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TOXIN AND REACTION CHANGES PRODUCED BY THE DIPHTHERIA BACILLUS IN CULTURE.*

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It has been known for many years that the initial reaction of the medium has a profound influence on the growth of the diphtheria bacillus and on the production of toxin. The earlier investigators, by the somewhat crude methods then available, made important observations on the changes in reaction which occur during the growth of the diphtheria bacillus, and were fully alive to the fact that reaction change and toxin production are intimately associated. They established several important facts; they showed that the reaction of the broth should be faintly alkaline before inoculation, that an early change which occurs on incubation is the formation of acid, and that this is followed by the production of alkali. They observed that when these changes occur toxin was usually produced in greater or smaller amounts.

The work of Sörensen (1909 [5]), and the more recent studies of Clark and Lubs (1917 [2]) have placed in the hands of present-day investigators the means of determining the true reaction of fluids by simple methods which yield accurate and easily reproducible results. It was inevitable that the old problem mentioned above, the importance of which is recognised as forcibly by the modern worker as it was by the pioneers, should be attacked again in the light of the new knowledge and by means of the new methods.

Of recent papers dealing with the problem of diphtheria-toxin preparation, few have aroused such interest as that of Bunker (1919 [1]). The reason for this is not far to seek, for according to his observations and experiments, a careful study of the daily changes in reaction during growth will furnish information of the most valuable kind relating to the production of toxin. Bunker shows that reaction conditions alone cannot govern toxin production; suitable food substance in the medium, the nature and activity of the strain and the shape and size of the container are among the other factors of importance. He plotted curves showing the reaction changes which occur when the bacillus grows on sugar-free media, and on media containing dextrose. The

* The substance of this paper was communicated to the Meeting of the Pathological Society of Great Britain and Ireland, at Cambridge, July 1, 1920. [Received for publication, June 8, 1922.]

media used in the experiments described below contained the fermentable substances normally occurring in muscle infusion, no attempt being made to remove these by preliminary treatment with *B. coli* or other organisms. As will be seen from the accompanying charts the curves showing reaction change during growth are of the same general type, and resemble very closely those recorded by Bunker. In all twelve cases the reaction curve was typical and toxin was formed; in ten out of the twelve cases high-grade toxin was produced at some stage of the experiment. The opportunity to study toxin formation in media in which the reaction curve was atypical did not occur.

Bunker studied the daily reaction change and toxin formation in media containing twenty different kinds of peptone, and plotted the results on a common scale; from this it was evident that the strongest toxins had occurred at a given range of P_H values on the respective curves, and he concludes "that it would be possible to establish a zone which would include all the strong toxins and outside of which no potent toxin would occur. This zone was arbitrarily fixed by inspection as lying approximately between P_H 7·85 and P_H 8·25." The significance of this conclusion is apparent. One of the chief difficulties encountered in routine work is to ascertain the time when toxin should be harvested. Bunker's work indicated a way in which this particular difficulty might be overcome.

When Bunker's paper appeared we were engaged in a study of the same subject on somewhat similar lines, but as some of our results differed from his, the experiments were repeated and extended with the object of following the daily changes in reaction and toxin production. Preliminary observations had indicated that reaction change and toxin production were influenced by three factors at least, viz., the peptone, the strain of the bacillus, and the volume of medium inoculated (and hence the size of container used). Bunker, indeed, points out that these are factors of importance which affect the results, but as the study of the phenomenon has yielded with us results which differ in some respects from his, a brief account of some of our experiments is given below.

Method of Experiment.

Bottles (capacity 500 c.c. or 5000 c.c.) cylindrical in shape, were used as containers. The smaller bottles contained 100 c.c. of medium, the larger ones 1000 c.c. After inoculation, samples were withdrawn aseptically at the end of 1, 2, 3, 4, 5, 7, 9 and 11 days, a smear being made and examined for morphology, contaminations, etc. To each sample withdrawn 0·5 per cent. carbolic acid was added, and after standing for twenty-four hours in the dark the P_H and toxicity were determined. From the data thus obtained, curves were plotted showing reaction change and toxin production during the course of each experiment.

