STUDIES IN TESTING INSECT REPELLENTS [1]

S. KASMAN,[2] L. A. O. ROADHOUSE,[3] AND GEORGE F WRIGHT [4]

The development of synthetic organic chemistry during the past century is due in large part to the development of simple, rapid, and precise chemical analyses. Synthesis proceeds at a rate that is dependent on the application of these methods. However, synthesis of chemicals of biological interest is frequently hindered by the delays and the crudities of some biological assays. In efforts to correlate the functional groups in organic chemicals with their insect-repellent properties, we have encountered such difficulties. For example, the human arm test, in which the forearm covered with 1 gram of repellent is exposed to biting insects (Roadhouse, in preparation), cannot be carried out economically at the rate at which pure chemicals can be synthesized for testing.

The ultimate evaluation of a repellent designed to protect humans against biting insects, especially mosquitoes, must be carried out with human subjects. However, it does not follow that the interim testing, during which the successful repellent is being devised, should be carried out with humans. There are several good reasons why other types of test should be used:

1. Preparation of the 1 gram of unproved repellent required for the human arm test usually entails much more time and expense than preparation of a smaller quantity.

2. The human arm test is time-consuming, since it often requires the services of the test subject during half a day.

3. Individual idiosyncrasy may vitiate the tests unless enough humans are used, in which case the expenditure of candidate repellent becomes inordinately high.

4. Dietary and environmental variations in as complex an animal as the human may cause wide variation in results from individual to individual, and from day to day.

5. Candidate repellents may have unsuspected toxicity.

For several reasons, the use of an olfactometer does not yet satisfy the requirements of a test that would enable a correlation to be made between the results of the test and the chemical groupings so that new repellents might be synthesized. First, it is not a rapid analytical method. Second, it does not take into account the essential function of a repellent as a barrier between the insect and the subject to be protected. It defines only the effect of the repellent vapor, without the surface from which that vapor must arise. Third, it does not define tactile repellency.

The human arm test as it is carried out in the laboratory is deficient as a means of complete evaluation of a repellent, since it does not measure environmental variations such as exposure to sunlight, perspiration, and wind. Because of this deficiency, we believe that it is unnecessary to use humans in the initial laboratory screening tests. Therefore we have sought to use animals that are more easily available for testing purposes, are more uniform, and are expendable for tests with types of substances not previously examined. We have chosen guinea pigs as test animals to ensure reproducibility of results in different laboratories.

---

[1] Contribution No. 3070, Division of Entomology, Science Service, Department of Agriculture, Ottawa, Canada. Studies performed in the laboratories of Science Service, Ottawa, in co-operation with the Defence Research Board, Department of National Defence, Ottawa; and the Department of Chemistry, University of Toronto, Toronto, Canada.

[2] Technical Officer, Defence Research Board, Ottawa, Canada, when these studies were performed.

[3] Associate Entomologist, Veterinary and Medical Entomology Unit, Division of Entomology, Ottawa, Canada.

[4] Professor, Department of Chemistry, University of Toronto, Toronto, Canada.

Not only are guinea pigs useful for the standardized repellency test, but they can be used also for investigation of their attractiveness to insects through change in diet, and may be sacrificed to evaluate the physiological effects of repellents on the skin. The method outlined herein is for mosquitoes, but may be adapted to other biting insects.

TESTING WITH GUINEA PIGS: It is of prime importance that the animal be kept immobile while a definite, fixed area of its back is exposed to mosquitoes. This immobility has been attained by fitting the guinea pig into a tapered 10-inch length of Visking HS sausage casing[5] in which a hole is cut to expose an even surface of skin. In this slippery enclosure the animal soon learns the futility of trying to escape and rests quietly.

For fitting the animal, a 20-inch calibrating length of casing 4⅛ inches in diameter is folded along one side, so that the narrow end of the flat casing is 1½ inches wide, and a 3:14 taper is made along the length. The fold is secured with Scotch tape. The unfolded edge is calibrated in 1-inch divisions with a wax pencil. Smearing of these markings is prevented by covering them with Scotch tape.

