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AUTHORITY

Biological Defense Research Lab ltr dtd 13 Sep 1971

TRANSLATION NO. 202

DATE: July 1968

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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

[redacted] #202

Quantitative studies of typhus fever rickettsiae pathogenic for mice.

by Johs. Ipsen.

From the National Serum Institute, Kopenhagen, Biologic Standardization Division. Schweizerische Z. f. Path. u. Bakt. Vol. 7, No. 3(1944),pp 129-151.

First report.

Method of measurement of viral quantities in infected organs.

The production of typhus fever vaccines in countries where the disease does not occur is hindered by the difficulty of obtaining fresh strains of classic European typhus fever. Importation from abroad is very tedious, especially under wartime conditions, and the virus is not durable and degenerates easily after a succession of animal and egg passages. On the other hand, certain murine strains of typhus fever are considerably more stable and can be stored for a long time in a frozen state, contrary to the classic strains.

Since immunization with murine virus (Rickettsia mooseri), according to the data contained in the literature (Mooser, Otto and Wohlrab et al.), also bestows a relative immunity to European typhus fever, and since work with murine virus is most convenient in the nordic countries, we have, at the National Institute, directed our attention specifically to the production of vaccine with this virus as starting material.

In a tentative publication (Ipsen, 1943) I have reported on our method for production of inactivated vaccine from the livers of mice infected with murine typhus and have mentioned the considerations which led us to this method. A detailed description of this method of obtaining typhus fever vaccines will be published in a subsequent paper.

In the following, a presentation is made of a method for the quantitative evaluation of virulent rickettsiae contained in organs. It is to be assumed that the higher the content of rickettsiae in the starting material, the more effective will be the vaccine. A sure and economical method for the computation of the degree of virulence is necessary in order to choose those conditions under which the greatest rickettsial concentration is obtained.

The seeding of plates cannot be utilized for the counting of rickettsiae, which do not grow on artificial media, and microscopic observation of smear preparations only allows us to judge whether the number has exceeded a certain limit, without offering a sure quantitative estimate.

As in the case of ultra-visible viral types, here too, one is limited to the titration of suspensions of the infectious material and to the determination of the smallest effective dosis by way of suitable test animals; the degree of virulence can then be expressed as the number of smallest reaction-causing doses per volume unit. Certain strains of R. mooseri cause a lethal infection in mice and are therefore particularly suited for quantitative

examinations of t[illegible]s fever virus, since one is enabled to titrate the number of smallest lethal [illegible]ses contained in an organ emulsion by way of intraperitoneal injection of mice.

Strains and methods.

We used two strains pathogenic for mice. One (Virus murin Laigret Nr.88) comes from the Pasteur Institute, Tunis, where it had been used by Laigret for the production of live vaccine. This strain was sent to us by the National Bacteriological Laboratory, Stockholm. We received the other one from the National Institute for Experimental Therapy, Frankfort/Main. This strain is called "Mexican virus" because it was isolated by Mooser in Mexico in 1927. It is this strain that is called R. mooseri in the papers of Otto and Wohlrab.

Concerning the effect on mice, we did not notice any difference between the two strains. After receiving them we maintained them through numerous passages. The brains of infected mice were injected intraperitoneally, and after the death of the mice the brain was removed at once and either immediately continued intraperitoneally or stored at -16°C. At this temperature the virulence remains unchanged for months.

The organs whose degree of virulence is to be measured (the yolk sac, mouse brain, mouse liver) are removed under sterile conditions and shaken in a bottle with glass beads until they are divided. Then a suitable volume of saline solution is added, and from this emulsion the desired dilutions are prepared with saline in the usual manner.

The principle of the method of measurement.

