# VARIATIONS IN THE VIRULENCE OF DIFFERENT STRAINS OF *BACILLUS DIPHTHERIAE.*

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The occurrence of non-virulent strains of bacilli, which are indistinguishable except by animal experiment from virulent diphtheria bacilli, makes it especially interesting to investigate the varying pathogenicity of this bacillus. Owing to their complete lack of pathogenicity for laboratory animals, the exact relationship of the non-virulent types of *B. diphtheriae* to the virulent forms of this organism, cannot yet be considered as settled, although Arkwright (1910) has succeeded in obtaining a slight but very definite development of antitoxin in the blood of a horse immunised with non-virulent strains.

In this paper no cognisance will be taken of the non-virulent forms. The experiments here recorded deal solely with the variations in virulence which are found to exist among strains which exhibit a definite pathogenicity for guinea-pigs.

The pathogenicity or virulence in a general sense has been examined by means of "whole" broth cultures containing both bacilli and the toxin formed by them, but an attempt has also been made to determine whether such differences in lethal power as occur between different strains are due chiefly to differences in the amount of toxin in the cultures, or to differences in the virulence proper of the bacillus, *i.e.* the activity of the bacillus after inoculation in the toxin-free state into the animal body.

## *Difference in the Pathogenicity of different strains.*

Many writers on this subject have recorded great variations in the pathogenicity of different strains, *e.g.* v. Behring (1901), Bardach (1895), Martin, L. (1898), Pennington (1907), and the matter is thoroughly

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discussed by Graham-Smith and Dean in *The Bacteriology of Diphtheria*, (Nuttall and Graham-Smith (1908)).

Graham-Smith (1904) and Cobbett (1901), on the other hand, found a remarkable uniformity in the lethal dose of the strains which they isolated during two large epidemics of diphtheria at Cambridge. Cobbett out of 68 virulent strains, found only two which when recently isolated did not kill a guinea-pig of 250 grms. in two or three days, when 0·1 c.c. of a two-day broth culture was injected subcutaneously. (In four instances only, 0·5 c.c. was the smallest dose used.) The two strains of exceptionally low virulence were rapidly raised to the same degree of pathogenicity as the remainder, after one or more subcultures in broth.

Graham-Smith amongst 88 virulent strains isolated, found one which did not kill within two or three days with the smallest dose used. The doses injected varied from 0·1 to 0·3 c.c. but the majority of strains killed in a dose of not more than 0·2 c.c. With only one strain, 0·1 c.c. did not kill the experimental animal in 12 days, but the growth was very poor and subculture in broth did not materially increase its vigour.

It is to be noted, however, that these writers do not record any observations as to variations in virulence above these limits, *i.e.* when smaller doses than 0·1 c.c. were used. Moreover all the strains were isolated during two epidemics in the same town and their work shows the high degree of virulence of *B. diphtheriae* during these two epidemics.

Williams (1902) also noted the occurrence of strains of low virulence which attained a higher grade of virulence when the growth on broth had been improved by subculture.

Smith, Th. and Walker, E. (1896) tested the virulence of different strains by estimating the accumulation of toxin in broth cultures, and came to the conclusion that the virulence of different strains was very uniform. They did not, however, test the pathogenicity of cultures containing bacilli, and their conclusions are remarkable when one considers the enormous differences in the toxin-yield from broth cultures which have been obtained with different, or even the same, strains by many workers, *e.g.* Park (1896), Dean (1908), Madsen (1907).

I have not infrequently met with strains of low pathogenicity. In a school epidemic which I investigated (Arkwright, 1908), of 13 virulent strains which were isolated, only four were of such a degree of virulence that 0·1 c.c. of a two-day broth culture killed a guinea-pig of

250 grms. within four days and 2 c.c. in 48 hours. Three other strains when given in a dose of 0·1 c.c. killed the animal in ten days whereas a dose of 2 c.c. was fatal within four days. The remaining six strains were non-lethal when 0·1 c.c. was injected, but a dose of 2 c.c. was fatal within four and a half days.

