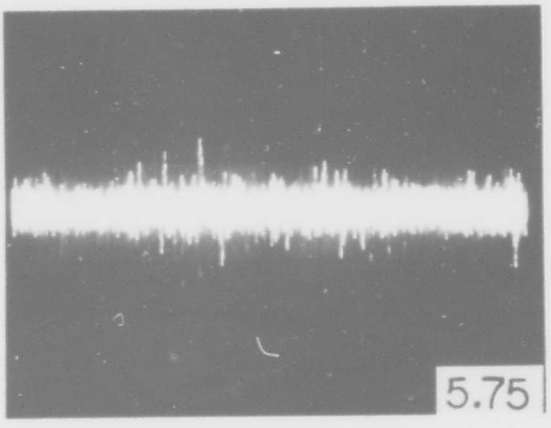
Figure 1a shows the electrodes short circuited in saline. This condition is the zero or noise level. Figure 1b shows the electrical activity of the ganglia at pH 7.5. Figure 1c shows this activity at pH 5.75. These pictures show the spontaneous discharge patterns produced by an unstimulated preparation. The pictures at pH 7.5 and 5.75 are very similar. At pH 5.5, however, (Figure 1d), a definite increase in the firing rate is observed. At pH 5.0 (Figure 1e) activity of the preparation is even further increased. All these saline solution were buffered at the respective pH's with tris(hydroxymethyl)aminomethane acetate.



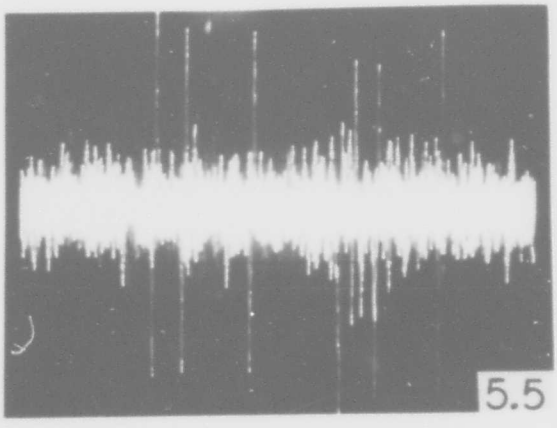
a. Electrodes short circuited (baseline).



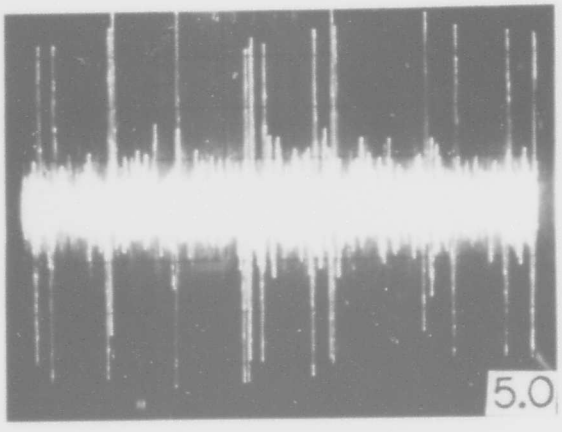
b. Electrical discharge at pH 7.5.



c. Electrical discharge at pH 5.75.



d. Electrical discharge at pH 5.5



e. Electrical discharge at pH 5.0.

Figure 1

EFFECTS OF pH ON THE SPONTANEOUS FIRING RATE
OF THE ISOLATED COCKROACH CENTRAL NERVOUS SYSTEM

Thus, acidity itself affects the firing rate, even in the absence of CO_2. However, the addition of CO_2 to saline causes the pH of the solution to decrease to values at which the rates of spontaneous firing due to pH alone are exceeded. The study of this effect is important in establishing a baseline for the stimulatory effects of CO_2 alone, without complications introduced by pH changes.

In order to overcome this difficulty, the CO_2 is bubbled into a solution containing 0.05 M sodium bicarbonate ($NaHCO_3$). Under this condition, the pH never goes below 6.0. However, since this buffer concentration is too great for the salt balance of the saline solution, the CO_2-treated buffered saline was always diluted 1:10 with nonbuffered saline to give a concentration of 0.005 M $NaHCO_3$ which is well tolerated by the cockroach preparation. In this way, it is possible to bubble CO_2 into saline alone, or into saline containing GABA, to form GABA-CO_2 complexes, without exceeding the pH limits which stimulate the preparation.

Bicarbonate was chosen over other possible buffers because, by the common ion effect, bicarbonate ion in solutions minimizes the hydration of molecular CO_2. This is advantageous, since GABA binds only molecular CO_2 (ref. 7).

Another difficulty encountered was choosing a time interval over which to count the discharges of the preparation, whether in control or in test situations. These discharges are counted using an electronic frequency counter. However, the frequency counters that are commercially available only allow for a 1-sec counting period (with 1-sec off) or a 10-sec counting period (with 1-sec off). The 1-sec counting period appeared to give erratic results from one counting interval to the next. Also, the actual counting time was only 50% of the total time over which observations were made. Though the actual counting time was over 90% of the total observation time with the 10-sec counting period, some resolution was lost in using this long counting time, and slow or fast changes in the responses of the preparation to the treatment were masked.

Therefore to resolve the counting time problem, a time-switch device was constructed and put in series with the counter. This permitted a counting interval of 5-sec with 1 sec for recycling between 5-sec counts. This counting period appears to be ideal, since actual counting proceeds for nearly 85% of the total observation time. The counting period is thus not too short, but short enough so that good resolution of the responses of the ganglia is retained. In all the experiments to be described, the 5-sec counting interval and bicarbonate-buffered saline were utilized.

The experimental setup for recording discharges from the isolated central nervous system of the cockroach is shown in Figure 2.

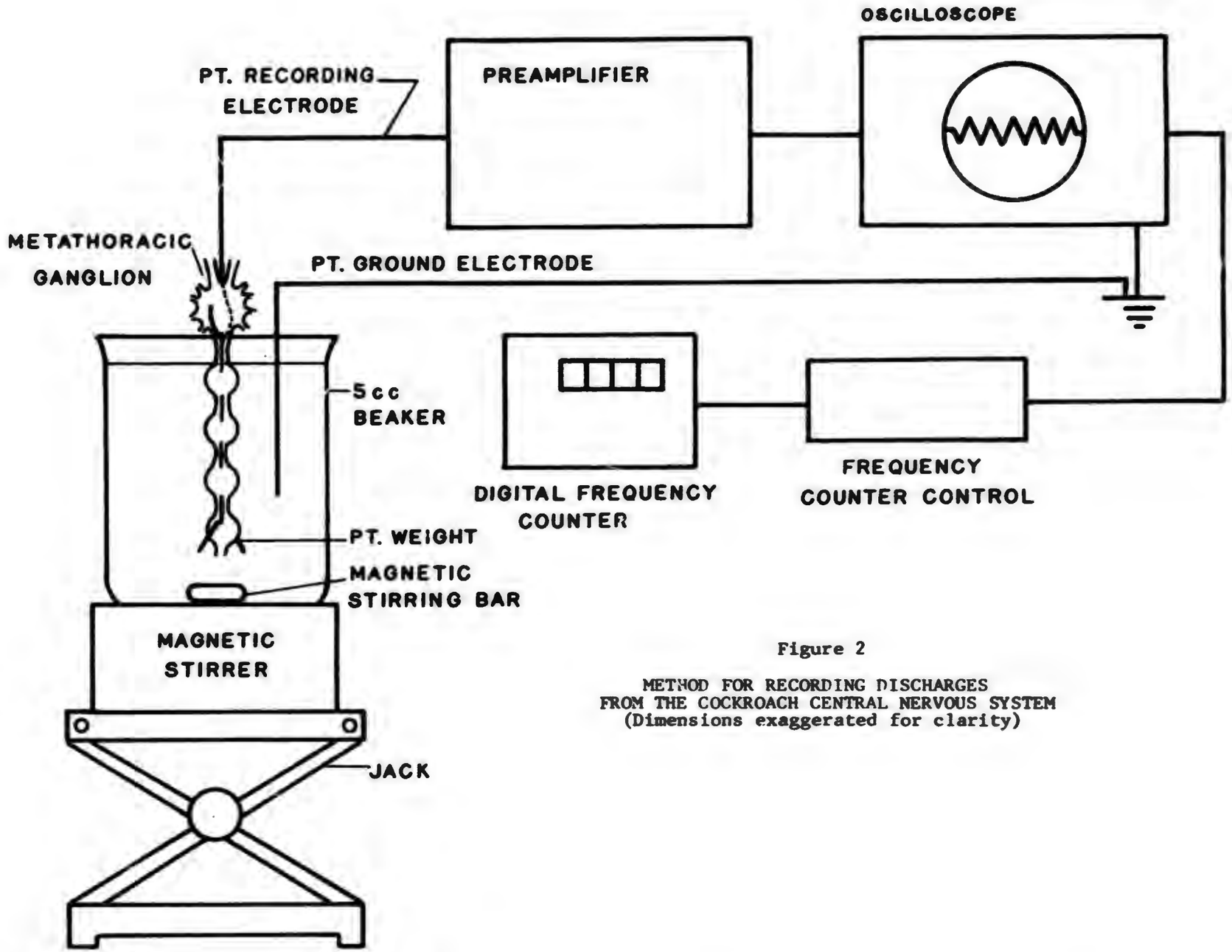


Figure 2

METHOD FOR RECORDING DISCHARGES
FROM THE COCKROACH CENTRAL NERVOUS SYSTEM
(Dimensions exaggerated for clarity)

The metathoracic ganglion (3rd thoracic ganglion) together with 4 abdominal ganglia are dissected from the insect. The metathoracic ganglion is hung from a platinum hook that is the recording electrode. Another small platinum wire serves as a weight for the bottom of the ganglia chain, so that it is kept in an extended condition. The 4 abdominal ganglia are immersed in saline in a 5-cc beaker or in the test solution together with a platinum ground electrode, and the spontaneous discharges can be observed on the oscilloscope. (The recording electrode with the 3rd thoracic ganglia does not enter the solution). The oscilloscope output is connected to the counter control previously mentioned (to give 5-sec counts) and thence to the digital frequency counter (Hewlett Packard Model 5221A).

When the solutions are tested, the supporting jack is lowered, thus leaving the cockroach preparation hanging in air. A different 5-cc beaker containing the test material is put into place. Occasionally, 0.5 cc of a test solution is put into 4.5 cc of saline to give a 1:10 dilution, as previously mentioned. In these cases, the mixture is stirred with the magnetic stirrer before the jack is raised, then the ganglia chain is replaced into the solution by raising the jack. This arrangement is simple, convenient, and quick. The preparation usually is active for many hours, and a number of experiments can be performed on a single preparation.

