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Report No. IITRI C222-6
(Quarterly Progress Report)

DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

Headquarters
U.S. Army Medical Research and
Development Command
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Attention: Major Lee Roy J. Jones

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IITRI Project No. C222
Contract No. DA-49-193-MD-2281

August 1 to October 31, 1963

I. INTRODUCTION

The object of this program is to develop an internally administered insect repellent. During this report period, testing of benzamide compounds was continued. In addition, studies on the distribution of diethyltoluamide-C^{14} (DEET-C^{14}) in mice, as determined by whole-body autoradiography and radioassay techniques, were initiated.

II. TESTING OF BENZAMIDES AS MOSQUITO REPELLENTS

A semiautomatic procedure was adopted to pick pupae from a mosquito pond and to isolate the female from the male pupae. The apparatus was designed wit' the assistance of Dr. K. S. Rai, Department of Biology, University of Notre Dame, and was constructed by the Fine Tools and Research Apparatus Company. In principle, the device consists of two glass plates with a finite

space between them. At the top end the plates are separated by approximately 3.5 mm and at the bottom end by less than 1 mm. A mixture of larvae and of male and female pupae is poured into the space between the two plates from the top, and washed down with water. The larvae, the smallest in size, are trapped near the bottom; the male pupae, the next in size, are found in the middle; and the female pupae are separated in the top layer. The bottom opening between the plates is then increased by a lift screw until the larvae are washed through and collected in a receiver, but the male pupae are trapped near the bottom. By repeating the operation, the female pupae are trapped at the bottom and then washed into a receiver. This procedure proved to be time-saving when a large number of pupae were separated.

The bioassay procedure employed in the in vivo testing was reported in Report No. IITRI-C222-3 and C222-5. In addition, the following in vitro test system was developed and standardized during this period. The apparatus consisted of 24 cylindrical glass tubes 1 in. deep which were closed at one end with a membrane to hold blood in a constant-temperature water bath. The glass cylinders were held in metal tubes built into the water bath. The closed ends of the cylinders protruded from the bath and rested on the screen of the cages containing the test mosquitoes. The mosquito cages were maintained at room temperature, and the blood was maintained at 34°C during the exposure period.

Baudruche caping skine was the membrane used to close the ends of the glass tubes. The skin was obtained from Long and Long, Belleville, New Jersey, boiled for 1 hr before use to soften it and to remove any preservatives used by the manufacturer.

The blood used in these studies was outdated, citrated whole human blood obtained from a nearby hospital. It was kept under refrigeration and was heated to the desired temperature just before use. The compound to be tested and Tween 80 were added to the blood, and the mixture was homogenized. Two mg of Blanchophore^{R} dye and 2 μc of radioiodinated serum albumin (RISA-I^{131}, Abbott Laboratories) per ml of blood were added as indicators.

Female Aedes aegypti mosquitoes were starved for 48 hr and deprived of water for 24 hr before testing. The mosquitoes, 50 in each cage, were kept at 25 $\pm$ 1°C and exposed for 1 hr to the membrane-covered end of the glass cylinders each containing 15 cc of blood at 37°C. After exposure, the mosquitoes were killed by chilling, and the amount of biting was determined by the same method used in the in vivo testing.

Table 1 shows the repellent properties of intraperitoneally injected benzamide derivatives. The data show that none of the compounds tested prevented mosquito biting significantly. Table 2 shows the effect of benzamide derivatives admixed with blood on mosquito feeding in vitro. A concentration of 1 mg/ml of all the

Table 1

EFFECT OF BENZAMIDE DERIVATIVES[1] INJECTED INTRAPERITONEALLY IN MICE ON IN VIVO MOSQUITO FEEDING

Benzamide Derivative	Time Between Treatment and Testing	Blood Ingested, µl/50 Mosquitoes	% Mosquitoes Which Fed
Control	--	155	74
		142	69
		170	83
		227	89
N, N-Diethyl-m-toluamide	5 min	116	65
	1 hr	186	80
		253	88
	2 hr	155	74
	4 hr	203	87
		158	70
N, N-Diethyl-m-n propylbenzamide	5 min	159	82
	1 hr	193	86
		257	94
	2 hr	157	84
		207	94
	4 hr	159	83
		162	66
N, N-Diethyl-m-isopropylbenzamide	5 min	161	82
	1 hr	171	83
	2 hr	174	83
		165	91
	4 hr	161	78
	4 hr	141	83
N, N-Diethyl-m-n amylbenzamide	5 min	177	94
	1 hr	189	88
	2 hr	244	96
	4 hr	137	69
		141	77
N, N-Diethyl-2,6-dimethylbenzamide	5 min	153	79
	1 hr	168	75
		225	88

Table 1 (cont.)

