KNOWN FACTORS CAUSING VARIATIONS IN RESULTS OF INSECT REPELLENT TESTS [1]

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Before the work of Granett (1940) techniques for testing insect repellents showed little uniformity. Since that time most investigators have attempted to standardize their methods and, with slight modification, have accepted the method used by Granett. English workers (Christophers 1947) paid special attention to the standardization of testing conditions, and the effectiveness of different materials was compared on the basis of number of bites received at selected intervals rather than on time to the first bite as in the Granett technique. Travis (1947) used this same method of comparison.

Although the results of tests have varied considerably, it has been possible to study and compare these variations and to determine some of the factors causing them. Most of the data presented in this paper were assembled from records at the Orlando, Fla., laboratory of the Bureau of Entomology and Plant Quarantine. These data will help the reader to understand better why there have been such wide variations in the results of tests previously published by the laboratory.

A review of the literature shows that wide ranges in repellent times seem to be a characteristic of tests with repellents against biting insects. Pijoan *et al.* (1946) made some special tests in which the range in repellent time was small, but the urgency of the search for better repellents during the last war did not permit delays to investigate such small variations.

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Most of the tests discussed in this paper were made with the standard repellents—6–12, dimethyl phthalate, Indalone, and mixture 6–2–2 (6 parts of dimethyl phthalate and 2 parts each of 6–12 and Indalone). The repellents were applied on research subjects at the rate of ¼ teaspoonful to the arms (elbow to wrist) and ⅜ teaspoonful to the legs (knee to ankle). In laboratory tests only the arms were treated and exposed at intervals of 20 or 30 minutes until a bite was received. In the field both arms and legs were treated and exposed continuously in areas with high insect populations. Normally the tests were not terminated until there was at least a second bite. Under cage conditions the biting rates on an untreated arm were 50 to 75 per half minute for *Aedes aegypti* (L.) and 5 to 20 per half minute for *Anopheles quadrimaculatus* Say. Except for a few tests, field tests against *Aedes taeniorhynchus* (Wied.) and *Aedes sollicitans* (Walk.) were not conducted unless the biting rate was 20 or more per half minute on an untreated leg; rates of 100 + were common. Against *Stomoxys calcitrans* (L.), the biting rates were 38 to 94 per half minute. All materials were evaluated on the basis of the repellent time (time from application of the chemical to the first bite).

Throughout the testing program there was close liaison between the laboratory and Philip Granett, who up to the war period had conducted the most precise repellent tests. When a repellent was compared with the same standard in both laboratories, the repellent ratio was usually the same, even though Granett made most of the tests on his own arms and had long repellent times, and the tests at Orlando were made on the arms of several individuals and the repellent times were shorter.

Arm-and-Leg Tests.—In field tests it has been customary to treat both the arms and legs of test subjects to increase the number of materials a given group could evaluate. An adjustment for the difference in the size of the limbs was made by applying one and a half times as much repellent to legs as to arms. Exposures were equalized by having the subjects squat every few minutes and hold their arms at the same level as the legs.

A statistical analysis of the data from the first field trip showed that there were no significant differences in the results obtained on the different limbs. This observation was confirmed by applying Indalone to both arms and legs of six subjects and exposing the appendages to *Aedes sollicitans*. The average repellent time for these tests was 143 ± 18.8 minutes for the arms and 146 ± 19.0 minutes for the legs. The difference required for significance was 44 minutes. The data from other field trips were not analyzed statistically, but at no time were variations noted that could be attributed to the different limbs.

Differences Between Subjects.—Early in the testing program repellents were found to be considerably more durable on some subjects than on others. Perhaps the most striking difference was observed in the tests with dimethyl phthalate against *Aedes aegypti*. Although the average repellent time for dimethyl phthalate was 247 minutes in 3,406 laboratory tests against this species, three subjects were located who were only partially protected. These subjects always received a few bites immediately after treatment. No other extreme differences were observed with any other subject or against any other species of biting insect.

Table 1 includes data from tests against four mosquito species. Since the tests were not made specifically to demonstrate differences between subjects, the 6 subjects analyzed were chosen because they had the largest series of records. Against *Aedes aegypti* and *Anopheles quadrimaculatus* tests with dimethyl phthalate provided data over a 2-year period. During that time the same 3 subjects exposed arms treated with dimethyl phthalate on 20 different days to *Aedes aegypti* and on 28 days to *Anopheles quadrimaculatus*. Against *Aedes taeniorhynchus* no records were available for large series of tests in which the same subjects were treated with the same repellents; therefore a field trip was selected in which each of 4 subjects was treated with the same 10 repellents. Included in this series were one subject on whom the repellents were effective for only a short time, one subject on whom repellents were very lasting, and two who were considered average. Data on the *Aedes sollicitans* mentioned in the arm-and-leg tests are also summarized.

