THE MEASUREMENT OF BACTERIAL VIRULENCE AND OF CERTAIN ALLIED PROPERTIES, WITH SPECIAL REFERENCE TO THE VIRULENCE OF *B. AERTRYCKE.*

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(With 6 Charts and 12 Diagrams.)

Introduction.

In connection with recent investigations on the spread of bacterial infection among mice, the possible effect of variations in the virulence of the infecting organism has, inevitably, formed one of the problems for study.

A consideration of the results of experiments, designed to define certain of the conditions which regulate the spread of infection among mice, has led Topley and his co-workers to suggest that changes in the infective powers of the bacteria probably play a definite part in determining the course of events during the epidemic spread of disease (Topley, 1919, 1923, Topley and Ayrton, 1924 *b*).

These experiments were not, however, especially designed to permit the observation of variations in bacterial virulence, other conditions being kept constant, and the conclusions based upon them must therefore be regarded as purely inferential and tentative. Webster, on the other hand, has submitted this particular problem, as far as it concerns mouse-typhoid, to direct and extensive experimental study (Webster, 1923 *a*, *b*, *c*, *d*, *e*, 1924) and has concluded, from the results obtained, that variations in bacterial virulence play no essential part in the epidemic spread of this disease, but that the course of events is fully determined on the one hand by the distribution of the bacterial parasite among the population at risk, and by the susceptibility of the individuals comprising that population on the other.

He would attach particular importance to the latter factor, and especially to an inherent and apparently non-specific resistance, which he regards as varying in an orderly manner among the members of an in-bred strain of mice, and which determines the form of the mortality curve which results when an adequate sample of such a strain is submitted to the action of a constant dose of the infective organisms.

He finds, moreover, that different strains of mice display different degrees of this natural resistance, so that constancy of strain of the hosts becomes a factor of the first importance in any series of comparative experiments.

Webster, indeed, not only regards variations in virulence as unimportant in the epidemic spread of mouse-typhoid, but believes that such variations are of infrequent occurrence, and that they cannot be produced by the means which are commonly employed in the laboratory for this purpose.

He has noted that different strains of mouse-typhoid bacilli, several of which on the grounds of their fermentative and serological reactions would be classed as *B. aertrycke*, may show constant differences in virulence; but these strains were derived from different sources, and had no historical connection one with another.

Taking any one of these strains, Webster failed to demonstrate any significant change in virulence as the result of prolonged cultivation in the laboratory, or of repeated animal passage carried out in a number of different ways. These results will be considered in greater detail in a later section of this report, and for the moment we would merely note his main conclusions.

These conclusions are clearly of fundamental importance, since, if we can regard fluctuations in bacterial virulence as a negligible factor in the epidemic spread of *B. aertrycke* infection, the problem presented in attempts to study this question by the method of direct experiment will be very greatly simplified. At the same time, their very importance renders their confirmation particularly urgent.

As a preliminary step in the re-investigation of this question it was desired to determine the accuracy of the methods in common use for the measurement of the virulence of such an organism as *B. aertrycke*.

The present report deals with this technical problem and with certain results which have been obtained when the method finally adopted was applied to the study of variations in the virulence of this organism.

The meaning of the term "virulence," or rather its lack of definition as usually employed, has been discussed by Topley (1923) in an earlier report. It was there suggested that the term "virulence," if used at all, should be employed in a general sense so as to include the lethal action of such an organism as the diphtheria bacillus. The term is indeed habitually employed for this purpose, in direct contradiction to the definition of virulence which is stated or implied in most discussions of this bacterial character. For the particular property of rapid and widespread multiplication within the tissues, leading to death of the host within a determined time limit it was suggested that the term "intraglisence" might be employed. In this report the term "virulence" is used in the general sense of poisonous, sanctioned alike by derivation, convenience and clearness of definition. The criterion of virulence is taken as the death of the host, and one organism is regarded as more virulent than another if (*a*) it kills the same proportion of hosts when administered in a smaller dose, (*b*) it kills a larger proportion of hosts when administered in the same dose, or (*c*) it kills the same proportion of hosts in a shorter time when administered in the same dose. These criteria are, of course, based on the assumption that the host factor can be kept constant,

or that fluctuations in resistance are so distributed among the host population as to allow the ordinary statistical tests for significance to be applied, when an adequate sample of hosts is employed for experimental purposes. The main purpose has been to decide which of these criteria it is best to employ. The first criterion is that of the minimal lethal dose. It is the criterion habitually employed in determining the toxicity of drugs or of bacterial toxins, and also in determining the virulence of such organisms as *Streptococcus haemolyticus*, *Pneumococcus*, or the *B. anthracis*, which kill by giving rise to a rapidly fatal septicaemia.

A somewhat extensive review of the literature of this subject has caused us to feel considerable surprise at the very inadequate basis of statistical data on which much of such work has been placed. The number of test-animals employed has, in the majority of cases, been curiously small, and no attempt has been made to evaluate the probable error involved in the results actually obtained. There is usually no clear statement as to the proportion of the test animals which should succumb to the effects of the minimal lethal dose, and indeed the number of animals employed for each test dose, often two or three, makes it clear that no such proportions could, in fact, have been determined. We have been left with a feeling of considerable scepticism as regards the validity of many of the statements put forward in regard to this question, except in those cases in which the critical dose appears to be so sharply defined that a relatively small decrease will cover a range in the curve describing change of mortality with dose, over which the mortality falls from a value in the neighbourhood of 100 per cent. to a value in the neighbourhood of 0 per cent. How often the conditions are, in fact, so favourable for easy measurement, we have at the moment no adequate means of judging. We cannot enter here into any detailed discussion of the general problem, but in the course of many enquiries from other workers we have been permitted to see certain unpublished results obtained by Dr Trevan and we would refer the reader to his paper, now in course of publication, for a consideration of the various points involved. We should like to express our thanks to Dr Trevan for his kindness in affording us this information.

Any degree of suspicion which attaches itself to the results obtained by the usual methods of measuring the minimal lethal dose of a chemical poison, a bacterial toxin, or an organism which kills by causing a rapidly fatal septicaemia, is, however, enormously increased when we come to deal with such an organism as *B. aertrycke*, using the mouse as host. *B. aertrycke* is a natural pathogenic parasite of the mouse, causing a well-defined disease, which may, under natural conditions of contact infection, lead to acute death, to a subacute infection producing typical lesions and ending in death or recovery, or to a chronic infection which may lead to a fatal issue after a prolonged period, or may persist for many months without causing any apparent departure from normal health.

The following questions at once present themselves for solution. How

define a minimal lethal dose of *B. aertrycke*, taking the mouse as our test animal? If we can arrive at a clear definition of such a standard, can its value be measured with reasonable accuracy by any technique which is practicable for the purposes of experimental work?

The problem is mainly a statistical one. We must know how the course of the infection is, in fact, modified by varying the dose of the infecting bacterium, before we can select a suitable time-interval, within which death must occur in order to be regarded as significant, and the proportion of mice which must die within this time-interval to yield a standard result. With this knowledge we shall also be in a position to judge the degree of approximation within which we can determine the dose which will bring about this result in a sample of mice of any particular size.

THE TYPES OF INFECTION CAUSED BY THE ADMINISTRATION OF *B. AERTRYCKE* TO MICE.

Before commencing the present investigation a review was made of all the available data dealing with experiments in which living cultures of *B. aertrycke* were administered to mice. For this purpose the figures were abstracted from the protocols given in the following reports: Webster, 1922, 1923 *a, b, c, d, e, f, g*, 1924; Topley and Ayrton, 1924 *a, b*; Lange, 1921, 1924; and the records of several series of unpublished experiments carried out in this laboratory were added.

The actual doses administered were converted, as accurately as the data permitted, into terms of numbers of living bacteria, and were tabulated separately according as the administration was carried out intraperitoneally or *per os*.

In tabulating the doses a logarithmic scale was employed, using dose intervals based on the same scale. All doses, for instance, falling between $10^{3\cdot5}$ and $10^{4\cdot5}$ were classed as 10^4 and so on.

A series of curves were drawn (see Chart I) showing the frequency of death on each day, following an intraperitoneal inoculation with *B. aertrycke*, up to and including the 21st day. Curves were drawn (*a*) for all doses, (*b*) for dose Group A, comprising doses of 10^7 and 10^8, (*c*) for dose Group B, 10^6, and (*d*) for dose Group C, 10^3, 10^4 and 10^5. It will be seen at once that the curve for all doses is bimodal. There is a mode on the 1st day and another on the 4th. Comparing this curve with those for the different ranges of dosage, it is clear that the first day mode is given mainly by the deaths of those mice which received the largest doses, the great majority of which died within 24–72 hours after inoculation.

The second mode is much less clearly marked, and the curve falls away gradually and irregularly on either side, but the general form of this part of the curve is quite evident.

We are clearly dealing with two kinds of infection, a rapidly fatal form, which kills within 24–72 hours and a subacute form which, when fatal, tends

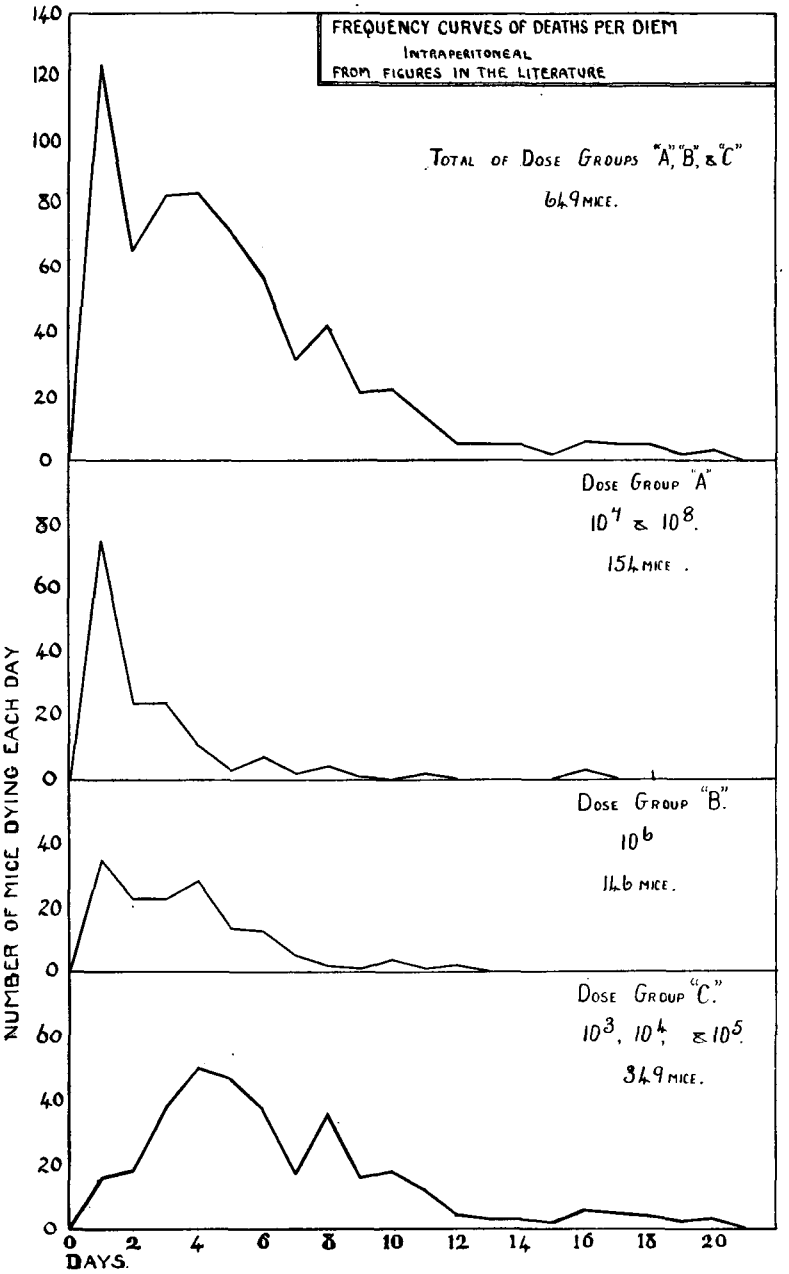

Chart I.

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to cause death somewhere between the 3rd and 12th day. The deaths after the 14th day are relatively so few as to be negligible.

The figures were then tabulated, for the different doses, to show (*a*) percentage mortality within 3 days, (*b*) percentage mortality within 14 days, (*c*) average time to death of all mice which died within 14 days, (*d*) average time to death of all mice which died between the 3rd and 14th day. These figures are given in Table I and the mortality rates are shown in Chart II.

Table I. *Intraperitoneal.*

Figures from the literature.

