# DISCONTINUOUS VARIATION IN THE VIRULENCE OF *BACT. AERTRYCKE* MUTTON.

## By G. S. WILSON.

*From the Division of Bacteriology and Immunology,
London School of Hygiene and Tropical Medicine.*

(With 6 Charts.)

WORKING with *Bact. aertrycke* Mutton, Lockhart (1926) observed a significant fall in the virulence of two strains of different origin that were being sub-cultured daily in broth. In one strain the virulence, as tested by intraperitoneal injection of batches of 20 mice, fell so that during a period of 7 months the mortality was reduced from 100 per cent. to 35 per cent.; in the other strain it fell during the same period from 50 per cent. to 25 per cent. In both strains the fall in virulence, in so far as it was revealed by the method of testing, appeared to occur fairly suddenly. The first strain showed a fall in virulence within a month from 100 per cent. to about 75 per cent.; it remained stable at this level for a period of 5 months, and then fell again during the next month to 35 per cent. The second strain remained at 50 per cent. for 3 or 4 months, and then dropped to 25 per cent., at which level it remained for the following 3 months.

This comparatively sudden fall in virulence suggested to us the possibility that some bacterial dissociation had occurred, resulting in the replacement of the original virulent organisms by variants of much lower virulence. On the other hand it seemed possible to explain the phenomenon on the assumption that the virulence of the constituent organisms of the culture was gradually falling, and that not until it reached a given point on the downward curve did it become evident by the four-dose method that Lockhart was using.

In order to decide which of these alternatives—if either—was true, we determined to repeat Lockhart's experiments, testing, however, the virulence not only of the whole culture but also of a certain proportion of its constituent organisms.

## Virulence Experiment 1.

The general technique employed was as follows:

A stock strain of *Bact. aertrycke* Mutton was used that was kept in stab agar culture and subcultured once a month. The virulence of this control strain has been tested at frequent intervals during the last 2 or 3 years, and has been found to be relatively constant. During the period covered by this experiment it was tested 28 times by intraperitoneal inoculation of batches of 20 mice; with a dose of about 50–100 organisms the average number of

mice killed was 12·1, the standard deviation being 3·13. This strain was seeded into a casein digest broth culture, and was subcultured daily, except on Sundays. (During the first 14 weeks, it was subcultured every day, Sundays included.) At the start of the experiment the broth culture was plated out on agar; 10 single colonies were picked off at random and seeded each on to a separate agar slope; at the same time an agar slope was seeded from the whole culture. After 24 hours' incubation at 37° C., each agar slope was washed off with sterile Ringer's solution, the suspension standardised to match an opacity tube containing 500 million *Bact. aertrycke*, and then diluted 1–200,000; 0·5 c.c. of this dilution was injected intraperitoneally into 20 mice. Since we have previously shown (Wilson, 1926) that in a 24-hour culture of *Bact. aertrycke* only about 50 per cent. of the organisms are viable, it was estimated that each mouse inoculated with this dose would receive about 1000 living organisms; the actual number it did receive was calculated from a roll-tube count made on the suspension used for injection. A comparison of these counts will show that the number of organisms injected varied from 444 to 2702; this variation in dosage is not of an order likely to make any appreciable difference in the results obtained (Lockhart, 1926). In order to make certain of the purity of each culture, the Ringer suspension was plated out on MacConkey's medium, and was put up against aertrycke Mutton group and type agglutinating sera. The mice used came from the normal stock, which was recruited from four or five different dealers, each of whom has supplied us with mice of his own breeding for some years past. Their weight was from 17–23 grm. Mice of all colours were used, with the exception of albinos, which according to Pritchett (1926) are somewhat more resistant to mouse-typhoid than coloured mice. After injection each mouse was kept in a separate cage. If it died within 14 days, cultures of the heart's blood and spleen were taken on to MacConkey's agar, and the spleen was dropped into a tube of broth. Non-lactose fermenters that appeared on the MacConkey plates were picked off into broth, and tested against a group and type aertrycke Mutton serum; if no growth occurred on the plates, then the spleen broth was plated out. Mice that survived 14 days were killed, and their spleen seeded into broth. All tubes that grew within 5 days were plated out, and any non-lactose fermenters were tested against the two sera mentioned. Each week during the first 3 months, and thereafter at infrequent intervals, the virulence of the whole culture was tested. After 1 month, and again after 4 months, the virulence of 10 constituent colonies of the culture was likewise tested.

The results can now be considered in detail.

On April 21st, 1927, the original stock culture was tested for virulence, together with 10 of its constituent organisms, labelled W 1 A to W 1 J.

From Chart 1 it will be seen that the whole culture, WC 1, killed 19 out of 20 mice. The single colony cultures may be classified into two groups; those of the first group, W 1 C, E, I, H and J, killed from 9 to 14 mice, whereas those of the second group killed from 16 to 20 mice. It will be noticed that

most of the deaths in the first group occurred during the second week, and that most of the deaths in the second group occurred during the first week. This will be more evident from Table I, in which the average expectation of life up to 14 days is calculated.
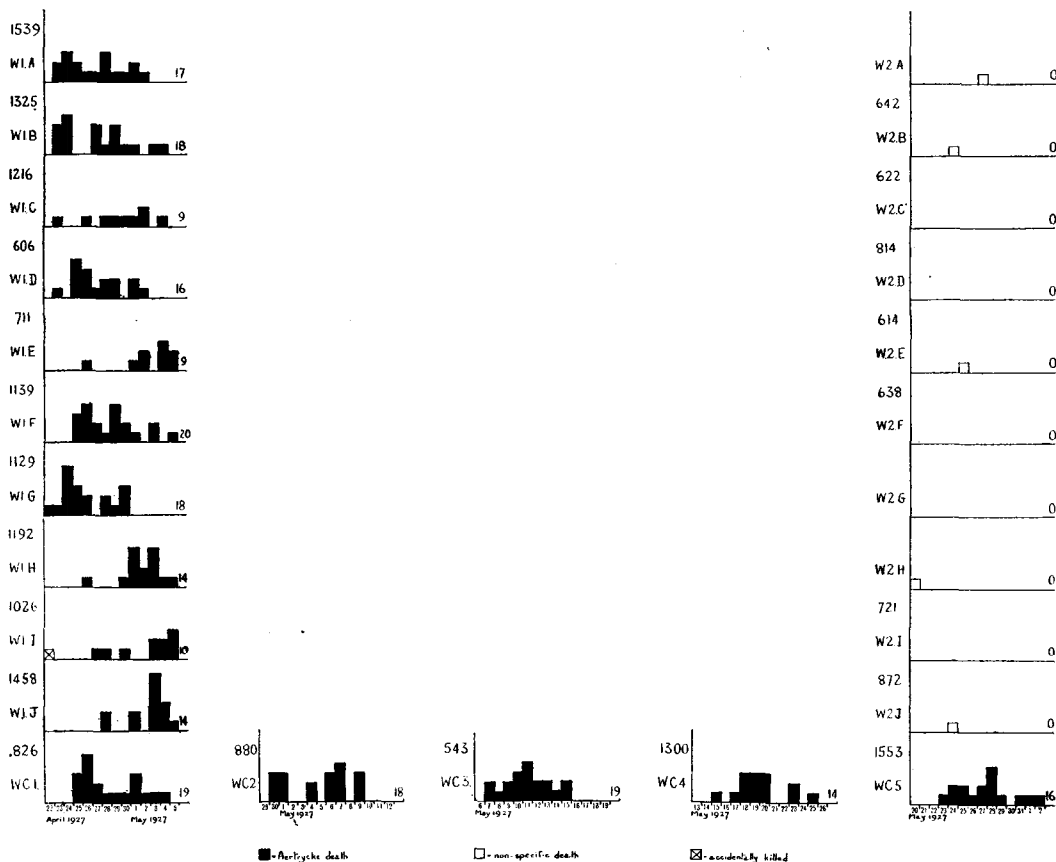


Chart 1. Virulence Experiment 1.

Each square represents the death of a single mouse. The number placed to the left of each series, immediately above the experimental group number, refers to the number of organisms injected into each animal. The number placed to the extreme right records the number of specific deaths in the series.

Though the specific deaths appear to form a graded series running up continuously from 9 to 20, the survival times show a discontinuous variation, indicating that the apparent gradation in mortality is illusory. We may regard Group 1 as consisting of 5 cultures, each capable of killing about 12 mice in 12 days; and Group 2 as consisting of 5 cultures, each capable of killing about 18 mice in 7 days. The virulence of the whole culture is similar to, and appears to be determined by, the virulence of the Group 2 series of organisms. Since the colonies were picked off at random it is probable that organisms belonging to Groups 1 and 2 were present in about equal numbers in the

Table I. *Specific deaths and average expectation of life up to* 14 *days of mice in Exp.* W 1 A–J (*Vir. Exp.* 1).

| | Specific deaths | Average expectation of life in days |
|---|---|---|
| Whole culture ... ... ... | 19 | 7·65 |
| Group 1. W 1 C ... ... ... | 9 | 11·5 |
| W 1 E ... ... ... | 9 | 12·9 |
| W 1 I ... ... ... | 10 | 12·63 |
| W 1 H ... ... ... | 14 | 11·75 |
| W 1 J ... ... ... | 14 | 12·15 |
| Arithmetic mean ... ... ... | 11·2 | 12·19 |
| Standard deviation ... ... ... | 2·215 | 0·523 |
| Standard error of mean ... ... | 1·035 | 0·234 |
| Group 2. W 1 D ... ... ... | 16 | 7·8 |
| W 1 A ... ... ... | 17 | 7·15 |
| W 1 B ... ... ... | 18 | 6·95 |
| W 1 G ... ... ... | 18 | 5·85 |
| W 1 F ... ... ... | 20 | 7·4 |
| Arithmetic mean ... ... ... | 17·8 | 7·03 |
| Standard deviation ... ... ... | 1·326 | 0·699 |
| Standard error of mean ... ... | 0·593 | 0·313 |
| Standard error of difference of means | 1·208 | 0·3905 |
| Observed difference of means ... | 6·6 | 5·16 |
| $\dfrac{\text{Observed difference}}{\text{Standard error of difference of means}}$ | 5·46 | 13·21 |

whole culture. This experiment seems to show that the original stock culture contained at least two different types of organism; one type was highly virulent and killed rapidly; the other was less virulent and killed slowly.