The hair is removed completely from the back and sides of the animal between the shoulders and the rump with an electric clipper fitted with a 0000 blade head. It is important that this clipping be as thorough and as close as possible; however, the skin must not be scarred or scratched in the process. Loose hair is brushed away, and the animal with the legs drawn up and hind end first is placed into the calibrating casing. The animal is then shaken firmly into the tapered calibrating casing (Fig. 1). The center of the circular area to be exposed and the position of the end of the rump are recorded to within 1/10 to ⅕ inch.

The pig is removed and a fresh 10-inch length of casing is laid with its edge even with the markings on the calibrated casing, which has its tapered edge toward the right side of the operator. The right-hand end of the short length of casing is made to overlap the recorded position of the end of the rump by 1 inch. The lower right-hand corner of the short length of casing is then folded to correspond with the taper of the calibrating casing, and is secured to itself with Scotch tape. (If the diagonal crease extends the full 10-inch length of the casing, then it should be abandoned in favor of the next smaller diameter of casing.) A semicircular pattern, 6.68 cm. in diameter (the circular exposure area is 35 sq. cm.), is then placed on the upper, straight edge of the casing with its center coinciding with the recorded position of the area to be exposed, as measured by the calibrating casing. The casing is then cut so as to provide the aperture of 35 sq. cm. Finally a closing fold of ½ inch is made and taped at the narrow end of this 10-inch length of casing; a small triangle is cut out at the lower corner for disposal of urine and feces.

The animal is slipped into the prepared casing in the same position that it was fitted into the calibrating casing, care being taken that its backbone lies along the line of the natural crease of the casing. The pig must be shaken firmly into place; if the casing splits, it may be reclaimed for use by repair with Scotch tape. The guinea pig in the casing is held during the test in one of two troughs constructed from two pieces of cardboard. These pieces are cut as shown in Figs. 4 and 5. The flaps of the larger pieces are folded and stitched at coinciding edges and the seams covered with Scotch tape. The smaller piece of cardboard is then bowed along the long axis and fitted inside this box with its straight end against the rectangular back end of the box; the re-

[5] Visking High-Stretch casing (Visking Ltd., Lindsay, Ont.) is packaged in 200 flat lengths, each 20 inches long. For economy, these lengths are normally cut in half, although the occasional active or large animal may require a 12-inch rather than a 10-inch length. The available ones useful for this test are those 3¼, 3¾, and 4⅛ inches wide. These diameters accommodate animals weighing between 350 and 1000 grams.

entrant shape of the front end of the box thus forms it into a trough, which is stitched and taped into position. These troughs maintain the guinea pig at an angle of 30° from the horizontal position so that the animal is unable to shift position or to climb out of the casing. One of the troughs is kept inside the mosquito cage, and the other serves for repose outside the cage during the period between the exposures to mosquitoes.

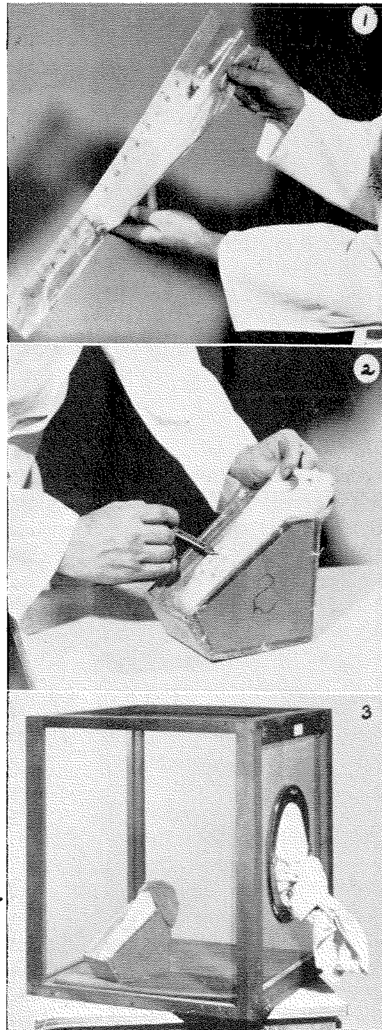

FIG. 1. Measuring of guinea pig in calibrating casing.