Dose	No. of mice at risk	Percentage mortality		Average time to death of mice which died between	
		3rd day	14th day	1st and 14th days	4th and 14th days
10^7	154	75·4	100·0	2·2 days	7·6 days
10^6	149	53·2	98·0	3·5 ,,	6·4 ,,
10^5	211	26·5	95·7	5·7 ,,	8·0 ,,
10^4	125	10·4	76·5	7·2 ,,	8·6 ,,
10^3	59	3·8	86·6	9·8 ,,	10·8 ,,
10^2	43	—	46·4	9·6 ,,	9·9 ,,

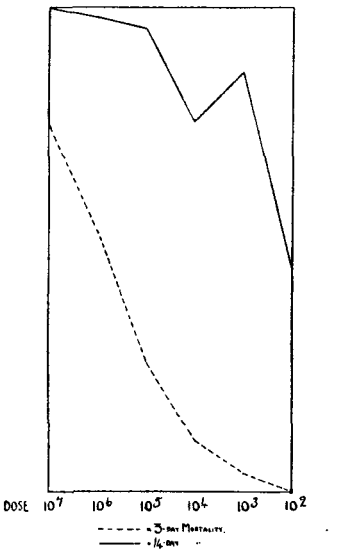

Chart II. Percentage mortality. Intraperitoneal.
From figures in the literature.

Decrease in dose is associated with decrease in mortality, whether the time of observation is limited to 3 or to 14 days, but the rate of decrease in mortality is very slow, so that large variations in dosage will have relatively little effect on the percentage death rate. The fall is most rapid with the 3-day deaths between 10^7 and 10^5, and with the 14-day deaths between 10^3 and 10^2. This question will, however, be discussed in more detail in connection with our own experiments.

The effect of variations in dosage on the average time-to-death is clearly very slight, if we exclude those mice which died on or before the 3rd day. If, on the other hand, we include *all* mice which died there is an increase in the average time-to-death with decrease in dose. This is because, as the dose falls, a decreasing proportion of mice succumb to the acute form of the infection, but if this latter type of infection be excluded and we confine our attention to the subacute disease (the ordinary form of mouse-typhoid under natural conditions) we find that the size of the infecting dose has little effect on the *duration* of life, which is probably determined by many different factors.

Charts III, IV and Table II have been constructed in an exactly similar fashion for those mice to which *B. aertrycke* was administered *per os*. They

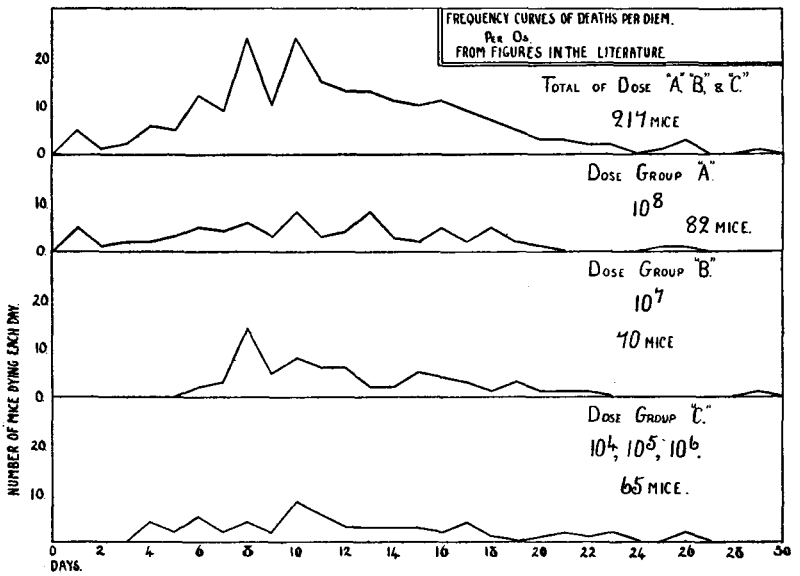

Chart III.

need little comment. The acute form of fatal infection occurs very exceptionally. The frequency curves of death occurring on successive days after infection are essentially similar, irrespective of dose, and are all of the same general type as that obtained with small intra-peritoneal injections. The effect of decrease of dose on percentage mortality is even less than in the case of intraperitoneal injections and variation of dosage has still less effect on the average time-to-death. In the case of *per os* administration the 42nd day is substituted for the 14th day.

During the present investigation large numbers of mice have been inocu lated intraperitoneally with accurately graded doses of *B. aertrycke*. In all cases the strain employed has been grown in broth for 24 hours at 22° C. A number of agar slopes have been inoculated from this broth culture and grown for 18 hours at 22° C. The growth on these slopes has been washed

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off in sterile Ringer's solution, and diluted to a bacterial content of 500 million to the c.c. by comparison with a set of standard turbidity tubes. From this bacterial suspension the required dilutions have been prepared, and in all cases the bacterial content has been confirmed by plating from the actual dilutions used for inoculation.

The error has seldom exceeded 20 per cent. and has in no case been greater than 43 per cent. In all cases the dilutions have been so graded that the required inoculum has been contained in 0·25 c.c. of the bacterial suspension.

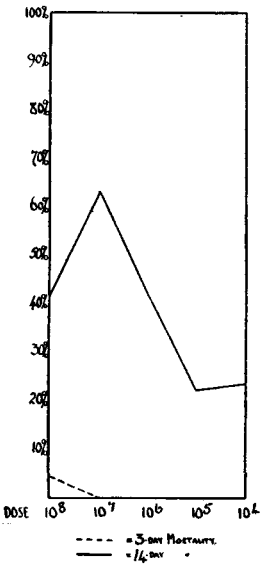

Chart IV. Percentage mortality per os.
From figures in the literature.

Table II. *Per Os.*

Figures from the literature.

Dose	No. of mice at risk	Percentage mortality		Average time to death of mice which died between	
		3rd day	42nd day	1st and 42nd days	4th and 42nd days
10^8	198	4·03	41·4	12·4 days	13·9 days
10^7	111	—	63·0	12·6 ,,	12·6 ,,
10^6	111	—	42·0	15·3 ,,	15·5 ,,
10^5	36	—	22·2	12·5 ,,	12·5 ,,
10^4	43	—	23·5	9·6 ,,	12·0 ,,

In a large number of experiments four groups of mice containing an equal number of individuals have been inoculated with 10^7, 10^5, 10^3 and 10 *B. aertrycke*.

Including all experiments of this type, which will be referred to later in this report, and several other experiments of the same type which have been carried out in this department, the results obtained with 1600 mice, which have been treated in this way, are available for analysis.

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From these data Charts V and VI and Table III have been constructed in exactly the same way as for the results obtained by collecting the available literature. The main features of both are essentially similar.

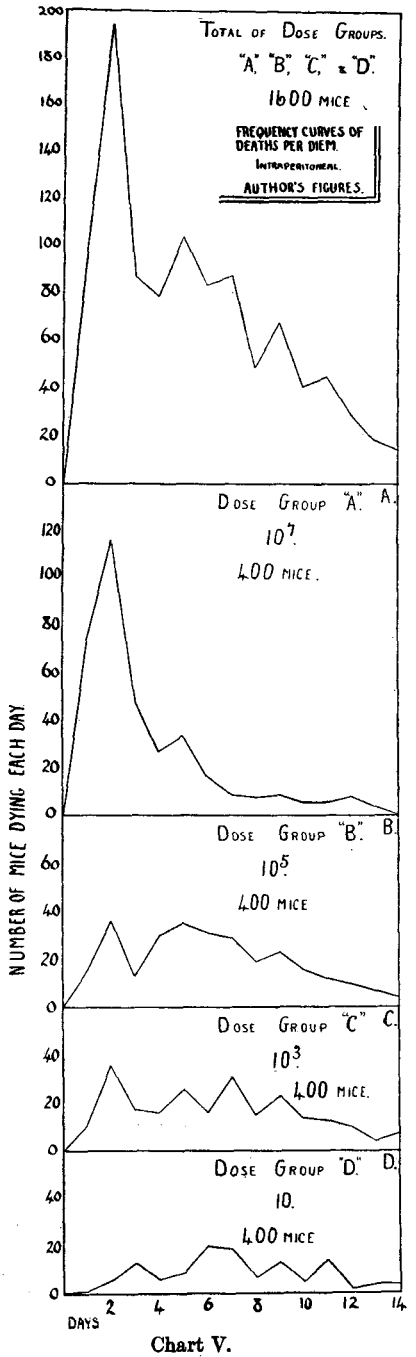

Chart V.

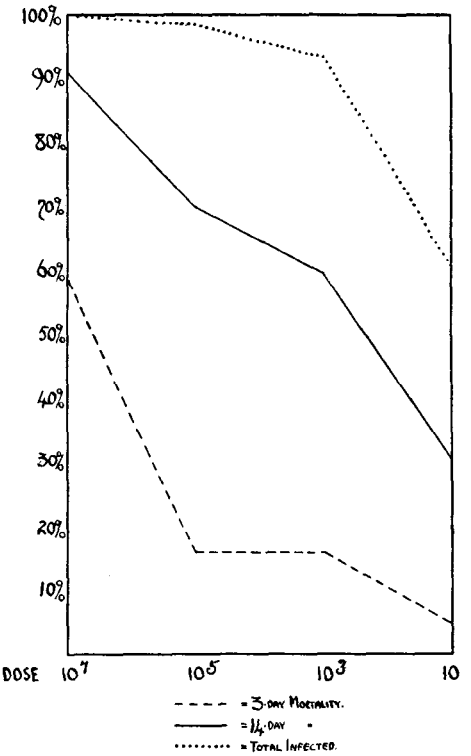

Chart VI. Percentage mortality and percentage infection. Intraperitoneal. Author's own figures.

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Table III. *Intraperitoneal.*

Author's own figures.

Dose	No. of mice at risk	Percentage mortality		Average time to death of mice which died between	
		3rd day	14th day	1st and 14th days	4th and 14th days
10^7	400	58·50	90·75	3·60 days	6·74 days
10^5	400	16·00	70·00	6·16 ,,	7·47 ,,
10^3	400	16·00	59·75	6·36 ,,	7·92 ,,
10	400	5·00	30·75	7·17 ,,	8·06 ,,

We may, then, by the intraperitoneal inoculation of *B. aertrycke* into mice produce two essentially different phenomena. A massive infection, leading to a fatal issue within 24–72 hours, or a subacute infection which when fatal usually results in death between the 4th and 12th days. As regards the second type of infection the size of the initial dose has a relatively slight effect in determining the duration of the fatal illness. As regards the acute type of infection our data do not allow us to draw any accurate conclusions respecting the relation between size of dose and time-to-death, since the observations have been recorded in terms of days, whereas it would be necessary to make records of observations at intervals of a few hours if this relation was to be measured. Our figures suggest that there is a definite association between large dosage and rapid death within the 3-day limit, but the point is of no importance in regard to the main problem under discussion.

In our own series of experiments an additional point has been noted. All mice which died during the 14 days, which was the period adopted for observation, were submitted to a post-mortem examination; cultures being prepared from the heart and spleen; and the organisms recovered were identified as *B. aertrycke* by agglutination. Those mice which remained alive on the 14th day were killed and submitted to a post-mortem examination modified as follows. If any obvious lesions were present the usual bacteriological examination was carried out, but if, as was usually the case, nothing abnormal was found a portion of the spleen was transferred to a tube of nutrient broth, incubated for 48 hours at 37° C. and then if growth were present plated on to McConkey's medium. Any non-lactose fermenting colonies were tested by agglutination. Thus we were able to determine the proportion of surviving mice which were suffering from a latent infection. As was to be expected in the light of earlier observations (Topley, Wilson and Lewis, 1925) this proportion proved to be a high one. A third type of reaction was thus added to the acute fatal infection and the subacute fatal infection, namely infection persisting for 14 days without death of the host. The significance of this type of reaction will be discussed later.

We may discuss here the method we have adopted in presenting our results. As mentioned above, we have recorded three factors in each sample of mice tested, the number dying within 3 days, the number dying within 14 days and the total number infected. We shall give reasons for regarding the second factor as the significant one in comparing the fate of different samples.

Webster has suggested the use of a mortality curve for purposes of such comparisons, in the course of experimental infection carried out *per os*, and he apparently attaches importance to the form of the curve in comparing the results of different experiments.

Neither a study of Webster's records, nor the data afforded by our own experiments, appear to us to afford any evidence in favour of the use of such a curve for this purpose. It is quite certain that, using any number of mice between 20 and 50, the form of the curve will vary widely and in a random manner. It may well be, however, that some significance which has escaped our notice may attach to the actual distribution of death in time, with a given sample of mice. For this reason we have included in this report a series of records which show the day of death of each mouse, for the great majority of the experiments. We have preferred the use of a tabular form of diagram to that of curves, having personal experience of the difficulty which may be encountered in attempting to extract numerical results from reports in which curves without tables are employed. Since, however, the inclusion of these diagrams in the body of the paper has certain disadvantages, we have relegated the majority of them to an appendix, and have retained in the report itself only those in which the results obtained are most conveniently shown in this form. For the rest we have abstracted the most important numerical results and give these in tabular form.

