THE EFFECT ON THE VIRULENCE OF *BACT. AERTRYCKE* OF CULTIVATION IN ATMOSPHERES CONTAINING VARYING PROPORTIONS OF OXYGEN.

By G. S. WILSON.

(*From the London School of Hygiene and Tropical Medicine.*)

(With 12 Text-figures.)

Working with *Bact. aertrycke*, Lockhart (1926) obtained evidence that daily sub-cultivation in broth under aerobic conditions led sooner or later to a fall in the virulence of this organism for mice. Repeated intraperitoneal passage through mice, on the other hand, resulted either in no change, or in a significant increase in virulence. It appeared to me possible that the difference in these results might be determined, to some extent at least, by the dissimilar oxygen pressures to which the organisms were exposed during growth. The present paper records a series of experiments in which an attempt was made to determine the effect, on the virulence of *Bact. aertrycke*, of cultivation in atmospheres containing varying proportions of oxygen.

Technique.

Throughout this investigation a single strain of *Bact. aertrycke* was employed, which was isolated from a mouse epidemic about the year 1924. For two years or so after its isolation it was kept in narrow stab agar tubes and sub-cultured from time to time; for the last three years it has been sub-cultured at monthly intervals, the culture being incubated for 18 hours at 37° C., and then stored in a dark cupboard at room temperature. When a virulence test was to be carried out on this control strain, a sub-culture was made in 5 c.c. of casein digest broth, contained in a $\frac{5}{8}$ in. test-tube; after 24 hours at 37° C. the culture was diluted with sterile Ringer's solution till there were approximately 100 viable bacilli in 0·5 c.c. of the suspension; this quantity was then inoculated intraperitoneally into twenty parti-coloured mice of about 17–23 grm. in weight. Counts of the viable bacilli were made on the actual suspension used for inoculation. All mice that died during the next fortnight were examined *post mortem*, cultures being taken from the heart's blood and spleen; mice that survived a fortnight were killed, and spleen cultures taken. No mouse was considered to have been specifically infected with *Bact. aertrycke* unless this organism was isolated from the tissues and identified by agglutination. During the three years Oct. 1926–Oct. 1929, fifty-four virulence tests were made on this control strain; the average number of specific deaths resulting was 12·71 ± 3·39.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

In the experimental series of sub-cultures this strain was grown in broth, *p*H 7·4–7·6, at 37° C. for 24 hours, and then transferred to a fresh tube of medium. Except in the aerobic series, in which 6 × $\frac{5}{8}$ in. tubes containing 5 c.c. of broth were used, the cultures were made in 6 × $\frac{3}{4}$ in. tubes containing 7 c.c. of broth. A fresh sub-culture was made every day, except on Sundays, and each experiment was continued, as a rule, till the strain had been carried through about ninety passages; some experiments, however, were continued for a year or more. Virulence tests were carried out at intervals, by injection of about 100 viable bacilli intraperitoneally into a batch of twenty mice.

For anaerobic cultivation the platinum black method, originally described by Laidlaw (1915), was employed.

For cultivation in atmospheres containing varying proportions of oxygen an apparatus was devised in which the gaseous mixture to be employed was made up in a gas jar, and driven over by water pressure into the tube containing the broth culture, which was incubated in a water bath held at 37° C. by a thermostat. The gas was led into the culture through a fine capillary tube reaching to the bottom of the test-tube, and was bubbled slowly through the medium. The escaping gas was conducted through a delivery tube, which was in connection with a Haldane gas analysis apparatus. Gaseous mixtures containing less than 21 per cent. of oxygen were made by mixing air with nitrogen from a cylinder; mixtures containing more than 21 per cent. of oxygen were made by mixing cylinder oxygen with cylinder nitrogen. The gases were not purified, but were always passed through 10 per cent. KOH solution to remove the CO_2. The average amount of gas bubbled through the culture was about 5 litres a day, except over the week-ends, when only half this quantity *per diem* was used. The apparatus worked very satisfactorily, and was capable of running for 48 hours without attention.

Interpretation of Results.

The determination of differences in virulence between the experimental and the control cultures has proved very much more difficult than was originally anticipated. It was thought that the information desired would be gained by performing four or five virulence tests at suitable intervals on the experimental culture, and comparing the results by the usual standard deviation of the difference method with the average virulence of the control culture. Unfortunately it was assumed that the virulence of the control culture would not be subject to more than minor variations, and it was therefore expected that occasional tests would be satisfactory for estimation of the mean virulence. Before the work had progressed very far it was realised that this assumption was unjustified. Marked variations in the apparent virulence of this strain were encountered from time to time, rendering it evident that, for the accurate comparison of the control and the experimental cultures, virulence tests would have to be conducted simultaneously. It appears probable that the variation in the percentage mortality following the inoculation of the control

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

strain was due not so much to an alteration in its virulence as to fluctuations in the susceptibility of the mice; evidence for this contention has been brought forward as the result of tests carried out over a period of three years (Wilson, 1930 *b*).

The data collected previous to the time at which the experimental and the control cultures were tested simultaneously are not so well suited for analysis as those collected subsequently, and they have had to be treated by a rather different method. The problem of determining the effect of an experimental procedure on the virulence of a strain, when samples of less than thirty animals are employed, may be put briefly as follows:

Given a small number of tests on the experimental culture a and a small number of tests on the control culture b, it is desired to test the significance of the difference between the a and b results in the following cases:

Case I. When the a and b experiments are available in pairs.

Case II. When pairs are available as before, but in addition there is greater knowledge of one of the universes, *e.g.* a.

Case III. When pairs are not available, but only scattered and non-corresponding samples from the two universes.

Case I has been treated by "Student's" method as modified by Fisher (1928, pp. 105 and 106). Suppose that there are N pairs of observations on a and b; then there will be N differences between each a and its corresponding b. Let x denote any one of the differences. The mean of these differences is ascertained from the formula

$$\bar{x} = \frac{\Sigma(x)}{N},$$

where Σ = the sum of all the N values of x. The square of the standard deviation of the sample of N differences is then found from the formula

$$s^2 = \frac{\Sigma(x - \bar{x})^2}{N - 1},$$

and the value of t is calculated from the formula

$$t = \frac{\bar{x}\sqrt{N}}{s}$$

(Fisher's t = "Student's" z multiplied by the square root of the number in the sample = $z \times \sqrt{N}$).

Using Fisher's table, and taking $n = N - 1$, the value of P corresponding to the ascertained value of t is found. P, it will be noted, gives the probability of a difference of either sign as large as, or larger than, this arising by chance. For example, $P = 0{\cdot}01$ means that so large a difference could be expected only once in 100 trials by pure chance. In this paper, values of P less than 0·05 are regarded as significant. The usual criterion of three times the probable error is equivalent to $P = 0{\cdot}043$.

Case II has been treated in the same way as Case I, the additional knowledge of one of the universes being neglected. The reason for this is that the

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

standard deviation of the differences depends on the correlation between the a's and the b's, as well as on the separate variations of the a's and the b's; and since this correlation can be found only from the pairs, it is not of much help to bring in the extra knowledge of the variation of a in circumstances that may be different.

Case III has been treated by finding the standard deviation of the mean difference, on the hypothesis that there is no correlation between the a's and the b's (Fisher, 1928, pp. 107, 108). This method will probably, and, if correlation does exist certainly, give too large a value for the variation of the difference; hence a difference that is really significant may not appear to be so. Suppose that there are N_1 a's and N_2 b's. The mean of the a's is found from the formula

$$\bar{a} = \frac{\Sigma(a)}{N_1},$$

and the mean of the b's from the formula

$$\bar{b} = \frac{\Sigma(b)}{N_2},$$

where Σ means the sum of all values.

The square of the standard deviation is found from the formula

$$s^2 = \frac{\Sigma(a-\bar{a})^2 + \Sigma(b-\bar{b})^2}{N_1 + N_2 - 2},$$

and t from the formula

$$t = \frac{\bar{a}-\bar{b}}{s}\sqrt{\frac{N_1 N_2}{N_1 + N_2}}.$$

Taking $n = N_1 + N_2 - 2$, the value of P corresponding to the value of t is ascertained from Fisher's table.

It may be reasonably objected that the method of treatment adopted here is not strictly applicable to the cases in question, since this method is based on the assumption that the causes responsible for producing a difference between the two universes are effective throughout the whole series of observations. In point of fact, however, the differences between the control and the experimental cultures did not always become obvious till the latter part of the experiment. If it is desired to ascertain whether a significant decrease or increase in virulence of the test cultures did actually occur, it is clear that the pairs selected for comparison should be taken from observations made subsequent to the appearance of the first definite difference; otherwise the observations of the later part of the experiment will be largely counterbalanced by those of the earlier part, and a truly significant difference may be missed.

On examination of the Charts reproduced in this paper it will be seen that this method of treatment is likely to lead to an erroneous result in only one particular case—Exp. 2 of the 1 per cent. oxygen series. An additional computation has, therefore, been made in this experiment, using the observations made after the first definite fall in virulence had occurred.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

Experimental Work.

A. *Daily sub-cultivation under anaerobic conditions.*

Exp. 1. Commenced on 16. i. 26 and terminated on 10. iii. 26. The results are given in Table I. Since no observations were made on the control strain, it is impossible to do more than conclude that there was no evidence of a decrease in virulence under anaerobic conditions during a period of sub-cultivation lasting for about two months. It may be noted, however, that in a parallel experiment, in which the strain was cultivated aerobically, there was a very marked fall in virulence (see Table VIII).

Table I.

Anaerobic culture. Exp. 1.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days up to 14 days
4. ii. 26	16	105	14	**14**	6	5	19	9·9
24. ii. 26	33	52	16	**16**	4	1	17	8·6
10. iii. 26	45	44	17	**16**	3	3	19	7·6
Arithmetic mean		67	—	**15·3**	—	—	—	8·7

Exp. 2. Commenced 14. x. 26 and terminated on 1. xii. 26. The results are given in Table II. The average number of specific deaths caused by the anaerobic culture was 15·2, by the control culture 12·0. P lies between 0·2 and 0·1; the increase in virulence of the anaerobic culture cannot therefore be regarded as significant.

Table II.

Anaerobic culture. Exp. 2.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
19. x. 26	4	67	14	**14**	6	5	19	7·95
28. x. 26	12	39	15	**15**	5	4	19	10·2
4. xi. 26	17	47	13	**13**	7	5	18	9·65
18. xi. 26	29	46	19	**19**	1	0	19	6·5
1. xii. 26	40	53	15	**15**	5	3	18	7·65
Arithmetic mean		50	—	**15·2**	—	—	—	8·39
Control culture.								
28. x. 26	—	91	7	**7**	13	11	18	11·75
4. xi. 26	—	50	16	**15**	4	4	19	9·0
18. xi. 26	—	55	13	**13**	7	6	19	9·8
30. xi. 26	—	30	13	**13**	7	5	18	9·25
Arithmetic mean		56	—	**12·0**	—	—	—	9·95

Treated as Case I, $P = <0{\cdot}2$ and $>0{\cdot}1$.

Exps. 3, 4. These two experiments, which were conducted simultaneously, were commenced on 13. vi. 28 and terminated on 26. ix. 29. The results are given in Table III and Fig. 1. The average number of specific deaths caused by the anaerobic culture in Exp. 3 was 13·3, in Exp. 4 13·4, and by the control

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

strain 13·4. P in each case approximates to unity, showing that there is no evidence of either a rise or a fall in the virulence of the anaerobic cultures.

Table III.