	2 hr	160	76
		161	85
	4 hr	175	88
		179	79
N, N-Diethyl-2, 4-dimethylbenzamide	5 min	178	88
	1 hr	141	57
		213	74
	2 hr	139	65
		142	68
	4 hr	156	64
		87	38
-do-[2]	5 min	252	91
	1 hr	260	93
		272	80
	2 hr	300	98
		257	94
	4 hr	240	78
		292	100
Control[2]	0	307	91
		317	100
N-m-Toluyl morpholine	1 hr	169	92
		198	88
	2 hr	105	62
	4 hr	167	80
		169	88

[1]All the drugs were given in a dose of 300 mg/kg except the morpholine, which was given in 500 mg/kg.

[2]Experiment was repeated since initial results were not clear.

Table 2

EFFECT OF BENZAMIDE DERIVATIVES
EMULSIFIED IN BLOOD ON IN VITRO MOSQUITO FEEDING

Benzamide Derivative	Concentration, mg/ml of Blood	Number of Replicates of 50 Mosquitoes	% Mosquitoes Which Fed, Mean
Control	--	4	98.1
	--	4	91.1
N, N-Diethyl-m-toluamide	1.0	3	5.4
	0.1	3	91.7
N, N-Diethylbenzamide	1.0	2	0.9
	0.1	2	63.2
N, N-Diethyl-m-nitrobenzamide	1.0	2	17.5
	0.1	2	81.5
N, N-Diethyl-m-n-propylbenzamide	1.0	2	0.9
	0.1	2	94.8
N, N-Diethyl-m-isopropylbenzamide	1.0	2	0.0
	0.1	2	87.5
N, N-Diethyl-p-isopropylbenzamide	1.0	2	0
	0.1	2	90.9
N, N-Diethyl-m-n-amylbenzamide	1.0	2	0
	0.1	2	87.2
N, N-Diethyl-2, 4-dimethylbenzamide	1.0	2	4.5
	0.1	2	92.2
N, N-Diethyl-2, 6-dimethylbenzamide	1.0	2	0
	0.1	2	89.2
N, N-3, 4, 5-trimethoxybenzamide	1.0	2	0.9
	0.1	2	91.8
N-m-Toluyl morpholine	1.0	2	60.7
	0.1	2	84.3

derivatives except N-m-toluylmorpholine completely prevented bloodsucking. However, the effectiveness of these derivatives was lost when their concentration was reduced to 0.1 mg/ml.

III. DISTRIBUTION OF DEET-C^{14} IN MICE

N-N-diethyl-m-toluamide-carboxy-C^{14} (DEET-C^{14}), with a specific activity of approximately 5 μc/mg was obtained from Nuclear Research, Inc. On request, the manufacturer provided a tracing from a vapor-phase chromatographic analysis (4-ft Apiezon L column, column temperature 195°C, attenuation 10), which showed one peak.

Female Swiss albino mice were injected intravenously with 50 mg of DEET-C^{14} per kg of body weight. The mice were sacrificed by instant freezing in a -78°C mixture of acetone and dry ice. The distribution of radioactivity was studied by radioassay and whole-animal autoradiography.

A. Radioassay Technique

For radioassay, the grossly visible anatomical structures of interest were dissected from frozen mice and homogenized in distilled water by using Teflon-glass homogenizers. Blood samples were drawn by orbital bleeding before sacrifice of the mouse. The homogenate was directly plated on planchets. The amount of tissue sample, determined by the weight after drying the planchet, was less than 8 mg/planchet. The samples were dried at room temperature. The radioactivity was determined as counts per minute by using a Geiger-Mueller open-window gas-flow counter.