There are a few points with regard to these more complete diagrammatic records which may be mentioned here. A study of these will show that the three variables, 3-day deaths, 14-day deaths and total-infecteds, do not fluctuate in any constant relation with one another. In particular it is quite evident that a strain of *B. aertrycke* which is of very low virulence may persist in the tissues of mice for 14 days or more, giving rise to a latent infection, even when introduced into the tissues in quantities so small as 10 bacilli. There is a suggestion, it can be put no higher, that some strains may be particularly potent in causing a rapidly fatal infection without of necessity causing a high total mortality over the 14-day period.

We feel strongly, however, that to demonstrate any such minor differences within a total number of fatalities, much larger samples of mice would have to be employed.

Variations in Host-resistance.

Before proceeding to a discussion of the results obtained in attempts to measure the virulence of different strains of *B. aertrycke* it is necessary to consider how far our results have been affected by variations in the inherent resistance of the hosts.

It has been noticed that Webster attaches the greatest importance to this factor, believing that it is essential, when comparing the virulence of different bacterial cultures to employ a single strain of in-bred mice.

A careful review of Webster's papers, and those of his collaborators,

suggested, however, that the variations in mortality encountered in testing different samples of mice taken from a single in-bred strain were so large, that the conclusion that significant differences in natural resistance have been demonstrated between different strains of mice could not be accepted without a survey of the data by simple statistical methods.

The very thorough investigations of Webster and his co-workers has provided a large mass of data for analysis of this kind, and we have utilised the figures provided in two recent reports by Pritchett (1925), who has studied, month by month, the mortality following the *per os* administration of a fixed quantity of a broth culture of *B. aertrycke* to samples of mice belonging to five separate in-bred strains, including the strain propagated for so long at the Rockefeller Institute and four other strains, referred to as Bagg, Hagedoorn, Lathrop and Little, originally obtained from other sources but in-bred at the Institute during the experimental period. The investigation was actually carried out over a period of twelve months from September 1923 to September 1924; but as will be seen, on reference to Pritchett's reports, it was not until December 1923 that sufficient mice were available for the full sample of 50 mice (the number employed in each monthly batch) to be tested in the case of each of the five strains. It would appear from the reports that the number forming each individual sample, during the following ten months, varied within narrow limits, the standard number of 50 being sometimes slightly exceeded. In the following analysis it has been assumed that each batch tested was of the same size, but variations of the order indicated in the reports would not affect the significance of the results.

In Table IV we have tabulated the percentage mortality observed in each test throughout the ten months available for analysis, and have calculated the mean and the standard deviation for the ten monthly tests with each strain of mice and for each monthly test with all five strains. It will be found that the standard deviations are considerable, and that except in the case of

Table IV. *Percentage Mortalities.*

Abstracted from papers by Pritchett, I. W. (1925).

Month	Rockefeller Institute mice	Bagg mice	Hagedoorn mice	Lathrop mice	Little mice	Mean	Standard deviation
December	66	64	32	64	88	62·8	17·9
January	78	66	74	76	76	74·0	4·2
February	90	74	96	82	96	87·6	8·52
March	78	68	92	80	86	80·8	7·93
April	58	88	86	78	76	77·2	10·56
May	86	91	100	91	90	91·6	4·59
June	52	48	63	80	70*	62·6	11·68
July	40	44	56	78	62	56·0	13·78
August	64	71*	71	76	80	72·4	5·46
September	80	78*	88	94	92	86·4	6·27
Mean	69·2	69·2	75·8	79·9	81·6		
Standard deviation	15·1	14·3	20	7·86	10·1		

* Figures marked thus are approximate, being given differently on different charts.

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the low mortality observed during the months of June and July the differences separating the values of the means are of the same order as their standard deviations.

In Table V we have tabulated the same series of figures, not as absolute percentage mortalities, but as relative mortalities. The mortality among the Rockefeller mice has been taken as unity for each month in the case of the figures given on the left-hand side of the table, and the July mortality as

Table V. *Relative Mortalities.*

Abstracted from papers by Pritchett, I. W. (1925).

	Taking Rockefeller Mice as Unity					Taking July Mortality as Unity							
Month	Rock. Inst. Mice	Bagg Mice	Haged. Mice	Lath. Mice	Little Mice	Rock. Inst. Mice	Bagg Mice	Haged. Mice	Lath. Mice	Little Mice	Mean	Standard Deviation	Probability
December	1·0	0·97	0·48	0·97	1·33	1·65	1·45	0·57	0·82	1·42	1·18	0·413	0·275
January	1·0	0·85	0·95	0·975	0·975	1·95	1·50	1·32	0·97	1·23	1·39	0·326	0·776
February	1·0	0·82	1·07	0·91	1·07	2·25	1·68	1·71	1·05	1·55	1·65	0·383	0·319
March	1·0	0·87	1·18	1·03	1·10	1·95	1·55	1·64	1·03	1·39	1·51	0·302	0·677
April	1·0	1·52	1·48	1·34	1·31	1·45	2·00	1·54	1·00	1·23	1·44	0·335	1·000
May	1·0	1·06	1·16	1·06	1·05	2·15	2·07	1·79	1·17	1·45	1·73	0·371	0·204
June	1·0	0·92	1·21	1·54	1·35	1·30	1·09	1·125	1·03	1·13	1·13	0·90	0·0024
July	1·0	1·10	1·40	1·95	1·55	1·00	1·00	1·00	1·00	1·00	1·00	0	0·0006
August	1·0	1·11	1·11	1·19	1·25	1·60	1·61	1·27	0·97	1·29	1·35	0·239	0·478
September	1·0	0·97	1·10	1·18	1·15	2·00	1·77	1·57	1·205	1·48	1·60	0·268	0·296
Mean	1	1·02	1·11	1·21	1·21								
Standard deviation	0	0·193	0·257	0·311	0·167								
Probability	—	0·772	0·769	0·494	0·223								

unity for each strain in the case of the figures given on the right-hand side of the table. We have again tabulated the means and standard deviations, which show that the ratio of the standard deviations to the differences between the means of the observed mortalities is, in general, so high that, among all the mean mortalities observed, the differences noted between the average mortalities of all strains for the months of June and July, when compared with the average mortalities for other months, can alone be regarded as significant. We have added to this table a row and a column which give the probabilities[1] that variations from the mean of the monthly- or strain-mortality ratios as great as or greater than those actually observed, will arise as the result of random sampling.

It is clear that the differences in the average strain mortality are in no case significant. The odds against such differences occurring as the result of chance is in no case as much as 4 to 1; in three out of four cases it is less than 2 to 1.

In the case of the differences between the mean monthly mortalities the differences observed in the case of the June and July death rates are alone significant, but with the question of seasonal variation in susceptibility we are not here concerned.

[1] For the calculation of these we are greatly indebted to Miss E. M. Newbold. Calculated from "Student's" Tables for Deviations in The Means of Small Samples, *Biometrika*, XI. pp. 414 et seq.

We are, then, unable to agree with Pritchett's conclusion that "clear-cut differences in the susceptibility of these strains to the infection have been shown to exist." Our own conclusions would be that no significant differences in susceptibility have been demonstrated and that these experiments afford no grounds for the belief that the use of a single in-bred strain of mice for comparative virulence tests will appreciably lessen the chance of error, due to variations in the response of individual hosts to infection, which is inherent in all experiments of this nature. To confirm the conclusions arrived at by this survey of Pritchett's results, a single series of experiments was carried out as follows. From our own normal stock, 180 mice were selected which were obtained from three different breeders, 60 being derived from each source. Each batch of 60 was arbitrarily divided into three batches of 20 and each mouse was inoculated intraperitoneally with 1000 *B. aertrycke*. The results are shown in Diagram I, in the form which has been adopted throughout this report and in Table IX, Series A. The day of death of each mouse which died of *B. aertrycke* infection is recorded, and also the presence or absence of infection in the mice which remained alive on the 14th day.

It will be seen that, although the mean values for the 3-day death rates, 14-day death rates and total-infected rates are different for the three strains of mice they are no greater than the differences existing between similar rates among the batches of any one strain. The test for significance which will be discussed later in this report, when applied to the greatest observed difference between the 14-day mortalities occurring in these three batches of 60 mice gives a value of 2·2. This is well below the value of 3·0 taken as the limit of significance, and hence there is no valid evidence of any inherent difference in resistance to *B. aertrycke* infection.

Throughout the experiments to be recorded in the remainder of this report we have employed mice coming from the breeders referred to above, from two other breeders and from the strain which has been bred in this Department during the last three years. We have noted in our records all cases in which mice from different sources have been used during a single large comparative experiment, and have distributed them so that all the mice of any individual sample were derived from one source.

Where the mortality among a series of samples receiving one strain of *B. aertrycke* has been compared with the mortality among another series of samples receiving another strain of this organism, we have included in each series the same number of samples derived from any one breeder. The results of such tests have, in all cases, confirmed our conclusion that no significant differences in resistance can be observed between the different strains of mice.

We should then, until fresh evidence pointing in another direction is brought forward, regard differences of strain of any of the mice employed in such experiments as a point of minor importance, provided of course that they have not been selectively bred from resistant or susceptible mice, and

do not come of an infected stock in which acquired or selective resistance may have been developed.

We may add that we have used throughout young adult mice weighing between 17 and 22 grammes.

KEY TO DIAGRAMS.

5	= Day of death.
■	= Survived but infected.
†	= Died not infected.
	= Survived not infected.

= Outline dividing acute from chronic death.

= Outline dividing survivors and non-infected dead from infected dead.

D^3 = Those dying acute death.

D^{14} = Those dying chronic death.

I. = Total number infected.

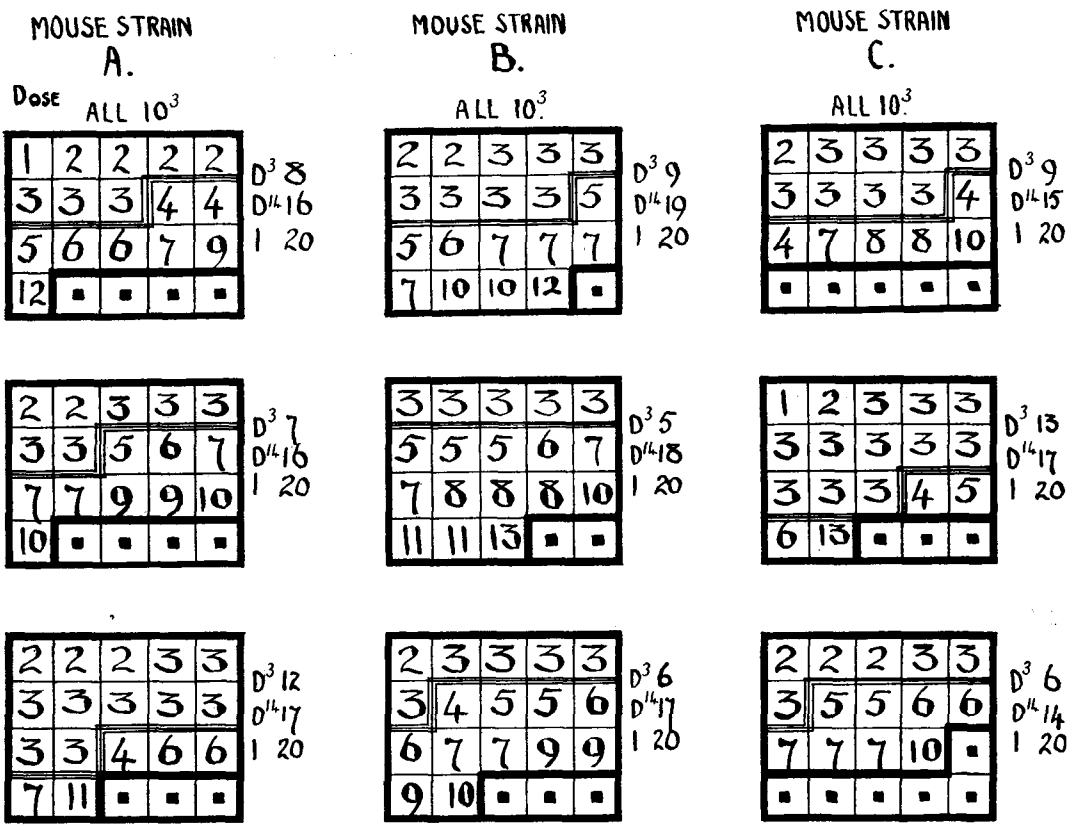

Diagram I.

The Minimal Lethal Dose.

The methods which have been adopted in carrying out the intraperitoneal inoculations and in observing and recording the fate of the inoculated mice have already been indicated in sufficient detail and we may proceed to

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consider, on the basis of the results obtained, the possibility of measuring the virulence of a given strain of *B. aertrycke*, in relation to the number of mice available for the test, and the degree of approximation to truth which may be expected to result under the limitation of number imposed.

