THE RELATION BETWEEN THE AGE AND THE VIRULENCE OF CULTURES OF *B. AERTRYCKE* (MUTTON).

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(With one Chart.)

Working with *Streptococcus haemolyticus*, Felty and Bloomfield (1924) concluded from intraperitoneal injection of mice that organisms in the logarithmic and stationary phases of reproduction are more virulent than in the decline phase. A careful examination of their protocols leads one to doubt the justice of this conclusion. In the three experiments that are related, the numbers of mice receiving similar doses are 6, 4 and 2 respectively—numbers that we consider inadequate to serve as a basis of comparison. The method of estimating the virulence of the organisms according to their minimal lethal dose is likewise open to fallacy. For a full discussion of this subject the reader is referred to a paper by Lockhart (1926).

Felty and Bloomfield, moreover, assert that at the commencement of the decline phase there is a sudden fall in virulence. For this, again, the evidence is unsatisfactory. In one experiment a culture 26 hours old that proved virulent in a certain dose is said to be in the stationary, and another of the same age that proved avirulent in a similar dose is said to be in the decline phase. As no growth curve was worked out for each culture, it is difficult to understand on what basis this statement rests.

Their results are open to a final objection. Not one of their experiments was repeated under the same conditions. Either a different strain was used, or it was passed through a mouse to raise its virulence, or some other disturbing feature was introduced. We are therefore led to conclude that though, on the whole, the evidence relating the virulence of a culture to its phase of reproductive activity is suggestive, it is inadequate to serve as a proof of this contention.

In the work that we are about to describe we have taken up this problem, endeavouring to avoid some of the errors to which the experiments of Felty and Bloomfield were exposed. In particular we have replaced, as our standard of comparison, the minimal lethal dose by the percentage mortality amongst samples of 20 mice receiving comparable doses.

The Virulence of Broth Cultures of B. aertrycke (*mutton*).

Exp. 1. A flask of 50 c.c. of casein digest broth, previously warmed to 37° C., was inoculated with 10 drops of a 17-hour broth culture at 20° C. of *B. aertrycke* (mutton). The flask was incubated at 37° C. and at varying

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intervals samples of the culture were withdrawn. These were counted by the roll-tube method (Wilson, 1922) and were used after preliminary dilution for the intraperitoneal inoculation of mice. Of the four consecutive hundredfold dilutions that were made, 0·5 c.c. was injected into four batches of five mice. The primary dilution was so adjusted as to obtain as close an approximation as possible to the doses given in the different parts of the experiment. After injection the mice that died were examined in the usual way by cultivation of the heart and spleen upon MacConkey plates, with subsequent identification of the organisms by type and group agglutinating sera. All mice that were alive at the end of 14 days were killed, their spleens being transferred to broth to ascertain if they were specifically infected. Chart I shows the growth curve of the culture, and Table I the resulting mortality of the mice.

