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Rapid histamine assays: a method and some theoretical considerations

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Summary

1. An automatic method for doing assays on superfused isolated tissues has been developed using the Autoanalyser sample turntable.

2. Responses in the range used for assays are independent of the preceding responses when elicited at 1 min intervals. A longer interval was found to be necessary when using the conventional immersion method.

3. The index of precision was similar to that reported for other superfusion methods.

4. The assay method behaved as though it were assaying histamine selectively in two samples of material released by the anaphylactic reaction of passively sensitized guinea-pig lung. Responses to anaphylactically released material were also independent of the preceding response at 1 min intervals.

5. Some theoretical considerations are discussed concerning the arrangement of doses, and the interpolation of results, in 2+1 dose assays. The object was to maximize speed while still allowing a valid estimate of the potency and its error.

Introduction

The assay of fifty or more histamine containing solutions a day is a routine problem in work on the mechanism of anaphylactic sensitization. An automatic assay apparatus of the sort described by Boura, Mongar & Schild (1954) is used in many laboratories, the assays being arranged in a variety of statistically dubious ways in the interest of speed. The methods to be described are the result of attempts to maximize output and to minimize statistical sin.

Part 1. *Use of the Autoanalyser sampler for assays by superfusion*

It has been found convenient to use the Autoanalyser sampler II (Technicon Limited, Chertsey, Surrey) to supply samples to a superfused segment of guinea-pig ileum.

Methods

The arrangement is indicated in Fig. 1. The sampler consists of a turntable fitted with forty cups, each loaded with 0·8–1·0 ml. of unknown histamine-containing solution, or up to 10 ml. of standard histamine solution. A peristaltic pump applies histamine solution to the ileum for 10 s; for the rest of the cycle the pump inlet

is transferred to a wash reservoir (near the turntable) containing Tyrode solution. A suitable flow rate was found to be 4 ml./min. The sequence of operations is controlled by a Technicon timing cam which was suitably modified. The solutions pass through narrow bore polythene tube except for a section of 1/16 inch bore, 1/16 inch wall Tygon tube (U.S. Stoneware Co., Ohio ; V. A. Howe Limited, London) in the peristaltic pump. The polythene tube passes through a jacket circulated with water at 32° C and then to a nozzle close to the thread attaching the ileum to a transducer. The temperature of the solution at the nozzle was about 28° C. Usually responses were recorded with a Grass FT03 isometric transducer. Isotonic recording was done with a Sanborn 7DCDT-1000 transducer.

Results

Dose response curves

The dose response curves using isotonic and isometric recording are shown in Fig. 2. Doses were given in random order (five random blocks were usually done). Isotonic recording gave a slightly steeper curve but was less convenient with the

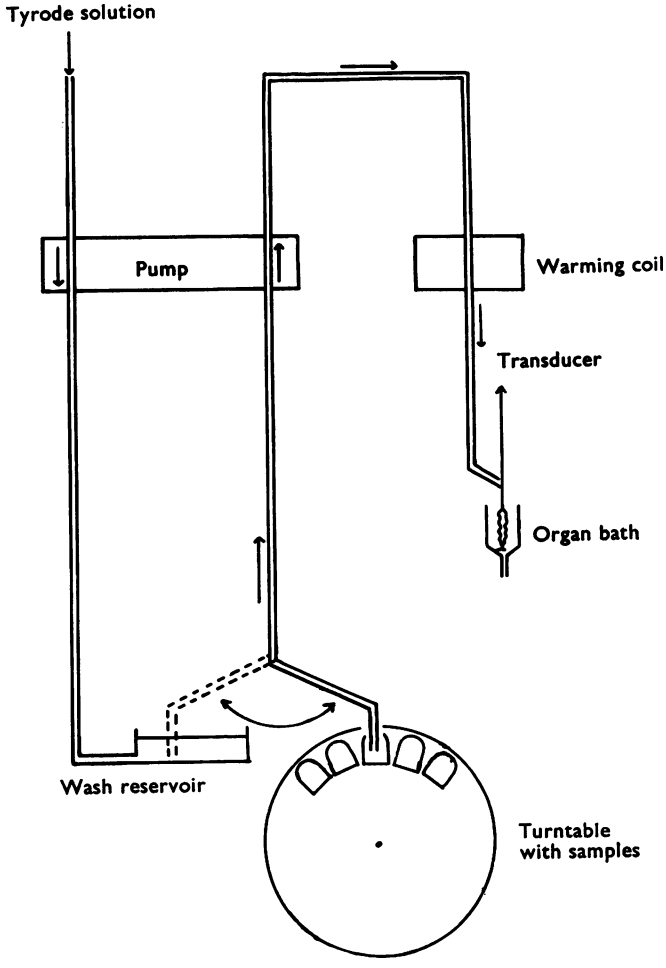


FIG. 1. Diagram of superfusion assay method.

