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The Error of Determination of Toxicity

J. W. Trevan

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The Error of Determination of Toxicity.

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CONTENTS.

I. *The Meaning of the "Minimal Lethal" (or Effective) Dose.*

The determination of toxicity is usually given quantitative expression by the statement of a minimal lethal dose. The common use of this expression in the literature of the subject would logically involve the assumptions that there is a dose, for any given poison, which is only just sufficient to kill all or most of the animals of a given species, and that doses very little smaller would not kill any animals of that species. Any worker, however, accustomed to estimations of toxicity, knows that these assumptions do not represent the truth. How widely different is the real state of affairs, however, is not, I think,

sufficiently recognised. The fact that the "minimal lethal dose," whether calculated for unit weight, or for surface area, or on any other basis, varies widely for different species has, perhaps, led to the looseness of its definition for any one species. For the accurate standardisation, by biological methods, of drugs which are not available in chemically pure form, it is essential to establish a more accurate definition of such terms as "minimal lethal dose," "minimal effective dose," etc.

Fig. 1 gives the results of the injection of four poisons into animals. The abscissæ are proportional to the doses injected, the scale obviously differing

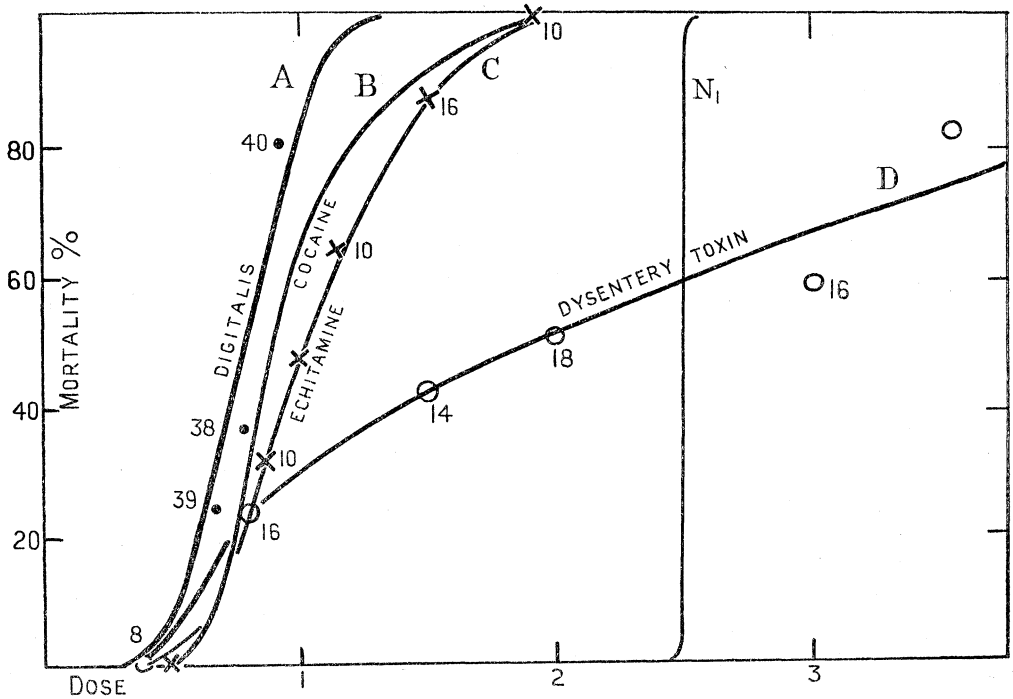

Fig. 1.—Mortality-dose curves (characteristics) of four poisons. A.—Digitalis on frogs. B.—Cocaine on mice. C.—Echitamine on Mice. D.—Dysentery toxin on mice (O'Brien, Sudmersen and Runge). The dose scale has been adjusted for each, so that the steepest parts of the curves are above one another. The numbers attached to the observed points represent the number of animals used to determine that point. N_1 represents the "ideal" characteristic (see text).

for the different drugs, and the ordinates give the percentage mortality for each dose injected. The number attached to each observed point represents the number of animals injected for its determination. The curves represent percentage mortalities produced by the subcutaneous injection of tincture of digitalis into frogs, by the intravenous injection of cocaine hydrochloride into

mice (see also fig. 2 and Table I), by the intravenous injection of echitamine into mice, and by the injection of dysentery toxin into mice, the data for the last being taken from O'Brien, Sudmersen and Runge (1924). A similar curve is given later (fig. 7) for the percentage of convulsions produced in mice by increasing doses of insulin, the data being obtained by the use of large numbers of animals. Shackell (1925) has published a number of similar curves, relating percentage mortalities to varying doses of different poisons, in a wide range of species. It is suggested that the curve expressing the percentage of mortality, or of some other limiting biological effect, produced by varying doses of a drug on animals of a certain species, shall be called the " characteristic" for that particular drug, effect and species. Thus, the curve relating the percentage of convulsions produced in mice to varying doses of insulin, would be termed the characteristic for the production of convulsions in mice by insulin.

Table I.—Mortalities after different doses of cocaine hydrochloride injected intravenously into mice kept at room temperature. Doses varied in proportion to weight. Variations of dose obtained by increasing or diminishing the volume of the 0·1 per cent. solution of hydrochloride injected. Dose given in terms of dose for 20 grammes of mouse.

Dose for 20 grammes.	Number of mice injected.	Number killed.	Percentage Mortality.
Mgms.			
0·8	20	20	100
0·7	24	20	84
0·7	20	17	
0·6	48	37	78·2
0·6	30	24	
0·5	65	31	55·25
0·5	30	19	
0·4	30	5	15·93
0·4	39	6	
0·3	20	0	0

It is quite clear from these curves that the lack of accurate definition of the term " minimal lethal dose " or " minimal effective dose " may lead to widely different estimates of the toxicities of any of these four poisons. The value indicated by different authors as the " minimal lethal dose " may be either the dose just sufficient to kill only an occasional animal, or that which kills 50 per

cent., or that which is just large enough to kill all the animals. The first probably approximates to what is sometimes called the "maximum tolerated dose," and the last is the "certainly lethal dose." These two doses are approximately in the ratio of 1 to 4·8 for digitalis on frogs, 1 to 3·85 for cocaine on mice, 1 to 5·5 for echitamine on mice, and about 1 to 20 for the dysentery toxin on mice. It is clearly necessary to define more closely what is meant by "minimal lethal dose," if the term is used as a measure of toxicity.

The choice of such a measure will be rendered easier by considering the nature of a dose-mortality "characteristic." Shackell has pointed out that these curves are integrated frequency or percentile curves. The sloping curves owe their shape to the fact that different individuals of a given species require different quantities of the poison to kill them. The curves are obtained by injecting groups* of animals with different doses of the poison under investigation, noting the percentage mortality at each dose, and drawing a smooth curve through the points so obtained.

The animals which die in any group, such as those represented by the point labelled 48 in fig. 2, are not only those in that group for which the dose injected is that just sufficient to kill, *i.e.*, those for which it is the individual lethal dose, but also those for which the dose just sufficient to kill is smaller than the dose injected. The smaller the range of variation among the individual lethal doses, the more nearly would the curve approximate to a vertical straight line, such as N_1 in fig. 1.

It is impossible to determine directly for single animals the "individual lethal dose," but approximations to its determination may be obtained in two ways.

(1) Increasing doses, separated by sufficient time intervals, might be administered to the group of animals, and the dose noted at which each animal dies. If the increments of dose were sufficiently small, an individual lethal dose could thus be determined for each animal. Variations, however, probably occur in the individual lethal dose from day to day, which would invalidate the method, and the intervals between the successive doses necessary to prevent the effects of partial accumulation, would render the method impracticably consumptive of time.

(2) A method of continuous slow infusion, such as that used in the Hatcher cat-method for digitalis, gives an approximation to the individual lethal dose.

* In the following discussion, to avoid confusion, the words "groups of animals" are used in the statistical sense of "random sample," whilst the word "sample," where used, refers only to a sample of poison.

The dose which finally kills is somethat larger, however, than the true individual lethal dose, if there is a latent period in the action of the drug.

