174. THE BIOLOGICAL ASSAY OF TESTICULAR DIFFUSING FACTOR

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In a series of papers Madinaveitia [1938; 1939, 1, 2; 1940, 1, 2, 3] has recently described investigations into diffusing factors that have been prepared as concentrates, mainly from bull testicle, but also from "Padutin" (Bayer), a commercial preparation of kallikrein, from bacterial cultures, from snake venom and from leech extracts. It has been suggested by Chain & Duthie [1939], and the view has been supported by Favilli [1940] and by McClean & Hale [1940], that diffusing activity may be due to mucinase, which they have found present in similar concentrates.

Confirmation of this view must depend on the possibility of making quantitative estimations of diffusing activity and comparing the results with the mucolytic activities shown by the same materials. The method described by Madinaveitia [1938] for measuring the diffusing activity of his preparations has been stated by him [1939, 2] to be insufficiently precise to permit the rapid progress of his investigations and is thus obviously not suited without modification to the more stringent requirements of comparison with mucolytic activity, which can be determined with some accuracy.

Both for the purpose of such comparisons and also to make possible more rapid advance in concentrating and purifying the diffusing factor itself, it seemed to us desirable, therefore, to attempt an improvement in the method of quantitatively measuring diffusing activity. We accordingly set ourselves the task of so modifying the technique laid down by Madinaveitia as to permit the conduct of biological assays in the strict sense of the term, that is, the comparison of activity with that of a stable standard preparation. Generally speaking, this can be done by means of the "four-point assay"; two doses of the standard and two doses of test material are used, the dose levels, the dose ratio and the number of responses to each dose being chosen so as, if possible, to permit calculation of potency ratios having fiducial limits ("probable limits of error") of between 60–70 % and 150–130 %. Calculation is carried out by the method of Irwin [1937] in a somewhat simplified form.

For the procedure adopted by Madinaveitia reference may be made to his publications. We have investigated a number of the factors likely to contribute to the inaccuracy of the test and have modified the procedure, not only in the light of the information so obtained, but also in certain directions that seemed to us likely to help towards the same end. For example, we have introduced planimetric measurement of the area of bleb, which is traced in Indian ink on large glass microscope slides. We have also paid special attention to preparation of the haemoglobin indicator solution, with a view to eliminating any traces of diffusing factor that might be present in the horse blood, thus reducing to a

minimum both the bleb size produced by the haemoglobin solution alone and its variance.

Inspection of Madinaveitia's curves suggested to us that differences in bleb size due to different doses did not increase materially after 20 min. from the time of injection, and our own observations confirmed this. We have, therefore, for assay purposes made all our measurements of bleb size after that interval.

Exploratory tests with quantities of testicular extract in the ratio 2 to 1 showed that the mean areas of 6 or 8 blebs gave differences that were generally significant. It was considered necessary to attempt further to measure the influence, if any, on bleb size of the position of the injection on the back of the animal and of the order (in time) of injection, as well as to estimate the contribution made by animal variation to the error of the method. Expert statistical advice was accordingly sought,[1] and as a result it was decided to carry out an experiment with six rabbits and six positions for injection on the back of each rabbit and to base the experiment on a Latin square design.

A 6×6 Latin square was therefore selected by the procedure described by Fisher & Yates [1938]. Table 1 illustrates the plan of the experiment. Arabic numerals (1 to 6) refer to the animals, letters (a to f) to the position on the backs of the animals and Roman numerals (i to vi) to the order of injection. Thus each animal was injected once and once only in each position and each position was the rth to be injected (r=i, ii, ..., vi) in only one animal. Also, the sites (a to f) were so arranged that three of them (a, c and e) lay near the animals' vertebrae, while the other three (b, d and f) occupied more lateral positions. In Table 1 are also shown the actual bleb sizes (in square centimetres) 20 min. after the injection.

