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EXPERIMENTAL DESIGN FOR BIOASSAY OF A MATERIAL INDUCING STRONG TACHYPHYLACTIC EFFECT (ANAPHYLATOXIN)

BY

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Anaphylatoxin, prepared by incubation of rat plasma with agar or starch, stimulates guinea-pig ileum by releasing histamine (Rocha e Silva and Aronson, 1952; Rocha e Silva, 1952a; Rothschild and Rocha e Silva, 1954). The effect is therefore indirect, and repetition of the same dose of activated plasma produces less and less effect until the preparation is desensitized to it. This tachyphylactic effect, though strong, is progressive and bound to follow certain rules, if the assay is done in an accurate way. However, the possibility of comparing two different preparations of anaphylatoxin by applying them alternately to the same piece of intestine would give very inaccurate results on account of the enormous bias introduced by the tachyphylactic effect. Even with randomized blocks as utilized for histamine (Schild, 1942), oxytocin (Holton, 1948) or bradykinin (Rocha e Silva, 1952b), the bias introduced by the tachyphylactic effect of anaphylatoxin is so strong that no accurate potency ratio could be derived. The responses depend greatly upon the position of the dose in the sequence of treatments. For instance, the response to the first dose can be 20 times that of the same dose applied after several treatments. Moreover, one of the prerequisites for a sound bioassay has been supposed to be the independence of all responses on the same animal or preparation. However, the literature shows the possibility of treatment comparisons even where a correlation between responses might be expected (Finney, 1955). Following a suggestion by Finney (1955) we have tried several designs in order to test the possibility of setting up a (2+2) assay for a quantitative estimation of anaphylatoxin. Much to our surprise, a Latin square design in which columns were separate pieces of gut, appeared to correct for the bias introduced by the tachyphylactic effect, allowing a reasonable estimate of the ratio of potency between " standard " and " unknown."

The possibility of segregating a sum of squares corresponding to the tachyphylactic effect affords a useful means of studying phenomena such as those occurring in anaphylaxis, or as a consequence of the action of animal venoms or histamine releasers, in which the pharmacological effects depend upon the liberation of active substances from an exhaustible stock bound to the tissues of the organs involved. Other analogous phenomena, such as the hypertensive effect produced by the release of hypertensin following the injection of renin, could possibly be studied more accurately by employing the experimental design described in this paper.

METHODS AND DESIGN

Biological Preparation.—Pieces of intestine from a bled guinea-pig were washed with Tyrode solution and distributed in separate beakers containing Tyrode solution. After storage in the ice-box for varying times, the pieces were suspended in a 10 ml. chamber and the experiments started after 10 min. To test the sensitivity of the gut, a standard dose of histamine was added a few times, and then the four doses of anaphylatoxin were added at intervals of 3 min. Four different doses of anaphylatoxin were assayed upon the same fragment, forming one " Group." The sequence of additions was that indicated in the next paragraph. The anaphylatoxin solutions were prepared as described previously (Rothschild and Rocha e Silva, 1954) by adding an optimum amount of agar to a heparinized sample of rat plasma. After incubation for 30 min., the material was centrifuged and kept frozen until used. In the following experiments " standard " and " unknown " are dilutions of the same plasma. All other conditions of the assay are the same as those described in previous publications.

Experimental Design.—Any attempt to devise a (2+2) assay for potency estimations, using a material that stimulates guinea-pig ileum, should aim at obtaining the responses to the four doses of standard and unknown (S_1, S_2, T_1, and T_2) in a single piece of ileum. Fortunately, desensitization (tachyphylaxis) to anaphylatoxin

is sufficiently slow to allow a complete sequence of four treatments to be assayed upon the same piece of ileum. It became, therefore, possible to confound "intestines" variation with group variation, so that the potency estimation could be derived from "intestines" comparisons. Similarly, by employing the Latin square design, the tachyphylactic effect was confounded with the "order of additions" (rows). By so doing the random error became reasonably low when compared with many biological assays—such as those for vitamins or insulin.

Although several Latin squares have been used most of the experiments were done with the following standard square:

Groups: I	II	III	IV
A	B	C	D
B	A	D	C
C	D	A	B
D	C	B	A

Treatments were allocated to the letters thus: A=S_1, B=S_2, C=T_1, D=T_2, where S_1 and S_2 indicate the lower and higher doses of the "standard," and T_1 and T_2 the corresponding doses of the "unknown." Although in the present experiments treatments have not been randomly allotted to the letters for each assay, such a procedure would be advisable for routine assays. The ratio $S_2/S_1=T_2/T_1$ was 2.00, unless otherwise indicated, and the ratio $T_1/S_1=T_2/S_2$ was arbitrarily made 1.5 or 1.00, as indicated.

A piece of ileum taken randomly from one guinea-pig was allotted to each group of a square; each square was thus performed with four pieces of gut from a single guinea-pig.

If we represent the totals for each dose by the corresponding letters S_1, S_2, T_1, and T_2, the three important contrasts:

$$\theta=-S_1-S_2+T_1+T_2$$
$$\varphi=-S_1+S_2-T_1+T_2$$
$$\psi=+S_1-S_2-T_1+T_2$$

will permit the calculation of the three parts into which the sum of squares corresponding to the variation due to "Doses" can be split: "Between samples"=$\theta^2/16$, "Slope"=$\varphi^2/16$ and "Deviation from parallelism"=$\psi^2/16$, each with one degree of freedom. The mean squares "Between samples" and "Slope" are also indicated by the two squares D^2 and B^2, respectively. The validity of the assay corresponds to an expectation of $\psi=0$, or to a variance ratio $\psi^2/16s^2$ smaller than 5.99 (F) at a probability level of 5%, and 6 degrees of freedom. Contrariwise, the variance $\varphi^2/16=B^2$ must be as large as possible when compared with the Error variance (s^2) of the assay. The value of $\theta^2/16=D^2$ will depend upon the artificial conditions of the dummy assay, in which the ratio "Unknown"/"Standard" is arbitrarily chosen. This ratio was taken as 1.50 in the present experiments except in one (Expt. 3, Table III) where it was 1.00.