The *distribution* of the individual lethal doses in one of the forms of the usual

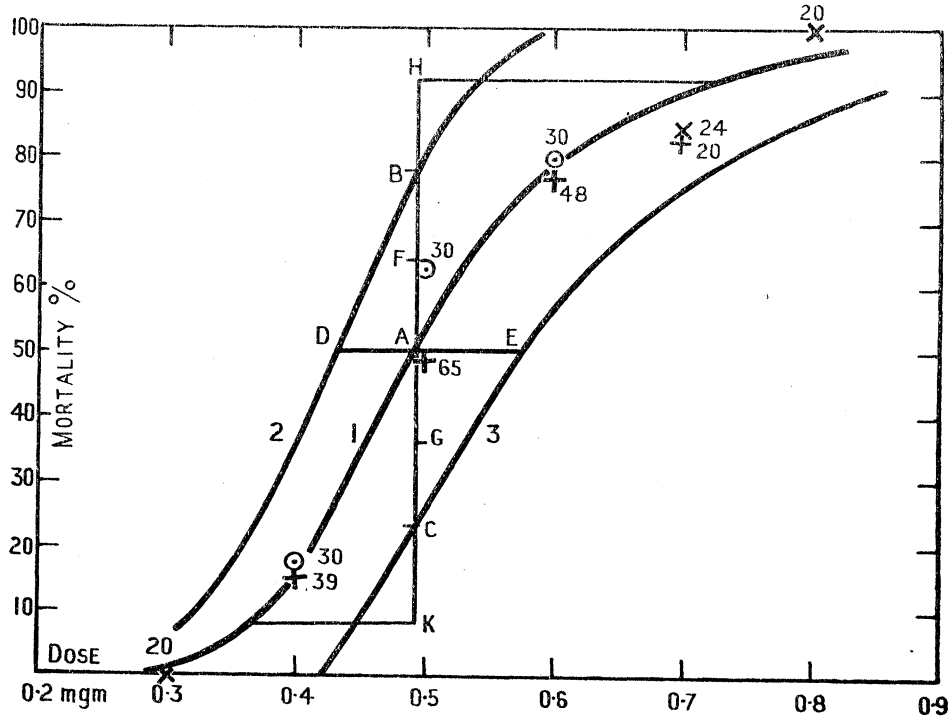

FIG. 2.—Characteristic for cocaine (A) from fig. 1, by intravenous injections into mice. Observed points 0 – 0 obtained six months later than + – + and × – × twelve months later. The curves through E and D represent limits of the mortality which any dose may be expected to give in 369 cases out of 370, when injected into 30 mice. KH represents the same limits for 0·505 mgm. using only 10 mice, and GF the limit using 120 mice. DE represents the limits of the doses that may give 50 per cent. mortality in 30 mice.

bell-shaped frequency curve can be approximately determined by plotting the differences in percentage mortality for successive small dose intervals against the respective doses as abscissæ. The differences have been measured for the smooth curve drawn through the observed points for the killing of mice by cocaine hydrochloride (fig. 2, middle curve). The figures obtained are tabulated in Table II. The increment in percentage mortality is given in the second column of this table against the corresponding interval of dose given in the first column. Fig. 3 is constructed by plotting the numbers in column 4 against

the mid-points of the dose-intervals to which they correspond. The frequency distribution, as will be seen, is asymmetrical.

Table II.—Frequency distribution of individual lethal doses of cocaine for mice, from smoothed curve, fig. 2.

Dose Interval.	Mid-point of dose Interval (d).	Difference of Percentage Mortality (f).	$f \times d$.
0·2 — 0·3	0·25	1	0·25
0·3 — 0·4	0·35	15	5·25
0·4 — 0·5	0·45	38	17·11
0·5 — 0·6	0·55	25	13·76
0·6 — 0·7	0·65	11	7·15
0·7 — 0·8	0·75	6·5	4·88
0·8 — 0·9	0·85	2·5	2·125
0·9 — 1·0	0·95	1·0	0·95
		S (f) 100	S (fd) 51·475

A.—Arithmetical mean $= \frac{S(f \times d)}{S(f)} = 0{\cdot}515.$

B.—Median $= 0{\cdot}4895.$

C.—Mode $= A - 3\ (A - B) = 0{\cdot}439.$

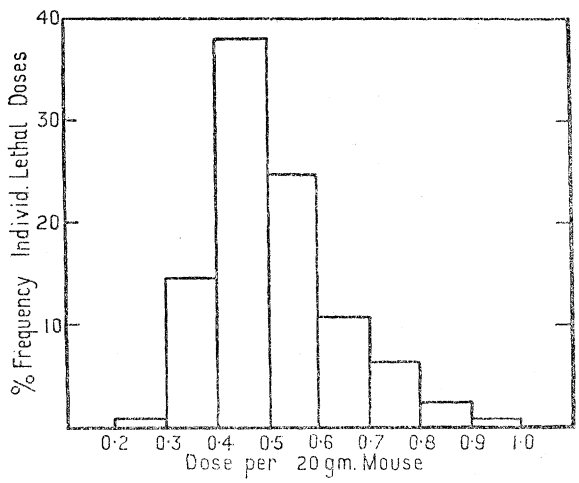

Fig. 3.—Distribution of individual lethal doses for cocaine when injected intravenously into mice; derived from the smoothed curve of fig. 2.

Animals with individual lethal doses between 0·45 and 0·5 mgm. per 20 gram body weight occur more frequently than animals with individual lethal doses in any other dose interval, *i.e.*, the " mode " of the frequency distribution is between 0·45 and 0·5 mgm. The frequency of the occurrence of animals

with higher individual lethal doses than these falls off less rapidly than that of animals with lower individual lethal doses. This type of asymmetry, with the mode on the side of the smaller doses, seems to be the commonest amongst the poisons so far studied. The greatest asymmetry yet observed is that of the curve for the killing of mice by dysentery toxin; for many drugs it is of the same order as that shown for the lethal action of cocaine hydrochloride on mice, and the dispersion of the distribution is not very widely different. For the action of digitalis on frogs it is almost symmetrical.

We may return now to the consideration of the particular dose in this range which affords the best estimate of the toxicity of a drug, taking the lethal action of cocaine hydrochloride as an instance. The logical method is to take, as an index, the toxicity of the drug for the average animal of the species. There are three different values which can be taken as expressing the "average" of a series of figures such as these:—(1) The arithmetic mean of all individual doses. (2) The median, *i.e.*, the particular value of the individual lethal dose which divides all animals of the variety used into two groups of equal size, and which when injected into an exceedingly large group would therefore kill exactly 50 per cent. (3) The mode—already referred to above, the individual lethal dose which occurs most frequently, and which is the dose at which the inflexion, or steepest portion, occurs in the S-shaped mortality curve of fig. 1.

The arithmetic mean can be calculated approximately from Table II, by multiplying the mean of the figures of column 2 by the figures in column 3, adding the products so obtained (set out in column 4), and dividing this sum by the sum of column 3, which is, of course, 100. The mean individual lethal dose of cocaine for mice so obtained is 0·515 mgm. The median is obtained by reading off from the smoothed mortality curve the dose necessary to kill 50 per cent. It is 0·490 mgm. It may be obtained also by the following method. The sum of the differences of percentage mortality (Table 2) from 0·2 to 0·4 is 16. The median is therefore a dose which is between 0·4 and 0·5 mgm. and is greater than 0·4 by $\frac{50 - 16}{38} \times 0{\cdot}1$, 38 being the percentage difference of mortality between doses of 0·4 and 0·5, and 0·1 being the dose interval between 0·4 and 0·5. This works out at 0·0895, so that the median individual lethal dose is 0·4895. The mode, as already pointed out, is between 0·4 and 0·5 mgm. and is approximately obtained by the following formula (Yule):—

$$\text{Mode} = \text{Mean} - 3\,(\text{Mean} - \text{Median}).$$

Calculated in this way for cocaine it is equal to 0·439 mgm. It is the point

of the maximum slope of the characteristic. The mean can also be calculated in a similar manner from the actual observed percentage mortalities (Column 4 in Table I) by treating the differences in these percentages as above. The mean lethal dose so calculated is 0·516 mgm., the median 0·486 mgm. and the mode 0·426 mgm.

For the degrees of asymmetry which are usual in dealing with most poisons (the case for dysentery toxin is rather exceptional), the median differs from the arithmetic mean by an amount which is within the error of determination, when small numbers of animals are used. So that, although the arithmetic mean is theoretically the best representative of a series of variables, no great disadvantage is incurred by using the median of the individual lethal doses to fix a toxicity. It has the advantage also over the mode and the arithmetic mean, in that the whole mortality curve need not be mapped out for its determination ; all animals available can be injected in groups with doses around the median, the median being approached by trial in successive groups until the dose which kills 50 per cent. is " bracketed."

I would suggest that the term " minimal lethal dose," with its variable meaning, should be dropped altogether, and that toxicity should be stated primarily in terms of the " median lethal dose," that is the dose which kills 50 per cent. of a large group of animals. As a convenient abbreviation I would suggest for this the symbol LD 50, which will be used in the following discussion. For doses which kill other proportions of large groups of animals it is convenient to use the analogous symbols LD 75, LD 25, for doses which kill 75 per cent., 25 per cent., and similarly for doses killing other proportions. There are other reasons, discussed later, of a statistical nature, for preferring the use of a dose in the neighbourhood of the average lethal dose.