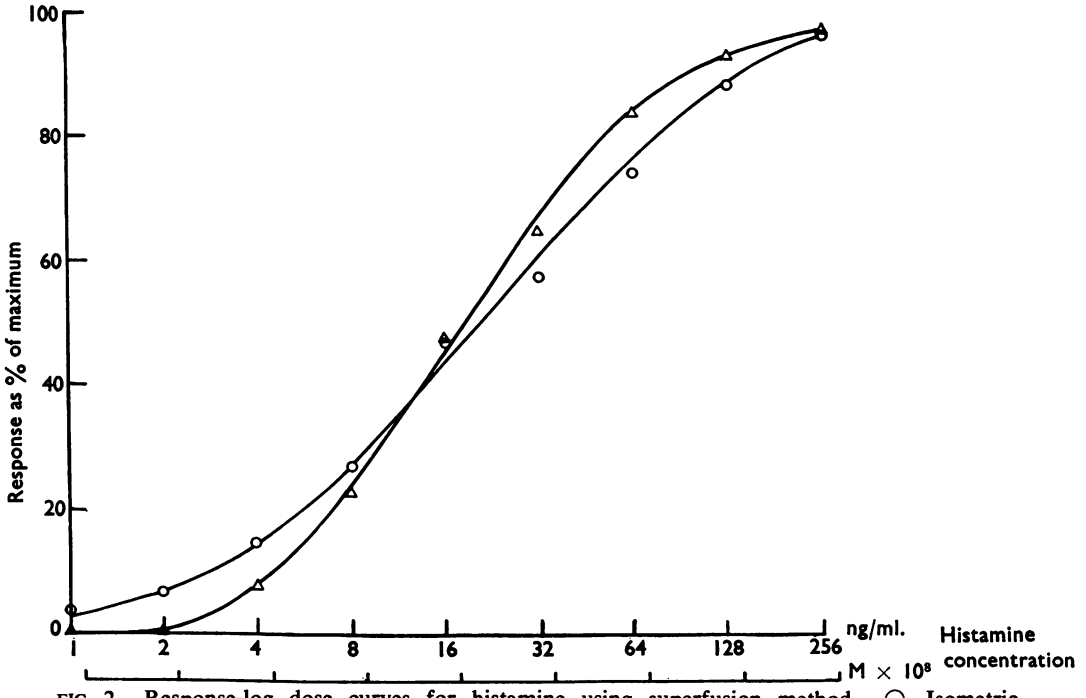


FIG. 2. Response-log dose curves for histamine using superfusion method. ○, Isometric transducer (1·0 g resting tension); △, isotonic transducer (1·0 g tension).

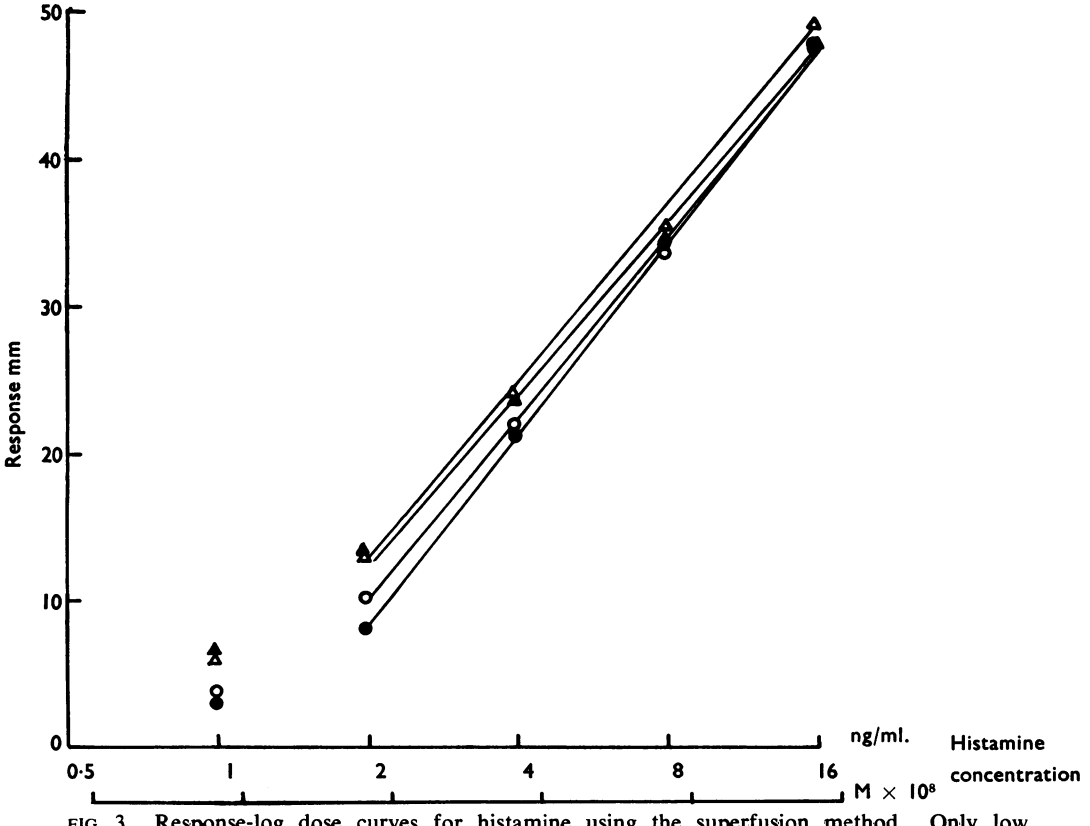


FIG. 3. Response-log dose curves for histamine using the superfusion method. Only low doses were given. Isometric transducer. Number of samples per hour: △ 60, ▲ 70, ○ 90, ● 110.