Repellent time in the *aegypti* tests varied so much that the difference between subjects was just under statistical significance; with *Anopheles quadrimaculatus* the difference between subjects was significant. One subject, however, experienced the shortest repellent time in tests against both *Aedes aegypti* and *Anopheles quadrimaculatus*, the respective averages being 202 and 118 minutes. In the *taeniorhynchus* tests considerable difference existed between subjects, which included two men who usually provided the extremes for the repellent range in a group of tests. One man had an average repellent time of 298 minutes, which was 2.2 times as long as the "short-term" man, who had an average of 133. The greatest difference between men occurred in the *sollicitans* tests, the average repellent time on one man being 267 and on another only 80 minutes.

This chemical had been tested early in the program and was found to show little repellency against *Aedes aegypti*. Since it was a poor repellent and a good organic solvent, it was used to dissolve various solid materials for testing as repellents. After a number of solids suddenly appeared effective, it was discovered that a second lot of this chemical was more effective as a repellent than the first. In three paired tests with the two lots, the first lot gave an average repellent time of only 21 minutes whereas the second was effective for 132 minutes.

REPELLENT TIME BASED ON FIRST AND THIRD BITES.—Although it has been customary to evaluate repellents on the time to the first bite, at Orlando tests are normally continued in the laboratory to the second bite and in the field to the third bite. An analysis of the data, however, indicated that results were only slightly more precise when they were evaluated on the basis of bites subsequent to the first. Therefore all the repellent records are based on the time to the first bite. Data to demonstrate this point are taken from a field trip in which 12 subjects were treated with the same 3 repellents—2-phenylcyclohexanol, 2-cyclohexylcyclohexanol, and a mixture of these two. The average repellent time and standard error to the first bite were 183 ± 20, 148 ± 18,

TABLE 1.—Variations in repellent time against mosquitoes on different subjects treated with dimethyl phthalate, except as noted.

Species	*Tests on each subject*	*Average repellent time (in minutes) on indicated subjects*							*Difference required for significance*
		1	*2*	*3*	*4*	*5*	*6*	*All*	
	Number								*Minutes*
Anopheles quadrimaculatus	28	145	118	229	...	...	...	164	49
Aedes aegypti	20	295	202	287	...	...	...	261	61
Aedes sollicitans	4	117	267	147	105	150	80	144	77
Aedes taeniorhynchus [1]	10	298	133	209	208	...	...	212	60

[1] Tests with 10 repellents.

SUBSAMPLE DIFFERENCES. — Although different subsamples of the same chemicals frequently varied in effectiveness, the most striking variation of this type was experienced with two lots of diethyl phthalate.

and 173 ± 21; to the third bite 215 ± 17, 200 ± 20, and 201 ± 18. In neither case was there any significant difference in effectiveness between the repellents, and they remained in the same order of effectiveness regardless of which bite was used for the evaluations. In addition, the variations between tests were only slightly less for the third than for the first bite.

EFFECT OF SWEAT.—In tests made on subjects wet with sweat D. M. Jobbins (personal communication), Pijoan *et al.* (1946), and the Orlando group found that repellent time was much longer on dry skin than on moist skin. In fact the subjects on whom repellents were not durable were usually those with a moist skin; likewise, the repellents were usually more durable on subjects with dry skin.

In comparative tests at Orlando with some of the more promising compounds on sweaty and moist skin, the subjects for the sweat tests remained in an air-conditioned room maintained at 90° F. and 90 percent relative humidity, and exercised at intervals to maintain an extreme sweat condition. These tests were made during July, August, and September of 1945, and because of the prevailing temperatures and humidities there was little difference between the sweaty and the moist skin.

The results with the different repellents against *Aedes aegypti* were too variable to permit a general conclusion (table 2). The greatest reduction in repellent time that might have been attributable to sweating was obtained with diisopropyl tartrate.

With five of the compounds a longer repellent time was obtained on wet skin than on moist skin, but with at least two of these materials, isobutyl sulfone and 2, 5, 7-trimethyl-3-octyne-2, 5-diol, the differences were clearly not significant. Greater differences in repellent time could be expected under more contrasting conditions.