The first two contrasts presented above permit the calculation of the logarithmic ratio of potency (M):

$$M=\frac{I\theta}{\varphi}=\frac{ID}{B}$$

where $I=\log(S_2/S_1)=0.301$. In Expt. 2 of Table III, $I=0.352$ because the ratio between the larger and the smaller doses of the standard, and those of the unknown, was 2.25. Likewise, the slope of the mean regression line will be given by the formula: $b=2\varphi/16\,I$ and the variance of the slope $s_b^2=2s^2/16\,I^2$. The standard deviation in terms of the log dose, indicated by $\lambda=s/b$, can measure the efficiency of the assay (Gaddum, 1941); alternatively, the reciprocal of its square ($1/\lambda^2$) could be taken as a measure of the "inherent precision" of the assay.

For the calculation of the error of M, the simplified formula

$$s_M=\frac{sI\sqrt{B^2+D^2}}{B^2}$$

was used and the fiducial limits of the ratio of potency calculated in the conventional way, by taking the antilogs ($M\pm s_M t$), where $t=2.45$ for a probability level of 5% and 6 degrees of freedom (Fisher and Yates, 1943). When the ratio b/s_b was lower than 7.8, the exact formula for s_M was employed (Bliss, 1952) and the fiducial limits calculated by the formula:

$$CM\pm ts_M\sqrt{C}$$

where $C=B^2/(B^2-s^2t^2)$.

The more elaborate calculation of S_M and the fiducial limits was applied to Square IV, Expt. 1; Squares II and III, Expt. 2; and Squares I and II, Expt. 3, of Table III.

For the calculation of the weighted mean, $\bar{M}$, and the corresponding $s_{\bar{M}}$, as well as the number of degrees of freedom in $s_{\bar{M}}$ (D.F. in $s_{\bar{M}}$) the schedule indicated by Bliss (1952, p. 579) was applied. The test of homogeneity of variances was Bartlett's test as described by Snedecor (1946). In general, the notation adopted in this paper is that given by Emmens (1948) and Finney (1952).

For the test of linearity, a Latin square design was also adopted, but the ratio of successive doses was 1.50. The splitting of the sum of squares corresponding to "Doses" was performed by utilizing the polynomial coefficients for doses.

In each experiment, to test the linearity, four squares were assayed in a sequence, and the 4×4 pieces of intestine of four different guinea-pigs (one guinea-pig for each square) were randomized as described in another section (Results). Attempts to cut as nearly identical pieces as possible were seldom successful, but it does not appear to be essential for the design of the assay.

Coefficient of Desensitization (Δ).—The simplest assumption about the mechanism of tachyphylaxis is that each dose (A, for instance) will affect the responses to any subsequent dose (B, C, or D) by a certain value, $\Delta\alpha$, that will reduce the expected responses β, γ, and δ given by the following doses B, C, and D, respectively. The residual effects introduced by the tachyphylactic

phenomenon in the first column of the Latin square presented above would be indicated by the following expressions:

Group I $(\alpha),(\beta-\Delta\alpha),(\gamma-\Delta\alpha-\Delta\beta)$, and $(\delta-\Delta\alpha-\Delta\beta-\Delta\gamma)$, and similar expressions would be obtained from the other columns of the square.

Now, if the responses are assembled according to the doses, the totals for each dose will be:

$$T_A=4\alpha -2\Delta\beta -2\Delta\gamma -2\Delta\delta$$
$$T_B=4\beta -2\Delta\alpha -2\Delta\gamma -2\Delta\delta$$
$$T_C=4\gamma -2\Delta\alpha -2\Delta\beta -2\Delta\delta$$
$$T_D=4\delta -2\Delta\alpha -2\Delta\beta -2\Delta\gamma$$

From the contrasts, (I) $-1\ -1\ +1\ +1$ and (II) $-1\ +1\ -1\ +1$, calculated with these totals, the following expressions are obtained:

$$\text{Contrast (I)}\ \theta+\frac{\theta\Delta}{2}=\theta\left(1+\frac{\Delta}{2}\right)$$

$$\text{Contrast (II)}\ \varphi+\frac{\varphi\Delta}{2}=\varphi\left(1+\frac{\Delta}{2}\right)$$

It is therefore obvious that the ratio of the two contrasts I and II, giving the logarithmic ratio of potency (M), is independent of Δ, if the assay is designed as a Latin square. Therefore, M can be calculated directly from the experimental data, by simply applying the formula:

$$M=\frac{I\,\theta}{\varphi}=\frac{I\,[\text{Contrast I}]}{[\text{Contrast II}]}$$

The close agreement between theoretical and experimental M, derived from the assays described below, is a good indication of the reliability of the assumptions involved in the mathematical development described above. The model adopted has been confirmed by the analysis of the experimental results.

Results

Estimates of Potency.—The possibility of deriving a reasonable ratio of potency by applying a (2+2) assay to such apparently variable data as the responses to different doses of anaphylatoxin is a good indication that the tachyphylactic effect is corrected by the experimental design adopted. In the five squares of Expt. 1, Table III, the ratio of potency T/S was made 1.50, by simple dilution of the activated plasma, the undiluted one being taken as "unknown" and the dilute one as "standard."