FIG. 2. Applying drops of repellent to exposed area of skin.

FIG. 3. Guinea pig in completed assembly in testing cage.

The casing-enclosed animal in a trough is treated with 0.05 ml. of repellent from a 0.25-ml. hypodermic syringe equipped with a fine (No. 28) needle. The repellent is distributed in about 10 small drops symmetrically on the exposed area of animal skin (Fig. 2). The drops of repellent are then spread rapidly and evenly with the finger tip; the periphery of the area must be covered slightly underneath the casing in order that the mosquitoes will not find an untreated surface.

The open end of the casing is then covered with a length of women's nylon hose to protect the guinea pig's face and ears from the mosquitoes (while still allowing access of light and air) so as to ensure its repose. The assembly is quickly transferred to the trough inside the mosquito cage (Fig. 3), which contains about 1000 males and females of *Aedes aegypti* (L.) in approximately equal numbers.

An initial 3-minute exposure is then made, and the number of landings and the number of bites involving ingestion of blood are recorded. The animal is then removed to the trough outside the cage, where it remains for 27 minutes until the next exposure. The test is finished when three bites are observed within one, two, or three consecutive 3-minute exposure periods.

The validity of the test obviously depends on the activity of the mosquitoes and the avidity with which the females seek a blood meal. When more accurate evaluation is desired, the guinea pig is exposed without repellent, and the biting rate (30–90 per minute) is determined about one minute after the animal is inserted into the cage. Ordinarily we dispense with this evaluation; instead, the activity of the mosquitoes is judged by the number that land on the treated area but do not bite. A landing rate of 15–35

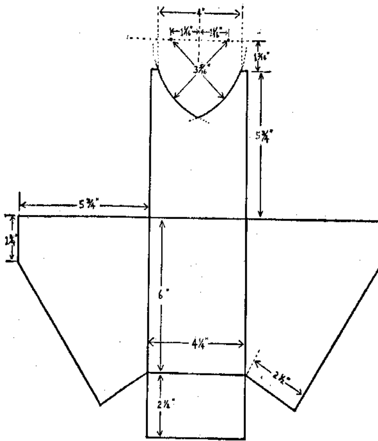

Fig. 4. Diagrammatic pattern of outer part of box for retention of guinea pig in tests of repellents.

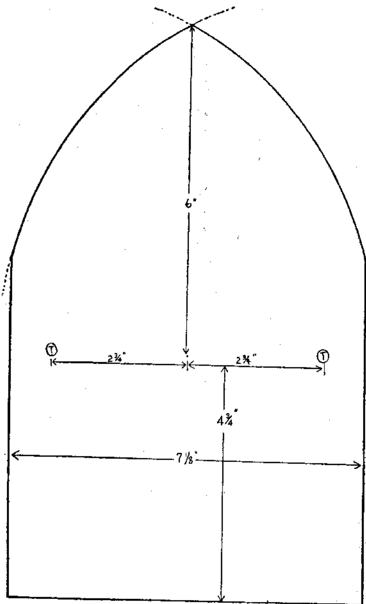

Fig. 5. Diagrammatic pattern of trough of box for retention of guinea pig in tests of repellents.

per minute is considered satisfactory. Only when this rate drops to 10 per minute are the mosquitoes considered to be too sluggish or satiated for reliable results. Until a precise differentiation between vaporous and tactile repellency is desired, this criterion seems satisfactory.