The intracutaneous method of Römer and Sames (1909 [4]), as developed by Glenny and Allen (1921 [3]) in these laboratories was used for determining the toxicity of the different samples. Without the use of this method the expenditure

in guinea-pigs would have been very large. In the twelve experiments described below, 410 separate tests were carried out to fix 99 points on 12 curves; but since six to twelve tests may be carried out on each guinea-pig the number of animals used was not excessive. The "skin test dose" or "$L_r/500$ dose" was determined for each sample of toxin. This dose may be defined as the smallest volume of toxin which, when mixed with $\frac{1}{500}$ of a unit of antitoxin, produces a typical positive reaction when injected intracutaneously into the shaven flank of a guinea-pig, the reading being taken about forty hours after injection. The values thus obtained were used for plotting the curves in the accompanying charts.

Results.

Experiment I.—Reaction change and toxin produced by the same strain of *B. diphtheriæ* (Park Williams No. 8) when grown on media containing different varieties of peptone.

The peptones chosen for study were Parke Davis Bacteriologic Peptone, Difco Proteose Peptone and Witte Peptone. A stock of horse-flesh infusion, containing 0·5 per cent. common salt was used, 2 per cent. solutions of the different peptones being prepared. In each case the reaction was adjusted to P_H 8 before final sterilisation. All the bottles were sterilised in the same autoclave. The strain was acclimatised to the different kinds of media before being used in the experiment. Just before inoculation a sample of medium was withdrawn from each of the experimental bottles and the P_H determined. It will be observed that the fall in P_H is different in the three cases.

The volume of broth inoculated was 100 c.c. in each case, the containers being 500 c.c. bottles supported on their sides on specially constructed wooden platforms.

The results of this experiment are shown in Chart I. The type of reaction curve is the same in all three cases and, as Bunker has shown, is characteristic under the conditions of this experiment—that is, when the bacillus is grown on media containing fermentable substances. There is first a production of acid, followed by a production of alkali, so that in two days the reaction has become markedly alkaline—P_H 7·9 to 8·2. Alkali production continues, quickly at first and then more slowly to a limiting concentration which is not widely different in the three cases, the P_H being 8·65 to 8·8. The reaction curves for Parke Davis Bacteriologic Peptone and Difco Proteose Peptone are almost identical; the curve for Witte Peptone is of the same type and shape, but the values for P_H are lower throughout.

The values recorded after twenty-four hours' growth do not represent the most acid points reached during growth. Subsequent experiments, in which more numerous observations were made during the first twenty-four hours, showed that the point of maximum acidity was reached at an earlier stage when such small volumes of broth were inoculated. With Parke Davis Bacteriologic Peptone P_H values as low as 7·1 have been recorded after twelve hours' growth of *B. diphtheriæ*.

The curves showing toxin production exhibit many interesting features. The amount of toxin formed varies with the kind of peptone used, being small in the case of Witte and much greater in the other two cases. Toxin production is rapid; a little toxin could be demonstrated in the case of Witte Peptone after twenty-four hours' growth, while in the other two cases the amount formed even after so short a time is very large. Moreover, the rate of toxin production is different, the maximum being reached in two days in two cases and in three days in the third case. The subsequent fate of the toxin produced also varies. With Difco Proteose Peptone the maximum is reached in three days and there

is no decline in value during the next eight days' incubation; during this time the P_H of the medium is between 8·6 and 8·8. In the case of Witte Peptone the maximum is reached in two days, when the P_H is 7·9, maintained until the fourth day (P_H 8·4) and then declines a little, the P_H rising slowly to 8·65 during the last seven days of incubation. The most interesting change occurs in the medium containing Parke Davis Bacteriologic Peptone. Here the maximum is reached in two days when the P_H is 8·2. The value of the toxin then falls rapidly and on the fifth day is very small and there is a further decline in value

