IITRI

Report No. IITRI-L6021-7
(Quarterly Progress Report)

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Headquarters
U.S. Army Medical Research
and Development Command
Office of the Surgeon General
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IITRI Project L6021
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I. INTRODUCTION

In previous reports (IITRI-L6021-5 and IITRI-L6021-6) a hypothesis that could explain the mechanism of attraction of mosquitoes to warm-blooded animals was presented. In brief, the hypothesis stated that gamma-aminobutyric acid (GABA), a substance known to inhibit transmission of nerve impulses across certain synaptic junctions in animal species, may also play an inhibitory role in the nervous system of mosquitoes. It was further proposed that GABA can combine with carbon dioxide (CO_2), and that the GABA-CO_2 complex may no longer possess the synaptic inibitory power of GABA alone. Support for the hypothesis was obtained when it was found by amino acid analysis that GABA exists in aqueous whole-body extracts of mosquitoes and that GABA combines with CO_2.

During this report period additional work was carried out:

(1) Certain GABA analogues were tested for mosquito repellency.

(2) The crayfish intestine preparation was further studied to detect the effects, if any, on inhibition of contractions by GABA with and without the presence of bicarbonate (HCO_3) ion.

(3) Preliminary work was begun on the crayfish abdominal stretch receptor preparation, which may be a more sensitive assay for detection of GABA-CO_2 complexes than the intestine preparation.

(4) Experiments were initiated to determine the effect of temperature on GABA-CO_2 complexes.

(5) Amino acid analysis were performed on separated heads and bodies of mosquitoes.

(6) Visits were made to the University of Tennessee School of Pharmacy and to the Gainesville Laboratories of the U.S. Department of Agriculture, to discuss mutual problems and to observe testing methods.

II. MOSQUITO REPELLENCY OF GABA AND GABA ANALOGUES

If our hypothesis on the mechanism of GABA action is correct, GABA or its chemical analogues when volatilized in the vicinity of a mosquito should alter the mosquito's biting behavior. To test this approach, gamma-amino-n-butanol (City Chemical Corp., New York) and gamma-aminobutyraldehde diethylacetal (Aldrich Chemical Co., Milwaukee) were tested as mosquito repellents. The electronic recording method was used for assay, and statistical analyses were performed as described in reports IITRI-L6021-4 and IITRI-L6021-5. During the course of these tests 23 control experiments were carried out, and the control groups were pooled. The mean for the controls was $\overline{P + E} = 87.63$. 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. Due to these differences, the threshold values for n_i, i.e., the values of $\bar{P} + \bar{E}$ which may not be exceeded if repellency of the test compound is significantly different from control values at the 95% confidence interval, is 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 1.

Table 1

THRESHOLD VALUES FOR Ni

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	59.96

GABA itself was found to have no significant repellency, even at a concentration of 10 mg per square inch 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 per square inch of skin was considerably repellent. The degree of repellency was about the same order of magnitude as that of m-diethyl toluamide (DEET). At a concentration of 0.1 mg per square inch of skin repellency was no longer evident. The repellency effects of GABOH were not very long lasting, and disappeared after about 5 hr (Table 2). This may be due to a high rate of evaporation of this compound.

Table 2

REPELLENCY OF GABA AND GABOH

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Mosquitoes Engorged (E) %*	$\bar{P} + \bar{E}$, %	Significant at 95% Confidence Level
GABA	10.0	44.5	38.9	106.5	No
	10.0	100.0	29.6		
	1.0	33.3	56.9	126.5	No
	1.0	100.0	63.0		
Gamma-amino-n-butanol	1.0	0	0		
	1.0	0	0		
	1.0	0	0	0	Yes
	1.0	0	0		
	1.0	13.6	0		
	0.1	92.9	37.7	119.4	No
	0.1	92.3	15.9		
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

*30-50 mosquitoes were exposed in each test.

Gamma-aminobutyraldehyde diethylacetal, also a liquid, was quite repellent at a concentration of 10 mg per square inch of skin. It also exhibited a significant, though lower, repellency at a concentration of 1 mg per square inch of skin. It was ineffective as a repellent at a concentration of 0.1 mg per square inch of skin (Table 3).