Evaluation of Repellency Time: With mosquitoes, a single bite within the test exposure of 3 minutes is not considered indicative of the breakdown of protection. It may be due to resistance of a single hardy mosquito. On the other hand, the failure of the repellent may become appreciable before the next test period occurs. Consequently the time, T, during which the repellent is considered effective is calculated according to the equation

$$T = (30)n + 30/(b + 1)$$

where n is the number of 3-minute exposures before a single exposure with at least 3 bites or before successive exposures with at least 3 bites, and b is the number of bites that occur during the first exposure that contributes to a total of at least three bites within that or succeeding exposures. During this evaluation, care must be taken not to count a group of mosquitoes all biting in a short line or space. Such behavior is almost certain evidence of uneven distribution of repellent and the test should be repeated. If three bites occur in the first exposure, T equals zero.

Determination of Amount of Repellent: Since all repellents used in this study are liquids, the amount of repellent is expressed in terms of volume. The variation in time of protection according to the volume of repellent distributed on the guinea pig has been determined for dimethyl phthalate. This was accomplished by applying various volumes of dimethyl phthalate to the exposed area of the guinea pig. Some of the smaller volumes were applied as 10 per cent solutions in 95 per cent ethanol to facilitate even distribution of the substance; larger volumes were applied as the pure liquid. Plotting the repellency times against the

corresponding volumes of dimethyl phthalate used, showed that the relationship was approximately linear up to the 0.05 ml. volume and then it tended to level off. The 0.05 ml. volume gave protection for approximately the same time as in the arm test and is the smallest volume that can be readily applied evenly on the exposed area of the guinea pig.

COMPARISON OF HUMAN ARM WITH GUINEA PIG TEST: More than 100 compounds have been used in a study of the two tests. For many of these the repellency times were too small to afford a valid comparison. The remainder are listed in Table 1, with physical constants given in Table 2 when it has been necessary or feasible to determine them. The evaluation of repellency by the arm test has been reported elsewhere (Roadhouse, in preparation), and the values obtained have been used for comparison when considered reliable. In general, the protection time for guinea pigs is slightly less than for the human arm. This difference is not unexpected in view of the higher body temperature of the small animal (Oppenheimer and Pincussen, 1925, pp. 376–377),

TABLE 1.—Comparison of repellents in human arm and guinea pig tests

Expt. No.	Compound	Boiling point °C	mm.Hg.	Protection time min. Human arm	Guinea pig
G-1	dimethyl phthalate	136.5–138.5	8	200	185
G-2	2-methyl-1-phenyl-2-hydroxy-propanone-1	119–121	11	185	160
G-3	4-phenyl-1,3-dioxane	177–179	104	245	186
G-4	ethyl 2-hydroxydecanoate	110–115	7.5	160	125
G-5	ethyl mandelate	118–120	7	275	300
G-6	benzyl lactate	122–125	7	345	280
G-7	1-phenyl-1-hydroxypropanone-2	115–117	7	335	345
G-66	1-phenyl-2-hydroxypropanone-1	108–110	7	330	225
G-9	1-phenyl-1-methylpropanediol-1,2	ca. 130	7	305	338
G-11	cinnamyl acetate	128	7.5	120	135
G-12	1-phenyl-1,3-diacetoxypropane	153–154	7	5	0
G-14	1-phenylpropanediol-1,3	92–94	7	135	95
G-15	ethyl 2-methoxyphenylacetate	ca. 128	8	105	147
G-16	2-ethylhexanediol-1,3	119.2–120.4	7	200	244
G-17	n-butylacetanilide	128.5–129.0	7	375	375
G-18	dicyclohexylketone	133.0–134.5	8	340	310
G-19	dicyclohexylcarbinol	m.p. 35°		0	0
G-20	saturated solution of G-19 in G-18			187	186
G-22	70% 2-cyclohexylcyclohexanol + 30% 2-phenylcyclohexanol	123.9–128.6	7	345	345
G-23	2-allyl-4-methoxyphenol	127.2–128.0	7	405	370
G-24	2-propenyl-4-methoxyphenol	136.2–140.2	7	400	375
G-25	2-allylhydroquinone dimethyl ether	108–110	7	156	127
G-26	tert-butylhydroquinone dimethyl ether	108.1–110.2	7	0	0
G-27	1-phenyl-2-methylpropanol-2	94–94.5	12	95	65
G-42	ethyl phenoxyacetate	131–133	13	45	75
G-43	1-phenyl-2-methyl-2-methoxy-propanol-1	ca. 125	14	188	160
G-49	geraniol acetate	122–124	16	75	45
G-50	linalyl acetate			5	45
G-52	benzyl glycolate			165	165
G-55	2-phenylcyclohexanol	142–146	15	165	135
G-59	hexahydro-4,4-endomethylene-indanone-5	112	15	35	15
G-63	2,2,4-trimethyl-1-acetoxy-pentanol-3	118–119	15	115	66
G-64	N,N-dicyclohexylimidazolidine	125–126	0.5	460	465
G-68	1,4-diacetoxydecahydronaphthalene	179	16	38	46
G-69	ethyl isopentylacetoacetate	135	16	75	45
G-70	2,2-dimethylpentanediol-1,5	ca. 147	16	0	0
G-71	2,2,4-trimethylpentanediol-1,3	123	16	310	315
T-50	eugenol methyl ether			40	100
T-90	benzyl 2-acetoxydecanoate			98	65
T-91	benzyl 2-acetoxypropionate			105	96
T-104	cinnamyl alcohol			248	225
T-181	1-phenyl-2-methylpropanediol-1,2			98	40
T-187	1-phenyl-2-methylpropanol-1			128	96
T-203	2-N-n-butylaminocyclohexanol			150	128