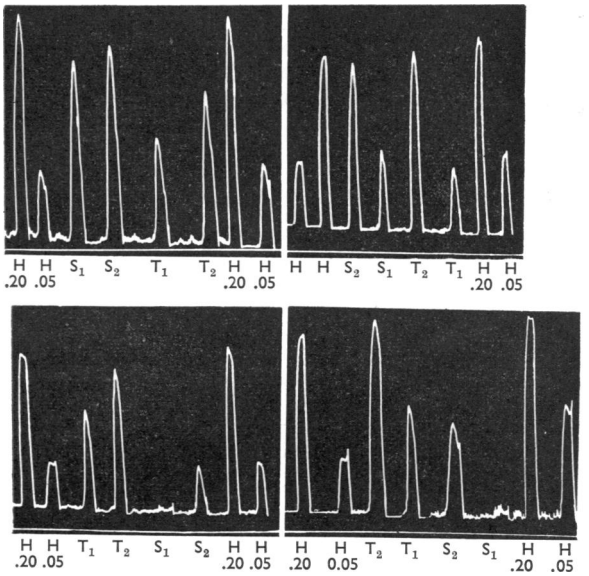

FIG. 1.—Responses of the guinea-pig intestine (four pieces) to four doses of anaphylatoxin. Latin square design for a (2+2) assay. H, histamine solution 1 : 2 millions. S_1, S_2, T_1, and T_2 are the doses of the "standard" and "unknown," respectively.

The data corresponding to square I are presented in detail (Fig. 1 and Table I) and a summary of the five squares is presented in Table III.

Table I shows the responses in millimetres given by the four doses added in the order indicated in (a), where the groups (columns) represent different pieces of ileum and the rows the "order of addition." In (b), the responses have been classified according to "doses."

Simple inspection of Table I shows how strong is the tachyphylactic effect. The smaller dose (S_1) of the standard gives a response of 63 mm. when

TABLE I

RESPONSES (MM.) OF FOUR PIECES OF GUINEA-PIG ILEUM TO DOSES (S_1 AND S_2) OF THE STANDARD AND TO DOSES (T_1 AND T_2) OF THE UNKNOWN

The experimental sequences are given in (a). In (b) the responses are assembled by doses

(a)					(b)				
Groups: I	II	III	IV	Totals T_R	Doses/ Groups	S_1	S_2	T_1	T_2
$S_1=63$ $S_2=69$ $T_1=36$ $T_2=54$	$S_2=58$ $S_1=28$ $T_2=63$ $T_1=22$	$T_1=35$ $T_2=49$ $S_1=2$ $S_2=18$	$T_2=68$ $T_1=39$ $S_2=33$ $S_1=3$	224 185 134 95	I II III IV	63 28 2 3	69 58 16 33	36 22 35 39	54 63 49 68
Totals T_G 222	171	102	143	638	Totals T_D	96	176	132	234

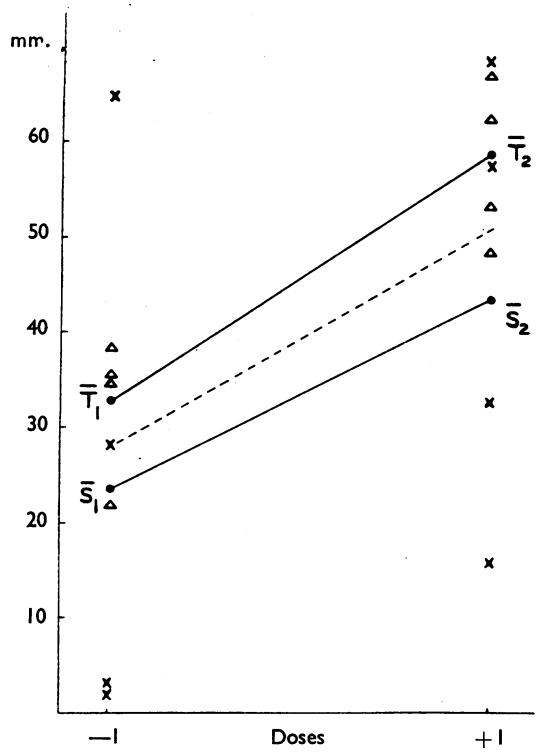

FIG. 2.—Regression lines for unknown (T_1, T_2), standard (S_1, S_2), and mean (dotted line). Individual responses to unknown (△) and standard (x). Abscissae, factorials for doses.

applied first (Group 1) and only 3 mm. when applied last (Group IV). The tachyphylactic effect is obviously eliminated from the totals for doses (T_D) from which a reasonable ratio of potency T/S=1.43 could be calculated. Fig. 2 shows the two regression lines for standard and unknown as well as the average regression line, and again it is impressive to note the enormous scatter of the individual data about the means.

By the analysis of variance presented in Table II, the large sums of squares corresponding to variation due to " Groups " and to " Order of additions " (" Rows ") can be isolated and subtracted from the " Total " leaving a residual sum for " Error " that is quite acceptable in this kind of biological assay. The mean square corresponding to " Deviation from parallelism " is of the order of magnitude of the Error variance and therefore non-significant.

Table III presents the most important statistics calculated from the data obtained in the 5 Latin squares of Expt. 1. All but one (Square IV) of the potency ratios fall inside of the fiducial limits, and only once (Square V) was the deviation from parallelism significant at the 5% level.

Expt. 2 of Table III is the same as Expt. 2 of Table VI, in which the four doses were considered as a sequence with an interval of log 1.5=0.176. To derive a ratio of potency, the alternate doses were arbitrarily taken as S_1, S_2, T_1, and T_2, with a ratio T/S=1.5 and $S_2/S_1=T_2/T_1=2.25$.

Expt. 3 of Table III includes four squares in which the ratio T/S=1.00 and the ratio $S_2/S_1=T_2/T_1=2.00$.

Test of Linearity.—The same Latin square design was utilized throughout the following experiments. Two series of 4 squares each were submitted to a test of linearity. The calculation of the theoretical equations was made routinely by using polynomial coefficients (Emmens, 1948).

Randomization of the pieces of guinea-pig ileum was done, as usual, by taking four pieces of intestine from the same guinea-pig and distributing them at random in four beakers containing Tyrode solution and keeping them in the icebox. For the four squares of each experiment, another Latin square design was chosen at random to allocate each fragment to each group of the main design in such a way that no repetition of " age " of the fragment to the same line was allowed.

For the test of linearity, four doses were used: 0.02, 0.03, 0.045, and 0.0675 ml., the constant ratio between the doses being 1.5. This range of doses was well tolerated by the pieces of gut, and good responses were obtained (Table IV).