Table 1

	Animals					
Positions	1	2	3	4	5	6
a	iii	v	iv	i	vi	ii
	7·9	8·7	7·4	7·4	7·1	8·2
b	iv	ii	vi	v	iii	i
	6·1	8·2	7·7	7·1	8·1	5·9
c	i	iii	v	vi	ii	iv
	7·5	8·1	6·0	6·4	6·2	7·5
d	vi	i	iii	ii	iv	v
	6·9	8·5	6·8	7·7	8·5	8·5
e	ii	iv	i	iii	v	vi
	6·7	9·9	7·3	6·4	6·4	7·3
f	v	vi	ii	iv	i	iii
	7·3	8·3	7·3	5·8	6·4	7·7

The total sum of squares of deviations from the mean of all the observations is 30·3599 and the analysis of variance is as follows:

	Sums of squares		Degrees of freedom		Mean squares	
Animals	12·8333		5		2·5667	
Position	3·8332		5		0·7667	
Order	0·5632	17·5266	5	30	0·1106	0·5842
Error	13·1302		20		0·6565	
Total	30·3599		35		(0·8674)	

[1] The statistician concerned wishes to remain anonymous; we must nevertheless express to him our sincere thanks for his assistance.

The effects of position on the animal and of order of inoculation are not significant, and the last three components of variation may therefore be combined to give an error variance of 0·5842. The estimate of the portion of the total variance due to animal variation is therefore (2·5667–0·5842)/6 = 0·3304.

An unbiased estimate of the total variance is therefore (0·3304 + 0·5842) = 0·9146. Of this 36 % is due to animal variation and 64 % to general experimental error. It is also clear that the mean of all the median blebs (positions *a*, *c* and *e*) does not differ significantly from the mean of all the lateral blebs (positions *b*, *d* and *f*); the values are 7·36 and 7·38, with $t = 0·7$ and $P = 0·45$.

The result of the experiment shows that it will be advisable, whenever possible, when two different preparations of diffusing factor are to be compared, to inoculate the pairs of doses to be compared into each of several animals, so that animal variation may be eliminated from the error of the result. It is clear that the site of the blebs on the animals' backs—within the range used in the Latin square experiment—and serial position in time have no significant effect and need not be controlled, but that uniform distribution of test doses over all the animals used is essential if the error of a test is to be kept minimal.

The ability to distinguish significantly between the means of six or eight responses to doses in the ratio of 2 to 1 is, however, generally considered a satisfactory achievement in the biological assay of hormones, vitamins and so on, and can be made the basis of four-point assays having not more than the desired fiducial limits.

Investigation of several varieties of rabbit were also made, with a view to using animals giving not only the maximum discrimination between doses in a given ratio but also the smallest variance in response to a given dose. At the same time we examined the range of doses over which it should be possible to conduct "four-point" assays, that is, the range over which the relationship between response and logarithm of dose did not depart significantly from linearity.

In general, it is necessary to use large rabbits, weighing at least 2 kg., having non-pigmented skins (a condition independent of fur colour) fairly, but not excessively thin. We have found no variety better than the Belgian Hare-Flemish Giant cross, although we have used Himalayan, Dutch, Polish and other breeds. Chinchillas are the least satisfactory.

With the animals and the technique outlined, a range of doses giving bleb-sizes from 4 to 8 sq. cm. has been found to satisfy the necessary requirements, the bleb-size given by the indicator solution being always below 4 and generally below 3·5 sq. cm. The mean control bleb-size is about 3·3 sq. cm. and the variance of the indicator bleb-sizes is very low.

The following is a summary of the procedure now used by us:

Suitable rabbits—preferably crosses between Belgian Hare and Flemish Giant, but in any event animals with thin non-pigmented skins—are shaved, with or without previous depilatory treatment (such as application of the paste described by Zacho [1939] for use on guinea-pigs). Almost the whole of the back and sides can be shaved, permitting production of 12 or even more blebs on each animal.[1] Injection is made with a 240 intradermal Agla needle, on the day following shaving.

The indicator solution is prepared from centrifuged fresh horse blood; the corpuscles are washed 4–5 times with fresh isotonic saline. The saline is decanted and distilled water is added to give 100 ml. of solution with the corpuscles from 100 ml. of blood. Laking must be complete, as judged microscopically, and after

[1] One of us (T. R. M.) has recently been able to accommodate 21 blebs on each of four rabbits.

