Changes in the culture medium during the growth of B. diphtheriæ / by A.F. Watson and U. Wallace.

Reprinted from THE JOURNAL OF PATHOLOGY AND BACTERIOLOGY, Vol. XXVII., 1924

Changes in the Culture Medium during the Growth of *B. diphtheriæ*

BY

A. F. WATSON AND U. WALLACE

From the Wellcome Physiological Research Laboratories, Beckenham

CHANGES IN THE CULTURE MEDIUM DURING THE GROWTH OF *B. DIPHTHERIÆ.*

A. F. Watson and U. Wallace.

From the Wellcome Physiological Research Laboratories, Beckenham.

I. Changes during the growth of a powerfully toxigenic strain of the bacillus.

Many efforts have been made by various investigators to isolate the toxic principle of culture filtrates of *B. diphtheriæ* with the object of elucidating the mechanism of toxin production and the nature of the toxin molecule. Brieger and Frankel (1890), for instance, used precipitating agents such as ammonium sulphate, alcohol, etc., and were able to demonstrate the existence of toxin in the precipitates. Later, Brieger and Boer (1896) attempted to isolate double compounds with zinc salts. Dean (1908), reporting on these and similar experiments, states "a substance of great physiological activity is not obtained by any of the methods which have been described." More recently Banzhaf (1920), by adding 65 per cent. alcohol to the slightly acidified toxic filtrates obtained a precipitate which on drying contained 40,000 guinea-pig fatal doses per grm. of material. When the preparation of high-grade diphtheria toxin was more difficult than it is now, Glenny and Walpole (1915) attempted the artificial conversion of low-value toxins to those of high titre by a precipitation method. These workers found that on acidification with dilute acetic acid culture filtrates of the bacillus from which salts had been removed by pressure dialysis in a collodion membrane developed a slight precipitate which contained most of the toxin. This technique has not been widely adopted for practical purposes, but the method has recently been of value in throwing light on what would appear to be one of the fundamental changes which takes place in an efficient medium during the growth of the bacillus and on the normal relationships of growth to toxin production.

Some attempt is here made to link up three important changes in nitrogen metabolism which take place during the growth of a powerfully toxigenic strain of *B. diphtheriæ*. Further, it is shown that by a simple technique it is possible to obtain a substance of marked physiological activity from culture filtrates made from a medium prepared by a modification of the Douglas method. For this type of work the Douglas medium with the modifications introduced by Hartley (1922) and the authors (1923) offers many advantages. It is readily made, is easily reproducible, and if properly controlled is reliable for the production of filtrates of constant toxicity over long periods of time. Although some of the work described in the present paper was done with "peptone" medium it was found that the tryptic digest medium mentioned above afforded such a satisfactory basis for the study of diphtheria toxin production that most of the results were obtained with this medium. Media made by this method usually contain little or no precipitated material after autoclaving, and are, therefore, very suitable for the quantitative determinations of the weight of bacterial bodies produced after varying time periods. The relationships between

bacterial nitrogen and toxin, between nitrogenous material precipitable by glacial acetic acid and growth, and finally between amino nitrogen and growth are dealt with in some detail. Throughout the work Park-Williams' No. 8 strain has been employed, and for testing the toxicity of the various filtrates the intracutaneous method of Romer and Sames (1909) and Glenny and Allen (1921) has been used. This technique is capable of a considerable degree of accuracy and has proved very useful for the preliminary testing of filtrates. The Lr/500 dose may be defined as "the smallest volume of toxin which, when mixed with one five-hundredth of a unit of antitoxin, produced a typical positive reaction when injected into the shaven flank of a guinea-pig, the reading being taken forty hours after injection" (Glenny and Allen (1921), Hartley (1922)).

1. The relationship between the amounts of bacterial bodies produced by *B. diphtheriæ* on the Douglas medium and the amount of toxin in solution at any moment.