Anaerobic culture. Exp. 3.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
19. vii. 28	30	32	18	**18**	2	2	20	5·45
1. viii. 28	41	20	11	**11**	9	6	17	8·9
15. viii. 28	53	185	19	**19**	1	1	20	7·1
2. x. 28	93	375	11	**11**	9	9	20	10·25
17. x. 28	105	87	14	**13**	6	5	18	10·7
30. x. 28	117	128	5	**3**	15	10	13	12·45
20. xii. 28	160	223	16	**16**	4	4	20	7·5
21. i. 29	188	173	12	**12**	8	8	20	8·95
26. ii. 29	218	123	8	**8**	12	10	18	10·65
26. iii. 29	242	197	17	**17**	3	3	20	6·85
16 iv. 29	260	97	8	**8**	12	12	20	11·05
28. v. 29	297	184	15	**15**	5	5	20	9·55
13. viii. 29	362	102	18	**18**	2	1	19	5·95
26. ix. 29	400	245	17	**17**	3	3	20	6·2
Arithmetic mean		155	—	**13·3**	—	—	—	8·68

Anaerobic culture. Exp. 4.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
19. vii. 28	30	65	17	**17**	3	3	20	4·95
1. viii. 28	41	178	10	**10**	10	7	17	9·35
15. viii. 28	53	177	19	**19**	1	1	20	5·35
2. x. 28	94	405	11	**11**	9	9	20	10·4
17. x. 28	106	126	14	**14**	6	6	20	7·15
30. x. 28	117	120	8	**5**	12	10	15	11·85
20. xii. 28	160	37	17	**17**	3	3	20	6·95
22. i. 29	188	183	15	**15**	5	4	19	7·6
26. ii. 29	218	157	13	**13**	7	7	20	8·6
26. iii. 29	242	176	10	**10**	10	10	20	10·3
16. iv. 29	260	111	9	**8**	11	11	19	11·35
28. v. 29	297	316	15	**15**	5	5	20	8·85
13. viii. 29	362	54	19	**19**	1	1	20	5·2
26. ix. 29	400	390	16	**15**	4	4	19	6·05
Arithmetic mean		178	—	**13·4**	—	—	—	8·14

Control culture.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
13. vi. 28	—	109	11	**11**	9	8	19	9·6
19. vii. 28	—	88	16	**16**	4	4	20	6·55
1. viii. 28	—	109	12	**12**	8	7	19	9·15
15. viii. 28	—	118	15	**15**	5	4	19	8·0
2. x. 28	—	157	13	**13**	7	7	20	10·1
16. x. 28	—	85	17	**17**	3	3	20	7·1
30. x. 28	—	108	13	**12**	7	6	18	9·0
20. xii. 28	—	118	18	**18**	2	2	20	7·85
22. i. 29	—	147	14	**14**	6	6	20	8·65
26. ii. 29	—	97	6	**6**	14	13	19	12·4
26. iii. 29	—	163	14	**14**	6	6	20	8·65
16. iv. 29	—	136	8	**8**	12	11	19	11·45
28. v. 29	—	60	14	**14**	6	5	19	9·2
13. viii. 29	—	89	20	**20**	0	0	20	5·4
26. ix. 29	—	48	9	**9**	11	9	18	9·85
Arithmetic mean		109	—	**13·4**	—	—	—	8·86

Treated as Case I, for Exp. 3, $P = <0\cdot9$ and $>0\cdot8$.

„ „ Exp. 4, $P = >0\cdot9$.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

Summary. Four experiments were performed, two of them lasting for over 15 months. The results showed that, when sub-cultured daily under anaerobic conditions, the organisms remained approximately constant in virulence.

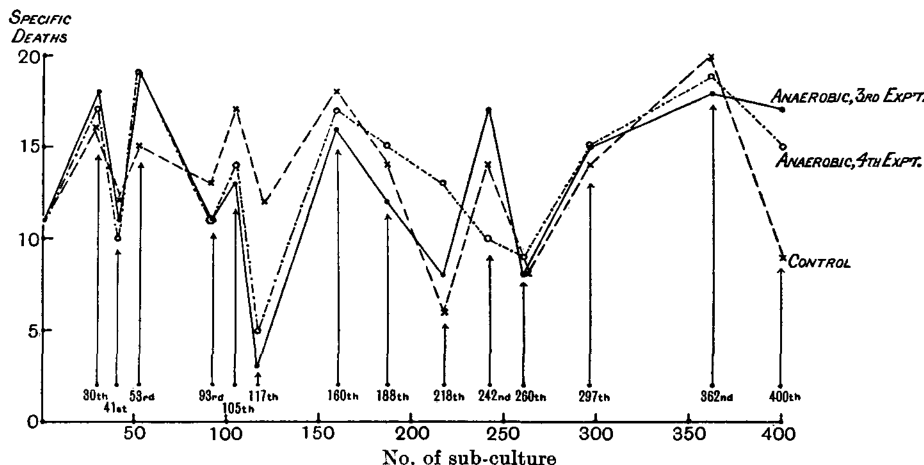

Fig. 1. Daily anaerobic culture. Exps. 3, 4. In Figs. 1–11 specific deaths are plotted along the axis of ordinates, the number of the sub-culture along the axis of abscissae.

B. *Daily sub-cultivation in* 1 *per cent. oxygen, the gas being bubbled through the medium.*

Exp. 1. Commenced on 27. v. 26 and terminated on 5. viii. 26. In this experiment the four-dose method was used for testing virulence (Wilson, 1926). The results are given in Table IV. The average number of specific deaths caused by the 1 per cent. O_2 culture was 10·75, by the control strain 15·0. P lies between 0·1 and 0·05, so that the decrease in virulence of the experimental strain cannot be regarded as significant.

Table IV.

1 *per cent. oxygen culture. Exp.* 1.

Date	No. of sub-culture	Lowest dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
17. vi. 26	18	96	14	**12**	6	6	18	7·2
2. vii. 26	31	26	11	**11**	9	7	18	9·6
15. vii. 26	42	52	13	**13**	7	5	18	9·25
5. viii. 26	60	99	7	**7**	13	10	17	9·75
Arithmetic mean		68	—	**10·75**	—	—	—	8·95
Control culture.								
27. v. 26	—	77	14	**14**	6	6	20	6·95
5. viii. 26	—	67	17	**16**	3	2	18	5·95
Arithmetic mean		72	—	**15**	—	—	—	6·45

Treated as Case III, $P = <0{\cdot}1$ and $>0{\cdot}05$.

Exp. 2. Commenced on 9. ii. 28 and terminated on 24. v. 28. In this experiment the usual one-dose method was used for testing virulence. The

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

results are given in Table V and Fig. 2. The average number of specific deaths caused by the 1 per cent. O_2 culture was 9·0, by the control strain 12·6. *P* lies between 0·1 and 0·05, so that the decrease in virulence of the experimental culture cannot be regarded as significant.

Table V.

1 per cent. oxygen culture. Exp. 2.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
24. ii. 28	13	105	13	**13**	7	7	20	9·45
8. iii. 28	24	118	17	**17**	3	3	20	7·5
23. iii. 28	37	33	18	**18**	2	2	20	10·75
12. iv. 28	54	48	9	**9**	11	9	18	11·75
27. iv. 28	67	92	1	**0**	19	17	17	14·0
4. v. 28	72	117	6	**6**	14	11	17	12·65
15. v. 28	82	114	7	**7**	13	13	20	11·9
24. v. 28	90	48	2	**2**	18	12	14	13·1
Arithmetic mean		84	—	**9·0**	—	—	—	11·39
			Control culture.					
9. ii. 28	—	71	12	**12**	8	8	20	11·2
24. ii. 28	—	83	13	**13**	7	5	18	9·15
8. iii. 28	—	90	15	**15**	5	5	20	7·5
23. iii. 28	—	91	18	**16**	2	2	18	8·95
12. iv. 28	—	34	13	**13**	7	7	20	9·7
27. iv. 28	—	71	11	**11**	9	9	20	10·45
15. v. 28	—	92	12	**12**	8	5	17	9·65
24. v. 28	—	36	9	**9**	11	9	18	10·7
Arithmetic mean		71	—	**12·6**	—	—	—	9·66

Treated as Case II, $P = <0·1$ and $>0·05$.
Treated as Case II, and using the last four pairs of observations only, $P = <0·05$ and $>0·02$.

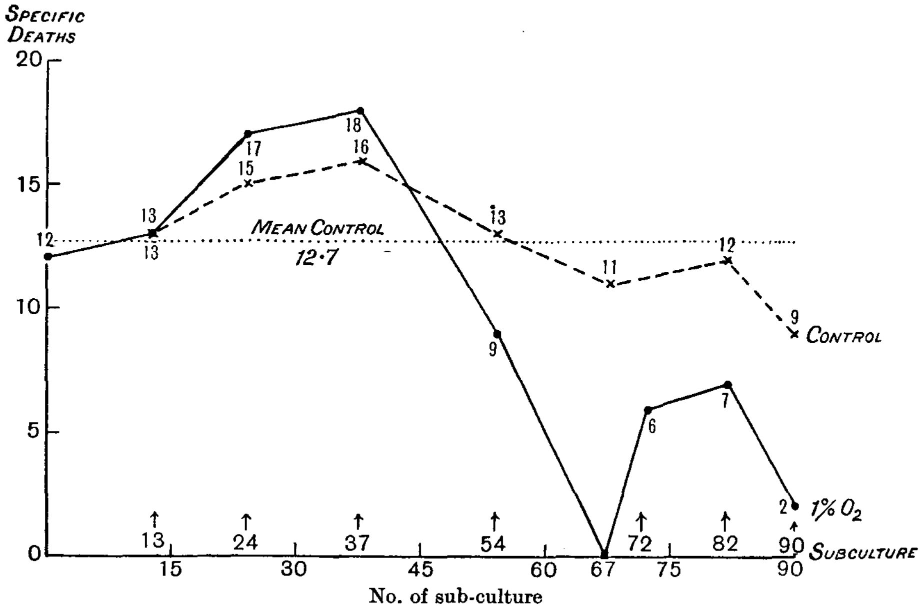

Fig. 2. Daily bubbling culture in 1 per cent. oxygen. Exp. 2.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

As previously mentioned, the use of all the pairs of observations in this experiment is not strictly justifiable. Reference to Fig. 2 will show that the first definite fall in virulence of the 1 per cent. oxygen culture did not occur till the fifty-fourth sub-culture, and that subsequent to this there was a marked difference between the two cultures. If only the last four pairs of observations are considered, then the value of P is found to lie between 0·05 and 0·02, indicating that the fall in virulence which did ultimately occur was truly significant.

Exp. 3. Commenced on 13. vi. 28 and terminated on 16. x. 28. The results are given in Table VI and Fig. 3. The average number of specific deaths caused

Table VI.

1 per cent. oxygen culture. Exp. 3.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
19. vii. 28	31	312	12	**12**	8	7	19	8·85
1. viii. 28	42	117	11	**11**	9	7	18	10·1
15. viii. 28	54	181	13	**13**	7	5	18	8·9
2. x. 28	95	90	4	**4**	16	10	14	13·45
16. x. 28	107	63	5	**4**	15	6	10	12·25
Arithmetic mean		153	—	**8·8**	—	—	—	10·71
Control culture.								
13. vi. 28	—	109	11	**11**	9	8	19	9·6
19. vii. 28	—	88	16	**16**	4	4	20	6·55
1. viii. 28	—	109	12	**12**	8	7	19	9·15
15. viii. 28	—	118	15	**15**	5	4	19	8·0
2. x. 28	—	157	13	**13**	7	7	20	10·1
16. x. 28	—	85	17	**17**	3	3	20	7·1
Arithmetic mean		111	—	**14·0**	—	—	—	8·42

Treated as Case I, $P = <0{\cdot}1$ and $>0{\cdot}05$.

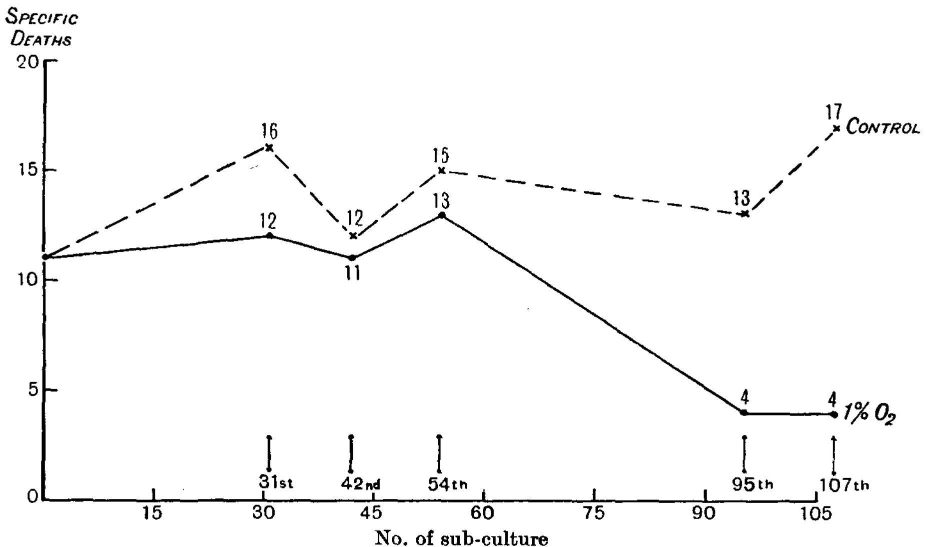

Fig. 3. Daily bubbling culture in 1 per cent. oxygen. Exp. 3.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

by the 1 per cent. O_2 culture was 8·8, by the control culture 14·0. *P* lies between 0·1 and 0·05, so that the decrease in virulence of the experimental culture cannot be regarded as significant.

Summary. Three experiments were performed. In each experiment the 1 per cent. O_2 culture appeared to be less virulent than the control culture, but in no case was the difference significant, except in Exp. 2, when the observations made in the latter part of the experiment only were taken into account. From the results of the three experiments taken in conjunction, however, there is little doubt that daily sub-cultivation in 1 per cent. oxygen over a period of three to four months does lead to a definite fall in virulence. The significance of this loss, as estimated by the method here employed, is partly obscured by the fact that the fall in virulence did not occur till comparatively late in each experiment, so that the lower values of the later experiments are largely counterbalanced by the higher values obtained earlier.