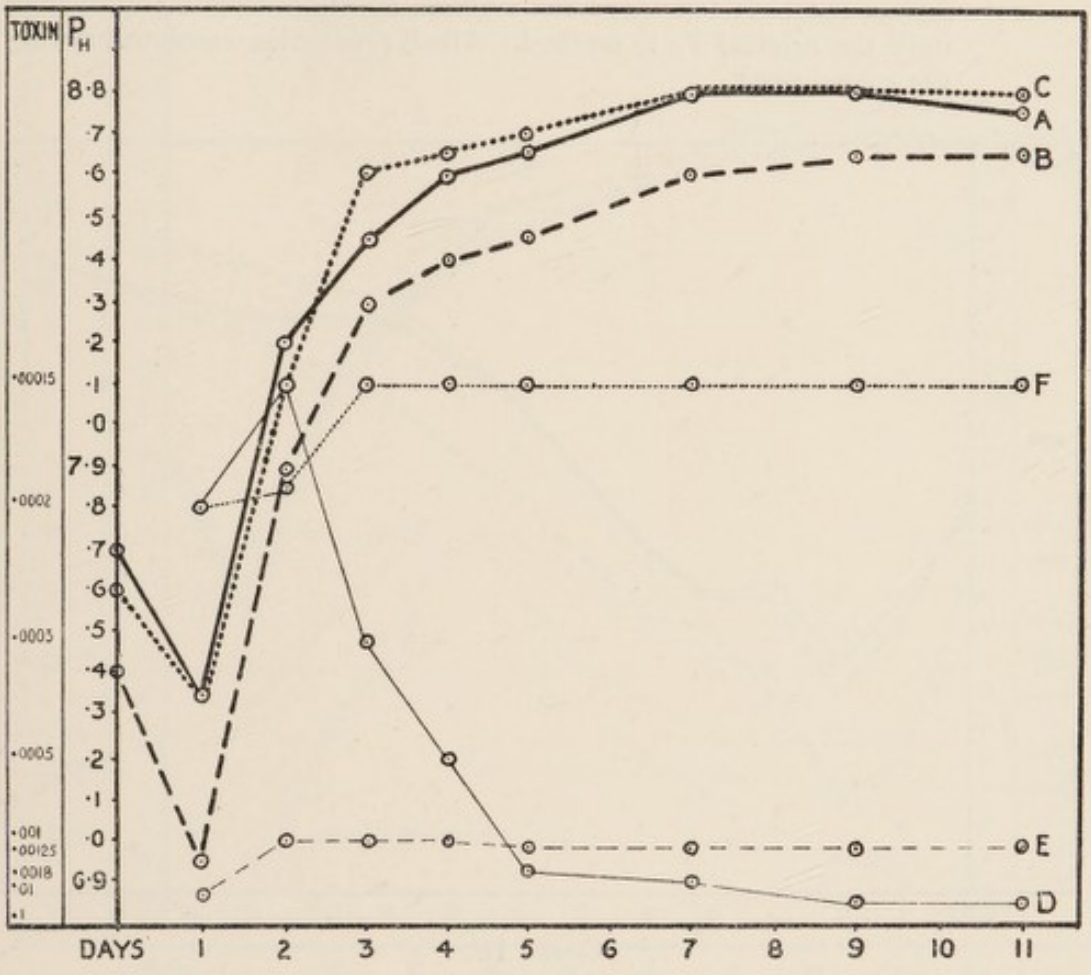

CHART I.

A = Reaction changes, Parke Davis Bacteriologic Peptone.
B = Reaction changes, Witte Peptone.
C = Reaction changes, Difco Proteose Peptone.
D = Toxin production, Parke Davis Bacteriologic Peptone.
E = Toxin production, Witte Peptone.
F = Toxin production, Difco Proteose Peptone.

during the next six days. On the third day the P_H is 8·45 and on the last day of the experiment the P_H is 8·75.

Experiment II.—This was carried out in precisely the same way as the previous one, with the exception that the volume of broth inoculated was in each case 1000 c.c., the containers being double Winchester quart bottles supported on their sides on wooden platforms.

The results of this experiment are shown in Chart II.; the main points may be summarised as follows:—

(1) The type of reaction curve is of the same general character as in the previous experiment.

(2) The similarity between the reaction curves for Parke Davis Bacteriologic Peptone and Difco Proteose Peptone again is very marked. The curve for Witte Peptone is of the same type as the other two, but the P_H values are lower throughout.

(3) The curves are still rising at the end of the experiment, and apparently the maximum alkalinity attainable has not been reached in fourteen days.