During the preparation of very large volumes of high-grade toxin on this medium it has been found that good growth of the bacillus is usually accompanied by good toxin production. This is not characteristic for all types of media, and it appeared probable that for this medium, which when properly controlled apparently supplies all the nutritional requirements of the organism, a fairly constant relationship existed between the weight of organisms produced during growth (or the amount of nitrogen in this material) and the toxin in solution.

As a preliminary experiment the weights of film on 100 c.c. of the medium were plotted against toxin in solution after varying time periods. Fig. 1 shows these results. The technique of the experiment was carried out as follows. Eight hundred c.c. of medium were distributed into eight cylindrical bottles of 500 c.c. capacity, 100 c.c. per bottle, and all inoculated on the same day with the same strain. In order to trace the changes that occur during growth it is possible to alter the technique in two ways (1) by taking samples periodically from the same bottle, (2) by removing a bottle each time for a sample. The first will obviously give the more accurate results, but in practice it was found much easier to follow the second method which was adopted throughout. There is plainly the possibility that individual bottles vary and that alterations in the curves may be due only to the variation between individual bottles. It became evident as the investigation proceeded that this factor of individual variation did not vitiate the results, for no result here recorded rests on one experiment only. In repeated experiments the same types of curve were obtained. There may be variations between duplicate bottles, but in control experiments it was found that the variations under the conditions of the experiment were very small.

Bottles were withdrawn after definite time intervals up to eleven days, 0·5 per cent. phenol added, and the film weights, the toxicity by the intracutaneous method and the P_H of the filtrates determined.

To obtain the weights of film material the cultures were filtered through a weighed Gooch crucible and washed with 25 c.c. of distilled water after which the washings gave no precipitate on evaporation. The crucible was then dried *in vacuo* over sulphuric acid and left until consecutive weighings gave constant figures. This method gave good comparative results although the technique was somewhat tedious. It will be seen from fig. 1 that a fairly constant relationship exists between the weight of film at any moment and the amount of toxin in solution. At a P_H of 8·6 after seventy-two hours' growth other factors come into play involving the partial destruction or modification