At that time it seemed possible that this prevalence of strains of low virulence, might be causally connected with the large number of completely non-virulent strains found in the same epidemic (seven strains were non-virulent out of 20 strains which were isolated), and it seemed not unlikely that different degrees of virulence might prevail in different epidemics.

In the case of a small epidemic in a school from which I have recently examined material, five cases of diphtheria occurred. From these patients three strains of *B. diphtheriae* were isolated and tested for virulence. All were found to be of low virulence, when tested on several occasions. Although a large dose of 2 c.c. or 2·5 c.c. killed a guinea-pig rapidly, and a control animal to which antitoxin was given was unaffected, yet 0·1 c.c. proved to be a sublethal dose.

In another series of 37 virulent strains from various sources 25 were lethal when 0·1 c.c. and the remainder when 2·5 c.c. were injected.

*Method of estimating pathogenicity.*

In estimating the pathogenicity[1] of a strain of *B. diphtheriae* by the injection of "whole" two-day cultures (bacilli and toxin together) several difficulties were met with.

No doubt the exact composition of the broth in which the bacilli are grown makes a considerable difference in the results obtained by injecting whole cultures, and occasionally strains which are generally of low pathogenicity attain a much higher grade when grown on a different batch of broth, but when this happens there is sometimes reason to believe that the strains of high pathogenicity in cultures made at the same time, and in the same broth, are correspondingly exalted. Graham-Smith and Cobbett attach great importance to the use of sugar-free broth, but other workers have found that so long as the broth culture remains alkaline in reaction, the presence of a small amount of sugar does not hinder the formation of toxin (*e.g.* Smith, Th., Park and Williams).

[1] In this paper when referring to my own work the term "Pathogenicity" is used when "whole" broth cultures, "Toxigenicity" when toxin free from bacilli, and "Virulence" when washed bacilli, have been employed for injection.

In this investigation the cultures in broth were found to remain alkaline. The broth employed was made from bullock's heart with 1 % of Witte's peptone added, and rendered just alkaline to litmus paper. That the variations in the broth used were not an important cause of the differences in the results with the different strains, was shown by the fact that all degrees of virulence were found with the same brew of broth.

The period of incubation was two days at 37° C.

*Estimation of the amount of growth in broth.*

The growth in broth is obviously not always equally great when the same strain is used, and with different strains the amount and character of the growth varies very much.

Williams, Cobbett and Graham-Smith attribute the low virulence of their exceptional strains to the scanty growth in broth when they were recently isolated. In the case of my strains of low pathogenicity the growth was usually, but not always, small, and was not less than that of some strains possessing a higher lethal power. Training the bacilli to grow in peptone broth will sometimes increase the pathogenicity somewhat, but it is possible that other changes than increased ability to grow on artificial media may be induced in this way.

In order to estimate the amount of growth 10 c.c. of the bouillon cultures were centrifuged in a tube drawn out to a small calibre in its lower half and graduated in hundredths of a c.c. in this part. The amount of growth as shown by the deposit measured in this way was not proportionate to the virulence.

It was not found possible to count the bacilli in a broth culture on account of the clumping of most strains.

The following strains were used:

| | | |
|---|---|---|
| Strain No. I. | 11 Bb | Isolated 24 Jan. 1911 from a diphtheria convalescent. |
| II. | 66 | ,, 2 Feb. 1911 from a school "carrier." |
| III. | 134 X | ,, 21 Oct. 1909. Diagnosis swab. |
| IV. | 134 Z | ,, 21 Oct. 1909. ,, ,, |
| V. | 19 AT | ,, 25 Feb. 1911 from a diphtheria convalescent. |
| VI. | 21 AN | ,, 25 Feb. 1911 ,, ,, ,, |
| VII. | R 50 | ,, 6 Dec. 1910. Case of diphtheria. |
| VIII. | R 4 | ,, 7 Dec. 1910. ,, ,, |
| IX. | 166 | Skin infection by *B. diphtheriae*. |
| X. | 27 | Isolated 14 Mar. 1911. Diphtheria convalescent. |
| XI. | Dkth | ,, 12 Jan. 1911. ,, ,, |
| XII. | NE 30 | ,, 16 Mar. 1911. ,, ,, |
| XIII. | R 70 | ,, 31 Mar. 1911. Case of diphtheria. |

The majority of the strains were chosen on account of their low virulence, but the extremes of virulence met with are represented. Some of the strains have been examined at considerable intervals of time with the object of ascertaining whether the degree of virulence remained fairly constant.

