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QUANTITATIVE ESTIMATION OF THE POTENCY OF DIGITALIS BY THE CAT METHOD IN RELATION TO SECULAR VARIATION.*

BY C. I. BLISS[1] AND J. C. HANSON.

Determinations of the acute toxic dose of a drug or poison follow one of two experimental procedures. The first and more common method is to administer different, predetermined dosages to successive groups of animals, so that each individual in a group receives the same dose. The percentage mortality from a given dose is the measure of effect and from a series of such dosages and percentages the dosage-mortality curve and the median lethal dose can be computed. These same values are obtained directly in the second, more specialized procedure, in that the lethal dose is determined separately for every individual. An example is the cat unit for cardiac glucosides, where the drug is infused into the venous system so slowly that the latent period between infusion and cardiac failure is presumably negligible in comparison with the total time of injection. The results, however, are sometimes computed so as to obscure the similarities between the two procedures. Moreover, there are seldom safeguards for secular fluctuations in susceptibility which are known to complicate many comparisons of the LD50 based upon the first method. Such safeguards have been observed for several years in tests of "Digiglusin" (Digitalis Glucosides, Lilly) by the cat method at the Lilly Research Laboratories, and it is of interest to examine the bearing of these results upon the so-called "cat unit" for cardiac glucosides and related drugs.

EXPERIMENTAL PROCEDURE.

The experimental procedure followed the general method first described by Hatcher and Brody (9). Digitalis extract was infused into the femoral vein of the etherized cat at the rate of 1 cc. per minute until the heart stopped. An assay required eight cats, all from the same source, four on the "Digiglusin" Standard and four on a new sample of extract which had been evaluated previously by the one-hour frog method as described in the U. S. Pharmacopœia. In conducting a test, four cats were etherized and prepared on four animal boards for the infusion of one of the two preparations in dilutions of 1:33$^1/_3$, the entire process requiring from 10 to 20 minutes. When

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all were ready, infusion was started in all four cats within a minute or two of each other. The four animals tested in parallel were exposed equally, therefore, to environmental influences.

From the data accumulated in the course of these tests, 52 series have been selected for statistical analysis. Since all of these were recorded by the junior author, any differences between series cannot be attributed to observer's bias. The analysis has been further restricted to the group in each series infused with the laboratory standard of "Digiglusin," which is kept in a refrigerator at 40° F. and checked for potency every three months, so that all solutions had presumably the same potency. The 52 assays covered a 19-month period from January 1937 to July 1938, inclusive, and were spaced at irregular intervals through this period. Animals for testing were not selected as to sex and of the 208 cats, 130 were males and 78 were females. They varied in weight from 1.7 to 3.5 Kg. and averaged 2.5 Kg. At a constant infusion rate of 1 cc. per minute, the infusion period varied from 38 to 144 minutes with a mean (geometric) of 72 minutes. The weight of each cat and its individual lethal dose in cc. (×0.1) have been transformed to logarithms and are given in full in Table I. These represent the record upon which the computations are based.

Comparison of the Variation between Series with That within Series.—The presence or absence of a secular variation in the individual lethal dose of digitalis for cats can be tested by comparing the variation between assays with that within assays. A significantly larger average difference between groups of four cats than between the individuals comprising these groups would show that secular changes were present. Such a comparison can be made readily by the analysis of variance, a procedure that is especially suitable when the units in the computation follow the normal curve of error. (Many authors have shown that although the distribution of the individual lethal dose is commonly skewed, the logarithm of the dose is usually distributed symmetrically and normally. For this reason the original records in Table I have been transformed to logarithms as a preliminary stage in the analysis.)

TABLE I.—BASIC RECORD ON EACH INDIVIDUAL CAT IN 52 ASSAYS WITH STANDARD "DIGIGLUSIN," SHOWING THE LOGARITHMS OF THE BODY WEIGHT IN KG. AND OF THE DOSE PER CAT IN 10 CC. UNITS OF EXTRACT.