The virulence of the whole culture was tested at weekly intervals (cf. Chart 1). At the fourth testing, WC 4, only 14 deaths occurred. As the virulence appeared to be falling, it was decided to test a fresh series of single colony cultures. Accordingly the 26th daily subculture was plated out on agar, the single colonies were picked off on to agar slopes, and tested for virulence as before. The whole culture, seeded on to an agar slope from the 27th daily subculture, was tested simultaneously. The result was unexpected. All 10 single colony cultures, W 2 A–J, proved completely avirulent; not a single specific death occurred amongst the 200 mice used. On the other hand the whole culture killed 16 out of 20 mice. It seems clear from this experiment that the constitution of the whole culture must have undergone a radical alteration since its first testing 4 weeks previously. Daily subculture in broth appears to have caused a replacement of most of the initially virulent organisms by others that were completely devoid of virulence. It is clear that not all the virulent organisms can have been replaced; otherwise the whole culture itself would have proved avirulent. The fact that it killed nearly as many mice as at the commencement of the experiment suggests that the presence of a comparatively small proportion of virulent organisms in a culture may suffice to render it virulent. Since each of the 10 colonies tested was avirulent, the virulent organisms in the whole culture probably did not exceed 10 per cent. of the total number, and may have been considerably less than this.

The whole culture tested during the next 3 weeks (cf. Chart 2, WC 6, 7

and 8) showed no signs of falling in virulence. It seemed to us possible however that if a smaller dose was used, a fall in virulence might be detected. We had learnt from other work that a fully virulent culture kills nearly as many mice in a dose of 50 as in a dose of 1000. But if the supposition was correct that the whole culture contained only a small proportion of virulent organisms, then a decrease in dosage from 1000 to 50 might make a considerable difference in the result. To test this, the 55th subculture was injected in two doses; WC 9 *a* received 1042 organisms, and WC 9 *b* 50 organisms. The result confirmed our anticipations. In the former group 19 specific deaths occurred, in the latter only 3. The interpretation that we put upon this experiment is as follows: the last time single colony cultures were tested, it appeared that at least 90 per cent. of the organisms in the culture were avirulent. Supposing that when the ninth whole culture test was made the position was approximately the same, somewhere about 10 per cent. of the organisms in the culture were probably virulent. The mice given the higher dosage would therefore receive about
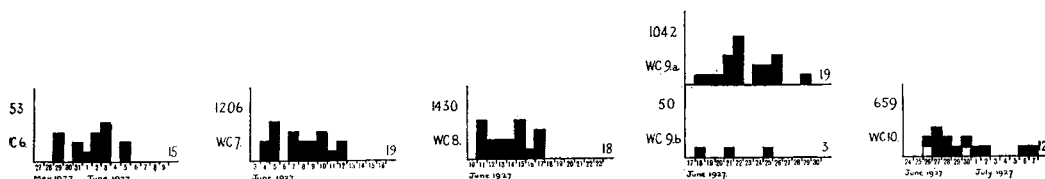


Chart 2. Virulence Experiment 1 (cont.).

100 virulent organisms—possibly less; the number received was sufficient to kill nearly every mouse. The mice given the smaller dosage, however, would receive probably not more than 5 virulent organisms, and possibly less. It would, therefore, be largely a matter of chance whether any given mouse received sufficient virulent organisms to overcome its natural resistance. As a matter of fact, only 3 mice receiving the smaller dose died. This experiment seems to show that when a culture is of moderate virulence, the dose in which it is injected may be of considerable importance, not because there is a close relation between proportional increase in the number of virulent bacteria administered and proportional increase in mortality, over the range in which an effective dose of such bacteria is given, but because, as the total dosage decreases, a limit may be reached at which no virulent organisms are included in the inoculum. When it is of high virulence, then the dose is of comparatively small importance; we have tested highly virulent cultures, and have found them capable of killing 90–100 per cent. of mice in a dose of 30 to 50 organisms. It shows further that in order to detect small alterations in the virulence of a culture, it is imperative to adjust the dose to a suitable level or series of levels. Judging by the higher dose alone in this experiment, one would not have suspected that WC 9 was any less virulent than the original culture, WC 1. Yet this culture killed on an average 12 mice in a dose of 50 to 100 organisms, whereas WC 9 *b* killed only 3.

The whole culture was tested again at weekly intervals.

WC 10 killed only 12 mice, but the two following cultures, WC 11 and 12 (cf. Chart 3), killed 19 mice. WC 13 was injected in 3 different doses. WC 13 *a* received 1531, WC 13 *b* 329, and WC 13 *c* 83 organisms; the deaths respectively were 16, 15 and 15. The control culture, from which the original seeding was made, was injected at the same time into a batch of 20 mice in a dosage of 80 organisms; 13 of the mice died. From this experiment it would appear
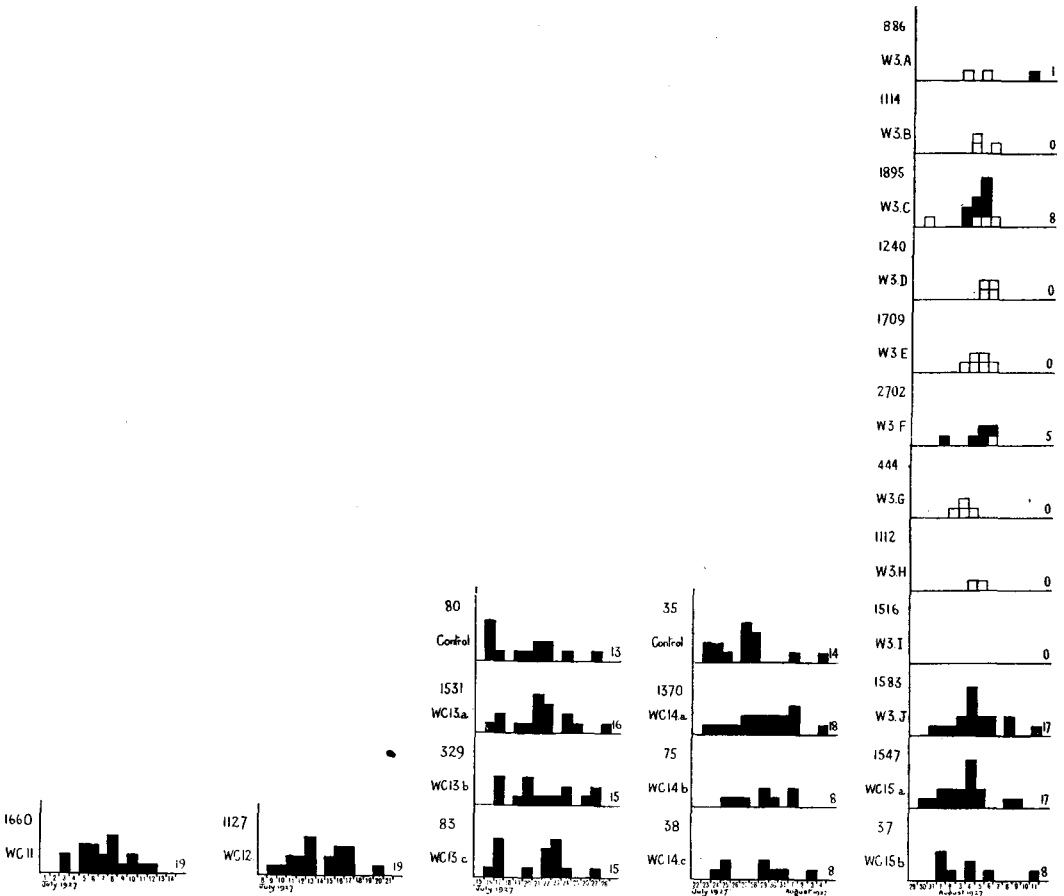


Chart 3. Virulence Experiment 1 (cont.).

that the virulence of the whole culture had risen till it was approximately the same as that of the original strain. A diminution in dosage from 1500 to 80 organisms made no appreciable difference to the result.

The following week the whole culture was again tested in three different doses. WC 14 *a* received 1370, WC 14 *b* 75, and WC 14 *c* 38 organisms; the deaths respectively were 18, 8 and 8. The control culture, tested at the same time in a dose of 35 organisms, killed 14 mice. Here, judging from the effect of the smaller doses, the whole culture seems to have suffered a diminution in virulence since the previous week. With the higher dosage, however, no fall was apparent.

The following week a third analysis of the constituent organisms of the culture was made. The 95th subculture was plated out on agar, 10 single colonies were picked off at random, seeded on to agar, and tested as before. The whole culture was tested at the same time in a high and a low dose; the deaths from these two doses were 17 and 8 respectively; that is to say, the virulence of the whole culture was apparently the same as it was the previous week. Amongst the mice inoculated with the single colony cultures a number of non-specific deaths occurred, some of them due to Morgan's bacillus. Deaths from this organism and from other non-lactose fermenters are not uncommon in our experience during the summer months (Wilson, 1927). The mice dying from aertrycke infection can be divided into three groups (cf. Table II).