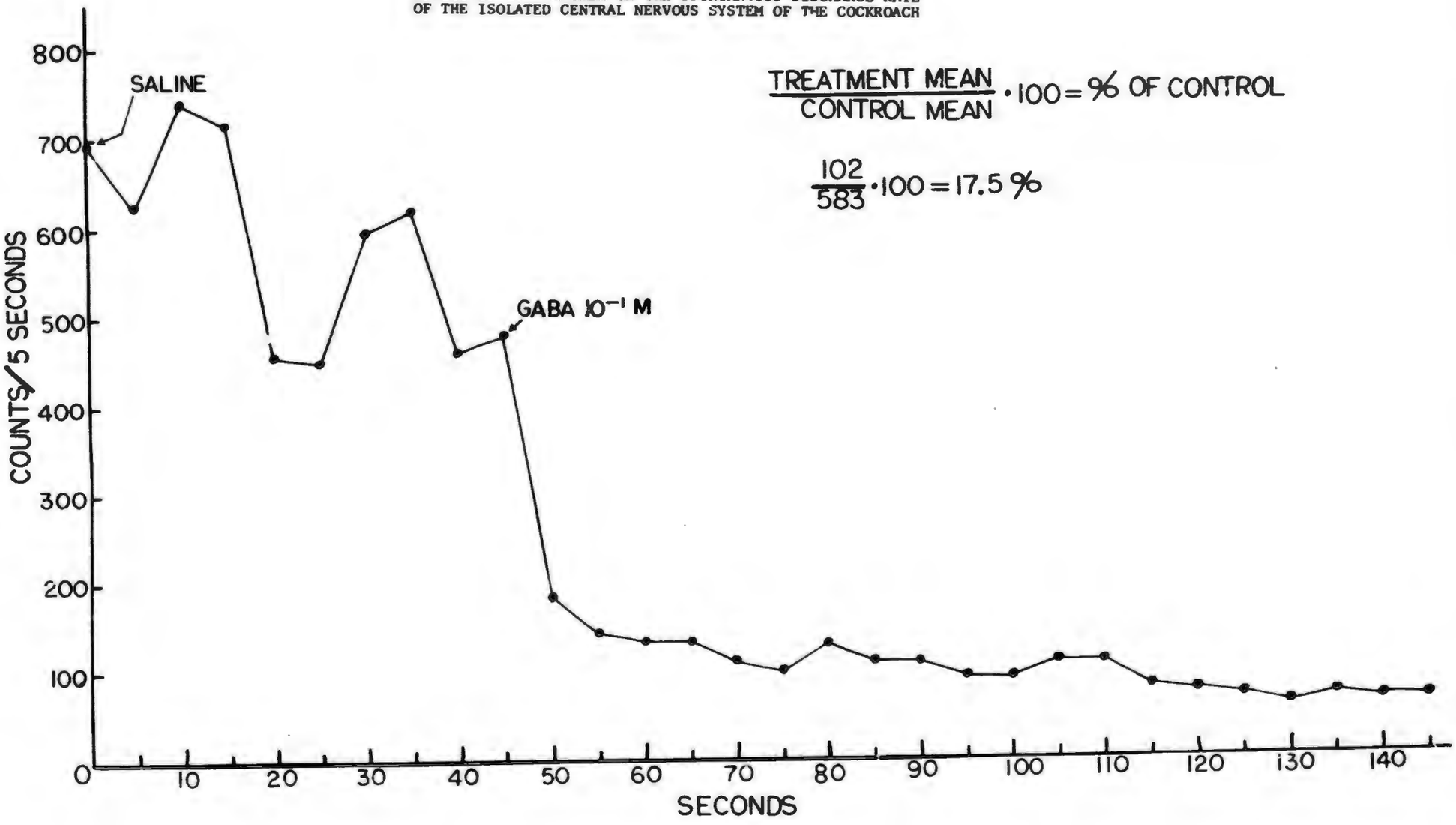
Figure 3 shows an experiment that was performed using 10^{-1} M GABA and makes apparent that GABA in this concentration greatly inhibits the spontaneous discharges of this preparation. With this GABA concentration, activity falls to only 17.5% of the control (untreated) value.

Figure 4 shows the effect of 10^{-1} M glutamate on central nerve cord of the cockroach. The stimulatory action of this substance is quite evident. Treatment with glutamate causes the spontaneous electrical discharge rate to increase to 417% of the control.

Figure 5 shows the effect of a 0.1 M GABA-CO_2 complex on the spontaneous rate of nervous discharge in this preparation. It is evident that the inhibition is considerably less, being 93% of the control value. The figure also shows that inhibition increases with time. This increase is probably due to the spontaneous release of CO_2 from the GABA-CO_2 complex, thus making more free GABA available to produce inhibition. This phenomenon is not observed with a 10-fold-lower GABA concentration (10^{-2} M GABA-CO_2). This is probably due to the relatively lower amount of free unassociated GABA available for exerting inhibitory effects (in terms of the absolute amount of free GABA available at equilibrium) at the lower concentrations.

Figure 3

EFFECT OF 10^{-1} M GABA ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH



$$\frac{\text{TREATMENT MEAN}}{\text{CONTROL MEAN}} \cdot 100 = \%\ \text{OF CONTROL}$$

$$\frac{102}{583} \cdot 100 = 17.5\ \%$$

Figure 4

EFFECT OF 10^{-1} M GLUTAMATE ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH

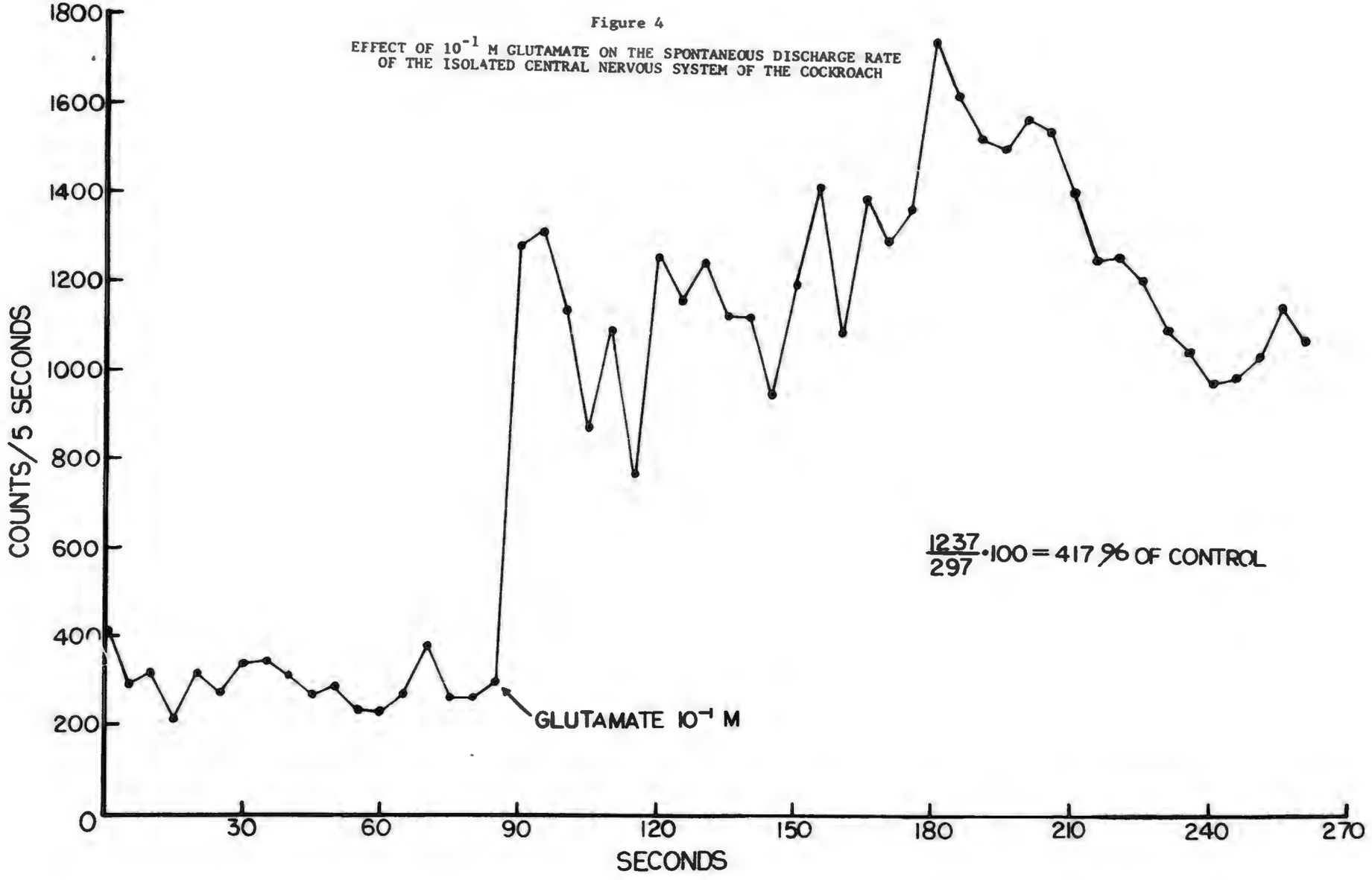


Figure 5

EFFECT OF 10^{-1} M GABA-CO_2 COMPLEX ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH

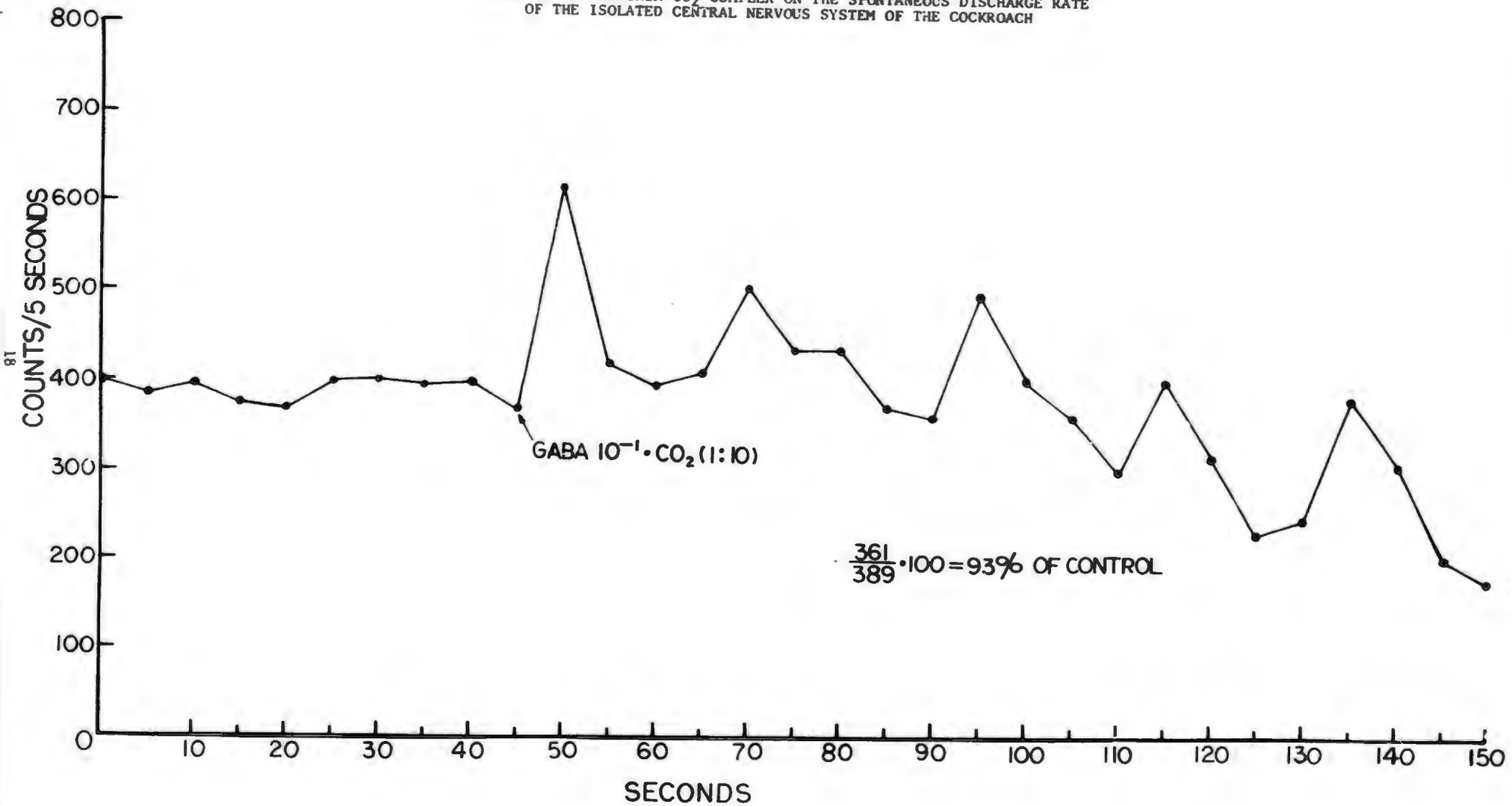


Figure 6 shows the effects of various GABA concentrations on the spontaneous discharge rate of the preparation. These preliminary experiments show that GABA may be somewhat stimulatory at concentrations between 10^{-6} and 10^{-4} M, but inhibition sets in at 10^{-3} M and higher concentrations.