C. *Daily sub-cultivation in* 5 *per cent. oxygen, the gas being bubbled through the medium.*

Exp. 1. Commenced on 25. i. 27 and terminated on 10. v. 27. The results are given in Table VII and Fig. 4. The average number of specific deaths caused by the 5 per cent. oxygen culture was 5·0, by the control culture 16·0. *P* lies between 0·05 and 0·02, so that the fall in virulence of the experimental culture may be regarded as significant.

Table VII.

5 *per cent. oxygen culture. Exp.* 1.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
3. iii. 27	32	69	8	**8**	12	10	18	10·65
18. iii. 27	45	89	6	**6**	14	11	17	12·7
5. iv. 27	60	58	12	**12**	8	6	18	10·6
28. iv. 27	80	138	1	**1**	19	11	12	13·9
10. v. 27	90	77	4	**3**	16	14	17	12·6
Arithmetic mean		86	—	**5·0**	—	—	—	12·09

Control culture.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
3. iii. 27	—	72	18	**17**	2	2	19	7·3
18. iii. 27	—	83	11	**11**	9	8	19	10·3
5. iv. 27	—	45	19	**19**	1	1	20	6·4
10. v. 27	—	163	17	**17**	3	3	20	8·0
Arithmetic mean		91	—	**16·0**	—	—	—	8·0

Treated as Case II, $P = <0{\cdot}05$ and $>0{\cdot}02$.

Summary. Only one experiment was performed. The result showed a significant fall in virulence of the 5 per cent. oxygen culture.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

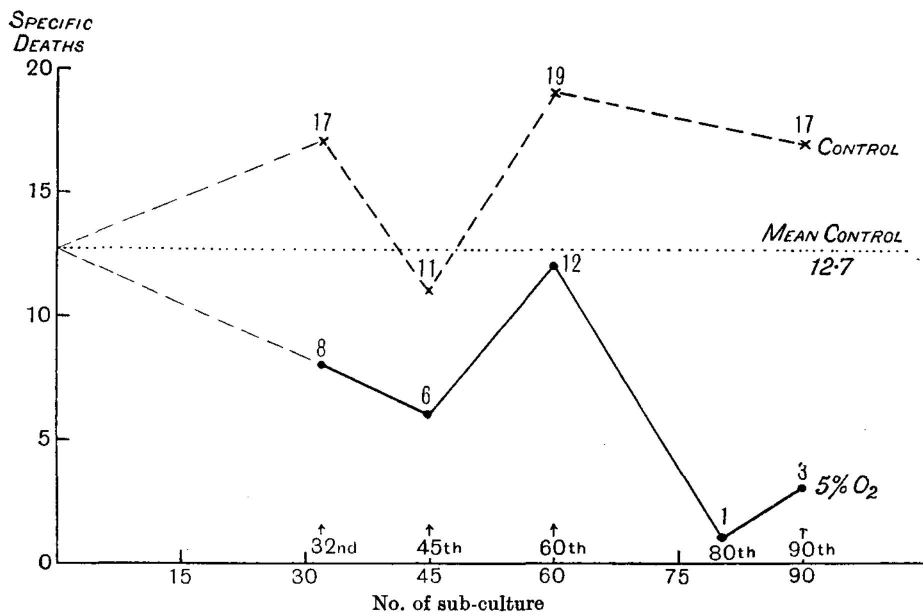

Fig. 4. Daily bubbling culture in 5 per cent. oxygen. Exp. 1.

D. *Daily sub-cultivation under ordinary aerobic conditions.*

Exp. 1. Commenced on 16. i. 26 and terminated on 9. iv. 26. The four-dose method of virulence testing was used. The results are given in Table VIII. The average number of specific deaths caused by the aerobic culture was 4·0, by the control culture 14·5. The value of P is less than 0·01, so that the fall in virulence of the experimental culture may be regarded as significant.

Table VIII.

Aerobic culture. Exp. 1.

Date	No. of sub-culture	Lowest dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
25. iii. 26	58	16	7	**4**	13	12	16	11·25
9. iv. 26	70	13	6	**4**	14	9	13	10·65
Arithmetic mean		—	—	**4**	—	—	—	10·95
Control culture.								
18. xi. 25	—	18	15	**15**	5	5	20	6·65
9. iv. 26	—	13	14	**14**	6	5	19	7·75
Arithmetic mean		—	—	**14·5**	—	—	—	7·2

Treated as Case III, $P = <0{\cdot}01$.

Exp. 2. Commenced on 15. xii. 26 and terminated on 25. vi. 29. The single dose method of virulence testing was used. The results are given in Table IX and Fig. 5. On 17. ix. 27 the daily sub-cultivations had to be unavoidably discontinued for a fortnight; during this time the strain was kept in a stab agar tube; the sub-cultures in broth were resumed on 3. x. 27. During the latter part of the experiment the control culture tests given in

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

the table are those most nearly corresponding in time to those of the aerobic culture. The average number of specific deaths caused by the aerobic culture

Table IX.

Aerobic culture. Exp. 2.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
28. xii. 26	13	35	10	**10**	10	6	16	10·9
18. i. 27	34	40	3	**2**	17	7	9	13·75
11. iii. 27	79	79	3	**3**	17	3	16	13·6
10. v. 27	130	242	2	**2**	18	17	19	13·5
15. vii. 27	186	66	2	**1**	18	9	10	13·75
16. v. 28	436	66	1	**1**	19	7	8	13·65
16. x. 28	568	105	1	**0**	19	3	3	13·7
27. xi. 28	601	73	0	**0**	20	3	3	14·0
8. i. 29	637	128	0	**0**	20	1	1	14·0
9. iv. 29	713	50	0	**0**	20*	0	0	14·0
25. vi. 29	780	54	0	**0**	20†	2	2	14·0
Arithmetic mean		85	—	**1·7**	—	—	—	13·53
Control culture.								
16. xii. 26	—	40	12	**12**	8	6	18	10·1
21. xii. 26	—	31	12	**12**	8	5	17	9·6
28. xii. 26	—	52	12	**12**	8	7	19	9·6
3. iii. 27	—	72	18	**17**	2	2	19	7·3
18. iii. 27	—	83	11	**11**	9	8	19	10·3
5. iv. 27	—	45	19	**19**	1	1	20	6·4
10. v. 27	—	163	17	**17**	3	3	20	8·0
14. vii. 27	—	80	13	**13**	7	7	20	8·65
15. v. 28	—	92	12	**12**	8	5	17	9·65
16. x. 28	—	85	17	**17**	3	3	20	7·1
30. x. 28	—	108	13	**12**	7	6	18	9·0
20. xii. 28	—	118	18	**18**	2	2	20	7·85
22. i. 29	—	147	14	**14**	6	6	20	8·65
16. iv. 29	—	136	8	**8**	12	11	19	11·45
4. vii. 29	—	121	15	**15**	5	4	19	7·75
Arithmetic mean		92	—	**13·9**	—	—	—	8·76

Treated as Case III, $P = <0{\cdot}01$.

* 2 mice accidentally killed on 10. iv. 29. † 1 mouse accidentally killed on 26. vi. 29.

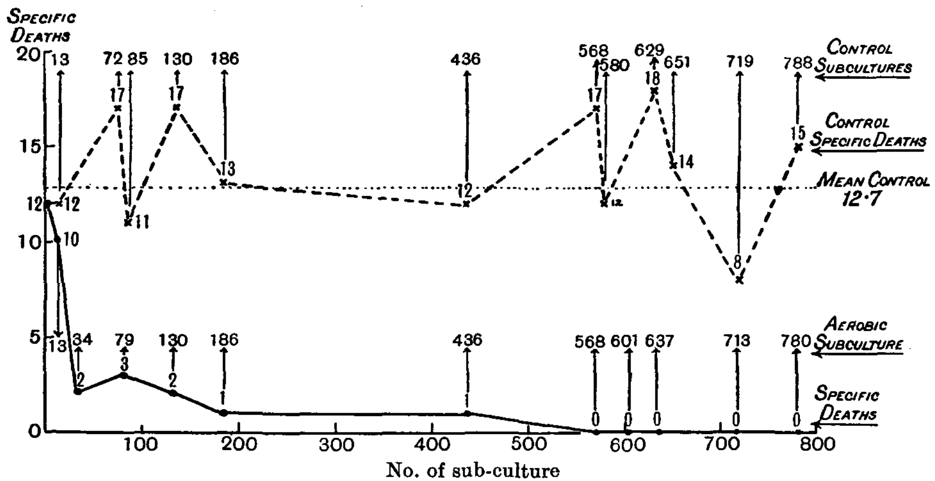

Fig. 5. Daily aerobic culture. Exp. 2.

Note. The control sub-culture figures merely indicate that tests of the control strain were made at times corresponding to those particular numbers of the aerobic sub-culture series.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

was 1·7, by the control culture 13·9. The value of P is less than 0·01, so that the fall in virulence of the experimental culture may be regarded as significant.

Summary. Two experiments were performed. In each case the virulence of the aerobic culture fell rapidly. In the second experiment, which was continued for 2½ years, the culture became so avirulent that it not only failed to kill any mice, but it became almost completely non-infective.

E. *Daily sub-cultivation in* 21 *per cent. oxygen, the gas being bubbled through the medium.*

Exp. 1. Commenced on 25. i. 27 and terminated on 10. v. 27. The results are given in Table X and Fig. 6. The average number of specific deaths caused

Table X.

21 *per cent. oxygen culture. Exp* 1.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
3. iii. 27	32	56	6	**6**	14	9	15	12·4
18. iii. 27	45	40	1	**1**	19	10	11	13·65
5. iv. 27	60	123	7	**5**	13	10	15	11·9
28. iv. 27	80	128	3	**2**	17	12	14	13·8
10. v. 27	90	40	5	**4**	15	10	14	12·85
Arithmetic mean		77	—	**3·6**	—	—	—	12·92
Control culture.								
3. iii. 27	—	72	18	**17**	2	2	19	7·3
18. iii. 27	—	83	11	**11**	9	8	19	10·3
5. iv. 27	—	45	19	**19**	1	1	20	6·4
10. v. 27	—	163	17	**17**	3	3	20	8·0
Arithmetic mean		91	—	**16·0**	—	—	—	8·0

Treated as Case II, $P = <0·01$.

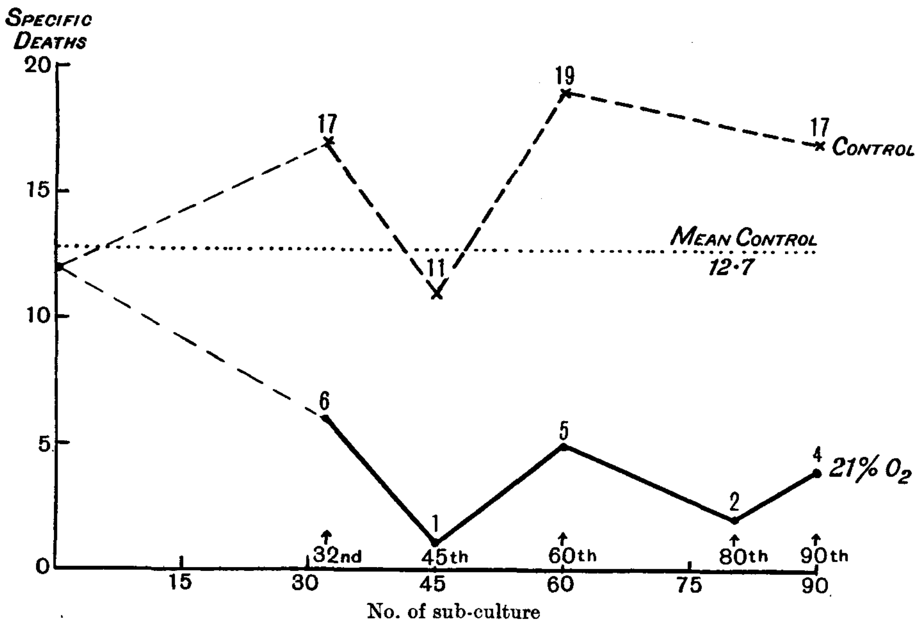

Fig. 6. Daily bubbling culture in 21 per cent. oxygen. Exp. 1.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

by the 21 per cent. O_2 culture was 3·6, by the control culture 16·0. The value of P is less than 0·01, so that the fall in virulence of the experimental culture may be regarded as significant.

Summary. Only one experiment was performed. The result showed a rapid and significant fall in virulence of the 21 per cent. oxygen culture.