According to the commonly used method for the determination of the smallest lethal dosis the agent is injected in diminishing doses, and thus the dosis is found which kills the animals with a known factor of certainty. Detailed biometric investigations of the last decade (Trevan, Gaddum, van der Waerden et al.) have clarified the rules of this method and have shown that the most feasible determination of the dosis would be by logarithmic intervals; thus the dosis which causes 50% mortality (LD 50) could be ascertained with the greatest positiveness. The principle is the same, regardless of whether one is dealing with pharmacological preparations, bacterial toxins or living bacterial cultures. The certainty with which LD 50 can be determined varies considerably with the different agents. This depends on the relationship between the resistance variability of the mice and the nature of the agent. Expressed as the deviation of the logarithm of the smallest lethal dosis for individual animals, we found the following variations in resistance, applicable to our mice: with neosalvarsan about 0.08, with tetanus toxin about 0.15, and with live Coli culture about 0.25 —— to name a few examples.

It was found that the error in the determination of LD 50 is very large for R. mooseri. In the proximity of LD 50 it is necessary to increase the dosis at least tenfold in order to attain an appreciable elevation of the percentage of mortality. As will be shown later, the variation of resistance, expressed as above, is 1.5-3.0. This huge variability requires that a fairly large number of mice be injected with doses around LD 50 in order to attain

a fair degree of certainty, and if one is not oriented beforehand as to the degree of virulence, one may use up test animals without results, or one is forced to repeat the measurement at a time when the virulence of the material may have abated.

The death time of mice injected with virulent material containing R. mooseri varies from 1 to 13 days. Although the variation is fairly extensive, a certain interdependency between death time and dosis can still be noted. For the purpose of measuring bacterial toxins, where a definite interdependency between death time and dosis was observed, I have worked out theoretic rules for the utilization of death time as a measure of toxidity (Ipsen, 1941). By the use of this principle a much more economical exploitation of the test results was attained than was possible with the determination of LD 50. We therefore tried to apply the same method to the measurement of the virulence of R. mooseri.

The principle is as follows: It is assumed that a constant relation exists between average death time and log dosis, so that all curves drawn at the same scale for average death time and log dosis have the same form for the same agent. Such curves, resulting from tests with preparations of diverse concentrations, may be merged by parallel displacement along the axis of dosis. From several tests covering a large dosis area, a standard curve may then be formed of the average death time and log dosis, which is characteristic for the agent in question. Accordingly, a dosis letalis media (m.l.d.) is defined as the dosis which causes a certain average death time. On the standard curve the logarithm of the number m.l.d., corresponding to a desired average death time, could then be read. If this logarithmic factor is called f(T), where T is the average death time caused by a dosis D, and d is written instead of m.l.d., we get, according to the definition of f(T)

$$f(T) = \log D/d \qquad (1)$$

$$\text{or} \quad \log d = \log D - f(T) \qquad (2)$$

Accordingly, m.l.d. is found thus: dosis D is injected, death time T is observed, the corresponding f(T) is found by means of the curve or a table, and f(T) is subtracted from log D.

The dosis-death time curve for R. mooseri.

If this principle is to be applied to the measurement of the virulence of material containing R. mooseri, one must first ascertain that the following requirements are met:

1. The establishment of a constant relation between average death time and the logarithm of the dosis of the infectious material must be possible.

2. The logarithmic time equivalence f(T) must be normally distributed among a group of mice, all of which have received the same dosis, and the deviation from this normal distribution must be constant and independent of the dosis.

3. This deviation must be the same as the deviation of the resistance (logarithm of the smallest lethal dose).

Ad 1. Data for the establishment of a standard curve were derived from 23 titrations of diverse organs of infected mice. A few organs were effective down to dilutions of 1:10^{-12}. We used the titration scale 10^{-1}, 10^{-2}, 10^{-3} etc. and designate the dosis by the logarithm of the degree of dilution, which we call pD (analogous to the designation pH).

$$pD = -\log D$$

pD = 10 therefore means that the dosis is 10^{-10} ccm.