Figure 7 shows the effects of various glutamate concentrations on the spontaneous discharge rate of the preparation. In this preliminary experiment stimulatory effects were not evident until a glutamate concentration of 10^{-2} M was achieved. At 10^{-1} M, glutamate stimulates to over 400% of the control values.

We previously described the steric relationships between glutamic acid, GABA-CO_2, and N-acetyl GABA (ref. 3). Since GABA-CO2 bears such a close chemical resemblance to glutamate, we theorized that in order to show that a GABA-CO_2 complex is indeed noninhibitory, or even stimulatory, we had to use a preparation that was stimulated by glutamate. Kerkert (et al, ref. 8) showed that contractions of the coxal muscle of the cockroach were stimulated by glutamate and even postulated that glutamate may be the actual excitatory transmitter in this insect. This is why we chose the isolated central nervous system of the cockroach to demonstrate the effects we were studying. The steric relationships between GABA-CO_2, N-acetyl GABA, and glutamic acid are shown in Figure 8.

It is apparent from Figure 8 that these substances closely resemble each other. On this basis we reasoned that N-acetyl GABA may also be stimulatory to the insect's central nervous system. Figure 9 shows the effects of N-acetyl GABA at various concentrations on the spontaneous discharge rate of the cockroach preparations. The activity profile of this substance in causing an increase in spontaneous discharge is very similar to that of glutamic acid, though in these preliminary results it appears to be not quite as stimulatory.

Thus a substance that is substituted in the same position in the GABA molecule (at the amino group position) as the CO_2 in the GABA-CO2 complex, is not inhibitory. This result was correctly predicted on the basis of the hypothesis.

Figure 10 shows the effects on the discharge rates of various concentrations of GABA-CO2 and CO_2 alone. These preliminary results show that a GABA-CO_2 complex at 10^{-3} M GABA is stimulatory. On the other hand, a GABA-CO_2 complex at 10^{-2} M GABA is more stimulatory than the GABA-CO_2 10^{-3} M. GABA-CO_2 10^{-1} M showed inhibition, probably for the reasons previously discussed.

Figure 6

EFFECT OF VARIOUS CONCENTRATIONS OF GABA ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH

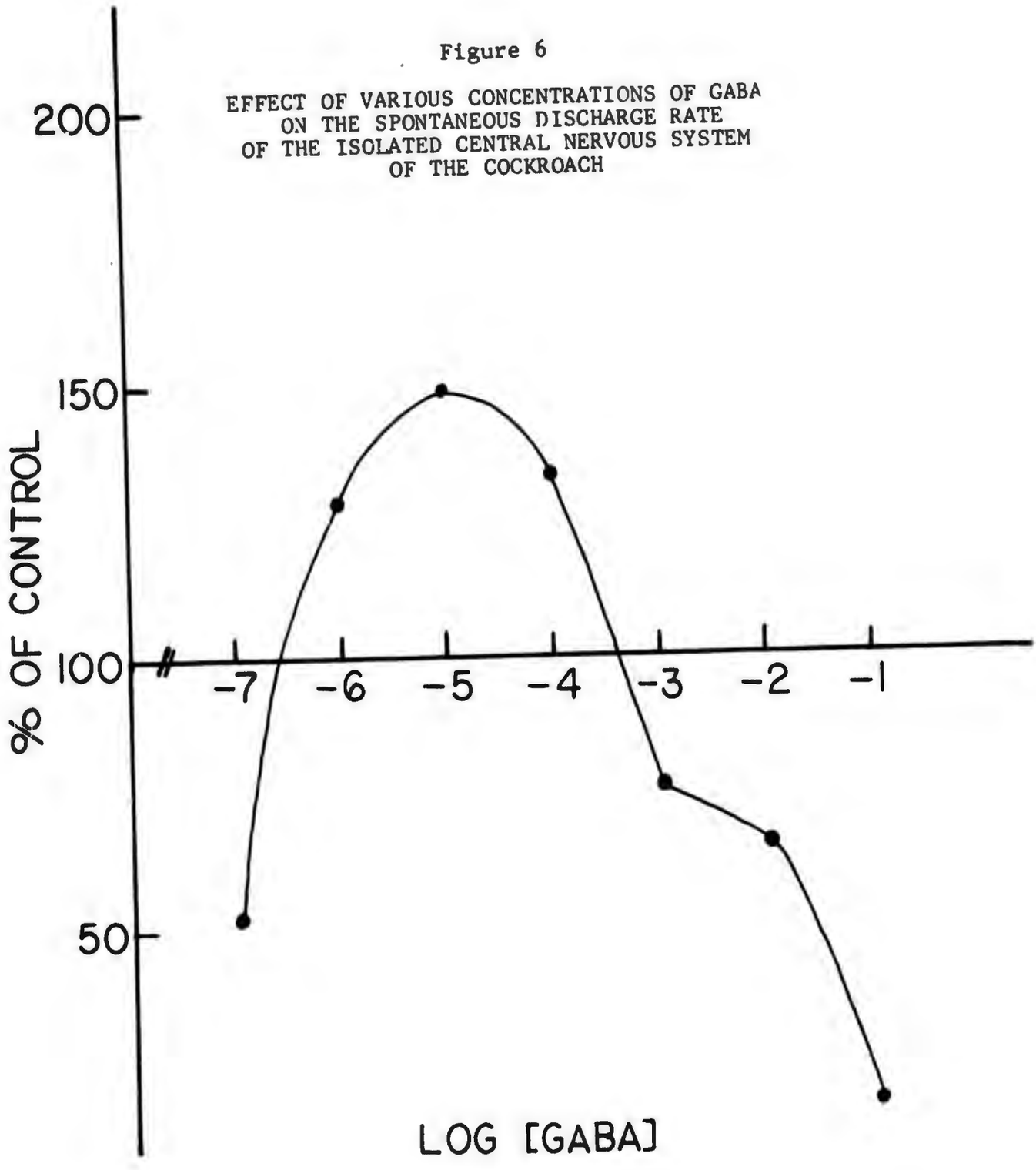
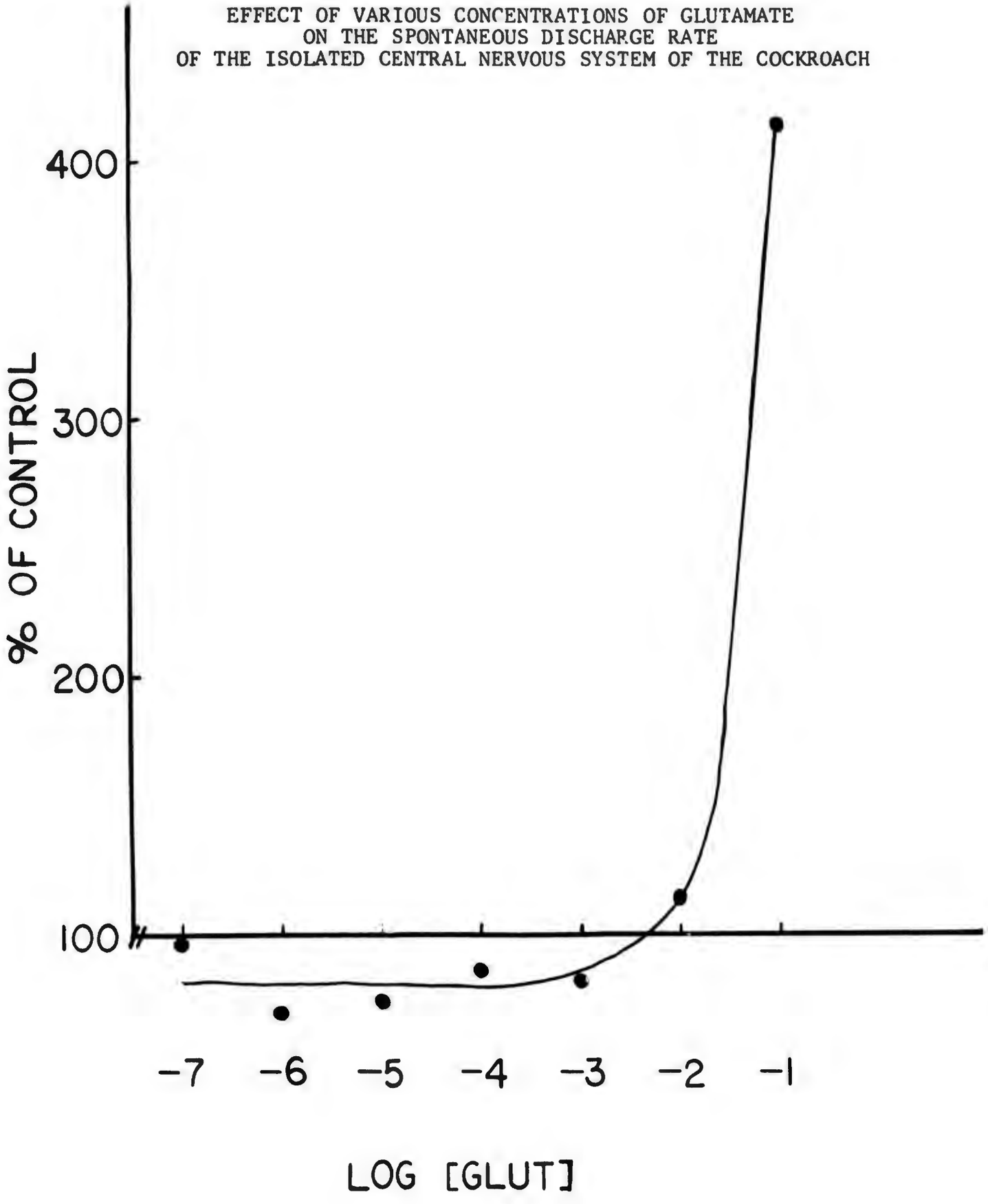


Figure 7

EFFECT OF VARIOUS CONCENTRATIONS OF GLUTAMATE
ON THE SPONTANEOUS DISCHARGE RATE
OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH



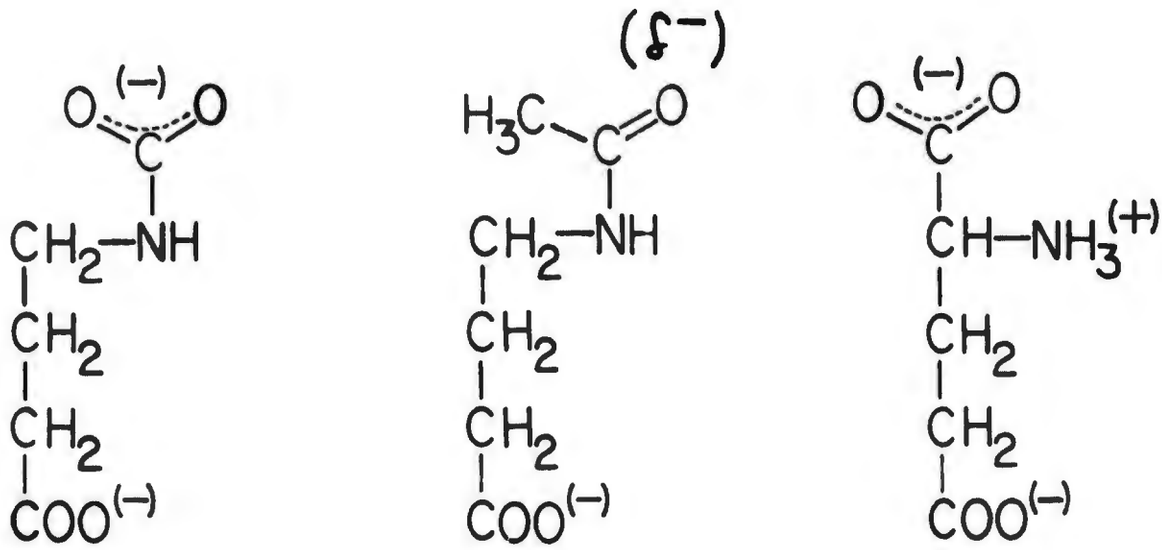


Figure 8

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

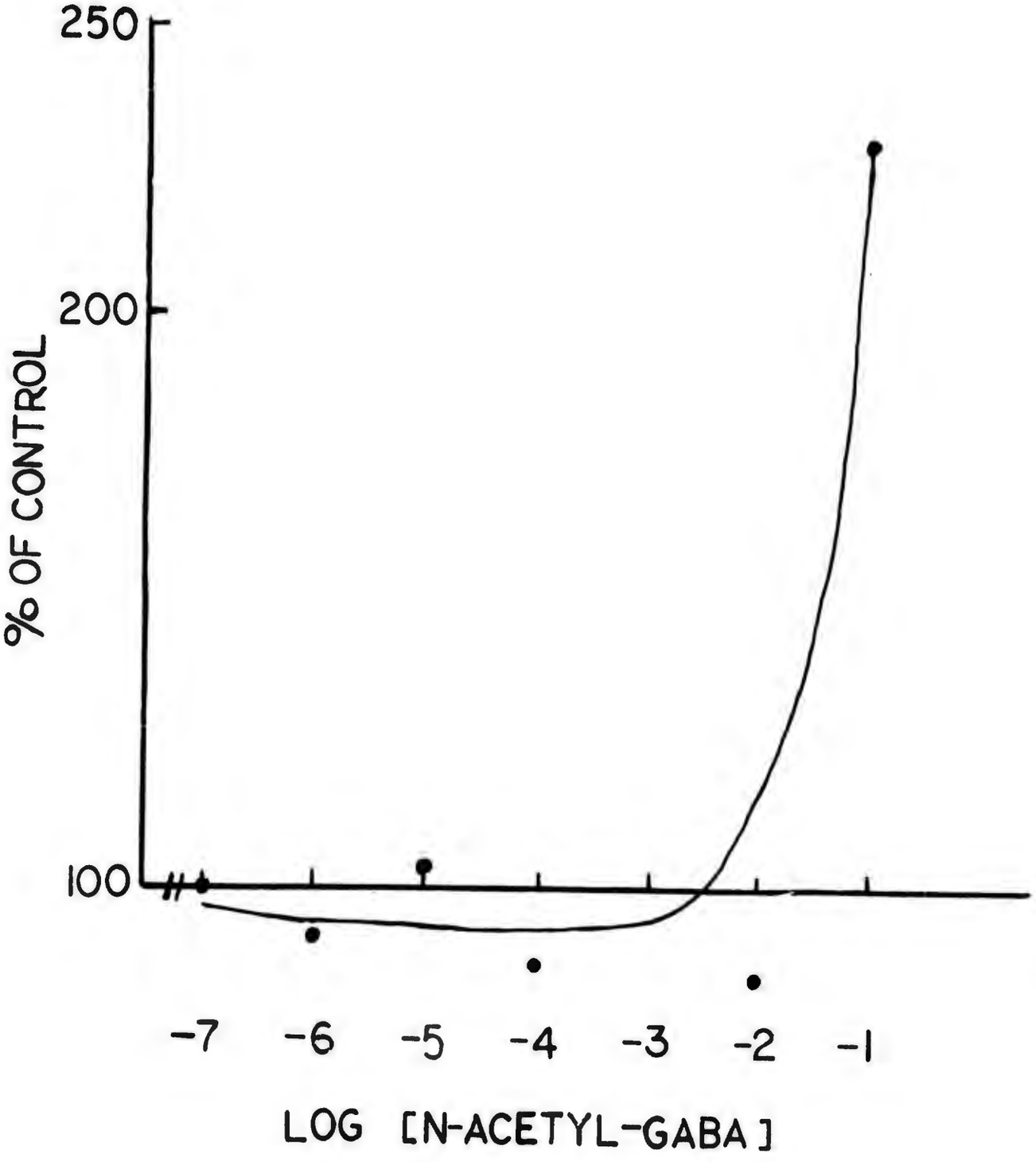


Figure 9

EFFECT OF VARIOUS CONCENTRATIONS OF N-ACETYL GABA
ON THE SPONTANEOUS DISCHARGE RATE
OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH

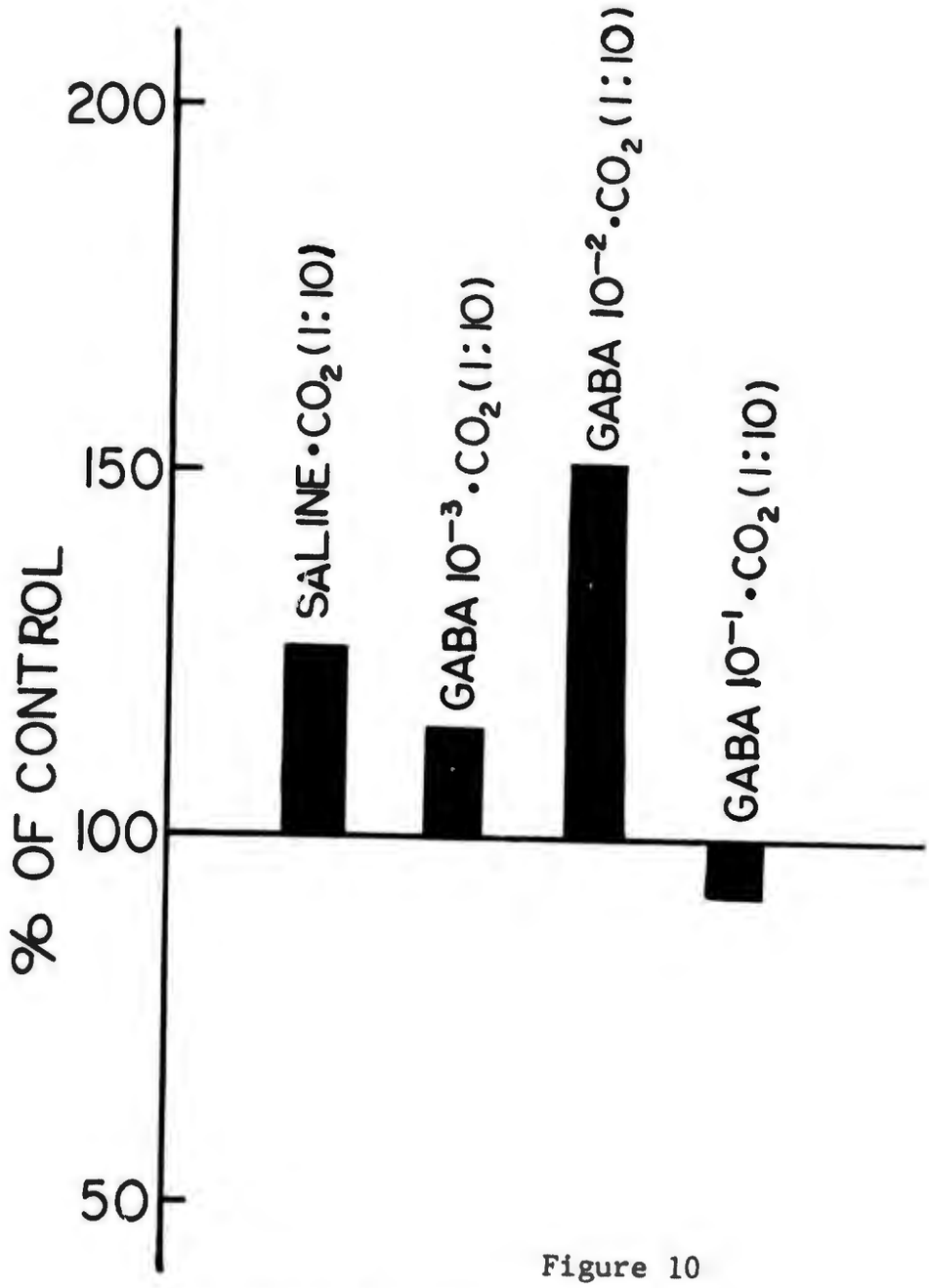


Figure 10

EFFECT OF SOME CONCENTRATIONS OF GABA-CO_2 AND CO_2 ALONE ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH

Figure 11 shows essentially these same results in another preparation. Here the inhibitory power of free GABA can be directly compared to the stimulatory power of GABA-CO_2 complexes. Again it is apparent that a concentration of free GABA (10^{-2} M) that ordinarily causes a greater inhibition than GABA-10^{-3} M, is actually more stimulatory when combined with CO_2 than a 10^{-3} M GABA solution. GABA-CO_2 10^{-1} M again shows inhibition, though it is more inhibitory in the absence of CO_2 than in its presence.

More experimentation is needed to confirm and further study these results. If these same trends continue to be observed, they will show that a GABA-CO_2 complex is excitatory. Then, a unified concept may emerge that describes the mechanisms by which water vapor, CO_2, and warmth emanating from a potential host interact with the nervous system of a mosquito to guide the insect's host-seeking and attack behavior.

III. CIRCUIT MODIFICATIONS OF NEW "BITOMETER-TIMER"

In the previous annual report (ref. 3), detailed descriptions of the circuitry and use of a new electronic "bitometer-timer" were presented. This instrument yields a direct readout on electronic digital counters for the total time of the repellency test and the total time during which mosquitoes bite a host mouse. These time parameters are important for assessing the repellency of test compounds utilizing the statistical and computerized methods developed (ref. 3).

In utilizing the "bitometer-timer," it became obvious that relay arcing was causing a problem in the circuitry. Relay arcing caused a slight but definite delay in the turnon point of the bite-time timer when biting began, and a similar delay (hysteresis) in the turnoff point of this timer when biting stopped. This difficulty was overcome with the circuitry modifications described below. The bite-time timer now switches on and off at precisely the preset desired point, and the entire apparatus is exceptionally stable for an electronic instrument of this sensitivity. No external shielding is required since AC components are internally bypassed. Only the modified circuitry is included in this report (Figure 12, 13) and should be substituted for Figures 6 and 7 (pp. 36 and 37) in our previous annual report (ref. 3).

Figure 12 shows the modified circuitry of the power supply and timer circuit.