II. *The Accuracy of Determination of the Lethal Dose.*

It is generally admitted that determinations of toxicity require the use of large numbers of animals, if any degree of accuracy is to be obtained, but very few attempts have been made to determine the relation between the numbers of animals injected and the possible error. If there were no differences between the individual lethal doses, the final fixation of the toxic dose would depend on the injection of two animals only, one with a dose x, and another with a dose $x + \delta x$, where δx is a small fraction of x, and repetition of the experiment would always give the same result, within the limits of error of measurement of the dose and the weights of the animals. The conditions encountered in practice are quite different. Suppose two doses of cocaine hydrochloride,

x and $x + \delta x$, where δx has the same significance as above, are injected into a pair of mice. Then if the dose x fails to kill, and the dose $x + x\delta$ kills, it will be found that the value of x, on different pairs of mice, varies from about 0·3 to about 0·9 mgm. Further, it will occasionally happen that the dose x will kill, and the dose $x + \delta x$ will not kill. Nor can it be said that, if both animals die, the dose is greater than the certainly lethal dose, which would be true for the ideal case discussed above.

These discrepancies arise, of course, from the fact that in an assembly of mice with different individual lethal doses, it is a matter of chance which particular mouse is chosen for injection. For instance, there is approximately one chance in ten that a mouse injected with a dose of 0·38 mgm. will die, and the chance that a dose will kill does not become negligible until the dose is reduced to about 0·28 mgm. At the other end of the scale, the chance of survival does not become negligible until the dose reaches 0·85 mgm. to 0·9 mgm.

On the other hand, if sufficiently large groups of animals are injected at each dose, increasing the dose will always give rise to increasing mortality, and the observed mortalities will fall more and more closely on a smooth mortality curve (such as those shown in figs. 1 and 2) as the size of the groups increases. If an attempt is made to measure the size of a dose of cocaine by its toxicity, with a pair of mice, the death of one of these, together with the survival of the other, will only indicate that the dose of cocaine injected is somewhere between 0·3 and 0·9 mgm., whereas when a very large group of animals is injected, the percentage mortality amongst them will indicate the exact size of the dose, to within a few units per cent.

The problems which present themselves at this point are: (1) What is the size of the animal group which must be used to attain any given degree of accuracy? and (2) What particular dose, in the whole range of the mortality curve, can be determined with the least expenditure of animals? Let us begin with the median lethal dose, LD 50 as defined above, which is 0·490 mgm. for cocaine on mice, and make the assumption that the smoothed curve represents the curve which would be obtained with indefinitely large groups. If this dose is injected into groups of, say, 30 mice, the number that die in each group will vary. It will only occasionally happen that exactly fifty per cent. will die. On the other hand, the chance that the whole of a group of 30 will survive, or that the whole of a group of 30 will die, after the injection of that dose, is so small that it can be neglected, provided that the group of mice has been chosen at random. The actual range of the number of deaths to be expected can be calculated from the well-known formula for the standard deviation:—

$$\text{Standard deviation} = \sqrt{pqN}$$

where p is the probability of death, q that of survival (both p and q in this case being 0·5), and N the number of animals used. This works out at 2·74, and the probability is approximately 0·9973 (say 370 to 1) that, in any group of 30 injected with a dose of 0·490 mgm., not more than $15 + 3 \times 2{\cdot}74$, and not less than $15 - 3 \times 2{\cdot}74$ animals will die. If 371 groups of 30 are injected, it is probable that only one group will show a number of deaths outside the limits $15 \pm 8{\cdot}22$, or, expressed as a percentage, $50 \pm 27{\cdot}4$. This probability is large enough to be taken, and is usually taken for practical purposes, as certainty. About 19/20 of the observed number of deaths will lie between the limits $15 \pm 5{\cdot}48$, and half of them will lie between the limits $15 \pm 1{\cdot}823$ ($= 2/3$ standard deviation). The limits corresponding to three times the standard deviation are plotted on the diagram in fig. 2 at B and C, AB being equal to 27·4 per cent.

Similar deviations have been worked out for the percentage mortalities from about 5 per cent. to about 95 per cent., substituting for p and q the respective proportions corresponding to each mortality. The points so obtained have been plotted on fig. 2, and the smooth curves 2 and 3 drawn through them. These curves are very rough at the extremities for the following reasons. For the small percentage death-rates (or survival rates) at the ends of the curves the deviation of mortality becomes very asymmetrical, deviations in the direction of larger death rates being more frequent at the lower end of the curve, and in the direction of smaller death rates being more frequent at the upper end. They can, however, be used to determine the errors, at least to a first approximation. These curves can be used to determine the accuracy with which the size of a dose of cocaine hydrochloride can be measured by its toxic effect on a single group of 30 mice. Such an operation is, of course, of little practical importance in dealing with cocaine, but this drug is used for illustrative purposes, and the results apply, with appropriate modification of scale, to all determinations of lethal dose, whatever drug is used. Cocaine is chosen as an example because it is obtainable in a stable crystalline form.

If 30 mice were injected with a dose of cocaine of unknown size, and 15 died, it is clear that of all possible dimensions the dose might have, 0·490 mgm. would be the most likely. But it is also clear from what has been said above, that the chances are small that it was exactly 0·490 mgm. Accepting the probability 0·9973 as practical certainty, it might be as low as 0·430, the point D on the diagram in fig. 2 being the upper limit of the mortality deviation for this dose, or it might be as high as 0·576 mgm., the point E on the diagram corresponding to the lower limit of the deviation for this dose. The range of possible

doses which might give rise to a mortality of 50 per cent., represented as a percentage of the middle dose of the range, is

$$\frac{0{\cdot}576 - 0{\cdot}430}{0{\cdot}490} \times 100 = 29{\cdot}8 \text{ per cent., or approximately } \pm 15 \text{ per cent.}$$

More accurately, the positive error is $\frac{0{\cdot}576 - 0{\cdot}490}{0{\cdot}490} \times 100 = 17{\cdot}5$, and the negative error $\frac{0{\cdot}490 - 0{\cdot}430}{0{\cdot}490} \times 100 = 12{\cdot}2$ per cent.

The ratio of the highest dose which will give a mortality of 50 per cent. to the lowest dose which will give that mortality is $\frac{0{\cdot}578}{0{\cdot}430} = 1{\cdot}34$.

By drawing horizontal lines in this manner through the curves at mortalities of 10 per cent., 20 per cent., 30 per cent., etc., the errors associated with the determination of the value of doses giving these mortalities have been estimated. The results are given in Table III. The last column gives the ratio of the

Table III.—Dose errors for cocaine hydrochloride at different mortality rates in a group of 30 mice.

No. of Animals dying out of 30.	Percentage Mortality Rate.	Percentage Error of Dose.		Ratio of Highest to Lowest Dose giving each Mortality Rate.
		Positive.	Negative.	
0	0	66·8	100	0·0
1	3·3̇	30·3	10·6	1·48
3	10	21·3	13·3	1·405
6	20	17·5	12·2	1·34
9	30	16·1	12·5	1·320
12	40	16·8	11·4	1·318
15	50	17·75	12·2	1·34
18	60	19·3	12·6	1·36
21	70	22·1	14·3	1·415
24	80	23·1	18·15	1·51
27	90	20·1	23·75	1·58
29	96·6̇	11·2	29·6	1·60
30	100	Indefinite.	66·6̇	>1·6

highest and lowest doses for each mortality rate. It will be seen that the range of dose deviation, as shown by this ratio, is smallest for a mortality rate of 40 per cent. and is practically constant from 20 to 60 per cent. For more symmetrical curves this range will extend to higher mortality rates. The deviation becomes indefinitely large at the ends of the curves. Therefore the

median lethal dose has further advantages over those already stated, in that it is in the neighbourhood of doses with the minimum relative error of determination, whereas the "certainly lethal dose" and the "maximum tolerated dose" have indefinitely large errors. The indefiniteness of the error at the ends of the characteristic is clearly due to the fact that, if either all die or all survive of a group of animals injected with an unknown dose of the drug, the information supplied by the event is merely that the dose is, in the one case, indeterminately greater than that represented by a certain point on the characteristic, or, in the other case, indeterminately less than that represented by another point, and the actual magnitude of these limiting doses depends on the number of animals used.

The use of larger or smaller groups of animals will, of course, lead to smaller or larger errors. Fig. 4 gives the deviation of mortality to be expected in

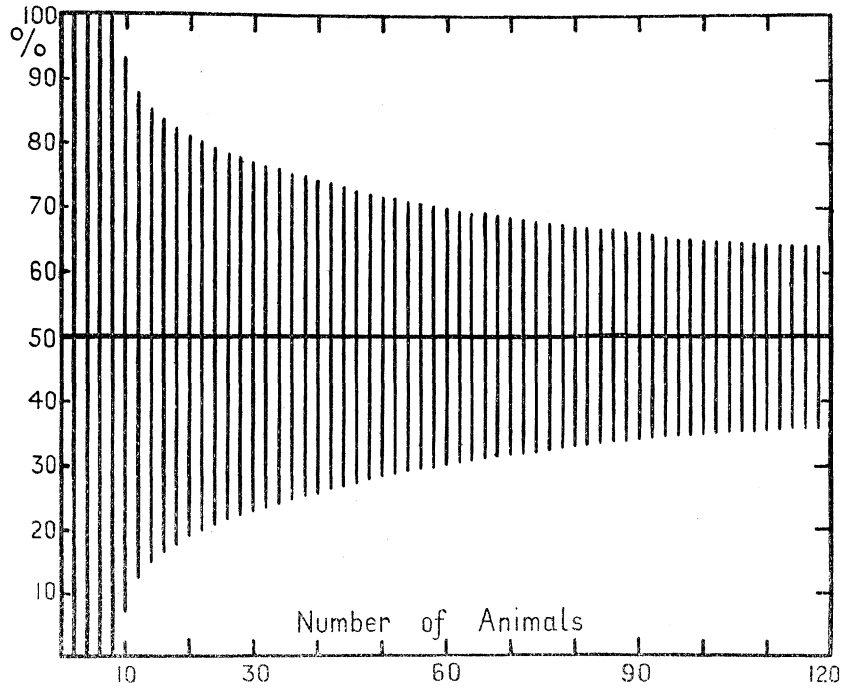

Fig. 4.—Limits of percentage mortality obtainable by chance when the average lethal dose causing 50 per cent. mortality is injected into different sized groups of animals. The limits are represented by the lines as percentage of the number of animals used, and correspond to ± three times the standard deviation of the mortality.

different sized groups of animals, when injected with the median lethal dose of *any* drug; the deviation depending only on the chance of death or survival.