transducer available, so isometric recording was used routinely. The dose response curve obtained using the conventional immersion method of assay with isotonic recording on a smoked drum was very similar to the isotonic curve shown in Fig. 2. With both immersion and superfusion methods, sensitivity is increased if low doses only are given. This is what would be expected from the interactions between responses discussed below. In almost all assays the log dose-response curve was

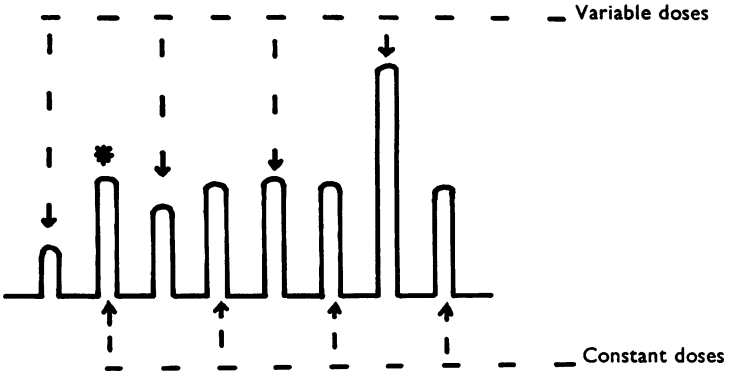


FIG. 4. Arrangement of doses for interaction experiments. All responses are expressed as a percentage of the mean response to the constant dose (marked with asterisk) following the lowest variable dose.

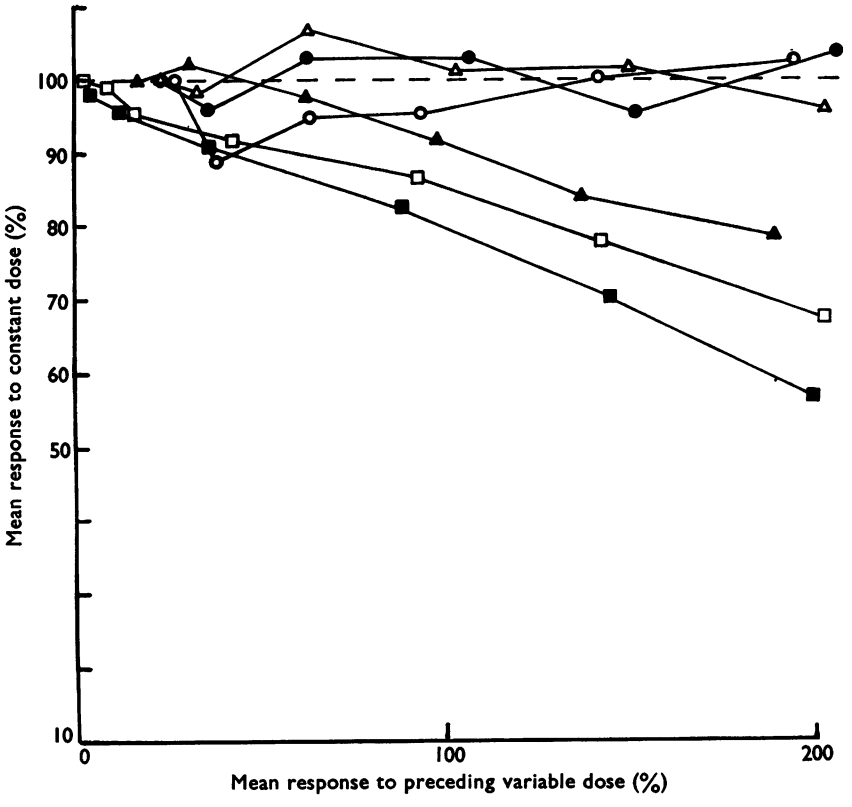


FIG. 5. Interaction between responses to histamine at 40 (○), 50 (●), 60 (△), 70 (▲), 90 (□) and 110 (■) samples per hour with the superfusion method and an isometric transducer.