The results of injection of the "whole" two-day cultures are shown in Table I.

In this table the dose stated to be the M.L.D. is that of a two-day broth culture which caused death within four days. In another column is shown the sub-lethal dose, *i.e.* the highest dose which allowed the animal to survive. The approximate amount of deposit from 10 c.c. of the culture is also shown. The guinea-pigs were all between 240 and 250 grms. in weight. The doses used were usually 0·01, 0·02, 0·1, 0·5 and 2·5 c.c.

In Table II are recorded a few of the observations in which several strains of different degrees of virulence were tested on the same day in the same broth, showing that large variations in virulence were obtained with the same quality of broth.

In considering the results shown in Table I, it is seen that of the seven strains, I, II, IX, XI, V, VI, VII, each of which was examined on several occasions, four, viz. I, II, IX and V, remained fairly constant in pathogenicity. Of these I and II were of high pathogenicity, having a very small M.L.D. varying at most from 0·01 to 0·02 c.c. in the case of strain I, and from 0·02 to 0·1 c.c. in the case of strain II. The other two constant strains IX and V were of low pathogenicity, the M.L.D varying from 0·5 to 2·5 c.c. Of the three remaining strains (XI, VI and VII) which were examined on more than two occasions, the M.L.D. varied from 2·5 to 0·1 c.c. Strain X was tested on only one occasion. Strain VIII was tested twice and on both occasions was of low pathogenicity.

The amount of deposit obtained by centrifuging 10 c.c. of culture varied for different strains between 0·0025 and 0·02 (1 to 8), but the amount of deposit was not proportionate to the pathogenicity of the culture, for the M.L.D. of culture for these strains varied from 2·5 to 0·01 (250 to 1). Thus strains I and II gave deposits only varying from 0·005 to 0·02 c.c. (1 to 4) and the M.L.D. for the same two strains varied from 0·1 to 0·01 c.c. (100 to 1).

In the case of strain IX the amount of deposit varied from 0·0025 to 0·005 c.c. with a M.L.D. never less than 0·5 (tested on four occasions). In the same way strain V gave a deposit of 0·0025 to 0·0075 c.c. but the M.L.D. was never less than 0·5 c.c.

TABLE I.