Tests were made against *Anopheles*

TABLE 2.—Protection obtained against *Aedes aegypti* with various repellents applied to wet skin.

Repellent	Tests on wet skin [1]	Repellent time	
		Average	Reduction under moist-skin tests
	Number	Minutes	Percent
Acetoacetic acid, cyclohexyl ester	8	84	Increase
Anisyl alcohol	4	141	14
Bicyclo [2.2.1]-5-heptene-2,3-dicarboxylic acid, *cis-*, dimethyl ester	6	148	36
Cinnamic acid, isopropyl ester	4	76	38
Cyclohexaneacetic acid, alpha-cyano-, ethyl ester	4	138	31
Cyclohexanecarboxylic acid, 1-hydroxy-, cyclopentyl ester	2	143	6
1,2-Cyclohexanedicarboxylic acid, diethyl ester	4	164	7
m-Dioxane, 4-(*p*-methoxyphenyl)-5-methyl-	4	118	55
1,3-Dioxolane, 2-hexyl-4-methoxymethyl-	4	133	40
1,3-Dioxolane, 5-methyl-5-nitro-2-propyl-	2	150	25
Ethanol, 2,2'-thiodi-, diacetate	4	152	36
Hydracrylic acid, beta-phenyl-, ethyl ester	6	122	40
Hydrocinnamic acid, alpha,beta-epoxy-beta-methyl-, ethyl ester	10	101	47
Isobutyl sulfone	2	158	Increase
dl-Malic acid, dibutyl ester	4	144	63
Mandelic acid, ethyl ester	4	108	52
2-Naphthol, 1,2,3,4-tetrahydro-	3	192	28
3-Octyne-2,5-diol, 2,5,7-trimethyl-	2	187	Increase
Phthalic acid, dimethyl ester	32	91	52
Phthalimide, *N*-butyl-1,2,3,6-tetrahydro-	6	171	Increase
Propionic acid, diester with 1,5-pentanediol	4	137	14
Succinamic acid, *N,N*-diethyl-, propyl ester	2	143	49
Succinamic acid, *N,N*-dipropyl-, ethyl ester	4	153	Increase
Succinic acid, alpha-cyano-beta-methyl-, diethyl ester	2	173	21
Tartaric acid, diisopropyl ester	5	84	65

[1] With most of the materials several times as many tests were run on moist skin as on wet skin.

quadrimaculatus with the same materials, but because the results were so variable it was concluded that sweating made little or no difference on the effect of the repellents.

Differences Between Broods.—Although the repellent time of a given compound varied considerably in the laboratory, little divergence from the average was seen in the field until the second year of the testing program. In June 1943 the average repellent time was 214 minutes for a total of 35 tests with dimethyl phthalate against *Aedes taeniorhynchus.* A month later, and after a new brood of mosquitoes had emerged, the average was only 50 minutes for 25 tests with the same species and the same repellent. None of the other repellents were very effective against this brood and low repellent times prevailed until another large brood emerged in September. With the September brood, dimethyl phthalate was again effective, with an average of 213 minutes, and the repellent times for other compounds were similar to previous averages. Low repellent times were experienced also with all repellents, including the standards, at least one time in 1944 and in 1945. Biting rates and meteorological conditions were comparable during all these tests. The only explanation that can be given for these variations in repellent times is that the broods of mosquitoes differed in their reaction to repellents.

In the laboratory *Aedes aegypti* showed considerably less variation than *Anopheles quadrimaculatus.* When the average repellent times for 12 bimonthly periods were compared, *Aedes aegypti* had a pooled average of 251 ± 11 minutes and *Anopheles quadrimaculatus* of 134 ± 17 minutes.

Tests were made in which the larvae and adults of *Anopheles quadrimaculatus* were reared and fed in different ways to discover the causes for variations in the repellent times. No encouraging leads were obtained, and from time to time, in spite of any changes in rearing, feeding, and testing techniques, the repellent time would vary widely from the average. Terzian and Stahler (1949), however, reported differences in the biting rates of *Anopheles quadrimaculatus* when they varied (1) the proportions of males to females in the test cages and (2) the population density of the larvae in the rearing pans. Usually the periods of either long or short repellent times extended over several days to several weeks and were not a day-to-day variation.

Biting Rates.—The effect of different biting rates on repellent times was mentioned by Bacot and Talbot (1919) but Granett (1938, 1940) demonstrated that repellent times decreased as biting rates increased, both in the laboratory and in the field. Therefore, repellent tests were not conducted in the Orlando laboratory unless the biting rates were high.