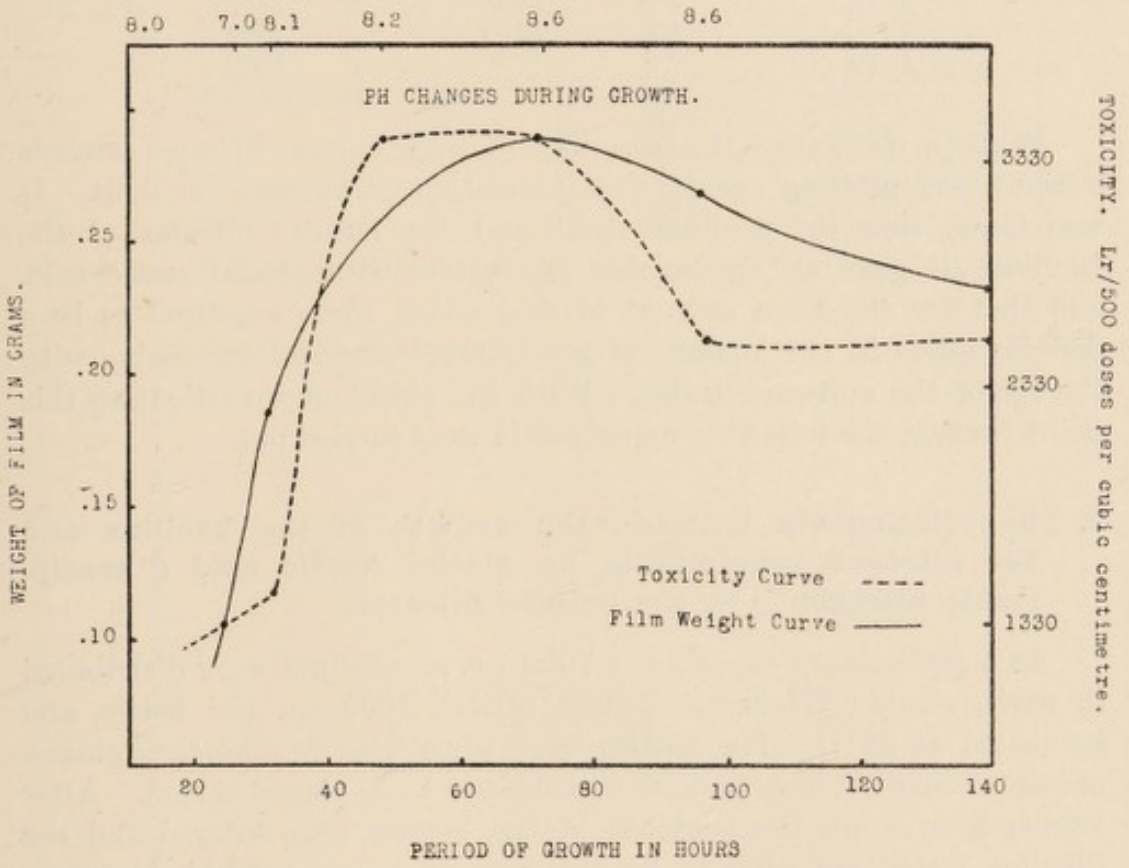

FIG. 1.

of the toxin in solution and autolysis of cells. The film weight curve passes through a maximum apparently at the same point as the toxicity curve.

Table I. shows the results obtained when 200 c.c. of this medium are employed. For this experiment the film material was collected on a small filter paper and washed with 250 c.c. of physiological saline, after which the washings contained no detectable traces of nitrogen. The total nitrogen in the film material was then estimated by Kjeldahl's method in the usual way. The figures show the same sort of relationship as is indicated in fig. 1. It seems, therefore, that for this type of medium there is a fairly close relationship between the amount of bacterial bodies present (or the amount of nitrogen in the solid matter) and the toxin in solution at any moment.

TABLE I.

The changes in the amount of nitrogen in the bacterial bodies during the growth of B. diphtheriæ.

Period of growth.	P_H of filtrate.	Amount of nitrogen in bacterial bodies.	Toxicity. Lr/500 doses per c.c.
0 days.	8·0	...	0
19 hours.	7·0	13·35 mgrms.	667
23 ,,	7·1	15·36 ,,	1000
45 ,,	7·3	38·08 ,,	2857
3 days.	8·3	37·13 ,,	2857
4 ,,	8·3	38·50 ,,	2857
6 ,,	8·5	33·77 ,,	2500
7 ,,	8·6	...	2500

While investigating the behaviour of these culture filtrates towards various precipitating agents one interesting point came to light. It was found that the medium itself and the culture filtrates of the bacillus all gave an appreciable precipitate with glacial acetic acid, and that for the same amount of acid added there appeared to be a definite grade in the amount of precipitated material increasing with the age of the culture filtrates. With the object of investigating this point further, the following experiments were carried out.

2. The relationship between the growth of the bacillus and the nitrogen precipitable by glacial acetic acid ("precipitable nitrogen") in the culture filtrates.

As a preliminary experiment 7000 c.c. of medium were distributed in seven double Winchester quart bottles, 1000 c.c. per bottle, and incubated at 36° C. The bottles were sown with *B. diphtheriæ*, taken out after definite time periods and 0·5 per cent. phenol added. After twenty-four hours the contents of the bottles were filtered and the filtrates tested for toxicity by the intracutaneous method, hydrogen ion concentration and "precipitable nitrogen" in solution. For the quantitative estimation of the "precipitable nitrogen" the following technique was found to give good results. Five hundred c.c. of the filtrate were treated with 5 c.c. of glacial acetic acid and the vessels placed in boiling water for ten minutes. The solution was then allowed to cool and filtered at room temperature. The precipitate was washed with 500 c.c. of glass-distilled water after which the washings contained no detectable traces of nitrogen. The total nitrogen in the precipitate was then estimated by Kjeldahl's method.