The death time is given in days. The animals were injected in the morning, between 10 and 12 o'clock. Death time 5 signifies that the animal died on the morning of the 5th day; -- death time 5 1/3, that the animal was dead on the evening of the 5th day. Each test was entered in a point diagram, pD as abscissas and the death time as ordinates. For each mouse the death time was indicated by a point. Death time 15 was entered for surviving mice.

Subsequently the diagrams were placed on a glass pane illuminated from below, one on top of the other, in such a way that the abscissas covered each other; the curves were then moved along this axis until the points showed the smallest possible deviation. The displacement was made up to the next whole pD unit, and the abscissa values on each individual diagram were then corrected with reference to a common zero point. Now a consolidated diagram could be drawn, the coordinates of which are given in table 1.

Table 1.

Death times of mice after administration of different doses. The results of 23 titrations are compiled with reference to a common zero point of the dosis logarithms (pD). The figures represent the number of mice that succumbed in the indicated time.

Beobachtungstag - day of observation
Überlebend - surviving
Totalanzahl - total number.

The diagram is not very clear, because there are numerous points with identical coordinates. Off hand, it would be difficult to draw a curve through all these points whose average distance from the curve should be as short as possible; therefore a number of average values should first be obtained. It would be easiest to calculate an average death time for each dosis, but since it is not permissible to assume a priori that the death times represent a normal distribution, another method was chosen which also led to the desired result. On the basis of test data a mortality/pD curve was established for each day of observation, and from this, LD 50 was calculated for each day, i.e. the pD value which gives 50% mortality on the corresponding day (**). These values (pD 50) are listed at the bottom of table I.

(**) Footnote: The detailed calculation of pD 50, in addition to other biomathematical descriptions of this paper, are collected in a "biomathematical appendix," to which the interested reader is referred. In the following, the results are summed up in order to facilitate the presentation.

If the day of observation is contrasted with pD 50, points are obtained which are situated on a curve that suddenly rises from the 8th day on; it is straight between the 3rd and 7th day; prior to the 3rd day the progression is steeper.

Fig. 1. (self-explanatory).
Fig. 2. Mexican strain. Tunesian strain.

It can be demonstrated that these points deviate less than the error in measurement from a curve with an equation of

$$\left(\frac{D}{d} - 1\right) \cdot 2^{\frac{1}{g}T} = K \qquad (3)$$

which can be derived from the formula of bacterial reproduction. $\frac{1}{g}$ is found to equal 8,434 and $K = 10^{19.911}$ (see below).

We now determine the death time equivalence f(T), which is found in (1) and (3):

$$f(T) = \log \frac{D}{d} = \log \left(K \cdot 2^{-\frac{1}{g}T} + 1\right) \qquad (4)$$

from which f(T) can be calculated for T over 3 days. In Fig. 1 the extended line shows values of f(T), calculated according to (4). It is apparent that f(T) = 0 corresponds to the vertical asymptote ($T = \infty$). The dosis letalis media therefore is defined as that dosis which reveals 50% mortality after an "infinite" period of observation. The dotted part of the curve (Fig.1) has been drawn only approximately and may serve for the reading of f(T) for $T < 3$ days.

The first requirement -- the establishment of a regular relation between average death time and log dosis -- therefore has been met with an equation.

Ad 2 and 3. Next it is demonstrated statistically that f(T) is distributed normally and reveals the same constant deviation as log d. Hence it follows that log d = log D - f(T) also is distributed normally. This formula therefore can be used for the calculation of m.l.d. for each mouse, and the average value of these individual observations is the most closely approaching value for virulence. Assuming pV = -log d, where pV is the power of 10 which corresponds to the number m.l.d. per ccm of material, then we get from (2)

$$pV = pD + f(T). \qquad (5)$$

If, for example, pV = 6 is found for the suspension of a virulent mouse brain, then it contains 10^6 = 1 million m.l.d. per ccm.

The practical method for the computation of the degree of virulence of animal organs infected with Rickettsia mooseri.