(4) The reaction changes occur much more slowly in this experiment. The maximum acidity was recorded at the end of forty-eight hours and during the next four days the reaction slowly became more alkaline until the original P_H is reached. Alkali production continued during the second week.

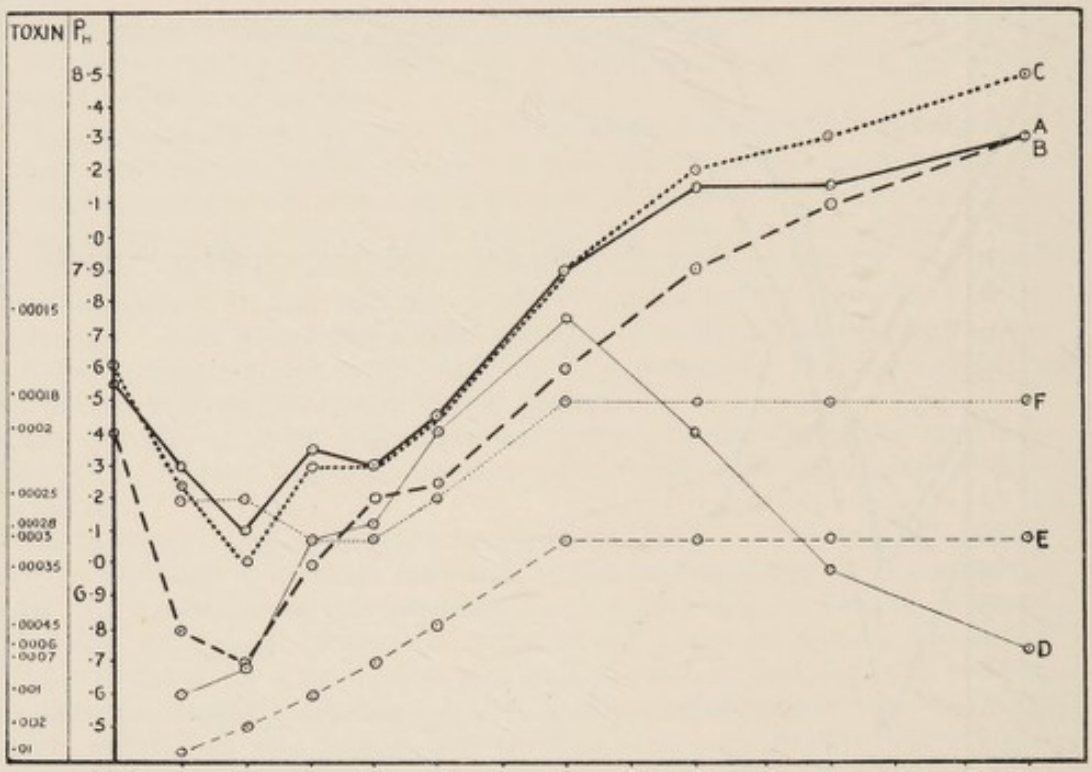

CHART II.

A = Reaction changes, Parke Davis Bacteriologic Peptone.
B = Reaction changes, Witte Peptone.
C = Reaction changes, Difco Proteose Peptone.
D = Toxin production, Parke Davis Bacteriologic Peptone.
E = Toxin production, Witte Peptone.
F = Toxin production, Difco Proteose Peptone.

(5) As before, toxin production is different in the three cases. The curve for Witte Peptone rises slowly to a maximum which is reached on the seventh day, and the value is maintained during the second week. Toxin production in medium containing Parke Davis Bacteriologic Peptone is, however, different. The curve rises steadily to a maximum which is reached on the seventh day, and then the value declines steadily during the second week.

Experiment III.—This was carried out to study the reaction changes and toxin production which occur when different strains of *B. diphtheriæ* are grown on the same medium under identical conditions.

The medium consisted of 2 per cent. Parke Davis Bacteriologic Peptone dissolved in infusion made from horse flesh and prepared in the usual way.

The strains used were Park Williams No. 8, Strain 78 and Strain 82; these were acclimatised before use. They were chosen as they were all proved toxin-producers, and the Parke Davis Bacteriologic Peptone was chosen in order to determine whether the deterioration phenomenon which had been observed in the two previous experiments was peculiar to the Park Williams No. 8 strain.