Table II. shows the results of this experiment. As usual with this type of medium, film formation was very heavy, the film material from one bottle after six days' growth containing 180 mgrms. of nitrogen. It will be seen that during the growth of the bacillus there is a steady accumulation of "precipitable nitrogen." As soon as growth is

complete this "precipitable nitrogen" content of the filtrate tends to remain steady (fig. 2). For this experiment the bacillus was grown on 600 c.c. of medium for periods up to five weeks.

TABLE II.

The accumulation of "precipitable nitrogen" in solution as growth continues and toxin accumulates.

Age of culture.	P_H.	Toxicity. Lr/500 doses per c.c.	Amount of nitrogen in the filtrate precipitable by 1 per cent. glacial acetic acid.
0 days.	8·0	...	9·66 mgrms.
2 ,,	7·0	1818	29·12 ,,
4 ,,	5·3	2857	38·08 ,,
6 ,,	8·1	3333	46·48 ,,
8 ,,	8·15	5000	50·68 ,,
10 ,,	8·2	5000	57·40 ,,
13 ,,	8·5	5000	61·88 ,,

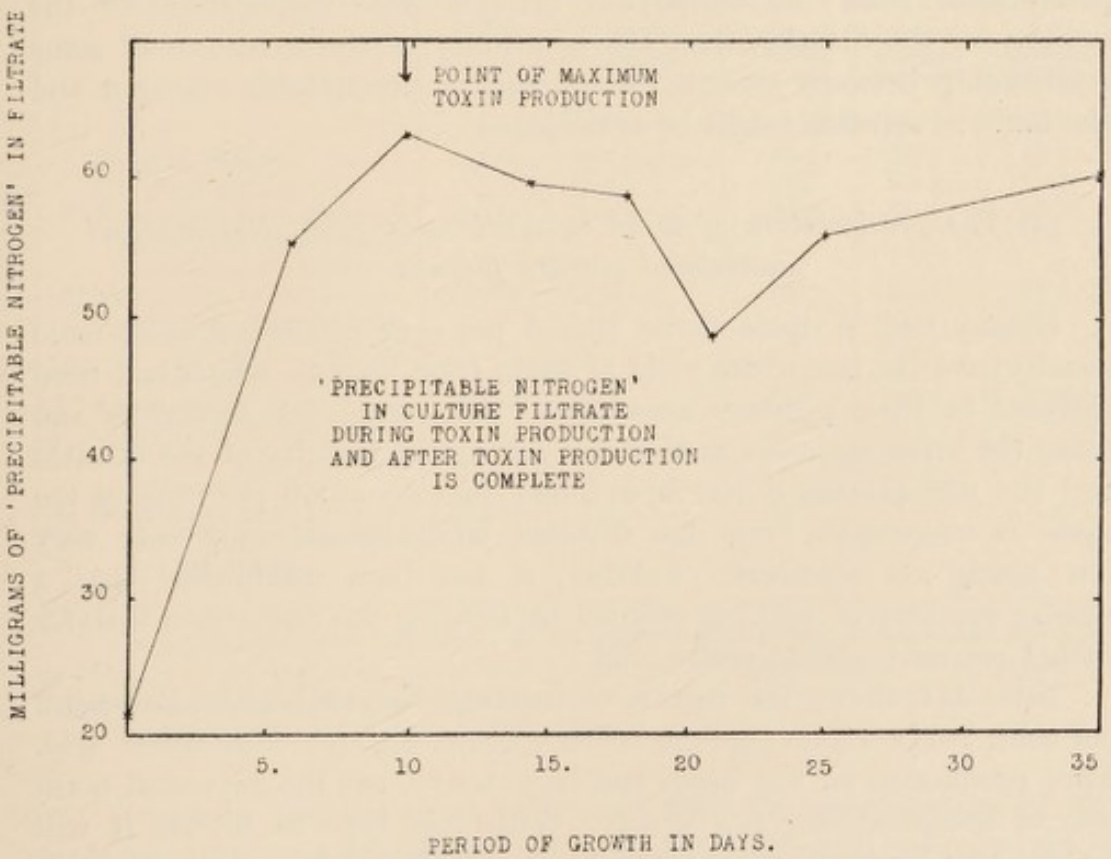

FIG. 2.

Having established this fact, a large number of culture filtrates of *B. diphtheriæ* were tested, and it was found that filtrates from media on which growth had been normal all gave a precipitate with glacial acetic acid. The precipitate from filtrates of media on which growth had been poor was much less in quantity and frequently negligible. These facts appeared interesting in the light of the experiments of

Glenny and Walpole (*l.c.*), who found that low-grade toxins could be artificially converted to those of high titre by the re-solution of the precipitate produced by dilute acetic acid treatment of the filtrates, after they had been submitted to pressure dialysis in a collodion membrane. These workers were apparently unable to obtain any appreciable precipitate from the undialysed filtrates, possibly owing to the fact that their low-value toxins had been made from media on which growth had been poor, and therefore the filtrate gave an appreciable precipitate with acetic acid only when the salts had been removed by dialysis. Two fields of inquiry were now opened up. It appeared from the above experiments that the Glenny-Walpole method of concentration might be shortened if the addition of 1 per cent. glacial acetic acid to the undialysed filtrates gave as good a yield of toxin as the treatment of the dialysed filtrates with the more dilute acid. In the second place, there appeared to be some fundamental connection between this precipitable nitrogenous material and the growth of the bacillus. If it could be shown that this material precipitable from the undialysed filtrates were responsible for the toxicity of the filtrates, then the possibility of the existence of some relationship between growth, the amount of precipitable nitrogen, and the toxin in solution might be anticipated.