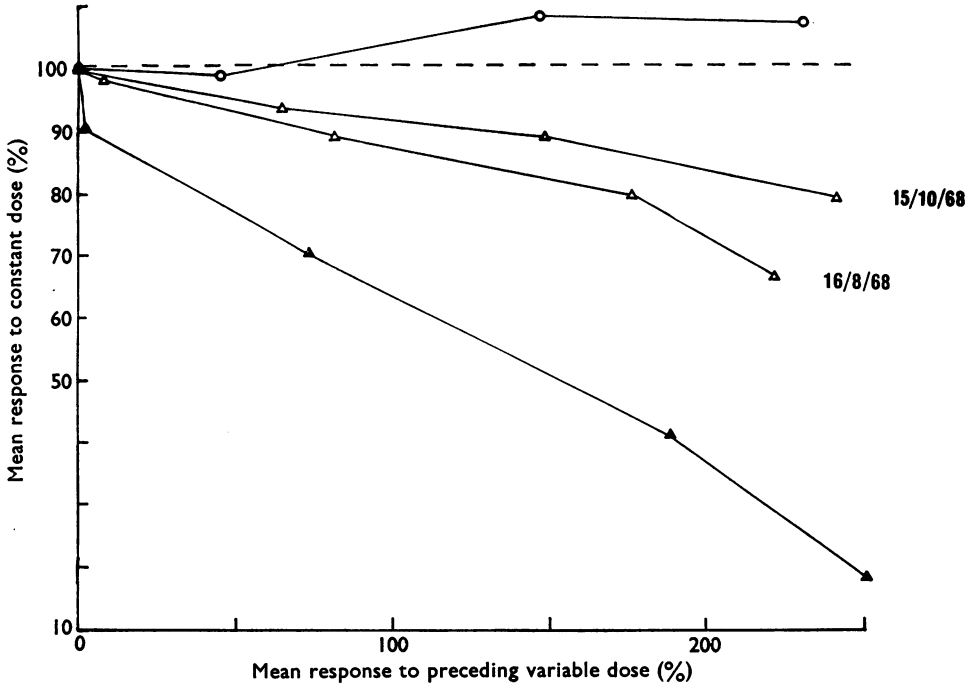


FIG. 6. Interaction between responses to histamine at 40 (○), 60 (△) and 90 (▲) samples per hour with the immersion method and isotonic recording.

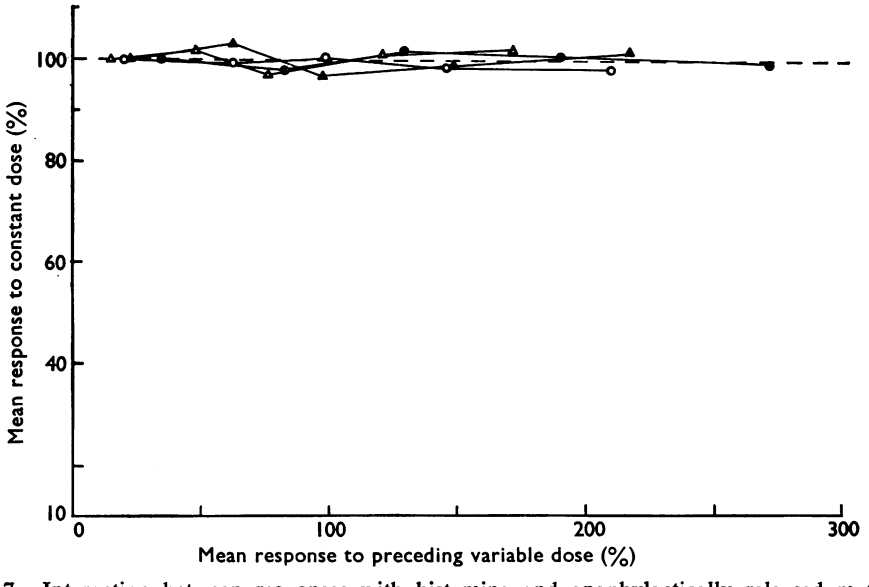


FIG. 7. Interaction between responses with histamine and anaphylactically released material (ARM) at sixty samples per hour with the superfusion method.

	Variable dose	Constant dose
○	Histamine	Histamine
●	Histamine	ARM
△	ARM	Histamine
▲	ARM	ARM

sufficiently straight to be used with histamine concentrations down to 2×10^{-8}M ($\simeq$2 ng/ml.) as long as large doses were not given. Fig. 3 is typical.

The index of precision of the log dose response line, $\lambda = s/b$ (s^2 = error variance from the analysis of variance, b = slope using $\log_{10}$ concentration as dose metameter) was in the range 0·02 to 0·07 (eight experiments) for the superfusion method. Similar values were found by Adam, Hardwick & Spencer (1954) and by Cambridge & Holgate (1954).

Interaction between responses

It is necessary (or at least very desirable) that the size of a response should not depend on the size of previous responses. This is discussed in greater detail in Part 2. In fact there is a tendency for the response following a large dose of histamine to be depressed, but this effect can be reduced to a negligible size by leaving a sufficient interval between doses.

The interaction between responses was investigated by giving alternately a constant dose (usually 4 ng/ml.$\simeq 4 \times 10^{-8}$M) and a variable dose, as illustrated in Fig. 4. The variable doses were usually 0·5, 1·0, 2·0, . . . , 16·0 ng/ml. ($\simeq$0·5 etc. $\times 10^{-8}$M), given in random order (five random blocks were usually done). The highest

