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THE ODOROUS ATTRACTANT OF THE AMERICAN COCKROACH, PERIPLANETA AMERICANA (L.)

II. A BIOASSAY METHOD FOR THE ATTRACTANT*

BY D. R. A. WHARTON, GAIL LORENZ MILLER, AND MARTHA L. WHARTON

(From The Pioneering Research Laboratories, United States Army Quartermaster Corps, Philadelphia)

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INTRODUCTION

In the preceding study (2) of the behavior of the male American roach, *Periplaneta americana*, towards the specific odorous attractant emitted by virgin females it was found that the response was proportional to the log of the concentration over a wide range. It was also found that roaches could safely be tested daily, but that considerable variation in response could occur over either short or long intervals of testing. In spite of this variability in response, similar groups of roaches from the same colony could be depended upon to behave alike under comparable conditions.

The need for an accurate method of assay for the attractant, to be used in connection with fractionation and purification studies, motivated the present work. It seemed possible, from the information given above, that a method could be developed that would have acceptable limits of error. This report deals with a consideration of several different methods, especially with one which we refer to as the counterpoint test. By means of this test, results having a statistical error of ±40 per cent at a level of significance of 95 per cent are shown to be obtainable.

Materials and Methods

The technique of testing the roaches was that described in our previous paper (2). One hundred and forty roaches were used in each test. They were distributed in 15 jars but were considered as a single group or as two groups of 70 each, depending upon the requirements of the test.

The source of attractant was a concentrated petroleum ether extract, preparation P-3-C, also described previously. This stock solution was stored in the refrigerator and proved to be relatively stable over a period of months. It was of such potency

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that under the stated conditions of the test (2), a $\frac{1}{25}$ dilution produced responses in approximately 40 per cent of the roaches. For convenience, the $\frac{1}{25}$ dilution was assumed to possess a potency of 100 units per ml.

RESULTS

Three different methods of assay were examined: (1) a direct "spot" test against a standard curve; (2) a "50 per cent end-point" test; (3) a "counterpoint" test.

The Spot Test.—The idea of this test was that a solution of the attractant of unknown strength might be assayed simply by determining the percentage

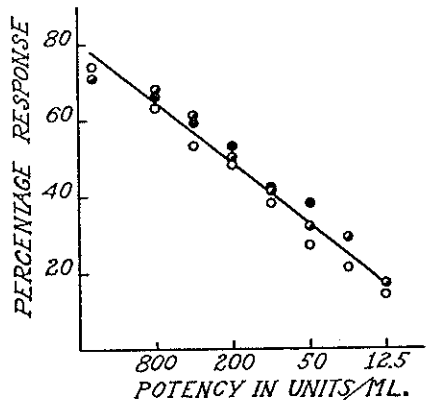

FIG. 1. Line fitted to data of three tests in descending series showing response–log potency relationship.

response of the roaches to some selected dilution of the unknown and relating that figure to its corresponding potency as revealed by a standard curve prepared especially for the purpose. To obtain the required curve, several descending series of titrations with the stock solution of the attractant and with a single group of 140 roaches were carried out on successive days, yielding the results shown in Fig. 1. A straight line was fitted to the data by the method of least squares, and a standard curve was thus made available for estimating potencies in "spot" tests on unknowns.

No concerted effort was made to determine the accuracy attainable in the spot test since it was realized from the start that when numerous tests were to be made, variability in the response of the roaches from test to test would preclude high reliability. The spot test was, therefore, to be considered useful for purposes of rough approximation only. It is, nevertheless, highly valuable in

this function since it makes possible the choice of favorable dilutions of unknowns to be used when more refined methods of assay are applied.

The 50 Per Cent End-Point Test.—This test, which was developed by Reed and Muench (1), seemed to offer another convenient method of assaying the attractant and one which should give results more accurate than those given by the simple spot test. As required by the method, it was assumed that roaches responding to any given dosage would also respond to all higher dosages; further, that roaches failing to respond to any given dosage would also fail to respond to all lower dosages. Cognizance was taken of the fact that such a condition would not necessarily obtain when individual roaches were considered. When the over-all response of a large group was considered, however, it appeared that the assumption was justified. The test was performed by titration of the attractant in a descending series using a single group of 140 roaches. The method of calculation was that of Reed and Muench (1).