| Strain and date of isolation | Dates of experiments | Deposit from 10 c.c. of culture | M.L.D. of "whole" culture | Day of death | Sublethal dose | Result of do. |
|---|---|---|---|---|---|---|
| No. I | 4 Feb. 1911 | — | 0·1 | Second | — | — |
| 24 Jan. 1911 | 11 Feb. ,, | — | 0·02 | Third | — | — |
| | 16 Feb. ,, | 0·015 | 0·01 | Third | — | — |
| | 15 May ,, | 0·005 | 0·02 | Second | — | — |
| No. II | 9 Dec. 1909 | — | 0·1 | — | — | — |
| 2 Feb. 1909 | 3 Feb. 1911 | — | 0·1 | Third | 0·02 | Large swelling. |
| | 9 Mar. ,, | 0·01 | — | — | — | — |
| | 15 May ,, | 0·02 | 0·02 | Third | — | — |
| No. IX | 13 Jan. 1911 | — | 2·5 | Second | 0·1 | Almost nil. |
| 11 Jan. 1911 | 22 Feb. ,, | — | — | — | 1·5 | Death 5th day. |
| | 24 Feb. ,, | — | 0·5 | Third | 0·25 | Death 10th day. |
| | 9 Mar. ,, | 0·0025 | — | — | — | — |
| | 6 Apr. ,, | — | 0·5 | Second | 0·1 | Death 5th day. |
| | 20 Apr. ,, | 0·005 | — | — | — | — |
| | 28 Apr. ,, | 0·0025 | 0·5 | Second | 0·1 | Death 8th day. |
| No. X | 20 Mar. 1911 | — | 0·1 | Second | — | — |
| 14 Mar. 1911 | 27 Mar. ,, | 0·003 | — | — | — | — |
| No. XI | 18 Jan. 1911 | — | 2·5 | Third | 0·1 | Lived. |
| 12 Jan. 1911 | 6 Apr. ,, | — | 0·5 | Fourth | 0·1 | Death 7th day. |
| | 28 Apr. ,, | 0·003 | 0·5 | Second | 0·1 | Lived. |
| | 15 May ,, | 0·003 | 0·1 | Third | — | — |
| No. V | 6 Apr. 1911 | — | 0·5 | Second | 0·1 | Lived. |
| 25 Feb. 1911 | 20 Apr. ,, | 0·0075 | 2·5 | Fourth | 0·5 | Lived. |
| | 28 Apr. ,, | 0·0025 | 0·5 | Second | 0·1 | Death 5th day. |
| No. VI | 11 Mar. 1911 | — | 2·5 | Second | 0·1 | Lived. |
| 25 Feb. 1911 | 6 Apr. ,, | — | 0·1 | Fourth | — | — |
| | 20 Apr. ,, | 0·005 | — | — | — | — |
| | 28 Apr. ,, | 0·0025 | 0·1 | Second | — | — |
| No. VII | 10 Dec. 1910 | — | 2·5 | Second | 0·1 | Lived. |
| 7 Dec. 1910 | 14 Dec. ,, | — | — | — | 2·0 | Lived. |
| | 5 Apr. 1911 | — | 2·5 | Second | 0·5 | Lived. |
| | 21 Apr. ,, | 0·005 | — | — | — | — |
| | 15 May ,, | 0·003 | 0·1 | Third | — | — |
| | 15 May ,, | — | 0·5 | Fourth | — | — |
| No. VIII | 12 Dec. 1910 | — | 2·5 | Second | 0·1 | Lived. |
| 7 Dec. 1910 | 5 Apr. 1911 | — | 2·5 | Second | 0·5 | Death 5th day. |
| | 21 Apr. ,, | 0·005 | — | — | — | — |

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## TABLE II.

*Observations on different strains on the same date showing that the broth used was not the cause of differences in virulence.*

A.

| Strain | Date of experiment | Deposit from 10 c.c. of culture | M.L.D. whole culture | Day of death | Deposit from 10 c.c. of emulsion | M.L.D. original emulsion of bacilli | Day of death | Sublethal dose original emulsion | M.L.D. standard emulsion | Day of death | Sublethal dose standard emulsion |
|---|---|---|---|---|---|---|---|---|---|---|---|
| No. I | 15 May '11 | ·005 | 0·02 | Second | 0·009 | 0·02 | Second | — | 0·02 | Second | — |
| No. II | ,, | ·02 | 0·02 | Third | 0·01 | 0·1 | Third | 0·02 | 0·1 | Third | 0·02 |
| No. XI. | ,, | ·003 | 0·1 | Third | 0·015 | 0·33 | Third | 0·066 | 0·5 | Third | 0·1 |
| No. VII. | ,, | ·003 | 0·1 | Third | 0·003 | 1·5 | Third | 0·3 | 0·5 | Third | 0·1 |

B.

| Strain | Date of experiment | M.L.D. whole culture | Day of death | Sublethal dose |
|---|---|---|---|---|
| No. VII | 5 Apr. 1911 | 2·5 | Second | 0·5 |
| No. XII | ,, | 0·5 | Fourth | 0·1 |
| No. XIII | ,, | 0·1 | Second | — |
| No. VIII | ,, | 2·5 | Second | 0·5 |

C.

| Strain | Date of experiment | M.L.D. standard emulsion | Day of death | Sublethal dose |
|---|---|---|---|---|
| No. I | 6 Mar. 1911 | 0·01 | Second | — |
| No. XI | ,, | 4·0 | Third | 1·0 |

The variation in deposit for the same strain was not proportionate to the changes in pathogenicity, *e.g.* strain V on the occasion on which the deposit was largest (0·0075 c.c.) had a M.L.D. of 2·5 c.c., whereas on another occasion with a smaller deposit of 0·0025 to 0·003 c.c. the M.L.D. was actually smaller, viz. 0·5 c.c.