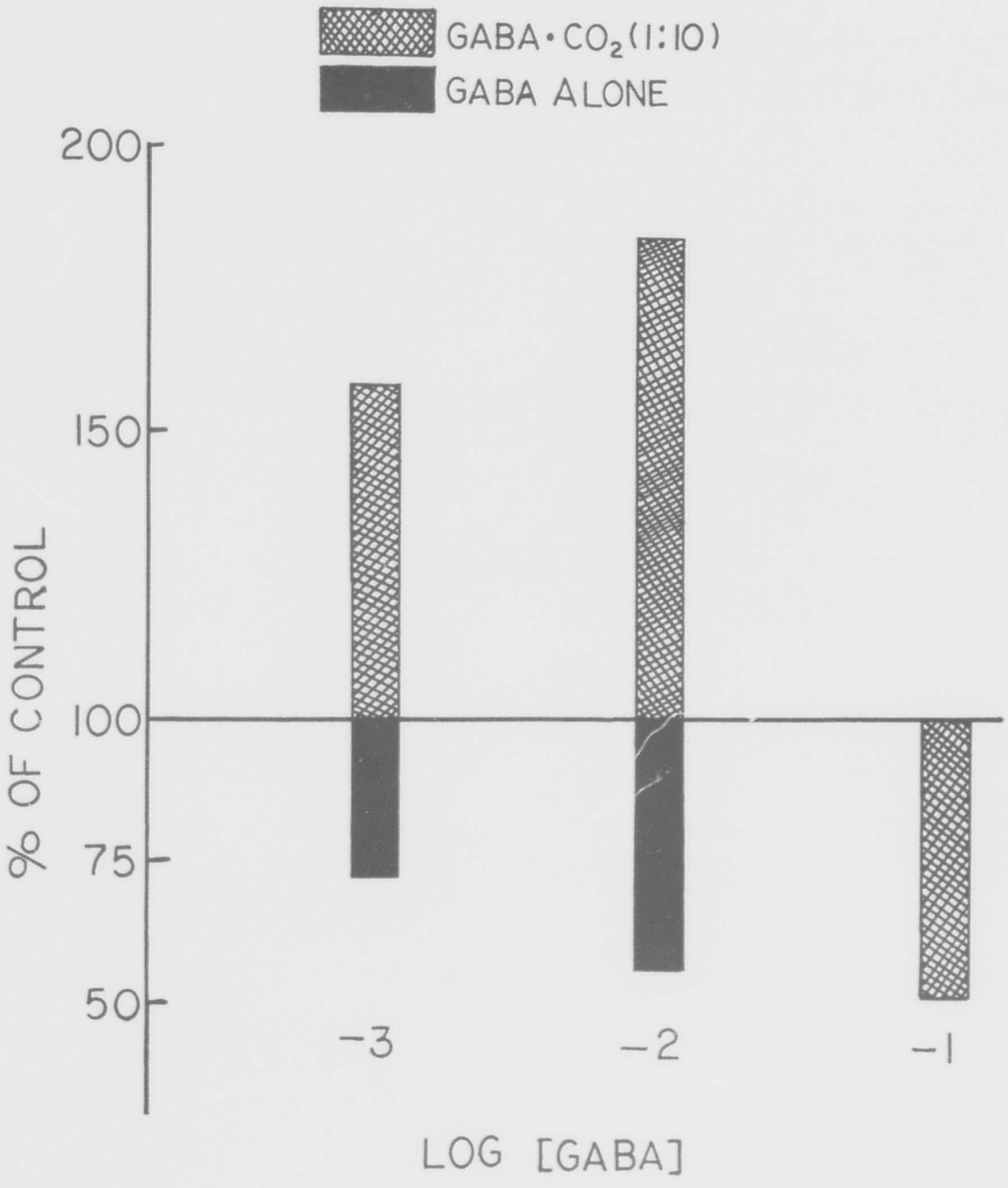


Figure 11

COMPARATIVE EFFECTS OF GABA ALONE AND CORRESPONDING GABA-CO_2 COMPLEXES ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH

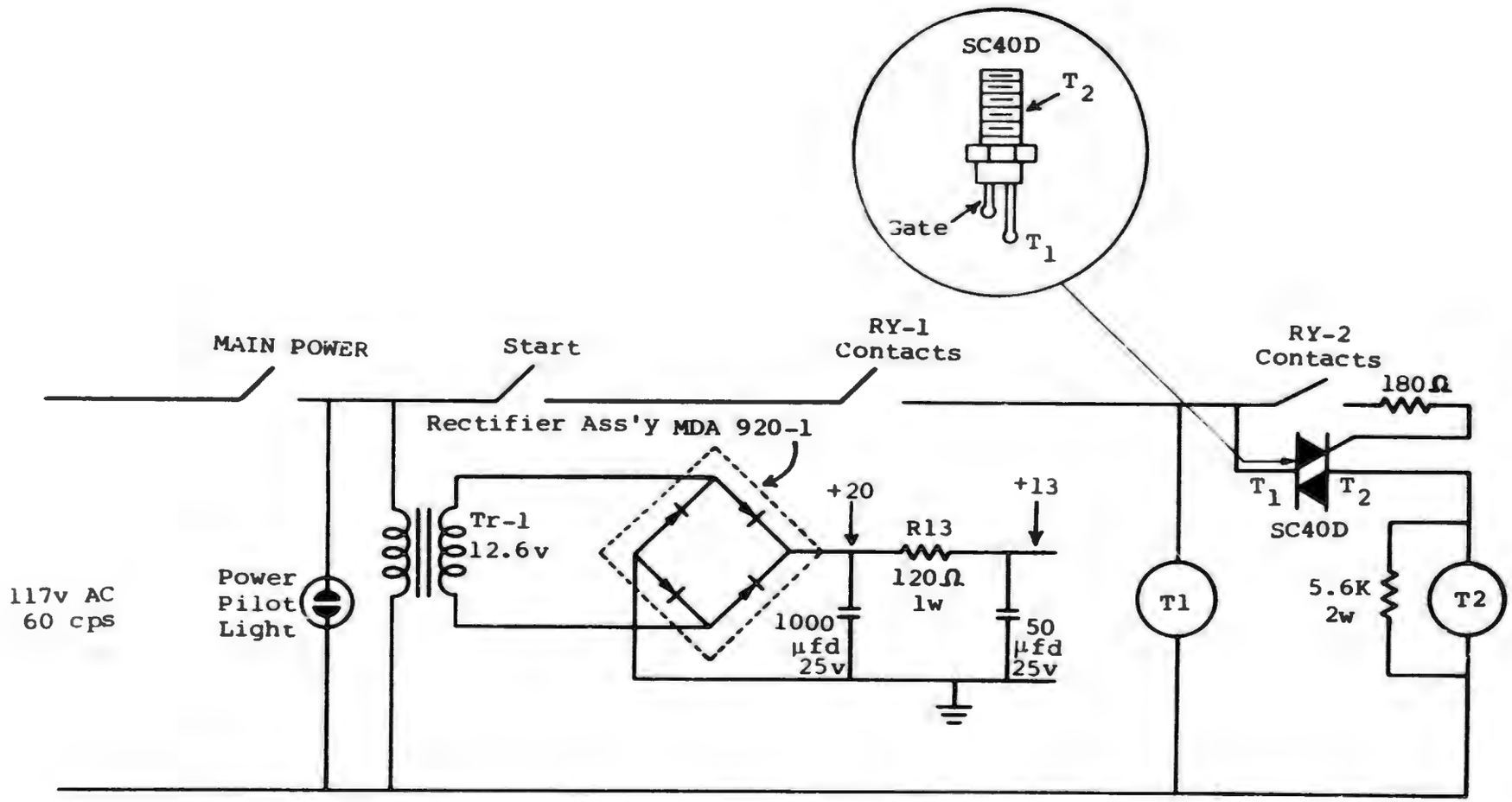


Figure 12

MODIFIED POWER SUPPLY AND TIMER CIRCUIT

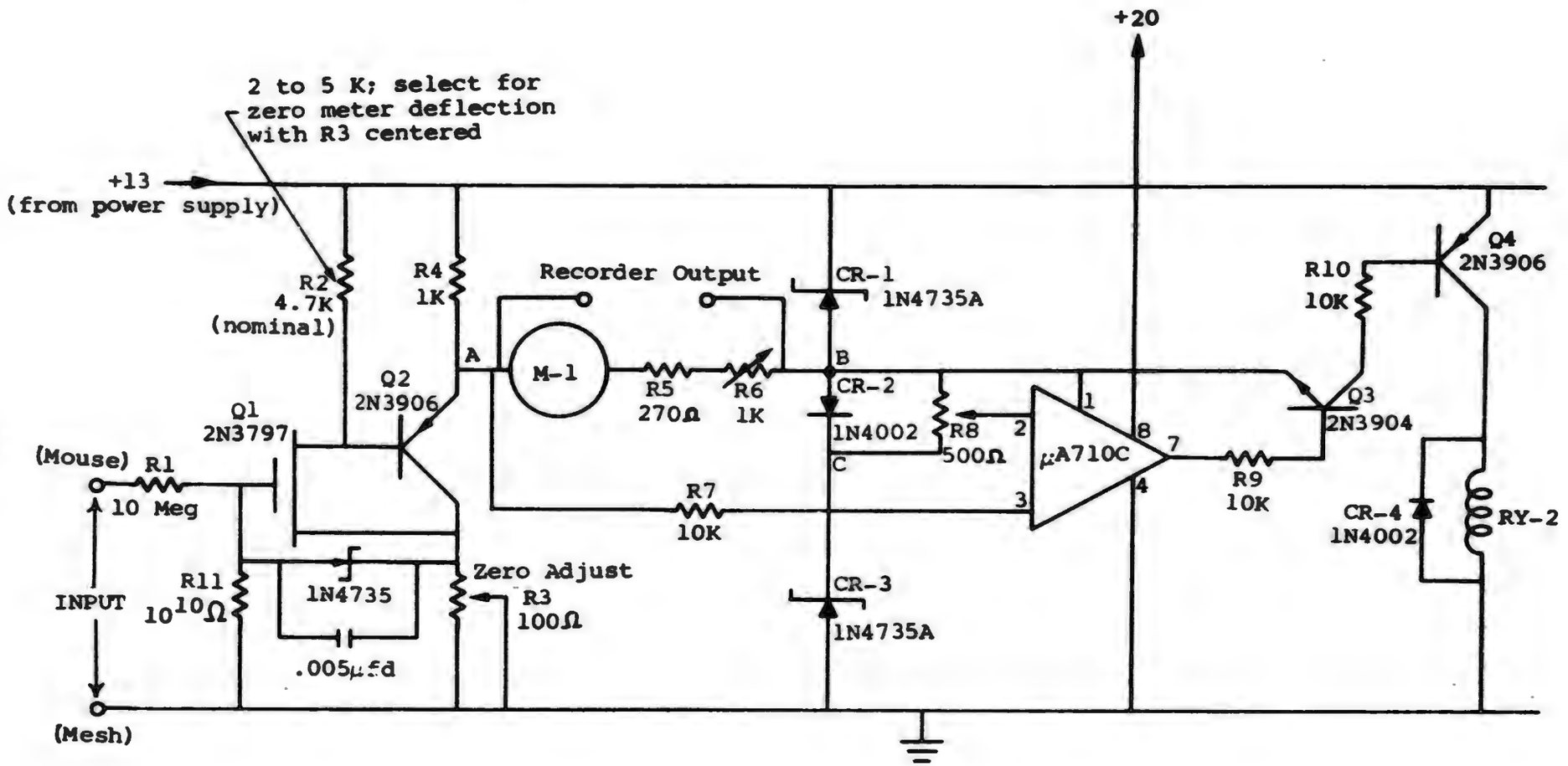


Figure 13

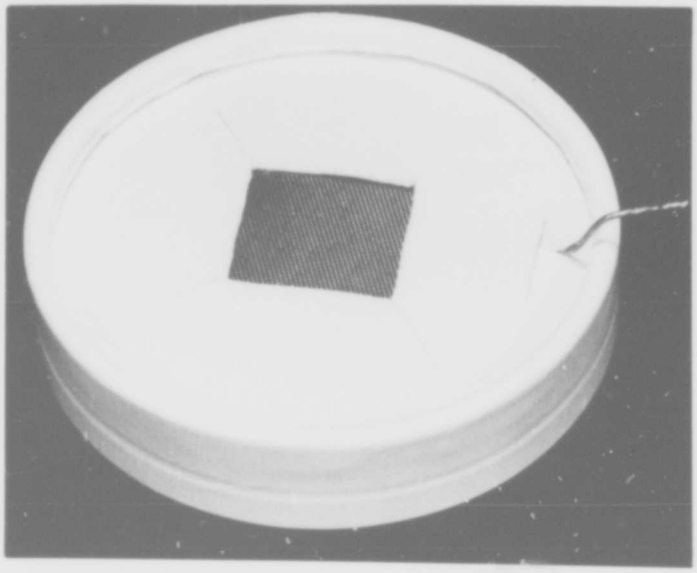
MODIFIED FIELD EFFECT VOLTMETER CIRCUIT

In this modification, relay RY-2 becomes energized when a mosquito (or mosquitoes) complete the circuit. The closing of the relay triggers the silicone-controlled triac switch (General Electric type SC40D) that starts timer T2, and the total bite time is recorded. Thus, the bite-time timer is energized by a silicone-controlled switch instead of directly by the relay. This modification effectively stopped the arcing problems, and the timer now responds instantaneously to the mosquito bites. This modification has tremendously improved the sensitivity and precision of the instrument. The field-effect voltmeter circuit (Figure 13) was also modified. A diode (IN4735) and a capacitor (.005 μfd) were inserted in the circuit of the field-effect transistor to protect it against accidental damage.