ERRATA

VOL. 34, Nos. 10 and 11, page 1467

line 1 *after the words* 'in suspension'

add 'Sodium chloride is then added to the clear solution to give molar concentration'

centrifuging there must be no unbroken cells in suspension. This solution will keep in the refrigerator for 2 or 3 weeks. Any indicator solution producing in doses of 0·2 ml. blebs as large as 4 sq. cm. (in 20 min.) should be discarded.

The amount of indicator solution used is always 0·2 ml.; with this is mixed the required dose of diffusing factor, made up to 0·1 ml. with distilled water, giving a total of 0·3 ml. for injection.

Injections should be so arranged that each animal receives the same number for each test dose: with large animals the number can be three, and there will also be room for one or two control blebs. Injections should be so arranged in time that the first blebs to be made do not encroach on the area required for those made later: there is still an increase in bleb-size after 20 min., but there is no difficulty in avoiding overlap with proper arrangement of doses in time and space.

The calculation of variance, standard error of mean bleb-size and potency ratio follows the now well-known procedure of Irwin [1937]. There is, however, a departure from the usual condition, in which means are calculated from a series of observations normally distributed round the mean. As we have found that the differences between bleb-sizes on the same animal are less than the differences between mean bleb-sizes on different animals for the same dose, the distribution of the whole of the observations will depart from normal if marked differences exist between animals, especially with the small number used—three or four. It is therefore the more important to use animals showing as nearly as possible the same mean response. This is best achieved by careful selection for skin-thickness and by having available one or two animals besides those actually to be used in the assay. As soon as the first blebs have been observed, any abnormally sensitive or insensitive animal can be rejected and one or more of the reserves brought into use. We have, however, seldom found this necessary. A suitable modification of statistical procedure, by analysis of variance, would make it possible to deal with any markedly heterogeneous distribution shown by the responses.

Certain experimental records will now be quoted to illustrate the kind of result obtainable.

(1) Two doses of a concentrate were tested; they were in the ratio of 2 to 1. Each dose was used to produce four blebs on each of two rabbits. The results were as follows:

	Bleb area (sq. cm.)	
Animal	Low dose	High dose
1	4·0, 4·5, 5·4, 5·7	6·8, 7·1, 6·8, 7·1
2	4·8, 5·0, 4·2, 5·5	5·8, 6·6, 7·3, 5·9
Mean	4·89	6·68
S.E. of mean	0·220	0·196

For the difference between the means, t has a value of six, corresponding with $P \ll 0{\cdot}001$. Another animal of the chinchilla type was used to produce six blebs with the lower dose; these had a mean area of only 3·7 sq. cm., indicating that the animal was less sensitive than the thinner-skinned rabbits used for the comparison. In this test, doubling the dose produced an increase of 1·8 sq. cm. in mean bleb-size; the value of b, the slope of the response curve, is therefore $\frac{1.8}{\log 2} = 6$.

(2) A powdered concentrate was tested in doses of 1·6 μg. and 3·2 μg. on two rabbits, each receiving three injections of each dose. The mean bleb values were 7·08 and 8·55 sq. cm.; for this difference the value of t was 3·05 ($P = 0{\cdot}015$). The slope of the response curve was 4·85, rather less than in the preceding experiment; ceiling values of diffusing factor were possibly being approached. Polish rabbits

were used in this experiment and they may respond to a somewhat lower dose level than the preferred cross, owing to their smaller size, which is also a disadvantage in itself.

(3) A certain batch of material (*A*) had been used in preliminary tests as a provisional standard preparation: it was desired to replace this with another batch (*B*) of which considerably larger supplies were available. As it was only possible to hazard a guess at the relative potencies of *A* and *B*, it was decided to test the latter at four dose levels compared with two dose levels of the former. The results are shown in Table 2.

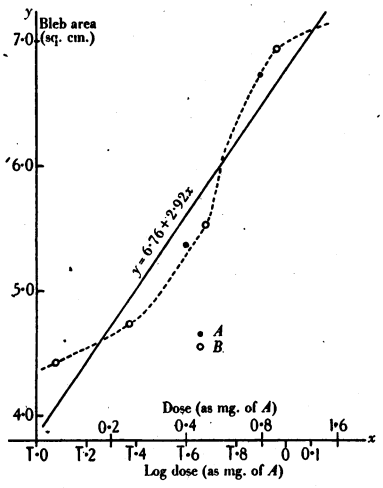

Fig. 1.