Asay Number.	Date.	Logarithm of Body Weight in Cat Number. 1.	2.	3.	4.	Logarithm of Dose in Cat Number. 1.	2.	3.	4.
1	1/13/37	0.388	0.382*	0.323*	0.379	0.919	0.900	0.699	0.905
2	1/26/37	0.394*	0.498	0.406	0.414	0.866	0.910	0.871	0.712
3	1/27/37	0.341*	0.378	0.323*	0.416*	0.736	0.708	0.735	0.816
4	2/ 2/37	0.415*	0.370	0.458*	0.399*	0.841	0.808	0.932	0.827
5	2/ 4/37	0.378*	0.362*	0.369*	0.339*	0.906	0.769	0.778	0.736
6	2/23/37	0.454	0.365*	0.443*	0.351*	0.944	0.946	0.949	0.949
7	2/26/37	0.447	0.370*	0.427	0.463*	0.782	0.695	0.921	0.821
8	3/ 1/37	0.465*	0.463	0.500	0.514	0.982	0.975	0.962	0.948
9	3/ 4/37	0.474	0.380	0.359*	0.391	0.834	0.752	0.861	0.888
10	3/ 5/37	0.479*	0.472	0.403	0.487	0.853	1.000	1.003	0.997
11	3/25/37	0.346	0.332	0.448	0.473	0.927	0.739	0.967	0.970
12	4/ 6/37	0.459*	0.350*	0.332*	0.498	0.799	0.658	0.672	0.845
13	4/19/37	0.418	0.456	0.328	0.521	0.795	0.763	0.776	0.938
14	4/26/37	0.401*	0.334	0.515*	0.424	0.963	0.794	0.981	0.856
15	5/ 7/37	0.536	0.483	0.518	0.501	1.002	0.946	1.076	1.047
16	5/18/37	0.384*	0.383*	0.459	0.323*	0.918	0.867	1.023	0.625
17	6/ 2/37	0.379	0.401	0.380	0.384	0.769	0.762	0.710	0.768
18	6/ 8/37	0.329*	0.354	0.447	0.366	0.893	0.823	0.892	0.750
19	6/17/37	0.480	0.390	0.431	0.383*	0.904	0.823	0.849	0.818
20	6/22/37	0.437	0.486	0.325	0.399*	0.916	0.924	0.712	0.967
21	10/26/37	0.425	0.365*	0.379*	0.313*	0.897	0.670	0.699	0.793
22	10/26/37	0.324*	0.301*	0.325	0.355*	0.704	0.828	0.775	0.647
23	1/19/38	0.337*	0.328*	0.399	0.412	0.777	0.782	0.760	0.865
24	2/ 1/38	0.348*	0.361*	0.359*	0.418	0.804	0.838	0.835	0.883
25	2/ 2/38	0.399*	0.468*	0.326*	0.418*	0.901	0.947	0.857	0.990

TABLE I.—BASIC RECORD ON EACH INDIVIDUAL CAT IN 52 ASSAYS WITH STANDARD "DIGIGLUSIN," SHOWING THE LOGARITHMS OF THE BODY WEIGHT IN KG. AND OF THE DOSE PER CAT IN 10 CC. UNITS OF EXTRACT. (*Continued from page 522.*)

Assay Number.	Date.	Logarithm of Body Weight in Cat Number. 1.	2.	3.	4.	Logarithm of Dose in Cat Number. 1.	2.	3.	4.
26	2/ 3/38	0.331	0.431*	0.404*	0.451	0.879	0.983	0.862	0.965
27	2/15/38	0.365*	0.280*	0.354	0.315*	0.825	0.707	0.693	0.679
28	2/18/38	0.470	0.413	0.445	0.316	0.974	0.968	0.906	0.772
29	2/22/38	0.348	0.375*	0.374*	0.304*	0.814	0.939	0.957	0.817
30	2/28/38	0.342*	0.406*	0.336*	0.318*	0.816	0.831	0.799	0.932
31	3/16/38	0.505	0.334	0.515	0.488	0.848	0.699	0.939	0.938
32	3/22/38	0.393	0.387	0.461	0.414	1.009	0.867	0.901	0.782
33	3/29/38	0.416	0.387	0.416	0.369	0.971	0.891	0.980	0.841
34	4/ 4/38	0.414	0.337*	0.367	0.454	0.877	0.829	0.889	1.036
35	4/ 5/38	0.228	0.247	0.343	0.430	0.737	0.659	0.821	0.949
36	4/19/38	0.434	0.442	0.243	0.331	0.950	0.908	0.785	0.875
37	4/22/38	0.355	0.344	0.502	0.492	0.821	0.813	1.092	0.971
38	4/28/38	0.473	0.482	0.301	0.397*	0.941	0.904	0.843	0.780
39	4/29/38	0.281	0.454	0.280*	0.371	0.814	1.003	0.767	0.833
40	5/ 9/38	0.374	0.317	0.332	0.329	1.018	0.772	0.867	0.822
41	5/26/38	0.326	0.368	0.395	0.538	0.717	0.978	0.983	1.098
42	5/27/38	0.447	0.461	0.529	0.462	0.931	0.925	1.158	0.927
43	6/ 1/38	0.424*	0.373*	0.394	0.452	0.923	0.812	0.914	0.921
44	6/15/38	0.348*	0.450	0.529	0.545	0.839	0.769	0.909	0.958
45	6/22/38	0.360*	0.400*	0.490	0.440	0.849	0.920	0.971	0.881
46	6/23/38	0.446	0.360*	0.318	0.333	0.946	0.822	0.758	0.818
47	6/28/38	0.350	0.367*	0.497	0.417	0.743	0.792	0.853	0.799
48	6/29/38	0.461	0.269*	0.475	0.246*	0.962	0.707	0.958	0.699
49	7/ 5/38	0.395	0.427	0.383	0.292*	0.886	0.880	0.912	0.653
50	7/ 7/38	0.333*	0.334	0.313*	0.376	0.738	0.580	0.828	0.915
51	7/12/38	0.411	0.446	0.318*	0.332*	0.826	0.973	0.699	0.838
52	7/15/38	0.504	0.258*	0.361	0.472*	0.952	0.677	0.713	1.009