Table V shows a typical analysis of variance of the data of Table IV, and Fig. 3 shows the theoretical lines (in full) as well as the individual lines corre-

TABLE II
ANALYSIS OF VARIANCE OF THE DATA OF TABLE I

Source of Variation:		Sum of Squares	Degrees of Freedom	Variances	F	P
Total		7,231·75	15			
Between doses	Samples	552·25	1	552·25 (D^2)	12·3	<0·05
	Regression	2,070·25	1	2,070·25 (B^2)	45·0	<0·01
	Dev. Parallel	32·50	1	32·50	0·7	
Between groups		1,904·25	3	634·75	14·2	<0·05
Between rows		2,405·25	3	801·75	17·9	<0·01
Error (residual)		267·25	6	44·81		

TABLE III

SUMMARY OF THREE EXPERIMENTS FOR ESTIMATION OF POTENCY BY THE (2+2) ASSAY

Expt.	Square	Ratio (Found)	Slope (b)	Error Variance (s^2)	Stand. Deviation (s)	λ (s/b)	s_M	Fiducial Limits	Variances (Mean Squares)		
									Stand. x Unkn.	Slope	Parallel.
1	I	1·43	74·5	44·80	6·70	0·090	0·050	1·89–1·08	552·25	2,070·25	33·05
	II	1·35	74·2	67·50	8·21	0·111	0·060	1·89–0·96	361·00	1,980·25	56·25
	III	1·34	56·25	18·60	4·32	0·77	0·041	1·70–1·06	203·06	1,139·06	39·06
	IV	1·75	47·70	34.60	5·89	0·123	0·087	3·62–1·18	650·25	900·00	132·25
	V	1·29	63·33	25·70	5·07	0·080	0·043	1·65–1·07	196·00	1,444·00	144·00
	Means:	1·37					0·031	1·59–1·18			
		1·50 (real)									
		$\bar{M}$=0·1365; D.F. in $S_{\bar{M}}$=21.5									
		Homogeneity of variances: χ^2=2·26 0·50<P<0·70									
		Homogeneity of M: χ^2=2·22 0·70<P<0·80									
2	I	1·33	54·1	19·00	4·36	0·079	0·034	1·61–1·10	202·06	1,620 06	10·25
	II	1·51	50·5	74·33	8·62	0·170	0·101	3·83–0·93	324·00	1,260·25	36·00
	III	1·42	61·4	63·17	7·95	0·130	0 072	2·41–0·99	361·00	1,980·50	1·00
	IV	1·39	76·2	29·17	5·40	0·071	0·033	1·67–1·21	663·06	2,943·06	0·06
	Means:	1·37					0·026	1·56–1·21			
		$\bar{M}$=0·1375; D.F. in $s_{\bar{M}}$=14·0									
		Homogeneity of variances: χ^2=2·67 0·30<P<0·50									
		Homogeneity of M: χ^2=1·21 0·70<P<0·80									
3	I	0 91	37 2	102·16	10·10	0·279	0·143	3·14–0·17	18·06	885·06	5·06
	II	1·11	87·0	177·43	13·30	0·153	0·078	2·14–0·62	52·29	2,093·06	52·06
	III	1·19	72·1	17·61	4·20	0·058	0·031	1·42–0·99	105·06	1,743·06	52·56
	IV	0·91	47·1	20·20	4·50	0·095	0·048	1·19–0·69	14·06	798·06	7·53
	Means:	1·09					0·032	1·26–0·92			
		1·00 (real)									
		$\bar{M}$=0·0327; D.F. in $s_{\bar{M}}$=11·6									
		Homogeneity of variances: χ^2=2·286 0·50<P<0·70									
		Homogeneity of M: χ^2=3·01 0·30<P<0·50									

sponding to each group (intestines). It is certainly remarkable that such an enormous variation in the data forming the group sequences corrects itself in the means of the responses to the 16 doses applied in the Latin square design, so as to give a curve that does not deviate significantly from linearity.

In another series of four squares similarly designed, a striking confirmation of the findings in the first experiment was obtained. Fig. 4 presents the four theoretical lines calculated by the least square method and the experimental points corresponding to the means of the responses for each dose. Table VI presents the most relevant data from the 8 squares utilized for the test of linearity, including the respective equations obtained by applying the method of least squares.

Estimate of the Coefficient of Desensitization.—The previous assumption about the mechanism of tachyphylaxis—that each dose will affect the responses to any subsequent dose by a certain residual value ($\Delta\alpha$) that will reduce the expected response to the following dose—would find a reasonable foundation if we assume that each addition will reduce the histamine store of the guinea-pig ileum by a fraction dependent upon the magnitude of the dose previously applied. Since

TABLE IV

EXPERIMENTAL DATA OF THE RESPONSES TO FOUR DOSES OF ANAPHYLATOXIN USING FOUR DIFFERENT PIECES OF ILEUM (GROUPS)

Groups:	I	II	III	IV	Totals T_R	Doses/ Groups	A	B	C	D	Totals T_G
	D 62	A 50	C 62	B 36	210	I	5	17	48	62	132
	C 48	B 46	D 61	A 17	172	II	50	46	52	52	200
	B 17	C 52	A 3	D 39	111	III	3	19	62	61	145
	A 5	D 52	B 19	C 10	86	IV	17	36	10	39	102
Totals: T_G	132	200	145	102	579	Totals: T_D	75	118	172	214	579