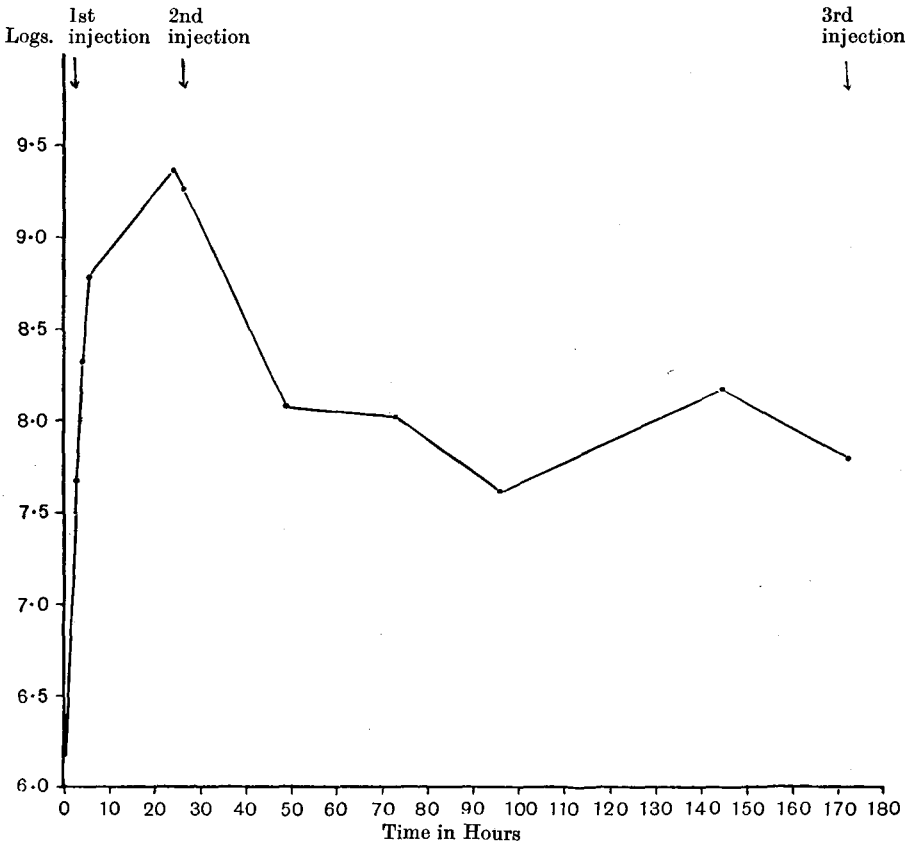

Chart I. Curve showing numbers of Viable Bacilli in Experiment 1.

The growth curve (Chart I) shows that the first series of injections was made during the logarithmic phase of growth, the second just after growth had reached its maximum, and the third during the phase of gradual decline. The mortality of the mice dying in each part of the experiment, 16, 18 and 18, reveals no alteration in the virulence of the culture over a period of 172 hours.

Table I.

A. 2 *hrs.* 40 *min. culture.*

No. of mice injected	Dose of viable organisms	Specific deaths	Killed	Spleens infected
5	22,000,000	5	0	0
5	220,000	5	0	0
5	2,200	3	2	2
5	22	3	2	2
20		16	4	4

B. 26-*hour culture.*

5	41,000,000	5	0	0
5	410,000	5	0	0
5	4,100	4	1	1
5	41	4	1	1
20		18	2	2

C. 172-*hour culture.*

5	17,000,000	5	0	0
5	170,000	5	0	0
5	1,700	5	0	0
5	17	3	2	0
20		18	2	0

The Virulence of Agar Cultures of B. aertrycke (*mutton*).

Exp. 2. The previous experiment was repeated on agar cultures, using such modifications as were necessitated by the change in medium. An $18\frac{1}{2}$ hour broth culture at 20° C. was used for seeding three nutrient agar slopes, one loopful being spread over each slope. After incubation at 37° C. for 5 hours, one of the cultures was withdrawn, the growth washed off with sterile Ringer's solution, and standardised by opacity to a strength of 500 million organisms per c.c. On this suspension a count of viable organisms was made. For purposes of injection it was diluted one-third, and from this 100-, 10,000- and 1,000,000-fold dilutions were made. The mice were inoculated intraperitoneally as before with 0·5 c.c. of these dilutions. A similar procedure was repeated with the other two agar cultures at 24 and 168 hours after seeding, the only difference being a variation in the primary dilution of the 500 million suspension to enable approximately similar numbers of bacilli to be given in each part of the experiment. The results are shown in Table II.

From a series of counts that we made at this stage on agar cultures put up under conditions similar to those used in this and in the following experiments, we found that from 4 to 9 hours after inoculation the organisms are in the logarithmic phase of reproduction, and that after this they progressively diminish in numbers till the culture becomes sterile. As the method by which these counts were made requires some explanation, we have reserved its description for another paper (Wilson, 1926).