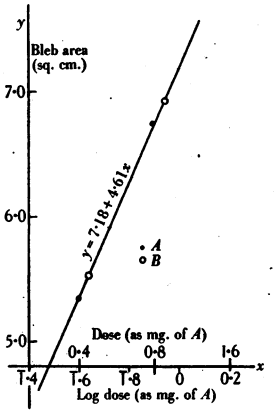

Fig. 2.

Table 2

Sample	Dose μg.	No. of blebs	Bleb area (sq. cm.) Mean	Stand. dev.	Coeff. of var.	S.E. of mean	For differences *t*	*P*
A	0·4	12	5·36	0·95	18·0%	$0·27_5$	3·03	0·008
	0·8	12	6·75	1·26	19·0%	0·36		
B	0·1	12	4·42	0·64	14·5%	$0·18_5$	1·13	0·25
	0·2	12	4·74	0·75	16·0%	$0·21_5$	2·08	0·05
	0·4	12	5·52	1·06	19·0%	$0·30_5$	3·40	0·003
	0·8	12	$6·92_5$	0·95	14·0%	$0·27_5$		

Table 3

Assay calculated from	Total no. of blebs	Slope of combined dosage-response curve	Potency ratio *B* : *A*	Fiducial limits of dose ratio *P* = 0·95	*P* = 0·99
2 doses *A*	72	2·92	$1·20_5$	0·82–1·78	0·72–2·01
4 doses *B*	—	—	—	(67·5–148%)	(59·5–167·5%)
2 doses *A*	48	4·61	$1·09_5$	0·81–1·48	0·74–1·62
2 doses *B*	—	—	—	(74–135·5%)	(67–149%)

If the responses are all taken into account and the potency ratio is calculated by means of a dosage-response curve derived by the method of least squares, a result is obtained that is shown graphically in Fig. 1. The value of *b*, 2·92, is

much less than found in Exps. 1 and 2. Moreover, it is clear that the departure from linearity of the six plotted points indicates that the sigmoid nature of the dosage-response curve has persisted even when response is plotted against log dose. This phenomenon is discussed by Emmens [1940] in a forthcoming publication; it shows that the range of responses found is too wide to justify them all being used to construct the regression line for response on log dose. Accordingly the result of the assay has been recalculated, omitting the responses to the two lower doses of *B*. Graphically, this is shown in Fig. 2. Linearity is here completely restored, because the four responses fall on that part of the dosage-response curve where response is a linear function of log dose.

If the two assays—"six-point" and "four-point" respectively—be calculated according to Irwin's method, results are obtained as shown in Table 3. It is clear, as the plotted curves also indicate, that reducing the number of observations by one-third—that is, eliminating those that invalidate calculations by the method of least squares—has not only brought about some change in the found potency ratio, but has also considerably reduced the error of the test. Obviously, had 72 blebs been available for use in the "four-point" assay, the fiducial limits would have been still further reduced. It will be noted that the "four-point" assay gives to *b* a value of 4·6, rather less than that obtained in Exp. 1, but not significantly different from that obtained in Exp. 2.

(4) In this experiment we assayed a sample of Dr Madinaveitia's standard preparation (*M*), of which he very kindly supplied us with a sample. Having no foreknowledge of its potency in relation to our own standard, we tested both at three dose levels, using five rabbits, with the results shown in Table 4.