Table II. *Specific deaths of mice in Exp.* W 3 A–J (*Vir. Exp.* 1).

|  | Specific deaths |
| --- | --- |
| Whole culture | 17 |
| W 3 B | 0 |
| W 3 D | 0 |
| W 3 E | 0 |
| W 3 G | 0 |
| W 3 H | 0 |
| W 3 I | 0 |
| W 3 A | 1 |
| W 3 F | 5 |
| W 3 C | 8 |
| W 3 J | 17 |

Some of the single colony cultures are for all practical purposes completely avirulent; two of them, W 3 F and C, are slightly virulent, killing 5 and 8 mice respectively, and one, W 3 J, is fully virulent, killing as many mice as the whole culture. Here we have evident discontinuous variation in the virulence of the organisms constituting the whole culture. Though, of course, a sample of 10 colonies is insufficient to give one exact figures, the suggestion is that roughly speaking 10 per cent. of the organisms in the whole culture were fully virulent, 20 per cent. were slightly virulent, and 70 per cent. were completely avirulent. It is interesting to note that this composition is very different from that of the original culture, which consisted of about equal numbers of moderately and of fairly highly virulent organisms. Yet each culture killed about the same number of mice when tested in a dose of 1000. It would appear that as with WC 5 the virulence of the whole culture is determined by the most highly virulent of its constituent organisms. There is no evidence to suggest that the virulence of the whole culture is decreased by the admixture of 70 per cent. of avirulent with the 10 per cent. of virulent organisms, unless the rather small death-rate following the injection of the lower dose is taken as pointing in this direction. This, however, is hardly permissible as the figure is well within the limits of normal variation of the control culture.

We were rather surprised that in the 3 months during which daily subculture had been carried out, the virulence of the whole culture had not fallen significantly. We decided, however, to continue the experiment, testing the

**20–2**

whole culture at less frequent intervals. A test made on August 4th resulted in the death of 15 mice (Chart 4).

Owing to the pressure of other work a fourth test could not be made till the end of January 1928; the culture, however, still proved virulent, killing 18 out of 20 mice. At the next test, on April 25th, not a single mouse died. During the 3 months since its last testing the culture had become completely avirulent. A further test made on May 15th showed that it was practically avirulent; only 4 deaths occurred after a full dosage, and those not till very late. The control culture, tested in a small dose on each of these occasions, displayed its usual virulence. In a final test made on August 10th the culture again proved completely avirulent. This result confirms Lockhart's observations. In his experience daily subcultivation in broth led to a fall in virulence in about 3 months; in our experience a period of 12 months may be necessary before this occurs.
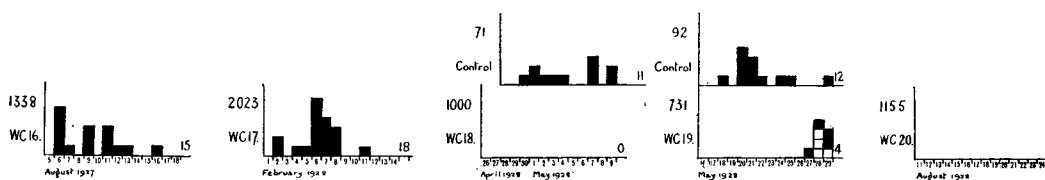


Chart 4. Virulence Experiment 1 (cont.).

Reviewing this experiment, we see that daily subculture in broth led in about 4 weeks to a marked change in the composition of the culture, the great majority of the virulent organisms present at the start being replaced by completely avirulent ones. When next analysed, 10 weeks later, the composition of the culture was again changed. The majority of the organisms were still avirulent, but besides these a certain number of slightly virulent, and a few fully virulent organisms were present. No more analyses were made, but after a further period of 9 months the virulence suddenly fell, and the culture, which previously killed 15 to 19 mice, killed in one test 4 mice, and in another test none at all. When finally tested 3 months later it proved completely avirulent, failing to kill a single mouse. It is apparent, therefore, that during the period of the experiment the composition of the culture underwent considerable alterations, and that after a period of about one year its virulence fell almost to extinction.

The full protocols of Exp. 1 are recorded in Table III.

### Virulence Experiment 2.

The general technique of this experiment was much the same as of the previous one. The main difference was that the culture, instead of being incubated under aerobic conditions, was incubated in an atmosphere containing 1 per cent. of oxygen and 99 per cent. of nitrogen; this mixture of gases was bubbled through the broth continuously to the amount of about 5 litres a day. Subcultures were made every day except on Sundays. The reason why

## Table III. *Virulence Exp.* 1.

| Date | Exp. No. | No. of mice injected | Dose | Agglutination Group | Type | Died | Specific deaths | Killed | Spleens infected | Total infected | Mean expectation of life in days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 21. IV. 27 | WC 1 | 20 | 826 | + + | — | 19 | 19 | 1 | 1 | 20 | 7·65 |
| ,, | W 1 A | 20 | 1539 | + + | — | 17 | 17 | 3 | 3 | 20 | 7·15 |
| ,, | W 1 B | 20 | 1325 | + + | — | 18 | 18 | 2 | 2 | 20 | 6·95 |
| ,, | W 1 C | 20 | 1216 | + + | — | 9 | 9 | 11 | 11 | 20 | 11·5 |
| ,, | W 1 D | 20 | 606 | + + | — | 16 | 16 | 4 | 4 | 20 | 7·8 |
| ,, | W 1 E | 20 | 711 | + + | — | 9 | 9 | 11 | 10 | 19 | 12·9 |
| ,, | W 1 F | 20 | 1139 | + + | — | 20 | 20 | 0 | 0 | 20 | 7·4 |
| ,, | W 1 G | 20 | 1129 | + + | — | 18 | 18 | 2 | 2 | 20 | 5·85 |
| ,, | W 1 H | 20 | 1192 | — | + + | 14 | 14 | 6 | 6 | 20 | 11·75 |
| ,, | W 1 I | 20 | 1026 | + + | — | 10 | 10 | 9 | 9 | 20 | 12·63 |
| ,, | W 1 J | 20 | 1458 | — | + + | 14 | 14 | 6 | 6 | 20 | 12·15 |
| 28. IV. 27 | WC 2 | 20 | 880 | + + | — | 18 | 18 | 2 | 1 | 19 | 7·4 |
| 5. v. 27 | WC 3 | 20 | 543 | + | + | 19 | 19 | 1 | 1 | 20 | 6·35 |
| 12. v. 27 | WC 4 | 20 | 1300 | | | 14 | 14 | 6 | 6 | 20 | 9·35 |
| 19. v. 27 | WC 5 | 20 | 1553 | + | — | 16 | 16 | 4 | 3 | 19 | 9·5 |
| ,, | W 2 A | 20 | * | + + | — | 1 | 0 | 19 | 13 | 13 | 13·7 |
| ,, | W 2 B | 20 | 642 | + + | — | 1 | 0 | 19 | 1 | 1 | 13·6 |
| ,, | W 2 C | 20 | 622 | + + | — | 0 | 0 | 20 | 17 | 17 | 14·0 |
| ,, | W 2 D | 20 | 814 | + + | — | 0 | 0 | 20 | 2 | 2 | 14·0 |
| ,, | W 2 E | 20 | 614 | + + | — | 1 | 0 | 19 | 15 | 15 | 13·6 |
| ,, | W 2 F | 20 | 638 | + | — | 0 | 0 | 20 | 13 | 13 | 14·0 |
| ,, | W 2 G | 20 | * | + | — | 0 | 0 | 20 | 9 | 9 | 14·0 |
| ,, | W 2 H | 20 | * | + + | — | 1 | 0 | 19 | 17 | 17 | 13·4 |
| ,, | W 2 I | 20 | 721 | + + | — | 0 | 0 | 20 | 15 | 15 | 14·0 |
| ,, | W 2 J | 20 | 872 | + + | — | 1 | 0 | 19 | 16 | 16 | 13·6 |
| 26. v. 27 | WC 6 | 20 | 1153 | + + | — | 15 | 15 | 5 | 5 | 20 | 8·4 |
| 2. VI. 27 | WC 7 | 20 | 1206 | + + | — | 19 | 19 | 1 | 1 | 20 | 6·2 |
| 9. VI. 27 | WC 8 | 20 | 1430 | + + | + | 18 | 18 | 2 | 2 | 20 | 5·75 |
| 16. VI. 27 | WC 9 a | 20 | 1042 | + + | — | 19 | 19 | 1 | 0 | 19 | 7·25 |
| ,, | WC 9 b | 20 | 50 | + + | — | 3 | 3 | 17 | 11 | 14 | 12·7 |
| 23. VI. 27 | WC 10 | 20 | 659 | + + | — | 14 | 12 | 6 | 5 | 17 | 8·8 |
| 30. VI. 27 | WC 11 | 20 | 1660 | + + | + + | 19 | 19 | 1 | 1 | 20 | 7·55 |
| 7. VII. 27 | WC 12 | 20 | 1127 | + + | + | 19 | 19 | 1 | 1 | 20 | 7·35 |
| 14. VII. 27 | WC 13 a | 20 | 1531 | + + | + | 16 | 16 | 4 | 3 | 19 | 8·6 |
| ,, | WC 13 b | 20 | 329 | + + | + | 15 | 15 | 5 | 4 | 19 | 9·2 |
| ,, | WC 13 c | 20 | 83 | + + | + | 15 | 15 | 5 | 3 | 18 | 8·65 |
| 21. VII. 27 | WC 14 a | 20 | 1370 | + + | + + | 18 | 18 | 2 | 2 | 20 | 8·45 |
| ,, | WC 14 b | 20 | 75 | + + | + + | 8 | 8 | 12 | 3 | 11 | 11·5 |
| ,, | WC 14 c | 20 | 38 | + + | + + | 8 | 8 | 12 | 10 | 18 | 11·35 |
| 28. VII. 27 | WC 15 a | 20 | 1547 | + + | + + | 17 | 17 | 3 | 3 | 20 | 7·55 |
| ,, | WC 15 b | 20 | 37 | + + | + + | 8 | 8 | 12 | 10 | 18 | 11·1 |
| ,, | W 3 A | 20 | 886 | + + | + | 3 | 1 | 17 | 8 | 9 | 13·25 |
| ,, | W 3 B | 20 | 1114 | + + | + | 3 | 0 | 17 | 16 | 16 | 13·05 |
| ,, | W 3 C | 20 | 1895 | + + | — | 12 | 8 | 8 | 6 | 14 | 9·8 |
| ,, | W 3 D | 20 | 1240 | + | + + | 4 | 0 | 16 | 9 | 9 | 12·9 |
| ,, | W 3 E | 20 | 1709 | + + | + + | 6 | 0 | 14 | 12 | 12 | 10·65 |
| ,, | W 3 F | 20 | 2702 | + + | + + | 6 | 5 | 14 | 5 | 10 | 12·05 |
| ,, | W 3 G | 20 | 444 | + | + + | 4 | 0 | 16 | 7 | 7 | 12·4 |
| ,, | W 3 H | 20 | 1112 | + | + + | 2 | 0 | 18 | 11 | 11 | 13·35 |
| ,, | W 3 I | 20 | 1516 | + + | + + | 0 | 0 | 20 | 13 | 13 | 14·0 |
| ,, | W 3 J | 20 | 1583 | + + | + + | 17 | 17 | 3 | 3 | 20 | 8·55 |
| 4. VIII. 27 | WC 16 | 20 | 1338 | + + | + + | 15 | 15 | 5 | 5 | 20 | 7·4 |
| 31. I. 28 | WC 17 | 20 | 2023 | + + | + + | 18 | 18 | 2 | 2 | 20 | 7·0 |
| 25. IV. 28 | WC 18 | 20 | 1000 | — | + + | 0 | 0 | 20 | 11 | 11 | 14·0 |
| 15. v. 28 | WC 19 | 20 | 731 | + | + + | 8 | 4 | 12 | 4 | 8 | 13·7 |
| 10. VIII. 28 | WC 20 | 20 | 1155 | + | + + | 0 | 0 | 20 | 10 | 10 | 14·0 |