Strain XI twice gave a deposit of 0·003 c.c. but on one occasion the M.L.D. was 0·5 c.c. and on the other 0·1 c.c.

It appears, therefore, that neither in regard to different strains, nor to different observations on the same strain, were the comparatively small variations in the amount of deposit proportional to the pathogenicity of the culture.

The difference in pathogenicity between the strains was much greater than could be accounted for by the difference in the amount of growth in the respective cultures.

Although the degree of pathogenicity of the same strain varied from time to time, the difference between the strains for the most part remained well marked.

*The independent variation of Bacilli and Toxin as regards Lethal power in two-day broth cultures.*

Workers who have investigated the relation of virulence to toxigenicity of different strains of *B. diphtheriae* have usually made use of 24-hour broth or young agar cultures in estimating the virulence, and filtrates from broth cultures of about a week old, for testing the amount of toxin produced. They have come to the conclusion that the same bacillus may be highly virulent and yet of feeble toxigenic power or the reverse (Martin, L., 1898, Behring, 1901). In the tests recorded in the present communication the virulence and toxigenicity have both been examined by means of a two-day broth culture.

It seemed that from the point of view of virulence it was of more interest to know the relation, as regards lethal power, between the toxin and bacilli in the same culture. Moreover it seemed unlikely that the amount of toxin accumulated in a 7-day broth culture had any very intimate relation to the pathogenicity of a particular strain, as the experimental animal injected with bacilli or culture usually dies within three days. Also the exact composition of the broth probably has a special effect on the accumulation of toxin independently of its formation.

*Methods of testing the Toxigenicity and Virulence.*

A measured quantity of the culture was centrifuged and the clear fluid poured off and recentrifuged. The resulting clear fluid was tested for its toxin-content.

The deposit after the first centrifuging was washed with salt solution, and after again centrifuging the deposit was made up to the original volume. The M.L.D. of the resulting bacterial emulsion was taken as a criterion of "Virulence proper."

The doses of toxin or bacterial emulsion employed were 0·01, 0·02, 0·1, 0·5 and 2·5 c.c. and occasionally doses with smaller intervals were used.

In order to reduce the dose of bacilli to a uniform measure a standard bacillary emulsion was used of such a strength that 10 c.c. of emulsion, on centrifuging, yielded a deposit of 0·01 c.c. of bacilli. The M.L.D. of bacilli could then be stated as a dose of such a standard emulsion. In a few instances a standard emulsion was actually made, and used for

injecting the animals, but usually the original emulsion was used and the dose of standard emulsion was calculated from the amount of deposit given by 10 c.c. of the original emulsion.

In Table III the M.L.D. of the original emulsion of washed bacilli made up to the same volume as the culture, and of the standard emulsion are shown, and the amount of deposit from 10 c.c. of the emulsion used is also shown.

TABLE III.