Each sample was taken in duplicates and counted at least twice to more than 1000 counts in order to limit the mean probable error due to the random distribution time of the disintegration to less than 3.3%. To determine the background rate, empty planchets were counted. The counting instruments were housed and used in an air-conditioned room.

Radioassay data on the blood drawn from the same mice at several time intervals after intravenous administration of the DEET-C^{14} are given in Table 3. The radioactivity rapidly disappeared from the blood. This may be due to penetration into the tissues and to excretion. In order to ascertain qualitatively the contribution made by the kidney in excreting the radioactivity, renal excretion was prevented in a mouse by ligating the ureters. The radioactivity of the blood drawn from this mouse indicated that the kidney was the major organ of excretion for DEET-C^{14} (Table 4).

The radioactivity of the various tissue samples was calculated on the basis of counts per minutes (CPM) per mg of wet weight (Table 5). These values were corrected for self-absorption and were then converted into ratios by dividing by the radioactivity present in 1 ml of blood of the same animal (Table 6). The data thus corrected showed less variation, since the distribution of the test compound in a particular tissue was calculated in relation to its concentration in the blood.

Table 3

DISTRIBUTION OF RADIOACTIVITY IN BLOOD OF MICE AFTER INTRAVENOUS INJECTION OF DEET-C^{14}

Time after Injection	CPM/ml of Blood		
	Mouse 1	Mouse 2	Mouse 3
2 min	3[illegible]3,887	299,436	410,109
15 min	302,732	302,084	316,999
30 min	247,017	240,370	241,505
1 hr	194,274	103,703	216,160
2 hr	90,463	23,832	265,066
4 hr	21,076	10,484	29,344
8 hr	--	--	1,373
24 hrs	Not significant	519	27

Table 4

DISTRIBUTION OF RADIOACTIVITY IN BLOOD OF URETER-LIGATED MOUSE AFTER INTRAVENOUS INJECTION OF DEET-C^{14}

Time after Injection	CPM/ml of Blood
1 min	306,800
30 min	196,960
1 hr	276,960
2 hr	283,560
4 hr	320,600
5 hr	317,360

Table 5

DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF MICE AFTER INTRAVENOUS INJECTION OF DEET-C^{14}

	CPM/g of Wet Tissue[2]						
	Time After Injection						
Tissue	2 Min	15 Min	30 Min	1 Hr	2 Hr	4 Hr	24 Hr
Lung	233,000	218,000	200,000	61,700	31,900	15,300	N.S.
Heart	176,000	155,000	130,000	34,300	29,900	6,200	N.S.
Spleen	147,000	109,000	121,000	84,500	8,910	6,800	N.S.
Adrenal	203,000	283,000	150,000	43,500	23,300	6,200	N.S.
Kidney	196,000	363,000	496,000	247,000	118,000	60,300	1600
Liver	291,000	320,000	254,000	119,000	524,000	41,200	700
Skelatal muscle	116,000	175,000	93,400	32,100	27,800	5,200	N.S.
Brain	137,000	119,000	85,200	28,500	5,700	2,900	N.S.
Spinal cord	76,500	65,200	51,600	19,100	4,000	3,900	N.S.
Biaphragm	167,000			14,300		8,000	____
Blood[1]	201,630	200,366	199,833	61,266	23,433	17,233	519
Urine[1]	7,500	1,721,000	39,200	1,356,000	6,854,000	--	472,496

[1] Counts per minutes per ml. [2] Mean of 3 Mice.
N.S. = Not significant.

Table 6

DISTRIBUTION OF TISSUE RADIOACTIVITY AS RELATED TO BLOOD RADIOACTIVITY IN MICE AFTER INTRAVENOUS INJECTION OF DEET-C^{14}