We may first discuss the method of determining the minimal lethal dose, and expressing the virulence as the reciprocal of this value. Certain aspects of the problem have already been considered and we will now submit the matter to more detailed analysis. The term "minimal lethal dose" (M. L. D.) is itself sufficiently vague. It is commonly defined as the smallest dose of a given poison, toxin or bacterial culture which will kill an animal of a given species, and of a given weight, within a fixed time interval. The obviously false assumption that all animals of that weight and species will react similarly to the particular material under test is implied in the definition, but is disregarded to a very varying extent in practice. No standard number of test-animals, nor any standard mortality rate has, however, been laid down for the determination of the M. L. D.

From the definition it might be assumed that one M. L. D. should produce 100 per cent. mortality among samples of animals inoculated with that dose, but we know that in such a case a smaller dose would have been sufficient to kill many of them. A perusal of any considerable number of protocols shows that, in practice, the M. L. D. has been taken as that dose of the material under test which kills the majority of the animals inoculated with that dose within the standard time-interval. The criteria employed have been altogether loose and the number of animals inoculated with any one dose has usually been less than ten, often only two, so that little statistical significance could in any case attach to the actual proportion of deaths observed.

There has, in fact, been another assumption underlying the employment of this technique. It has been assumed, perhaps unconsciously in many cases, that the ratio of decrease in the death rate to decrease in the dose is high, so that a relatively small decrease in dose will cover the range over which the death rate changes from 100 per cent. to zero. The dose which kills *all* test animals, under the standard conditions, is assumed to be a low multiple of the dose which kills *none*.

There are not many protocols to be found in the literature which will enable us to test how far this assumption is valid in particular cases, but we may quote two examples.

The first (Craw and Dean, 1907) concerns the titration of diphtheria toxin, and is reproduced in Table VI.

It will be seen that a reduction in dose of toxin from 0·8 to 0·4 c.c. reduces the mortality from 100 per cent. to zero. Over the middle of this range a reduction in dose from 0·65 to 0·5 c.c. reduces the mortality from 70 to 13 per cent. If we take a M. L. D. to represent the dose which will kill an *average* animal under the standard conditions, and assume this to be equivalent to the dose which will kill *half a given sample* of such animals, selected at

Table VI. *Free Diphtheria toxin estimation.*

Craw, J. A. and Dean, G. (1907).

C.c. of toxin	No. of guinea-pigs	Survivors	
		Actual	Percentage
1·2 –0·8	10	0	0
0·8 –0·75	15	1	6·6
0·75–0·7	33	5	15·0
0·7 –0·65	20	6	30·0
0·65–0·6	32	17	53·0
0·6 –0·5	16	14	87·0
0·5 –0·4	10	10	100·0
0·4 –0·3	8	8	100·0
0·3 –0·2	5	4	80·0

random, we can clearly estimate the M.L.D. from such a protocol as that given in the table with a fair approximation to truth.

The second example is concerned with the standardisation of dysentery toxin (Sudmersen, Runge and O'Brien, 1924) and is reproduced in Table VII.

Table VII. *Anti-dysentery (Shiga) serum. Standardisation.*

Sudmersen, H. J., Runge, B. F. and O'Brien, R. A. (1924).

Mgm. of toxin	No. of guinea-pigs dying	No. living	Percentage death-rate
0·1	1	0	100
0·05	1	0	100
0·04	6	0	100
0·03	5	1	83
0·025	13	3	81
0·02	13	3	81
0·015	8	6	57
0·01	9	9	50
0·0075	5	7	42
0·005	4	12	25
0·0025	0	8	0

The range in dosage covered by a decrease in mortality from 100 per cent. to zero is here 0·05 to 0·0025 c.c.: while halving the dose of diphtheria toxin produces this 100 per cent. fall in mortality, the dose of dysentery toxin must be reduced to $\frac{1}{20}$th to produce the same result. Over the middle of this range the fall of mortality with fall of dose is more rapid, a decrease in dose from 0·02 to 0·0075 c.c., a threefold decrease, reduces the mortality from 81 to 42 per cent., but clearly the approximation to truth, in determining the M.L.D. of a specimen of dysentery toxin, will be far less close than in the case of a diphtheria toxin, assuming that the same number of test animals be used in each case. By employing an adequate number of animals it would, of course, be theoretically possible to determine the actual percentage mortality with any given dose to any required degree of approximation, but this takes us outside the region of practical laboratory politics.

We may now consider the results obtained during the present investigation, in the intraperitoneal inoculation of 1600 mice with graded doses of living suspensions of *B. aertrycke*. Four doses only were administered, containing 10^7, 10^5, 10^3 and 10 bacilli respectively, 400 mice being inoculated with each dose (see Table III).

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Several strains of *B. aertrycke* were employed but in each case equal numbers of mice were inoculated with each dose so that the results may be added together.

Taking as our standard result deaths within 14 days we find that by decreasing the dose from 10^7 to 10, that is one million times, we decrease the percentage death rate from 91 to 31. The slope of the curve appears to be slightly steeper towards either extremity than in the middle of its range, but this irregularity in slope may well be a chance result of this particular series of tests, and the exact form of the curve is in any case unimportant from our present point of view. It needs no statistical analysis to see that the ratio of the probable error of the percentage mortality, observed for any given dose, to the difference to be expected between mortality with that dose and with doses one hundred times larger or smaller, will be large, unless very great numbers of mice are used in testing each dose. There is clearly no practical possibility of determining to any useful degree of approximation the dose which kills any given percentage of mice within 14 days.

In regard to the 3-day deaths the case is a little better, since the death-rate curve slopes somewhat more steeply between the dose values 10^7 and 10^5, but it is bad enough. This hundredfold decrease in dose gives a decrease in mortality from 58·5 to 16 per cent. and the above remarks need little modification. Moreover, the power of *B. aertrycke* to produce this rapidly fatal effect is not the character which we particularly desire to measure, in trying to estimate the potentiality of a given strain to produce fatal infection in mice under natural conditions.

It may be stated with some confidence that the M. L. D. of a strain of *B. aertrycke*, using the mouse as host, cannot be determined under the limitations imposed by practical laboratory technique, and that this measure cannot therefore be employed in estimating virulence.

In considering the general problem of comparing the virulence of two strains of a given bacterium, it was suggested above that as an alternative to comparison in terms of the M. L. D. we could compare the virulence in terms of the relative mortality rates with a single fixed dose. We could clearly employ a fixed series of graded doses for the same purpose, measuring in each case the total death rate among two equal samples of mice over a fixed time interval.

The essential point of such a method is that all the mice used in testing the virulence of a given strain are treated as a single group and, hence, with a given number of mice the statistical significance of the proportion of deaths observed is greater than would be the significance of the mortality observed in any one of the small sub-groups, which would be employed in testing a series of falling doses, in an attempt to determine the M. L. D. by the latter method. We cannot hope in this case, more than in the other, to obtain a result which will enable us to say that the virulence of one strain is so many times greater than that of another without using an impossible number of

mice. The problem to be solved is whether or no, by using a reasonable number of test animals, we can determine that one strain is significantly more or less virulent than another.

We may first consider the problem from the purely statistical point of view.

Let us assume that we employ two samples, each containing n mice, and that we administer to each mouse of one sample a given dose of bacterial emulsion A, and that to each mouse of the other sample we give the same dose of emulsion B, and that the observed deaths, over a fixed time interval number a in one case and b in the other, then the observed difference in the number of deaths is $a-b$, and the mean mortality rate is $\frac{a+b}{2n}$. Since we have no other criterion we must take this as representing the true chance of dying on the further assumption that there is no effective difference in the samples or strains, and that the distribution of deaths is due to chance. We may then put $p_0 = \frac{a+b}{2n}$, where p_0 is the chance of dying, and $q_0 = 1 - p_0$ where q_0 is the chance of survival.

The standard deviation (σ) of the difference $a - b$ will be given by

$$\sigma = \sqrt{2n p_0 q_0}$$

and the ratio of the observed difference to this standard deviation by

$$\frac{a-b}{\sqrt{2n p_0 q_0}}.$$

Using the standard deviation in place of the probable error, which is equal to the standard deviation multiplied by 0·67449, the odds against a difference as great or greater than three times this value being observed as the result of random sampling is 369 to 1. This figure does not, however, hold if the sample is small, or if the mean chance of death $\left(\frac{a+b}{2n}\right)$ is in the near neighbourhood of 1 or 0 (Greenwood, 1913). By adopting the more stringent criterion of three times the standard deviation instead of three times the probable error, the chance of the observed differences being arrived at by errors of random sampling is very small.

For the purposes which we have in view it would be necessary to determine the relative virulence of large numbers of strains of *B. aertrycke*, and a method therefore which involved the use of a hundred or of fifty mice for each virulence test would be of small practical value.

We have, therefore, arbitrarily taken twenty mice as the number for each test sample. Since the standard deviation is proportional to the square root of the number of test animals the use of twenty-five or thirty mice would make relatively little difference in the significance of the result, while it would greatly increase the expenditure of mice over a large number of tests.

Table VIII shows the ratio of the observed difference to the standard deviation for all possible combinations of deaths in the two samples, which

Table VIII. *To show the ratio of the Observed Difference to the Standard Deviation for deaths in two samples each containing 20 mice.*

Deaths in the second sample	Deaths in the first sample																					
	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1	0
20	—	—	—	—	—	2·39	2·66	2·91	3·16	3·41	3·65	3·90	4·14	4·39	4·64	4·90	5·16	5·44	5·72	6·02	6·33
19	—	—	—	—	—	—	—	2·37	2·65	2·92	3·19	3·45	3·71	3·98	4·25	4·52	4·80	5·08	5·38	5·69	6·02
18	—	—	—	—	—	—	—	—	2·19	2·48	2·76	3·04	3·31	3·59	3·87	4·16	4·45	4·75	5·06	5·38	5·72
17	—	—	—	—	—	—	—	—	—	2·07	2·36	2·65	2·94	3·23	3·52	3·81	4·12	4·43	4·75	5·08	5·44
16	—	—	—	—	—	—	—	—	—	—	1·99	2·29	2·58	2·88	3·18	3·48	3·79	4·12	4·45	4·80	5·16
15	—	—	—	—	—	—	—	—	—	—	1·63	1·94	2·24	2·54	2·85	3·16	3·48	3·81	4·16	4·52	4·90
14	—	—	—	—	—	—	—	—	—	—	—	—	1·91	2·22	2·53	2·85	3·18	3·52	3·87	4·25	4·64
13	—	—	—	—	—	—	—	—	—	—	—	—	—	1·90	2·22	2·54	2·88	3·23	3·59	3·98	4·39
12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·91	2·24	2·58	2·94	3·31	3·71	4·14
11	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·94	2·29	2·65	3·04	3·45	3·90
10	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1·63	1·99	2·36	2·76	3·19	3·65
9	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·07	2·48	2·92	3·41
8	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·19	2·65	3·16
7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·37	2·91
6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·66
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	2·39
4	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

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give results approximating to significance. Since the samples are small it will be wise to regard no difference which is less than three times the standard deviation as definitely significant. Values for many possible observed differences which are well below this standard of significance have been omitted.

It will be seen from the table that, if we actually observe 15 deaths in one sample of twenty mice and 10 in another the observed difference is only 1·63 times its standard deviation, and may well have resulted from the play of chance and random sampling. If, however, we find 15 mice die in one sample of twenty and 5 in another the observed difference is 3·16 times its standard deviation, and is very unlikely to be the result of chance, assuming that the usual conditions required for statistical comparisons are fulfilled.

In the course of the present investigation we have performed four series of experiments the results of which may be tested from this point of view and which are summarised in Table IX. In one experiment already referred to

Table IX. *Four series of experiments.* 20 *mice in each sample. Each mouse being inoculated intraperitoneally with* 10^3 B. aertrycke.

All mice in each series received the same culture.

Series	No. of samples	Sample No.	3-day deaths	14-day deaths	Total infected
	9	1	8	16	20
		2	7	16	20
		3	12	17	20
A		4	9	19	20
See also		5	5	18	20
Diagram I		6	6	17	20
		7	9	15	20
		8	13	17	20
		9	6	14	20
	5	1	6	19	20
B		2	4	20	20
See also		3	6	19	20
Diagram A^{II}		4	5	20	20
		5	8	19	20
	5	1	1	5	16
C		2	1	2	16
See also		3	1	4	11
Diagram B^{II}		4	1	3	13
		5	1	5	16
	5	1	10	18	20
D		2	4	17	20
See also		3	3	13	20
Diagram C^{II}		4	9	16	20
		5	3	13	20

(Series A) see Diagram I, nine samples, each of 20 mice, were inoculated with the same dose of the same strain of *B. aertrycke*, and we have stated our reasons for accepting the 180 mice as a homogeneous sample. Taking the 14-day deaths, the most important test, we find our greatest difference in mortality is that between 19 and 14 deaths a difference which on reference to Table VIII is seen not to be significant. Taking the 3-day deaths the greatest difference lies between 5 and 13, a difference which closely approaches the limit of significance. Taking the total-infected rate the observed difference is zero.