Control tests made at the same time

| Date | Exp. No. | No. of mice injected | Dose | Agglutination Group | Type | Died | Specific deaths | Killed | Spleens infected | Total infected | Mean expectation of life in days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 14. VII. 27 | Control | 20 | 80 | + + | + + | 13 | 13 | 7 | 7 | 20 | 8·65 |
| 21. VII. 27 | ,, | 20 | 35 | + + | + + | 14 | 14 | 6 | 4 | 18 | 8·4 |
| 27. IV. 28 | ,, | 20 | 70 | + + | + + | 11 | 11 | 9 | 9 | 20 | 10·45 |
| 15. v. 28 | ,, | 20 | 90 | + + | + | 12 | 12 | 8 | 5 | 17 | 9·65 |
| 1. VIII. 28 | ,, | 20 | 109 | + + | + + | 12 | 12 | 8 | 7 | 19 | 9·15 |

\* Tubes for counting accidentally destroyed.

*Note.* Between 21. IV. 27 and 1. VIII. 28 the control culture was tested 28 times, and killed on an average 12·1 mice, the standard deviation being 3·13. The tests recorded here refer merely to those that were made at the same time as tests of the experimental culture.

an atmosphere of 1 per cent. oxygen was chosen was because daily subculture in this gas had been found in other experiments to lower the virulence gradually. Incubation in this gas was maintained for $3\frac{1}{2}$ months; as the virulence had not then fallen completely, 1 per cent. oxygen was replaced by 21 per cent. $O_2$, which was bubbled through in the same quantity. This gas had been found to lower the virulence fairly rapidly; and in effect it did succeed in lowering the virulence of this strain almost to the point of extinction within about 6 weeks.

At the commencement of the experiment the control culture—the same as that used in Exp. 1—was plated out, 10 colonies were picked off on to agar
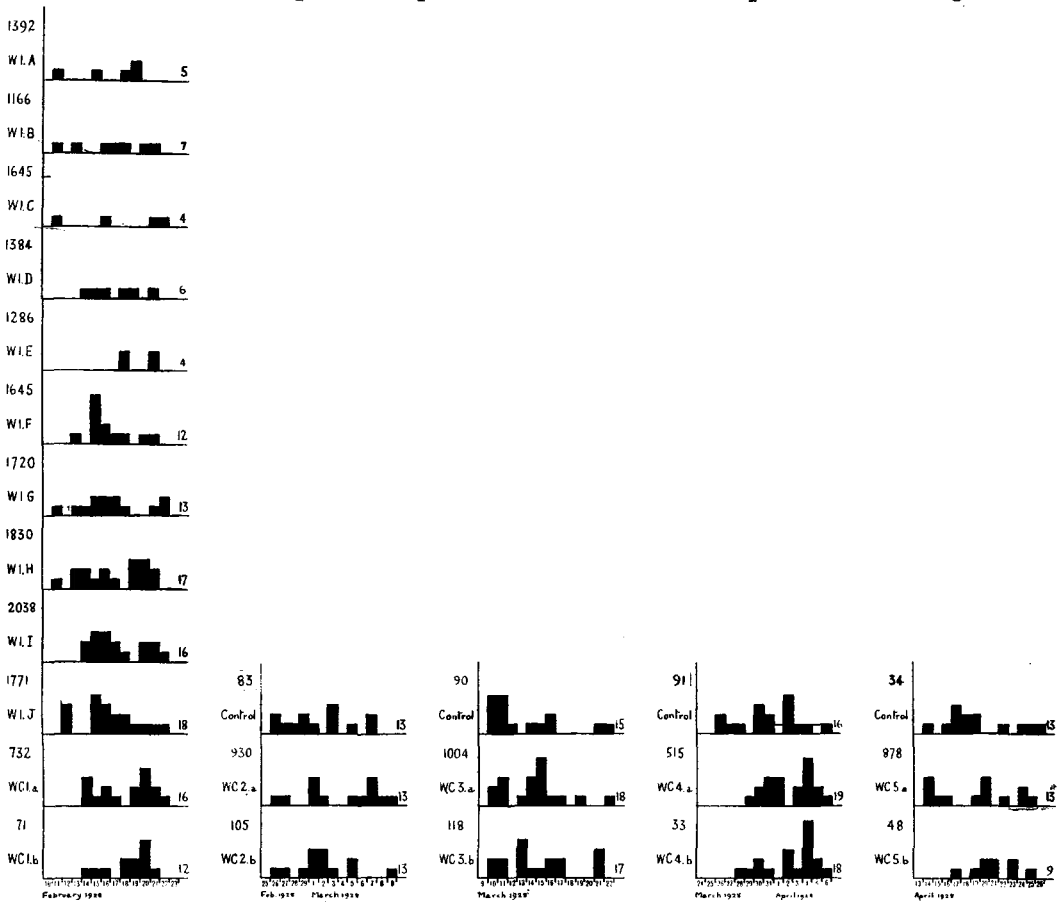


Chart 5. Virulence Experiment 2.

slopes, and these were injected intraperitoneally each into a batch of 20 mice in a dose of about 1000 organisms. The whole culture was injected simultaneously in two doses, one of about 1000 and one of about 100 organisms. The larger dose was prepared from an agar slope put up in the same way as the single colony cultures; the smaller dose was prepared from the first subculture in broth. At each subsequent testing the control culture was tested simultaneously. For this purpose a 24-hour broth-culture, seeded from

the stock agar stab, was diluted, and injected in a dose that was comparable with the lower dose of the experimental culture. This technique was maintained throughout the experiment. All counts were made on the actual suspensions used for inoculation.

The whole culture proved moderately virulent (cf. Chart 5), killing 16 mice in the higher dose and 12 in the lower. Of the single colony cultures 5, W 1 F–J, proved of about the same virulence as the whole culture, and 5, W 1 A–E, proved to be only of slight virulence, killing 4 to 7 mice. A difference in the survival times of the mice injected in the two groups was also noticeable (cf. Table IV).