	Ratio of CPM/g of Wet Tissue to CPM/ml of Blood[1]							
	Time After Injection							
	DEET-C^{14}							I^{131} Serum Albumin[2]
Tissue	2 Min	15 Min	30 Min	1 Hr	2 Hr	4 Hr	24 Hr	10 Min
Lung	1.15	1.08	0.97	0.98	1.46	0.86	N.S.	.55
Heart	0.87	0.75	0.64	0.55	0.64	0.34	"	.23
Spleen	0.72	0.82	0.56	1.66	0.37	0.41	"	.13
Adrenal	1.00	1.12	0.73	0.84	0.83	0.34	"	.22
Kidney	0.96	1.83	2.49	4.04	5.25	4.05	"	.22
Liver	1.45	1.62	1.34	1.92	2.34	2.45	"	.20
Skeletal muscle	0.57	0.85	0.46	0.57	0.80	0.33	"	.04
Brain	0.68	0.59	0.40	0.39	0.22	0.16	"	.03
Spinal cord	0.38	0.28	0.27	0.32	0.13	0.25	"	.02
Diaphragm	0.80	--	--	0.27	--	--	"	
Urine[3]	0.034	12.0	0.208	26.0	226	--	$>10^3$	

N.S. = Radioactivity was not significant in tissues.

[1]Mean of 3 mice

[2]Lal et al, to be published.

[3]CPM/ml of Urine (1 mouse) CPM/ml of Blood

These ratios were compared with data obtained with RISA-I^{131}. The albumin was distributed only in the vascular compartment of the organs in this time period. Any substance confined to the blood alone should give data comparable to those obtained with albumin. Since the DEET-C^{14} values exceeded those of albumin, penetration into the extravascular compartment of the tissues is suggested.

The radioactivity was highest in the liver and next highest in the lung just after intravenous injection. Fifteen min after injection the radioactivity increased in the Liver, adrenal gland, kidney, and skeletal muscle. All the other organs declined in radioactivity at this time and in all subsequent time periods studied. Thirty min after administration the radioactivity progressively declined except in the kidney. Radioactivity increased in the kidney except at 24 hr after injection when most activity had already passed into the urine. In most instances the brain and the spinal cord contained the lowest radioactivity. Initially the activity in the spinal cord was lower than that in the brain, but with the passage of time this discrepancy narrowed.

In short, the radioactivity after DEET-C^{14} injection was distributed throughout all the organs but rapidly excreted, mainly by the kidney. Some excretion occurred through the gall bladder. Whether the radioactivity in the gastrointestinal tract was due to the excretion from the gall bladder or to direct

excretion by the gastrointestinal tract walls or both cannot be quantitatively determined from the present experiment.

B. Whole-Animal Autoradiography

The mice were frozen 2 min, 30 min, 1 hr, 2 hr, 4 hr, and 24 hr after injection and mounted on microtome stages by using a chilled, saturated solution of carboxymethylcellulose. Counting and sectioning were done in a -8 to -12°C cold room. Sagittal sections, 30 to 30 μ thick, were cut with a heavy microtome (Jung, model K) and taken up on Scotch tape. The frozen sections were freeze-dried and exposed to x-ray film (Kodak dental x-ray film occlusal ultraspeed). Afteradequate exposure, the film was developed and fixed.

The autoradiograms were compared with the original untreated sections. Whenever necessary, the sections were stained for further identification. Sections from mice injected with unlabeled DEET were processed for autoradiography by the above procedure. This assured the absence of any chemical artifact produced by the DEET.

Figures 1 to 5 are photographs from typical autoradiograms. However, the results described below are based upon the examination of many autoradiograms.

Two min after intravenous administration of DEET-C^{14}, radioactivity was found in all the organs studied. The highest radioactivity was in the blood, the nasal secretion, the kidney cortex and the villi of the small intestines, and the next highest was in the liver. The cardiac and skeletal muscle, the brain, and the adrenal cortex were lower than the liver. The bone and the eye lens did not show any radioactivity.

Thirty min after administration, the blood, liver, nasal secretion, urine, and kidney showed the highest radioactivity and the myocardium, skeletal muscle, intestines, skin, and hair follicles showed the next highest radioactivity. The brain, bone marrow, salivary gland, thymus and spleen showed intermediate radioactivity, but the bone did not show any.

Two hrs after administration, the kidney pelvis, gall bladder, and urine showed the highest radioactivity, and the liver, blood, kidney cortex, and intestinal contents showed the next highest. Intermediate radioactivity was found in the cranial nerve trunks. Among the organs of low activity were the brain, spleen, myocardium, skeletal muscle, and bone marrow.