F. *Daily sub-cultivation in* 40 *per cent. oxygen, the gas being bubbled through the medium.*

Exp. 1. Commenced on 11. v. 27 and terminated on 17. vi. 27. The results are given in Table XI. On the only occasion on which the 40 per cent. oxygen culture was tested it caused nineteen deaths; the mean number of deaths caused by the control culture was 14·0. The experiment was stopped at an early date because it was found that the 40 per cent. oxygen culture contained Gram-negative bacilli in bundles, which were not agglutinated by an *aertrycke* antiserum. It was thought that the culture had become contaminated, and it was therefore discarded. Subsequent work rendered it probable that the organisms had passed into a pure O form, and hence were not agglutinated by group and type *aertrycke* serum.

Table XI.

40 *per cent. oxygen culture. Exp.* 1.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
3. vi. 27	20	70	19	**19**	1	1	20	5·95
Control culture.								
10. v. 27	—	163	17	**17**	3	3	20	8·0
3. vi. 27	—	206	11	**11**	9	8	19	10·85
Arithmetic mean		185	—	**14·0**	—	—	—	9·43

Exp. 2. Commenced on 9. vi. 27 and terminated on 20. ix. 27. As the final testing of the culture could not be performed on this date, it was put into a

Table XII.

40 *per cent. oxygen culture. Exp.* 2.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
23. vi. 27	12	118	13	**13**	7	7	20	9·25
15. vii. 27	29	96	12	**11**	8	6	17	10·55
10. viii. 27	52	62	16	**13**	4	3	16	9·75
13. x. 27	87	75	14	**14**	6	?	?	10·25
Arithmetic mean		88	—	**12·8**	—	—	—	9·95
Control culture.								
3. vi. 27	—	206	11	**11**	9	8	19	10·85
23. vi. 27	—	90	11	**10**	9	7	17	9·55
8. vii. 27	—	80	7	**7**	13	12	19	11·6
14. vii. 27	—	80	13	**13**	7	7	20	8·65
21. vii. 27	—	35	14	**14**	6	4	18	8·4
10. viii. 27	—	44	6	**6**	14	9	15	12·05
13. x. 27	—	32	5	**5**	15	9	14	12·45
Arithmetic mean		81	—	**9·4**	—	—	—	10·51

Treated as Case II, $P = <0{\cdot}1$ and $>0{\cdot}05$.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

stab agar tube, and tested for virulence, along with the control culture, on 13. x. 27. The results are given in Table XII and Fig. 7. The average number of deaths caused by the 40 per cent. oxygen culture was 12·8, by the control

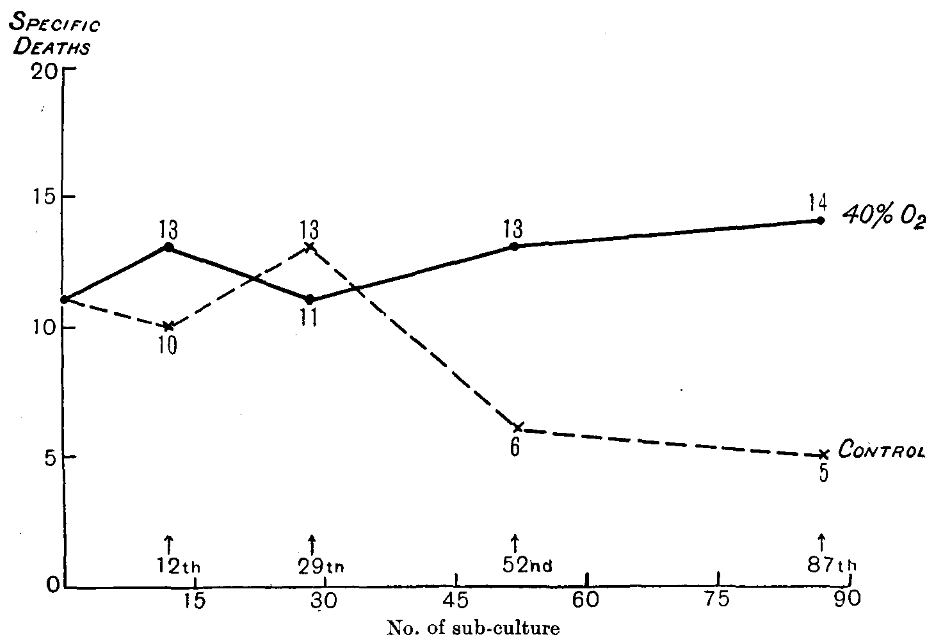

Fig. 7. Daily bubbling culture in 40 per cent. oxygen. Exp. 2.

culture 9·4. P lies between 0·1 and 0·05, so that the rise in virulence of the experimental culture cannot be regarded as significant. In this, as in the preceding experiment, the 40 per cent. oxygen culture became inagglutinable, and passed into what was presumably a pure O form (see Exp. 3).

Exp. 3. Commenced on 13. x. 27 and terminated on 27. i. 28. The results are given in Table XIII and Fig. 8. The average number of specific deaths caused by the 40 per cent. oxygen culture was 15·0, by the control culture 11·8. The value of P is less than 0·01, indicating that the increase in virulence of the experimental culture is significant. In this experiment, as in the preceding two experiments, the 40 per cent. oxygen culture became inagglutinable. It was found that, though no agglutination occurred with group or type sera, the culture was agglutinated by an *aertycke* O serum. By inoculation of a rabbit with the culture a serum was obtained that agglutinated *Bact. aertrycke* in its O form, but had no action on the flagellar antigens. Examination of the culture showed that it consisted of non-motile organisms. It would appear therefore that, as the result of cultivation in 40 per cent. oxygen, the organisms had passed into the non-motile O form. This change rendered it very difficult to ascertain whether the mice that died or were killed were specifically infected, because the organisms recovered were inagglutinable with the usual group and type sera. Until the nature of the

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

antigenic change was appreciated, it was found necessary to replate the cultures several times before they manifested even a slight degree of agglutination with these sera.

Table XIII.

40 *per cent. oxygen culture. Exp.* 3.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
20. x. 27	6	64	17	**17**	3	3	20	8·7
4. xi. 27	19	80	16	**12**	4	4	16	9·0
16. xi. 27	29	29	13	**10**	7	6	16	9·85
7. xii. 27	47	160	16	**12**	4	4	16	9·9
21. xii. 27	59	124	19	**19**	1	0	19	7·05
4. i. 28	71	103	18	**18**	2	2	20	7·55
19. i. 28	84	96	18	**17**	2	2	19	8·25
27. i. 28	91	138	17	**15**	3	2	17	7·7
Arithmetic mean		99	—	**15·0**	—	—	—	8·50
Control culture.								
13. x. 27	—	32	5	**5**	15	9	14	12·45
20. x. 27	—	110	14	**14**	6	6	20	8·9
4. xi. 27	—	95	8	**8**	12	11	19	10·75
16. xi. 27	—	58	13	**13**	7	7	20	8·5
7. xii. 27	—	178	8	**8**	12	11	19	11·35
21. xii. 27	—	107	14	**14**	6	5	19	8·35
4. i. 28	—	160	16	**16**	4	4	20	9·4
19. i. 28	—	128	13	**13**	7	7	20	10·2
27. i. 28	—	112	15	**15**	5	5	20	9·2
Arithmetic mean		109	—	**11·8**	—	—	—	9·90

Treated as Case I, $P = <0{\cdot}01$.

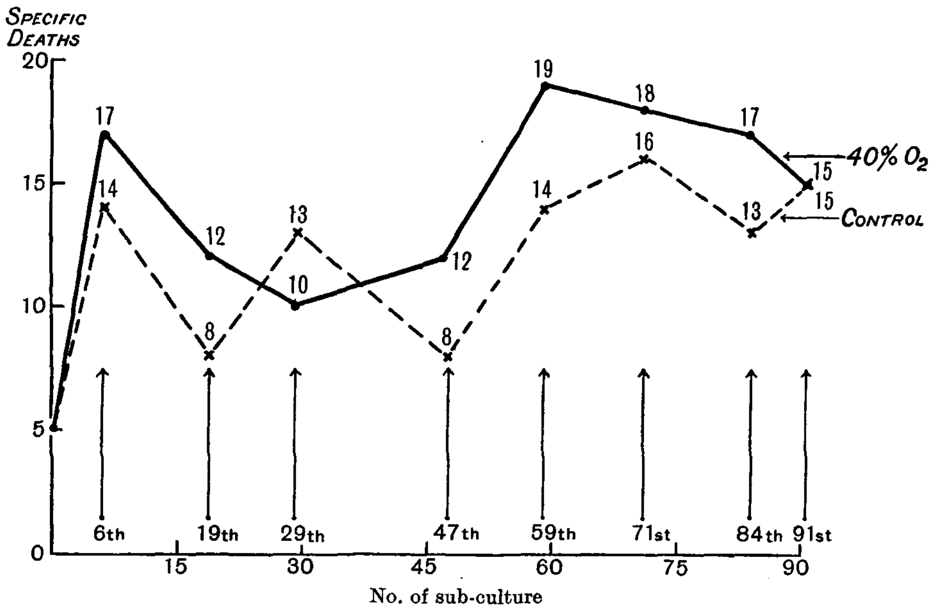

Fig. 8. Daily bubbling culture in 40 per cent. oxygen. Exp. 3.

Summary. Three experiments were performed, one of them lasting, however, for only a month. In each case an apparent rise in virulence occurred,

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

and in one case this was definitely significant. It appears, therefore, that cultivation in 40 per cent. oxygen leads to an increase in virulence of *Bact. aertrycke.* The interesting observation was made that cultivation under these conditions led to the replacement of the initial motile type of bacilli by organisms of the non-motile O form.

G. *Daily sub-cultivation in* 75 *per cent. oxygen, the gas being bubbled through the medium.*

Exp. 1. Commenced on 11. v. 27 and terminated on 17. vi. 27. The results are given in Table XIV. On the only occasion on which the 75 per cent. oxygen culture was tested it caused sixteen deaths; the mean number of deaths caused by the control culture was 14·0. The experiment was discon-

Table XIV.

75 *per cent. oxygen culture. Exp.* 1.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
3. vi. 27	20	60	16	**16**	4	4	20	8·25
Control culture.								
10. v. 27	—	163	17	**17**	3	3	20	8·0
3. vi. 27	—	206	11	**11**	9	8	19	10·85
Arithmetic mean		185	—	**14·0**	—	—	—	9·43

tinued because the organisms became inagglutinable by group and type *aertrycke* sera, and it was thought that the culture had become contaminated. Subsequent work rendered it probable that the organisms had passed into a pure O form.

Exp. 2. Commenced on 9. vi. 27 and terminated on 10. viii. 27. The results are given in Table XV. The average number of specific deaths caused

Table XV.

75 *per cent. oxygen culture. Exp.* 2.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
23. vi. 27	12	116	19	**19**	1	1	20	7·2
8. vii. 27	25	32	11	**11**	9	7	18	9·3
15. vii. 27	30	80	18	**11**	2	2	13	8·15
10. viii. 27	53	87	7	**7**	13	8	15	11·95
Arithmetic mean		79	—	**12·0**	—	—	—	9·15
Control culture.								
3. vi. 27	—	206	11	**11**	9	8	19	10·85
23. vi: 27	—	90	11	**10**	9	7	17	9·55
8. vii. 27	—	80	7	**7**	13	12	19	11·6
14. vii. 27	—	80	13	**13**	7	7	20	8·65
21. vii. 27	—	35	14	**14**	6	4	18	8·4
10. viii. 27	—	44	6	**6**	14	9	15	12·05
Arithmetic mean		89	—	**10·2**	—	—	—	10·18

Treated as Case II, $P = <0{\cdot}3$ and $>0{\cdot}2$.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

by the 75 per cent. oxygen culture was 12·0, by the control culture 10·2. P lies between 0·3 and 0·2, so that the rise in virulence of the experimental culture cannot be regarded as significant. In this, as in the preceding experiment, the 75 per cent. oxygen culture became inagglutinable, and passed into what was presumably a pure O form. As it was thought that the culture was contaminated, the experiment was prematurely brought to an end.

Exp. 3. Commenced on 13. x. 27 and terminated on 27. i. 28. The results are given in Table XVI and Fig. 9. The average number of specific deaths caused by the 75 per cent. oxygen culture was 16·4, by the control culture 11·8. The value of P is less than 0·01, so that the rise in virulence of the experimental culture may be regarded as significant.

Table XVI.

75 per cent. oxygen culture. Exp. 3.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
20. x. 27	6	58	19	**19**	1	1	20	6·65
4. xi. 27	19	87	14	**13**	6	6	19	9·55
16. xi. 27	29	100	8	**8**	12	11	19	11·55
7. xii. 27	47	138	15	**15**	5	4	19	8·85
21. xii. 27	59	102	17	**17**	3	3	20	7·9
4. i. 28	71	99	19	**19**	1	1	20	7·1
19. i. 28	84	125	20	**20**	0	0	20	8·4
27. i. 28	91	142	20	**20**	0	0	20	6·45
Arithmetic mean		106	—	**16·4**	—	—	—	8·31

Control culture. (Same as in Table XIII.)