The figures are obtained by substituting the requisite number for N in the formula for three times the standard deviation, for groups larger than 20. The deviations for values of N below 20 are obtained from the terms of the binomial expansion $(0{\cdot}5 + 0{\cdot}5)^N$. The deviation plus or minus is half the number of those terms (symmetrically disposed about the middle term), of which the sum is 0·9973 of the sum of all the terms of the series. For values of $N = 20$ the number of these terms practically coincides with $6\sqrt{pqN}$. The ordinates are percentage mortality, the abscissæ the number of animals in a group injected with the average lethal dose. The theoretical mortality is the same for every group number—50 per cent. The black lines, with their mid-points at 50 per cent., represent the deviations for different group numbers of animals, expressed as percentage of the group number. The percentage mortality in any group of animals, resulting from the injection of LD 50, may therefore be expected to fall somewhere on the black line corresponding to the number of animals in the group. Thus the percentage mortality to be expected if 5 animals are used may be between 0 per cent. and 100 per cent., if 10 animals, on an average 4 per cent. to 96 per cent., if 120 animals, 36 per cent. to 64 per cent., and so on; the length of the black lines diminishing as the square root of the number of animals.

The percentage error of measurement of *dose* caused by these deviations is less, and will, of course, depend on the slope and shape of the characteristic. Not only so, but the error in dose diminishes with increasing size of group more rapidly than the deviation in mortality when the number of animals used is less than about 30. This is illustrated in fig. 2, where the deviations of mortality for groups of 10 (HK), 30 (BC), and 120 (FG) animals are plotted.

The deviations of dose corresponding to these deviations of mortality are obtained by drawing horizontal lines at the ends of the mortality deviation. The points where these lines cut the characteristic give the range of dose deviation for each size of group of animals. It will be seen that the deviations for 120 and 30 animals fall wholly on the part of the characteristic which is practically a straight line, whereas that for 10 falls outside the straight portion of the characteristic. The dose error thus calculated differs from that on p. 493 in that this gives the range in dose error for a fixed dose, in this case the average lethal dose, whereas the dose error on p. 493 is that corresponding to a fixed mortality. The dose error for a fixed mortality will increase in a similar manner but not quite to the same extent.

In consequence, the increase in error of the average lethal dose, produced by diminishing the size of animal group from 120 to 30, is proportional to the

percentage mortality deviation plotted in fig. 5; *i.e.*, usually proportional to the square root of the number of animals used. Below this, the dose error

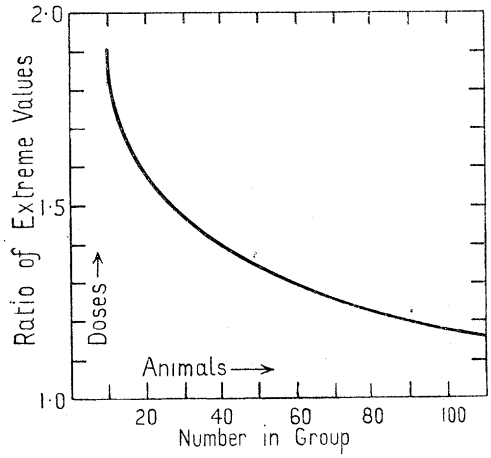

FIG. 5.—Ratio of extreme values assigned to an average lethal dose of cocaine by the use of different sized groups of mice.

increases more rapidly than the mortality errors given in fig. 4 because of the increasing curvature of the characteristic.

An important practical point emerges from these considerations. A group of about 30 animals is near the optimum size for the determination of an average lethal dose. A significant increase of accuracy above that given by this group number is only obtained by the use of greatly increasing numbers of animals; whilst diminution of the group number below 30 leads to a very rapidly increasing error. The ratio of the cost of the test to the accuracy obtained reaches a minimum in the neighbourhood of this group number. This applies to all those drugs so far tested.

The relatively rapid increase in dose error with animal groups of a size less than 20 to 30 is shown in fig. 5. The points on the curve give the ratios of the extreme values which might be assigned to a median lethal dose if the value of the dose were estimated by reading from the characteristic the dose corresponding to the mortalities actually obtained with such groups. This ratio becomes indefinitely large when the group number diminishes below 10.

A simple formula will give an approximation to the error of determination of doses in the neighbourhood of the median lethal dose, once the slope of the "straight" part of the characteristic is obtained. By determining the LD 25 and the LD 75 on a fairly large number of animals, this slope is obtained with sufficient accuracy for most drugs.

Let N be the number of animals injected with a dose near the LD 50, D the deviation of dose (reckoned as a percentage of the dose) corresponding to three times the standard deviation of the mortality, K the ratio of the percentage increase of dose to percentage increase in mortality, and p the probability of survival, and q that of death, with the dose injected, p and q being 0·5 for the average lethal dose.

Then let three times the standard deviation of deaths $= d$.

$$d = 3\sqrt{pqN}.$$

Let d be reckoned as percentage of N $= d'$.

$$d' = \frac{3\sqrt{pqN}}{N} \times 100.$$

Since $D/d' = K$, for parts of the curve which approximate to straight lines, then

$$\frac{D\,N}{300\sqrt{pqN}} = K. \qquad \frac{D\sqrt{N}}{300\sqrt{pq}} = K.$$

$$\sqrt{N} = \frac{300\,K\sqrt{pq}}{D}$$

$$N = \frac{90{,}000\,K^2\,pq}{D^2}. \qquad (1)$$

For cocaine, the value, for the median lethal dose, of K is 0·510 and for N = 30, the error of the average lethal dose is

$$D^2 = \frac{90{,}000 \times (0{\cdot}510)^2 \times 0{\cdot}5 \times 0{\cdot}5}{30},$$

$$D = \pm 14{\cdot}05,$$

as against + 17·75 or − 12·2, as worked out by the graphic method (p. 493). The formula applies to values of D not larger than about 20 per cent., provided that only the "straight" part of the characteristic is involved in the calculation. For very small values (up to 5 per cent.) of D, it is applicable to any part of the curve—the value of K being the ratio of the percentage increment of dose at the point under investigation to the percentage increment in mortality.

The estimate of the accuracy of the determination of the toxicity clearly depends on the liability of casual groups of animals of sufficient size to give characteristics of the same shape, and average lethal doses of the same magnitude. Observations on the toxicity of cocaine over a period of more than twelve months have shown that at different intervals the percentage mortality for any given dose does not vary significantly from that given in the curve.

The series of points marked with vertical crosses in fig. 2 were obtained about six months before those marked with circles, and those marked with diagonal crosses about twelve months later, and the difference between these two series is well within the significant limits. So that, in experiments demanding the knowledge of the toxicity of cocaine (as, for example, in the exploration of possible antidotes for cocaine poisoning) the expected mortality for any given dose can be read from the curve, for casual samples of mice, not specially selected. The injections must be made in a similar manner to those used for the determination of the curve, namely :—

(1) The dose must be proportional to the weight of the animal, and be injected intravenously.

(2) The concentration of the solution injected must be such that 0·5 c.c. contains 0·5 mgm.

(3) Variations of dose, whether necessitated by the weight of the animal, or made for the purpose of obtaining different points of the curve, must be obtained by altering the total volume of solution injected.

Unless these conditions are satisfied, the characteristic given will not represent the relation between dose and mortality. The evidence for this will be given in a further paper on the toxicity of cocaine and certain of its derivatives.