* Indicates females, those without are males.

Dosages are ordinarily expressed in mg. of drug per Kg. of body weight, although it is usually specified that the experimental animals should not exceed certain weight limits. This specification alone shows that the practice of dividing the dose per animal by its weight is essentially a convenient approximation. Ideally, every cat should be of exactly the same size, so that no correction for body weight would be necessary, but this, of course, was not the case. Not only did the four cats comprising any one group vary in size, but the average weight of different groups varied almost as much, from 2.1 to 3.2 Kg. An apparent variation in susceptibility might be attributed to differences in body mass unless it were shown that the usual dosage ratio corrected these differences satisfactorily in this particular series of tests. The difficulty has been solved by the analysis of covariance, using the logarithm of the dose per cat as the criterion of digitalis effect and the logarithm of body weight as a concomitant measure to be equalized in the calculation. By this procedure the variation in weight was corrected on the basis of the best-fitting straight line relating log-dose and log-weight *within* groups of cats that were tested simultaneously.

The mode of computation has been described in detail by R. A. Fisher (6), so that it need only be summarized here in presenting the results. All determinations of the lethal dose per individual cat (in logarithms) may be considered as independent estimates of one value which is represented best by the mean log-dose, 0.8577. The variation about this mean, measured by the total sum of the squared deviations, includes two components, that due to the differences between the 52 group means and the general mean and the reminder due to departures of the four individual records in each assay from its own group mean. The sums of the squares of these differences are listed in the Column Y^2 of Table II and equivalent values for the body weights, W^2,

and the product WY are given in the two preceding columns. From WY and W^2 within assays the slope of the straight line relating log-dose to log-weight has been computed as $b = \frac{WY}{W^2} = 1.085$, which was then used to correct the variation in the log-dose (Y^2) for inequalities in body weight. When the corrected or "reduced" sum of Y^2, 0.6535, was divided by the reduced degrees of freedom,

TABLE II.

Analysis of Data in Table I by Covariance, where W is the Logarithm of the Body Weight and Y is the Logarithm of the Dose Per Cat, Both in Terms of Deviations from Their Respective General Means.

Variation.	Degrees of Freedom.	Sums of W^2.	Sums of WY.	Sums of Y^2.	Regression Coefficient. b.	Reduced Sum of Y^2.	Reduced Mean. Y^2.
Between assays	51	0.35140	0.35319	0.88491	.....	0.53130	0.010418
Within assays	156	0.56657	0.61496	1.32092	1.08540	0.65345	0.004216
Total	207	0.91797	0.96815	2.20583	1.05466	1.18475	.....

155, the quotient, 0.004216, measured the net variance for the individual dose in logarithms exclusive of differences between days. The corresponding term for the variance between successive assays or groups of four cats was 0.010418 or more than twice as large. To find whether the variation between assays was significantly larger than that within assays, the variance ratio, "F" in Snedecor's terminology (11), was computed from the two corrected variances as $\frac{0.010418}{0.004216} = 2.471$. By reference to a table giving the expected values of the variance ratio at $P = .001$ (7), the observed ratio was clearly larger than that which would be exected at $n_1 = 51$ and $n_2 = 155$, so that there is less than 1 chance in 1000 that the cats in this series did not vary in susceptibility from one assay to another to a greater extent than from one individual to another on the same day. There can be no doubt, therefore, that under these experimental conditions digitalis can be assayed with greater precision if the sample is always compared with the standard on the same day by means of cats from the same source.