In this experiment the volume of medium was, in each case, 100 c.c. The results, summarised below, are shown in Chart III.

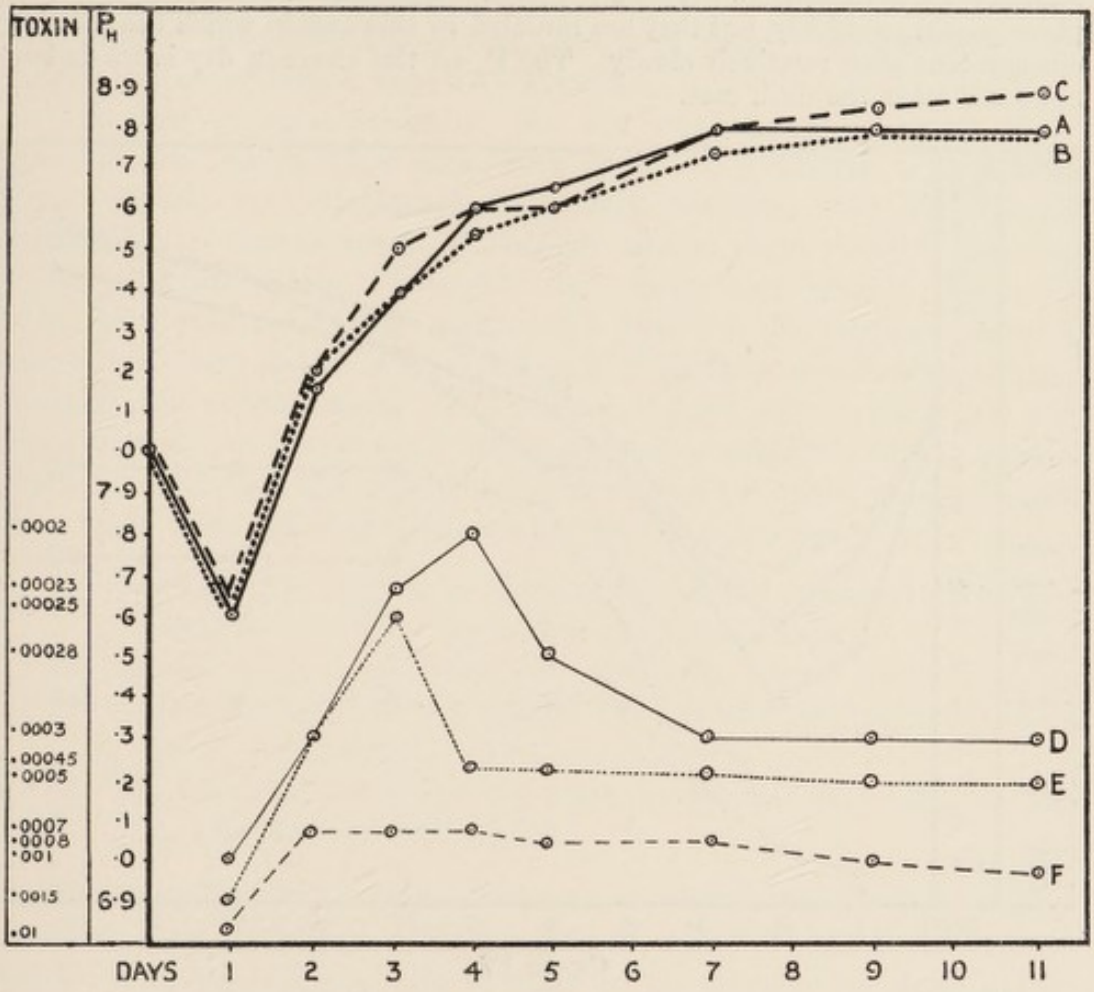

CHART III.

A = Reaction changes, Strain Park Williams No. 8.
B = Reaction changes, Strain No. 78.
C = Reaction changes, Strain No. 82.
D = Toxin production, Strain Park Williams No. 8.
E = Toxin production, Strain No. 78.
F = Toxin production, Strain No. 82.