To test the reliability of the method as applied to the present problem, the different sets of data of Fig. 1, representing titrations carried out with the same solution and the same roaches on different days, were used as bases of calculation. The values for the 50 per cent end-points were found to be at 166, 179, and 219 units per ml., respectively. Further studies, carried out over an extended period of time, showed that under certain conditions the reproducibility was less favorable than this. For example, after a period of rest of 12 days, a group of roaches gave an abnormally high response, resulting in an apparent 50 per cent end-point at only 104 units per ml. During successive days of intensive testing, the responsiveness decreased and end-points were found at 192, 200, 248, and 298 units per ml., respectively. Such variation, following long intervals of rest, or during the course of intensive daily testing, has been confirmed repeatedly. It is clear that it would contribute directly to errors in the application of the 50 per cent end-point test, the magnitude of which would be outside acceptable limits for the purpose of the assay. The variations are undoubtedly expressions of a type of adaptation or fatigue and recovery therefrom which are operable over extended intervals of time.

The Counterpoint Test.—The variability in the response of the roaches, which was encountered when tests were carried out at widely different times, suggested that more satisfactory results could be obtained by making comparisons of standards and unknowns on the same day and, preferably, at the same time. It did not seem feasible to attempt a complete titration of a standard followed by that of an unknown, or *vice versa*, because of the inevitable effect of adaptation or fatigue and because of the prohibitive length of time required for such tests. On the contrary, by alternating the standard with the unknown in successive rounds of the test, it was expected that the influence of adaptation or fatigue on the response of the roaches to the standard on the one hand and the unknown on the other would be more nearly equalized; and, further, by restricting the range of the titrations to only four different dilutions of the standard and of the unknown, the samples could be titrated concurrently and within a reasonable length of time. A preliminary design of this method of assay together with sample data, is presented in Table I. As may be seen, titrations of standard and unknown are carried out in "counterpoint" manner, in descending series. The data from a counterpoint test are first recorded as percentages of roaches responding to the various dilutions of the standard and the unknown. The two sets of data thus obtained are then each fitted by the

TABLE I

Preliminary Design of Counterpoint Test

Round No.	Sample tested*	Response
		per cent
1	Standard	47
2	Unknown	38
3	Standard/2	39
4	Unknown/2	34
5	Standard/4	32
6	Unknown/4	26
7	Standard/8	28
8	Unknown/8	21

* The starting potency of the standard was 400 units per ml.; that of the unknown, 200 units per ml.

method of least squares to lines of regression of the form $Y = a + bX$, in which Y is the percentage response, X is a function of the potency,

$$-\frac{\text{Log relative potency}}{\text{Log 2}},$$

and a and b are constants. For simplicity of calculation, the X values for the potencies in each series of four successive twofold dilutions, are taken as 1, 2, 3 and 4, respectively. The level of response at which the standard and unknown are compared is then taken as the mean response to the standard and the unknown at their respective mid-potencies. This level is ascertained by setting X equal to 2.5, which represents the mid-potency, solving for Y in the equations of the lines of regression for the standard and for the unknown, and calculating the mean of the Y values thus obtained. By substituting the mean value of Y in the equations for the lines of regression, the desired X values are obtained. The difference, obtained by subtracting the X value for the standard from the X value for the unknown, is multiplied by the log of the dilution factor (log 2) to give the log of the ratio of the potency of the unknown to that of the standard. The antilog gives the ratio. Understanding of the steps involved may be aided by the graphical representation in Fig. 2 of the data of Table I.

To test the counterpoint method over a wide range of potencies, five different dilution series of attractant, considered as unknowns (stock extract diluted twofold through four dilutions from 400, 200, 100, 50, and 25 units per ml., respectively), were each tested against a fixed dilution series of attractant considered as a standard (stock extract diluted twofold through four dilutions from 100 units per ml.). The theoretical ratios of potencies of the unknowns to that of the standard were, therefore, 4, 2, 1, 0.5, and 0.25, respectively. The results of the tests are shown opposite Experiment 1 of Table II. It will be noted from the table that when the unknowns were stronger than the standard the observed ratios tended to be lower than expected, while when the unknowns

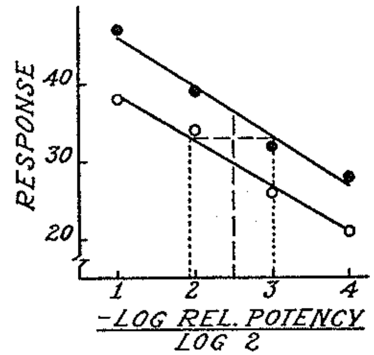

FIG. 2. Graphical representation of data of counterpoint test of Table I showing points of reference for calculation of potencies. ● standard; ○, unknown.

were weaker than the standard the ratios tended to be too high. These results were confirmed by those of Experiment 2 in which the initial potencies of the standard and the unknown were four times those in Experiment 1.