| Strain and date of isolation | Date of experiment | Deposit from 10 c.c. of emulsion | M.L.D. of original emulsion | Day of death | Sublethal dose | Result of do. | M.L.D. of standard emulsion | Sublethal dose of standard emulsion | Result of do. |
|---|---|---|---|---|---|---|---|---|---|
| No. I | 16 Feb. '11 | — | 0·01 | Third | — | — | — | — | — |
| 24 Jan. '11 | 6 Mar. ,, | 0·01 | 0·01 | Second | — | — | 0·01 | — | — |
| | 20 Mar. ,, | — | 0·01 | Third | — | — | — | — | — |
| | 15 May ,, | 0·009 | 0·02 | Second | — | — | 0·02 | — | — |
| No. II | 9 Dec. '09 | — | 0·1 | — | — | — | — | — | — |
| 2 Feb. '09 | 3 Feb. '11 | — | 0·1 | Third | 0·02 | Lived | — | — | — |
| | 9 Mar. ,, | 0·01 | 2·0 | Second | 0·5 | Lived | 2·0 | 0·5 | Lived. |
| | 15 May ,, | 0·01 | 0·1 | Third | 0·02 | Lived | 0·1 | 0·02 | Lived. |
| No. IX | 24 Feb. '11 | — | >1·0 | — | 1·0 | Death 10th day | — | — | — |
| 11 Jan. '11 | 9 Mar. ,, | 0·01 | 0·1 | Fourth | 0·02 | Lived | 0·1 | 0·02 | Lived. |
| | 20 Apr. ,, | 0·005 | 0·5 | Fourth | 0·1 | Lived | 0·17 | 0·03 | Lived. |
| No. X | 27 Mar. '11 | 0·004 | 0·05 | Fourth | — | — | 0·02 | — | — |
| 14 Mar. '11 | | | | | | | | | |
| No. XI | 10 Feb. '11 | — | >3·0 | — | 3·0 | Lived | — | — | — |
| 12 Jan. '11 | 20 Apr. ,, | 0·0075 | 2·5 | Third | 0·5 | Lived | 1·5 | 0·3 | Lived. |
| | 6 Mar. ,, | — | — | — | — | — | 4·0 | 1·0 | Lived. |
| | 15 May ,, | 0·015 | 0·33 | Third | 0·066 | Lived | 0·5 | 0·1 | Lived. |
| No. V | 20 Apr. '11 | 0·003 | 0·5 | Fourth | 0·1 | Lived | 0·17 | 0·03 | Lived. |
| 25 Feb. '11 | | | | | | | | | |
| No. VI | 16 Mar. '11 | — | >4·0 | — | 4·0 | Lived | — | — | — |
| 25 Feb. '11 | 20 Apr. ,, | 0·003 | ·5 | Second | 0·1 | Lived | 0·17 | 0·03 | Lived. |
| No. VII | 21 Apr. '11 | 0·003 | >2·5 | Seventh | 2·5 | Death 7th day | >0·83 | 0·83 | Death 7th day. |
| 6 Dec. '10 | 15 May ,, | 0·003 | 1·5 | Third | 0·3 | Lived | 0·5 | 0·1 | Lived. |
| No. VIII | 21 Apr. '11 | 0·005 | 2·5 | Third | 0·5 | Lived | 1·25 | 0·25 | Lived. |
| 7 Dec. '10 | | | | | | | | | |

Table III shows that the M.L.D. of standard emulsion varied for the same strain at most from 0·1 to 2·0 c.c. in the case of No. II, but so large a M.L.D. as 2 c.c. was quite unusual for this strain. Leaving this one observation out of consideration the greatest variation for a single strain was in the case of No. XI which on different occasions gave a M.L.D. of 4 c.c. and 0·5 c.c. (variation of 8 to 1). The differences amongst the different strains, if one takes the occasions of least difference, ranged from 0·5 to 0·01 (50 to 1), or if one takes the greatest differences, from 4 to 0·01 (400 to 1).

The nine strains fall roughly into three groups as regards their virulence as tested by the M.L.D. of standard emulsion.

(1) Two strains, I and X, had a M.L.D. of 0·01 to 0·02 c.c.

(2) Three strains, IX, V and VI, had a M.L.D. of 0·1 to 0·17 c.c.

(3) Three strains, XI, VII and VIII, whose M.L.D. was 0·5 c.c. or more.

Strain II, whose M.L.D. varied from 2·0 to 0·1 c.c., should probably be included in the second group; this strain had been tested for pathogenicity on several previous occasions and the M.L.D. was never more than 0·1 c.c. The occasion on which the M.L.D. of original emulsion and standard emulsion was 2·0 c.c. was altogether exceptional.

The M.L.D. of standard emulsion and of original emulsion of the same strain were not widely different when they were both examined on the same occasion. This is merely another way of stating that the differences in deposit yielded by the original emulsions of the different strains were not very large, and further that variations in the inherent properties of the bacillus were of more importance when determining the M.L.D. than variations in the number of bacilli present.

There is no evidence to show whether the greater virulence of some strains is due to increased resistance to the destructive powers of the animal body, greater rapidity of multiplication in the body or a greater ability to make toxin from the body fluids.

In Table IV the M.L.D. of toxin and that of the emulsion of washed bacilli made up to the original bulk of culture after centrifugalising, are shown together. The ratios of the minimal lethal doses of original emulsion and of the standard emulsion to the M.L.D. of toxin occurring in the same culture, are given in order to show the relative values of the bacilli and toxin as lethal agents in two-day cultures.