Three similar series of tests are recorded in Table IX, Series B, C and D, and in Diagrams A^{II}, B^{II} and C^{II} in the Appendix. In all these five samples each mouse received a dose of 10^3 *B. aertrycke*. Taking the 14-day deaths the greatest differences between different samples, in each of the three series, are between 19 and 20 deaths, 5 and 2 deaths and 18 and 13 deaths respectively. Reference to Table VIII shows that none of these differences is significant. For 3-day deaths the greatest differences are between 8 and 4 deaths, 10 and 3 deaths and zero; again none of these is significant. For the total-infecteds the greatest difference is that between 16 and 11 in Series C, the remaining series showing no difference. We find then that the results obtained by actual experiment indicate that the conclusions based on statistical consideration are borne out in practice. In no case does the observed difference in mortality between two samples of twenty normal mice, each inoculated with the same dose of the same strain of *B. aertrycke*, exceed the value, which, on statistical grounds, we should regard as significant.

In the case of the 14-day deaths this value is never approached. In the case of the 3-day deaths it is closely approached in one instance, a point we shall refer to later. In the case of the total number of mice infected no difference approaching significance is observed.

We have stated that we might replace the single dose administered to each sample of twenty mice by a series of graded doses. We might, for instance, give five mice of each sample 10^7, five 10^5, five 10^3 and five mice 10 bacilli. The statistical conditions are altered, but need not be considered here, since the standard deviation is in this case slightly less than that given by the formula considered above and the test for significance given in Table VIII will therefore hold.

The results of the tests will, in this case, be weighted by the fact that, with a strain of average virulence the five mice receiving the largest dose will almost certainly die, and the five mice receiving the smallest dose will probably live. We might, *a priori*, expect that tests carried out in this way would give somewhat more consistent results than tests performed by inoculating each mouse of each sample with the same dose of 10^3 bacilli. The best criterion of the relative merits of the two methods is, however, actual experiment.

Seven series of tests are available for discussion, the results of which are recorded in Table X and Diagrams D^{I-II}, E^{I-II}, A^{I}, B^{I}, C^{I} in the Appendix.

In the first two series (A and B) six samples, each of twenty mice, were given four graded doses of the same strain of *B. aertrycke*, containing 10^7, 10^5 10^3 and 10 bacilli respectively. In the remaining five series (C, D, E, F and G) five samples each of twenty mice were employed.

In the case of the 14-day deaths the greatest observed differences are between 20 and 18 deaths, 7 and 3, 18 and 13, 10 and 6, 17 and 14, 12 and 8, and 17 and 10 deaths. Reference to Table VIII shows none of these results to be significant. For the 3-day deaths the greatest differences are between

10 and 6 deaths, 1 and 0, 9 and 5, 5 and 3, 7 and 5, 4 and 1, and 5 and 2. Again no difference is significant. As regards the total-infected mice the greatest differences are those between 20 and 18, 19 and 13, 20 and 19, 18 and 14, 18 and 17, 19 and 15, and 19 and 16. None of these differences is significant.

Table X. *Seven series of experiments. 20 mice in each sample. Each sample receiving the same set of graded doses, given intraperitoneally, of* B. aertrycke.

All mice in each series received the same culture.

Series	No. of samples	Sample No.	3-day deaths	14-day deaths	Total infected
A See also Diagram D^{I}	6	1	4	10	16
		2	5	8	15
		3	5	9	18
		4	3	10	15
		5	3	6	14
		6	3	10	17
B See also Diagram D^{II}	6	1	7	17	18
		2	5	16	18
		3	6	14	18
		4	5	15	17
		5	7	14	18
		6	6	16	18
C See also Diagram E^{I}	5	1	3	12	19
		2	1	9	17
		3	4	11	18
		4	1	8	15
		5	2	10	16
D See also Diagram E^{II}	5	1	3	17	18
		2	5	10	16
		3	2	15	19
		4	3	11	19
		5	3	13	16
E See also Diagram A^{I}	5	1	7	20	20
		2	10	19	19
		3	6	18	20
		4	8	18	19
		5	7	19	20
F See also Diagram B^{I}	5	1	1	3	16
		2	1	6	13
		3	0	5	16
		4	1	4	19
		5	0	7	17
G See also Diagram C^{I}	5	1	5	14	20
		2	9	13	19
		3	7	13	20
		4	8	14	19
		5	8	18	19

Comparing these two methods, then, there would appear to be little to choose between them. We have, for the purpose of the further experiments recorded in this report, adopted the method of graded doses since it seemed possible that differences in the mode of action of different strains might be recorded in this way, which would escape observation by the single-dose method. We are not, however, convinced that it offers any particular advantages.

We may note that the figures for the 3-day deaths appear to be subject to occasional variations, though these are exceptional, when the same culture

was tested on different samples of mice by the graded dose method, but in one instance only did the difference approach significance.

These results are not surprising. *B. aertrycke* kills mice under normal conditions by inducing a subacute fatal infection, even though a second type of infection of a rapidly fatal septicaemic form can be shown to occur. The factors which determine the type of infection which results in any given instance may well be of a different kind from those which determine death or recovery. It is possible, for instance, that factors connected with mechanical injury during inoculation may play a part. In any event the arbitrary limit of three days cannot be expected to correspond to a sharp natural division between the two types of infection, and the only logical time limit to adopt would appear to be that within which the full lethal power of the parasite has an opportunity to declare itself. For all practical purposes, as we have shown in the frequency curves, the 14-day limit fulfils these conditions. We may note that Webster has, in his many experiments, adopted the method of measuring virulence by inoculating each individual of a given sample of mice with the same dose, using the intrastomachal route and observing the resulting percentage mortality during a fixed time-interval.

THE VIRULENCE OF DIFFERENT STRAINS OF *B. AERTRYCKE*.

Using the method we have outlined above we have compared the virulence of different strains of *B. aertrycke*. The first comparison was made between strain A 52, one of our own stock strains derived from a mouse dying of *B. aertrycke* infection, and strain "Ellinger" which was kindly forwarded to us by Professor Neufeld from the Robert Koch Institute. We should like to express our thanks to Prof. Neufeld for his kindness in sending it to us. The result of this first comparison is recorded in Table XI and Diagram F (Appendix). Here and elsewhere the results as regards 3-day deaths, 14-day deaths and total-infecteds are recorded in the diagram, but we shall confine our attention mainly to the 14-day deaths, and test the significance of the results by reference to Table VIII.

Table XI. *Comparisons of virulence of strain "Ellinger" and strain* A 52 *in terms of the fourteen-day mortality.*

Date	No. of mice in each sample	14-day mortality "Ellinger" Total	"Ellinger" Percentage	A 52 Total	A 52 Percentage	Ratio of observed difference to its standard deviation
12 xi. 24	20	20	100	10	50	3·65
5 xii. 24	20	19	95	10	50	3·19
12 xii. 24	120	92	76·7	53	44·17	5·14
27 i. 25	100	66	66	50	50	2·29
26 v. 25	20	13	65	4	20	2·95
10 vi. 25	20	7	35	5	25	—

In this first comparison, carried out on November 12th 1924, the Ellinger strain gave 20 deaths and the A 52 strain 10 deaths. The difference in favour

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of the Ellinger strain is significant. Thereafter those two strains were sub-cultured daily, from broth to broth, and comparative tests were carried out at irregular intervals, subcultures being made to agar slopes and dealt with as described above.

The strains were retested on December 5th, 1924, the Ellinger strain giving 19 deaths and the A 52, 10 deaths; again the difference in favour of the Ellinger strain is significant. On December 12th the strains were tested on 120 mice apiece (Table XI and Diagram D). Each 120 mice were arbitrarily divided into six groups of twenty mice, in order to test the standard deviation of the means observed in the different groups, but for the present purpose each 120 may be regarded as a single group. The deaths were 92 with the Ellinger and 53 with the A 52 strain. The ratio of the difference to its standard deviations, given by the formula $\frac{a-b}{\sqrt{2npq}}$ is 5·14, which difference is therefore significant.

The two strains were retested on January 27th 1925, 100 mice being used for each strain (Table XI and Diagram E). The mice were grouped as before, and again may be treated as one sample.

The Ellinger strain gave 66 deaths, the A 52 strain 50. These figures, tested on the formula just given, show a ratio to the standard deviation of 2·29 which is not significant. It will, in fact, be noted that the percentage difference observed in the previous test was less marked than that observed in the first two tests performed (Diagram F). In fact this difference steadily fell during the progress of the first four tests shown in Table XI. It is probable that the continued fall would not have been detected if the two later tests had not been concerned with such large samples. It will further be noted that the decrease in the difference of the virulence was due to a loss of virulence on the part of the Ellinger strain, the A 52 strain remaining more or less constant.

The strains were compared again on May 26th, 1925, samples of 20 being employed. The Ellinger strain gave 13 deaths, the A 52 strain 4 deaths. The difference in favour of Ellinger is just below the limit of significance. The great decrease in the virulence of the A 52 strain will be noticed and will be referred to later.

The last comparative test was carried out on June 10th, 1925, samples of 20 mice being employed. The Ellinger strain gave 7 deaths, the A 52 strain 5 deaths. Here we have definitely no significant difference. The virulence of the Ellinger strain had sunk to the level of the A 52.

It would appear that these two strains of *B. aertrycke*, coming from different laboratories and with an entirely independent history, presented at the time of the first comparative test significantly differing degrees of virulence. On this point, then, our results are in accord with those of Webster (1923 *a*), who found that strains of *B. aertrycke* which came from different sources showed different degrees of virulence.

It would not, however, appear that the different degrees of virulence are

inherent characters of these strains, since, as the result of repeated subculture, they both suffered a loss of virulence which abolished the distinction between them in this respect. We shall, however, return to this point again. With the exception of the Ellinger strain all the strains tested during the course of the investigation had a common history. They were all derived from mice dying during the epidemics which Topley and his colleagues have studied during the past seven years, and from mice experimentally infected by various routes dying during the course of subsidiary investigations. They have all been derived, many of them by devious paths, from the original strain isolated in 1918.

There are not many experiments available for considering the differences in virulence between such strains, but we may give a few instances, confining ourselves as before to the 14-day deaths.

The first series of tests, recorded in Table XII, have been carried out by Topley and his collaborators during the last six months in connection with various experiments not yet recorded. It will be seen that, judged by the usual statistical method, the extreme differences are clearly significant, and several other differences closely approach the standard of significance.

Table XII. *Results of virulence tests on different strains of* B. aertrycke, *each from a common source but with a different subsequent history.*

Each strain tested by intraperitoneal inoculation of 20 mice with graded doses containing 10^7, 10^5, 10^3 and 10 bacilli.

Strain	Recent history	14-day mortality
1	Isolated from heart's blood of a mouse dying during an epidemic ...	20
2	Isolated from faeces of mouse which had been fed with *B. aertrycke* ...	20
3	Isolated from heart's blood of a mouse dying during an epidemic ...	19
4	Isolated from faeces of mouse fed with *B. aertrycke*	19
5	Isolated from faeces in similar manner to strain 4	18
6	Isolated from heart's blood of mouse dying during an epidemic	16
7	Isolated from faeces of mouse fed with *B. aertrycke*	12
8	Isolated from faeces in similar manner to strain 7	11
9	Laboratory strain isolated about 12 months ago from heart's blood of a mouse dying after being fed with *B. aertrycke*. Since then occasionally subcultured in agar stabs	10

As regards the present investigation there are three experiments (Table IX, B, C and D) and Diagrams A, B, and C (Appendix), in which samples of 100 mice were tested with three different laboratory strains of *B. aertrycke*. Strain A 123 gave 94 deaths, strain A 52 gave 25 deaths and strain A 60 gave 72 deaths. The differences between A 52 and the other two strains are clearly significant, but A 52 had by this time lost much of its original virulence as the result of day to day subculture. As regards the difference between the deaths caused by strains A 123 and A 60, the ratio of the difference to its standard deviation is 4·14, which is a significant figure.

Thus we see that different strains of *B. aertrycke*, coming from a common source within a period of a few years but with a different history as regards their contact with a series of normal hosts, may show significant variations in virulence.

Loss of Virulence with continued Subculture in Broth.

The strain of *B. aertrycke* A 52 which has been repeatedly tested throughout the course of this investigation, has been subcultured daily from broth to broth during the whole period, incubation being carried out at 22° C. It may be added that there has never been the slightest indication of the occurrence of a rough variant; on all media the strain has been consistently smooth.

In Table XIII the results are given of the tests on this strain carried out on different dates, the 14-day deaths alone being tabulated.