It occurred to us that this failure of the roaches to appreciate the full difference between the potency of the standard and the unknown was probably due (*a*) to the fact that the standard, being presented first, exerted a depressing influence upon the unknown because of adaptation, and (*b*) to the continuous effect of adaptation from round to round, which would blunt the responsiveness of the roaches under any circumstances. A means of obviating the problem of order of presentation, which is so general a source of error in bioassays of this type, was found by using a "cross-over" device. This principle employs two matched groups of roaches tested according to the design of Table III. It has the merit of neutralizing errors resulting from the order of presentation of the test samples and also, at the same time, errors due to unavoidable differences in responsiveness of two groups of roaches. On the basis of the previously ob-

TABLE II

Comparison of Theoretical and Found Ratios of Potency in the Counterpoint Test, Using the Preliminary and Final Designs of the Test

Experiment No.	Potency tested		Ratios of potency		
	Standard	Unknown	Theoretical ratio	Found ratio	Error
	units per ml.	*units per ml.*			*per cent*
	Data obtained with preliminary design of test				
1	100	400	4.00	2.84	−29
	100	200	2.00	1.45	−28
	100	100	1.00	0.82	−18
	100	50	0.50	0.51	+2
	100	25	0.25	0.29	+16
2	400	1600	4.00	1.56	−61
	400	800	2.00	1.32	−34
	400	400	1.00	0.90	−10
	400	200	0.50	0.47	−6
	400	100	0.25	0.34	+36
	Data obtained with final design of test				
3	100	400	4.00	3.62	−10
	100	200	2.00	1.78	−11
	100	100	1.00	1.12	+12
	100	50	0.50	0.53	+6
	100	25	0.25	0.34	+36
4	200	800	4.00	2.93	−27
	200	800	4.00	3.84	−4
	200	400	2.00	1.62	−19
	200	400	2.00	2.25	+13
	200	400	2.00	1.95	−3
	200	200	1.00	1.13	+13
	200	200	1.00	0.88	−12
	200	100	0.50	0.40	−20
	200	100	0.50	0.51	+2
	200	100	0.50	0.59	+18
	200	50	0.25	0.28	+12
	200	50	0.25	0.18	−28
	200	50	0.25	0.32	+28
5	400	1600	4.00	3.16	−21
	400	400	1.00	0.90	−10

served synchronous behavior of different groups of roaches (2), it appeared safe to assume in this connection that the direction and degree of change in responsiveness with respect to any consecutive set of rounds would be practically the same for both groups.

To test the validity of the cross-over principle, apart from its incorporation into the counterpoint test, the data of Fig. 3 (*b*) of our previous paper (2) were used. Since all the rounds of this series were made with the same solution, individual rounds could arbitrarily be considered either as tests of standards or of unknowns. When, without introduction of the cross-over device, the first round was considered to be a test of the standard, the second that of the unknown and when, as a replicate, the third round was considered to be a test of the standard, the fourth that of the unknown, etc., and a total of 10 replicates was obtained, a mean error of 6 per cent in percentage response resulted. When, on the other hand, the roaches were divided into two matched groups and, according to the cross-over principle, the test of round 1 of group 1 and that of round 2 of group 2 were considered to be those of the standard, while the test of round 2 of group 1 and that of round 1 of group 2 were considered to be those of the unknown, etc., the mean error was reduced to 3 per cent. In confirmation

TABLE III

Design of the Cross-Over Principle

Round No.	Group 1	Group 2
1	Standard	Unknown
2	Unknown	Standard

of these results, the data of Fig. 3 (*c*) of our previous paper were similarly analyzed and found to show, without application of the cross-over principle, a mean error of 17 per cent, but with the principle an error of only 2 per cent.