When the diethylacetal was hydrolyzed at 4°C overnight in an acetone solution containing a few drops of 0.1 M hydrochloric acid, the resulting compound, presumably gamma-aminobutyraldehyde, was repellent at concentrations of 1.0 and 0.1 but not significantly repellent at 0.01 mg per square inch of skin. The butyraldehyde is obviously a closer analogue of GABA than the acetal, since the carbonyl function is free. It is a better repellent than the acetal, and also exceeds the repellency of GABOH. Table 2 shows that the repellency of the aldehyde lasted for at least 5 hr at a concentration of 1.0 mg per square inch but was not longer apparent after 24 hr.

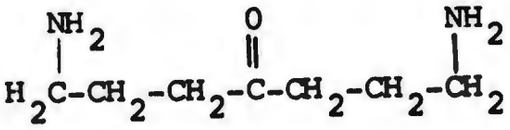
It is premature to draw firm conclusions at this time. The data are insufficient, and the actual degree of hydrolysis of the diethylacetal under the conditions used is uncertain. In future work the acetal will be hydrolyzed by the usual method, i.e., heating in aqueous acidified solution. It is probably correct to conclude, however, that these GABA analogues considerably affect mosquito-biting behavior.

Table 3

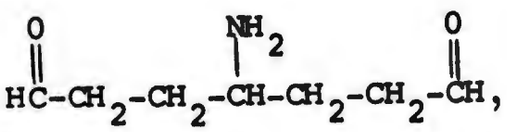
REPELLENCY OF GAMMA-AMINOBUTYRALDEHYDE DIETHYLACETAL AND GAMMA-AMINOBUTYRALDEHYDE

Compound	Conc. on Mouse, mg	Time Displaced (P), %	Mosquitoes Engorged (E) %	- - P + E %	Significant at 95% Confidence Level
Gamma-aminobutyraldehyde diethylacetal	10.0	0	0		
	10.0	0	0	2.5	Yes
	10.0	0	0		
	10.0	10.0	0		
	1.0	0	0		
	1.0	81.5	18.8	37.2	Yes
	1.0	11.2	0		
	0.1	88.0	39.5	127.5	No
2-hr retest	0.1	57.3	55.6	112.9	No
5-hr retest	1.0	47.6	4.4	52.0	No
24-hr retest	1.0	37.7	22.5	60.2	No
	10.0	48.8	20.0	100.8	No
	10.0	94.5	38.1		
Gamma-aminobutyraldehyde, hyrolyzed	1.0	0	0	0	Yes
	1.0	0	0		
	0.1	0	0	0	Yes
	0.1	0	0		
	0.01	59.2	13.1	43.1	No
	0.01	10.0	3.9		
5-hr retest	1.0	16.8	9.7	26.5	Yes
24-hr retest	1.0	52.2	7.4	59.6	No
	0.1	45.5	32.0	77.5	No

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



or



which are "double-edged" GABA analogues, as well as many others based on the GABA structure may be designed, which 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. GABA has been found to be present in many insects (ref. 1,2) and to play a role in the normal physiology of these insects. Further work with these analogues is in progress.

III. BIOASSAY OF GABA-CO_2 COMPLEXES

In a previous attempt (Report IITRI-L6021-6) to substantiate the hypothesis that a GABA-CO_2 complex does not inhibit nerve transmission, as does GABA alone, we performed an experiment with the hind gut of a crayfish. This preparation was originally described by Florey (ref. 3). When GABA alone or a GABA solutin that had CO_2 bubbled through it for 5 min before the assay

was added to the Florey crayfish intestine preparation, inhibition of both spontaneous as well as acetylcholine-produced contraction of the crayfish intestine was observed. Therefore we could not confirm our hypothesis by this method. The assay was performed at room temperature, and it was thought that dissociation of GABA-CO_2 complexes at room temperature together with constant aeration in the assay system may have contributed to the failure of these experiments, as CO_2 probably escaped rapidly under these conditions.