Table XIII. *Repeated determinations of the virulence of strain* A 52 *during the period of daily subculture in broth.*

Date	No. of mice in each sample	Total deaths in 14 days	Percentage deaths in 14 days
12 xi. 24	20	10	50
5 xii. 24	20	10	50
12 xii. 24	120	53	44·17
27 i. 25	100	50	50
4 ii. 25	20	12	60
23 iii. 25	20	5	25
29 iv. 25	20	6	30
5 v. 25	100	25	25
26 v. 25	20	4	20
10 vi. 25	20	5	25

It will be noticed that the differences observed between the individual samples of twenty mice are below the limit of significance, but their cumulative effect is undoubted. Moreover the difference between the samples of 120 and 100 mice tested on 12 xii. 24 and 27 i. 25, and the sample of 100 tested on 5 v. 25, is significant, the ratio of the observed difference to its standard deviation in the latter case being 3·8.

It will be observed that the loss of virulence was not gradual but sudden. From 12 xi. 24 to 4 ii. 25 there was no significant variation. Between 4 ii. 25 and 23 iii. 25 the virulence dropped about half its former value, as judged by the percentage mortality among a given sample of mice. From 23 iii. 25 to 10 vi. 25 the virulence remained constant at its new level. There is, therefore, no need in this case to discuss the effect of any possible seasonal variation in host resistance, to which reference has been made in considering Pritchett's results, and this question may be left to some future report.

Table XI gives similar information for the Ellinger strain. There can be no doubt as to the loss of virulence. It would appear to have been more gradual and constant than in the case of A 52, but the Ellinger series does not contain a sufficient number of samples for us to be certain on this point. We do not think the seasonal variation of resistance hypothesis can bear much relation to this matter, since in the June 1925 our A 60 strain killed 72 per cent. of the mice into which it was inoculated.

It may fairly be concluded from two series of experiments that strains of *B. aertrycke* may undergo a marked decrease in virulence as the result of

repeated subculture on ordinary laboratory media, this decrease occurring suddenly in some cases, and perhaps more gradually in others. This loss of virulence is not associated with any other observed change in the character of the bacterium, such as that from the smooth to the rough condition. The decrease in virulence is not, indeed, of that striking order, from high virulence to almost complete avirulence, which has been shown to be associated with the change from smoothness to roughness (Topley and Ayrton, 1924).

It would seem to follow that the difference in virulence between two such strains as "Ellinger" and A 52 is not due to any inherent characteristics, but is an expression of the environmental history of the particular series of strains. On this point, then, we should differ fundamentally from Webster (1923 *a*), who was unable to demonstrate any loss of virulence as the result of prolonged subculture in the laboratory.

INCREASE OF VIRULENCE AS THE RESULT OF PASSAGE.

We have seen that different strains of *B. aertrycke* derived within recent years from a common source but differing in their subsequent history, show significant differences in their virulence for the mouse, as tested by intraperitoneal inoculation. It is clearly desirable to determine whether or no it is possible to raise the virulence of any given strain by the usual method of animal passage. Webster, as we have seen, failed to produce such a result during an extensive series of experiments.

It will be generally admitted that the assumption underlying all passage experiments is that we are proceeding by a process of selection. We inoculate into a given animal a large number of bacteria, which we assume to vary continuously or discontinuously in that character which we call virulence. We may recover from that test animal, at death or before, those bacteria which have been able to multiply and maintain themselves in the tissues of the host. Each new passage will, theoretically, form a new step in the selective process, and finally we hope to isolate those bacteria which are best fitted to maintain themselves in the tissues of the host, and produce those changes which will eventually result in the death of that host. It is not easy, *a priori*, to determine the particular method of passage which is best calculated to assist this selective process. If we inoculate a series of mice with a fixed dose of *B. aertrycke*, ought we to perform the next passage from the tissues of the first mouse which dies, or from a mouse dying at some later date? It might be argued that in the former case we should be carrying on that strain of the bacterium which had proved itself most capable of producing a rapidly fatal infection; but it might also be argued with equal force, that the rapid death might be due to some adventitious cause and that, in any case, the strain would not have been subjected to the selective action of the tissues of the host to the same extent as in the case of an animal which had survived several days.

It appears to us that there are, in reality, no data to justify any *a priori*

hypothesis, and that experiment alone will give an answer. It may, however, reasonably be urged that it is not justifiable to assume that the same process which will exalt the virulence of such an organism as the *Pneumococcus* will necessarily exalt the virulence of *B. aertrycke*, or that, because it fails to

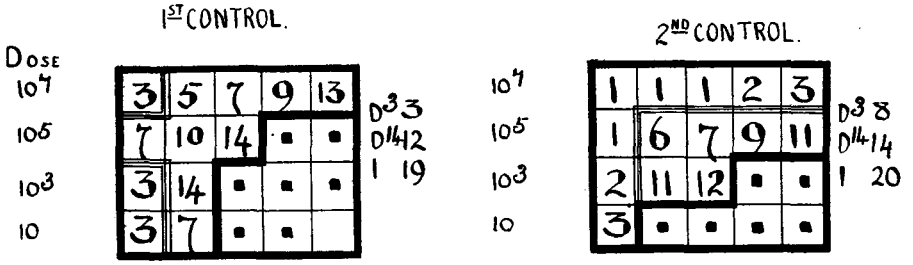

Diagram II. Passage.

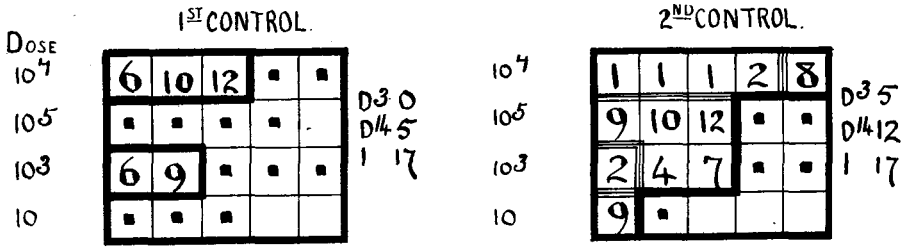

Diagram III. Passage.

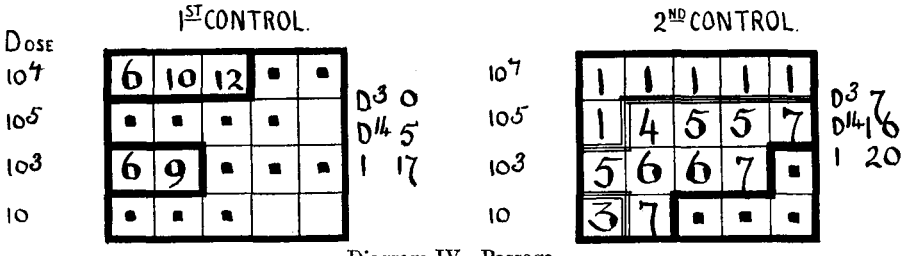

Diagram IV. Passage.

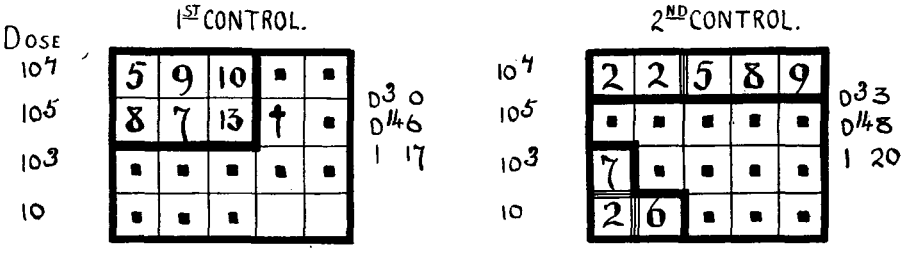

Diagram V.

do so, *B. aertrycke* is an organism where virulence is a constant which is unchangeable.

We have performed a number of passage experiments with the following results. The general course of the experiments are recorded in Diagrams II,

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III, IV, V and VI. The virulence test on the culture which formed the starting point of each series is given in the usual form, the particular series of passages employed is briefly indicated, and the virulence test on the final culture is shown in the same form as the first.

The results of the passage experiments are given below and cross references made to the protocols in Diagrams II, III, IV, V and VI.

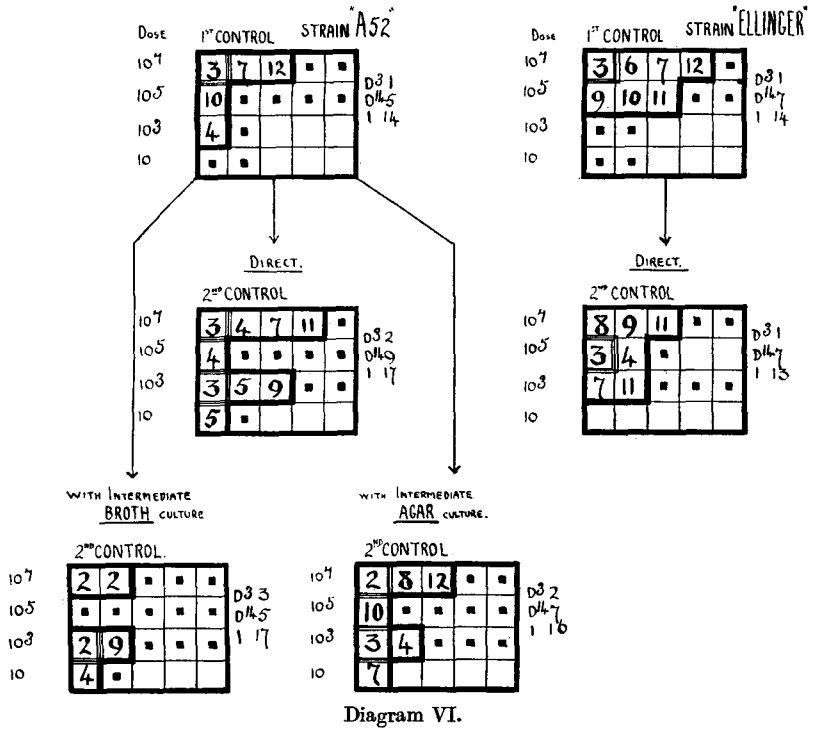

Diagram VI.

PASSAGE EXPERIMENTS.

27 i. 25. *Passage Experiment I. See Diagram II.*

Passage (1). 20 mice were inoculated with graded doses (10^7–10) of a strain A 52 from an agar culture from the heart blood of a mouse of Control I (see diagram), dying on the 7th day.

Passage (2). 20 mice were similarly inoculated from a mouse of Passage (1), dying on the 5th day.

Passage (3). 6 mice were inoculated with a suspension of heart's blood direct from a mouse of Passage (2), dying on the 8th day. 2 mice receiving blood diluted with Ringer's solution 1 in 1, 2 mice blood diluted 1 in 200, 2 mice receiving blood diluted 1 in 20,000.

Passage (4). 6 mice were inoculated similarly to Passage (3), from a mouse in that series, dying on the 7th day.

Control II. 20 mice were inoculated with graded doses (10^7–10) from an agar culture from the heart blood of a mouse in Passage (4), dying on the 1st day.

Control I gave 12 14-day deaths, and Control II 14 14-day deaths. There was therefore no significant alteration in virulence as a result of this experiment.

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23 iii. 25. *Passage Experiment II. See Diagram III.*

Passage (1). 10 mice were inoculated with a dose of 10^3 of strain A 52 from a broth culture, Control I being inoculated at the same time from the same inoculum.

Passage (2). 10 mice were inoculated with a dose of 10^3 from an agar culture from the heart blood of a mouse in Passage (1), dying on the 4th day.

Passage (3). 10 mice were inoculated similarly from a mouse in Passage (2), dying on the 7th day.

Passage (4). 10 mice were inoculated similarly from a mouse in Passage (3), dying on the 2nd day.

Passage (5). 10 mice were inoculated similarly from a mouse in Passage (4), dying on the 4th day.

Passage (6). 10 mice were inoculated similarly from a mouse in Passage (5), dying on the 6th day.

Passage (7). 10 mice were inoculated similarly from a mouse in Passage (6), dying on the 8th day.

Passage (8). 10 mice were inoculated similarly from a mouse in Passage (7), dying on the 10th day.

Passage (9). 10 mice were inoculated similarly from a mouse in Passage (8), dying on the 10th day.

Passage (10). 10 mice were inoculated similarly from a mouse in Passage (9), dying on the 11th day.

Control II. 20 mice were inoculated with graded doses (10^7–10) from an agar culture from the heart blood of a mouse in Passage (10), dying on the 8th day.

Control I gave 5 14-day deaths, and Control II 12 14-day deaths. Reference to Table VIII shows the ratio of this difference to its standard deviation as 2·24, the change in virulence is therefore not significant but there is a suggestion of increased virulence.