(a) *The precipitation of toxin by acetic acid from dialysed and undialysed culture filtrates.*

Glenny and Walpole found that 3 per cent. of normal acetic acid usually gave the maximum yield of toxin from filtrates which had been dialysed in their pressure apparatus. Employing this technique and using the intracutaneous method for testing the toxicity of the filtrates and the precipitates, it has been found that about 60 per cent. of the toxin is recoverable from the filtrates, whilst occasionally only very low yields are obtained. Further, it has been established that a similar recovery of toxin is effected by treating the undialysed filtrates with 1 per cent. glacial acetic acid.

Table III. shows the results of treating dialysed and undialysed 2 per cent. Parke Davis peptone broth culture filtrates with acetic acid. Film production on the broth had been heavy, but the harvested toxin was of comparatively low grade. With this type of filtrate it will be seen that nearly as much nitrogen is precipitated from the undialysed filtrates with 17 per cent. normal acetic acid as from the dialysed with 3 per cent. normal acid, and further, that the solution of the precipitated material in equal quantities of dilute alkali to a P_H of 8·4 gives solutions of a similar potency, although some loss of toxin has occurred. From these results and experiments of a similar nature, it would appear that provided the growth on the broth has been normal the preliminary dialysis of the filtrates may be dispensed with. In no case,

however, where the acetic acid treatment has been carried out at room temperature has the whole of the toxin been recovered from the original filtrate by this method. Normally about 60 or 70 per cent. appears to be recoverable whether dialysed or undialysed filtrates are used. An interesting feature of table III. is that it is shown how, starting with a culture filtrate of the bacillus, it has been possible by precipitation, re-solution, and subsequent dialysis in a collodion membrane to get a final solution which while containing only 14 per cent. of the original nitrogen is ten times as toxic as the original filtrate. It is also clear from these experiments that the precipitable nitrogen, whatever its nature, is not composed entirely of "toxin," since by dialysis it is possible to remove more than half the nitrogen and yet retain considerably more than half the toxin.