We therefore attempted to repeat this assay at near 0°C temperature, using known amounts of sodium bicarbonate as the GABA-CO_2 complexing agent instead of unknown quantities of absorbed gaseous CO_2. Figures 1 and 2 show the results of some representative experiments. The experimental conditions are described in the figures. It is obvious that confirmation of the hypothesis could still not be obtained with the intestine preparation under these new conditions. Both GABA alone and compounds that are presumably GABA-CO_2 complexes inhibited the gut contractions.

Although there are other premutations of the test system that we shall try before abandoning the gut preparation, it does not appear hopeful that this preparation is adequate to either prove or disprove the hypothesis. Smooth muscle, as a relatively undefined tissue, may not be sensitive to GABA-CO_2 complexes; continuous aeration may dissociate the GABA-CO_2 complex even at low temperature and in the presence of excess bicarbonate.

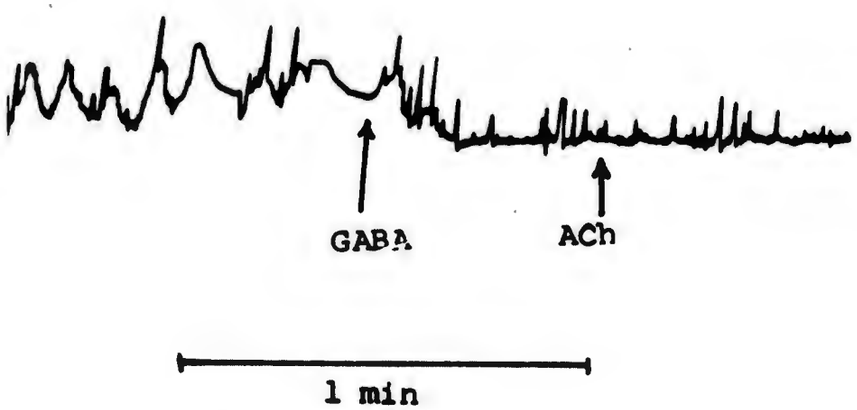


Figure 1

INHIBITION OF CONTRACTIONS OF CRAYFISH INTESTINE BY GABA. Note immediate decrease in amplitude upon additions of 10^{-3}g/ml of GABA. Addition of acetylcholine (10^{-3}g/ml) did not overcome the inhibition. Tests performed at 4°C.

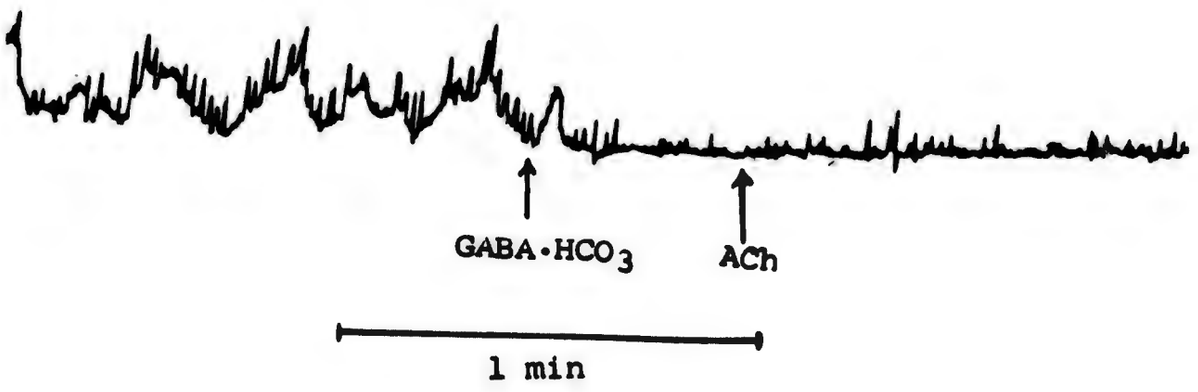


Figure 2

INHIBITION OF CONTRACTIONS OF CRAYFISH INTESTINE BY GABA-CO_2 COMPLEX. 1 ml GABA (10^{-3}g/cc) mixed with 1 cc of 0.3 M $NaHCO_3$ (25 x 10^{-3}g/cc) before addition. Acetylcholine (10^{-3}g/cc) did not overcome the inhibition. Tests performed at 4°C.