23 iii. 25. *Passage Experiment III. See Diagram IV.*

Control I of this series is the same as in Experiment II above, and the passages are made from Passage (5) of that experiment, the organisms from a culture from the heart blood of the mouse dying on the 6th day in that series being passed rapidly through 10 mice by direct inoculation of a suspension in Ringer's solution of the heart blood of one mouse into the peritoneal cavity of the next. These passages were carried out at 24 hour intervals, the mouse being killed with ether if it was not dead inside that time interval.

On two occasions during the series a culture from the heart blood was held for two days in stab-agar before inoculating it into the next mouse of the series.

Control II consisting of 20 mice inoculated with graded doses gave 16 14-day deaths as against 5, in Control I. Table VIII shows the ratio of this difference to the standard deviation to be 3·48 which means a significant raising of the virulence.

Although the significant increase in this experiment had only been attained after direct mouse-to-mouse passage, it will be noted that the greater part of this increase had been attained by the slower method of passage adopted in Experiment II. The following series of direct mouse-to-mouse passages was carried out in order to test the direct method.

29 iv. 25. *Passage Experiment IV. See Diagram V.*

A culture of the A 52 strain which was undergoing day to day broth subculture was tested as Control I with the result shown in the diagram; 0·5 c.c. of the same broth culture was inoculated intraperitoneally into a mouse, and with a series of rapid mouse-to-mouse passages as described in Experiment III the state of the virulence at the end of 10 passages was found to have attained no significant increase. Control I giving 6 14-day deaths, and Control II 8 14-day deaths.

10 vi. 25. *Passage Experiment V. See Diagram VI.*

A. A strain of A 52 was tested with the result shown in the diagram. From a mouse in Control I dying on the 3rd day a strain was isolated as in the previous experiments and with this three series of ten passages each were carried out.

In one series the passages were of the direct mouse-to-mouse type, in the second the heart blood was cultured in broth between each passage and in the third it was cultured on agar between the passages. The results in the final controls show no significant increase in virulence.

B. A culture of the Ellinger strain was tested by the direct method with the result shown in Diagram VI. No increase in virulence was acquired.

To summarise this series of experiments it has been found in one series that a significant raising of the virulence has resulted from animal passage, and a second series of passages has given a suggestive but inconclusive result.

It does not appear, however, that such an increase can be brought about with any regularity, and in particular the direct mouse-to-mouse passage which is so successfully employed for raising the virulence of such an organism as the *Pneumococcus*, appears to be a most uncertain method in the case of *B. aertrycke*. It will be clear that the results obtained in the present series of investigations lead to conclusions which are diametrically opposed to those put forward by Webster. It is not altogether easy to account for the difference between our own results and those which he has recorded, but a review of the protocols contained in his reports, in the light of the experience gained in the present investigation, has convinced us that the methods which he employed in testing the virulence of strains by intraperitoneal inoculations were inadequate to detect minor but possibly significant degrees of difference. This criticism, however, does not apply to his experiments on virulence as tested per os. We may note here that the same criterion must apply to many other records of attempts to determine the virulence of such organisms as *B. aertrycke*, including, for instance, certain observations of Topley and Ayrton (1924). The wide and striking difference in virulence between the rough and smooth varieties may be accepted as proved, but the comparison of the number of the different serological types of the smooth variety must be regarded as statistically unsound in the light of our added knowledge, though no differences in virulence were, in fact, observed.

From the experiments here recorded we should conclude that the virulence of any given strain of *B. aertrycke* is not constant in degree, that it may certainly undergo a marked decrease as the result of repeated subculture in the laboratory, that it may, under certain circumstances, be increased by animal passage and that several different strains, derived from a single parent strain but with different subsequent histories, may show significant differences in virulence.

We should therefore conclude that fluctuations in the virulence of *B. aertrycke* certainly occur. It does not follow that they play a significant part in the epidemic spread of mouse-typhoid. They may or may not do so, but Webster's conclusions that virulence may be regarded as a constant factor

in this disease, and can therefore be disregarded in attempts to determine the significance of different variables in controlling the spread of infection, is clearly not valid.

It will be evident from the records of our experiments, that we have so far not succeeded in controlling, or in varying at will, the virulence of a given strain of *B. aertrycke*. This is a problem for the future and its solution is urgently required as a preliminary to the further study of many problems in the epidemiology of mouse-typhoid.

Conclusions.

1. It is impossible to determine accurately the minimal lethal dose of a culture of *B. aertrycke*, if we limit ourselves to any reasonable number of test animals.

2. The virulence of two strains of *B. aertrycke* may be compared by observing the percentage mortality resulting among two test samples of mice after the intraperitoneal inoculation of a single constant dose, or of a constant series of graded doses.

3. From records drawn from the literature, and from the results of our own experiments, it is concluded that there is no significant difference in the reaction to infection of different strains of mice, obtained from various sources.

4. The statistical conditions which must be fulfilled, if the observed differences are to be regarded as significant, are described; and a table is provided for testing significance in samples of 20 mice.

5. From the results of experiments carried out by the method described it is concluded that:

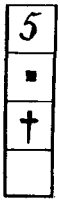
(*a*) Different strains of *B. aertrycke* may differ in virulence.

(*b*) A single strain of *B. aertrycke* may decrease materially in virulence as the result of repeated subculture.

(*c*) The virulence of a single strain of *B. aertrycke* may be significantly increased as the result of animal passage.

The author wishes to take this opportunity of acknowledging his great indebtedness to Prof. W. W. C. Topley and to Dr Major Greenwood. To the former for his unfailing kindness and encouragement during the course of this work and for his constant interest in its progress; and to the latter for the time and trouble he has spent, assisted by Miss E. M. Newbold, in verifying and correcting the statistical part of this report.

KEY TO DIAGRAMS.



= Day of death.

= Survived but infected.

= Died not infected.

= Survived not infected.

= Outline dividing acute from chronic death.

= Outline dividing survivors and non-infected dead from infected dead.

D^{3} = Those dying acute death.

D^{14} = Those dying chronic death.

= Total number infected.

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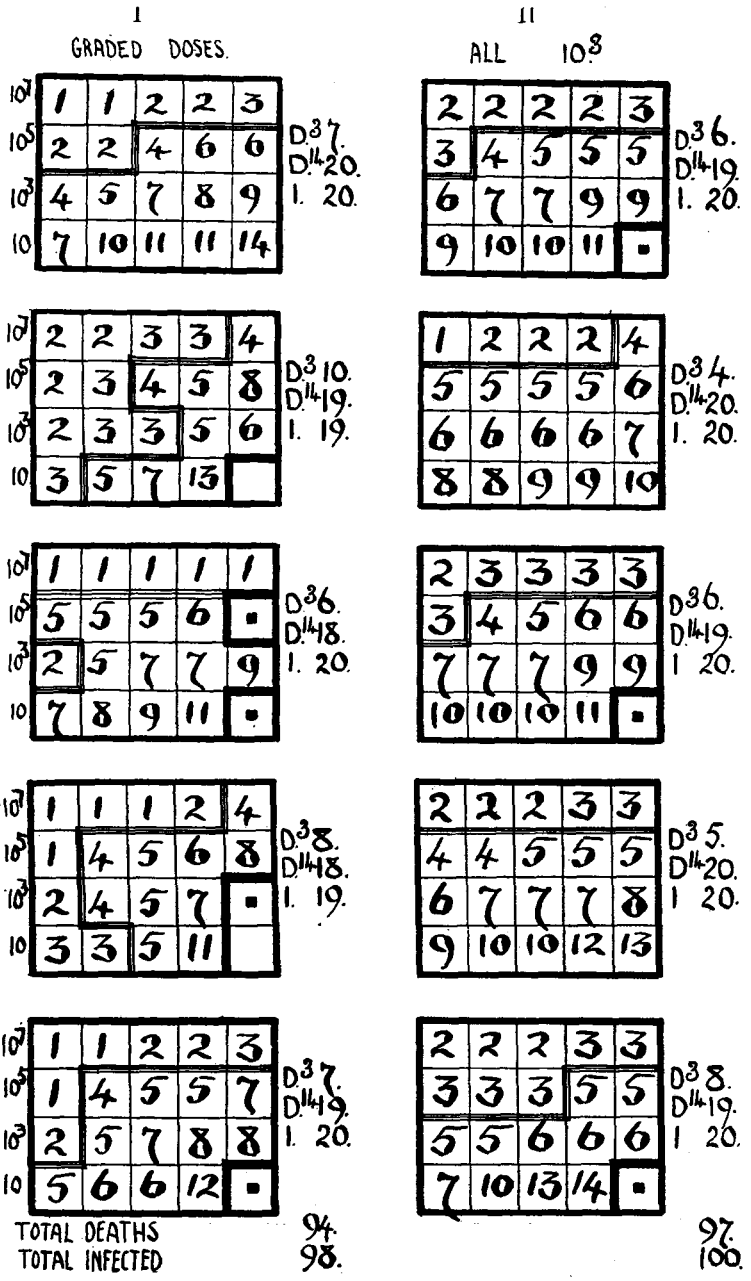

Diagram A.

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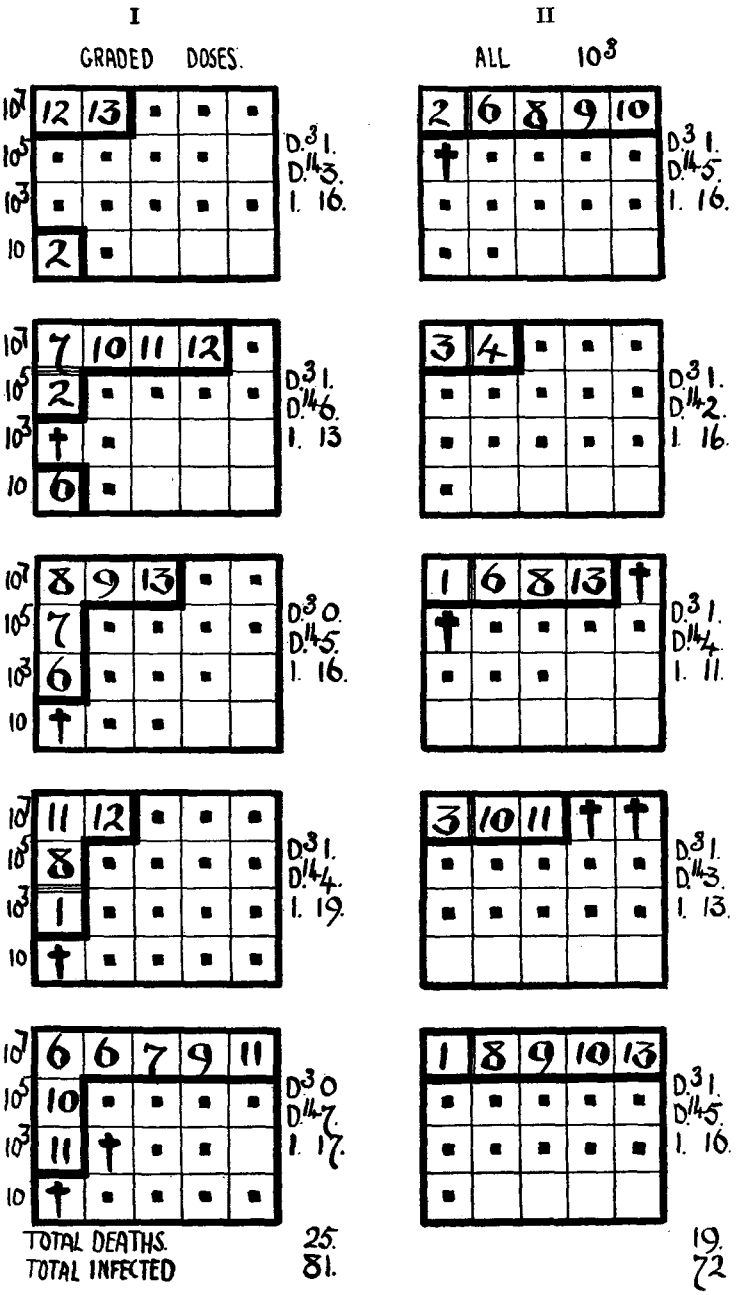

Diagram B.