The ratio of the M.L.D. of the bacilli to that of the toxin obtained from the same culture was most commonly as 1/1; this was the case for 7 out of 11 strains examined, and shows that the lethal power of the

toxin and bacilli in a two-day culture are usually equal. This relation was curiously maintained, even when the same strain yielded cultures of high and low pathogenicity on different occasions, as in the case of strain VI.

TABLE IV.

*Comparison of the lethal power of washed bacilli and toxin in the same cultures (two days' growth).*

| Strain | Date of experiment | M.L.D. of original emulsion | M.L.D. of standard emulsion | M.L.D. of toxin | Ratio of M.L.D of original emulsion to M.L.D. of toxin | Ratio of M.L.D. of standard emulsion to M.L.D. of toxin |
|---|---|---|---|---|---|---|
| No. I | 11 Feb. 1911 | 0·01 | 0·01 | 0·5 | 1/50 | 1/50 |
| ,, II | 3 Feb. ,, | 0·1 | — | 0·1 | 1/1 | — |
| ,, ,, | 9 Mar. ,, | 2·0 | 2·0 | 0·5 | 1/0·25 | 1/0·25 |
| ,, III | 21 Dec. 1909 | 0·1 | — | 0·1 | 1/1 | — |
| ,, IV | 21 Dec. ,, | 0·1 | — | 0·1 | 1/1 | — |
| ,, V | 20 Apr. 1911 | 0·5 | 0·17 | 2·5 | 1/5 | 1/15 |
| ,, VI | 16 Mar. ,, | 4·0 | — | 4·0 | 1/1 | 1/3 |
| ,, ,, | 20 Apr. ,, | 0·5 | 0·17 | 0·5 | 1/1 | 1/3 |
| ,, VII | 21 Apr. ,, | >2·5 | >0·83 | 2·5 | 1/1 | 1/3 |
| ,, VIII | 21 Apr. ,, | 2·5 | 1·25 | 2·5 | 1/1 | 1/2 |
| ,, IX | 9 Mar. ,, | 0·1 | — | 1·0 | 1/10 | — |
| ,, ,, | 20 Apr. ,, | 0·5 | — | 2·5 | 1/5 | — |
| ,, X | 27 Mar. ,, | 0·05 | 0·02 | 1·0 | 1/20 | 1/50 |
| ,, XI | 10 Feb. ,, | >3·0 | 4·0 | 3·0 | 1/1 | 1/0·75 |
| ,, ,, | 20 April ,, | 2·5 | — | 2·5 | 1/1 | — |

On the other hand the M.L.D. of bacillary emulsion of strains I, V, IX and X was smaller than that of the toxin, the ratio $\frac{\text{M.L.D. bacilli}}{\text{M.L.D. toxin}}$ being represented by the fractions $\frac{1}{50}$, $\frac{1}{5}$, $\frac{1}{10}$ or $\frac{1}{5}$ and $\frac{1}{20}$ respectively, so that, *e.g.* in the case of strain I, the bacilli in the culture had actually fifty times the lethal power of the toxin.

When the M.L.D. of standard emulsion is compared in the same way with the M.L.D. of toxin, none of the strains showed an equal value of standard emulsion and toxin. In two cases, *e.g.* strains II and XI, the M.L.D. of standard emulsion of bacilli, was actually slightly greater than that of the toxin, but more commonly the M.L.D. of toxin was the greater. For example in the cases of strains VI, VII and VIII the ratio $\frac{\text{M.L.D. of standard emulsion}}{\text{M.L.D. of toxin}}$ was equal to $\frac{1}{3}$, $\frac{1}{3}$ and $\frac{1}{2}$ respectively, and in strains I and X the ratio was represented by $\frac{1}{50}$ in each case.

This method of comparison emphasises the fact that in a two-day culture the lethal value of the bacilli equals or may greatly exceed that

of the toxin, and that variation in the pathogenicity of the whole culture depends on the bacilli more than on the toxin.

Some strains, therefore, were apparently far better producers of toxin in the body than in vitro, perhaps on account of their greater resistance to the adverse influences met with in the body or on account of their more rapid multiplication there.