Treated as Case I, $P = <0{\cdot}01$.

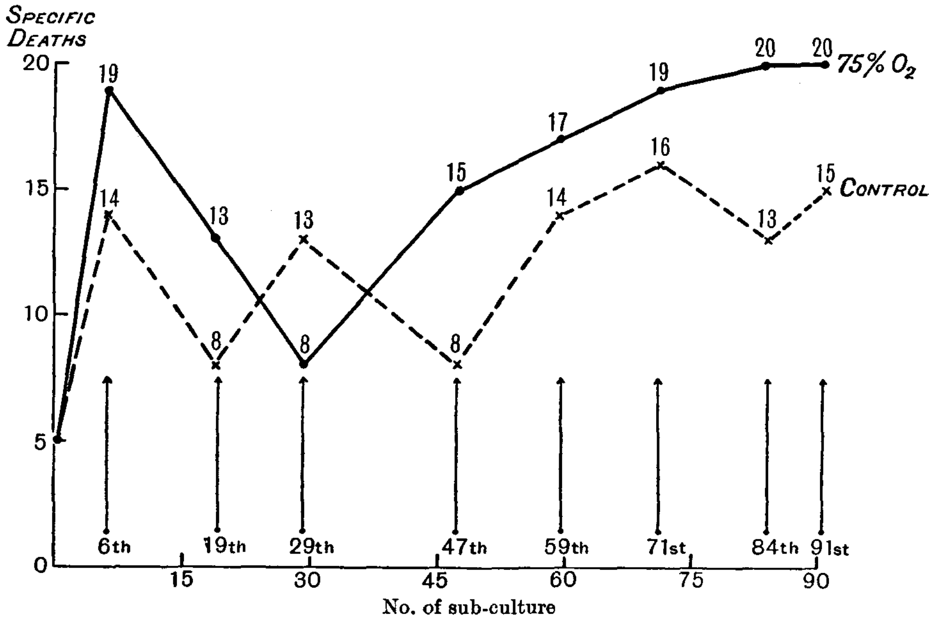

Fig. 9. Daily bubbling culture in 75 per cent. oxygen. Exp. 3.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

In this experiment, as in the two preceding experiments, and as in the three experiments with 40 per cent. oxygen, the experimental culture became inagglutinable with group or type sera. The organisms, however, reacted with an *aertrycke* O serum; microscopically they proved to be non-motile.

Summary. Three experiments were performed, one of them lasting for only a month, and another for only a little over two months. In each case an apparent increase in virulence occurred, and in one case this was definitely significant. It appears, therefore, that cultivation in 75 per cent. oxygen leads to an increase in virulence of *Bact. aertrycke.* As with the experiments in which 40 per cent. oxygen was used, it was found that cultivation in 75 per cent. oxygen led to the replacement of the normal organisms by organisms of the non-motile O form.

H. *Daily sub-cultivation in* 100 *per cent. oxygen, the gas being bubbled through the medium.*

Exp. 1. Commenced on 5. x. 26 and terminated on 18. i. 27. The results are given in Table XVII and Fig. 10. The average number of specific deaths caused by the 100 per cent. oxygen culture was 15·6, by the control culture 12·0. P is less than 0·1, but greater than 0·05, so that the rise in virulence of the experimental culture cannot be regarded as significant.

Table XVII.

100 *per cent. oxygen culture. Exp.* 1.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
26. x. 26	18	83	11	**10**	9	9	19	10·05
2. xi. 26	24	87	16	**16**	4	4	20	9·45
30. xi. 26	48	52	19	**19**	1	1	20	7·05
21. xii. 26	66	87	19	**19**	1	0	19	6·85
18. i. 27	91	62	14	**14**	6	5	19	10·3
Arithmetic mean		74	—	**15·6**	—	—	—	8·74
Control culture.								
28. x. 26	—	91	7	**7**	13	11	18	11·75
4. xi. 26	—	50	16	**15**	4	4	19	9·0
18. xi. 26	—	55	13	**13**	7	6	19	9·8
30. xi. 26	—	30	13	**13**	7	5	18	9·25
16. xii. 26	—	44	12	**12**	8	6	18	10·1
21. xii. 26	—	31	12	**12**	8	5	17	9·6
28. xii. 26	—	52	12	**12**	8	7	19	9·6
Arithmetic mean		50	—	**12**	—	—	—	9·87

Treated as Case III, $P = <0·1$ and $>0·05$.

Exp. 2. Commenced on 27. i. 28 and terminated on 15. v. 28. The results are given in Table XVIII and Fig. 11. The average number of specific deaths caused by the 100 per cent. oxygen culture was 18·7, by the control culture 13·4. The value of P is less than 0·01, so that the rise in virulence of the experimental culture may be regarded as significant.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

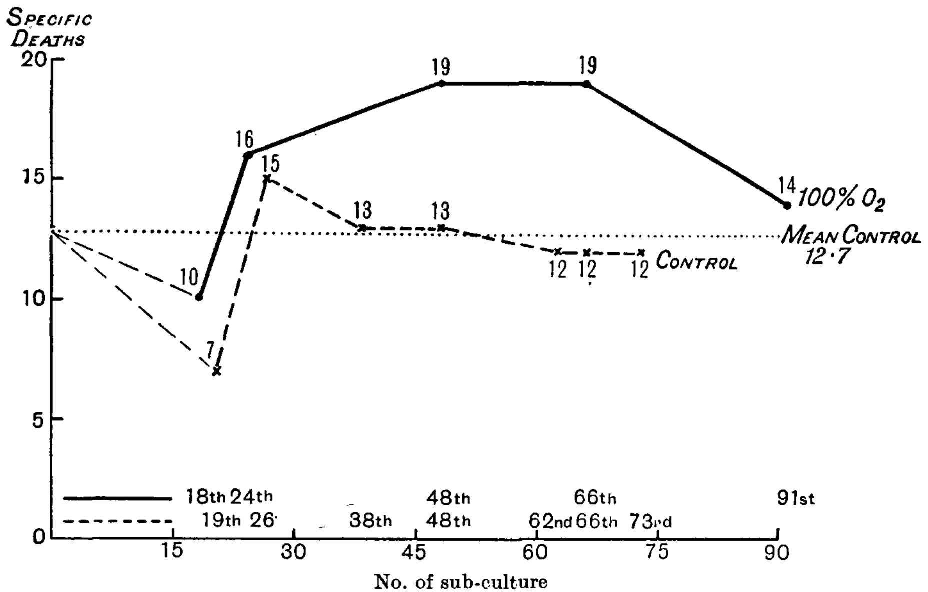

Fig. 10. Daily bubbling culture in 100 per cent. oxygen. Exp. 1.

Summary. Two experiments were performed. In each there was an apparent rise in the virulence of the experimental culture, and in one case this was definitely significant. It may be concluded therefore that cultivation in pure oxygen leads to an increase in the virulence of *Bact. aertrycke*. It is interesting to note that daily sub-culture in 100 per cent. oxygen did not lead, as in the

Table XVIII.

100 *per cent. oxygen culture. Exp.* 2.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
24. ii. 28	24	36	18	**18**	2	1	19	7·4
8. iii. 28	35	84	20	**19**	0	0	19	6·05
23. iii. 28	48	99	19	**19**	1	1	20	8·3
12. iv. 28	64	150	20	**20**	0	0	20	4·5
27. iv. 28	77	190	20	**20**	0	0	20	8·55
15. v. 28	92	149	16	**16**	4	2	18	8·55
Arithmetic Mean		118	—	**18·7**	—	—	—	7·23

Control culture.

Date	No. of sub-culture	Dose of living organisms	Deaths	Specific deaths	Killed	Specifically infected	Total infected	Mean expectation of life in days
27. i. 28	—	112	15	**15**	5	5	20	9·2
9. ii. 28	—	71	12	**12**	8	8	20	11·2
24. ii. 28	—	83	13	**13**	7	5	18	9·15
8. iii. 28	—	90	15	**15**	5	5	20	7·5
23. iii. 28	—	91	18	**16**	2	2	18	8·95
12. iv. 28	—	34	13	**13**	7	7	20	9·7
27. iv. 28	—	71	11	**11**	9	9	20	10·45
15. v. 28	—	92	12	**12**	8	5	17	9·65
Arithmetic mean		81	—	**13·4**	—	—	—	9·48

Treated as Case II, $P = < 0{\cdot}01$.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

40 and 75 per cent. oxygen series, to a replacement of the normal organisms by organisms of the non-motile O form.

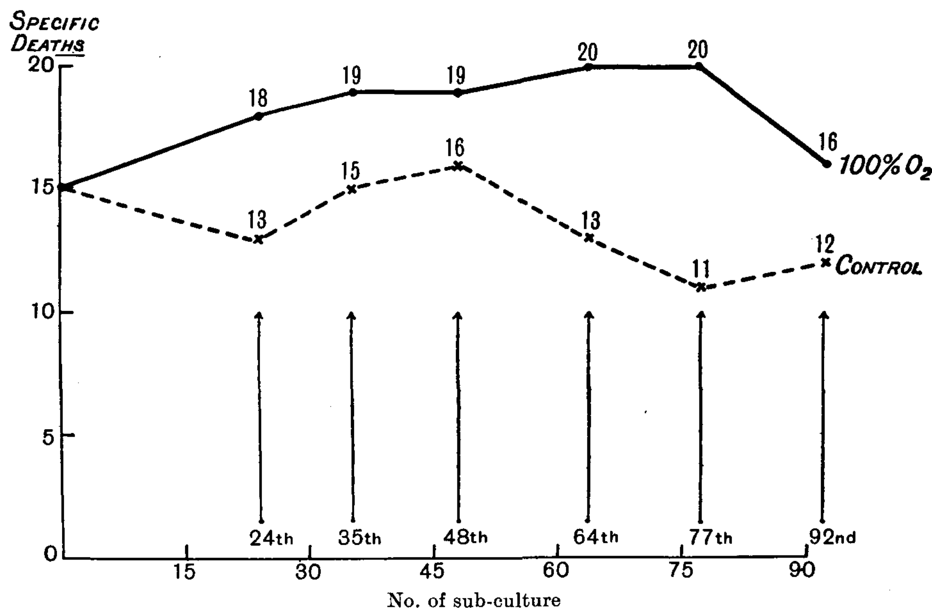

Fig. 11. Daily bubbling culture in 100 per cent. oxygen. Exp. 2.

Summary of the whole series of experiments.

In order to obtain some quantitative measure of the effect of growth in various atmospheres on the virulence of *Bact. aertrycke*, Table XIX has been compiled. A record is given in this table of the total and average number of specific deaths caused by the control and experimental cultures under each set of conditions, together with the differences in the arithmetic means. The figures used in the compilation of this table were taken from those employed in estimating the value of *P*, and refer exclusively to those experiments in which the virulence was tested by the single-dose method. The first experi-

Table XIX.

Comparison of control and experimental cultures.

		Control series			Experimental series			
Conditions of growth	Exp. no.	No. of tests	No. of specific deaths	Mean no. of specific deaths	No. of tests	No. of specific deaths	Mean no. of specific deaths	Difference of means
Anaerobic	2, 3, 4	18	236	**13·1**	18	249	**13·8**	+ 0·7
1 % O_2	2, 3	12	162	**13·5**	12	110	**9·2**	− 4·3
5 % O_2	1	4	64	**16·0**	4	29	**7·3**	− 8·7
Aerobic	2	12	166	**13·8**	11	19	**1·7**	− 12·1
21 % O_2	1	4	64	**16·8**	4	16	**4·0**	− 12·0
40 % O_2	2, 3	12	135	**11·3**	12	171	**14·3**	+ 3·0
75 % O_2	2, 3	12	137	**11·4**	12	179	**14·9**	+ 3·5
100 % O_2	1, 2	13	164	**12·6**	11	190	**17·3**	+ 4·7

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

ments in 40 per cent. and in 75 per cent. oxygen have been omitted, since they were discontinued after twenty sub-cultures, at a time when only a single estimation of virulence had been made. The third and fourth anaerobic experiments have been treated as one, the mean number of deaths caused by the two anaerobic cultures being compared with that caused by the single control culture.