In correcting the lethal dose per cat for variations in body weight, no arbitrary assumptions have been made as to the exact relation between the two factors. It is of interest, therefore, to test how nearly the correction computed from the data agrees with the conventional dosage ratio which it replaced. If computed by means of logarithms, the logarithm of the dosage ratio for an individual cat in mg. per Kg. would be $Y - W$. The equation used in the foregoing calculation was $Y - bW$, with b equal to 1.085 instead of 1 as is usually assumed. The regression coefficient b is equivalent in the size factor as defined by Bliss (1) to the exponent h in the expression $\frac{\text{mg.}}{(\text{Kg.})^h}$ when both drug or poison and body mass are expressed in original weight units. Several instances have been reported (4) in which h does not equal 1. In the present case, however, $h = 1.085 \pm 0.086$ which is not significantly greater than 1, so that the conventional dosage ratio would be justified here and its logarithm should lead to substantially the same results as have been obtained by covariance.

Nature of the Variation between Assays.—The above analysis demonstrates that the susceptibility of cats to digitalis varied significantly between assays. Was this secular change seasonal or fortuitous? In order to correct for variations in body weight, the logarithm of the individual lethal dosage ratio ($x = y - w$) has been computed for all of the cats in Table I. From these the mean log-ratio ($\bar{x}_p$) was determined for each of the 52 assays and plotted in Fig. 1 against the date of the test. It is evident from inspection that there was no consistent or marked seasonal trend over the two years, which is in agreement with the experience of previous workers (12). With the possible exception of the last few months of the experiment, the group means seemed to fall quite fortuitously above and below the general mean ($\bar{x}$), indicated by the horizontal broken line. If the variation were really random, the distribution of the means for individual assays should approach the normal curve of error. This has been tested by computing two constants which in the normal curve are equal to zero, that for skewness, $g_1 = -0.461 \pm 0.330$, and that for

kurtosis, $g_2 = -0.618 \pm 0.650$. Since neither of these differed significantly from zero, the observed variation in the means might well have been random. It is clear, therefore, that the absence of seasonal trends is no guarantee against pronounced secular variations in susceptibility.

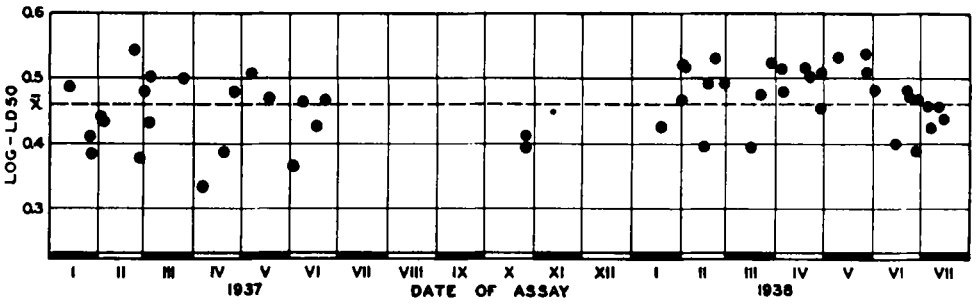

Fig. 1.—Relation between date of test and log-LD50 for digitalis in etherized cats. Each log-LD50 is the mean log-dose for four cats tested simultaneously.

In order to compare the relative magnitudes of the variation between and that within assays, the logarithmic dosage-ratios whose means are shown in Fig. 1 have been computed by the analysis of variance (Table III). As was to be expected, the two mean squares differed but little

TABLE III.

Analysis of Variance for the Logarithm of the Dosage-Ratio Computed from the Data in Table I, in Terms of the Deviations from the General Mean.

Variation.	Degrees of Freedom.	Sum of Squares.	Mean Square or Variance.	Standard Deviation.
Between assays	51	0.52992	0.010391	0.10193
Within assays	156	0.66478	0.004261	0.06528
Total	207	1.19470	....	...

from the equivalent "reduced" means obtained by covariance (Table II). The standard deviations in Table III, which are the square roots of the mean squares, show the relative magnitude of the errors affecting comparisons between groups of four cats on different days as contrasted with comparisons between cats within the same group.