Table IV. *Specific deaths and average expectation of life up to* 14 *days of mice in Exp.* W 1 A–J (*Vir. Exp.* 2).

|  | Specific deaths | Average expectation of life in days |
|---|---|---|
| Whole culture W 1 ... ... ... | 16 | 10·0 |
| Group 1.  W 1 C ... ... ... | 4 | 12·9 |
| W 1 E ... ... ... | 4 | 13·3 |
| W 1 A ... ... ... | 5 | 12·35 |
| W 1 D ... ... ... | 6 | 12·25 |
| W 1 B ... ... ... | 7 | 11·75 |
| Arithmetic mean ... ... ... | 5·2 | 12·51 |
| Standard deviation ... ... ... | 1·166 | 0·737 |
| Standard error of mean ... ... | 0·522 | 0·329 |
| Group 2.  W 1 F ... ... ... | 12 | 10·0 |
| W 1 G ... ... ... | 13 | 10·0 |
| W 1 I ... ... ... | 16 | 9·45 |
| W 1 H ... ... ... | 17 | 8·85 |
| W 1 J ... ... ... | 18 | 8·1 |
| Arithmetic mean ... ... ... | 15·2 | 9·28 |
| Standard deviation ... ... ... | 2·315 | 0·723 |
| Standard error of mean ... ... | 1·035 | 0·323 |
| Standard error of difference of means | 1·160 | 0·462 |
| Observed difference of means ... | 10·0 | 3·23 |
| $\dfrac{\text{Observed difference}}{\text{Standard error of difference of means}}$ | 8·63 | 6·99 |

An examination of this table shows an unmistakable discontinuous variation between the two groups of 5 single colony cultures. With one group the average number of deaths was 15·2, with the other 5·2; the corresponding survival times were 9·28 and 12·51 days respectively. As in Exp. 1, it will be noticed that the virulence of the whole culture was determined by the most virulent of its constituent organisms.

It is interesting to notice that the composition of the control culture at this date, February 1928, differed somewhat from that of the previous testing, April 1927. Reference to Chart 1 and Table I will show that it then consisted of about equal numbers of fairly highly virulent organisms, which killed rapidly, and of organisms of lower virulence, which killed slowly. Ten months later, there are two groups of organisms present in about equal numbers; the one is rather less virulent than the first group of the April testing and takes longer to kill; the other group is definitely less virulent than the second group
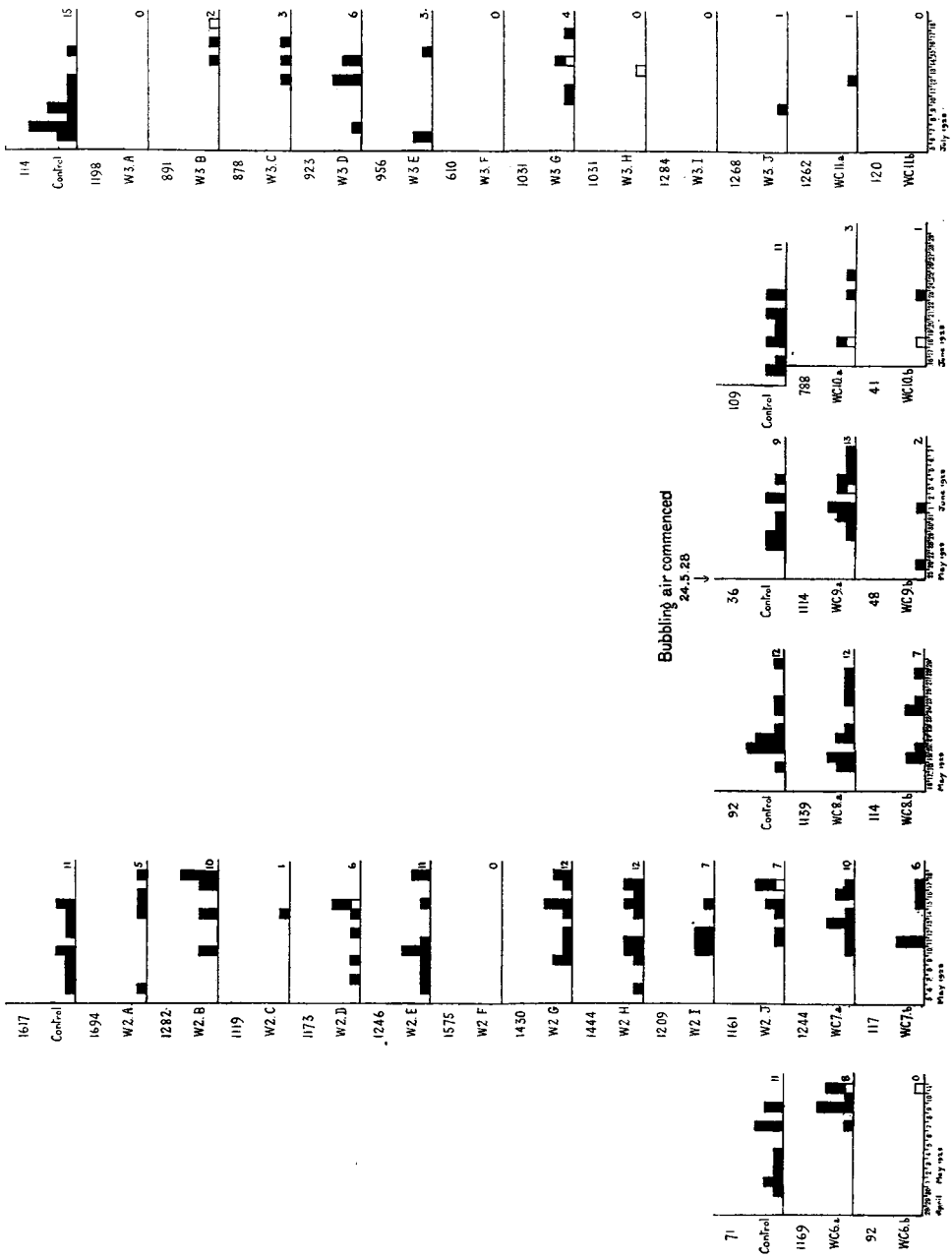
Chart 6. Virulence Experiment 2 (cont.).

of the April testing, but takes about the same time to kill. It looks as if the organisms composing the culture had received a shift towards a lower plane of virulence.

During the next 3 months the whole culture was tested four times, each time in two doses; and at the same time the control culture was tested—in the lower dose only (except on one occasion). The results, figured in Chart 5, show no definite indication of a fall in virulence.

At the sixth testing, however, WC 6 *a* and *b* (cf. Chart 6), a fall in virulence was quite evident. The whole culture in the large dose killed only 8 mice; in the small dose it killed no mice at all: the control culture killed the usual number. It was decided, therefore, to test another series of 10 colonies. These were picked off at random and tested in the usual way. The whole culture, WC 7 *a* and *b*, it will be seen, proved slightly more fatal than the previous week; in the larger dose it killed 10 and in the small dose 6 mice. The results of the single colony cultures were unusual; as seen in Table V they showed no obvious discontinuous variation; the deaths ranged from 0 to 12, and the survival times from 10·2 to 14·0 days.

Table V. *Specific deaths and average expectation of life up to* 14 *days of mice in Exp.* W 2 A–J (*Vir. Exp.* 2).

|  | Specific deaths | Average expectation of life in days |
|---|---|---|
| Whole culture WC 7 | 10 | 11·55 |
| W 2 F | 0 | 14·0 |
| W 2 C | 1 | 13·8 |
| W 2 A | 5 | 12·95 |
| W 2 D | 6 | 12·05 |
| W 2 I | 7 | 11·75 |
| W 2 J | 7 | 12·7 |
| W 2 B | 10 | 12·7 |
| W 2 E | 11 | 10·2 |
| W 2 G | 12 | 11·35 |
| W 2 H | 12 | 10·75 |

The interpretation of these figures is not easy. Superficially, it would appear that the constituent organisms of the culture are arranged in a regularly graded series from completely avirulent at one end of the scale to moderately virulent at the other. This may be the correct interpretation, but we have reason to doubt it. A careful study of numerous colonies from each of these strains revealed certain differences, which corresponded to some extent with the differences observed in their virulence. It is conceivable, therefore, that there were really two types of discontinuous variants in the culture, the one being slightly more virulent than the other. We do not wish to discuss this question here, as we are engaged at the moment in submitting the relationship between colonial appearance and virulence to special study. Suffice it to say that the differences in colonial appearance that we noted did not correspond to the differences between the rough and smooth types, as described by Arkwright (1921), and subsequent workers. All colonies tested gave perfectly homogeneous suspensions, even in 10 per cent. saline, produced a uniform turbidity in broth, and were not agglutinated by an aertrycke rough O serum.

The whole culture was tested twice more, and appeared to remain at about the same level of virulence. On May 24th, $3\frac{1}{2}$ months after the commencement of this experiment, the 1 per cent. $O_2$, which had been used for bubbling through the cultures, was changed to 21 per cent. $O_2$.

The first test after the change was made on June 15th—that is, 3 weeks later. The whole culture had then fallen considerably in virulence, killing in the larger dose only 3, and in the smaller dose only 1 mouse.

A final testing, together with 10 single colony cultures, was made on July 4th, 1928, 5 months after the commencement of the experiment and 6 weeks after the change-over to 21 per cent. $O_2$. The whole culture WC 11 $a$ and $b$ proved almost completely avirulent.

Table VI. *Specific deaths and average expectation of life up to 14 days of mice in Exp.* W 3 A–J (*Vir. Exp.* 2).

|  | Specific deaths | Average expectation of life in days |
|---|---|---|
| Whole culture WC 11 $a$ | 1 | 13·7 |
| W 3 F | 0 | 14·0 |
| W 3 H | 0 | 13·75 |
| W 3 A | 0 | 14·0 |
| W 3 I | 0 | 14·0 |
| W 3 J | 1 | 13·55 |
| W 3 B | 2 | 13·7 |
| W 3 C | 3 | 13·8 |
| W 3 E | 3 | 12·65 |
| W 3 G | 4 | 12·8 |
| W 3 D | 6 | 12·2 |

The single colony cultures (cf. Table VI) again showed no obvious discontinuous variation; most of them were avirulent or only slightly virulent. Examination of the colonies after the picking had been performed showed certain differences not unlike those observed at the second testing; in addition, however, two frankly rough variants, F and H, were observed. It seems possible, therefore, that the apparent gradation in virulence may have been illusory.