(1) The reaction curves are almost identical. They are characteristic and are similar to those shown in Chart I. The P_H rises to 8·2 in forty-eight hours, has become 8·6 on the fourth day, and reaches the high figures of 8·8 to 8·9 on the eleventh day.

(2) Toxin production varies with the three strains. In the case of Park Williams No. 8, the maximum point is reached on the fourth day, when the P_H is 8·6. The value falls during the next three days, the P_H rising meanwhile to 8·8; the toxin value is maintained at the lower level during the last four days of observation. In the case of strain No. 78, the maximum is reached on the third day when the P_H is 8·4

and falls during the next twenty-four hours. In the case of strain No. 82, the maximum is reached on the second day when the P_H is 8·2; the value is maintained until the fourth day (P_H 8·6) and then declines slightly during the last week.

Experiment IV.—This was similar in all respects to the foregoing one except that litre quantities of media were inoculated, the containers being double Winchester quart bottles.

The results are shown in Chart IV. Acid production varies with the three strains. These differences were not apparent in Experiment III. because the acid phase passed so quickly, but they are revealed in this case in which the reaction changes take place relatively slowly. The P_H on the eleventh day is 8·5 in two cases and 8·4 in the third case.

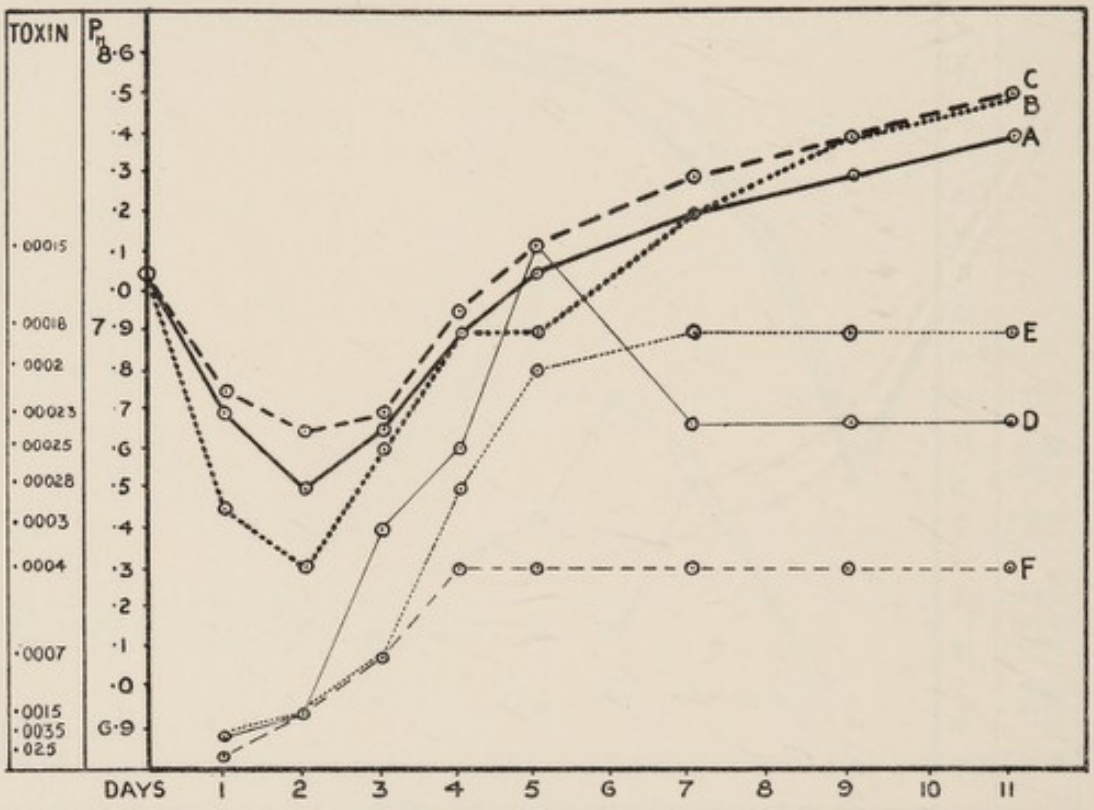

CHART IV.