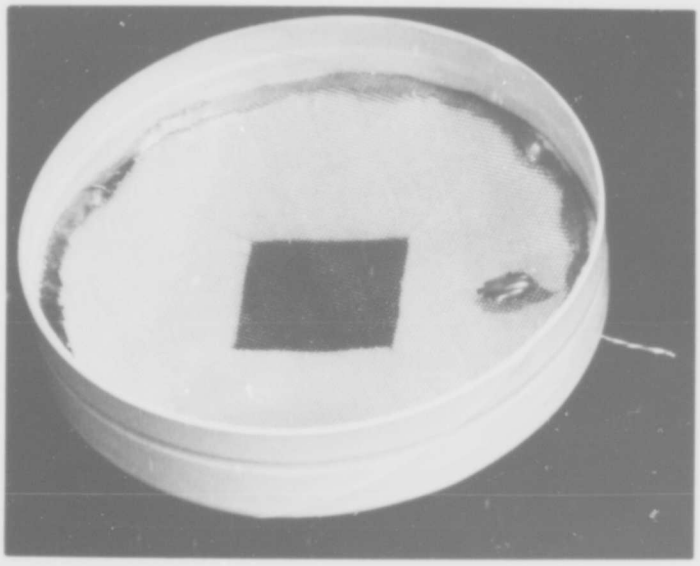
A methodological change was also introduced, in that stainless steel screens instead of bronze screens are now used in the electronic method. Stainless steel gives more uniform control values than bronze and is easier to clean. Also there is no possibility of the formation of copper sulfate ($CuSO_4$) or copper chloride ($CuCl_2$) on the mesh, which has been reported to inhibit the mosquitoes from biting through the mesh. The mesh is placed under the lid of a 1-pt ice cream container with the electrode protruding as shown in Figure 14a and 14b. A 1-sq in. hole is cut in the center of the lid to expose the treated area of the mouse to mosquitoes and mask the untreated areas.

IV. TESTS OF REPELLENTS

In our previous annual report (ref. 3) a rationale was presented by which chemical compounds meeting certain criteria were selected to be tested as repellents. These compounds were chosen because they bore certain relationships to the GABA molecule in terms of electronic distributions. In general, compounds that have within their molecules at least one electrophilic (electron-sparse) and one nucleophilic (electron-dense) center appear to be repellent to mosquitoes. GABA has one nucleophilic group (amino) and one electrophilic group (carboxyl).



a. Top Side



b. Under Side

Figure 14

PLACEMENT OF WIRE MESH INSIDE CONTAINER LID

It was hypothesized that compounds that are volatile analogues of GABA exert repellency by upsetting the "inhibition-activation balance" (ref. 2), possibly mimicking the inhibitory effects of GABA as the mosquito approaches a host. Many substances that were selected on this basis for repellency testing were found to be significantly repellent at low levels compared to parallel controls. This work was continued during this year, and the general approaches again appear to be substantially correct.

Table 1 shows the coded computer listing with the corresponding names and structures of compounds tested for repellency. Table 2 shows control data for the repellency tests, and Table 3 shows the repellency test data derived from the computer printout. The number labeled "upper bound" is the confidence level. An upper bound 100 is the 95% confidence limit. Numbers for the upper bound less than 100 indicate that the substance is significantly repellent at the 95% confidence level. The magnitude of the number indicates by what margin the 95% level of significance is proven for the repellency of a compound. A very low number indicates that the compound has better proof of repellency than a high number, although both are significant at the 95% level if both numbers are less than 100. Numbers above 100 for the upper bound indicate that the compound is not significantly different from the controls at the 95% level of confidence. This statistic thus permits a comparative ranking of compounds by their level of significance in terms of repellency. Uncertainties concerning the extremes of the distribution are ameliorated by this approach. A full description of the statistical methods and computer program is available (ref. 3).

Unless otherwise stated, all the following tests were done using the stainless steel meshes. All the data are in Table 3.

The compound m-diethyltoluamide (DEET) taken as a standard shows repellency at a high level of confidence (i.e., has a low upper bound) at 0.1 mg/sq in. of mouse skin, but is not significantly repellent at 0.01 mg/sq in.

4-Diethylamino-1-butanol and 4-dimethylamino-1-butanol are both about equally repellent and show significant repellency at 0.1 mg/sq in. The upper bound, however, shows that neither of these substances are as repellent as DEET at equal concentrations. N,N,-dibutyl formamide also shows repellency at 1.0 and at 0.1 mg/sq in., though again the upper bound at each of these concentrations is not as low as that for DEET. All three of the compounds mentioned above can be shown to have nucleophilic and electrophilic moieties in a single molecule.

Table 1

COMPUTER LISTING CORRESPONDING TO THE TEST COMPOUNDS

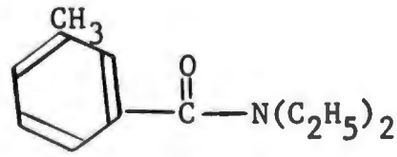
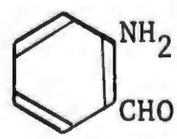
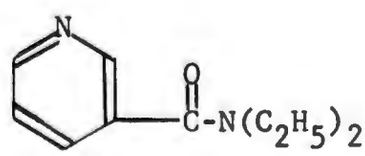
Computer Listing	Compound Name and Formula
DEET	M-diethyltoluamide 
4-DEA-1-BUTANOL	4-Diethylamino-1-butanol $(C_2H_5)_2N(CH_2)_3CH_2OH$
4-DMA-1-BUTANOL	4-Dimethylamino-1-butanol $(CH_3)_2N(CH_2)_3CH_2OH$
N,NDIBUTYLFORMAM	N,N-dibutylformamide $(C_4H_9)_2NCHO$
1-NH_2-GLYCEROL	1-Aminoglycerol NH_2-$(CHOH)_2CH_2OH$
4-AM-BUTANOL	4-Aminobutanol $NH_2(CH_2)_3CH_2OH$
2AMBENZALD	2-Aminobenzaldehyde 
2AMBENZALD(BRONZ)	Same as above with bronze mesh
ETNOLPROPNOLAMINE	Ethanolpropanolamine $CH_2(OH)CH_2NHCH_2CH_2CH_2(OH)$
NIKETHAMIDE	Nikethamide 

Table 1 (cont.)

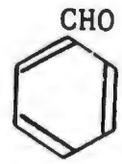
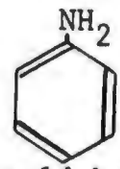
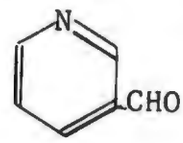
Computer Listing	Compound Name and Formula
BENZALDEHYDE	Benzaldehyde 
ANILINE	Aniline 
NICOTINALDEHYDE	Nicotinaldehyde 

Table 2

CONTROL DATA FOR REPELLENCY TESTS

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
CONTROL	-0.00000	77.50	80.47	157.97	
	-0.00000	70.33	73.08	143.91	
	-0.00000	38.24	96.73	134.97	
	-0.00000	62.75	35.85	98.59	
	-0.00000	35.85	41.58	77.43	
	-0.00000	47.17	51.20	98.37	
	-0.00000	70.73	75.67	146.40	
	-0.00000	34.78	79.41	114.20	
	-0.00000	39.22	79.30	118.52	
	-0.00000	19.23	60.33	79.56	
	-0.00000	32.69	56.37	89.06	
	-0.00000	39.39	65.18	104.57	
	-0.00000	32.73	50.17	82.89	
	-0.00000	50.00	92.63	142.63	
	-0.00000	51.28	84.33	135.62	
	-0.00000	24.53	49.75	74.28	
	-0.00000	38.78	42.21	80.98	
	-0.00000	77.78	35.55	113.32	
	-0.00000	26.92	77.18	104.10	
	-0.00000	36.00	75.91	111.91	
	-0.00000	37.50	63.10	100.60	
	-0.00000	61.76	61.09	122.85	
	-0.00000	42.00	65.37	107.37	
	-0.00000	47.06	86.35	133.41	
	-0.00000	77.36	77.93	155.29	
	-0.00000	23.53	90.31	113.84	
	-0.00000	53.06	70.67	123.73	
	-0.00000	15.09	28.07	43.17	
	-0.00000	56.00	52.87	108.87	
	-0.00000	85.71	73.28	159.00	
	-0.00000	90.38	90.20	180.58	
	-0.00000	74.47	80.27	154.73	
	-0.00000	40.43	73.83	114.26	
	-0.00000	20.75	33.20	53.95	
	-0.00000	67.35	78.05	145.40	
	-0.00000	48.15	85.65	133.80	
	-0.00000	23.08	66.13	89.21	
	-0.00000	61.70	66.45	128.15	
	-0.00000	34.04	71.78	105.82	
	-0.00000	20.00	45.70	65.70	
	-0.00000	38.30	83.81	122.11	
	-0.00000	80.43	98.34	178.77	
	-0.00000	54.55	92.56	147.10	
	-0.00000	65.96	72.75	138.71	

Table 2 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
	-0.00000	77.08	91.53	168.61	
	-0.00000	25.00	63.62	88.62	
	-0.00000	85.00	96.17	181.17	
	-0.00000	95.56	78.17	173.72	
	-0.00000	55.56	70.32	125.87	
	-0.00000	95.24	75.30	170.54	
	-0.00000	93.55	97.34	190.89	
	-0.00000	86.67	74.23	160.90	
	-0.00000	75.47	93.95	169.42	
	-0.00000	41.51	80.53	122.04	
	-0.00000	63.04	95.77	158.81	
	-0.00000	87.18	93.06	180.24	
	-0.00000	97.06	53.54	150.60	
	-0.00000	94.59	99.84	194.43	
	-0.00000	31.43	12.96	44.39	
	-0.00000	48.84	81.40	130.24	
	-0.00000	32.65	41.95	74.61	
	-0.00000	72.00	76.67	148.67	
	-0.00000	54.35	45.99	100.34	
	-0.00000	44.68	65.65	110.33	
	-0.00000	44.19	28.90	73.09	
	-0.00000	58.70	30.30	88.99	
	-0.00000	65.85	96.04	161.89	
	-0.00000	32.08	81.13	113.21	
	-0.00000	64.00	61.09	125.09	
	-0.00000	24.44	39.00	63.44	
	-0.00000	21.05	60.83	81.89	
	-0.00000	59.52	63.37	122.89	
	-0.00000	42.65	92.87	135.51	
	-0.00000	46.00	44.49	90.49	
	-0.00000	13.73	22.92	36.65	
	-0.00000	56.86	83.43	140.30	
	-0.00000	62.26	97.77	160.03	
	-0.00000	3.70	19.36	23.06	
	-0.00000	25.00	49.47	74.47	
	-0.00000	28.00	48.80	76.80	
	-0.00000	39.62	80.23	119.85	
	-0.00000	51.06	44.39	95.45	
	-0.00000	41.51	45.67	87.18	
	-0.00000	27.45	66.59	94.04	
	-0.00000	32.00	41.57	73.57	
	-0.00000	35.19	35.73	70.92	
	-0.00000	35.29	62.42	97.71	
CONTROL	-0.00000	50.48	66.61	117.09	100.0
		22.61	21.52	38.17	(108.0)UPPER BOUND

Table 3

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
DEET					
	1.00000	0.00	0.00	0.00	
	1.00000	0.00	0.00	0.00	
	1.00000	0.00	0.00	0.00	
DEET	1.00000	0.00	0.00	0.00	0.0
		-0.00	-0.00	-0.00	(33.2)UPPER BOUND
DEET					
	0.10000	0.00	0.07	0.07	
	0.10000	5.00	4.51	9.51	
	0.10000	0.00	0.00	0.00	
DEET	0.10000	1.67	1.52	3.19	2.6
		2.89	2.58	5.47	(36.1)UPPER BOUND
DEET					
	0.01000	9.80	65.90	75.70	
	0.01000	9.80	31.43	41.24	
	0.01000	50.94	77.47	128.41	
DEET	0.01000	23.52	58.27	81.78	70.9
		23.75	23.95	43.90	(108.0)UPPER BOUND
4-DEA-1-BUTANOL					
	1.00000	2.04	14.70	16.75	
	1.00000	0.00	0.00	0.00	
	1.00000	2.17	36.59	38.76	
4-DEA-1-BUTANOL	1.00000	1.40	17.10	18.50	13.5
		1.22	18.41	19.44	(44.1)UPPER BOUND