Reviewing this experiment, we see that at the start the control culture was of fairly high virulence, killing 16 mice in the larger and 12 mice in the smaller dose. In composition it consisted of about 50 per cent. of fairly highly virulent, and about 50 per cent. of rather lowly virulent organisms. After daily subculture in 1 per cent. bubbling oxygen for 3 months the culture showed a definite fall in virulence, the larger dose killing 8–10 mice and the smaller 0–6. Analysis of its composition showed that it contained organisms that varied from complete avirulence on the one hand to moderate virulence on the other. About $3\frac{1}{2}$ months after the commencement of the experiment, the 1 per cent. $O_2$ was replaced by 21 per cent. $O_2$; this change was followed in 3 weeks by a further fall in virulence; the larger dose now killed only 3 and the smaller dose only 1 mouse. At the close of the experiment 6 weeks after the commencement of the 21 per cent. oxygen the whole culture was almost completely avirulent; the culture consisted of organisms that varied from

### Table VII. *Virulence Exp.* 2.

| Date | Exp. No. | No. of mice injected | Dose | Agglutination Group | Type | Died | Specific deaths | Killed | Spleens infected | Total infected | Mean expectation of life in days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 9. II. 28 | WC 1 a | 20 | 732 | + + | + + | 16 | 16 | 4 | 4 | 20 | 10·0 |
| ,, | WC 1 b | 20 | 71 | + + | + + | 12 | 12 | 8 | 8 | 20 | 11·2 |
| ,, | W 1 A | 20 | 1392 | + + | + + | 5 | 5 | 15 | 15 | 20 | 12·35 |
| ,, | W 1 B | 20 | 1166 | — | + + | 7 | 7 | 13 | 13 | 20 | 11·75 |
| ,, | W 1 C | 20 | 1645 | — | + + | 4 | 4 | 16 | 15 | 19 | 12·9 |
| ,, | W 1 D | 20 | 1384 | — | + + | 6 | 6 | 14 | 14 | 20 | 12·25 |
| ,, | W 1 E | 20 | 1286 | — | + + | 4 | 4 | 16 | 16 | 20 | 13·3 |
| ,, | W 1 F | 20 | 1645 | + + | — | 12 | 12 | 8 | 8 | 20 | 10·0 |
| ,, | W 1 G | 20 | 1720 | — | + + | 13 | 13 | 7 | 6 | 19 | 10·0 |
| ,, | W 1 H | 20 | 1830 | + | + + | 17 | 17 | 3 | 3 | 20 | 8·85 |
| ,, | W 1 I | 20 | 2038 | — | + + | 16 | 16 | 4 | 4 | 20 | 9·45 |
| ,, | W 1 J | 20 | 1771 | + | + + | 18 | 18 | 2 | 2 | 20 | 8·1 |
| 24. II. 28 | WC 2 a | 20 | 930 | + | + + | 13 | 13 | 7 | 6 | 19 | 10·6 |
| ,, | WC 2 b | 20 | 105 | + + | + + | 13 | 13 | 7 | 7 | 20 | 9·45 |
| ,, | Control | 20 | 83 | + + | + + | 13 | 13 | 7 | 5 | 18 | 9·15 |
| 8. III. 28 | WC 3 a | 20 | 1004 | ± | + + | 18 | 18 | 2 | 2 | 20 | 7·05 |
| ,, | WC 3 b | 20 | 118 | + | + + | 17 | 17 | 3 | 3 | 20 | 7·5 |
| ,, | Control | 20 | 90 | + + | + + | 15 | 15 | 5 | 5 | 20 | 7·5 |
| 23. III. 28 | WC 4 a | 20 | 515 | + | + + | 19 | 19 | 1 | 1 | 20 | 10·3 |
| ,, | WC 4 b | 20 | 33 | + | + + | 18 | 18 | 2 | 2 | 20 | 10·75 |
| ,, | Control | 20 | 91 | + | + + | 18 | 16 | 2 | 2 | 18 | 8·95 |
| 12. IV. 28 | WC 5 a | 20 | 878 | + | + + | 13 | 13 | 7 | 6 | 19 | 9·45 |
| ,, | WC 5 b | 20 | 48 | + | + + | 9 | 9 | 11 | 9 | 18 | 11·75 |
| ,, | Control | 20 | 34 | + + | + + | 13 | 13 | 7 | 7 | 20 | 9·7 |
| 27. IV. 28 | WC 6 a | 20 | 1169 | + | + + | 9 | 8 | 11 | 11 | 19 | 13·35 |
| ,, | WC 6 b | 20 | 92 | + | + + | 1 | 0 | 19 | 17 | 17 | 14·0 |
| ,, | Control | 20 | 71 | + + | + + | 11 | 11 | 9 | 9 | 20 | 10·45 |
| 4. v. 28 | WC 7 a | 20 | 1244 | + | + + | 10 | 10 | 10 | 10 | 20 | 11·55 |
| ,, | WC 7 b | 20 | 117 | + | + + | 6 | 6 | 14 | 11 | 17 | 12·65 |
| ,, | Control | 20 | 1617 | + + | + | 11 | 11 | 9 | 9 | 20 | 10·05 |
| ,, | W 2 A | 20 | 1694 | + | + + | 5 | 5 | 15 | 13 | 18 | 12·95 |
| ,, | W 2 B | 20 | 1282 | + | + + | 10 | 10 | 10 | 9 | 19 | 12·7 |
| ,, | W 2 C | 20 | 1119 | + | + + | 1 | 1 | 19 | 19 | 20 | 13·8 |
| ,, | W 2 D | 20 | 1173 | + | + + | 7 | 6 | 13 | 13 | 19 | 12·05 |
| ,, | W 2 E | 20 | 1246 | + | + + | 11 | 11 | 9 | 8 | 19 | 10·2 |
| ,, | W 2 F | 20 | 1575 | + | + + | 0 | 0 | 20 | 20 | 20 | 14·0 |
| ,, | W 2 G | 20 | 1430 | + | + + | 12 | 12 | 8 | 8 | 20 | 11·35 |
| ,, | W 2 H | 20 | 1444 | + | + + | 12 | 12 | 8 | 8 | 20 | 10·75 |
| ,, | W 2 I | 20 | 1209 | + | + + | 7 | 7 | 13 | 12 | 19 | 11·75 |
| ,, | W 2 J | 20 | 1161 | + | + + | 8 | 7 | 12 | 12 | 19 | 12·7 |
| 15. v. 28 | WC 8 a | 20 | 1139 | + | + + | 12 | 12 | 8 | 8 | 20 | 9·75 |
| ,, | WC 8 b | 20 | 114 | + + | + + | 7 | 7 | 13 | 13 | 20 | 11·9 |
| ,, | Control | 20 | 92 | + + | + | 12 | 12 | 8 | 5 | 17 | 9·65 |
| 24. v. 28 | WC 9 a | 20 | 1114 | + | + + | 14 | 13 | 6 | 6 | 19 | 10·7 |
| ,, | WC 9 b | 20 | 48 | — | + + | 2 | 2 | 18 | 12 | 14 | 13·1 |
| ,, | Control | 20 | 36 | + + | + + | 9 | 9 | 11 | 9 | 18 | 10·7 |

21 % oxygen bubbling culture

| Date | Exp. No. | No. of mice injected | Dose | Agglutination Group | Type | Died | Specific deaths | Killed | Spleens infected | Total infected | Mean expectation of life in days |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 15. VI. 28 | WC 10 a | 20 | 788 | — | + + | 4 | 3 | 16 | 14 | 17 | 12·4 |
| ,, | WC 10 b | 20 | 41 | + + | + + | 2 | 1 | 18 | 3 | 4 | 13·15 |
| ,, | Control | 20 | 109 | — | + + | 11 | 11 | 9 | 8 | 19 | 9·6 |
| 4. VII. 28 | WC 11 a | 20 | 1262 | — | + + | 1 | 1 | 19 | 19 | 20 | 13·7 |
| ,, | WC 11 b | 20 | 120 | — | + + | 0 | 0 | 20 | 14 | 14 | 14·0 |
| ,, | Control | 20 | 114 | + + | + + | 15 | 15 | 5 | 4 | 19 | 7·0 |
| ,, | W 3 A | 20 | 1198 | — | + + | 0 | 0 | 20 | 20 | 20 | 14·0 |
| ,, | W 3 B | 20 | 891 | — | + + | 3 | 2 | 17 | 14 | 16 | 13·7 |
| ,, | W 3 C | 20 | 878 | — | + + | 3 | 3 | 17 | 14 | 17 | 13·8 |
| ,, | W 3 D | 20 | 923 | — | + + | 6 | 6 | 14 | 11 | 17 | 12·2 |
| ,, | W 3 E | 20 | 956 | — | + + | 3 | 3 | 17 | 9 | 12 | 12·65 |
| ,, | W 3 F | 20 | 610 | + | + + | 0 | 0 | 20 | 11 | 11 | 14·0 |
| ,, | W 3 G | 20 | 1031 | — | + + | 5 | 4 | 15 | 10 | 14 | 12·8 |
| ,, | W 3 H | 20 | 1031 | + | + + | 1 | 0 | 19 | 15 | 15 | 13·75 |
| ,, | W 3 I | 20 | 1284 | — | + + | 0 | 0 | 20 | 16 | 16 | 14·0 |
| ,, | W 3 J | 20 | 1268 | — | + + | 1 | 1 | 19 | 16 | 17 | 13·55 |

complete avirulence on the one hand to low virulence on the other. Since the last testing of single colony cultures there had been a replacement of the more virulent by less virulent organisms.