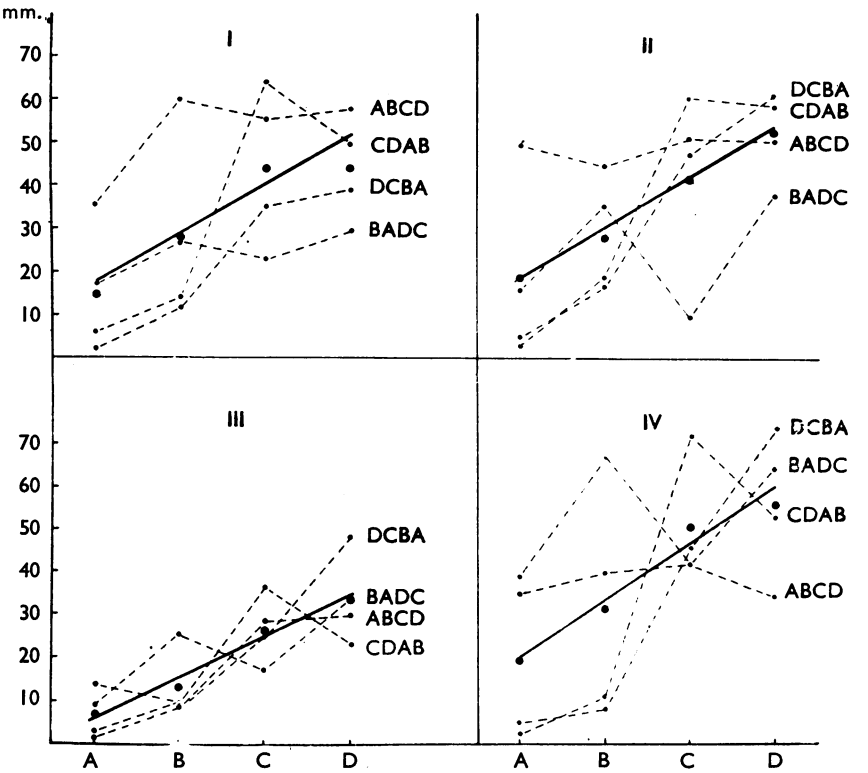

FIG. 3.—Tests of linearity. Dotted lines, responses to treatments upon the same piece of ileum (group). Larger dots: the means to each dose. Solid lines, the theoretical lines calculated by the least squares method.

there is no refilling of the histamine store—at least for the duration of each experiment—we can assume that this residual effect introduced by any previous addition of anaphylatoxin is permanent and cumulative. These assumptions, in good agreement with the experimental results, constitute the basis for the establishment of the model proposed in this paper.

The definition of Δ, the coefficient of desensitization, arises naturally from the model adopted in this paper (Methods and Design). If we take, for instance, the simplest case of repeated additions of the same dose of anaphylatoxin to a single piece of intestine, and if the full effect be represented by Y_0, the first response to that particular dose of anaphylatoxin and the subsequent effects are represented by the responses $Y_1 = Y_0 - \Delta Y_0$, $Y_2 = Y_0 - 2\Delta Y_0$, $Y_3 = Y_0 - 3\Delta Y_0$ and so forth, the series of responses showing a downward trend for each new addition of the same dose of anaphylatoxin. The experiment has shown that this sequence forms a linear regression; for convenience this can be called regression of " responses on the number of previous additions of anaphylatoxin " or of

TABLE V

ANALYSIS OF VARIANCE OF THE DATA OF TABLE IV

Source of Variation:	Sum of Squares	Degrees of Freedom	Mean Squares	F	P
Total	6,674·44	15			
Between doses	2,779·69	3	926·56	24·6	<0.01
„ rows	2,397·69	3	799·23	20 4	<0.01
„ groups	1,260·69	3	420·23	10·7	<0.05
Error (residual)	236·99	6	39·5		
Linearity	2,773·18	1	2,773·18	70·5	<0.01
Quadratic	0·06	1	0·06		
Residual	6·51	1	6·51		

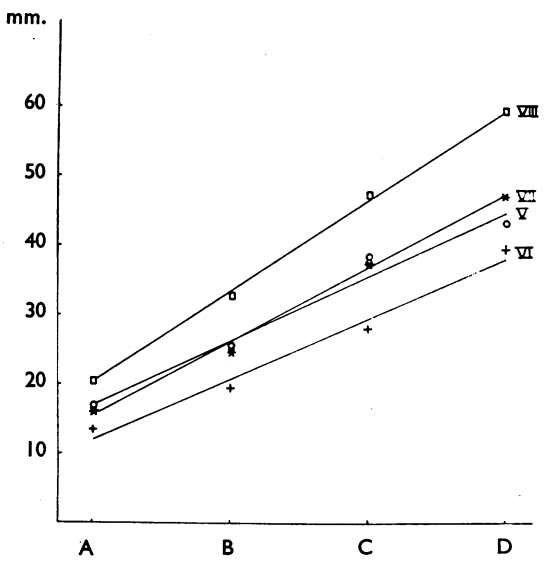

FIG. 4.—Tests of linearity. Theoretical lines calculated upon the means of the responses. Each line corresponds to a Latin square (Table VI).

" responses on levels of desensitization or tachyphylaxis."

When several doses are applied according to the schedule of the Latin square utilized for the test of linearity, the means of " Rows " can be employed for the calculation of Δ, since in this design the first " Row " (T_0) is composed of the responses to a first addition of each one of the four doses of anaphylatoxin, the second " Row " (T_1) of the responses following *one* previous addition of anaphylatoxin and so forth. It is clear that *levels of desensitization*, or the number of previous additions of anaphylatoxin, refer to the index of the totals for " Rows." The square given in Table IV can be taken as a model. If the regression " means of rows on levels of desensitization " are calculated by the least square method, Δ can be calculated as the quotient of the slope (b') of the line by the maximum ordinate (Y_0). This definition of Δ agrees well with the definition previously given. (In the following discussion, b' will always indicate the slope of the regression " responses on levels of desensitization," since b has been already utilized to indicate the slope of the regression " responses upon log dose of anaphylatoxin ").