These two standard deviations, however, are not direct indices to the relative importance of the two sources of variation. Although the mean square or variance within assays is independent of the differences between assays or groups, the variation between assays in Table III is compounded from both sources. Snedecor (10) and others have shown that when there are four units in each group, the mean square between groups or assays is equal to $4\,s_1^2 + s_2^2$ and that within assays to s_2^2. From Table III, $s_2^2 = 0.004261$ and $4\,s_1^2 + s_2^2 = 0.010391$ or $s_1^2 = 0.001532$. In terms of standard deviations the variation within assays, $s_2 = 0.0653$, would be augmented by the additional variation between assays, $s_1 = 0.0391$, unless comparisons between unknown and standard were restricted within four-cat groups. It is not sufficient, therefore, to test the standard digitalis extract occasionally between assays of unknown preparations as a control on the technique of a given laboratory or worker, as has been advocated (12). Tests with cats should be run in parallel, quite as is required in the frog assay.

The source of the variation between assays is to be sought in the origin of the successive batches of cats rather than in the environmental conditions of the testing laboratory which were relatively constant and hardly a major cause of variation. The cats were purchased from widely separated dealers who gathered them as needed and sometimes covered a considerable distance in filling a single order. Since they came from very heterogeneous sources, the cats in any one lot probably agreed better than cats from different lots when delivered to the laboratory. This simi-

larity extended to the susceptibility to digitalis and to body weight, which was significantly more uniform within than between assays. Once received at the laboratory the animals were used fairly promptly since cats do not thrive when confined in cages.

Relation of the Cat Unit to the Dosage-Mortality Curve.—The cat unit is usually computed as an arithmetic mean of the individual dosages, although the median lethal dose or LD50 is the preferred measure for dosage-mortality data. If the logarithm of the dosage ratio for individual cats were distributed normally, then the median lethal dose or LD50 for slow infusion of digitalis in etherized cats could be estimated best from the mean logarithm of the doses observed in any one test. Moreover, a demonstration that the log-dose is distributed normally in the present tests would give additional support to the use of logarithms in the above analyses of variance and of covariance.

The individual determinations were exposed to two unequal sources of variation, one between assays or groups of four and a second within these groups. In view of the random nature of the secular variation and the absence of any seasonal curve which could be used to correct the log-dosage-ratios of the successive groups, the ratio of the standard deviations in Table III provided the most satisfactory basis for adjusting the individual observations. A constant factor was added to each term in a group which reduced the departure of the group mean from the general mean to $\frac{0.06528}{0.10193} = 0.6404$ of the original observed difference. This corrected the individual values for secular variation so that the standard deviation based upon all 208 adjusted log-dosages was the same as that observed originally within assays. Since some of these corrections were positive and some negative, the mean log-dosage was unchanged.

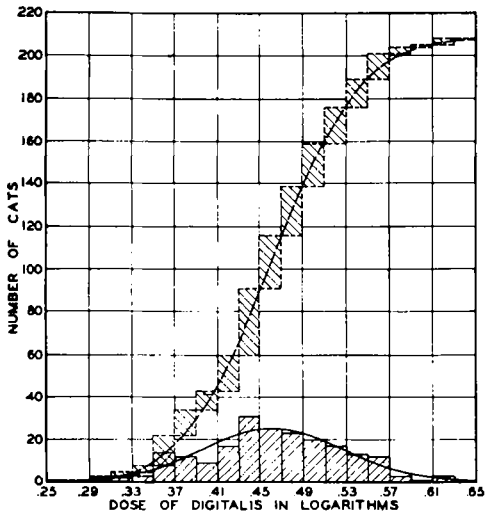

Fig. 2.—Initial and cumulative frequency distributions of individual lethal dosages in logarithms of standard "Digiglusin" in 10 cc. units per Kg. after correction for secular variation. The frequencies expected by the normal curve of error are shown by smooth curves.

The resulting distribution has been plotted as a frequency histogram along the base of Fig. 2, which shows the number of cats reacting in each dosage interval on the abscissa. The normal curve of error has been drawn over the histogram and it is apparent that the observed values agreed moderately well with expectation. The same data have been plotted in a cumulative form in the upper part of the diagram by moving each block vertically upward until its lower edge was contiguous with the upper edge of the preceding block. The normal curve in its cumulative, sigmoid form has been drawn over this block diagram as well and its similarity to the dosage-mortality curve in which percentage kill is plotted against log-dose is at once apparent.

The frequency histogram in the lower part of Fig. 2 is not unlike "Kurve 1" in van Wijngaarden's (12) paper on the digitalis assay in cats except that he plotted units of dosage rather than of log-dosage along the abscissa. This diagram has been quoted widely and seems to show that his original dosages were distributed normally. The figure, however, is a composite, covering different assays on different samples of digitalis so that the results based on more homogenous portions of this complex would be more nearly comparable to the evidence reported here. An analysis of these restricted records (based on his second and third diagrams) shows that the individual lethal dosages were not distributed symmetrically. When dosages were transformed to logarithms, this asymmetry largely disappeared, leading to the same conclusion as Fig. 2 of the present paper. His standard deviation, however, was 0.127 in contrast to the 0.0653 obtained here, so that our data were considerably more consistent than those reported by van Wijngaarden.