Examination of the table shows that cultivation under anaerobic conditions has no appreciable effect on the virulence of *Bact. aertrycke*; the slight increase in the mean number of specific deaths, + 0·7, is probably well within the limit of experimental error. The introduction of 1 per cent. of oxygen into the atmosphere in which the organisms are grown leads to a slight but definite decrease in virulence, − 4·3. Increase in the proportion of oxygen is accompanied by a further diminution of virulence, and it will be noted that,

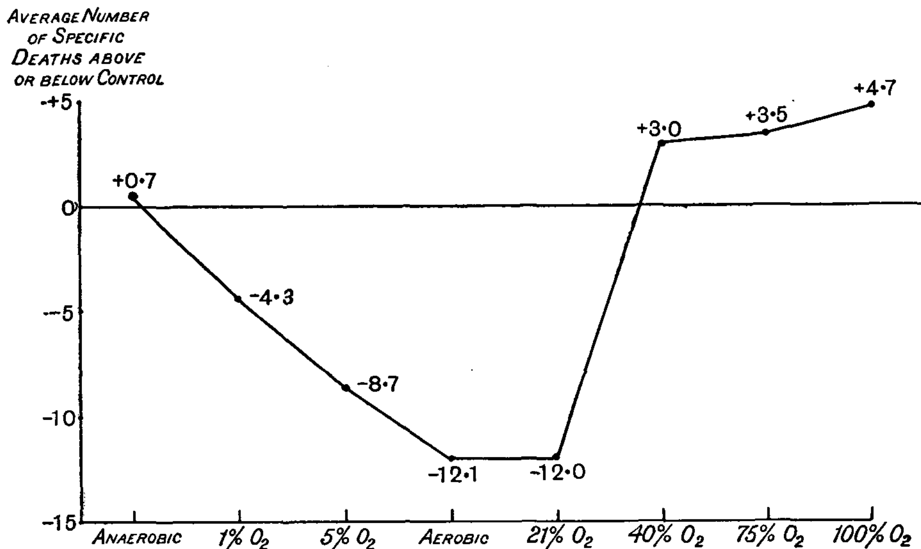

Fig. 12. Summary of whole series of experiments indicating the difference in the mean number of specific deaths in the gas cultures from those in the controls.

up to 21 per cent. oxygen, there is a relationship between the percentage of oxygen in the atmosphere and the decrease in virulence of the culture. Prolonged cultivation in air renders the organisms completely avirulent, as judged by the method here employed. But when the oxygen in the atmosphere is increased to 40 per cent., the virulence, instead of exhibiting an even more rapid fall than in air—as might have been expected—actually shows a rise, + 3·0. Further increase in the proportion of oxygen to 75 and 100 per cent. is accompanied by a gradual increase in the virulence of the culture. When the differences in the means are plotted, as in Fig. 12, a curve is obtained having one maximum, which corresponds to the mean virulence of the control culture, under anaerobic conditions, a second maximum in 100 per cent. oxygen, and a minimum in 21 per cent. oxygen.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

Discussion.

Before discussing the results obtained in these experiments we propose to consider the findings of other workers in regard to the effect of various artificial procedures in bringing about changes in growth and in virulence of different micro-organisms.

The effect of cultivation in different oxygen pressures on growth and virulence.

Wosnessenski (1884), following up preliminary work done by Bert and by Chauveau, grew the anthrax bacillus in flasks of broth incubated in air under a pressure greater than that of the normal atmosphere. At 35° C. the organisms grew well up to a pressure of thirteen atmospheres of air, and appeared, under these conditions, to become rather more virulent for guinea-pigs. But when the pressure was increased to fifteen atmospheres of air, growth ceased to occur, and the non-sporing bacilli died. The main interest of these experiments lies in demonstrating the sharply discontinuous effect on growth of different oxygen pressures.

Roux and Yersin (1890), working with the diphtheria bacillus, found that, when incubated at 35° C. in a thin layer of broth in a flask through which air was passed continuously, the organisms grew more rapidly than in a control, non-aerated flask. In the aerated flask the medium became acid in 36 hours, and alkaline again by the fourth day; in the non-aerated flask these changes in reaction occupied a fortnight. No attenuation of virulence was observed in the organisms in the aerated flask, when this was incubated at 35° C. for a month, but when the flask was incubated at 39·5° C. the culture became avirulent in a fortnight. That the increased temperature was not by itself capable of bringing about this result was shown by a control experiment with a non-aerated culture. The fall in virulence of the aerated culture at 39·5° C. occurred as a rule suddenly, not gradually. Thus in one experiment a sub-culture made on the fourteenth day was virulent for guinea-pigs, whereas one made on the sixteenth day proved to be avirulent. In order to examine the mechanism by which this fall occurred, the aerated culture was plated out at intervals on serum, and a number of individual colonies tested for virulence. It was found that some colonies were virulent, while others were avirulent for guinea-pigs; the proportion of avirulent to virulent colonies increased with the age of the culture, till eventually the culture consisted purely of avirulent organisms. So long as the whole culture contained some virulent organisms, sub-cultures made from it proved virulent, even though they contained also a high proportion of avirulent organisms; but once the virulent organisms had been replaced completely by avirulent organisms, sub-cultures proved avirulent. This undoubtedly explains why the fall in virulence of the whole culture appeared to occur suddenly. These results have been quoted at length because of their being amongst the first to demonstrate conclusively the discontinuous nature of bacterial virulence. Similar results have since been obtained with

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

cultures of *Bact. aertrycke* (Wilson 1928, 1930 *a*). It is clear that the term "virulence" as used in connection with the diphtheria bacillus is not identical with that used in connection with *Bact. aertrycke*, since in the former case virulence implies toxicity, in the latter invasive power. In both cases, however, the fall in virulence is dependent on the appearance of non-virulent variants, and in so far as this is concerned the two cases may be regarded as analogous.

Haffkine (1892), using Roux and Yersin's method, found that cultivation of the cholera vibrio at 39° C. in a constantly aerated atmosphere, subcultures being made every two to three days, resulted in a fall in virulence of the organisms.

Lubinski (1894) grew *Staph. aureus* on agar in an atmosphere of pure oxygen. The organisms formed no pigment under these conditions; and it is stated that after a single generation they lost their power of liquefying gelatin, and their virulence for rabbits, as tested by intraperitoneal inoculation. When cultivated anaerobically, growth was less abundant than under aerobic conditions, and no pigment was formed, but the organisms are said to have increased in virulence for rabbits.

The effect of high partial pressures of oxygen on the growth of a large number of different aerobic organisms was investigated by Moore and Williams (1909, 1911), and by Adams (1912). Most organisms grew as well in 50–90 per cent. of oxygen as in air, but the tubercle bacillus and staphylococci grew poorly or not at all under these conditions, while *Past. pestis* entirely failed to grow. The evidence indicated that these organisms were not actually killed by the high oxygen pressure, but were merely inhibited from growing. Karsner, Brittingham and Richardson (1923), using partial pressures of 83 to 99 per cent. oxygen, obtained, on the whole, similar results. They found that, though most organisms tested grew satisfactorily under these conditions, two strains of *Proteus vulgaris* were completely inhibited, and a strain of haemolytic streptococcus was partly inhibited from growing.

Müller (1912) studied the oxygen consumption of *Ps. fluorescens* and of *Bact. coli* in a synthetic medium. Working with *Ps. fluorescens* he compared the growth in a closed flask with that in an open flask in which the medium was aerated from time to time by shaking. Incubated at 20–22° C., the organisms grew in each flask at the same rate during the first 20 to 28 hours. At the end of this time the oxygen in the closed flask was completely exhausted, further multiplication ceased, and the organisms began to die off rapidly. In the open flask, on the other hand, growth continued till the number of organisms per c.c. was five to six times the maximum reached in the closed flask, and the ultimate cessation of active growth was succeeded by a prolonged stationary phase, followed by a slow decline. *Bact. coli* was found to use considerably less oxygen than *Ps. fluorescens*. In the aerated flasks the maximum number of organisms reached per c.c. was only about three times that in the closed flasks; moreover, in both flasks cessation of growth was followed by a long stationary phase.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

Wreschner (1921) observed that reversion of a non-capsulated variant of *M. tetragenus* to the parent capsulated form occurred more rapidly in serum broth incubated aerobically than in the same medium incubated under comparatively anaerobic conditions.

Novy and Soule (1925), working with a human strain of tubercle bacillus, grown on glycerol agar, found that within certain limits the amount of growth was proportional to the oxygen pressure. Maximal growth occurred under partial pressures of oxygen of 40–50 per cent. Above this value growth was less abundant, though some growth occurred even in 100 per cent. oxygen. In atmospheres containing less than 40 per cent. oxygen the growth was correspondingly retarded. No growth occurred under anaerobic conditions.

Webster (1924, 1925), studying the virulent D and the avirulent G forms of *Past. lepiseptica*, described by De Kruif (1921, 1922 *a* and *b*), found that no growth of the D type occurred under aerobic conditions in plain meat extract broth, *p*H 7·4, unless about 100,000 organisms were inoculated per tube. But if the broth tubes were boiled for 30 minutes prior to seeding, and sealed with vaseline, then growth occurred after the introduction of even minimal numbers of organisms. The same effect could be brought about by adding rabbit blood to the medium, or a synthetic iron compound with strongly catalytic properties. In aerobic broth cultures dissociation of the D → G type occurred readily, whereas in blood broth incubated aerobically, or in plain broth incubated under a low partial pressure of oxygen, dissociation was strongly inhibited. The G type grew readily in broth under aerobic conditions even when seeded in very small numbers. These results suggest that the virulence of *Past. lepiseptica* is best conserved under comparatively anaerobic conditions, or in a medium with a high reducing intensity. Incubation under aerobic conditions in a medium of low reducing intensity rapidly leads to a fall in virulence. The effect of high pressures of oxygen on the virulence of this organism was apparently not tested.

Soule (1928), working with *B. subtilis*, found that good growth occurred under high partial pressures of oxygen and only poor growth under low partial pressures. Dissociation of the S to the R form occurred best in 40 to 60 per cent. oxygen, and was completely inhibited in broth, and almost completely on agar, by 80 to 100 per cent. of oxygen. With pressures of oxygen below atmospheric, the S → R transformation increased with a decrease in the oxygen pressure. When the cultures were incubated in pure nitrogen, dissociation was very marked, but in nitrogen plus 6 per cent. CO_2 no dissociation occurred at all. The position with regard to this organism is somewhat confusing. According to Soule the S form is the normal type, and this dissociates into the R form. Both types are avirulent. But with *B. anthracis*, which closely resembles it, the R form appears to be the normal type, and the S form the variant type. The R form of *anthracis* is highly virulent; the S form is comparatively avirulent.

The gaseous requirements of *Br. abortus* are peculiar. The freshly isolated

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

bovine type of this organism, as Bang (1897) showed, will not grow on the surface of an agar medium incubated aerobically. If it is inoculated into an agar shake tube, it grows in the form of a band situated about 3–5 mm. below the surface. Bang interpreted this as indicating that the organisms required a lowered oxygen pressure. It was subsequently found by Huddleson (1921) that growth would occur under aerobic conditions on the surface of an agar medium provided 5 to 10 per cent. of CO_2 was added to the air. Studying this phenomenon more closely I obtained evidence that the formation of the band of growth below the surface in shake cultures was due likewise to the need of the organisms for CO_2 (Wilson, 1930 *c*). Analyses showed that CO_2 was given off by the organisms, even when no active growth was taking place. Provided a liberal inoculation was made, sufficient CO_2 was generated by the organisms to raise the CO_2 pressure to a point which permitted of growth commencing. No growth occurred at the surface of the shake culture, because the CO_2 evolved by the organisms was dissipated, and no growth occurred in the depths of the medium because the oxygen pressure was too low. Further work showed that surface growth would not occur in pure oxygen, unless about 10 per cent. of CO_2 was added. Under anaerobic conditions no growth occurred at all, even in the presence of CO_2.

Summary. It is difficult to summarise the effect of varying partial pressures of oxygen on the growth and the virulence of different organisms. *Past. lepiseptica* and *Br. abortus* are clearly peculiar in their reaction to oxygen, and certainly differ from each other in their respiratory mechanisms. The anaerobic bacteria likewise fall into a different class. Dealing with the remaining aerobic bacteria that have been studied, it would appear as if variations in oxygen pressure during growth had a very marked effect on dissociation, both of colonial characters and of virulence. The data are obviously insufficient to permit of any definite conclusions, but there is a general suggestion that incubation under anaerobic conditions and in high partial pressures of oxygen, 80 to 100 per cent., has a stabilising effect on the organisms, while incubation in pressures of oxygen nearer atmospheric has the reverse effect.

The effect of aerobic cultivation on bacterial variation.

It is not proposed to discuss at all fully the various means by which bacterial variation may be promoted, since these have been admirably summarised by Hadley (1927) in his monograph on "Microbic Dissociation." But I should like to draw attention to one particular method, namely that of prolonged incubation under aerobic conditions, especially in a fluid medium. A considerable amount of evidence has now accumulated to show that under these conditions most aerobic organisms undergo dissociation with the production, by pathogenic bacteria, of weakly virulent or completely avirulent variants.