Edwards and Kuffler (ref. 4) did not stir the GABA 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 a considerably more difficult one than the intestine, and we have obtained the consulting services of Dr. Sidney Katz, a neurophysiologist for performing experiments on crayfish abdominal stretch receptor. This work is presently in progress.

IV. EFFECT OF TEMPERATURE ON GABA-CO_2 COMPLEXES

This work was initiated as a result of the observation that heat dissociates GABA-CO_2 complexes. The effect of temperature is a central point in the hypothesis of the mechanism of mosquito attraction to mammals (see reports IITRI-L6021-5 and IITRI-L6021-6).

Our initial attempts to determine the effects of various temperatures on the stability of GABA-CO_2 complexes were unsuccessful for 2 reasons: The various GABA and blank (distilled water) solutions were not adequately protected from absorption of atmospheric CO_2 during experimental procedures; and the choice of sodium bicarbonate as the CO_2 donor substance was unfortunate.

Essentially, the protocol involved the addition of known sodium bicarbonate solutions to known GABA solutions at various temperatures. Any excess bicarbonate was precipitated with an excess of saturated barium hydroxide. The supernatant

resulting after centrifugation was heated in a boiling-water bath for 15 min in the presence of excess barium hydroxide to release the CO_2 bound to GABA. This second precipitate was considered to be GABA-bound CO_2. The same procedure was applied to distilled water blanks not containing GABA.

In both GABA-containing solutions and water blanks the precipitate after boiling was considerable. Apparently, all the unbound bicarbonate was not precipitated with the first addition of barium hydroxide. This was probably due to a considerable alkaline pH shift resulting from the formation of sodium hydroxide from the sodium ion originally present as bicarbonate. Thus, complete removal of free carbonate was prevented. When gaseous CO_2 was used as the CO_2 complexing agent for GABA, the blank solution showed little or no precipitate upon heating with excess barium hydroxide, while the GABA solution showed a considerable amount of the second precipitate when heated in a boiling water bath in the presence of excess barium hydroxide.

In the next series of experiments, gaseous CO_2 will be used as the GABA-complexing agent. To prevent absorption of atmospheric CO_2 during the procedures, the tests will be performed under a layer of paraffin oil. The amount of GABA-bound CO_2 precipitated as the barium carbonate will be quantitated by the method described by Roessler and Brown (ref. 5).

V. AMINO ACID ANALYSES OF MOSQUITO EXTRACTS

It was reported in IITRI-L6021-6, that GABA was the third most abundant free amino acid in water-soluble dialyzable extracts of mosquitoes. To localize the area of GABA concentration, the heads were separated from the bodies of 500 mosquitoes, and both parts were analyzed.

The extraction procedure was as follows. The tissue was homogenized in about 5 cc of distilled water in a glass homogenizer with a teflon pestle. 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 the solution. This material was separated by centrifugation and also discarded. The clarified supernatant was put into a dialysis bag and dialyzed with stirring at 4°C overnight. The dialysand (water external to the dialysis casing) was freeze-dried, the residue was redissolved in 1 cc of 0.1 N hydrochloric acid, and an aliquot of this solution was taken for amino acid analysis.

Perusal of the amino acid profile showed that fewer free amino acids were present in the head than in the body, a result undoubtedly due to the respective amounts of tissue present. GABA was present in both the head and body in approximately equivalent amounts. The amount of GABA relative to the concentration of other amino acids was considerably larger in the head than in the body. However, the total GABA in the head and in the body did not add up to the high concentration originally detected with the first whole-mosquito extract.

Another extract using the whole bodies of about 500 mosquitoes was repeated, and the total GABA content was again much lower originally observed.

The first batch of mosquitoes was older than the batches used for the above analyses, the age of the mosquitoes at the time of extraction may have been a factor. For the next amino acid analysis, a group of mosquitoes equivalent in age to the first group will be used.