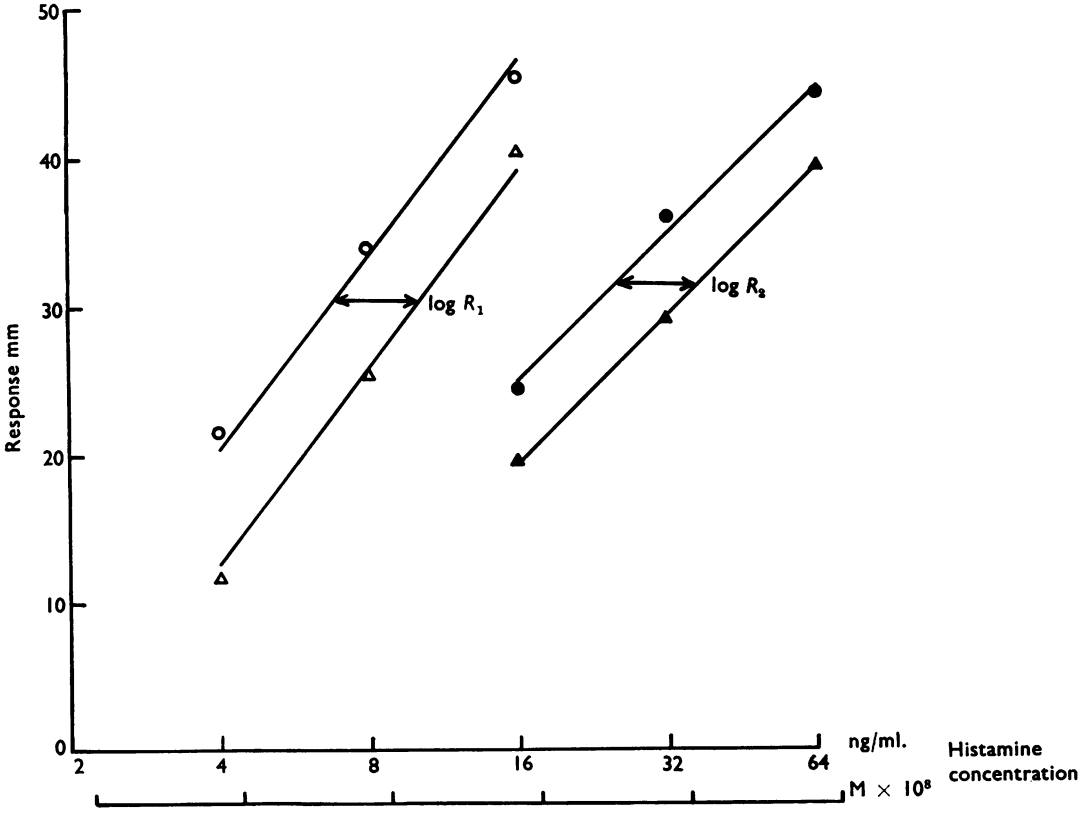


FIG. 8. Effect of mepyramine (3×10^{-9}M) on responses to histamine and anaphylactically released material (ARM) with the superfusion method and sixty samples tested per hour. ○, Histamine ; △, ARM ; ●, histamine + mepyramine ; ▲, ARM + mepyramine. $R_1 = 0{\cdot}666$, $R_2 = 0{\cdot}679$, $R_1/R_2 = 0{\cdot}981$; 95% gaussian confidence limits for R_1/R_2 0·815 to 1·18.

standard dose in most assays was 4 ng/ml. ($\simeq 4 \times 10^{-8}$M). All responses were expressed as a percentage of the mean response to the constant dose that followed the smallest variable dose, as indicated in Fig. 4.

The results in Fig. 5 show that if seventy or more doses were given per hour, the response to the constant dose was depressed to an extent depending on the size of the preceding dose. At sixty or fewer doses per hour the responses were essentially independent of each other. Figure 6 shows the results of similar experiments using the conventional immersion assay method. With this method it was found necessary to reduce the rate to forty samples per hour to ensure independence of the responses.

Use of the method with material released in anaphylaxis

Chopped perfused lung tissue from normal guinea-pigs was used as described by Colquhoun & Brocklehurst (1965). It was sensitized *in vitro* with 5·0 μg/ml. of specifically purified guinea-pig anti-(dinitrophenylbovine gamma globulin)γ_1-globulin for 2 hr, and challenged with 100 μg/ml. dinitrophenyl human serum albumin. The material released had both histamine-like and SRS-A-like activity. It was assayed without further processing. In the presence of mepyramine, 10^{-6}M, the dose of material had to be increased roughly 20–60 times to keep the size of the response the same as before mepyramine.

Figure 7 shows that there is no sign of interaction between responses at sixty doses per hour when anaphylactically released material is used either as the constant dose or as the variable dose or as both doses.

The superfusion assay method appears to detect only histamine in our material. It remains to be seen whether the specificity of the method for histamine extends to samples with, say, a lower ratio of histamine to SRS-A.

Figure 8 shows an estimation of the potency ratio (R) between the anaphylactically released material and pure histamine before (R_1) and after (R_2) mepyramine (3×10^{-9}M). For Fig. 8, $R_1/R_2 = 0{\cdot}981$ with 95% gaussian confidence limits 0·815 to 1·18.

In an experiment with a different sample the ratio was $R_1/R_2 = 1{\cdot}19$ with 95% gaussian confidence limits 0·996 to 1·43. There was no reason to believe that the log dose-response lines for anaphylactically released material and histamine were not parallel, or that the potency ratio between anaphylactically released material and histamine was altered by mepyramine.