A = Reaction change, Strain Park Williams No. 8.
B = Reaction change, Strain No. 78.
C = Reaction change, Strain No. 82.
D = Toxin production, Strain Park Williams No. 8.
E = Toxin production, Strain No. 78.
F = Toxin production, Strain No. 82.

Toxin production also varies, maximum values being observed on the fourth, fifth, and seventh days, when the values for P_H were 7·95, 8·05, and 8·2 respectively. A decline from the maximum value reached was observed in one case only in this experiment.

SUMMARY AND CONCLUSIONS.

Bunker's conclusions are based, primarily, upon a study of twenty different peptones tested at the same time in small flasks. It is evident that he obtained further confirmation of these results when working under other conditions, although his paper contains no detailed account of any such observations. Bunker proposes to substitute

reaction for time of incubation as a better criterion for determining when to harvest toxin. He maintains that when toxin is formed, whether the amount be small or large, the strongest toxin will be found when the P_H of the medium lies between 7·8 and 8·25, and that outside this reaction zone no potent toxin would occur. In order to ascertain whether this "rule" is of general application, we have studied toxin production under widely different conditions, the strain, peptone and volume of broth inoculated, all having been varied.

For the purpose of studying Bunker's rule it is convenient to divide the experiments into two groups.

The first group consists of the six experiments in which small quantities of media (100 c.c.) were used. (See Charts I. and III.) These results are considered separately, because, with such small quantities of media, reaction changes occur very rapidly and the P_H may increase from 7·8 to 8·25 in twenty-four hours. Hence, in order to obtain completely satisfactory data for the purpose, samples should have been taken every few hours while the reaction was passing through the critical zone. This was not done at the time and the point was not observed until the tests were complete and the charts plotted. Although the experiments are not so complete as one might have made them, the following results may be noted:—

(1) Bunker's rule applies in three cases, *i.e.*, the strongest toxin was found when the P_H lay within the critical zone.
(2) In the other three cases, the strongest toxins were obtained after the reaction had passed beyond the critical zone, when the P_H was 8·4, 8·6, and 8·6 respectively.
(3) The final reaction in all six experiments was very alkaline, the P_H ranging from 8·65 to 8·9.

The second group consists of the six experiments in which a litre of broth was inoculated. The conditions under which these experiments were carried out were similar in all respects to those under which toxin preparation is carried out on a large scale. Reaction changes take place slowly, and the data are more suitable for study and analysis. In all these cases Bunker's rule applies; the strongest toxins were obtained when the P_H lay—somewhere—between 7·8 and 8·25.

We are not quite clear, however, as to the manner in which Bunker's rule should be applied in practice, and as others may have experienced the same difficulty some discussion of the point is necessary. From our reading of Bunker's paper we assume that the reaction would be determined daily, and when the P_H had reached the critical zone (7·8 to 8·25) the toxin would be harvested. Now this zone of reaction is a wide one, and in a medium which is well buffered in this region change from one extreme to the other is very slow; in our experiments the time taken was from three to five days. Should the toxin be harvested immediately the P_H reaches 7·8—or a little more—or should

one wait until the reaction has reached the vicinity of $P_H = 8{\cdot}25$? The results are most diverse according as to whether the lower or upper limit is adopted. Thus, referring to the results shown in Chart II., toxin produced in medium containing Parke Davis Bacteriologic Peptone reached its maximum value when the P_H was 7·9; two days later the P_H was 8·15, but the value of the toxin had decreased by 25 per cent. In the other two cases shown on this chart maxima were recorded at P_H 7·9, and no decline in value occurred during the next week, although the P_H increased to 8·3 and 8·5 respectively. Clearly, from these experiments, it would appear that the rule should be to harvest the toxin as soon as the reaction reached a value of $P_H = 7{\cdot}9$. But if this rule is applied to the results shown on Chart IV. it will be seen that in two cases the toxin would have been harvested long before it had reached its maximum potency. In one case, two days later, the P_H was 8·05 and the toxin had reached its maximum potency; and in the other case the maximum was not reached until four days later when the P_H was 8·2. The results suggest that all parts of this wide reaction zone cannot be regarded as equally suitable for determining the stage at which incubation should cease. It is very probable that the final reaction chosen will depend upon the medium employed, and particularly on the peptone which it contains.