It is of importance to note that in this experiment the control culture was tested simultaneously with the experimental culture. Given in the lower dosage, that is about 50–100 organisms, it remained apparently constant in virulence; the highest number of mice it killed was 16 and the lowest 9—a difference well within the experimental error of testing. There seems to be no doubt, therefore, that the fall in the virulence of the experimental culture may be attributed to the peculiar conditions of subculture to which it was exposed.

The full protocols of Exp. 2 are recorded in Table VII.

### DISCUSSION.

On the basis of the experiments recorded in this paper we would put forward the following propositions:

1. A pure culture of *Bact. aertrycke* Mutton may contain variants showing markedly discontinuous variations in virulence, when tested by intraperitoneal inoculation of mice. It is important to understand that we are not dealing here with the phenomenon of roughness and smoothness—at least not in its generally accepted sense. The colonies that we studied were, with only two exceptions, of a smooth or finely granular type, and were often frankly indistinguishable from one another by the ordinary methods of examination. Side by side in the same culture were colonies which, when picked at random, were found to present marked differences in virulence. Observations of a similar nature were made by Amoss (1922). Working with the Mouse Typhoid ii strain, he found that of 6 single cell cultures from the same strain, 3 appeared to be more virulent than the other 3. One of these single cell strains—Mouse Typhoid ii A—he compared with the parent strain. Extracting the comparable figures from his tables, we find that he injected 80 mice intraperitoneally with each culture; the parent culture killed 65 mice in 100 hours and the single cell strain 51 mice. The observed difference between the two tests is 17·5 per cent.; this is 2·48 times the standard deviation—7·06 per cent.—and is therefore nearing the limit of significance. Amoss concludes that the results of these experiments "point to individual variation among the micro-organisms comprising the parent strain." With this conclusion we are in entire agreement.

2. When a given strain comprises organisms showing discontinuous variations in virulence, the virulence of the whole culture is similar to that of the most virulent variants. This proposition may be subject to quantitative limitations; our experiments were too few to indicate whether this is so or not. The second and third testings of single colony cultures in Exp. 1 (Charts 1 and 3) show that if even 10 per cent. or less of the constituent organisms are fully virulent, then the whole culture will itself prove fully virulent. What the lowest proportion of virulent organisms must be in order to insure the full virulence of the whole culture it is impossible to say; in any case it will

probably depend on the dosage. It is conceivable that 1 per cent. of virulent organisms might prove fatal in a dose of 1000, but not in 100. With the latter dose, owing to variations in sampling, it is almost certain that not every mouse in the batch would receive a single virulent organism; the results would therefore be irregular. We do not suggest that a single virulent organism is sufficient to cause death; quite possibly it is not. We do know, however, that a fully virulent culture often kills nearly every mouse in a dose of 30 bacteria (cf. Exp. 2, WC 4 b: Chart 5); we have met with several examples of this in experiments not recorded in this paper. It therefore seems likely that the number of highly virulent organisms necessary to cause death is a very small one. So long as the number of highly virulent organisms injected reaches or exceeds this "critical dose," as we may call it, the chances are that the mouse will die.

On purely *a priori* grounds it is conceivable that when a culture contains a high proportion of avirulent or only slightly virulent organisms along with a small proportion of fully virulent ones, the organisms of lower virulence may have a deterrent action on the multiplication of the fully virulent ones, or may actually exert some vaccinating effect on the animal into which they are injected. Since, in the subacute or chronic illness that generally follows inoculation of mice with comparatively small doses of *Bact. aertrycke* Mutton, death does not occur for from 3 to 14 days or so, there might be time enough for some degree of active immunity to develop. This possibility is strengthened by the evidence recently adduced by Topley and Greenwood and their associates (1928), that inoculation of mice with a relatively avirulent strain may afford them some protection against subsequent infection with a highly virulent strain. It is important to remember, however, in connection with these experiments, that the conditions were very different from those prevailing in our own work. It is true that the inoculation of the avirulent strain was made intraperitoneally, but subsequent infection with the virulent strain occurred by natural channels; moreover, there was a definite time interval separating the two infections. The two cases are not strictly analogous, and it may be that in order for an avirulent strain to protect against subsequent infection with a virulent strain, the time interval is of paramount importance. If, however, any vaccinating effect is operative when avirulent and virulent organisms are injected simultaneously, one would expect the deaths following inoculation of such a culture to be fewer than those following the inoculation of a culture consisting in the main of highly virulent organisms. This may be true, but there is nothing in our experiments definitely to indicate that it is so. For example, in Exp. 1 the whole culture killed nearly as many mice when over 90 per cent. of the constituent organisms were completely avirulent (WC 5), as when all the organisms were of moderate or full virulence (WC 1) (Chart 1). As this question is of some importance we decided to make an experiment to test it directly. Two agar slopes were seeded, one with a highly virulent culture *V*, and the other with an almost completely avirulent culture *Av*. They were incubated for 17 hours at 37° C., and were then washed off with

Ringer's solution, standardised to a given opacity, and diluted 1/500,000. Six batches of 20 mice were taken. Three batches, *A*, *B* and *D*, were inoculated intraperitoneally with varying doses of the virulent suspension; two batches, *C* and *E*, were inoculated with mixtures in varying proportions of the virulent and avirulent suspensions; the remaining batch, *F*, received the avirulent suspension only. The actual inoculum used was in all cases 0·5 c.c. A viable count was made on each suspension used for inoculation. The results are recorded in Table VIII.

### Table VIII.

| Exp. 3 | Date | No. of mice | Dose Virulent | Dose Avirulent | Agglutination Group | Agglutination Type | Died | Specific deaths | Killed | Spleens infected | Total infected | Mean expectation of life in days |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1. XII. 27 | 20 | 1849 | 0 | + | + + | 18 | 18 | 2 | 2 | 20 | 7·5 |
| B | ,, | 20 | 156 | 0 | | | 19 | 19 | 1 | 1 | 20 | 6·35 |
| C | ,, | 20 | 156 | 849 | | | 17 | 17 | 3 | 3 | 20 | 8·55 |
| D | ,, | 20 | 45 | 0 | | | 19 | 19 | 1 | 1 | 20 | 6·0 |
| E | ,, | 20 | 45 | 1021 | | | 16 | 16 | 4 | 3 | 19 | 8·35 |
| F | ,, | 20 | 0 | 992 | + | + + | 2 | 1 | 18 | 14 | 15 | 13·35 |

It will be seen that the virulent culture killed 18, 19 and 19 mice in doses of 1849, 156 and 45 organisms respectively. The culture, in fact, proved as virulent in the smaller doses as in the large dose. The avirulent culture in a dose of 992 organisms caused only one specific death. When virulent and avirulent cultures were mixed in the proportion of approximately 1 to 5, 17 mice died; when this proportion was reduced to 1 to 23, only 16 mice died. In each batch inoculated with the mixture of virulent and avirulent organisms the number of deaths was slightly reduced and the average expectation of life slightly increased. These differences, however, are clearly too small to be of significance. There is a mere suggestion that the effect of mixing avirulent with virulent organisms is to reduce the mortality rate, as compared with that due to inoculation of virulent organisms alone; but no definite conclusions can be reached from this single experiment. Possibly the vaccinating effect, if it does occur, is so weak that it could be determined with certainty only if experiments on very large numbers of mice were undertaken.

3. Whole cultures that are similar in virulence are not necessarily similar in constitution. This is merely a corollary to the previous proposition. It means to say that of two virulent whole cultures the one may consist almost entirely of fully virulent organisms, whereas the other may contain only a small proportion of such organisms. The virulence of the whole culture affords very little guidance to the virulence of the individual organisms that it contains, or to the proportion in which the different variants are present.

4. There is no apparent relationship between the possession of a type or group antigen and the degree of virulence of *Bact. aertrycke* Mutton. This has already been pointed out by Topley and Ayrton (1924). Reference to Tables III and VII will show that representatives of both varieties ranged from complete avirulence on the one hand to a high degree of virulence on the other.

5. There is no constant relationship between the virulence (power to kill) and the infectivity of a given strain of *Bact. aertrycke* when introduced directly into the tissues. It is true that, judged by the total number of infected mice in a given batch, highly virulent organisms are also highly infective; but the converse is not true. There are many examples in our experiments of strains that were completely or almost completely avirulent, and that yet proved highly infective. In Exp. 2, for example, strain W 2 F (Table VII) did not cause one specific death, yet at the end of a fortnight *Bact. aertrycke* was recovered from the spleen of every mouse in the batch.

6. Daily subculture of *Bact. aertrycke* Mutton from broth to broth with incubation at 37°, either under aerobic conditions or in an atmosphere of 1 per cent. or 21 per cent. oxygen maintained by bubbling gas continuously through the culture, results sooner or later in a complete fall in virulence. We do not wish here to discuss the optimum atmospheric conditions requisite for bringing about a fall in virulence; we hope to deal with this problem in a subsequent paper. But what we do wish to stress is that a complete fall in the virulence of this organism can be brought about by a simple process of subculture. The necessity for subculturing, we believe, lies in the opportunity it affords for growth and consequent variation. It seems probable that under a given set of conditions certain variants flourish more readily than others, with the result that one type of variant is gradually replaced in the culture by another type.