Roger (1890) observed that when a strain of *Streptococcus*, isolated from erysipelas, was sub-cultured serially in broth, its virulence for rabbits de-

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

creased. Todd (1928) recently made a similar observation. He found that, if serial sub-cultures of haemolytic streptococci were made either in broth or on agar, the normal highly virulent matt colonies were gradually replaced by glossy colonies which were permanently attenuated in virulence.

In the case of the pneumococcus Eyre and Washbourn (1897) noticed the development, in old broth cultures, of organisms which differed considerably from the original pneumococci, and which proved to be avirulent to mice and rabbits. Wright (1927) grew a strain of pneumococcus Type I in a glucose phosphate broth medium for a period of two months, sub-culturing it daily. At the end of this time the growth tended to be granular, and the culture was completely avirulent for rabbits inoculated intravenously with 1 c.c.

Stickdorn (1909) found that the virulence of *Erysipelothrix suis* diminished after numerous sub-cultures in broth and agar; finally its virulence for mice was lost altogether.

De Kruif (1921, 1922 *a* and *b*), working with *Past. lepiseptica*, found that in plain broth cultures the original virulent D colonies were gradually replaced by the avirulent G colonies. The G colonies did not appear till the second or third day of incubation, and then increased till after nine days they exceeded in numbers the original D colonies. Daily sub-culture in broth was not so successful in bringing about this result; G colonies were not found till the thirtieth sub-culture, and then they were present in only small numbers. But when the sub-cultures were made every two days, the relative proportion of G colonies increased rapidly. De Kruif's work was confirmed and amplified by Webster and Burn (1926 *a*).

With organisms of the Salmonella group numerous workers have noted the effect of daily sub-culture in broth, or of prolonged incubation of a single broth culture, in bringing about a replacement of smooth virulent by rough avirulent variants (Topley and Ayrton, 1923; Arkwright and Goyle, 1924; Savage and White, 1925; Lockhart, 1926; Jordan, 1926; Webster and Burn, 1927; Wilson, 1928, 1930 *a*). Similar observations have been made by Arkwright (1921) on *Bact. shigae*, by Shousha (1923) on *V. cholerae*, by Webster (1928) on Friedländer's bacillus, and by Hughes (1930) on *Past. aviseptica*. (For references to other bacteria, see Hadley, 1927.) It is of interest to note that Noguchi (1912) observed a sudden fall in virulence for rats and mice of *Trep. kochi* as the result of serial cultivation in his ascitic fluid kidney medium.

Summary. Cultivation under aerobic conditions, especially in a fluid medium, favours microbic dissociation, and leads to the replacement, in the case of pathogenic bacteria, of the initial virulent organisms by avirulent variants. The evidence available suggests that the agent responsible for inciting this dissociation is present in broth cultures after the stage of active growth has ceased, and that it is prolonged contact with this agent that favours the appearance of the rough avirulent variants.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

In vitro *methods of raising the virulence of micro-organisms.*

Reported observations on the production of the rough to smooth variation *in vitro*, and of the replacement of avirulent variants by virulent organisms, are few compared to those dealing with the reverse process.

Roger (1890), as related in the last section, found that serial cultivation in broth of a *Streptococcus* from erysipelas resulted in a decrease in the virulence of this organism; but he also found that if such a culture, which had become comparatively avirulent for rabbits, was sub-cultured serially in normal serum, its virulence was regained. Todd (1928), working with haemolytic streptococci, stated that matt colonies of attenuated virulence for mice could be restored to the fully virulent state by serial cultivation in normal human serum. It was impossible, however, by this method to render virulent the attenuated glossy colonies.

Wreschner (1921) was able by cultivation in serum broth to bring about a reversion of the non-capsulated type of *M. tetragenus* to the original capsulated type. Wreschner was working with whole cultures, and it appears probable that the culture of non-capsulated organisms with which he worked contained in reality a few capsulated cocci. When he used the same strain after it had been sub-cultured on agar for a long time, and had become completely avirulent, his attempts to restore its capsule formation and its virulence by growth in serum broth failed completely.

De Kruif (1922 *a*), though never succeeding in bringing about the reversion of the avirulent G to the virulent D type of *Past. lepiseptica*, found that the reverse process or D → G transformation, which occurred readily in broth cultures, hardly occurred at all in cultures in pure rabbit serum, indicating that the serum had a stabilising effect on the D type.

Griffith (1923) was able by serial cultivation in blood broth to bring about the reversion of certain rough avirulent cultures of pneumococci to the primitive smooth virulent type, but not of others. Pittman and Falk (1930), however, failed to raise the virulence of an avirulent strain of pneumococcus by sub-culture twice daily in broth media enriched with blood, glucose, or glucose and serum.

Soule (1928) succeeded in inducing the R → S transformation in *B. subtilis* by growing the organisms in flasks containing 300 c.c. of broth. After a fortnight's incubation at 33° C., about 10 per cent. of smooth organisms had appeared in the culture, as judged by plating experiments. The same R → S transformation was greatly hastened by the addition of 5 per cent. of normal rabbit serum to the broth; in this medium after eight days 50 per cent. of the organisms were of the smooth type. As previously mentioned the position of this organism is somewhat confusing, and it is not clear whether the R → S transformation is strictly analogous to that of the other organisms quoted in this section.

The experiments so far recorded are concerned with the value of serum

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

or blood in favouring the transformation of avirulent to virulent bacilli. Another series will now be described in which a different method was employed—namely, rapid sub-cultivation.

Wadsworth and Kirkbride (1918) found that daily sub-cultivation of pneumococcus Type I in broth led to a fall in virulence for mice, but that if the sub-cultures were made at eight-hourly intervals, the virulence of the organisms was retained intact. Felton and Dougherty (1924), pursuing this method, found that sub-cultivation at two-hourly intervals in nutrient broth rapidly rendered the pneumococcus avirulent, but that if the sub-cultures were made every eight hours in skimmed milk it was possible to restore the virulence to avirulent strains. In one experiment a single-cell culture of a rough strain, which was avirulent to mice in a dose of 1 c.c. of an 18-hour broth culture, was sub-cultured in milk at four-hourly intervals. After 150 transfers it was so virulent that the injection of a single coccus proved fatal. This method, however, was not uniformly successful, and certain strains were encountered which could not be raised in virulence. The effect of sub-culture in milk on the virulence of pneumococci appeared to depend considerably on the H-ion concentration. Starting with organisms of high virulence it was found that two-hourly sub-cultures in milk of *p*H 5·0, 6·0, and 7·0 had no apparent effect; but at *p*H 8·0 the virulence fell somewhat, and at *p*H 9·0 it showed a marked decrease. On the other hand, in broth, fall in virulence was more evident in media of a high than in those of a low H-ion concentration.

Jordan (1926), working with single-cell cultures of *Bact. paratyphosum* B, found that reversion of the avirulent R to the virulent S type could be partially accomplished by sub-culturing in broth at 12-hourly intervals. Smooth organisms began to make their appearance after two to three weeks, and after five weeks constituted about 20 per cent. of the organisms in the culture. One strain apparently proved refractory to this treatment.

Summary. The addition of serum or of blood to a fluid medium appears in some cases to favour the reversion of rough avirulent to smooth virulent organisms. The same effect can be occasionally produced by frequent subcultures, made two or three times a day, in a plain broth medium, or in a medium containing a natural animal protein such as milk. It is necessary to point out, however, that many experiments recording the success of the R $\rightarrow$ S transformation have been made with whole cultures which, though appearing to consist of the pure rough type, may quite well have contained a very small proportion of smooth or possibly intermediate colonies. The success of restoring the virulence to cultures of this type would seem to lie not in bringing about a reversion of the rough to the smooth type, but merely in increasing the proportion of smooth to rough organisms. This warning seems to be particularly apposite, when it is remembered that many workers who have claimed to bring about the R $\rightarrow$ S transformation, though succeeding with certain strains, have yet entirely failed with other strains. It would appear as if, at least with some bacteria, there were two different types of

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

rough colonies—one capable under appropriate conditions of being restored to the smooth virulent type, the other remaining permanently rough and avirulent. The evidence, as a whole, is, we consider, not yet sufficiently complete to render it certain whether a reversion of the true rough avirulent type to the smooth virulent type can be accomplished.

It may be added that attempts made by the author to restore the virulence to two attenuated cultures of *Bact. aertrycke*, one slightly virulent and the other completely avirulent, by daily sub-culture in pure oxygen over a period of eight months, failed completely.

In vivo *methods of raising the virulence of micro-organisms.*

The method of bringing about a rise in virulence of micro-organisms by passage through animals is too well known to warrant more than a brief notice. Numerous examples are quoted by Hadley (1927), to whose monograph reference may be made. But here again the same *caveat* may be entered as in the last section. Roux and Yersin (1890), working with the diphtheria bacillus, Wreschner (1921) with *M. tetragenus*, Griffith (1923) with the pneumococcus, Lockhart (1926) with *Bact. aertrycke*, and Todd (1928) with *Strep. haemolyticus* all encountered strains whose virulence could not be restored by animal passage; while Webster and Burn (1926 *b*), working with *Past. lepiseptica*, and Hughes (1930), working with *Past. aviseptica*, were unable to restore the virulence to any avirulent variants by this means. It appears therefore as if, once the organisms have reached the pure rough completely avirulent stage, attempts, either by *in vitro* or *in vivo* methods, to restore them to the smooth virulent type are likely to meet with failure.

Although it is obvious that the mere effect of selection will tend to favour the survival of the virulent organisms, it is interesting to enquire what mechanism in the animal body is responsible for increasing the virulence of attenuated strains of bacteria. Campbell (1926) has shown that the oxygen pressure in the subcutaneous tissues of mammals is generally about 20–30 mm. Hg, *i.e.* about 2·5 to 4 per cent. of an atmosphere. The results of most workers suggest that this pressure is likely to bring about a fall rather than a rise in virulence, but it must be remembered that the CO_2 pressure in the tissues is fairly high, 5·3 to 6·5 per cent., and it may be that the presence of this gas has some modifying action on the effect of the oxygen pressure. The conditions are complicated by the presence in the tissues of blood plasma; and it is not unreasonable to suppose that this substance may be mainly responsible for favouring the development of the smooth virulent type, since numerous workers have demonstrated the value *in vitro* of the addition of blood or serum to broth cultures in promoting the R $\rightarrow$ S variation.

In this brief summary of the factors favouring microbic dissociation, reference to such incitants as the bacteriophage and immune sera has been purposely omitted, since their mode of action is probably different from that of the other factors which we have considered.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

DISCUSSION OF THE EXPERIMENTAL RESULTS RECORDED IN THIS PAPER.

From Table XIX and Fig. 12, it appears that daily sub-cultivation of *Bact. aertrycke* under anaerobic conditions stabilises the virulence; that growth in partial pressures of oxygen varying from 1 to 21 per cent. leads to a fall in virulence, the fall increasing with the oxygen pressure; and that growth in high partial pressures of oxygen, from 40 to 100 per cent., brings about a slight increase in virulence, the increase being proportional to the oxygen pressure.

That these results are not due to any variations in dosage between the control and the experimental cultures is clear from the work of Lockhart (1926), Topley (1927), and Wilson (1930 *b*), all of whom have shown that small variations in dose have very little effect on the outcome of experimental inoculation of this organism into mice.

It was thought that a study of the growth curves of *Bact. aertrycke* under different partial pressures of oxygen might throw light on these results. Accordingly, a series of experiments was carried out according to a technique already described (Wilson, 1922). It is hoped to report the full results in a future paper; for the present purpose it is enough to extract the most important figures (Table XX).

Table XX.

Exp.	Viable bacilli At start	Viable bacilli (24 hr.)	Total bacilli (24 hr.)	Percentage of viable to total (8 hr.)	Percentage of viable to total (24 hr.)	pH (24 hr.)	pH (48 hr.)
Anaerobic *A*	380	350×10^6	520×10^6	65	68	7·4	7·6
,, *B*	340	360×10^6	510×10^6	58	71	7·4	7·6
,, *C*	950	380×10^6	520×10^6	68	73	7·4	(1)
1 % O_2 bubbling	1,340	1130×10^6	1180×10^6	81	95	8·0	8·5
5 % O_2 bubbling	1,050	2130×10^6	2380×10^6	88	90	8·3	8·6
Aerobic *A*	7,200	1260×10^6	1590×10^6	84	79	8·2	8·4
,, *B*	16,290	1180×10^6	1420×10^6	88	83	8·0	8·5
21 % O_2 bubbling *A*	20,740	3490×10^6	5050×10^6	76	69	8·6	8·6
,, *B*	1,360	3310×10^6	3880×10^6	72	85	8·5	8·6
100 % O_2 bubbling *A*	170	2750×10^6	8550×10^6	(2)	32	8·8	8·6
,, *B*	15,390	2280×10^6	8330×10^6	70	27	8·6	8·6
,, *C*	1,430	2550×10^6	6740×10^6	70	38	8·6	8·6

(1) Exp. stopped after 30 hours.
(2) No total count made at 8 hours.