Equation (5) shows that the degree of virulence pV of an organ emulsion may be measured by the addition of the dosis exponent pD to f(T), which

corresponds to the time which expired between injection and the animal's death. Since it is practically impossible to observe the exact moment of death, one is limited to observing the animals at set intervals and to assume an instant of death that lies medially between the two observations at which the animals were found to be alive or dead, respectively. This approximation is connected with coincidental errors only, which are insignificant in comparison to the variation in the animals' resistance.

We found it sufficient to observe the animals daily at 9 a.m. and 5 p.m., and to interpolate the f(T) values accordingly. It is not recommended to omit the observation at 5 p.m. It must be stressed that table II, below, solely requires readings at the two times specified, and that the animals were injected at about 11 a.m. If f(T) is desired for precise death times, formula (4) or table IX may be used.

Table II.

The logarithmic dosis equivalence
f(T)
for R. mooseri and mice.

Readings at 9 a.m. and 5 p.m. Injection at 11 a.m.

Tage - days
überlebend - surviving

Examples of the virulence measurement of infectious material.

When the described method is used, it is unimportant whether the material is titrated in different doses, or whether all mice receive the same dosis. The main thing is that the doses are sufficiently large to kill all mice.

First example: Titration in diminishing doses. The yolk sac of an egg infected with R. mooseri serves as material. The yolk sac was removed 6 days after injection and emulsified in 25 ccm saline solution.

Table III.

Measurement of the virulence degree of a yolk sac.
2 mice per dosis. Dosis in 0.5 ccm.

Verdünnung	- dilution
unverd.	- undiluted
Sterbezeit in Tagen	- death time in days
überlebend	- surviving
Summe	- total
Streuung	- deviation
Fehler von pV	- error of pV = ± 0.41.

The death time of each mouse is transformed into f(T) and pD is added thereto, according to the dosis received by the animal. The total of all values, divided by the number of dead mice, is the average value pV. The variation is computed in the usual manner on the basis of the deviation of the individual values f(T) $\neq$ pD from pV. The error in the determination of pV is the deviation, divided by the square root of the number of mice.

Second example: Measurement with a single dosis and ten mice. The material consisted of live virus in a dry vaccine, produced by Laigret's method. 20 brains of infected mice were mixed with ten sterile egg yolks and 125 g of a phosphate mixture. The mixture was dried in the exsiccator and reduced to a powder. Of the powder thus obtained (210 g), 25 mg in 0.5 ccm were injected intraperitoneally into each of ten mice. The measurement was conducted after the powder had been in cold storage for 10 months.

Table IV.

Measurement of the virulence of a "Laigret vaccine."

Sterbetag - day of death.

Average value of f(T) = pV = 3.67.
25 mg contain $10^{3.67}$ = 4700 dml.
1 mg = about 200 dml
deviation = 1.74
error in pV = $\neq$ 0.54.

One can use one or several doses for measuring; but the utilization of different dilutions does not yield a greater precision, since this is dependent only on the number of killed mice. If it is expected that the material may be only a little virulent, it is best to use only one dosis of undiluted material. Otherwise, when utilizing more virulent suspensions for the evaluation of the specificity of death cases, it may be of a certain value to produce a number of dilutions. It can be determined thereby, whether an elevation in death time, characteristic of typhus fever pathogenic for mice, occurs in conformance with decreasing doses. A mixed infection, which in most cases occurs only in connection with the strongest concentrations, will then betray itself by an irregular dosis-death time curve.

Table V.

Lung emulsion with mixed infections.
0.5 ccm of each dilution interperitoneally into 2 mice.

* infected with Corynebacteria

The test of table V shows that the first two doses, in comparison with later ones, caused death times that are too low: f(T) $\neq$ pD namely deviate by more than the twofold value of deviation of pV obtained from the following doses. Section yielded numberous Corynebacteria in the lungs and liver of those 4 mice which had been injected with the first two doses. For virulence determination, only the last values are used for computation in such cases.