A number of writers have shown that the sigmoid dosage-mortality curve can be plotted as a straight line by converting dosages to logarithms and percentage mortalities to probits or their equivalent (3). The same transformation has been used in the present case, the cumulated frequencies being changed to percentages and then to probits to obtain the rectilinear diagram of Fig. 3. The observed values have been fitted by a straight line which passes through the mean log-dose of the original observations at 5 probits with a slope equal to the reciprocal of the standard deviation within assays from Table III and has the equation $Y = 5.00 + 15.32\ (X - 0.4613)$, where Y is the mortality in probits and X is the dose of digitalis in logarithms. One could conclude from the graphic analysis that the logarithm of the individual lethal dose followed the normal distribution, but for confirmation the constants for skewness and kurtosis have been determined, neither of them varying significantly from zero ($g_1 = -0.136 \pm 0.169$, $g_2 = -0.150 \pm 0.336$).

The equivalence of Fig. 3 to the rectified dosage-mortality curve gives added support to the interpretation of the dosage-mortality curve first introduced by Gaddum (8). The difference between them is merely of experimental and computational technique. When the individual lethal doses are transformed to logarithms before computing the mean, the antilogarithm of this mean logarithm (the geometric mean) is the median lethal dose or LD50. In this series of tests the log-LD50 of standard "Digiglusin" for 208 etherized cats was 0.4613 or (since the log-dosages were in 10 cc. units) LD50 = 28.93 cc. of dilute tincture per Kg. when injected at the rate of 1 cc. per minute.

A Modified Procedure for Cat Assays.— It is evident from the above study with a single preparation that when an unknown sample is compared with the standard within groups of four cats tested in parallel, assays will have a smaller error than when the comparison is between such groups or sets. The procedure would be to infuse in any one set two of the cats, selected at random, with the unknown preparation and the remaining two with the standard. This is repeated with additional sets each of four cats until the required precision is attained. The basic unit for analysis is the logarithm of the dosage ratio for each cat in cc. per Kg. (or in 10 cc. per Kg.), which is here designated by the symbol x. The computation both of relative potency and of its error is facilitated by the use of logarithmic units.

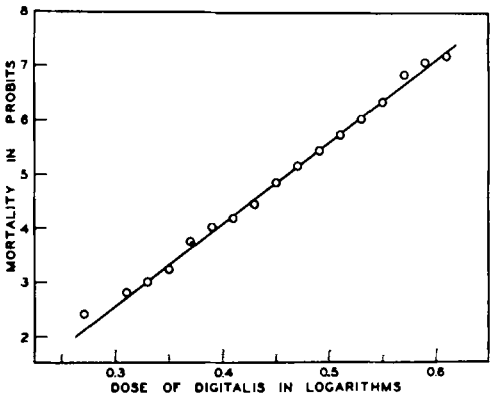

Fig. 3.—The cumulative curve of Fig. 2 after transformation from frequencies to probits.

The log-ratio of potencies, M, is given by the equation

$$M = \frac{D}{N'} \tag{1}$$

where D is the difference obtained by subtracting the sum of the log-doses for the unknown from the sum of the log-doses for the standard, disregarding the differences between groups of four cats or the number of groups involved, and N' is the total number of cats tested on each preparation. M gives the amount of standard required to produce the same response as one unit of unknown and can be changed to the logarithm of the percentage potency merely by adding 2. The antilogarithm of $M + 2$ is the percentage potency of the unknown sample.

In computing the standard error of M it is important to exclude any contributions from the differences between the four-cat groups since the inherent balance of the design has already excluded them from the estimate of M. The following equation for the standard error of M, s_M, has this characteristic:

$$s_M = 2\sqrt{\frac{S(x^2) - \frac{S(C^2)}{4} - \frac{D^2}{2N'}}{N'(3N' - 2)}} \tag{2}$$

where $S(x^2)$ is the sum of the squares of the original log-dosages for all cats on both standard and unknown, C is the sum of the four log-doses in any one group on both standard and unknown, which is squared and summed for all groups in the assay ($= S(C^2)$), and the other symbols have the same significance as in Equation 1. When there are only two groups each of four animals, Equation 2 can be simplified

$$s_M = \sqrt{\frac{S(x^2) - 0.125(T^2 + D_g^2 + D^2)}{10}}, \qquad (2a)$$

where T is the total of all log-dosages and D_g is the difference between groups obtained by subtracting the sum for the four cats in the second group from the sum in the four cats in the first group, disregarding differences between unknown and standard.