TABLE 2.—Refractive indices and densities of compounds G–1 to G–18 in Table 1

Expt. No.	Refractive Index*	Density**
G–1	1.5159	——
G–2	1.5314	$D^{24.2}$ 1.023
G–3	1.5288	$D^{25.1}$ 1.110
G–4	1.4372	$D^{25.6}$ 0.928
G–5	1.5141	$D^{24.9}$ 1.114
G–6	1.5147	$D^{24.5}$ 1.116
G–7	1.5356	$D^{25.3}$ 1.102
G–66	1.5361	$D^{22.7}$ 1.100
G–9	1.5361	$D^{25.2}$ 1.062
G–11	1.5389	——
G–12	1.4951	$D^{22.3}$ 1.098
G–14	1.5312	$D^{24.9}$ 1.019
G–15	1.5022	$D^{25.5}$ 1.113
G–16	1.4512	——
G–17	1.5148	$D^{26.5}$ 0.987
G–18	1.4845	$D^{25.3}$ 0.961

* Taken on *D* line at 20° C.
** At degrees Centigrade in comparison with water at 0° C.

since heat is an attractant to mosquitoes. Fig. 6 shows that these data are best described by the equation

$$T_p = T_a - 20$$

where T_p and T_a are protection times in minutes for the guinea pig and arm tests respectively. Though a few of the values differ by as much as 50 minutes, Fig. 6 shows that the majority of the values are included within the terms of this equation.

FATE OF THE APPLIED REPELLENT: A repellent may lose its effectiveness in three ways: it may evaporate, it may be absorbed through the skin, or it may be destroyed on the skin. These possible modes of loss have been studied in the guinea pig test.

The approximate rate of evaporation of dimethyl phthalate was determined by evaporation from filter paper. Whatman No. 50 (50 mm.) papers were suspended