In some experiments, similar to that in Fig. 8, there was statistical reason to believe that the histamine log dose-response curves were not parallel before and after mepyramine, presumably because of changes in the tissue with time. In others, deviations from parallelism were no greater than could be expected on the basis of experimental error. For an experiment of the latter sort the pA_2 for mepyramine-histamine was 9·2 and the pA_2 for anaphylactically released material was 9·3 (Arunlakshana & Schild (1959) found a pA_2 of 9·3 for mepyramine-histamine on guinea-pig ileum).

Part 2. *Design of rapid assays*

It is common for routine laboratory assays of large numbers of solutions to be designed in a way which defies any attempt to get a valid estimate of the experi-

mental error, or even of the unknown concentration. The design described below is far from ideal from the point of view of bioassay theory, but it is fast and avoids some of the common faults of fast methods.

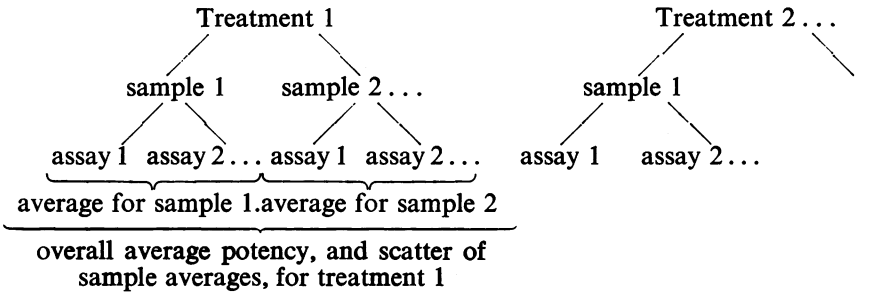
Allowance for interaction between doses

The arrangement of doses randomly (or in random blocks, latin squares, etc.) does not, of course, eliminate the requirement, for all conventional methods of analysis, that responses should not affect each other. Experimental designs have been described that allow for the effect of one response on subsequent responses (see, for example, Finney, 1964, pp. 291–293, 1956; Finney & Outhwaite, 1956). Various models for the interaction are possible, but, although there is some knowledge about the desensitization produced by large doses of histamine (Paton, 1961), there is not sufficient information to say which model is appropriate. The only interaction model suitable for experiments in which all responses are obtained from the same tissue is the residual effect model of Finney & Outhwaite (1956). Even if this model fitted the observations, the complexity of the design and analysis make it unsuitable for the sort of rapid assays under discussion. And even for assays over which more time can be spent, it may well be preferable (Finney, 1964, p. 293) to leave a sufficient interval between doses to ensure their independence, using, if necessary, an incomplete block arrangement (Finney, 1964, Chapter IX; Colquhoun, 1963).

Rapid (2+1) dose assays

Assays with two doses of standard (high standard = HS, and low standard = LS) and one of unknown (U) have been used routinely. Ideally each of the three doses should be given several, say n, times, all $3n$ doses being given in random order or in n random blocks of 3. Usually, however, the order is not randomized, to save time. Furthermore it increases the speed if standard and unknown are given alternately, thus: HS, U, LS, U, HS, U, LS, etc. The concentration of the unknown is then interpolated between the standards on each side of it (see below). This introduces another problem. Although responses have been shown in Part 1 to be independent of each other when elicited at a rate of not more than one a minute, the repeated assays on a sample will not be independent of each other, because one standard response is used for the unknown on each side of it. If a response to LS were excessively low, it would make the estimates of both the U preceding it and the U following it too high. This means that the scatter of repeated estimates of concentration on a single sample will not give a realistic estimate of the experimental error of the assay. The simple way out is to do two standards for each unknown, but this would increase the time taken by 50%. In order to obtain valid estimates of error without using two standards for each unknown, the method described below, which is suitable for experiments laid out like ours, has been used for routine assays. This method ensures that the estimate of scatter is obtained from observed means that are independent of each other.

In the sort of experiments with which we are concerned the samples to be assayed are from experiments in which various treatments (for example, antibody concentrations), each replicated several times, are tested in random order. On each of the replicate samples for a given treatment, several replicate assays (each consisting of standard, unknown, standard) are done using the alternate standard and unknown arrangement described above. The arrangement is as follows:



The replicate assays on each sample are done consecutively. The samples are assayed in the (randomized) order in which they were arranged in the experiment, or, preferably, in a newly randomized order. For each treatment, the several replicate assays on each sample (which are not independent) are averaged, and the scatter (for example, standard deviation) of these average figures (which have no responses in common and are therefore independent as required) is used as an estimate of the experimental error of the histamine release for the specified treatment. The estimate of error includes both the scatter of histamine contents of replicate samples and the scatter of replicate assays on each sample. An extra standard dose should be given between the set of assays on one sample and the set of assays on the next, so that there are no standard doses in common between the sets (often this will be ensured by the necessity for a trial dose to find an appropriate dilution of the sample).