Table 4

Sample	Dose μg.	No. of blebs	Bleb area (sq. cm.) Mean	Stand. dev.	Coeff. of var.	S.E. of mean	For differences *t*	*P*
M	0·2	10	4·25	0·80	18·7%	0·25		
	0·4	10	4·85	0·96	19·8%	0·30	1·5	0·15
	0·8	10	5·93	0·76	12·9%	0·24	2·8	0·01
B	0·2	10	5·04	1·12	22·3%	0·36		
	0·4	10	5·47	0·89	16·2%	0·28	0·95	0·35
	0·8	10	6·45	1·15	17·9%	0·36	2·2	0·04

Table 5

Sample	Total no. of blebs	$\bar{y}_2 - \bar{y}_1$	Mean variance	*b*	Units (G.L.)* per mg.	Fiducial limits *P* = 0·95	*P* = 0·99
A	4 × 12	0·17	1·134	4·61	1820	74–135·5%	67–149%
M	5 × 8	0·57	0·905	3·42	1370	63·5–157%	55–181%

* For assay purposes a "unit (G.L.)" is defined as the diffusing activity of 0·5 μg. of product *B*.

It is clear that the lowest dose of each standard is below the threshold, that is, gives a response on the tail of the dosage-response curve, where response is no longer proportional to log dose. These two responses were therefore ignored, and the potency ratio of *M* and *B* was calculated from the remaining four responses. For purposes of comparison, the results are shown in Table 5 along with the results of the evaluation of our first laboratory standard (*A*) in terms of the second, as described under Exp. 3.

It will be seen that the assay of *M* was rather less satisfactory than that of *A* (against *B* as standard in both instances). This is largely due to a low value of *b*,

for which the relatively poor discrimination shown between doses was responsible: doubling the dose—from 0·4 to 0·8 μg.—produced an increase in mean bleb size of only 1·03 sq. cm., whereas in Exp. 3 the same increase in dose had produced an increase of 1·4 sq. cm. in mean bleb-size.

(5) It was desired to examine the effect, if any, of including 0·5 % of phenol on the diffusing factor activity of an aqueous solution of a solid concentrate. A four-point assay, using the usual dose ratio of 2 to 1, was carried out on four animals, each of which received two injections of each dose. The results are shown in Table 6.

Table 6

		Bleb area	
Solution	Dose	Mean	$(\text{S.D.})^2$
Without phenol	Lower	5·81	0·781
	Higher	$7{\cdot}42_5$	0·197
With phenol	Lower	5·45	0·262
	Higher	7·24	0·344

A very low mean variance was found in this assay (the mean coefficient of variation was only 9·7) and this, combined with a high value of b (5·64) led to very low fiducial limits for the mean potency ratio calculated. The figures were

	Probable limits of error	
Ratio	$P=0{\cdot}95$	$P=0{\cdot}99$
0·894	79–127 %	83·5–120 %

In spite, however, of the low limits of error, it was found that the ratio of 0·894 did not depart significantly from 1—as can be seen from the values for $P=0{\cdot}95$; the chance of the 10 % difference being due to random sampling errors is 1 in 6.

These experiments seem to us to indicate that the level of accuracy obtainable with the technique described is not on the average less than that of other biological assays carried out on a comparable scale. There are, of course, variations from test to test, both in the values of b and in the mean variance of response, but such fluctuations in the gamble of animal assay are inevitable and can be urged no more cogently against one procedure than against another; the crucial test is the general level of error found over a series of assays. In this connexion it is pertinent to point out that bleb-size shows in various dosage groups a coefficient of variation ranging from 5·9 to 22·3 % with a mean (unweighted) value for 22 groups (216 blebs) of 14·3. A scattergraph shows little evidence of correlation between mean bleb size and the standard deviation of single observations in a group; the correlation coefficient for the two sets of values was indeed found to be only 0·35 (for 22 pairs, $P \doteq 0{\cdot}1$), suggesting that any other expression of diffusing effect—e.g. square root of bleb-size or logarithm of bleb-size—is unlikely of itself to lead to a reduction in the error of tests carried out by our present procedure.

Summary

A method is described for comparing the diffusing factor activities of pairs of preparations, with an estimate of the error of comparison, by measurement of the mean bleb-size produced in suitable rabbits, followed by the construction for each assay of a response-log dose curve by the method of least squares and calculation of the fiducial limits of the derived potency ratio of the preparations. The error is no greater than that found in other biological assays carried out and evaluated on comparable lines.

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centre of page	*for*	Ratio	$P=0{\cdot}95$	$P=0{\cdot}99$
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