There remains, however, the question of how much free amino acid is not extractable by our methods because of their non-specific binding to the discarded insoluble tissue. If we cannot resolve this question, an alternative is to measure GABA in these tissues by using enzymayic methods (ref. 6-9). Some workers (ref. 10) using enzymatic methods have obtained 95 to 100% recoveries of GABA added to brain homogenates. We therefore plan to investigate the GABA content of mosquitoes by enzymatic means.

VI. VISITS TO OTHER INSTALLATIONS

During this report period, visits were made to the University of Tennessee College of Pharmacy at the invitation of Dr. Andrew Lasslo and to the Laboratories of the U.S. Department of Agriculture at Gainesville, Florida.

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We became familiar with Dr. Lasslo's approaches toward development of an orally effective insect repellent. We agreed to use our electronic recording method to test some griseofulvin conjugates synthesized by his group. Table 4 shows the preliminary data. At the concentrations used, only compound A013 PADSII-15c showed significant repellency. One of the compounds was insoluble in acetone and was not tested. We hope to solubize this compound by using other solvents. We have also received larger quantities of these compounds for testing at higher levels. The results will be reported later.

Our main objective in visiting the Gainesville Laboratories was to observe their methods of repellency testing, since, though repellency estimates by the two methods generally agree, some discrepencies between our judgment and their judgment of repellency had been noted. We concluded that our respective testing methods may account for these discrepencies. Whereas the the USDA method presents the whole stocking-covered arm to a container full of mosquitoes, with only a small area covered with the test repellent, the IITRI method presents to the mosquito only the repellent-treated area on the mouse. The USDA method appears to be more accurate in terms of attractant and repellent interaction that may culminate in a landing, while the IITRI method is more sensitive in testing the repellent-treated area itself and is perhaps more quantitative in terms of the actual biting activity of the mosquito.

Table 4

REPELLENCY OF COMPOUNDS SUBMITTED BY UNIVERSITY OF TENNESSEE

Compound No.	Conc. on Mouse, mg	Time Displaced (P), %	Mosquitoes Engorged (E), %	P + E %	Significant at 95% Confidence Level
A003 PADS III 77e	9.3	66.7	15.7	82.4	No
A013 PADS II 15c	10.0	7.0	2.3	9.3	Yes
A013 PADS II 15c (immediate retest, same mouse)	10.0	0	0	0	Yes
A014 PADS III 77g	8.2	67.0	30.6	97.6	No
A016 PADS IV 78h	8.3	75.8	29.4	105.3	No
A018 PADS IV 65c	9.5	61.2	41.6	102.8	No
A017 PADS II 24 1	Insoluble in acetone (not tested)				

A combination of the two methods may provide the most accurate test system. For example, a repellent-treated area in the stocking test could be covered by the wire mesh and the human test subject wired into the electronic recording apparatus. This would be simple to do; an electrode covered with a potassium chloride paste can be used to complete the circuit to the human subject. In this way, only the mosquitoes landing on the mesh and biting the repellent-treated area would be registered by the electronic apparatus. Because of the size of the subject, such a test would probably have to be performed in an electrically shielded room to remove stray electromagnetic fields that can influence the sensitive electronic recording apparatus. We hope to test the feasibility of such an arrangement.

VII. FUTURE WORK

We shall continue to test GABA analogues as mosquito repellents and also to test their repellency when administered orally or parentally.

Further work with the crayfish intestine preparation and the crayfish stretch receptor preparation will be carried out. Cooperation with other laboratories participating in this work will be continued to further our mutual efforts in the development of an orally effective insect repellent.

VIII. PERSONNEL AND RECORDS

The consulting services of Dr. S. Katz in the dessection and preparation of the crayfish stretch receptor and the technical assistance of Miss Eileen M. Gross are gratefully acknowledged. Mr. Merl Kardatzke contributed to the statistical analysis of the data. All data are recorded in Logbook C16939 and also in the form of the actual chart recordings.

Respectfully submitted,

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