The final form of the counterpoint test with sample data, employing two groups of roaches and the cross-over principle, is presented in Table IV. Results obtained with its use at different ratios of potency of unknown to standard are shown opposite Experiments 3, 4, and 5 of Table II. Graphical representations of typical tests are shown in Fig. 3. It may be seen from Table II that the errors in the ratios found in Experiments 3, 4, and 5 were considerably lower on the average than those in Experiments 1 and 2 in which only one group of roaches was used and the cross-over principle was not applied.

The relationship whereby the roaches fail to evaluate the full difference in potencies of unknown and standard is still evident in the final results. It is probably due in some way to the continuous influence of adaptation from round to round, referred to in (*b*) above. In this connection we have considered other designs of test but have found none more likely to reduce the effect. It would appear to be irreducible except by approximating the unknown to the standard. On the other hand this error resulting from the effect occurs consistently over the entire range of ratios studied and is susceptible to statistical analysis.

When the logs of the found ratios of unknown to standard are plotted against the logs of the theoretical ratios a linear relationship results, as shown by Fig. 4 in which a line of regression is fitted to the data. This line is represented by the equation, $\log T = 0.012 + 1.086 \log F$, in which F corresponds to the found

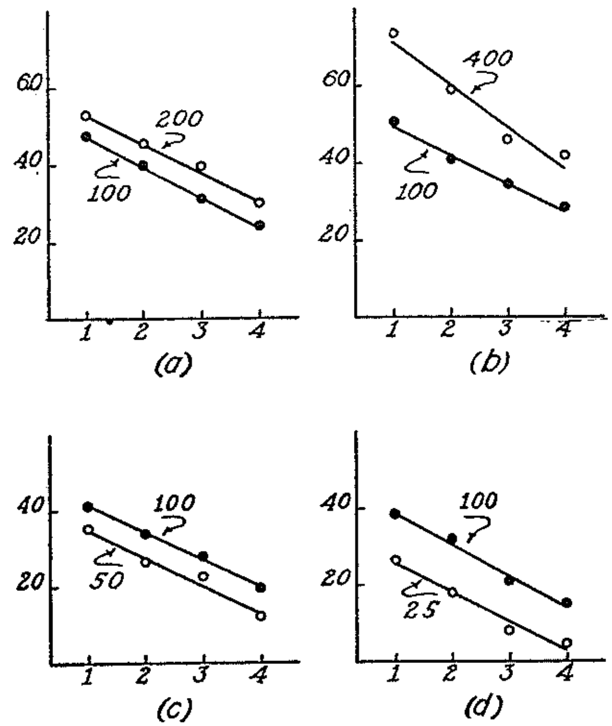

Fig. 3. Typical counterpoint tests at different ratios of potency of standard to unknown. ●, standard; ○, unknown. The values on the ordinates represent percentage response. Those on the abscissae represent the function:

$$-\frac{\text{Log relative potency}}{\text{Log 2}}$$

ratio, T the theoretical ratio. The scatter of data about the line appears independent of the log ratio, justifying the use of the data for calculation of a standard error of estimate. This was found to amount to ± 0.073 log unit. Twice this value, ± 0.146 log unit, corresponds to a percentage error in titer of ± 40 per cent at a level of significance of 95 per cent. The value of ± 40

per cent thus represents the range of error within which 95 per cent of the results can be expected to fall, under the conditions of the test. It also represents the extent of difference between standard and unknown which has a 95 per cent certainty of significance.

The use of a line of regression, such as that of Fig. 4, as an adjunct to the counterpoint test would appear entirely feasible. It provides a statistical basis for establishing the accuracy of the test and at the same time effectively reduces the error obtained.

The error obtained with the counterpoint test is necessarily lower than that obtainable by the other methods tested by virtue of the fact that the counter-

TABLE IV

Final Design of Counterpoint Test

Round No.	Group 1		Group 2		Average responses	
	Sample tested	Response	Sample tested	Response	Standard	Unknown
		per cent		*per cent*	*per cent*	*per cent*
1	Standard*	48	Unknown	54		53.0
2	Unknown*	52	Standard	47	47.5	
3	Standard/2	41	Unknown/2	45		45.5
4	Unknown/2	46	Standard/2	39	40.0	
5	Standard/4	33	Unknown/4	40		39.5
6	Unknown/4	39	Standard/4	29	31.0	
7	Standard/8	26	Unknown/8	27		30.0
8	Unknown/8	33	Standard/8	22	24.0	