III. *Periodic Variation of Median Lethal Dose.*

Unfortunately, however, several substances for which only biological methods of standardisation are available, do not give the same median lethal dose when large groups of animals are injected on different days. Some figures representing the difference in the median lethal dose as determined in the summer and

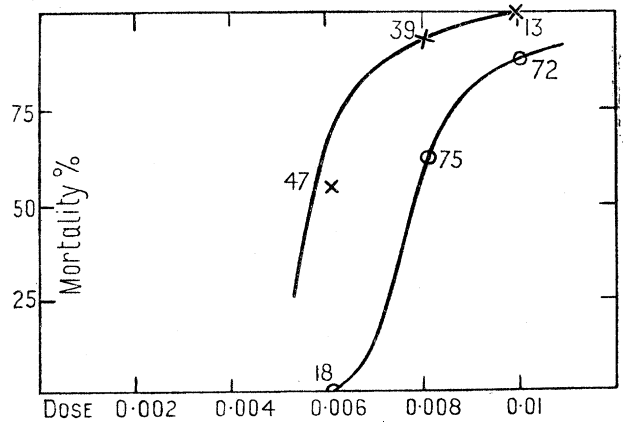

Fig. 6.—Diphtheria toxin characteristics for guinea-pigs in summer and winter (Sudmersen and Glenny).

in the winter, for diphtheria toxin, taken from a paper by Glenny and Sudmersen, are plotted in fig. 6. No alteration in composition of the toxin can account for these differences, for the summer curve is made up of two sets of figures obtained in two succeeding summers with the same toxin, the results agreeing in the two summers. Similar, but more rapid periodic variations occur in standardising the digitalis series by the injection of frogs. I have had most experience of this type of variation in the standardisation of insulin by determination of the convulsive dose in mice. The method and results are given in a contribution to a report to the Health Committee of the League of Nations (Trevan and Boock, 1926). Two characteristics out of five, obtained with the same sample of insulin on different dates, are shown in fig. 7, curves A and B. As will be seen, the

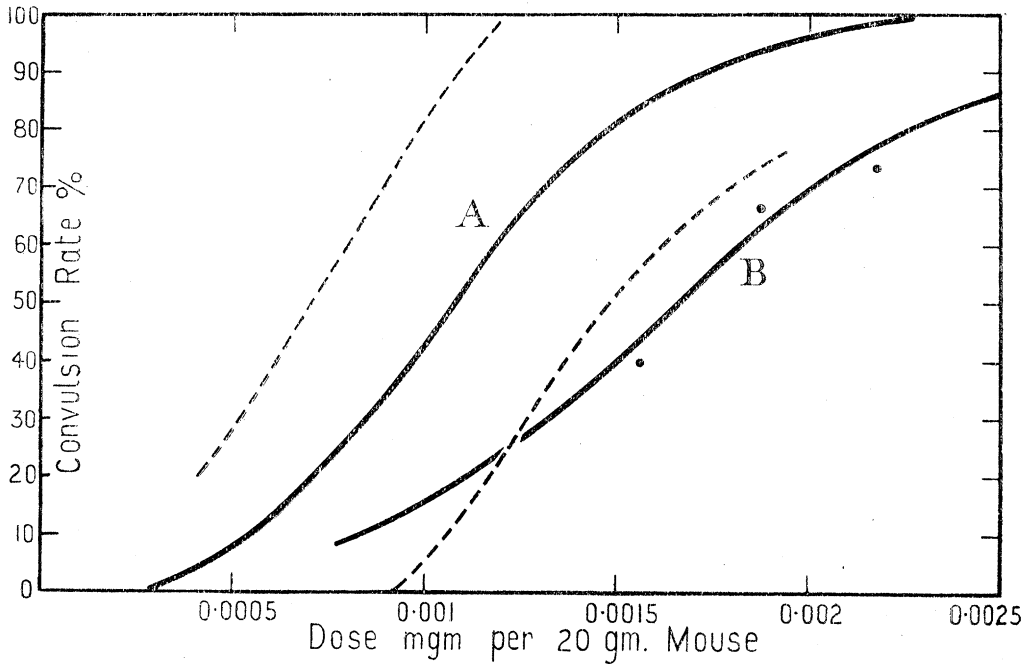

FIG. 7.—Insulin characteristics for mice on different days, A and B, and limits of possible errors when a "test" sample and a "standard" sample are injected into two groups of 30 mice each.

figures obtained on different occasions varied; in series B the median convulsive dose was 0·00155, whereas in the others, using about 900 mice, it was practically 0·00113.

In series B it will be seen that each of the results for groups of 30 animals lies outside the dotted line, which represents the limits of variation from the first curve for groups of 30 animals if the variation is solely due to errors of sampling.

(See later.) The displacement of curve B is therefore largely due to a decrease in susceptibility of the whole stock of mice to insulin. Variations of this order of magnitude are uncommon, and have, so far, not been traced to any specific factor. Similar variations in susceptibility to insulin have been described by Marks (1926) as occurring amongst rabbits. But even for the extreme case shown in curve B, although the median convulsive dose is 54 per cent. greater, the distribution of the individual convulsive doses around the mean is similar to the distribution on the days of greater susceptibility; so that, for example, the dose causing 30 per cent. of mice to convulse is also 54 per cent. greater, within the error due to the practical limitation of the size of the groups injected. This is shown in the figure, where the line B is constructed by increasing the abscissæ of the smoothed curve A by 54 per cent. It will be seen that the actual figures obtained, represented by the dots, do not deviate by a significant amount from the smoothed curve B. The ratio of percentage increment of dose to percentage increment of mortality for corresponding points is the same, whatever the median convulsive dose. Curves of the same shape are given by samples prepared in different ways. Since the median convulsive dose varies in this fashion, the curve cannot be used for the absolute determination of the value of an unknown sample in the manner described for cocaine. Any of these curves can, however, be used for the comparison of the activities of two samples, in one of the following ways :—

(1) The two samples can be injected over a wide range of doses, so that two characteristics, one for each sample, are obtained. The ratio of the abscissæ of the two characteristics gives the ratio of activities.

(2) The two samples can be injected into large groups of animals, at doses which by preliminary experiments are adjusted to give about 50 per cent. of convulsions for each sample. The convulsion rate with each sample is noted and the doses which gave these rates read off from one of the convulsion-dose curves, such as either of those in fig. 7. The ratio of these doses then gives the ratio of potency of the doses actually injected.

The error of such a determination, with a given number of animals, will be larger than that given for determinations of toxicity when no variation of average dose occurs. There will be a statistical error affecting the group injected with the standard preparation, as well as one affecting the group injected with the unknown. The standard deviation of the difference between the two mortalities obtained, expressed as a percentage, is given by the formula,

$$= 100 \sqrt{pq\left(\frac{1}{m}+\frac{1}{n}\right)}$$

where n is the number of animals in the group injected with the standard, m that of the group injected with the unknown, p and q the probabilities of death or survival (or occurrence or non-occurrence of convulsions, for insulin) with the standard dose.

It is easy to show algebraically that, for a given number of animals 2N, the standard deviation is a minimum when $m = n = \mathrm{N}$. The number of controls injected with the standard should therefore be equal to the number injected with the unknown, and the standard deviation of the difference between the two mortalities obtained becomes :—

$$100\sqrt{2pq/\mathrm{N}},$$

where N is the number of animals in each of the comparative groups.

The formula becomes then

$$\mathrm{M} = 180{,}000\ \mathrm{K}^2 pq/\mathrm{D}^2$$

(where D is the dose error corresponding to three times the standard deviation of the mortality).

For insulin, $\mathrm{K} = 0{\cdot}967$, and for $\mathrm{D} = 10$, and $p = q$ (median convulsive dose),

$$\mathrm{N} = \frac{180{,}000 \times (0{\cdot}967)^2 \times \frac{1}{4}}{100} = 451,$$

and for $\mathrm{N} = 30$, $\mathrm{D} = 37{\cdot}4$ per cent.

The deviations at different mortalities are given by the dotted curves for curve A, and give similar figures for the error.

The results of the injection of 27,000 mice with batches of insulin have been analysed to see how far the prediction of the frequency of deviations of a given magnitude are fulfilled. Table IV is the result of the examination of twelve of these batches. In the second column is tabulated the number of groups of 60 mice used for testing each batch, thirty mice in each group being injected with the batch under test and thirty with a standard preparation (Trevan and Boock, 1926). The convulsion rates were noted for the batch under test and the standard in each group of 60, and an estimate of the relative activity of the two samples obtained in the following manner. The doses are read which correspond to these convulsion rates on curves A or B (fig. 7)—either will give the same ratio of activities. The ratio of the doses so read from the curve is taken as the ratio of the activities of the doses actually injected, and by appropriate allowance for the dilutions injected, a value for unit quantity of the batch under test in terms of unit quantity of the standard insulin is obtained. The

Table IV.—Standard deviations of a series of insulin estimations, calculated by the mean square method from the observed deviations for each batch.

Batch.	Number of sets of 60 mice used.	Standard deviation of one observation as per cent. of mean.
1	27	14·3
2	13	13·04
3	18	14·8
4	23	12·93
5	20	11·78
6	12	12·17
7	14	12·23
8	18	10·35
9	21	13·18
10	20	12·51
11	27	13·85
12	17	10·73
		Average 12·65
		Calculated from shape of curve, as described in text = 12·45.

mean of these values is calculated for all the groups of sixty mice used on a batch, and the standard deviation for the result from a single group worked out for each batch by the method of mean squares. These standard deviations are given in column 3 as percentages of the corresponding mean. Since the convulsion rates in all these groups are in the neighbourhood of 50 per cent., the standard deviation so obtained should be in the neighbourhood of $\frac{1}{3}D = 12 \cdot 45$, assuming the mean value obtained for each batch is sufficiently near the true value of the batch. The actual values do not depart by significant amounts from this value.