This is perhaps as good a criterion as can be found of their virulence proper, and such strains may be correctly described as of higher virulence than strains in which the toxin production in vitro and in vivo are more equal.

### *Irregularities.*

The Minimal Lethal Dose of "whole culture," of toxin or of bacillary emulsion was not constant for individual strains, and this was not to be expected. There was, however, a very fair amount of agreement for most of the strains on the different occasions on which they were tested. Four strains of low virulence (Nos. V, VI, VII and XI) were retested after four passages through broth and in only one case (No. VI) did a smaller dose of the whole culture prove fatal.

The strains of low virulence which have been observed are widely separated from completely non-virulent strains. The difference may not, however, be much greater than that between strains of very high and very low virulence. The relation of the M.L.D. of a strain of very high virulence to one of low virulence may be expressed as a ratio of 1 to 50 or more. If the M.L.D. of a strain of low virulence bore the same ratio to the M.L.D. of a "non-virulent" strain, the latter would be lethal in a dose of 50 c.c. whereas a dose larger than 10 c.c. cannot be conveniently used.

### *Incidental observations.*

In order to find out whether any considerable part of the lethal effect of injecting washed bacilli was due to toxin clinging to the bacilli, toluol (on other occasions chloroform) was added to the emulsion of washed bacilli and also to the separated toxin. They were then kept at room temperature for 24 to 48 hours till all the bacilli in the emulsion were dead. The M.L.D. of toxin was then found not to have altered materially but the bacilli had become almost innocuous, and 30 to 40 times the dose of bacillary emulsion, which when living caused death, now caused hardly any disturbance. With a similar object, a small

amount of antitoxin was added to the bacillary emulsion and the mixture incubated for half an hour and then centrifuged, the bacilli again washed and the emulsion made up to the original volume. The M.L.D. of bacilli remained about the same as that of the emulsion before the antitoxin was added.

It was thought that possibly strains of low virulence, or strains with no pathogenic action, in reality formed toxin but had acquired the property of destroying it with greater rapidity than ordinary virulent strains. Two-day cultures of non-virulent bacilli and virulent bacilli grown together were, however, almost as lethal as pure cultures of virulent bacilli.

## CONCLUSIONS.

(1) The pathogenicity of different strains of *B. diphtheriae* when first isolated, as tested with two-day broth cultures, varies greatly (Minimal Lethal Dose varies as 400 to 1).

(2) There is a tendency for some strains of low virulence to increase slightly in pathogenicity in artificial culture, *e.g.* a fall of M.L.D. to $\frac{1}{5}$th of its original figure. The amount of growth in a two-day culture varies for different strains, but not in proportion to the lethal power of the culture.

(3) The pathogenicity of young two-day "whole" broth cultures of different strains of *B. diphtheriae*, even after the strains examined have been subjected to prolonged culture on artificial media, varies in a degree which may be expressed as 1 to 50.

(4) The virulence of washed bacilli from two-day broth cultures of different strains varies at least as much as the pathogenicity of whole cultures.

(5) The amount of growth in two-day cultures of different strains varies considerably but not in proportion to the pathogenicity of the culture.

(6) The M.L.D. of toxin in a two-day broth culture varies less for different strains than does the virulence of the bacilli in the same culture.

(7) The ratio of the M.L.D. of toxin in a two-day broth culture to that of the bacilli in the same culture may frequently be expressed as $\frac{1}{1}$ or M.L.D. toxin = M.L.D. bacilli, but the M.L.D. of bacilli may be $\frac{1}{50}$ that of the toxin. The M.L.D. of bacilli was only once observed to be greater than that of the toxin.

(8) The ratio of the M.L.D. of standard bacillary emulsion to the M.L.D. of toxin from the same culture as the bacilli, varies for different strains within about the same limits as the ratio of the M.L.D. of the original emulsion to that of the toxin. The M.L.D. of standard emulsion is, however, sometimes greater than that of the toxin. $\frac{\text{M.L.D. standard emulsion}}{\text{M.L.D. toxin}}$ varies for different strains from $\frac{1}{0{\cdot}25}$ to $\frac{1}{50}$.

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