The results of Exp. 2 suggest that the rapidly growing 5-hour culture, which killed 17 mice, was more virulent than the declining 168-hour culture, which killed only 11 mice. Statistically, however, this difference is insufficient

Table II

A. 5-*hour culture.*

No. of mice injected	Dose of viable organisms	Specific deaths	Killed	Spleens infected
5	17,000,000	5	0	0
5	170,000	5	0	0
5	1,700	5	0	0
5	17	2	3	3
20		17	3	3

B. 24-*hour culture.*

5	21,000,000	5	0	0
5	210,000	5	0	0
5	2,100	4	1	1
5	21	1	4	2
20		15	5	3

C. 168-*hour culture.*

5	18,000,000	5	0	0
5	180,000	3	2	2
5	1,800	2	3	3
5	18	1	4	1
20		11	9	6

to be significant. It is, moreover, invalidated by the circumstance that four of the remaining C mice were dying when they were killed. Had the experiment been continued for another day, the mortality of these mice would have been 15 instead of 11, thus approximating closely to that of the A mice.

Exp. 3. Counts of the total and viable organisms made on agar cultures showed that at the end of a week so many organisms had died that, in order to give sufficient living bacilli to render the doses comparable with those given in the earlier parts of the experiment, it was necessary to include large numbers of dead bacilli. It was thought possible that the simultaneous injection into mice of these dead organisms might have a toxic, or—less probably —a vaccinating, effect on the mice, and so interfere with the strict comparison between the virulence of organisms of different ages that we desired to make. An experiment was therefore planned in which the three series of mice should be injected with approximately equal numbers both of living and of dead bacilli. For this purpose a suspension of organisms in Ringer's solution was prepared by washing off several agar slope cultures of *B. aertrycke* (mutton) that had been incubated for a week at 37° C. and killed by heating at 55° C. for an hour. The number of bacilli was ascertained by counting on the Helber slide (Wilson, 1922). A calculated quantity of this suspension was then added to the suspension of the 5-hour and the 24-hour cultures, on each of which a total and viable count was made, so that the number of living and of dead organisms inoculated should be known with certainty. The results are shown in Table III.

Table III.

A. 5-*hour culture.*

No. of mice injected	Dose of dead organisms	Dose of viable organisms	Specific deaths	Killed	Spleens infected
5	420,000,000	16,000,000	5	0	0
5	4,200,000	160,000	5	0	0
5	42,000	1,600	3	2	2
5	420	16	2	3	3
20			15	5	5

B. 24-*hour culture.*

5	510,000,000	18,000,000	5	0	0
5	5,100,000	180,000	5	0	0
5	51,000	1,800	4	1	1
5	510	18	3	2	1
20			17	3	2

C. 168-*hour culture.*

5	650,000,000	15,000,000	5	0	0
5	6,500,000	150,000	5	0	0
5	65,000	1,500	4	1	1
5	650	15	2	3	1
20			16	4	2

If we compare the mortality of mice in Exp. 2, A and B, with that of mice in Exp. 3, A and B, we find that the addition of the dead organisms in the latter experiment made apparently no difference to the results. This is in accordance with our expectations. The five mice receiving the highest dosage die partly of toxaemia caused by the dead organisms, but in those receiving smaller doses the number of dead organisms is too small to exercise this effect. Nor is there any evidence of a protective action. Their presence can therefore be neglected. Amongst the C mice in Exp. 3, 16 out of 20 died, showing that the lower mortality amongst the corresponding mice in Exp. 2 was probably independent of any alteration in the virulence of the organisms.

Exp. 4. This was a replica of Exp. 2. The results are given in Table IV.

Table IV.

A. 5-*hour culture.*

No. of mice injected	Dose of viable organisms	Specific deaths	Killed	Spleens infected
5	7,000,000	5	0	0
5	70,000	4	1	1
5	700	4	1	1
5	7	3	2	2
20		16	4	4

B. 24-*hour culture.*

5	18,000,000	5	0	0
5	180,000	5	0	0
5	1,800	2	3	3
5	18	3	2	2
20		15	5	5

C. 168-*hour culture.*

5	10,000,000	5	0	0
5	100,000	4	1	1
5	1,000	3	2	2
5	10	5	0	0
20		17	3	3

The mortality of the three sets of mice A, B and C, is 16, 15 and 17 respectively. There is no evidence to suggest any fall in the virulence of the culture corresponding to its increasing age.

Table V summarises the results of all four experiments, which can be conveniently grouped together.

Table V. *Mortality of Mice in Exps.* 1 *to* 4.