Four hrs after injection, the urine, gall bladder, and kidney pelvis showed the highest radioactivity; the stomach and the intestinal contents were next highest. The liver and skin showed lower activity.

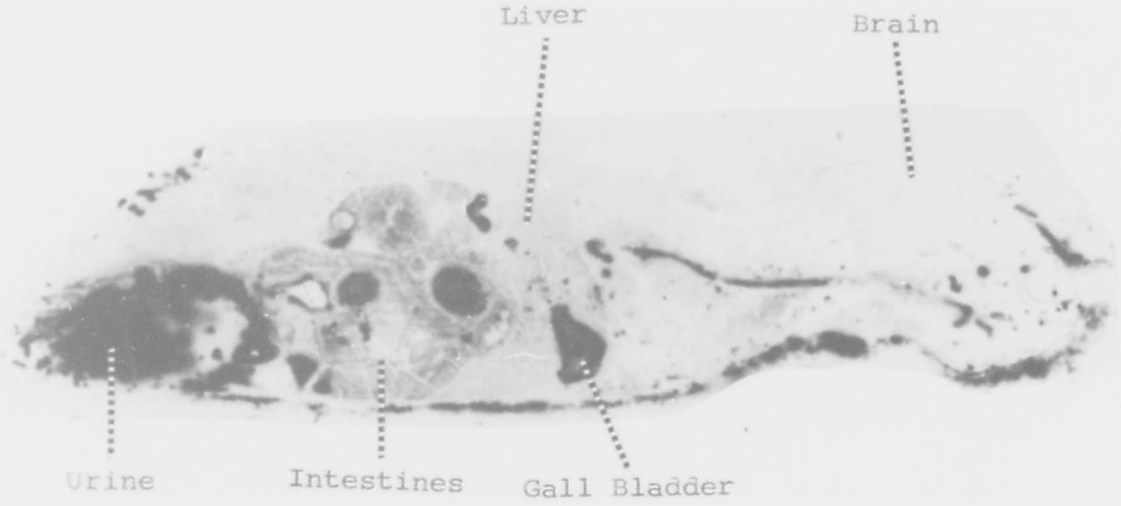

Figure 5. Distribution of Radioactivity 24 Hr After Intravenous Injection of DEET-C^{14} In Mice.

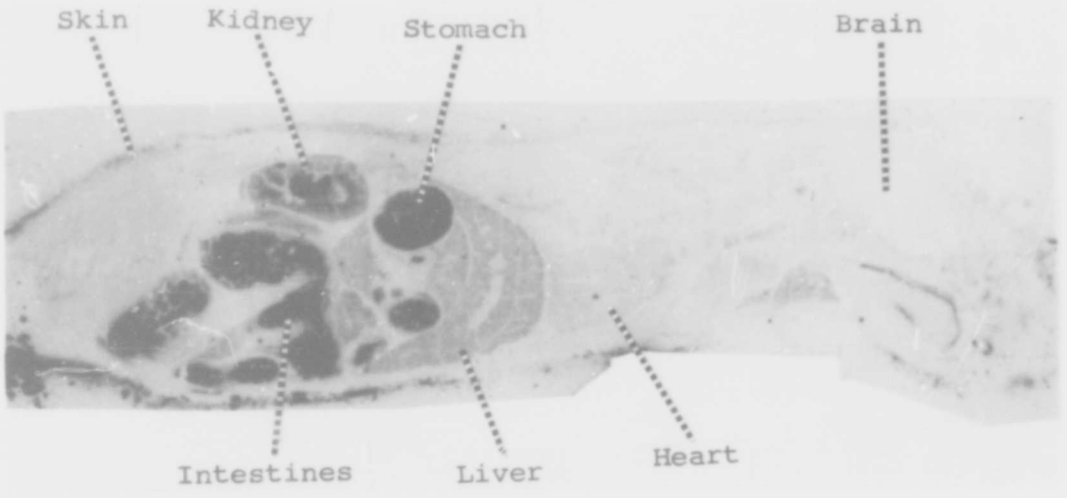

Figure 4. Distribution of Radioactivity 4 Hr After Intravenous Injection of DEET-C^{14} In Mice.