Table 3 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
4-DEA-1-BUTANOL					
	0.10000	5.77	49.67	55.44	
	0.10000	11.76	34.47	46.23	
	0.10000	40.00	95.12	135.12	
4-DEA-1-BUTANOL	0.10000	19.18	59.75	78.93	65.1
		18.28	31.56	48.88	(99.3)UPPER BOUND
4-DMA-1-BUTANOL					
	1.00000	8.33	31.96	40.29	
	1.00000	5.88	32.66	38.54	
	1.00000	3.92	17.62	21.54	
4-DMA-1-BUTANOL	1.00000	6.05	27.41	33.46	27.7
		2.21	8.49	10.36	(62.7)UPPER BOUND
4-DMA-1-BUTANOL					
	0.10000	9.80	30.91	40.72	
	0.10000	9.38	36.19	45.56	
	0.10000	23.81	57.51	81.32	
4-DMA-1-BUTANOL	0.10000	14.33	41.54	55.86	53.2
		8.21	14.08	22.18	(93.2)UPPER BOUND
N,NDIBUTYLFORMAM					
	1.00000	0.00	0.00	0.00	
	1.00000	2.17	8.37	10.54	
	1.00000	0.00	0.00	0.00	
	1.00000	26.53	65.70	92.23	
N,NDIBUTYLFORMAM	1.00000	7.18	18.52	25.69	17.6
		12.94	31.70	44.64	(44.4)UPPER BOUND

Table 3 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
N.NDIBUTYLFORMAM					
	0.10000	13.46	86.12	99.58	
	0.10000	13.16	63.90	77.06	
	0.10000	0.00	7.97	7.97	
	0.10000	5.66	26.00	31.66	
	0.10000	8.00	26.60	34.60	
	0.10000	0.00	0.00	0.00	
N.NDIBUTYLFORMAM	0.10000	6.71	35.10	41.81	48.2
		6.00	33.33	39.06	(80.7)UPPER BOUND
1-NH2-GLYCEROL					
	5.00000	10.00	82.73	92.73	
	5.00000	0.00	0.97	0.97	
	5.00000	7.69	98.57	106.26	
1-NH2-GLYCEROL	5.00000	5.90	60.76	66.65	77.1
		5.24	52.38	57.29	(127.5)UPPER BOUND
1-NH2-GLYCEROL					
	1.00000	54.35	97.04	151.39	
	1.00000	55.10	90.17	145.27	
	1.00000	55.10	95.08	150.18	
1-NH2-GLYCEROL	1.00000	54.85	94.10	148.95	129.3
		0.44	3.54	3.24	(165.7)UPPER BOUND
1-NH2-GLYCEROL					
	0.10000	60.42	86.70	147.12	
	0.10000	32.00	81.14	113.14	
	0.10000	15.63	73.63	89.26	
1-NH2-GLYCEROL	0.10000	36.01	80.49	116.51	100.1
		22.66	6.56	29.08	(135.5)UPPER BOUND

Table 3 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
4-AM-BUTANOL					
	1.00000	20.00	62.17	82.17	
	1.00000	0.00	21.66	21.66	
	1.00000	0.00	3.03	3.03	
4-AM-BUTANOL	1.00000	6.67	28.95	35.62	37.8
		11.55	30.23	41.37	(82.8)UPPER BOUND
4-AM-BUTANOL					
	0.10000	56.82	88.07	144.88	
	0.10000	8.33	45.48	53.81	
	0.10000	14.89	35.27	50.16	
4-AM-BUTANOL	0.10000	26.68	56.27	82.95	83.8
		26.30	28.01	53.67	(128.7)UPPER BOUND
2AMBENZALD					
	1.00000	1.96	11.37	13.33	
	1.00000	25.53	36.53	62.06	
	1.00000	4.35	16.28	20.63	
2AMBENZALD	1.00000	10.61	21.39	32.01	24.2
		12.97	13.34	26.28	(55.2)UPPER BOUND
2AMBENZALD					
	0.10000	24.00	90.62	114.62	
	0.10000	41.38	50.00	91.38	
	0.10000	11.63	46.77	58.39	
2AMBENZALD	0.10000	25.67	62.46	88.13	65.6
		14.95	24.44	28.25	(99.3)UPPER BOUND

Table 3 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
2AMBENZALD(BRONZ)	1.00000	0.00	0.00	0.00	
	1.00000	0.00	0.00	0.00	
	1.00000	0.00	0.00	0.00	
2AMBENZALD(BRONZ)	1.00000	0.00	0.00	0.00	0.0
		-0.00	-0.00	-0.00	(33.2)UPPER BOUND
2AMBENZALD(BRONZ)	0.10000	10.81	45.97	56.78	
	0.10000	0.00	0.00	0.00	
	0.10000	0.00	0.00	0.00	
2AMBENZALD(BRONZ)	0.10000	3.60	15.32	18.93	18.0
		6.24	26.54	32.78	(58.7)UPPER BOUND
2AMBENZALD(BRONZ)	0.01000	4.26	18.33	22.59	
	0.01000	0.00	0.00	0.00	
	0.01000	0.00	0.00	0.00	
2AMBENZALD(BRONZ)	0.01000	1.42	6.11	7.53	6.8
		2.46	10.59	13.04	(47.5)UPPER BOUND
ETNOLPROPNOLAMINE	1.00000	20.45	63.80	84.25	
	1.00000	0.00	1.63	1.63	
	1.00000	0.00	1.63	1.63	
	1.00000	0.00	0.00	0.00	
ETNOLPROPNOLAMINE	1.00000	5.11	16.77	21.88	19.6
		10.23	31.36	41.59	(54.3)UPPER BOUND

Table 3 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
ETNOLPROPNOLAMINE	0.10000	2.08	2.50	4.58	
	0.10000	14.55	63.63	78.18	
	0.10000	0.00	0.10	0.10	
ETNOLPROPNOLAMINE	0.10000	5.54	22.08	27.62	29.5
		7.87	36.01	43.84	(71.9)UPPER BOUND
NIKETHAMIDE	1.00000	2.33	20.72	23.04	
	1.00000	6.38	33.11	39.49	
	1.00000	0.00	0.00	0.00	
NIKETHAMIDE	1.00000	2.90	17.94	20.84	16.4
		3.23	16.73	19.84	(48.8)UPPER BOUND
NIKETHAMIDE	0.10000	16.67	58.34	75.01	
	0.10000	6.52	47.08	53.60	
	0.10000	9.52	72.30	81.82	
	0.10000	7.69	28.16	35.85	
NIKETHAMIDE	0.10000	10.10	51.47	61.57	43.4
		4.55	18.65	20.94	(68.5)UPPER BOUND
BENZALDEHYDE	1.00000	64.86	97.16	162.03	
	1.00000	12.00	80.50	92.50	
	1.00000	52.08	41.03	93.12	
BENZALDEHYDE	1.00000	42.98	72.90	115.88	98.0
		27.58	28.83	39.96	(132.3)UPPER BOUND

Table 3 (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS
ANILINE					
	1.00000	37.50	64.77	102.27	
	1.00000	13.21	57.23	70.44	
	1.00000	35.29	91.23	126.53	
ANILINE	1.00000	28.67	71.08	99.75	86.5
		13.43	17.86	28.13	(121.4)UPPER BOUND
NICOTINALDEHYDE					
	1.00000	1.85	1.47	3.32	
	1.00000	3.92	86.77	90.69	
	1.00000	8.33	31.53	39.87	
NICOTINALDEHYDE	1.00000	4.70	39.92	44.62	51.6
		3.31	43.26	43.88	(102.1)UPPER BOUND
NICOTINALDEHYDE					
	0.10000	7.69	57.29	64.99	
	0.10000	20.37	45.87	66.24	
	0.10000	33.33	58.58	91.92	
NICOTINALDEHYDE	0.10000	20.47	53.91	74.38	86.0
		12.82	7.00	15.20	(136.5)UPPER BOUND

Another compound was tested that possesses nucleophilic and electrophilic constituents on a single molecule, namely 1-aminoglycerol. This compound, however, showed no repellency at any concentration tested, up to 5.0 mg/cc. However, a test of 3-amino-1-propanol that was previously reported (ref. 3), showed significant repellency at 1.0 mg/sq in. 1-Aminoglycerol contains one nucleophilic group (amino) and three electrophilic (hydroxyl) groups in close proximity. 3-Amino-1-propanol on the other hand contains only one nucleophilic (amino) and one electrophilic (hydroxyl) group. Perhaps relative nucleophilicity and electrophilicity in a molecule is also important in determining repellency, and a ratio as high as 3 of the electrophilic/nucleophilic groups in a compound vitiates repellency.

4-Aminobutanol was tested and showed significant repellency at 1.0 mg/sq in. but not at 0.1 mg/sq in. These results substantially agree with those previously reported for amino alcohols when the bronze mesh was used (ref. 2,3).

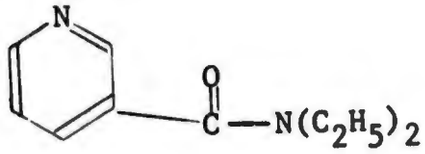
The compound 2-aminobenzaldehyde shows significant repellency at 1.0 and 0.1 mg/sq in. with the stainless steel mesh, but also shows repellency at 0.01 mg/sq in. with the bronze mesh. The upper bound at each concentration is lower when using bronze mesh than when using stainless steel mesh, which could reflect the presence of copper sulfate or copper chloride on the mesh surface, which may inhibit biting.

Ethanolpropanolamine was shown to be repellent at 1.0 mg/cc and 0.1 mg/cc at a fairly high level of the upper bound (54.3 and 71.9, respectively). This compound contains a hydroxyl group at each end of a six-member chain, with an amino group at position 3. Thus, the GABA-type conformation is essentially retained.

On the other hand, King (ref. 9) reported that 1,6 hexanediol is not repellent. We previously noted (ref. 3) that dihydroxy compounds where the two hydroxyl groups are in equivalent chemical environments are not repellent, whereas dihydroxy compounds where the hydroxyl groups are not in equivalent environments, such as in Rutgers 612 or 2,2,4 trimethylpentane-1,3-diol (TMPD), do show considerable repellency. We interpreted these findings in terms of the differences in the relative nucleophilicity and electrophilicity conferred upon the hydroxyls by the differing chemical environments in which they are located. The end hydroxyl group is much more acidic (i.e. less nucleophilic; has greater ability to lose a proton) than the one in the middle This is due to the greater electron input to the mid-position hydroxyl group from the rest of the molecule, thus intensifying its nucleophilicity and diminishing the probability of proton escape. It is well known that primary alcohols are more acidic than secondary or tertiary alcohols.

In the case of ethanolpropanolamine, the two end hydroxyls are in relatively equivalent environments, but an amino group is substituted by carbon-3. Thus a potent nucleophile is introduced into the molecule, and is "sandwiched" between 2 electrophiles (hydroxyl groups). This molecule exhibits good repellency.

We recently found nikethamide to be significantly repellent to mosquitoes at a concentration of 1.0 and 0.1 mg/sq in. Chemically, nikethamide is N,N-diethylnicotinamide (a derivative of nicotinic acid) and has the following structural formula:



The similarity in structure between this compound, DEET, and diethylbenzamide is striking. This drug is used therapeutically as a respiratory stimulant. The human oral, i.v. or i.m., dose is 1 to 5 cc of a 25% solution (ref. 10). The oral LD in rabbits is 650 mg/kg (ref. 10).