In a separate series of experiments, we have attempted a direct estimate of this coefficient of desensitization (Δ). For this purpose, the main argument of the previous design has been inverted and four doses of anaphylatoxin (the same as those employed in the sequences of Table VI) were added repeatedly to four pieces of ileum. Here, variation due to doses was confounded with group variation (inter-intestines) and therefore discarded in the corresponding analysis of variance. The sequences employed were the following:

Groups:	I	II	III	IV	Totals T_R	Means
Rows	A_0	B_0	C_0	D_0	T_0	Y_0
	A_1	B_1	C_1	D_1	T_1	Y_1
	A_2	B_2	C_2	D_2	T_2	Y_2
	A_3	B_3	C_3	D_3	T_3	Y_3

A typical set of responses is presented in Fig. 5. The totals of " Rows " represent the responses to the four doses of anaphylatoxin in different *levels of desensitization*. It is obvious that the first total T_0 is composed of the full responses to the four doses in a level (0) of desensitization; the following totals T_1, T_2, T_3 . . . correspond to the responses in 1, 2, 3 . . . levels of desensitization, due to the tachyphylactic effect. It is easy to conclude that the best method for the estimation of Δ, consists in the calculation of the regression lines, using as dependent variable the means Y_0, Y_1, Y_2 . . . corresponding to the totals and as independent

TABLE VI

SUMMARY OF ANALYSIS OF 8 SQUARES DESIGNED FOR TEST OF LINEARITY

Doses in ml.: A, 0·02; B, 0·03; C, 0·045; D, 0·0675

Expt.	Square	Variance of Error (s^2)	Stand. Deviation (s)	Slope (b)	Ratio ($s'b$) $=\lambda$	Theoretical Equations ($X=\text{Log}_{10}$ dose)	Mean Square Variances Corresponding to " Between Doses "		
							Linear	Quadrat.	Res.
1	I	57·72	7·60	59·8	0·13	$Y=1{\cdot}16+59{\cdot}8X$	2,111·5	189·0	85·12
	II	39·40	6·28	66·9	0·09	$Y=-1{\cdot}37+66{\cdot}9X$	2,773·1	0·06	6·51
	III	96·75	9·83	55·1	0·18	$Y=-12{\cdot}4+55{\cdot}1X$	1,881·8	0·25	36·45
	IV	48·91	6·99	75·0	0·09	$Y=-2{\cdot}37+75{\cdot}0X$	3,406·5	30·25	92·00
2	I	19·00	4·30	54·1	0·08	$Y=1{\cdot}00+54{\cdot}0X$	1,795·5	10·56	25·93
	II	74·33	8·62	50·6	0·17	$Y=-3{\cdot}07+50{\cdot}6X$	1,584·2	30·25	0·55
	III	63·17	7·96	61·4	0·13	$Y=-2{\cdot}80+61{\cdot}4X$	2,332·8	1·00	9·20
	IV	29·17	5·40	76·2	0·07	$Y=-2{\cdot}36+76{\cdot}2X$	3,604·6	0·62	0·88

Homogeneity of variances: $\chi^2=4{\cdot}134$. Degrees of freedom, 7. $0.70<$ [illegible] $<0{\cdot}80$.

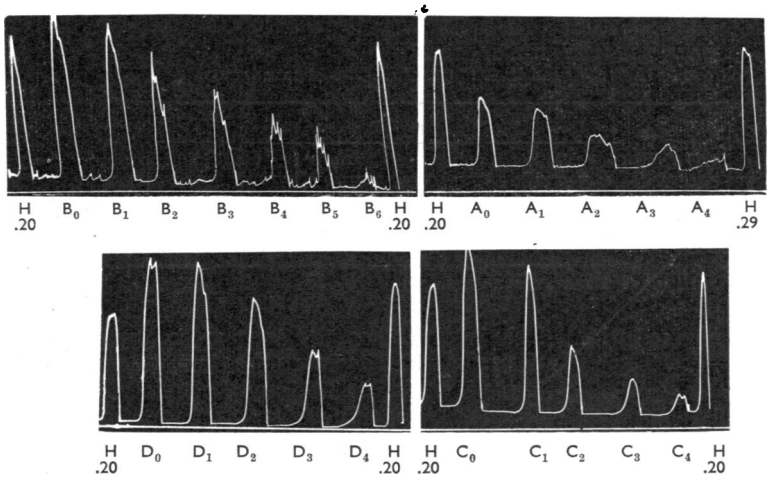

FIG. 5.—Design to calculate the regression "responses on levels of desensitization." To each piece of ileum the same dose of anaphylatoxin was repeatedly applied. H, 0.2 ml. histamine solution 1 : 2 millions.

variate the different *levels of desensitization* (0, 1, 2, 3 . . .).

In 3 different experiments so designed, the following totals were obtained:

Levels of desensitization:	0	1	2	3
Square No.				
I	145·5	110 5	82 0	57·0
II	184·5	142·0	101 0	69·0
III	160·5	142·0	103 0	59·0

The simplest inspection of these data shows that there is a regression of totals of "Rows" (or their corresponding means) upon the levels of tachyphylaxis. If the means are taken and the lines calculated by the least square method, the slopes will give a quantitative measure of the tachyphylactic effect, since the coefficient of desensitization (Δ) indicates the slope of the regression line (b') divided by the maximum ordinate (Y_0) of the theoretical line: $\Delta = b'/Y_0$.

The analysis of variance shows that the "linear" component of the variance due to the tachyphylactic effect is highly significant, and that the combined "Quadratic Residual" component is trivial and non-significant (Table VII). In Fig. 6, the means corresponding to the totals for "Rows" (T_R) are plotted against the four "levels of desensitization," showing the good fit of the experimental data with the regression lines. Table VII shows also the values of Δ, deduced from the experimental data. Its constancy is good evidence that the tachyphylaxis follows a regular course in different pieces of ileum taken from different guinea-pigs.