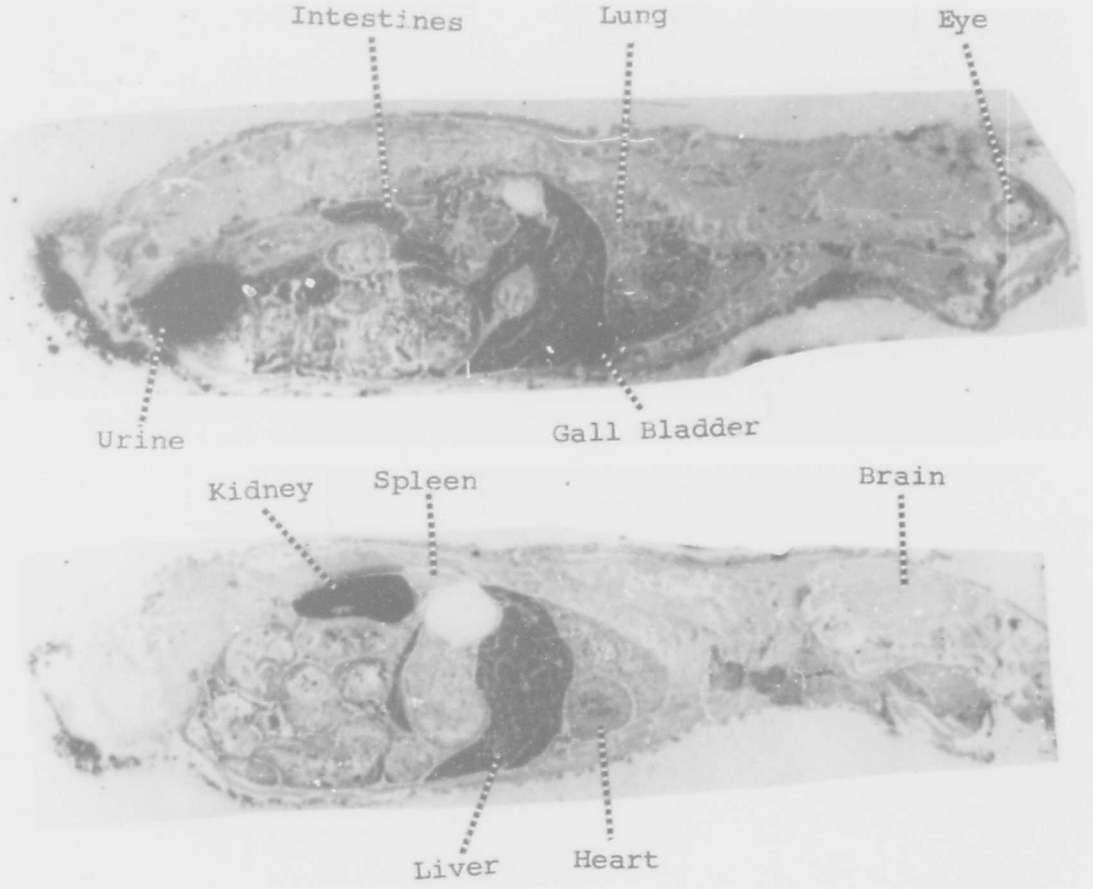

Figure 3. Distribution of Radioactivity 1 Hr After Intravenous Injection of DEET-C^{14} In Mice.