The figures therefore are in accord with the initial assumptions, that the conditions of random sampling have been observed for the mice used, and that the method of calculating the distribution of the errors from the characteristic is a sufficiently close approximation for practical purposes. The same point may be presented in a different form. For a series of 455 tests on groups of 60 mice, including those given above and others, the frequency of the occurrence of deviations corresponds closely with what would be expected in a normal distribution with a standard deviation of 12·45 per cent. of the mean. The figures are given in Table V. The calculated frequencies are obtained from a table of the normal distribution in terms of a standard deviation of 12·45 per cent. (*cf.* Fisher, p. 76). The frequency of errors not exceeding $\pm$ 37·4 per cent.

(= D) is 454 out of 455. The calculated frequency is 453·8. 436 times the error did not exceed ± 25 per cent. The calculated value is 435. 274 did not exceed ± 10 per cent. The calculated number is 263.

Table V.—Assay of insulin. Comparison of observed frequency of deviations with frequencies expected if the distribution were normal with a standard deviation of 12·45 per cent.

Percentage Deviation from Means of each Batch.	Frequency of Negative Deviations.		Frequency of Positive Deviations.	
	Observed.	Calculated.	Observed.	Calculated.
0 to 5	90	71·5	63	71·5
5 to 10	64	60	57	60
10 to 15	46	44·25	34	44·25
15 to 20	33	27·25	20	27·25
20 to 25	11	19·5	16	19·5
25 to 30	4	6·3	8	6·3
30 to 37·4	1·0	3·1	7	3·1
>37·4 (Actual value 42)	0	0·6	1	0·6

The figures are plotted in fig. 8. The dotted lines represent the normal distribution for a standard deviation of ± 12·45 per cent. and the full lines the observed frequencies.

Inspection of this figure suggests that the distribution actually obtained is not quite normal. There is an excess of values between 0 and — 5 per cent., a deficit for extreme values in the negative direction and an excess of extreme positive values. These facts suggest that the true form of the distribution is slightly skew, with the mode on the negative side of the mean between 0 and — 5 per cent. I have tested the significance of the variation from the normal distribution by calculating χ^2 (Fisher, Cap. 4) for the figures in Table V. The value of χ^2 after combining the frequencies of deviations above + 30 per cent. and also those below — 30 per cent. works out at 24·224; P is therefore 0·03, taking n as 13. A larger value of χ^2 would only occur about once in 35 such sets of 455 tests if the distribution were normal. The difference between the normal distribution and that obtained is probably therefore just significant. That the distribution is not quite normal depends in the end on the asymmetry of the insulin characteristic. The dose errors for a convulsion rate of 50 per cent. obtained from the horizontal distances between the characteristic and the dotted lines (fig. 7) are + 37·6 per cent. — 34·4 per cent., an asymmetry in the

same direction as and of a magnitude similar to that obtained. The deviation from the normal distribution is, however, not large enough to be of practical importance.

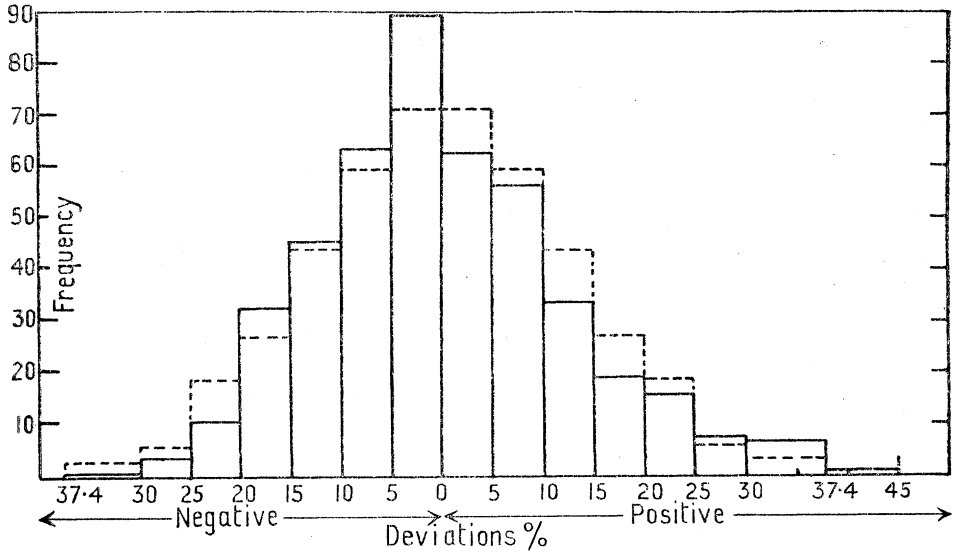

Fig. 8.—Frequency of deviations from mean value of estimation of value of insulin solutions on 450 groups of 60 mice, half injected with a "standard" insulin, and half with a "test" solution. The dotted lines represent a normal frequency curve of the same area, and with a standard deviation, corresponding to that calculated from the characteristic as explained in the text (12·6 per cent.). Some of these results are tabulated in Table V.

For diphtheria toxin, the value of K for the median lethal dose is 0·323. So at that dose the number of animals to be used, when the toxicities of two samples are compared with one another, is, for an error of ± 10 per cent. :—

$$N = \frac{180{,}000 \times (0{\cdot}323)^2 \times \frac{1}{4}}{100}$$

$$= 46{\cdot}8 \text{ in each group.}$$

For a 20 per cent. error, 11 to 12 animals in each group would suffice. The number of animals represented in fig. 6 render this calculation only very rough, but the order of accuracy attainable with the titration of diphtheria toxin on guinea-pigs is very much greater than that so far attained with the titration of insulin on mice.

The titration of diphtheria *antitoxin* against a "standard" toxin is considerably more accurate than that of toxin alone, for the following reasons. Antitoxin is measured by its neutralising power for toxin. Quantities of

about 100 "lethal" doses of toxin are injected into each guinea-pig used, but mixed before injection with a quantity of antitoxin which is varied for different animals until two mixtures are found, one of which kills, and one, with a slightly larger amount of antitoxin, fails to kill. The mixture which has only just not sufficient antitoxin to save the animals, say x c.c., is taken as containing about one lethal dose of toxin unneutralised, and the amount of antitoxin in it is considered to be sufficient to neutralise 99 lethal doses of toxin. The accuracy of this figure as a representative of the real number of lethal doses absorbed by x c.c. of the antitoxin depends on the number of animals injected with the mixture containing 1 lethal dose in excess. As already stated, 24 animals injected, 12 with the unknown and 12 with the standard, will determine a median lethal dose with an accuracy of $\pm$ 20 per cent. Under such conditions a mixture may be said to contain 1 lethal dose when it really contains as little as 0·8 or as much as 1·2, and the real equivalent in toxin of x c.c. of antitoxin is therefore between 98·8 and 99·2 lethal doses. If the neutralisation of toxin by antitoxin followed the laws of simple chemical proportion, these figures would represent the accuracy with which the amount of antitoxin present in a sample of serum could be titrated. Actually, the relationship between number of lethal doses absorbed and amount of serum added to a toxin is such that progressively more antitoxin is required to neutralise one lethal dose as complete neutralisation is approached. (Glenny, Pope and Waddington, 1925.) With a fresh toxin of small average lethal dose, the effect of this is to multiply the error calculated above by about 10 times, so that the final value of the serum in antitoxic units (1 antitoxic unit being equivalent to 100 MLDs.), will be 99 $\pm$ 2·0—an error of 2 per cent. For such degrees of accuracy, the toxin used should contain as many lethal doses as possible in 1 c.c., and as little toxoid, the degree of accuracy being proportional to the number of lethal doses.

The relatively high degree of accuracy of titration of antitoxin by this method has probably led, in the past, to some exaggeration in estimates of the accuracy of biological standardisation in general. It is clear that all antitoxic sera should be titrated by the determination of the amount necessary to protect an animal against the largest number of lethal doses it is possible to inject.

IV. *Error from using Limited Numbers of Animals.*

The practical problem as to what results are to be expected when the number of animals used is below 10, deserves special consideration. It is easy, though laborious, to work out from the terms of a binomial expansion the chances for any number of animals. Table VI gives the chances of distinguishing between

Table VI.—Limits of accuracy with 10 mice in two groups, one injected with LD 25, and the other with LD 75.