Comparison of two strains of R. mooseri.

The establishment of a standard curve and the computation of the function f(T) derived therefrom is based on tests with "virus murin Laigret No. 88" from Tunis. When we subsequently received another strain, "Mexican virus," from Frankfort/Main, it suggested itself to examine whether or not the dosis-death time curve of this strain would be identical to the first.

The brains of mice infected with one of these two strains were injected into 25 mice each, whose death time was transformed into f(T). It was noted that the deviation of f(T) was the same for both strains, even if a certain difference in virulence of the utilized material was demonstrated.

Table VI.

Comparison of the variation in death time of two strains of R. mooseri.

Dosis 1/200 brain intraperitoneally.
25 mice per strain.

Sterbetag	- day of death
Anzahl toter Mäuse	- number of dead mice
Mittelwert von f(T)	- average value of f(T)
Streuung von f(T)	- deviation of f(T).

The two distributions of f(T) are both normal, which was graphically represented with the aid of a probit or rankit diagram (see Ipsen and Jerne, 1944). f(T) therefore shows the same distribution upon infection with the two strains.

In addition, it was investigated whether the average death time and dosis of the two strains follow the same curve. Different doses of the strains were injected into 5 mice, whose death time was transformed into f(T) according to the discovered formula. The average values of f(T), contrasted with pD, must then follow a straight line with a slope of -1 (45°), as revealed by the equation (5).

Table VII.

Comparison between the dosis-death time curves of two strains of Rickettsia mooseri.

Dosis 0.5 ccm of the dilution intraperitoneally into 5 mice each.

Verdünnung	- dilution
Sterbetag	- day of death
Mittelwert von	- average value of
Streuung von	- deviation of
überlebend	- surviving

Table VII and Fig. 2 reveal that the two strains have the same dosis-death time curve. At any rate, the points average-f(T)/pD deviate approximately the same amount from a straight line with a slope of 1. A measure of the deviation from this line is obtained by computing the variation of the values f(T) / pD from pV. As depicted in table VII, the variations of both strains belong in the same quantitative category. The vatiation of the Tunisian strain used in the presentation of the f(T) table coincidentally is even greater than that of the Mexican.

It is shown in this connection that the principle of measurement presented here is completely valid for two mouse-pathogenic typhus fever strains of wholly different origin, although they had been maintained for years by animal passages at different institutes.

Biomathematical appendix.

1. Computation of pD 50.

For each of the test days 1-13 we were able to measure, with the aid of table I, what percentage of mice, in receipt of a certain dosis, had died up to a given day. We assumed that the logarithm of the smallest dosis lethal to a mouse on a given day is distributed normally. According to this assumption, which we shall test later, a symmetrical S-curve of the same form as the integrated curve of normal distribution would be formed, when the percentages of mortality are contrasted with pD. If the percentages of mortality are then transformed into probits (y), y and pD will be rectilinearly connected by the equation

$$y-5 = b\,(pD - pD\,50);$$

y, of course, is the abscissa of a normal distribution with deviation 1 and average value 5, b is the slope of the reaction line, and pD is the dosis exponent listed in table I. pD 50 therefore is the average number of a normal distribution with deviation $\frac{1}{b}$.