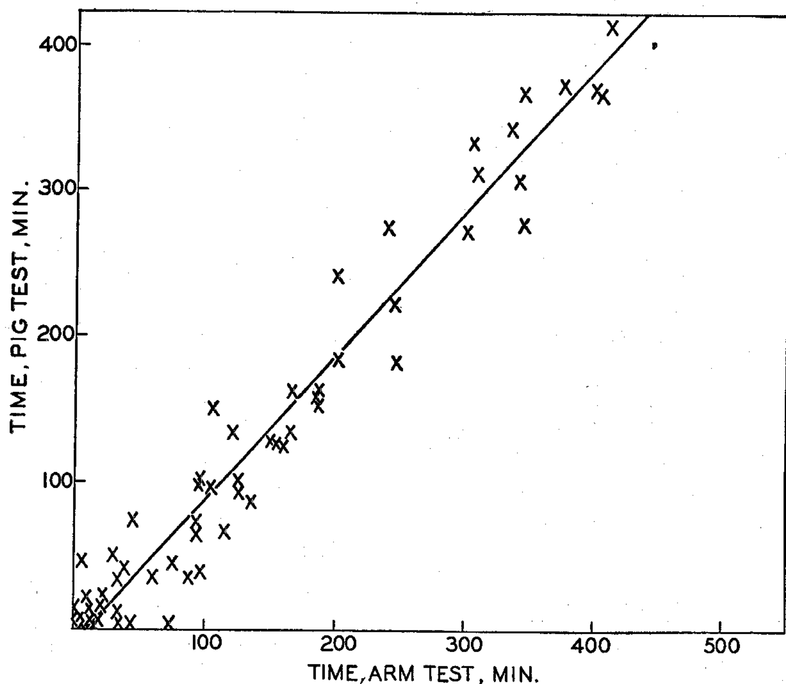

FIG. 6. Repellency times in guinea pig and human arm tests of various repellent compounds.

overnight from fine piano-wire hooks in an oven at 37° C. They were then allowed to cool to room temperature and weighed. Various amounts of dimethyl phthalate were then applied near the hooks and allowed to soak into the paper. The discs were reweighed, replaced in the oven, and withdrawn at intervals for reweighing to determine loss by evaporation. Table 3 summarizes the averages of five concurrent determinations.

The rate of evaporation is approximately linear for the first 300 minutes, indicating that it depends on the area of the surface and not on the quantity of repellent present. The data may be expressed by the equation

Weight lost by evaporation (in milligrams) = 0.0174 T (in minutes)

where the proportionality constant is a modulus of surface area, vapor pressure of dimethyl phthalate at 37° C., and adventitious factors such as amount of air circulated past the surfaces, structure of filter paper fiber, and room temperature during weighing periods. Despite these factors, which should tend to increase the evaporation rate by comparison with that occurring from the skin, it may be seen (G-1, Table 1) that the rate of loss by evaporation is much lower than the loss of protective action by dimethyl phthalate on the back of the guinea pig or on the human arm. Table 3 indicates that the loss by evaporation from filter paper is

$$\frac{(22.3 - 17.7) \text{ mg.}}{(265 \text{ min.}) (3.14 \times 2.5^2 \times 2 \text{ sq. cm.})} = 0.00044 \text{ mg. (min./sq. cm.)}$$

whereas the weight of dimethyl phthalate applied per square centimeter on the back of the guinea pig is

$$\frac{1.19 \text{ (density of DMP)} \times 0.05 \text{ ml.}}{35 \text{ cm.}^2 \text{ (area of skin)}} = 1.7 \text{ mg./cm.}^2$$

On the basis of evaporation, then, dimethyl phthalate should give protection for 1.7/0.00044, or 3900, minutes. Since this is about 20 times the observed duration of repellency, the loss of protection must therefore be due to hydrolysis of the ester or to absorption through the skin.

TABLE 3.—Evaporation rates of dimethyl phthalate

Time min.	Weight of dimethyl phthalate mg.	Loss %
0	22.30	0
107	20.67	7.2
190	18.92	15.1
265	17.70	20.6
350	17.24	22.6
1397	3.69	93.4