In one series of field tests the standard repellents were used against *Stomoxys calcitrans,* when the biting rate was only 1 to 4 per minute on an untreated leg. Most of the repellents were still effective after 5½ hours, when the tests were terminated. A few weeks later the same materials were tested against a population of flies sufficiently high to give biting rates of 38 to 94 in half a minute. Indalone was the only material that remained effective for as long as 2 hours. Dimethyl phthalate and 6–12 failed in less than an hour.[3]

A similar incident occurred in a field test against *Aedes taeniorhynchus* when biting rates were from 10 to 13 per minute. Tests were made with several mixtures, but the repellent times were far greater than had been expected and most of the tests were terminated without a bite. For example, when treated with 75 percent of Indalone in alcohol, 1 man received a bite within 90 minutes and 2 received none in 148 minutes; whereas, with a biting rate in excess of 20 in half a minute, the average repellent time was 52 minutes. Similar results were obtained with other materials tested at the same time.

[3] Travis, B. V., and Smith, A. L. Unpublished manuscript.

No quantitative studies were made in the laboratory, but it was found impractical to evaluate repellents when the biting rate fell below 10 bites per half minute.

DIFFERENCES BETWEEN SPECIES.—The effectiveness of repellents against biting insects is so variable that one cannot safely predict which materials will be the most effective. In general, however, 6–12 and mixture 6–2–2 have been found superior to dimethyl phthalate and Indalone when used against culicine mosquitoes. The chief value of Indalone is against *Stomoxys calcitrans*. One of the most striking reversals in effectiveness was the poor showing made by dimethyl phthalate against *Anopheles farauti* Laveran in the South Pacific. This material had been selected because of favorable results against *A. quadrimaculatus*. Conversely, 6–12, which was much less effective than dimethyl phthalate against *A. quadrimaculatus*, was much more effective against *A. farauti*. Data taken from other reports being prepared at Orlando illustrate these differences (table 3), and show that there was less variation with mixture 6–2–2 than with any of the other standard repellents.

OTHER VARIABLES.—In field tests the subjects moved about every few minutes so as to be frequently exposed to "fresh" mosquitoes. Although the biting rates on untreated appendages might show little difference between the new and the old location, the mosquitoes appeared to be more avid when first disturbed; the subjects often received punctures on treated appendages immediately after they moved to a new location.

When the data from different subjects were being studied, there seemed to be no significant difference in results due to the size of the arm or leg, in spite of the fact that large limbs received less repellent per unit area than did small limbs. Frequently longer repellent times were obtained on subjects having hairy arms and legs. The arms of one subject, on whom the repellents lasted the longest, were unusually hairy, but this did not account for the long repellent time as the results were the same after one arm was shaved.

SUMMARY. — Data are presented to demonstrate known factors that cause variations in results of insect repellent tests against *Aedes aegypti* (L.), *A. taeniorhynchus* (Wied.), *A. sollicitans* (Walk.), *Anopheles quadrimaculatus* Say, and *Stomoxys calcitrans* (L.). The duration of effectiveness of repellent compounds varied greatly on different persons and also against different species of mosquitoes. The most striking variation of this type was experienced with two subsamples of diethyl phthalate, one lot giving an average repellent time of only 21 minutes and the other 132 minutes. Repellent times also varied with different broods or lots of mosquitoes. Low biting rates caused an extension of repellent time, whereas in tests made on skin wet with sweat the repellent time was decreased.

TABLE 3.—Average repellent times (in minutes) of standard repellents against different species of biting insects.

Species	*6–12*	*Dimethyl phthalate*	*Indalone*	*Mixture 6–2–2*
		Laboratory tests		
Aedes aegypti	331	247	141	271
Anopheles quadrimaculatus	53	108	41	147
A. freeborni Aitken	466	447	274	416
A. punctipennis (Say)	477	447	282	455
A. farauti	388+	43	72	198
Stomoxys calcitrans	101	47	246+	189
		Field tests		
Aedes taeniorhynchus	272	160	145+	243
Mansonia spp.	406	243	...	336
Stomoxys calcitrans	46	38	192+	...

Variations also occurred because the insects were more avid when they were first disturbed. Although the repellent time to the third bite displayed less variability than the time to the first bite, there seemed to be no significant difference between the results obtained with the two methods of evaluation; neither was there a significant difference between arms as compared with legs, or between different sizes of appendages.

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