According to each y-value, a weight is formed, depending on the magnitude of the mortality percentage and the number of mice used in the computation of the mortality percentage. These weights are found in Fischer and Yates' Table XI.

pD 50 is then computed thus:

$$pD\,50 = \overline{pD} - \frac{1}{b}(\bar{y} - 5)$$

where $\overline{pD} = \frac{\Sigma pD \cdot w}{\Sigma w}$; $\bar{y} = \frac{\Sigma y \cdot w}{\Sigma w}$

In addition, the magnitudes

$$SK\,|\,pD\,|\,= \Sigma (pD)^2 \cdot w - \frac{(\Sigma pD \cdot w)^2}{\Sigma w}$$

$$SK\{y\} = \Sigma y^2 \cdot w - \frac{(\Sigma y \cdot w)^2}{\Sigma w}$$

$$SP\{pD \cdot y\} = \Sigma y \cdot pD \cdot w - \frac{\Sigma pD \cdot w \cdot \Sigma y \cdot w}{\Sigma w}$$

are computed and the $\frac{1}{b}$ found therefrom, since

$$\frac{1}{b} = \frac{SK\{pD\}}{SP\{pD \cdot y\}}$$

2. Proof of the hypothesis of the normal distribution of pD.

If the death time is to be used according to the present principle of measurement, it becomes necessary to prove whether it is probable that the dosis-death time curve corresponds to a normal distribution for all days of observation, and whether this distribution shows the same deviation on each day. For this purpose a common slope (beta) is found for all curves (y/pD), and a determination is made of the probability with which the points of the daily mortality curve deviate from a straight line with the slope beta.

Table VIII.

Computation of the common slope beta for all curves y/pD.

Sterbetag - day of death
Summe - total

Different values are listed in table VIII for the determination of beta for each day. From these, the average slope beta is found,

$$\beta = \frac{\Sigma SP\{pD \cdot y\}}{\Sigma SK\{pD\}} = 0.336$$

and now one can find the magnitude of probability in favor of the hypothesis that each one of the curves y/pD has the slope beta, by determining

$$\chi^2 = SK\{y\} + \beta(\beta \cdot SK\{pD\} - 2 \cdot SP\{pD \cdot y\})$$

for each day. This χ^2 is distributed like Pearson's χ^2, and a table of this distribution will reveal the probability of correctness of the hypothesis, when the number of the degrees of freedom (f), corresponding to the curve, are sought. The degrees of freedom in this case are the number of points on the curve minus 1.

In the next to the last column of table VIII the probabilities P are listed in per cent. Only in one case are they smaller than 5% (but bigger than 2%) and one is therefore justified in assuming that the logarithm of the

smallest lethal dosis is distributed normally, with the same deviation, regardless of the time of observation chosen.

3. Adjustment of T and pD 50 to a theoretical curve.

From among the many curves which could be adapted with a passable approximation to points whose coordinates are T and pD 50, it would be most feasible to choose one in the equation of which a plausible biological hypothesis is expressed.

The death time in connection with bacterial infections -- apart from individual variations -- depends on the time in which the injected dosis of bacteria is enabled to multiply into a quantity lethal for the animal. The multiplication of bacteria can be expressed by the exponential equation

$$x = x_0 \cdot 2^{\frac{1}{g}T} \qquad (6)$$

x_0 is the starting dosis and x is the number of bacteria after time T. g is the time in which the respective bacterium peproduces by binary fission. Cavalli and Magni conclude therefrom that a rectilinear correlation exists between the death time and log starting dosis of the bacteria, since (6) may be expressed in logarithmic form:

$$T = \frac{g}{\log 2}(\log x - \log x_0) \qquad (7)$$

assuming the animal dies when x has reached a certain value (K), and $g/\log = \gamma$ and $\log x_0 = -pD$, then

$$T = \gamma \cdot (\log K + pD) \qquad (7a)$$

This equation expresses the average relation between dosis and death time, and we can therefore anticipate that pD and T are connected rectilinearly. Such a rectilinear correlation has been demonstrated by Cavalli and Magni within a limited dosis range for some bacterial types (B. tuberculosis, B. anthracis, Pneumococcus type 1). An inspection of our data on T and pD 50 will reveal that the rectilinearity is valid only for the range from the 2d to the 3rd and the 7th day. Subsequently the death time is steeply elevated, ending asymptotically (see Fig. 1). We can include these asymptotes in equation (7) by substituting log (D/d-1) for log x_0, where d expresses the smallest dosis capable of reproduction in a mouse.

$$T = \gamma \cdot (\log k - \log (D/d - 1)) \qquad (8)$$

(8) reveals that T becomes infinitely large when D approaches d. When D is larger than 10d, then log (D/d-1) will approach log D/d; T and log D will then be connected rectilinearly. The equation is not valid for $T < 2$. In that sector we are limited to the values expressed by the curve connecting the empirically determined points.