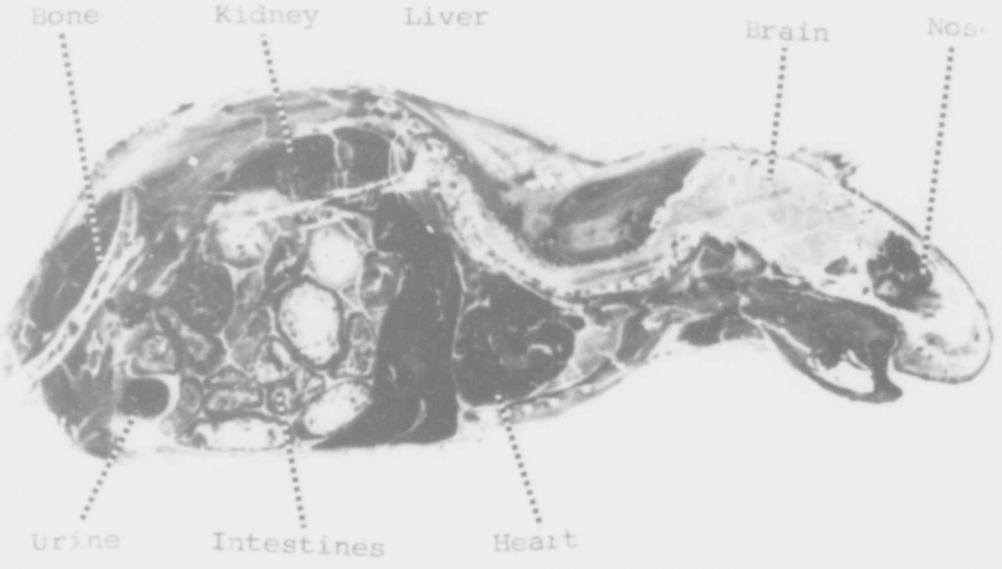

Figure 2. Distribution of Radioactivity 30 Min After Intravenous Injection of DEET-C^{14} In Mice.

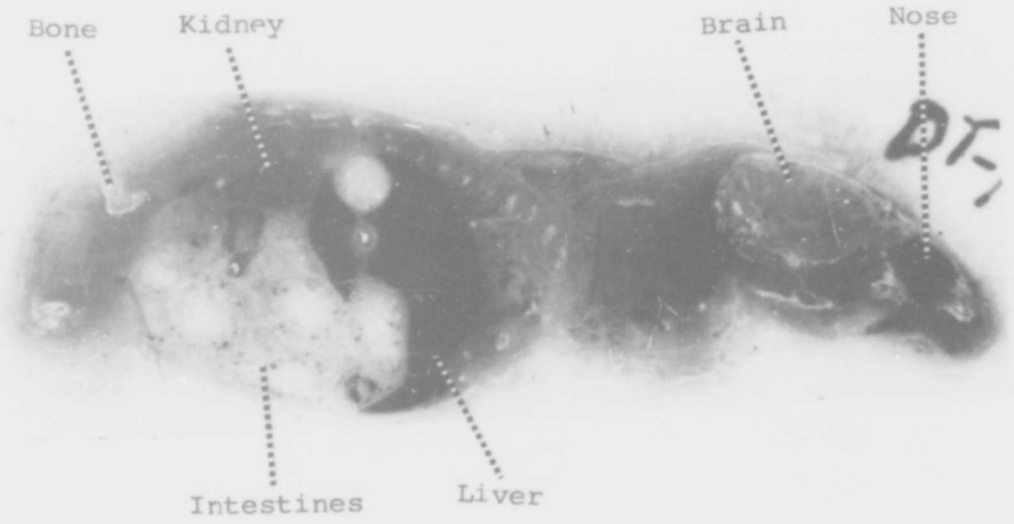

Figure 1. Distribution of Radioactivity 2 Min After Intravenous Injection of DEET-C^{14} In Mice.

Twenty-four hours after administration, the urine showed the highest activity, and the intestines werethe next highest. The liver, skin, and kidney also showed some activity.

IV. CONCLUSIONS AND FUTURE WORK

The benzamide derivatives were potent repellents when applied locally or mixed with blood for in vitro testing. However, when injected intraperitoneally in sublethal doses, no protection against mosquito biting was demonstrated. From the study of the distribution of radioactive diethyltoluamide in mice, it is obvious that the compound is very rapidly excreted from the body. During the period that it is in the body that amount excreted through the skin is not sufficient to repel mosquitoes. This is suggested by the fact that when the concentration in the in vitro tests was reduced to 0.1 mg/ml, the repellent properties of the blood were lost.

Further work on benzamide derivatives will be continued. In addition, several pyrethrine-like compounds will be tested since allethrine showed promising results in earlier work.

Stephen Ginocchio participated in the investigation. The data are recorded in Logbook C13755.

Respectfully submitted,

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HL/lp

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