* The starting potency of the standard was 100 units per ml.; that of the unknown, 200 units per ml.

point test enables a simultaneous comparison of the standard and the unknown, establishes a concurrent relationship between response and potency, reduces the errors introduced by the order of presentation, and provides correction for adaptation. This error, ± 40 per cent, is considered to be acceptable for a method of assay of the attractant. It applies to analyses made on unknowns differing in potency from the standard by as much as fourfold as well as to analyses in which unknowns closely approximate the standard. The spot test described earlier would provide a preliminary estimate of the potency of an unknown which would be an adequate basis for proceeding with the counterpoint test. The counterpoint test is easily executed, requiring approximately 2 hours for its completion. Finally, since it is amenable to statistical treatment, any desired accuracy is attainable depending only on the number of replicate tests carried out.

Recapitulation and Sample Calculation of the Counterpoint Test.—The following example, employing the data of Table IV, will serve to summarize the procedure of the counterpoint test. Two groups of 70 roaches each were used to test a standard and an unknown. The standard was the stock solution prepared in a potency of 100 units per ml. The same stock solution at a potency of 200 units per ml. was regarded as an unknown. Each sample was further diluted to give a series of four successive twofold dilutions. The percentage responses to the standard in each successive pair of rounds were averaged as shown in

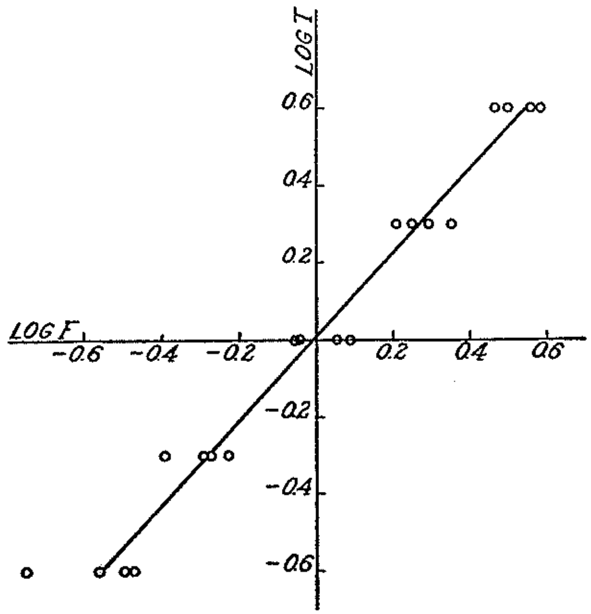

FIG. 4. Line of regression of data of counterpoint tests represented by log of found ratios *vs.* log of theoretical ratios.

the table and the data for the unknown were averaged in the same way. When the potencies of the four different solutions tested were ascribed the numerical values 1, 2, 3, and 4, respectively, the equation relating percentage response (Y) to potency (X) was found to be $Y = 55.5 - 7.95\ X$ for the standard, $Y = 60.8 - 7.50\ X$ for the unknown. Letting $X = 2.5$, representing the mid-potency, Y became 35.6 for the standard, 42.0 for the unknown. Letting $Y = 38.8$, which was the average of the values 35.6 and 42.0, X became 2.10 for the standard, 2.93 for the unknown. The difference obtained by subtracting the X value of the standard from the X value for the unknown is multiplied

by the log of the dilution factor (log 2) to give the log of the found ratio of the potency of the unknown to that of the standard, namely $\log F = 0.24985$. By substituting this value for log F into the equation of the line of regression, *i.e.* $\log T = 0.012 + 1.086 \log F$, the log of the theoretical ratio of potencies, log T, then equals 0.28334. The antilog of log T is 1.92. By multiplying this value for T by the known potency of the standard, 100 units per ml., the potency of the unknown is obtained, namely, 192 units per ml.

SUMMARY

A method has been developed for the assay of the odorous attractant of the cockroach, *P. americana*. The method, called the counterpoint test, employs two matched groups of roaches and provides for a simultaneous titration of standard and unknown. It has a statistical error of ±40 per cent at a level of significance of 95 per cent.

REFERENCES

1. Reed, L. F., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
2. Wharton, D. R. A., Miller, G. L., and Wharton, M. L., *J. Gen. Physiol.*, 1954, **37,** 461.