In maintaining the convertibility of a virulent into an avirulent strain, we are in disagreement with Webster (1923 a), who believes that, though different strains of this organism may vary in virulence, the virulence of any one strain remains unaltered and, so far as we understand him, unalterable. The reason for his adopting this position is not very clear. Following De Kruif (1921), he has himself shown (Webster, 1925) that a culture of *Pasteurella* may contain two types of variants, a D type, which is virulent, and a G type, which is avirulent. He has found that incubation under a low oxygen pressure favours the growth of the D form, and inhibits the D → G transformation. Moreover, he has recently succeeded (1927) in demonstrating variants of different virulence in cultures of *Bact. enteritidis*—an organism closely allied to *Bact. aertrycke*. He does not seem, however, to assign any importance to these variants in the natural spread of epidemic disease, or to believe that they are likely to arise in cultures kept under the usual laboratory conditions. Thus he says "the tests indicate that type-pure cultures from the common smooth colonies are of similar virulence, whether obtained from mice in epidemic or endemic periods, or from stock agar slants kept for 2 years at 4° C."

It is necessary to dwell on this difference, because it forms an essential part in the interpretation that Webster has put upon the results of experimental epidemics. He postulates that the virulence of *Bact. aertrycke* Mutton is fixed; and by choosing his mice of a given breed and weight, and by feeding them on a given dietary, he maintains that they too can be standardised. The

injection, therefore, of a given dose of organisms into a given number of mice produces the same effect; the reaction, in fact, is one between two standard reagents, and according to Webster the result is so constant that a standard curve can be plotted. He finds (1923 *b*) that when 100 mice are injected by the gastric route, 70 or 80 will die, 5 or 10 will become infected but will recover, and 20 or 30 will show no signs of infection at all. From this he concludes that 70–80 per cent. of the mice are highly susceptible, 5–10 per cent. moderately susceptible, and 20–30 per cent. completely resistant. This interpretation may be correct, but it is by no means necessarily so. It seems equally probable that the reaction of any given mouse to any given dose of organisms is influenced by so many small and uncontrollable factors that the result is determined by chance, and can be represented by a frequency curve.

The difficulty in deciding between these alternative hypotheses arises from the impossibility of carrying out repeated tests, of the same evidential value, on the same sample of mice. We know that if we toss 50 pennies a large number of times we shall, on the average, get 25 heads and 25 tails; but we know in this case that our result does not depend on the fact that 25 of the pennies are biased in the "heads" and 25 in the "tails" direction, but on the fact that each penny has an equal chance either way. We know this because we can take a single penny and by repeated tossings prove its absence of individual bias. We can, as Webster (1924) has done, reinoculate our survivors and show that they are now more resistant than normal animals, but we cannot tell whether this is because they were more resistant *ab initio*, or because they have been rendered more resistant by their previous experience. We do not doubt for a moment that mice vary in resistance—it would be surprising if they did not—our difficulty lies in assessing the relative weight to be attached to this variation among all the factors that determine death and survival. We do very seriously doubt whether it is possible to divide mice into such distinct categories as Webster has done, on the result of a single test. This doubt is increased by the fact that in long-continued epidemics of mouse typhoid almost every mouse whose fate can be definitely established does in the long run succumb to infection with *Bact. aertrycke*. It seems clear that resistance to natural infection is never absolute.

Let us suggest an analogy. Imagine the case of a golfer on a putting green which, instead of one hole, contains a hundred holes, arranged in a semi-circle at equidistant points. The golfer stands at the centre of the semicircle and makes a putt at each of the hundred holes in succession. We will presume either that he is a very good putter, or that the distance he stands from the hole is comparatively short. He therefore succeeds in holing his ball 70 times; 10 times he all but succeeds, and 20 times he misses the hole by a considerable distance. As we understand Webster, he would explain this result by assuming that the success of 70 of the putts depended on the peculiar receptivity of the particular holes that were aimed at; that the near approach to success of 10 of the putts was determined by a slight standoffishness, and the complete

failure of 20 of the putts by an unmistakable offensiveness on the part of the holes in question. In this analogy we are presenting a very simple case, and are presuming that the skill of the golfer remains constant throughout. In the corresponding experiment of injecting mice, the organisms injected are necessarily different. Even if they come from a single culture, they must present individual differences, and their reaction to the environment in which they are placed must vary correspondingly. The conclusion that Webster draws about the varying susceptibility of the mice seems to us to be unjustified. Let us repeat, we do not doubt that the resistance of individual mice does vary; but the view that it varies in the particular order and degree that Webster postulates is not in our opinion supported by adequate evidence. And unfortunately the crucial experiment to test it, namely the reinjection of the mice to ascertain if the result was the same as before, cannot be made because the mice are no longer normal animals.

The result obtained by the golfer, in our opinion, is determined by a multiplicity of small factors over which he has only imperfect control. The exact position at which his hands grasp the club, the delicate oculo-muscular coordination, the slope of the grass upon the green, the variation in the force and direction of the wind, and innumerable factors must play a part in deciding the success or failure of his putt. The result, that is to say, is determined by chance, and if plotted graphically would conform to an ordinary skew curve. The chances of success or failure are, of course, weighted by the skill of the golfer, but the final result is nevertheless determined to a considerable extent by chance. In a second series of putts the successes might total only 60, and in a third series they might reach 80, even though all the conditions that could be controlled were kept constant, and the skill of the golfer remained unchanged. The average figure 70, therefore, cannot be regarded as representing anything more than the result of the interaction of all the numerous factors concerned. From a single series of putts it is clearly unjustifiable to come to any conclusion about the possible existence of variations in size of the holes upon the green, much less about the order in which these possible variations are arranged. In the mouse-inoculation experiment there are similarly numerous factors that cannot be perfectly controlled. The amount of trauma inflicted by the gastric catheter, the quantity of suspension passing through the pylorus, the amount of food in the stomach, the acidity of the gastric contents, the rate at which bile is excreted, the absorptive capacity of the intestinal mucosa, the activity of the mesenteric circulation, the degree of peristaltic motion, the numbers and types of macro- and microphages available for defence, and numerous other factors which, while perhaps in themselves unimportant, are, when combined, responsible for determining the actual outcome of the infection. These factors are all subject to temporary disturbance, and it is for this reason that we doubt whether the survival of a mouse in any given experiment can be ascribed, as Webster apparently believes, to an inherent and permanent resistance rather than

*to the favourable combination of a number of factors liable to periodic fluctuation.*

The evidence we have brought forward in this paper merely helps to reveal the extraordinary complexity of a single factor in the mouse inoculation experiment. It is clearly wrong to regard a whole culture as a collection of homogeneous organisms. Any given culture consists of an extremely large number of individual organisms, which vary in one or more respects. This population is liable to serious fluctuations, and the proportions in which the *different types of organisms are present is variable from time to time. Certain* environmental conditions may so favour the growth of some of the types as to lead to their preponderance in the culture, and possibly to the ultimate displacement of all other types. Since these types differ in their virulence to mice, it is not surprising that the virulence of the whole culture is subject to alteration. The evidence we have before us suggests, however, that the replacement of a virulent by an avirulent type is a comparatively slow process, and therefore unless direct *ad hoc* experiments are made to determine the constitution of the culture, there may be a superficial appearance of constancy over a considerable period of time.

It would, of course, be quite unjustifiable to assume in the absence of adequate experimental data that the variations in virulence which have been demonstrated *in vitro* have any significance in the spread of natural infection. This problem still awaits final solution; and the observations that Webster has so far recorded yield no support to the view that such variations play a significant part in the epidemic spread of disease.

### Summary and Conclusions.

A given strain of *Bact. aertrycke* Mutton has been tested repeatedly for its virulence to mice, and on some of these occasions the virulence of 10 single colony cultures taken from this strain has likewise been tested. Between these single colony cultures such marked differences in virulence have been found as to constitute definite discontinuous variations. Side by side in the same culture there have been found virulent and avirulent organisms. Daily sub-culture in broth under certain atmospheric conditions resulted in the fall in virulence of the whole culture; this was accompanied by a replacement of the virulent organisms by organisms that were either completely avirulent or were only weakly virulent. The evidence suggests that the fall in virulence of the whole culture is not due to a simultaneous fall in the virulence of each of its constituent organisms, but to a replacement of the highly virulent organisms by organisms of a lower degree of virulence. During the process of replacement two or three different variants, showing discontinuous variations in virulence, may be demonstrated together in the same culture. The conclusions to be drawn from these findings, and their bearing on the interpretation of the results of experimental epidemiology, are discussed.

We would express our thanks to the Government Grant Committee of the Royal Society for financial aid in part of this investigation.

## REFERENCES.

AMOSS, H. L. (1922). *J. Exp. Med.* **36**, 25.
ARKWRIGHT, J. A. (1921). *J. Path. and Bact.* **24**, 36.
DE KRUIF, P. H. (1921). *J. Exp. Med.* **33**, 773.
LOCKHART, L. P. (1926). *J. Hygiene,* **25**, 50.
PRITCHETT, IDA W. (1926). *J. Exp. Med.* **43**, 161.
TOPLEY, W. W. C. and AYRTON, JOYCE (1924). *J. Hygiene,* **23**, 198.
TOPLEY, W. W. C., GREENWOOD, M., WILSON, J. and NEWBOLD, E. M. (1928). *Ibid.* **27**, 396.
WEBSTER, L. T. (1923 *a*). *J. Exp. Med.* **38**, 33 and 45.
—— (1923 *b*). *Ibid.* **37**, 231.
—— (1924). *Ibid.* **39**, 129.
—— (1925). *Ibid.* **41**, 571.
WEBSTER, L. T. and BURN, C. (1927). *Ibid.* **46**, 887.
WILSON, G. S. (1926). *J. Hygiene,* **25**, 150.
—— (1927). *Ibid.* **26**, 170.

21–2