Bunker states that potent toxins were not found when the reaction lay outside the critical zone (P_H 7·8 to 8·25), and that "irrespective of the peptone medium from which toxin was obtained, this relation held true in this experiment." In our experiments different results were obtained; deterioration occurred in seven cases, yet in the remaining five the toxin showed no deterioration in value although the reaction became more alkaline than P_H 8·25. (This point is dealt with more fully below.)

It is interesting to apply the rule to another series of results obtained during the routine preparation of toxin. This series consists of 67 toxins, the P_H and minimum lethal dose of each of which had been determined after filtration. The results are collected in Table I.

TABLE I.

Value of Toxin M.L.D.	Number of Toxins, the P_H of which was		
	Between 7·8 and 8·25.	Less than 7·8.	Greater than 8·25.
c.c 0·001 to 0·002 . . . (very high grade)	16	5	10
0·0021 to 0·004 . . . (high grade)	14	3	8
0·0041 to 0·007 . . . (low grade)	2	3	2
0·0071 to 0·01 . . . (usable)	2	2	0
Totals . .	34	13	20

Thus, of these 67 toxins, 56 (84 per cent.) may be classed as "very high grade" or "high grade." Of these 56, the P_H of 30 (56 per cent.) lay within the critical zone, but the P_H of 8 (14 per cent.) was less than 7·8, and the P_H of 18 (32 per cent.) was greater than 8·25. In these cases reaction changes during growth were not studied. It is possible that the 8 toxins which were harvested before the critical zone had been reached might still have been high-grade toxins at a later date when the P_H had reached the critical zone; and, similarly, the 18 toxins which were more alkaline than P_H 8·25 might still have been high-grade toxins at an earlier date before the reaction had passed beyond the critical zone. Hence, the data given in the above table cannot be held to invalidate the claims of Bunker, but the results show that the occurrence of potent toxin is not incompatible with a reaction outside the critical zone either on the acid or alkaline side. In the case of these 56 high-grade toxins the P_H varied from 7·5 to 8·9.

It has frequently been observed that toxin often deteriorates in value on prolonged incubation at 37° C. We were inclined, at one time, to attribute the variable results obtained during routine operations to the fact that some toxins were harvested before maximum potency had been reached, others when the toxin content was at a maximum, and others after deterioration had occurred. The earlier experiments, as well as those described above, were instituted partly with the object of studying this phenomenon of toxin deterioration. The main results may be summarised as follows:—

(1) Some deterioration in value occurred in seven of the twelve cases.

(2) In five of these cases deterioration was marked, and in all five instances Parke Davis Bacteriologic Peptone was the one employed.

(3) High-grade toxin was obtained in both experiments in which Difco Proteose Peptone was used, and in neither case did any decline in value occur on prolonged incubation.

(4) The strain may be a factor. Park Williams No. 8 strain was used in eight experiments. In four of these the medium contained Parke Davis Bacteriologic Peptone and deterioration occurred in all four cases. In two experiments the medium contained Difco Proteose Peptone: high-grade toxin was formed in each case and deterioration did not occur. In two experiments the medium contained Witte Peptone: the toxin produced was of less potency, and slight deterioration occurred in one case only.

Strains 78 and 82, grown on media containing Parke Davis Bacteriologic Peptone, produced toxin, the value of which deteriorated when small volumes were used; when grown on larger volumes, the toxin attained a maximum value which was maintained during prolonged incubation.

(5) Deterioration occurs more frequently, and in a more marked degree, when toxin is prepared in small volumes of media. Correlated with this is the fact that the degree of alkalinity attained when the bacillus is grown on small volumes of media is greater than when larger volumes are inoculated. It was thought, at first, that deterioration occurred as the result of the combined effect of a strongly alkaline reaction and prolonged incubation at 37° C., but these experiments show that this combination of circumstances leads to deterioration only when certain peptones and strains are used; under other conditions toxin appears to be quite stable.

It seems, therefore, that media can be prepared which, while yielding high-grade toxin, at the same time possess the very useful property of conserving the toxin which has been produced. In the composition of such media the peptone incorporated plays an important part.

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