The standard error, s_M, is used to answer the question: does the potency of the sample or unknown differ significantly from that of the standard? This is determined from the ratio $t = \frac{M}{s_M}$, which is then referred to a table giving the expected values of t for different levels of significance, such as that in the collection of tables by Fisher and Yates (7). If the observed t exceeds that expected by chance at odds of 1 in 20, the sample is judged to differ significantly in potency from the standard. The expected value of t depends upon the degrees of freedom (n) in the error which for any individual assay is given by the rule $n = \frac{(3N' - 2)}{2}$, where, as before, N' is the number of cats tested either with the standard or the unknown.

To determine the approximate standard error of potency in original units, a convenient rule for converting from logarithms is that given by Cochran (5) as

$$\text{s.e. of relative potency} = 2.3026\ (\text{antilog } M)s_M. \qquad (3)$$

For percentages this is multiplied by 100.

The individual log-dosages for three assays of "Digiglusin," each based upon two groups of four cats, are given in Table IV and may be used to illustrate the above calculation. From the

TABLE IV.

The Lethal Dose in Logarithms for Individual Cats in Three Bioassays of "Digiglusin" (Digitalis Glucosides, Lilly).

Assay Number.	Log. Dosage-Ratio (X) for Individual Cats in First Group.				Second Group.				Total (T).	Differences between Groups. 1st–2nd (D_g).	Solutions: Standard –Unknown (D).
	Unknown.		Standard.		Unknown.		Standard.				
1	0.375	0.461	0.387	0.421	0.574	0.455	0.540	0.536	3.749	−0.461	0.019
2	$\bar{1}$.986	$\bar{1}$.918	0.468	0.422	0.082	$\bar{1}$.880	0.542	0.383	1.681	−0.093	0.949
3	0.665	0.473	0.644	0.455	0.443	0.350	0.535	0.493	4.058	0.416	0.196

eight log-doses (x) in each row the total (T) and the differences between groups (D_g) and that between standard and unknown (D) have been computed and listed in the last three columns of the table, N' being 4 in all cases. Substituting the results for the first assay in Equation 1, $M = \frac{0.019}{4} =$ 0.00475, and in Eq. 2a, $s_M = \sqrt{\frac{1.795553 - 0.125(3.749^2 + 0.461^2 + 0.019^2)}{10}} = 0.03474$. Since M was smaller than its error, it is clear without further test that the potency of the first sample did not differ significantly from that of the standard. To convert to percentages, the antilogarithm of 2 + 0.00475 = 2.00475 was read from a table of logarithms as 101.1 per cent, while from Equation 3 its standard error was (2.3026)(101.1)(0.03474) = 8.10, so that this sample or unknown showed 101.1 ± 8.1 per cent of the potency of standard "Digiglusin." A similar calculation for the second assay led to $M = 0.4872 \pm 0.0604$, from which $t = \frac{0.4872}{0.0604} = 8.07$ and $n = \frac{12 - 2}{2} = 5$. The observed t was larger than that expected at $P = 0.001$, so that there was no question that the 307 ± 43 per cent potency of the sample was in excess of the standard. Following factory dilution of 2.8

times, it gave the results in the third assay of Table IV, from which a relative potency of 111.9 ± 18.4 per cent was computed.

The standard deviation of the log-dosage for standard "Digiglusin" from the records on 208 individual cats is equivalent to the slope of the standard dosage-mortality curve in alternate techniques of bioassay, the one being the reciprocal of the other. From the slope of a standard curve one can estimate the expected precision of a given experimental procedure. The standard deviation within assays, $s = 0.06528 \pm 0.00369$, can be used similarly to compute the expected variation in the estimate of potency. Even when both standard and unknown have exactly the same potency, most assays will fall somewhat above or below the expected value of $M = 0$ or 100 per cent potency. If in every group of four cats, two are tested with the standard and two with the unknown, the extent of the expected variation may be calculated from the standard deviation and a table of the statistic t. In Table V the limits are given in terms of the log-ratio of potencies, M, and in Table VI the same values after conversion to percentages. Statisticians usually require for significance that a divergence must be of sufficient magnitude that in the absence of a real difference it would not be expected to occur by chance oftener than once in 20 tests or assays. A determination of potency based upon only two groups of cats, for example, as in the assays shown in Table IV, could vary from 76 to 131 per cent and still pass the requirement for standard or 100 per cent potency.