The totals of "Rows" in the experiments of Table IV have also been used to calculate Δ, by the method described above (Table VIII). Here, too, the regression "responses upon levels of desensitization" is linear, since deviations from

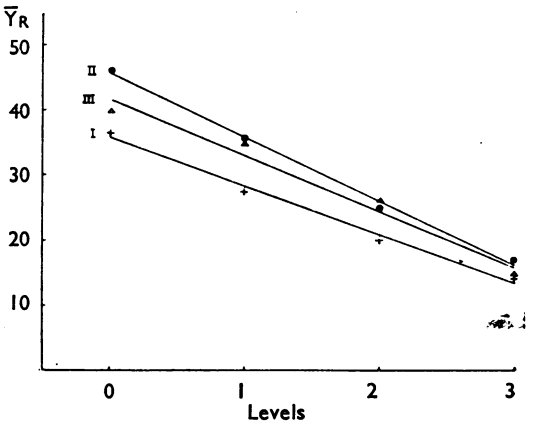

FIG. 6.—Regression lines "means of rows on levels of desensitization." Ordinates, means of "rows." Abscissae, number of previous additions of the same dose of anaphylatoxin. Each line represents the average of four doses.

TABLE VII

REGRESSION LINES AND ANALYSIS OF VARIANCE OF THE EXPERIMENTS DESIGNED TO ESTIMATE THE COEFFICIENT OF DESENSITIZATION

Expt.	Theoretical Lines	Coeff. Desens. (Δ)	Sum of Squares "Between *Levels*"			Error Variance
			Linear	Quadratic	Residual	
1	$Y=35·6-7·4X$	0·20	1,065·80*	5·64†	0·78†	31·59
2	$Y=45·5-9·7X$	0·21	1,876·95*	6·89†	0·66†	18·32
3	$Y=42\ 0-8·6X$	0 20	1,474·90*	40·64†	2·96†	33·08

*, Highly significant; †, non-significant.

TABLE VIII

DATA FROM EXPT. 2, TABLE IV

Regressions of "means of rows on levels of desensitization" and coefficient of desensitization

Expt.	Theoretical Lines	Coeff. Desens. (Δ)	Sum of Squares "Between *Levels*"			Error Variance
			Linear	Quadratic	Residual	
1	$Y=48{\cdot}8-11{\cdot}5X$	0·23	2,663·17*	51·56†	62·83†	19·00
2	$Y=43{\cdot}7-12{\cdot}7X$	0·28	2,953·45*	196·00†	7·00†	74·33
3	$Y=49{\cdot}8-11{\cdot}9X$	0·24	2,856·05*	36·00†	24·95†	63·17
4	$Y=61{\cdot}2-13{\cdot}6X$	0·22	3,712·81*	38·06†	47·13†	29·17

*, Highly significant; †, non-significant.

linearity were non-significant when compared with the Error variance.

Correction for Group Differences and for Δ.—Now, if we take $\Delta=0.22$ for Square IV, Table VIII, and apply a correction to each single response according to its place in the sequence of treatments, a corrected square can be obtained. Fig. 7 gives a graphical representation of this correction operating upon the individual columns of the square. In (a) the experimental data are presented without any correction; in (b) the individual data have been corrected for the tachyphylactic effect, considering $\Delta=0.22$; and in (c) a further correction for the "Group" differences has been applied. It is apparent that the initial scatter of the data about the means becomes corrected, giving an even distribution around the theoretical line.

DISCUSSION

The analysis of variance of the assays presented in this paper shows that the so-called (2 + 2) design can be successfully applied for potency estimations of a material inducing strong tachyphylaxis. The variation from this effect, being due to a systematic source, could be confounded with the order of additions in the common Latin square design, and its sum of squares eliminated from the experimental error, thus increasing the precision of the assay several-fold. If one recalculates the variance of the Error, in the experiment analysed in Table II, including the sum of squares corresponding to "Rows," with 9 degrees of freedom, the corresponding variance will be 296.9 and the standard deviation of a single response will be 17.2 instead of 6.67—a considerable decrease of from 6- to 7-fold of the "information" given by the assay. Deviations from parallelism in all assays studied only once attained the probability level of 5%, and in most assays the sum of squares corresponding to "Parallelism" was of the same, or of a lower, order of magnitude as the Error variance. On a special sequence of assays designed to test linearity, using 2 series of 4 squares each, there was no significant departure from linearity in any single square; only once (Square I, Table VI) the variance ratio (F) corresponding to the "Quadratic" term went up to 189.0/57.7=3.3, still non-significant at the 5% level of probability.

In a total of 13 squares (Table III), the ratio b/s_b was above 10.0 in five, above 7.5 in nine; and only in four squares (Square IV, Expt. 1; Square II, Expt. 2; and Squares I and II, Expt. 3) was this ratio too low for the application of the simplified formula for the calculation of the Error of M (s_M). Therefore, in more than 2/3 of the assays, the application of the simplified formula for s_M was well warranted by the intrinsic qualities of the assays. Considerations derived from the values of $\lambda=s/b$, the "standard deviation in terms of log dose," permit an assessment of the efficiency of each response. In this respect, the present assay is less

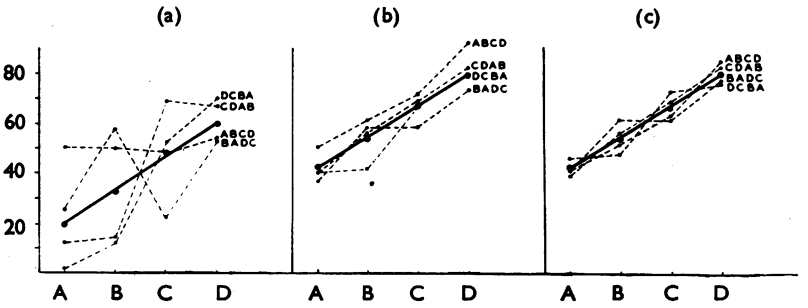

FIG. 7.—Corrections applied to the data of one square utilized in the test of linearity. (a) Without correction; (b) corrected for $\Delta=0.22$; and (c) corrected for Δ and for Group differences.

efficient than similar assays performed with histamine or bradykinin, for instance, in which λ tends to a value below 0.050. However, the precision of the assay (measured as the reciprocal of the Error variance) is about a quarter of that with histamine or bradykinin, but still above the precision of many biological assays with insulin or vitamins (for comparison, see Bliss and Cattell, 1943). Obviously, the precision of a method will depend on the care with which the biological preparation is handled, on accurate measurements of volumes, and on registration of the responses in each replication. This last source of variation is increased when a fresh preparation has to be set up for each new replication of the basic sequence of treatments. The histamine and bradykinin assays can be replicated as many times as necessary upon a single piece of intestine. In an assay involving a strong tachyphylactic effect we are fortunate to be able to assay a whole sequence of treatments (4) constituting a " Group " on the same piece of intestine, as in the present experiments. Then, by confounding inter-intestine variation with variation " Between groups," the precision is considerably increased. However, some inherent variation still remained and it could not be eliminated by the design adopted.