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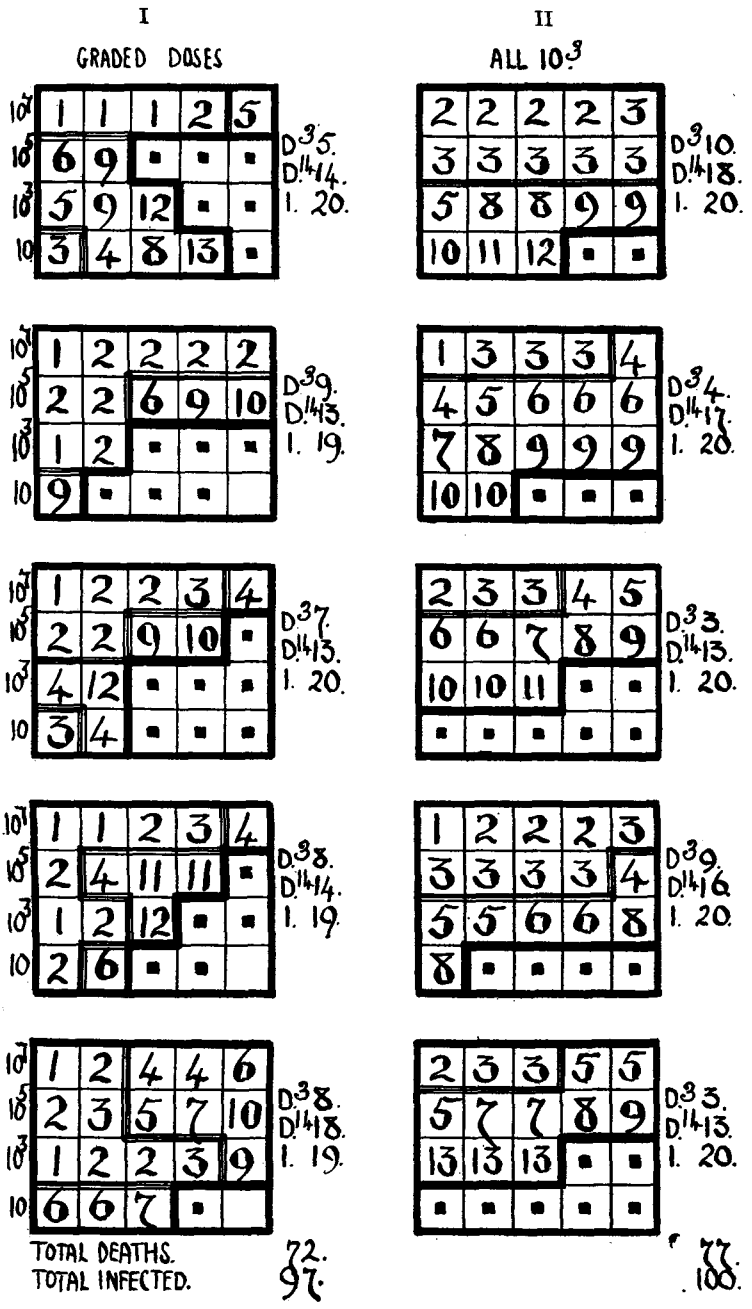

Diagram C.

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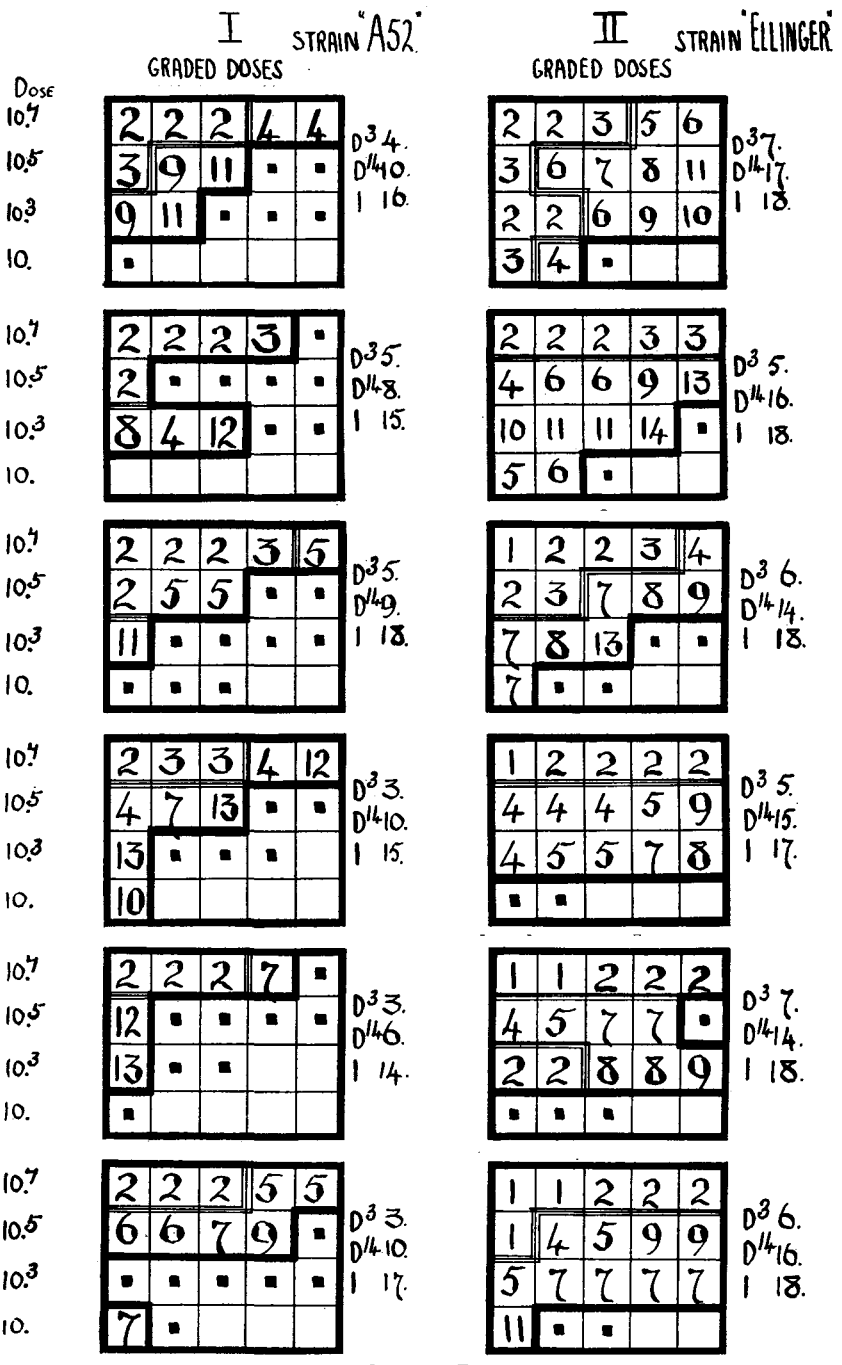

Diagram D.

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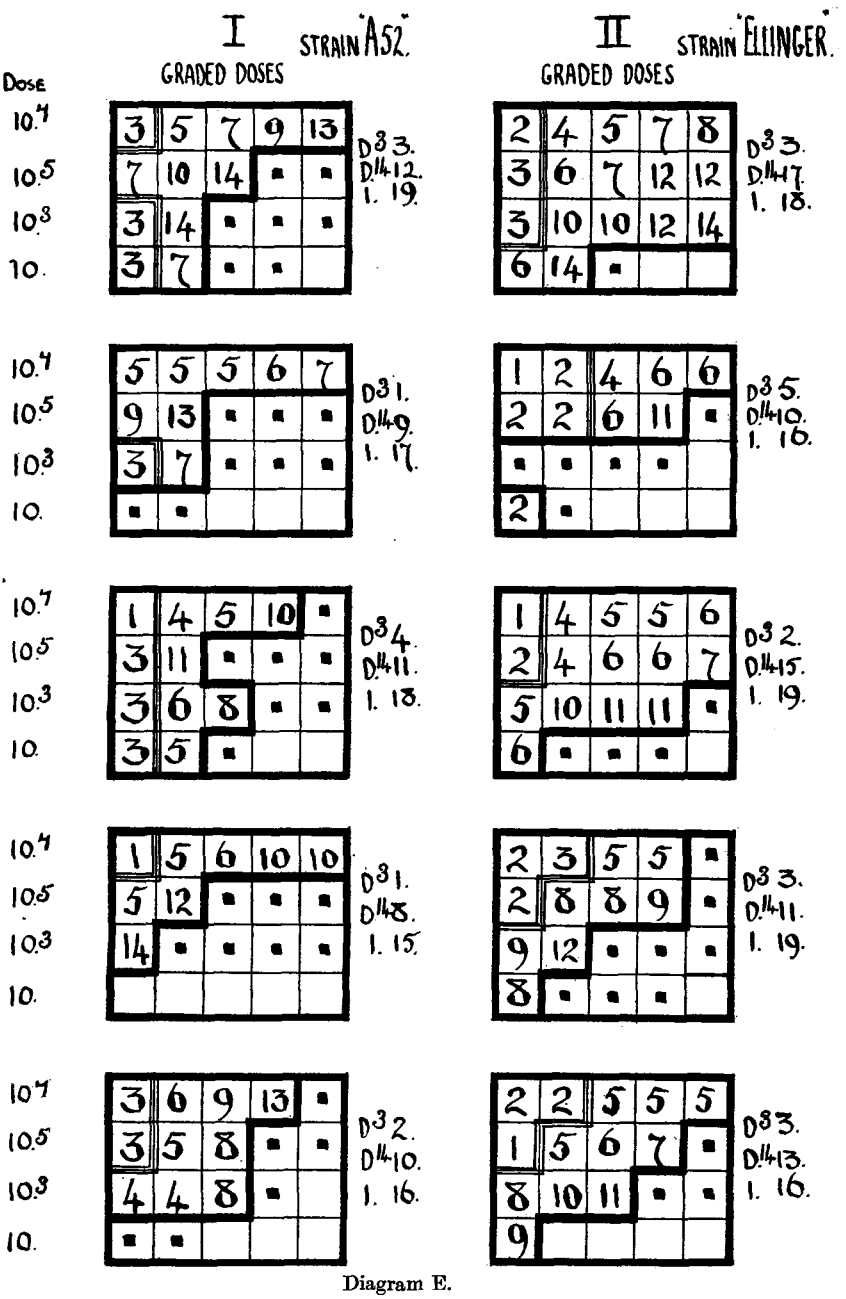

Diagram E.

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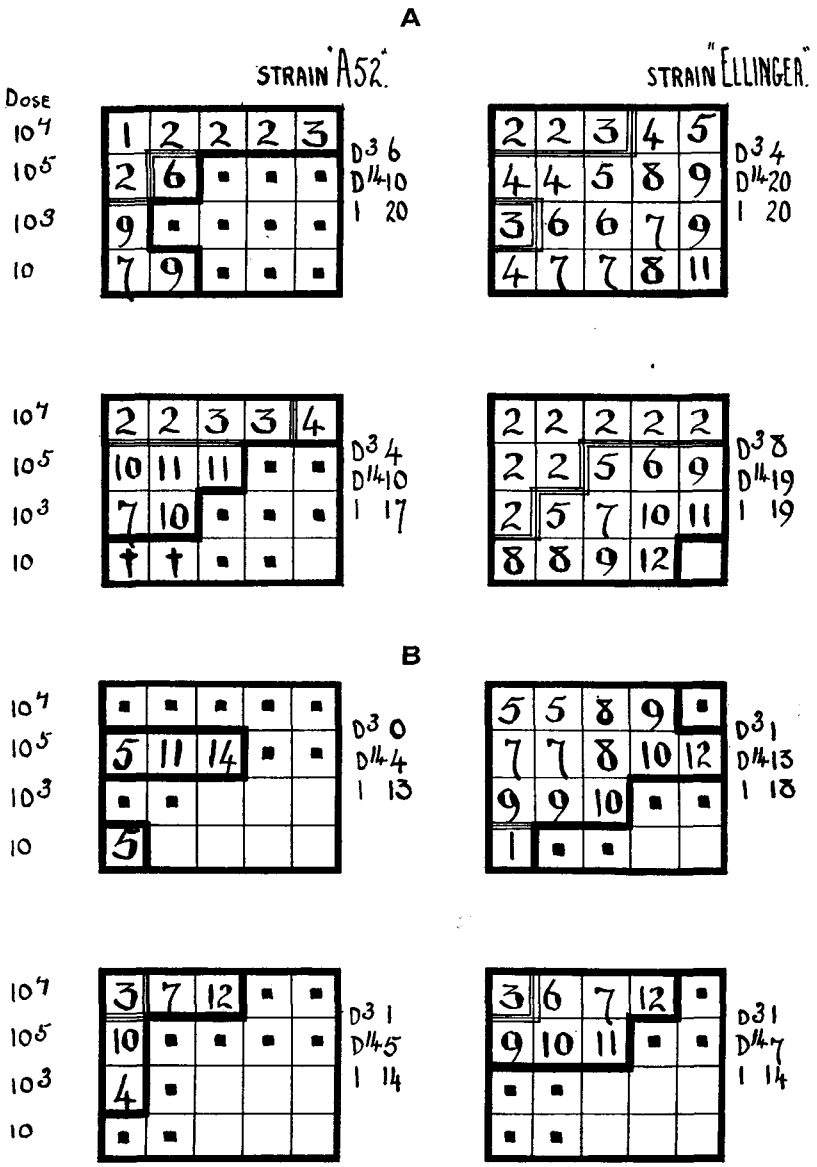

Diagram F.

Comparison of Virulence. "A" before and "B" after, 8 months subculturing.

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