By the transcription of equation (8) we find the formula mentioned previously for the computation of f(T)

$$f(T) = \log \frac{D}{d} = \log \left(K \cdot 2^{-\frac{T}{g}} + 1 \right). \qquad (9)$$

From the present data the parameters of equation (8), namely γ, K and d, are to be determined.

γ can be calculated with the aid of the 5 points between the 3rd and 7th day, in which sector the curve reveals a rectilinear relation between T and pD 50.

T	pD 50
3	0.19
4	1.76
5	4.73
6	7.64
7	9.92

According to the method of smallest squares,

$$\gamma = \frac{\Delta T}{\Delta pD} = \frac{1}{2,534}$$

and therefrom,

$$g = \gamma \cdot \log 2 = \frac{0.30103}{2.534} = \frac{1}{8.414}$$

is found.

K and d may be found by means of graphic, step-by-step adjustment; an arithmetic adaptation based on R.A. Fisher's "Method of Maximum Likelihood" is better, however.

From (9) we get

$$pD\,50 = pd + \log \left(K \cdot 2^{-\frac{T}{g}} + 1 \right). \qquad (9a)$$

where pD = -log d.

We must therefore ascertain the minimum for

$$\Sigma \left(pD50 - \log \left(K \cdot 2^{-\frac{T}{g}} + 1 \right) - pd \right)^2 \qquad (10)$$

That is, the differentiation occurs after pD and the differential quotient is expressed as zero:

$$2 \left[\Sigma\, pD50 - \Sigma \log \left(k \cdot 2^{-\frac{T}{g}} + 1 \right) - n \cdot pd \right] = 0$$

$$n \cdot pd = \sum pD50 - \sum \log\left(K \cdot 2^{-\frac{T}{g}} + 1\right) \qquad (11)$$

Then (10) is differentiated after K

$$2\left[\sum \left(pD50 - \log\left(K \cdot 2^{-\frac{T}{g}} + 1\right) - pd\right) \frac{1}{K + 2^{T/g}}\right] = C \qquad (12)$$

We then include $f(T) = \log(K \cdot 2^{-T/g} + 1)$ and $w = \frac{1}{K + 2^{T/g}}$ in (12) and obtain

$$\sum pD50 \cdot w - \sum f(T) \cdot w = \sum pd \cdot w. \qquad (12a)$$

From (11) we get

$$\sum pD50 \cdot w - \sum f(T) \cdot w = \frac{1}{n}\left(\sum pD50 - \sum f(T)\right) \cdot \sum w \qquad (13)$$

In equation (13) only the observed magnitudes pD 50 and T occur, as well as the computed constant g and the unknown K. A value of K must now be found which leads to the identity of both sides of equation (13).

It was found by relatively few approximate calculations that this requirement is met by

$$\underline{K = 10^{19.91}}$$

in which the exponent was determined with a precision of up to two decimal points.

(11) then yields

$$\underline{pd = 12.064}.$$

When the determined constants are included in equation (9), the time equivalence f(T) can then be computed for $T > 3$. The curve in Fig. 1 is drawn through these determined points; for $T < 3$ it has been extended according to the best estimates. Table IX contains a few coordinates for the construction of a standard curve.

Table IX.

f(T) table with the provision of exact observation of death times.

The difference between table IX and table II — as evidenced by what has been said previously — is solely a result of different modes of observation. Table IX presupposes a precise observation of the instant of death, while table II is based only on two daily observations, at 9 a.m. and 5 p.m.