The precision obtained with a single square can be satisfactory for many purposes. If we take the antilog of $s_M=0.050$ for the first assay of Table III, equal to 1.12, it would indicate a standard deviation of $\pm 12\%$ in the estimation of a single ratio of potency; if we take the antilog of $s_{\bar{M}}=0.031$, corresponding to the average $\bar{M}$ of the 5 squares (80 responses), the standard deviation will not exceed $\pm 7.4\%$. In general, if we assume that $\lambda=0.10$, we can calculate how many treatments (N) would be necessary to keep $s_{\bar{M}}$ below, say, 0.030. According to Bliss (1952):

$$N=\frac{4\lambda^2}{s^{2M}}$$

Substituting the data for the letters in this formula, N would be about 40, and if we make an allowance for the variation of λ, and taking $s_\lambda=0.03$, the safest number would be around 96 treatments or about 6 squares. Such a precision, bringing down the standard deviation to $\pm 7.2\%$, will probably not be necessary for a common assay of estimating the potency ratio of two preparations of anaphylatoxin. If one is satisfied with a standard deviation of $\pm 10\%$, corresponding to a $s_{\bar{M}}=0.042$, two squares would be theoretically enough.

Of interest for the study of the intimate mechanism of the tachyphylactic phenomenon is the possibility of estimating Δ, coefficient of desensitization. It was defined as the quotient of the slope of the line by the first response (Y_0) to a single dose of anaphylatoxin. In fact, we are dealing here with effects of " intrinsic " histamine (Dale, 1948), since the responses obtained by repeating the additions of the same dose of anaphylatoxin upon the same piece of ileum are due to successive levels of released histamine. What we have called the regression of " rows on levels of desensitization," or simply " responses on previous additions of anaphylatoxin," actually is a regression of " responses on *doses* of intrinsic histamine," and the fact that it is negative only indicates that the amounts of released histamine are smaller and smaller as tachyphylaxis sets in.

It might be useful to speculate further on the intimate nature of the regression " responses on levels of desensitization." The fact that it is linear, as indicated by the analysis of variance, shows that the process of release is of such a nature that the responses to the successive amounts of the released metabolite are linearly decreased following each new addition of the releasing agent. On this basis, it would be interesting to deduce an arbitrary *scaling* for doses of *intrinsic* histamine released at successive steps of the tachyphylactic effect. The simplest assumption concerning the mechanism of this phenomenon is that the " available " stock of the metabolite becomes more and more depleted and that any further dose of anaphylatoxin will release a certain percentage of the residual " available " stock. This assumption is a natural one, and warranted by previous experiments dealing, for instance, with the release of histamine from the rat's diaphragm by successive contacts with the same concentration of (+)-tubocurarine (Rocha e Silva and Schild, 1949). If it is so, the law regulating this intrinsic phenomenon could be expressed by the exponential

$$D=Ce^{-ax}$$

where x will be 0 or any positive integer from 1 to 3 or 4, until the amount of released histamine becomes too small to produce a measurable contraction, and a and C are constants. Now, if we take the natural logarithms of the doses of released histamine (D), as an expression of the responses obtained at the different levels of desensitization, we will have the equation:

$$Y=k\log_e(Ce^{-ax})=k\log_e C-akx$$

where k is a new constant, and Y the response in mm.

This equation must correspond to the linear relation between the responses and the number of previous additions of the same dose of anaphylatoxin, and therefore could be identified with the

experimental equations of Table VII, calculated by the method of least squares. It is clear that ak can be identified with the slope b' of these lines, and since $\Delta = b'/Y_0$ and $Y_0 = k \log_e C = (b'/a) \log_e C$ we can conclude that $\Delta = a/\log_e C$.

By simple substitution, the above equation can be presented in the form:

$$Y = b'/\Delta - b'x$$

If we take, for instance, the experimental equation for Square II (Table VII)

$$Y = 45.5 - 9.7x$$

it can be compared with the theoretical one deduced above, by making $b' = 9.7$ and $\Delta = 0.21$:

$$Y = 46.0 - 9.7x$$

showing a most excellent agreement between theory and experiment.

Summary

1. A (2+2) assay designed as a 4×4 Latin square is described to estimate a ratio of potency between two solutions (unknown and standard) of a material producing tachyphylaxis when assayed upon the guinea-pig ileum. The material utilized—rat's plasma activated with agar (anaphylatoxin)—has been shown previously to stimulate the guinea-pig ileum by release of histamine.

2. To test linearity of the log dose response curve, the same Latin square design was utilized. Deviations from parallelism and from linearity were almost always trivial and non-significant.

3. The theoretical model adopted, by assuming that each dose affects the responses of any subsequent addition of anaphylatoxin by a certain residual value, introduced the idea of a coefficient of desensitization Δ. The method for its calculation is described.

4. The negative regression " responses on number of previous additions of anaphylatoxin " indicating a linear downward trend of the responses to the same dose of anaphylatoxin has been understood as a linear regression of " responses on doses of *intrinsic* histamine." An arbitrary scaling of doses, $D = Ce^{-ax}$, of intrinsic histamine has been derived from considerations concerning the mechanism of release. By taking the natural logarithms of the doses of intrinsic histamine, as an expression of the responses $Y = \log_e D$, the negative linear regression so obtained could be identified with the regression of " responses on number of previous additions of anaphylatoxin."

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