Possible results. Mortality out of 5.			Probability of Occurrence.
A.—Experiments in which the doses will be placed in right order.			
Dose LD 25		LD 75.	
0/5	and	5/5	
	or	4/5	
	or	3/5	
	or	2/5	
	or	1/5	
1/5	and	5/5	
	or	4/5	
	or	3/5	
	or	2/5	
2/5	and	5/5	0·9215
	or	4/5	
	or	3/5	
3/5	and	5/5	
	or	4/5	
4/5	and	5/5	
B.—Experiments in which doses will be called equal.			
1/5	and	1/5	
2/5	and	2/5	0·0583
3/5	and	3/5	
4/5	and	4/5	
C.—Experiments in which LD 25 will be called greater than LD 75			
5/5	and	4/5	
	or	3/5	
	or	2/5	
	or	1/5	
	or	0/5	
4/5	and	3/5	
	or	2/5	
	or	1/5	
	or	0/5	0·0197
3/5	and	2/5	
	or	1/5	
	or	0/5	
2/5	and	1/5	
	or	0/5	
1/5	and	0/5	
D.—Experiments in which no result will be obtained.			
0/5	and	0/5	0·0005
5/5	and	5/5	

two doses of a drug, each injected into five animals, one of which would kill 25 per cent. of animals, if a sufficient number were injected, and the other 75 per cent. The table shows that the probability that the LD 75 will kill more than the LD 25 is approximately 0·9215 ; the probability that the same number of animals will die with each dose is 0·0583 ; the probability that more will die with the lower dose is 0·0197, and the probability is 0·0005 that either all will die or all will live with each dose. That is to say, it is only about 11·5 to one that the doses will be placed in their right order, 17 to one they will not be called equal, and about 47 to one that they will not be reversed in order (*i.e.*, on the average, once in 48 tests, the weaker may appear to be the stronger). If 5 animals injected with doses differing by an amount corresponding to these mortalities be used, it is not unlikely that the proper order of the two doses will be reversed in the result. The probability of the reversal is not greater than the limits usually taken to give " significant " differences. If the doses injected are the LD 10 and the LD 90, the chances are 318 to one that the doses will be placed by the test in the proper order; and 609 to one that they will not be reversed in order. For the purpose of standardisation it is clear that the range of error in using 5 animals is almost exactly the difference between the doses necessary to cause 10 and 90 per cent. deaths, and considerably greater than that between the doses causing 25 and 75 per cent. deaths.

Here again, the extent of the error, reckoned as a percentage of the dose injected, depends on the slope and shape of the characteristic. For example, it is possible to confuse doses of cocaine as widely separated as 0·38 mgm. and 0·7 mgm., by using only 5 animals at each dose.

V. *The Construction of a Characteristic Curve.*

The mapping of a characteristic should be done on a batch of animals as far as possible simultaneously. The shape of the curve can be assumed to be the same for groups with different LD 50, as is the case for the summer and winter groups in the diphtheria curves, and the two insulin curves. The part of the characteristic which is of most use for practical purposes is that between the LD 25 and the LD 75. The majority of the animals used should therefore be injected with doses of the drug which will give mortalities between 25 per cent. and 75 per cent. The accuracy of slope of the line drawn through the observed points will be the greatest if the animals are injected in two groups only, one with the LD 25, and the other with the LD 75, as nearly as possible, as will be shown later.

All the calculations given in sections II and III are subject to an error arising from the practical limitations to the number of animals which can be used for setting up the characteristic. It is a matter of considerable difficulty to arrive at an accurate estimation of the error of every part of the curve, but by making a certain number of assumptions a sufficient approximation can be made.

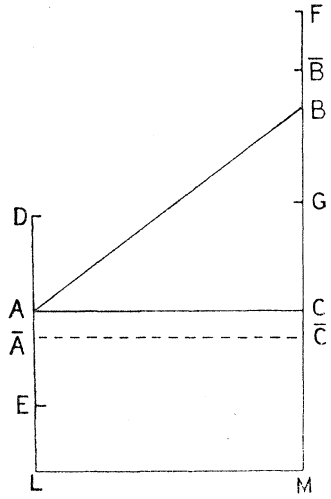

Fig. 9.

Let L and M (fig. 9) represent doses LD 25 and LD 75, and A and B the actually observed values of the percentage mortalities in the two groups injected respectively with these doses. Then A may lie anywhere in the range DE, which represents $\pm$ 3 times the s.d. of A on either side of the mean position $\bar{A}$, and similarly B anywhere in the range FG. As on p. 501 the standard deviation of the difference BC of percentage mortality is $100\sqrt{2pq/N}$, where $pq = 0{\cdot}75 \times 0{\cdot}25$.

Hence maximum value of BC = true mean value $\overline{BC} + 300\sqrt{2pq/N}$, and minimum value of BC = true mean value $\overline{BC} - 300\sqrt{2pq/N}$.

True value of $\frac{1}{K}$ at L $= \frac{\overline{BC}}{\alpha AC}$, where α is a constant equal to $\frac{100}{\text{dose LD 25}}$.

Therefore AC $= 50\,\bar{K}$, where $\bar{K}$ is the true value of K at LD 25. Therefore maximum observed value of

$$\frac{1}{K} = \frac{\text{maximum value of BC}}{\alpha AC} = \frac{1}{\bar{K}} + \frac{300\sqrt{2pq/N}}{50\,\bar{K}} = \frac{1}{\bar{K}} + \frac{6\sqrt{2pq/N}}{\bar{K}}.$$

Similarly minimum observed value of $\frac{1}{K} = \frac{1}{\bar{K}} - \frac{6\sqrt{2pq/N}}{\bar{K}}$.

For insulin, 2,460 mice were used altogether. Taking these for a first approximation as injected in two groups, at the LD 25 and LD 75, and K observed as 1·045, then

$$N = \frac{2460}{2} = 1230, \text{ and the error of } \frac{1}{K} = \pm \frac{6\sqrt{\left(\frac{2 \times \frac{1}{4} \times \frac{3}{4}}{1230}\right)}}{1{\cdot}045} = \pm 0{\cdot}102.$$

With K = 1 approximately, the error in K is $\pm$ 10 per cent., and since the

error of estimation of the dose varies directly with K, this estimated error is probably not more than 110 per cent. of its true value, or less than 90 per cent.

A glance at fig. 9 will show that if doses nearer together than LD 25 and LD 75 are used to determine the slope of the characteristic, the error of K will be increased. If doses further apart than these are used, errors are introduced, owing to the departure of the characteristic from its approximation to a straight line and to the asymmetry of the frequency distribution at the ends of the characteristic.

VI. *Factors which influence the Shape of the Characteristic.*

(*a*) *Multimodal variations.*—In the case in which there is under experiment an animal of which there are two or more varieties, such as male and female with different average lethal doses, but the same range of variability, characteristics with two modes will be obtained. Fig. 10 makes this clearer. Let A

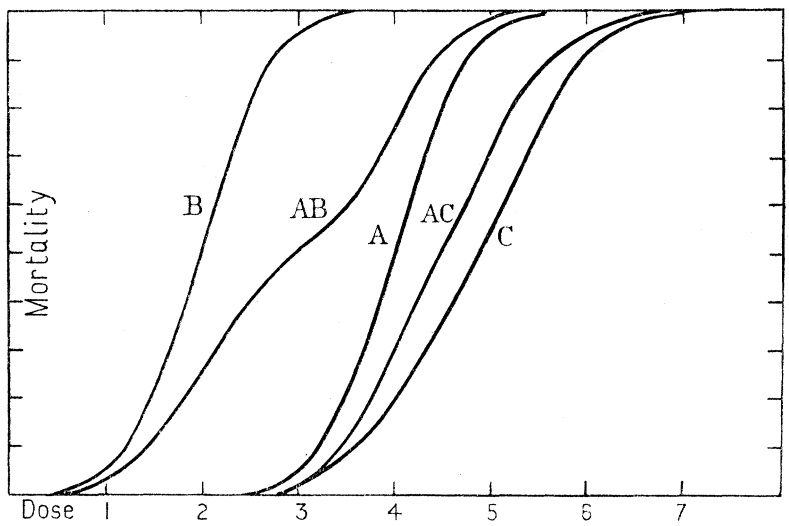

Fig. 10.

and B represent two symmetrical frequency curves, which, we will assume, represent the two varieties of animals. If a series of doses represented along the abscissæ are injected into large enough groups of animals, it is evident that, if the numbers of each variety are equal, a dose of 2·0 units will kill 50 per cent. of the more sensitive animals, and none of the less sensitive, which will correspond to 25 per cent. of the whole number of animals (there being equal numbers of more and less sensitive animals). At a dose of 3 units, there will be roughly

96 per cent. dying of the more sensitive, and 4 per cent. of the less sensitive, *i.e.*, 50 per cent. of the total, and so on. In this way, the curve AB is obtained, which has a kink in the middle. Now it is clear that, if only a relatively small number of animals are injected at each dose, for the purpose of determining a characteristic, the observed figures, after allowing for the statistical errors of limited numbers, will apparently fit a smooth characteristic of a slope intermediate between the two, as in the case of diphtheria toxin. Similarly, with A and C, and with combinations A, B, C.