4. Proof of the adaptability of all test data to the dosis-death time curve.

In the second chapter it was found that log D or pD is distributed normally around pD 50 with a constant deviation of 1/beta, therefore the distribution may be transformed to probit/log dosis lines of the formula

$$y - 5 = \beta \cdot (pD - pD\,50).$$

Since pD 50 = pd - f(T), one can see that f(T), for a certain dosis pD, is distributed normally with the deviation 1/beta, because these two equations together yield

$$y - 5 = \beta \left(f(T) - (pd - pD)\right). \qquad (14)$$

This may be proved graphically for the data of table I by entering f(T) for each pD in a probit or rankit diagram. This results in a number of straight lines all of which may be approximately adjusted to the slope beta = 0.336.

For the purpose of arithmetical computation of the degree of safety in this approximation we have chosen another method, utilizing the χ^2-proof of Pearson:

With the aid of equation (14) we were able to determine y for a certain day and a given dosis, and a probit table will show how large the percentage of mortality is which can be expected on the respective day of observation. From the number of mice in this group the expected number of dead up to this day or the expected number of dead between two moments of observation are determined. Care must be taken to choose time intervals large enough to allow for an expected number of dead mice of at least 5, as otherwise the χ^2 proof cannot be used. The expected number of dead is contrasted with the number of dead mice observed in the same period:

$$\chi^2 = \frac{(\text{observed number} - \text{expected number})^2}{\text{expected number}}$$

The sum of these 46 χ^2 values is 46.87 (see table X). The number of degrees of freedom is f = 42, since 4 bonds are placed on the observations at the start, i.e. one at the determination of deviation (1/beta), one at the computation of the curve's elevation (g), one at the determination of the curvature (K) and one at the computation of the curve's position (d).

$\sqrt{2\chi^2} - \sqrt{2f-1}$ is normally distributed with deviation 1.

$\sqrt{2 \cdot 46.87} - \sqrt{2 \cdot 42 - 1} = 0.57$; the test data deviate less than once the deviation from expected values. We may therefore validate the hypothesis that f(T) and pD 50 are connected rectilinearly, and that f(T) is normally distributed for an identical dosis, with constant deviation which is equal to the deviation of pD for the same observation time. Naturally it can be expected that this deviation varies for different mouse populations.

Table X.

The observed number of dead mice within the period indicated by vertical lines (whole numbers)

The expected number, computed according to equation (13), is indicated below with a decimal point.

Erwart. Mittelw. von f(T) - expected average value of f(T)
Totale Anzahl Muse - total number of mice
Tag - day

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5. The error in the determination of pV.

The error of pV is determined directly from the test, since pD $\neq$ f(T) is distributed normally around pV. The deviation, which is computed in the usual manner from the sums of the deviations squared, as a rule is somewhat smaller than the deviation obtained from the compiled data of table I, since this must be considered as a maximal value.

The deviation applicable to tables III and IV is computed directly from the test at 1.30 and 1.74, respectively. The error of pV is then found by division by the square root of the number of dead mice (in both cases 10).

Upon utilization of the death time method presented here a final result is obtained with the first measurement, without having to resort to an orientating test of the material. In addition, the error is smaller than that connected with the determination of LD 50. This, for instance, is revealed by table III, the data of which may also be used for the computation of LD 50. According to them, LD 50 lies approximately between -8 and -10. The error in this approximation, according to van der Waerden, is

$$\sqrt{0.5^2 - \frac{s^2 \cdot \delta}{n}} = \sqrt{0.5^2 - \frac{1.30^2 \cdot 2}{2}} = \sqrt{0.741} = 0.86$$

(s is the deviation and δ the logarithmic dosis interval; n is the number of animals per dosis).

Since the error in determining pV in the same test is only 0.41, the precision of measurement has been doubled by rational exploitation of disclosures offered by the death time of the animals.