It was found that the rate of growth during the logarithmic phase was much the same under all conditions, but that the actual number of organisms produced varied with the oxygen pressure. In cultures incubated anaerobically growth ceased comparatively early and the total number of organisms alive and dead at the end of 24 hours was only about 500 million per c.c. In 5 per cent. oxygen bubbling cultures growth continued for a longer period, and the total number of organisms after 24 hours was 2000 million. In 100 per cent. oxygen bubbling cultures growth continued still further, and the total number of organisms at the end of 24 hours was 8000 million. The proportion of viable to total organisms after 8 hours was much the same under all conditions, but

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

after 24 hours a very marked fall was observed in the 100 per cent. oxygen cultures.

It seems clear from these experiments that the growth phase can be prolonged by providing the organisms with a liberal supply of oxygen, but that when a very large number of organisms have been produced, even the supply of pure oxygen is insufficient to prevent a considerable proportion of them from dying. These results are similar in many respects to those obtained by Müller (1912).

Estimations of the H-ion concentration of the cultures were made at intervals by the capillator method, using phenol red and thymol blue as indicators. The results are probably accurate to within 0·2 *p*H. It was found that in all cultures there was a preliminary increase in the H-ion concentration, followed by a fall. Under anaerobic conditions this fall ceased at *p*H 7·6, but in the experiments in which oxygen was supplied it continued till the culture was markedly alkaline. The greater the amount of oxygen supplied, the more rapid was the development of alkalinity.

It is obviously premature to attempt to offer any explanation of the effect of different partial pressures of oxygen on the virulence of different organisms, but in so far as *Bact. aertrycke* is concerned the following alternative hypotheses are tentatively advanced.

(1) Under aerobic conditions, and under low partial pressures of oxygen generally, substances are formed in the medium, as the result of growth, which favour dissociation, and lead to the appearance of weakly virulent or completely avirulent variants. (For a description of these variants, which may be either smooth or rough, reference should be made to Wilson, 1930 *a*.) Under anaerobic conditions these substances either are not formed at all, or appear in amounts too small to be of significance. In cultures incubated in high partial pressures of oxygen, 40 to 100 per cent., these substances are formed, probably in large quantity, but owing to the high alkalinity of the medium and the abundant oxygen present, they undergo rapid destruction. This explanation is based on the known fact that many protein degradation products, particularly those with a phenolic grouping, prove markedly unstable in an alkaline medium in the presence of free oxygen. It may be that the time during which the organisms are exposed to these substances is a significant factor in the determination of bacterial dissociation; evidence has already been brought forward from the literature to suggest that this may be so. This explanation depends on the supposition that the agent responsible for inciting variation is formed outside the cell.

(2) The second hypothesis supposes that the maintenance of the organisms in the virulent state depends on the intracellular *p*H. It was pointed out by Jacobs (1920 *b*) that the H-ion concentration within the cell bears no necessary relation to that of the medium to which it is exposed. Working with certain protozoa, and with the flowers of *Symphytum peregrinum*, he (Jacobs, 1920 *a*, *b*) brought evidence to show that CO_2 was able to penetrate the living cell

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

very much more rapidly than other acids. Owing to this peculiarity it was found possible to produce a rise of intracellular acidity by immersing the cells in a slightly alkaline solution of CO_2 in $M/2$ $NaHCO_3$ almost as effectively as in a highly acid solution of CO_2 in distilled water. Working with the cat, Dale and Evans (1922) adduced evidence to suggest that the tonic action of the vasomotor centres is conditioned by the concentration of free CO_2 rather than by that of free H-ions in the arterial blood. The effect of CO_2 in permitting the growth of the bovine type of *Br. abortus*, in favouring the production of skin toxin and haemolysis by *Staph. aureus*, and in increasing the pigment production of *Chromo. prodigiosum* has been established by various workers (see Burnet, 1930). It would appear that the effect of the CO_2 is to produce a rise in the H-ion concentration within the cell.

Working with *Bact. coli* Stephenson and Whetham (1924) showed that this organism was unable to utilise lactic acid as its sole source of carbon under anaerobic conditions. In the presence of air lactic acid was consumed with the evolution of CO_2, but the rate at which this reaction occurred was greatly accelerated by increased oxygen pressure.

Reverting to the experiments described in this paper, it may be supposed that under anaerobic conditions the metabolism is such that the H-ion concentration within the cell undergoes no marked change. In the presence of air and of low pressures of oxygen generally, owing to the breakdown of the protein in the medium and the production of ammonium salts, the intracellular reaction becomes more alkaline; whereas in high pressures of oxygen the increased amount of CO_2 produced is sufficient to compensate for the production of ammonia, and the intracellular pH remains more or less at its original value.

Summary and conclusions.

1. A given strain of *Bact. aertrycke* was sub-cultured daily, except on Sundays, in 5–7 c.c. of casein broth, pH 7·4–7·6, under different partial pressures of oxygen. Most experiments were continued till about ninety subcultures had been made. Virulence determinations were made from time to time by intraperitoneal inoculation of twenty mice with about 100 living organisms, the control and the experimental cultures generally being tested simultaneously. The results showed that under anaerobic conditions there was no appreciable change in virulence. Under the usual aerobic conditions, and in partial pressures of oxygen varying from 1 to 21 per cent., the virulence declined, the extent and rapidity of the fall increasing with the pressure of oxygen supplied. In high partial pressures of oxygen, varying from 40 to 100 per cent., there was a slight increase in virulence, the increase being greatest in cultures incubated in pure oxygen.

2. Some of the literature dealing with incitants to microbic dissociation is discussed, particularly that dealing with variations in oxygen pressure.

3. In explanation of the experimental results obtained, a tentative

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

hypothesis is put forward with reference to the dissociation of *Bact. aertrycke*. It is suggested that under aerobic conditions, and under low partial pressures of oxygen generally, substances are formed in the medium, as the result of growth, which favour dissociation, and lead to the appearance of weakly virulent or completely avirulent variants. Under anaerobic conditions these substances either are not formed at all, or appear in amounts too small to be of significance. In cultures incubated in high partial pressures of oxygen, these substances are formed, probably in large quantity, but owing to the high alkalinity of the medium and the abundant oxygen present, they undergo rapid destruction. This explanation is based on the fact that many protein degradation products, particularly those with a phenolic grouping, prove markedly unstable in an alkaline medium in the presence of free oxygen.

As an alternative hypothesis it is suggested that the maintenance of the organisms in the virulent state depends on the intracellular *p*H. Under anaerobic conditions it is supposed that the metabolism is such as to lead to no marked change in the H-ion concentration within the cell. In the presence of air and of low pressures of oxygen generally, owing to the breakdown of the protein in the medium and the production of ammonia, the intracellular reaction becomes more alkaline; whereas in high pressures of oxygen the increased amount of CO_2 produced is sufficient to compensate for the production of ammonia, and the intracellular *p*H remains more or less at its original value. This hypothesis rests on the observation of Stephenson and Whetham (1924) that in high pressures of oxygen *Bact. coli* may produce considerably larger quantities of CO_2 from lactic acid than under ordinary aerobic conditions; and on the demonstration by Jacobs (1920 *b*) that, owing to the ease with which CO_2 is able to penetrate the living cell, the intracellular *p*H may differ markedly from that of the medium to which the cell is exposed.

4. It is of interest to note that *Bact. aertrycke*, when incubated under partial pressures of 40 to 75 per cent. of oxygen, rapidly passed into the non-motile O form.

In conclusion, I would express my thanks to Prof. M. Greenwood for assistance with the statistical treatment of this paper; to Prof. H. Raistrick for furnishing me with certain chemical data; and to the Government Grant Committee of the Royal Society for financial assistance in this investigation.

REFERENCES.

Adams, A. (1912). *Biochem. J.* **6**, 297.
Arkwright, J. A. (1921). *J. Path. and Bact.* **24**, 36.
Arkwright, J. A. and Goyle, A. N. (1924). *Brit. J. Exp. Path.* **5**, 104.
Bang (1897). *Zeitschr. f. Thiermed.* **1**, 241.
Burnet, F. M. (1930). *J. Path. and Bact.* **33**, 1.
Campbell, J. A. (1926). *J. Physiol.* **61**, 248.
Dale, H. H. and Evans, C. L. (1922). *Ibid.* **56**, 125.
De Kruif, P. H. (1921). *J. Exp. Med.* **33**, 773.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press

DE KRUIF, P. H. (1922 *a*). *J. Exp. Med.* **35**, 561.
—— (1922 *b*). *Ibid.* **35**, 621.
EYRE, J. W. and WASHBOURN, J. W. (1897). *J. Path. and Bact.* **4**, 394.
FELTON, L. D. and DOUGHERTY, KATHARINE M. (1924). *J. Exp. Med.* **39**, 137, 155.
FISHER, R. A. (1928). *Statistical Methods for Research Workers.* Oliver and Boyd, Edinburgh and London, 2nd ed.
GRIFFITH, F. (1923). *Rept. on Pub. Health and Med. Subjects, Ministry of Health, London,* No. 18.
HADLEY, P. (1927). *J. Infect. Dis.* **40**, 1.
HAFFKINE, W. M. (1892). *Compt. rend. Soc. Biol.* **4**, 635, 671.
HUDDLESON (1921). *Cornell Veterinarian,* **11**, 210.
HUGHES, T. P. (1930). *J. Exp. Med.* **51**, 225.
JACOBS, M. H. (1920 *a*). *Amer. J. Physiol.* **51**, 321.
—— (1920 *b*). *Ibid.* **53**, 457.
JORDAN, E. O. (1926). *J. Amer. Med. Assoc.* **86**, 177.
KARSNER, H. T., BRITTINGHAM, H. H. and RICHARDSON, M. L. (1923). *J. Med. Res.* **44**, 83.
LAIDLAW, P. P. (1915). *Brit. Med. J.* **1**, 497.
LOCKHART, L. P. (1926). *J. Hygiene,* **25**, 50.
LUBINSKI, W. (1894). *Centralbl. f. Bakt.* **16**, 769.
MOORE, B. and WILLIAMS, R. S. (1909). *Biochem. J.* **4**, 177.
—— (1911). *Ibid.* **5**, 181.
MÜLLER, A. (1912). *Arb. a. d. Kais. Gesundht.* **38**, 294.
NOGUCHI, H. (1912). *J. Exp. Med.* **16**, 199.
NOVY, F. G. and SOULE, M. H. (1925). *J. Infect. Dis.* **36**, 168.
PITTMAN, MARGARET, and FALK, I. S. (1930). *J. Bact.* **19**, 327.
ROGER, G. H. (1890). *Compt. rend. Soc. Biol.* **2**, 573.
ROUX, E. and YERSIN, A. (1890). *Ann. Inst. Pasteur,* **4**, 385.
SAVAGE, W. G. and WHITE, P. B. (1925). *Med. Res. Council, Spec. Rep. Ser.* No. 91, London.
SHOUSHA, A. T. (1923). *J. Hygiene,* **22**, 156.
SOULE, M. H. (1928). *J. Infect. Dis.* **42**, 93.
STEPHENSON, M. and WHETHAM, M. D. (1924). *Biochem. J.* **18**, 498.
STICKDORN, W. (1909). *Centralbl. f. Bakt.* **50**, 5.
TODD, E. W. (1928). *Brit. J. Exp. Path.* **9**, 1.
TOPLEY, W. W. C. (1927). *J. State Med.* **35**, 63.
TOPLEY, W. W. C. and AYRTON, MARY J. (1923). *J. Hygiene,* **22**, 234.
WADSWORTH, A. B. and KIRKBRIDE, MARY B. (1918). *J. Exp. Med.* **28**, 791.
WEBSTER, L. T. (1924). *Proc. Soc. Exp. Biol.* **22**, 139.
—— (1925). *J. Exp. Med.* **41**, 571.
—— (1928). *Ibid.* **47**, 685.
WEBSTER, L. T. and BURN, C. (1926 *a*). *Ibid.* **44**, 343.
—— —— (1926 *b*). *Ibid.* **44**, 359.
—— —— (1927). *Ibid.* **46**, 855.
WILSON, G. S. (1922). *J. Bact.* **7**, 405.
—— (1926). *J. Hygiene,* **25**, 142.
—— (1928). *Ibid.* **28**, 295.
—— (1930 *a*). *Ibid.* **30**, 40.
—— (1930 *b*). *Ibid.* **30**, 196.
—— (1930 *c*). Unpublished.
WOSNESSENSKI, J. (1884). *Compt. rend. Acad. des. Sci.* **98**, 314.
WRESCHNER, H. (1921). *Zeitschr. f. Hyg.* **93**, 74.
WRIGHT, H. D. (1927). *J. Path. and Bact.* **30**, 185.

https://doi.org/10.1017/S0022172400010585 Published online by Cambridge University Press