Interpolation of the unknown

The log dose response curve in Fig. 3 is sufficiently straight for the unknown concentration, z_U, to be estimated from

$$\log z_U = \log z_{LS} + \alpha \log D \tag{1}$$

that is,

$$z_U = z_{LS} \cdot D^{\alpha} \tag{2}$$

where z_{LS}=low standard concentration; $\alpha = a/b$ (see Fig. 9), the response to unknown minus response to low standard, as a proportion of the difference between the standard responses on each side of the unknown; and $D = z_{HS}/z_{LS}$, the ratio of high standard concentration to low standard concentration.

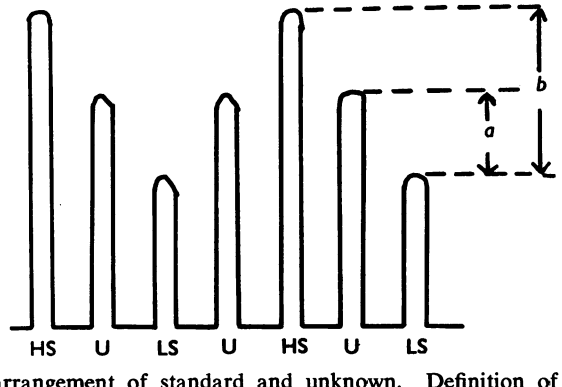


FIG. 9. Alternate arrangement of standard and unknown. Definition of quantities *a* and *b* used in discussion of interpolation.

This method will be called logarithmic interpolation.

It would save time if logarithms did not have to be used. Different laboratories have used different methods of achieving this. One method is to use a Perspex template, which allows logarithmic interpolation to be done graphically on the original record. Another method is to prepare tables of $D\alpha$ for all the commonly used values of D so the factor for use in equation 2 can be obtained without calculation, once α has been measured. Copies of such tables will be sent on request. It is the practice in some laboratories to guess α by eye from the original record. The

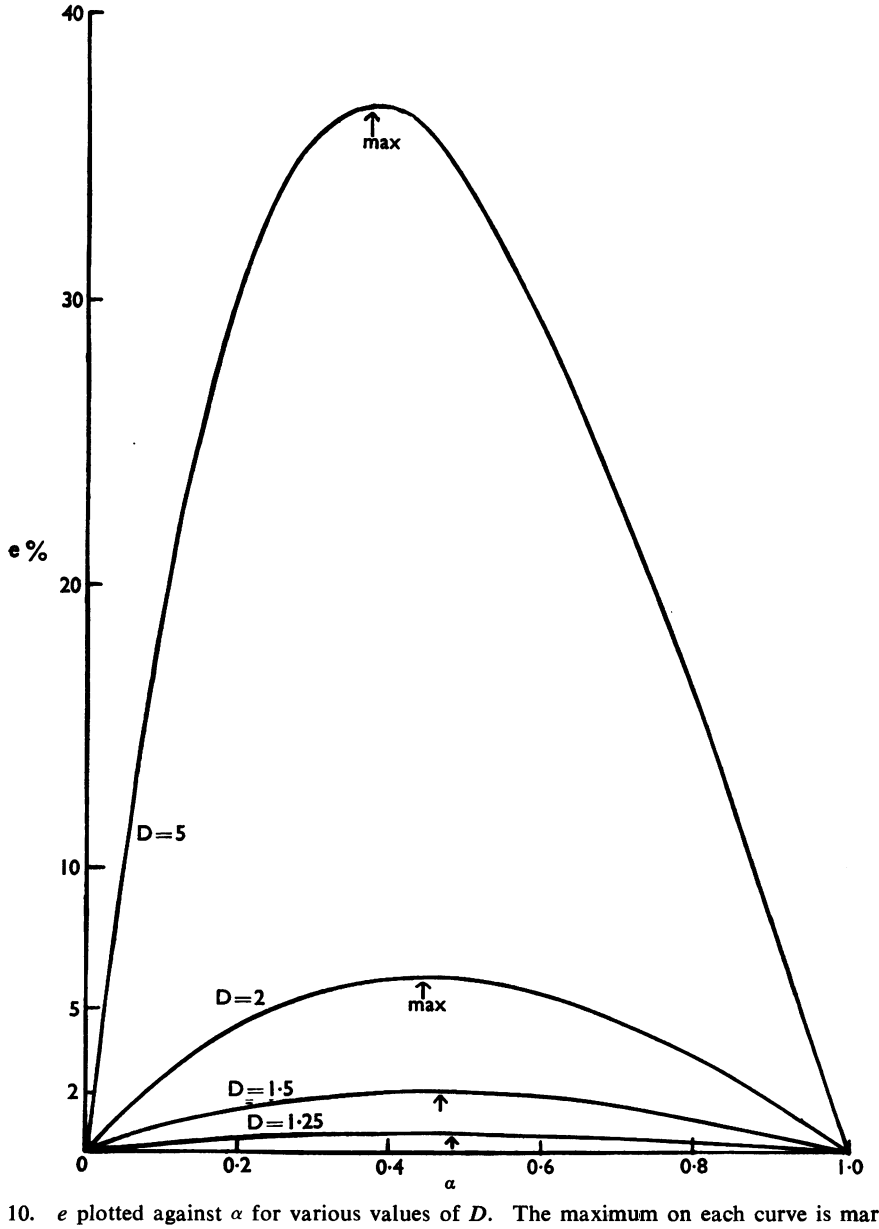


FIG. 10. e plotted against α for various values of D. The maximum on each curve is marked with an arrow.

guessed value of α can then be used in equation 2, with the aid of tables of $D\alpha$, or it can be used for linear interpolation, which can be done mentally using equation (3). Although this sounds crude, it has been shown (Colquhoun, 1964), by comparison with proper measurement, that this very rapid method can be surprisingly accurate. But it is, of course, necessary for anyone wishing to use this method to check that its accuracy is sufficient for his purposes.