Age of culture in hours	No. of mice injected	Specific deaths	Mortality %	Killed	Spleens infected
2¾ to 5	80	64	80·0	16	16
24 „ 26	80	65	81·2	15	11
168 „ 172	80	62	77·5	18	11

The percentage mortality of the three sets of mice is so close as to be surprising. It is noticeable however that of the surviving A mice all were specifically infected, whereas four of the B and seven of the C mice showed no evidence of infection. Whether this is due to a mere chance distribution of the resistance of the individual mice or to some difference in the invasive property of the organisms, it is impossible to say. We feel, however, justified in concluding from these experiments that, judging by the mortality amongst mice injected with similar doses of *B. aertrycke* (mutton) in different stages of reproductive activity, there is no evidence to suggest that any diminution of virulence occurs during the first week of growth, either in broth or in agar cultures.

Survival Time in Mice after Intraperitoneal Inoculation with Cultures of Different Ages.

Felty and Bloomfield (1924) asserted that not only did a young, actively growing culture of pneumococcus prove fatal to mice in smaller numbers than an older culture in the decline phase, but that the survival time of mice dying subsequently to injection was shorter with the younger than with the older culture. Though the evidence adduced for this contention was extremely meagre, it was thought advisable to ascertain from our experiments whether any prolongation of life was noticeable amongst the mice receiving the older cultures. For this purpose the average survival time of the mice dying within a fortnight has been calculated for each experiment. As the results are similar in each of the four experiments, they are summarised in Table VI.

Table VI. *Average Survival Time of Mice Dying after Injection in Exps.* 1 *to* 4.

	Survival time in days after			
Batch	Highest dose	100-fold dilution	10,000-fold dilution	1,000,000-fold dilution
A	1·5	6·0	9·2	8·2
B	1·4	6·0	7·4	8·7
C	1·4	7·4	7·2	9·4

It will be noticed that the average survival time of mice injected with the highest dose—generally about 10–20 million living bacilli—is 36 hours or less, whereas the survival time of mice receiving smaller doses is from 6–9 days. The difference between the mice receiving the highest dose and those receiving the next highest dose is far greater than that between the three sets receiving the lower doses. In fact it seems of little importance whether the mouse is injected with 20 bacilli or 200,000; in either case its average time of survival will be 6–9 days: but it is of great importance whether it is injected with 200,000 or 20 million bacilli; in the former case it will live for 6–9 days, in the latter for not more than 36 hours. This difference indicates that the cause of death in these two sets of mice is not the same. It suggests that the mice dying after the largest dose succumb to acute septicaemia, whereas those dying after the lower doses succumb to a subacute form of disease. A similar conclusion has already been reached by Lockhart (1926).

The figures in Table VI for the three groups of mice are so similar as to lend no support to the suggestion that organisms in the decline phase show a lag period when growing not only *in vitro*, but also *in vivo*.

Discussion.

The results obtained in this work are contradictory to those of Felty and Bloomfield. Arguing from them we do not, however, for a moment deny the correctness of these authors' findings, but we do consider that they stand all the more in need of support. It is possible, and indeed probable, that the factors governing the virulence of the *Streptococcus* and the *Pneumococcus* for mice are quite different from those operating in the case of an organism that is naturally pathogenic for these animals, such as *B. aertrycke* (mutton). This has already been pointed out by Webster (1925), who found no parallel to the conditions described by Felty and Bloomfield in such natural infections as mouse typhoid or rabbit snuffles and pneumonia. "On the contrary," he asserts, "in these infections equal numbers of virulent bacteria, incubated two hours or several days, induce similar responses, and the same quantities of avirulent organisms, a few hours or several months old, behave quite the same in the animal body." Webster concludes that there is no experimental evidence to support the hypothesis of a geometric rise and fall of bacterial virulence. With this conclusion we agree.

Summary and Conclusions.

(1) Four batches of 60 mice have been injected with comparable doses of *B. aertrycke* (mutton), taken from broth or agar cultures in different phases of reproductive activity.

(2) The mortality and the average survival time of the mice after injection varied so little amongst the different groups as to justify the conclusion that

no difference in the virulence of this organism is detectable during the first week of growth.

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