The extreme asymmetry of the dysentery toxin curve is possibly due to the presence of a proportion of individuals which have antitoxin in varying quantities in their serum, the much larger quantities of antitoxin being less frequent in proportion. For dysentery toxin, the sensitivity of the animal to *uncombined* toxin may be taken to be unaffected by the presence of immunity, so that the effect of the antitoxin would be to subtract an equivalent amount of toxin from the dose injected. In consequence, the characteristics for the groups of animals with the same degrees of immunity would be parallel. A series of parallel frequency curves has been drawn in fig. 11, *a*, the one on the extreme left representing the hypothetical characteristic of a non-immune population, and the oblique curve crossing them is the characteristic which would be obtained from a mixture of groups with different antitoxic content, the relative proportion of the various groups being indicated on the base line. So that the extreme asymmetry of the dysentery toxin curve is possibly due to the presence of animals with a different quantity of some neutralising substance in the blood stream. The majority of the animals must have little or none, to account for the steepness of the rise of the curve at low doses. If fewer were non-immune, the characteristic would approach a straight line drawn diagonally across the figure.

(*b*) *Mixtures of toxic effects on the same animal.*—The influence on the form of the characteristic can be predicted under certain conditions. If the drugs act on two separate structures, the susceptibility of which for the poison varies independently, or, if two different poisons acting on separate structures exist in the drug under consideration, the form of the characteristic can be arrived at from the characteristics for each independent effect by the construction given in fig. 11, *b*.

In this figure, A is the characteristic for one toxin, B that for another, and C that for a mixture of the two in equal parts. C is described in the following manner:—At a dose which contains 1 unit of toxin A, y_2 per cent. will die solely as the result of the injection of A, and, of the remainder, y_1 per cent. will

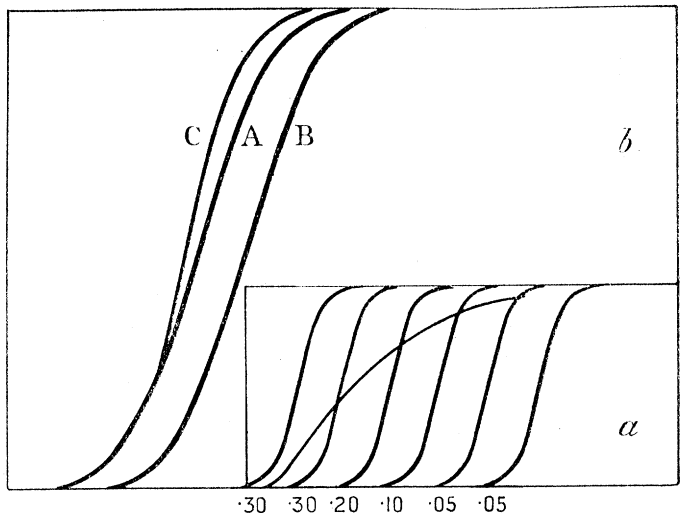

FIG. 11.

die solely as the result of the injection of B, corresponding to $\frac{y_1}{100}(100 - y_2)$ per cent. of the total number injected; therefore, in all, y_3 per cent. $= y_2 + \frac{y_1}{100}$ $(100 - y_2)$ per cent. will die, if the actions of the drugs are completely independent. As a result, the curve C is obtained, which is asymmetrical, in that the "spread" at low dose is greater than that at high. If the action of the two toxins is not independent, *i.e.*, if small doses of B make it easier for A to produce its effect, then the symmetry is not interfered with, but the whole curve is shifted to the left. The asymmetry will be at a maximum when two curves intersect at the LD 50, one being steeper than the other. If the toxins have widely varying median lethal doses, the characteristic obtained would, of course, be identical with the most toxic, if that were present in sufficient quantity.

Variations in the experimental conditions may produce changes similar to those discussed for racial changes. A comparison of the two characteristics for insulin at 37° C. and 28° C. shows that the approximation to a normal curve, produced by the increase in temperature, is very marked (Trevan and Boock, 1926).

It is apparent from these arguments that the more nearly the characteristic, under any given conditions of race, experimental conditions, etc., approaches the normal form, and the steeper it is, the more confidence in its reproducibility for the purposes of test can be obtained; but the best test for reproducibility is, of course, in the end, the experimental test. The number of animals for checking

reproducibility need not be as large as that necessary for the setting up of the characteristic.

VII. *Discussion.*

A few points of general interest remain to be cleared up.

(1) The form of a characteristic depends on the combined effect of a large number of different factors, some of which are controllable by alteration of experimental conditions (see, for instance, the remarkable effect of temperature on the response of mice to insulin—Trevan and Boock, 1926) and most of which are not. Among the former is probably the effect of heredity.

It is almost certain that animals inbred, after the fashion of the Wistar Institute rats, will give steeper characteristics than casual groups selected at random from the ordinary dealers' stocks. The effects of colour and weight on the insulin curve have, however, been found to be negligible (Trevan and Boock, 1926), and I do not think very *great* alterations are to be expected by selection of animals. Amongst the uncontrollable factors is the final relationship between dose and effect, on the organs especially affected by the drug, in producing its toxic effect, such as is discussed by Shackell, Gaddum (1926), Clark (1926) and others, using isolated organs. Such dosage-effect relationship must contribute to the complexity of the characteristics obtained, and drugs for which the basal dose-effect curve has a small angle of slope must tend to give characteristics of small angle of slope, and *vice versa*. It is, however, of great importance to realise that the characteristic is not solely a dosage-effect curve in this sense. Its shape depends also, and to a much greater extent, on the variability of the dosage-effect curve itself. Clark, for example, shows that the action of acetyl-choline on muscle may be represented fairly by the formula $Kx = \frac{y}{100 - y}$, where x is dose, and y the percentage of maximum effect produced, and without discussing here whether this agreement entails the acceptance of Clark's explanation of the relation between dose and effect, I may use his figures to explain what I mean. He found that K varied as much as 200 times from one heart to another, and it is this variation of K which chiefly determines the shape of the characteristic, and variations of this order will entirely mask the fundamental dosage-effect curve.

(2) The numbers of animals which are necessary for the determination of toxicity would appear from the above argument to be much larger than it is customary to use. It must be remembered, however, that these errors are worked out for single groups of the dimensions specified. The same number of

animals, split up into smaller groups, and injected with doses along the straight part of the characteristic, will give results which will have only a slightly greater error than that which is obtained by injecting the whole group with the average lethal dose. If, however, any of the smaller groups are injected with doses outside the straight part of the characteristic, the error is considerably increased for these doses. All estimations of toxicity on an unknown sample of a drug must begin by the injection of small groups of animals—two or three animals in each—with a series of varying doses. The doses, for economical working, should be separated by powers of two. Then, at the dose intermediate between those where the break from survival to death comes, a larger group should be put up in comparison with a standard. The size of this group depends on the accuracy with which the final result is required, and that depends on the slope of the characteristic. The number of animals for setting up the characteristic can be reduced by confining the determination of the characteristic to the ends of the straight part, roughly LD 25 and LD 75, and the error allowable in the value of 1/K may in general be as much as 20 per cent. The error of K will then be $+25$ per cent. or $-16{\cdot}6$ per cent., and an error of estimation calculated to be 10 per cent., may be anything between $8{\cdot}3$ per cent. and $12{\cdot}5$ per cent., which is sufficient for most purposes. For insulin, the value of N for setting up a characteristic with an error of K of $\pm$ 20 per cent. is 312, and the total number of animals twice this—624. For digitalis, the total number is 113. Once the characteristic is determined, the number of animals necessary depends on the accuracy desired. One thing should be insisted on, however, with great force, and that is, that unexpected or "discrepant" results must not be eliminated from the final average, except on the clearest evidence of a mistake in technique. If one result is so discrepant that its inclusion or not will make the difference between rejection or acceptance of a sample, the only way to deal with it is to repeat the experiment until the effect of the single discrepancy vanishes in the general average. It is of the utmost importance to observe this rule at all cost.

(3) Finally, with regard to standards in biological assay. For any new method of assay, it must be assumed that the median lethal dose is variable, and that, therefore, comparison must be made with a suitable standard preserved with suitable precautions against deterioration. There may be a minority of drugs which are assayable on the basis of a constant average lethal dose, and, for them, a statement for average lethal dose and the value of K, would be all that was needed for setting up a standard for any given worker, but even when the median lethal dose remains constant, the preparation of a standard is

necessary to correlate the work of different laboratories, and to eliminate the effect of variations in technique and the strain of animals in use.

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Studies on the Relation of Gonadic Structure to Plumage Characterisation in the Domestic Fowl.—III. *The Laying Hen with Cock's Plumage.*

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[Plate 39.]

Among the many fowls exhibiting abnormality of the sex characters which have been presented to this department by breeders sympathetic to the view that a study of the abnormal may lead to a better understanding of the normal processes of development and maintenance, have been two which were in every way similar to the cock which, in 1474, was sentenced by the magistrates of Basle to be burned at the stake "for the heinous and unnatural crime of laying an egg." It is recorded that the executioner on cutting open this cock found three more eggs within him, but this has been doubted by some, who have held that it was impossible and absurd, and that these eggs were not