The method of assay described, like most methods used for rapid routine assays, has no internal check on the linearity of the log dose-response curve. In fact when isolated tissues are near to the limit of their sensitivity the curve must be concave downwards, as seen at the foot of Figs. 2 and 3, so, in this region, the dose (rather than log dose)-response curve may be more nearly straight. If the dose-response curve were straight the unknown concentration would be estimated as

$$z'_U = z_{LS} + \alpha(z_{HS} - z_{LS}) \tag{3}$$

This method will be referred to as linear interpolation. It will now be shown that logarithmic interpolation (from equation 1 or 2) and linear interpolation (from equation 3) give almost the same answer if D is small. This means that, if D is small, the result of the assay will not be very sensitive to the sort of deviations from linearity that are likely to occur. It also means that linear interpolation can be used, as a sufficiently accurate approximation, in place of logarithmic interpolation, if it is convenient to do so.

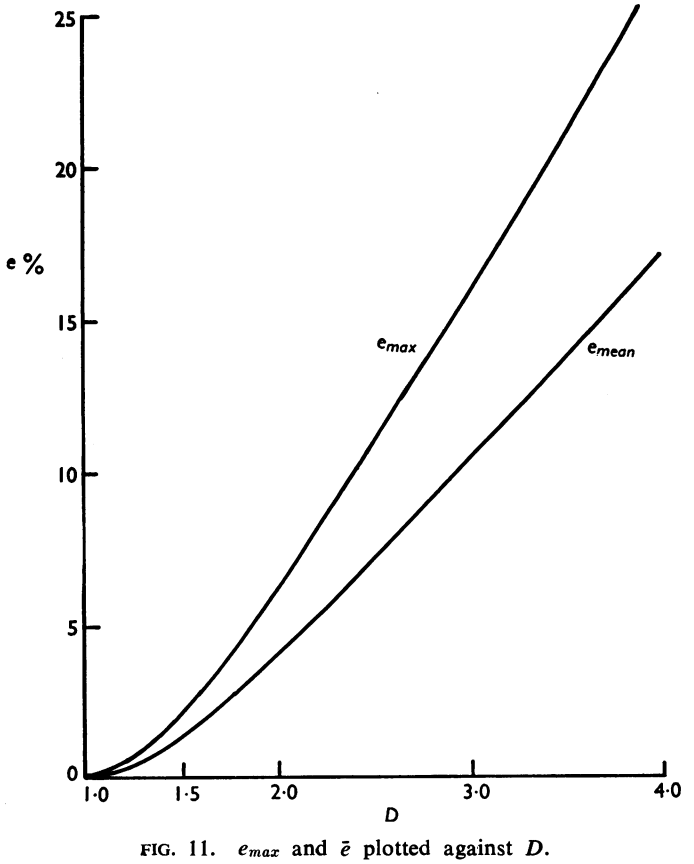


FIG. 11. e_{max} and $\bar{e}$ plotted against D.

The percentage (e) by which linear interpolation overestimates the result (z_U) given by logarithmic interpolation, $e=100\ [(z'_U - z_U)/z_U]$, is plotted against α in Fig. 10.

There is, of course, no difference ($e=0$) between z_U and z'_U when the response to unknown is the same as the response to one of the standards ($\alpha=0$ or $\alpha=1$). The maximum difference, e_{max}, occurs at α_{max} when the response to unknown is rather nearer to the low standard response than to the high standard response.

In Fig. 11 the maximum difference, e_{max} (marked with an arrow in Fig. 10), is plotted against D. The average difference, $\bar{e}$, which would be found if unknown responses fell equally often at various points between the standards, is also plotted in Fig. 11. Clearly if $D=1{\cdot}5$ the methods of interpolation agree well enough for most purposes, and if $D=2$ they agree well enough for approximate purposes.

The equations for the curves plotted in Figs. 10 and 11 are as follows:

$$e = 100\left[\frac{1+\alpha(D-1)}{D^{\alpha}} - 1\right] \tag{4}$$

This follows from equations (2) and (3). Substituting

$$\alpha_{max} = \frac{1}{\ln D} - \frac{1}{D-1} \tag{5}$$

for α in (4) gives e_{max}. Integration of (4) gives

$$\bar{e} = 100\left[\frac{(D-1)^2}{D(\ln D)^2} - 1\right] \tag{6}$$

We are very grateful to Professor D. J. Finney for his comments on a draft, and for suggesting the construction of tables of D^{α}.

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