Since the pharmacological and toxicological properties of nikethamide have already been defined, and it has already been orally administered to humans, it may be possible to test the effectiveness of this drug as an oral repellent in human volunteers. We have communicated with Dr. Howard I. Maibach in this regard, and he has agreed to test this compound in volunteers. The results of these studies will be reported when available.

The drug exerts its effects by increasing the sensitivity of the respiratory center to CO_2 (ref. 11). This last fact suggests special implications in terms of the possible mechanism of repellent action of the DEET type. It is possible that the receptor sites for CO_2 in the insect nervous system may be similarly affected, and the delicate inhibition-activation balance may be upset as the insect approaches its host (ref. 2). These areas will be further explored by monitoring the effects of compounds resembling DEET and GABA, with and without the presence of CO_2, on the spontaneous firing rate of the isolated central nerve cord of the cockroach.

Other substances tested were benzaldehyde and aniline. With these substances we were able to test the repellent effects of the nucleophilic amino group of aniline or the electrophilic aldehyde group of benzaldehyde alone on the benzene ring. Neither of these substances show significant repellency when either the nucleophilic or the electrophilic group alone is present on the ring. When both groups are present simultaneously, as in 2-aminobenzaldehyde, significant repellency at low levels of application can be demonstrated (Table 3).

Nicotinaldehyde was also tested for repellency. In this case a pyridine ring is substituted at the meta position with an aldehyde group. No significant repellency is seen at 1.0 mg/sq in. These results are similar to those of benzaldehyde, although the upper bound for nicotinaldehyde is lower. The nitrogen in the pyridine nucleus is apparently not sufficiently exposed to express its nucleophilic properties to the same extent that the aldehyde moiety expresses its electrophilic properties. Again the balance in relative nucleophilicity and electroplicity in the same molecule may not have been achieved, and repellency is not evident.

The formation of a very potent repellent was noted when 4-aminobutyraldehyde diethylacetal (ref. 2,3) was hydrolyzed in acid solution, then neutralized. We presume that the active compound in this mixture is the free 4-aminobutyraldehyde; however, no evidence for this assumption is available. Preliminary attempts to extract the repellent substance have been unsuccessful so far. Under industrial sponsorship (S. C. Johnson and Son, Inc.) the organic chemists at IITRI are currently attempting to synthesize, extract, and purify this repellent substance, and results will be reported when available.

V. SUMMARY AND DISCUSSION

A. GABA Hypothesis

A detailed exposition on the GABA hypothesis has been presented (ref. 2). Briefly, the hypothesis states that GABA, a substance known to inhibit the transmission of impulses across crustacean synapses and found exclusively in association with the nervous tissue of many mammalian and nonmammalian species, may also intermediate synaptic inhibition in a mosquito. We proposed that GABA combines with CO_2 and that the resulting GABA-CO_2 complex is a stimulant rather than a synaptic inhibitor as GABA is alone. Therefore in the vicinity of a potential host, where the CO_2 content is increased above the normal atmospheric CO_2 content, the mosquito is activated. This activation is hypothesized to underlie the host-seeking behavior of mosquitoes. GABA is therefore proposed to be the actual CO_2 receptor substance in nervous tissues of mosquitoes that eventually guides the mosquito to its host.

Evidence to support this hypothesis was obtained when we observed that GABA exists in aqueous dialyzable extracts of mosquitoes, that GABA binds CO_2 in aqueous solution or as a dry deposit in the presence of moisture, and that the GABA-CO_2 complex is easily destroyed by heat. The complex is rapidly dissociated as the temperature approaches that of a warm-blooded animal. We recently obtained physiological evidence that a GABA-CO_2 complex does not inhibit nervous transmission, but appears to simulate it.

B. Repellent Designs

On the basis of the GABA hypothesis, we reasoned that volatile analogues of GABA may repel mosquitoes by neutralizing the stimulatory effects of CO_2 emanating from a host, i.e., by causing neuroinhibition, and loss of host recognition. Our understanding of the problem was subsequently broadened from observing that certain well-known mosquito repellents such as DEET and Rutgers 612 are not structurally related to GABA, but that these compounds contain within their molecules certain nucleophilic and electrophilic relationships (ref. 3) that were correlated with the electronic configurations of GABA. We subsequently proposed that the electronic configuration within a molecule (rather than its chemical constitution) coupled with its ability to be volatilized under ordinary conditions determines its repellent properties.

Investigations were subsequently undertaken to test this proposition, and most of the accumulated experimental evidence supported these predictions. Substances, such as the presumed gamma-aminobutyraldehyde, o-aminobenzaldehyde, many substituted aminoaldehydes, amino-alcohols, and unsaturated alcohols (e.g., 3-butene-2-ol, ref. 3), that contain at least one nucleophilic and one electrophilic center within one molecule were essentially repellent to mosquitoes.

Further investigations conducted during this year using improved methods have confirmed the repellency of certain compounds previously tested and have shown that this approach in terms of electronic requirements for repellents continues to be valid. Thus, substances such as N,N-dibutylformamide, nikethamide, and ethanolpropanolamine show significant repellency at 1.0 or 0.1 mg/sq in. of mouse skin, while substances such as aniline, benzaldehyde, and nicotinaldehyde, which do not have within their molecules these electronic relationships, are nonrepellent. Therefore a rational basis in the search for potent, long-lasting repellents has been proposed, and the experimental findings continue to support the correctness of this approach.

C. Repellency Assay

The electronic method developed in the IITRI laboratories that detects and records the mosquito bite and permits the visualization of every phase of the mosquito bite, has been fully described in past reports (ref. 1) and publications (ref. 12 to 17). In applying this method to repellency bioassay, a statistical discriminant-function analysis showed that only two parameters are necessary to achieve a good separation of control (untreated) from test (repellent-treated) mice. The parameters are (1) the percentage of time that the recording stylus is displaced in the electronic recording during the 30-min test period (P), and (2) the percentage of mosquitoes out of approximately 50 mosquitoes employed in each test that engorge blood (E).

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

Compounds can be ranked for repellent efficacy as a function of concentration, and the value of the upper bound. Therefore, an accurate, sensitive, and efficient means for the assay of repellents was developed by this application of statistical principles and the utilization of an electronic recording apparatus.

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

VI. FUTURE INVESTIGATIONS

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

A. Further Evaluation of Our Repellency Testing Method

In view of the observations by Gouck and Smith (ref. 18) that mosquito age and time of day influences the biting rate of low-level repellent treated subjects, we have designed a comprehensive statistical plan to test this effect, as well as some other possible effects in our repellency assay method. This experimental design will allow for independent analysis of the following:

1. A reevaluation of our previous findings of day-to-day variations in control tests (day effect).

2. A comparison with the result of Gouck and Smith (ref. 18) showing the effect of mosquito age and time of day on avidity in the presence of low-level repellent treatment.

3. The consistency of the responses among the three "bitometer-timers" we use in our testing procedures.

4. The influence of the actual number of mosquitoes in the test upon the test results.

5. The possibility of variations among different batches of mosquitoes.

The test is arranged with 14 days of testing, not necessarily consecutive. Each test day will consist of 4 sets, 2 in the morning and 2 in the afternoon. Each set will utilize 2 repellent treated mice and 1 untreated control. Each set will also consist of 2 different ages of mosquitoes run in parallel at different times of day on the 3 available meters. Sixteen separate batches of mosquitoes will be utilized to provide mosquitoes of ages 2, 4, 6, 8, 10, 12, 14, and 16 days of age in a randomized block design. The complete design calls for 56 untreated controls and 112 repellent treated tests, and the results will be evaluated for the test and control observations. This experiment will supply a comprehensive basis for future evaluations or applications to mass screening of repellents.

B. Search for New Repellents

The search for new repellents based on specific demands in terms of molecular and electronic configurations of candidate repellents will continue. Commercially available materials will be obtained for testing, whenever possible, if they appear to fit the structural requirements. If certain promising structures are unavailable commercially, we will consult our organic chemists about the synthesis and the purification of these compounds.

C. Purification and Characterization of Hydrolysis Products of 4-Aminobutyraldehyde Diethylacetal

Our experiments have shown that when the diethylacetal of 4-aminobutyraldehyde is hydrolyzed with acid and neutralized, the resultant product is a potent mosquito repellent. We presumed that the active substance in the hydrolysis mixture is 4-aminobutyraldehyde. At present we have no evidence to support this assumption. Under industrial sponsorship (S. C. Johnson and Son, Inc.) the organic chemistry section is now attempting to purify and characterize the active repellent substance in

this mixture. The fractionation will be followed with bioassay for repellency at each step to observe the partitioning of the repellent activity.

D. Further Verification of GABA Hypothesis

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

The distribution of GABA in mosquitoes will be further investigated. The actual site(s) of GABA synthesis in tissue sections of nervous tissue can be histologically localized by a method recently made available (ref. 19). The method makes use of the reduction of a tetrazolium salt to insoluble formazan in the course of the metabolic pathway by which GABA is converted to succinic acid. To apply this histochemical method to the determination of the sites of GABA metabolism in mosquitoes, cold sectioning of mosquitoes will be performed, and the histochemical method will be applied. Studies will be conducted to localize the neural tissue in the mosquito in which GABA is synthesized, and attempts will be made to insert microelectrodes into the tissue in order to study the action potentials of the nerve fibers in the absence and the presence of mosquito-repellent vapors and CO_2. We may thus be able to demonstrate whether repellents cause neuroinhibition and whether CO_2 will evoke neural activity in these structures.

E. Maintenance of Mosquito Colony

Our colony of Aedes aegypti (L) mosquitoes and Periplaneta americana (L) cockroaches will continue to be bred and maintained for purposes of our research throughout the course of this work.

VII. CONCLUSIONS

Our investigations to date have included 3 areas of research:

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

2. Developing accurate statistically-based methods to evaluate mosquito repellency of test compounds. These methods employ a device that electronically records, and thus unequivocally documents the mosquito bite.

3. Testing the mosquito repellency of certain chemical structures that are related to the structure of GABA and exploring the mechanism of repellency. This work is directed toward obtaining a rationale to guide the discovery of more potent and longer-lasting repellents than those currently available.

We plan to continue using the unique theoretical and methodological approaches developed during the course of this work. The attempt to elucidate the basic physiological mechanisms governing the interactions of mosquitoes with their warm-blooded hosts, and the logical approaches to the development of new insect repellents engendered during this work appear to be bearing fruitful results. Continuation of this effort should substantially contribute to ultimate success in the important endeavor to achieve superior repellents for systemic or topical use.

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13 ABSTRACT The objective of this program is to develop mosquito repellents for topical or systemic use that will be more potent, longer lasting, and afford mor uniform protection than those currently available. The testing of compounds for mosquito repellency was continued. Repellency data was derived from an electronic "bitometer-timer" apparatus (which yields the percentage of total time of actual biting), and from the percentage of mosquitoes that engorged blood during the test period. The sum of these two parameters, the repellency index, was analyzed for statistical significance for differences from controls in a specialized digital computer program. Substances that resemble the structure of the neuroinhibitor, gamma-aminobutyric acid (GABA), in terms of their electronic distribution, are generally significantly repellent at low levels of application. Work continued on a hypothesis that could explain the neurochemical basis of a mosquito's attraction to warm-blooded hosts. It was theorized that when GABA, which is found in mosquitoes, combined with carbon dioxide, it is no longer neuroinhibitory, but stimulatory. The activation of mosquitoes in the presence of hosts is attributed to this chemical reaction. Evidence to support the hypothesis was obtained by investigation of the effects of GABA alone and GABA-CO_2 complexes on the spontaneous firing rate of the isolated central nervous system of a cockroach. GABA alone was inhibitory, while the GABA-CO_2 complex and its chemical analogues were stimulatory. Further studies of the physiological basis of repellency and attraction in mosquitoes are in progress.

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Mosquito						
Repellent						
Gamma-aminobutyric acid						
Carbon dioxide						
Neuroinhibitor						
Activation						
Electronic						
Bitometer-Timer						
Repellency index						
Computerized repellency assay						