To evaluate these losses, the normal benzoic acid content in the urine of a guinea pig, collected during 24 hours in a metabolism cage, was determined. Then 0.25 ml. (0.277 g.) of 1-phenyl-2-hydroxypropanone-1 (G-66, Table 1) was placed on the shaved back of the guinea pig, and the urine was collected during the next two 24-hour periods. The urine collected during one of the periods was made alkaline with 10 ml. of 0.4 N aqueous sodium hydroxide, and then warmed for 2 hours at 85° C. to hydrolyze any hippuric acid that may have been present. The hydrolyzate was filtered, acidified with a 4-ml. excess of 7 per cent hydrochloric acid, and then steam-distilled to collect 400 ml. of distillate. This distillate was extracted four times with a total of 60 ml. of chloroform. Evaporation of the chloroform left negligible residue from the samples collected during the 24 hours before application of repellent and during the second 24-hour period after its application. On the other hand, the residue from the urine sample collected during the 24-hour period after application of repellent was appreciable. It was sublimed at 750 mm. to yield 10 mg. melting at 112–118° C. This sublimate was proved by mixture melting point to be benzoic acid after it was resublimed to melt at 119–121° C. A 10-mg. yield represents 4.5 per cent of that theoretically available from the oxidation of 1-phenyl-2-hydroxypropanone-1. If this benzoic acid is the metabolic product from the ketone (G-66,

Table 1), absorption of repellent through the skin is a significant factor of loss.

INFRA-RED SPECTRA OF INSECT REPELLENTS: It has been suggested (Miles and Beck, 1947) that a substance is odorous because its vapor passing among the olfactory hairs selectively absorbs in the infrared spectral region so as to disturb the thermal equilibrium in the olfactory region. This disturbance is thought by Miles and Beck to initiate olfaction.

Since repellency must in part be related to the olfactory sense, we have partially tested this hypothesis by examination of the infra-red spectra (25μ—16μ) of the substances listed in Table 1 for which boiling point, refractive index, and density are reported. The Baird spectrograph used by Dr. Charles Hubely of the Defense Research Board (to whom we are indebted for these spectra) is not equipped for study of vapors, so that it must be assumed that spectra of the liquids are indicative of those in the vapor state. With this limitation we can affirm that no absorption band is present among these substances that is common to them all, or that is related to intensity to the observed insect repellencies of the compounds.

SUMMARY: A method of screening compounds as insect repellents with guinea pigs as the test animals is described. The repellency values obtained with the guinea pig are approximately the same as those obtained from human arm tests. The advantages of this technique are that a smaller quantity of the candidate material is required for each test and toxicity to the host is not a major consideration. In tests of dimethyl phthalate with guinea pigs more repellent was lost by absorption through the skin than by evaporation. Infra-red spectrographic analyses of several compounds in liquid form were of no value in predicting their effectiveness as insect repellents.

ACKNOWLEDGMENTS: The authors are grateful to Dr. C. R. Twinn, Head, Veterinary and Medical Entomology Unit, for assistance in the direction of these studies and in the preparation of the manuscript.

References

MILES, W. R. & L. H. BECK. 1947. Infra-red absorption in field studies of olfaction in bees. *Science*, 106:512.

ROADHOUSE, L. A. O. In preparation. Laboratory studies on insect repellency.

OPPENHEIMER, C. & L. PINCUSSEN [editors]. 1925. Reine und physiologische Physik. Physikalische chemie und biologische Anwendungen. Tabulae Biologicae 1. W. Junk, Berlin.

MOSQUITO SURVEY OF GUAM

STEPHEN M. K. HU, Sc.D.

Chief, Bureau of Mosquito Control, Department of Health, Territory of Hawaii

Since Hawaii became a crossroads for planes as well as the many ships of the Pacific, the island of Guam is only twelve hours away from our Territory. Hawaii is only nine hours by plane to California. Faster transportation increases the danger of the spread of diseases as well as of the vectors of diseases from the Pacific region to our West Coast.

A survey trip was taken from July 11 to August 18, 1952 to Guam and the Trust Territory.

On the way to Guam, brief stops were made on Midway and Wake Island. Both of these islands were once infested with mosquitoes until Hawaii's mosquito control personnel started a clean-up campaign for the Civil Aeronautics Administration.