TABLE V.

Expected Variation in the Log-Ratio of Potencies (M) when Both Standard and Sample Have the Same Potency. This Table Is Based upon a Standard Deviation of $s = 0.06528$ and May Be Used when the Observed s_M Falls within the Limits of the Last Column ($P = 0.05$).

Number of Groups in Assay.	Number of Cats on Each Solution (N').	The Observed M, Either + or −, May Reach or Pass This Level Once in 5 Assays.	10 Assays.	20 Assays.	50 Assays.	100 Assays.	Observed s_M Must Fall within Limits of.
2	4	0.068	0.093	0.119	0.155	0.186	0.009 – 0.083
3	6	0.053	0.070	0.087	0.109	0.126	0.016 – 0.060
4	8	0.044	0.059	0.072	0.089	0.101	0.017 – 0.048
5	10	0.039	0.051	0.063	0.077	0.087	0.017 – 0.041
6	12	0.036	0.046	0.056	0.068	0.077	0.017 – 0.037

Tables V and VI can be used to segregate the samples that differ in potency from the standard in excess of the narrower limits of 85 and 118 per cent with a minimum expenditure in the retesting of chance variants. For routine tests, two sets totaling eight cats would be used and all samples for which the log-ratio of potencies M does not exceed ± 0.068 passed as having standard potency in agreement with the frog assay. Samples differing by more than this would be held for further testing. If the combined result with one additional set of four agrees within $M = \cdot \pm 0.070$,

TABLE VI.

Expected Variation in the Observed Percentage Potency of the Unknown when Both Standard and Unknown Have the Same Potency.

Number of Groups in Assay.	Total Number of Cats.	The Observed Percentage Potency of the Unknown May Reach or Pass These Limits Once in 5 Assays.	10 Assays.	20 Assays.	50 Assays.	100 Assays.
2	8	85–117	81–124	76–131	70–143	65–154
3	12	89–113	85–118	82–122	78–129	75–134
4	16	90–111	87–115	85–118	82–123	79–126
5	20	91–109	89–113	87–116	84–119	82–122
6	24	92–109	90–111	88–114	85–117	84–119

such samples would also be passed as having corroborated the frog assay, the others being held for testing with a fourth group of cats. With four groups the limiting value is $M = \pm 0.072$ at odds of 1 in 20. Since this is the critical level statistically, any combined value for four groups of cats which by Equation 1 gave an observed potency below 85 or above 118 per cent would be classified as significantly different from the standard. This procedure would be valid, of course, only if the observed s_M for any given assay, as computed by Equation 2 or 2a, agrees with its expected values

within the limits of sampling error shown in the last column of Table V. These limits have been determined from the constants in Table VI of reference (2) and the s_M of any assay for which the log-dosages seem erratic should be checked before using the limiting values of M given in the table.

SUMMARY.

In the assay of digitalis extract by the cat method secular variations in susceptibility are usually disregarded, as if they were negligible in comparison with other sources of error. The variation within assays relative to that between assays has been isolated by covariance in data on the individual lethal dose of standard "Digiglusin" for 52 groups each of four cats tested simultaneously. By using the dose per cat as the dependent variate and the weight of the cat as a concomitant measure, both in logarithms, arbitrary corrections for body size which might impair the validity of the comparison were avoided. In the present case, however, the size factor did not differ significantly from unity, so that the logarithm of the conventional dosage ratio in cc. per Kg. gave substantially the same results. In either case the variation within assays was very significantly less than that between assays, the net standard deviation of the latter being 60 per cent as large as that of the former. The variation between group means was independent of the date of the test and apparently random, showing that the absence of a seasonal trend does not rule out secular variation.

When the individual values were corrected for secular variation, the distribution of the individual log-doses followed the normal curve of error and could be plotted in a form equivalent to the dosage-mortality curve of which it is a variant. Because of this relation, the mean log-dose provides the best estimate of log − LD50 and its antilogarithm, the median lethal dose, is recommended in place of the usual arithmetic mean.

A modified procedure of bioassay is proposed so that the variation between groups will not bias estimates of the log-ratio of potencies or its error either in the conduct of the experiment or in the calculation of the result. The computation is illustrated by numerical examples and tables are given to show the expected presion of the method.

Acknowledgment: The authors are indebted to Dr. K. K. Chen and to Mr. C. C. Hargreaves, of the Lilly Research Laboratories, for their suggestions and criticisms in the conduct of the tests and in the preparation of the manuscript.

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