TABLE III.

The acetic acid treatment of dialysed and undialysed culture filtrates of B. diphtheriæ *grown on 2 per cent. Parke Davis peptone broth.*

	Lr/500 doses per c.c. toxicity.	Total nitrogen in 10 c.c.	Original nitrogen, per cent.	Proteose nitrogen, per cent.	Amino-nitrogen, per cent.
Original filtrate . .	1,250	40·5 mgrm.	100·0	52·4	20·1
Dialysed filtrate . .	...	15·2 ,,	37·8	59·2	13·3
A* Precipitate from dialysed filtrate with 3 per cent. normal acetic acid.†	16,666	19·9 ,,	49·0	51·5	20·9
B* Precipitate from undialysed filtrate with 17 per cent. normal acetic acid.	16,666	17·6 ,,	43·2	71·6	11·4
C* Precipitate from undialysed filtrate with 7 per cent. normal acetic acid.	16,666	12·0 ,,	29·6	60·8	12·5
D Solution C after twenty-four hours dialysis against water.	12,500	5·6 ,,	14·0	...	...

* The precipitate from two litres of the original filtrate was in each case dissolved in 100 c.c. of 0·7 per cent. saline and 0·5 per cent. phenol added. This represents, therefore, a volume concentration of twenty.

† The Glenny-Walpole method of concentration.

It may be mentioned at this point that the precipitation of the toxic principle may be effected by most acids although the members of the acetic acid series, which are only feebly dissociated and therefore yield only small quantities of hydrogen ions, offer many advantages. Von Groer (1923) has recently demonstrated the fact that the addition of hydrochloric acid in appropriate amount to diphtheria toxin produces a precipitate which contains practically all the toxin. According to this worker, precipitation is practically complete at a P_H of 3·0 to 4·0 It would appear, therefore, that the toxic principle, whatever its

nature, is soluble in faintly alkaline solution but is unable to exist in solution at a P_H of 3·0 to 4·0 at any rate for most types of filtrates.

(b) *The influence of temperature of precipitation on the recovery of toxin from the filtrates of the bacillus.*

One of the most marked features of the acetic acid precipitation of either the dialysed or undialysed culture filtrates is the variability of the toxin recoveries. Occasionally as high a recovery as 80 per cent. would be obtained, whilst as frequently the recovery would fall as low as 30 per cent. After a certain amount of experimental work it was determined that these variations could be controlled to a considerable extent by carrying out the precipitation at low temperature. Table IV. shows one result which is typical of several others carried

TABLE IV.

The influence of temperature of precipitation on the recovery of toxin from filtrates of the bacillus.

Temperature of	Toxicity. Lr/500 doses per c.c.			Percentage of toxic doses recovered.
	Filtrates.	Reconstructed antigen.	Supernatant fluid.	
4° C.	4000	16,666	Nil.	83·3
17° C.	4000	6,666	Nil.	33·3
37° C.	4000	5,000	10	20·0

out along similar lines. For this experiment three litre-volumes of filtrate prepared from tryptic digest media were precipitated with 1 per cent. glacial acetic acid at 4° C., 17° C., and 37° C. respectively. The precipitate was washed in each case with 300 c.c. of distilled water at the corresponding temperature, and then dissolved in 200 c.c. of caustic soda to a P_H of 8·4.

It will be seen that at the lowest temperature the recovery of the toxin is greater than it is at the higher temperatures. In the other cases a large percentage of the toxin has been either lost or else so modified as to escape detection by the Lr/500 test. At a temperature of 4° C. the toxin molecule, whatever its nature, is less injured by the conditions of the precipitation than it is at higher temperatures. Although experiments of this type have been carried out many times, the complete recovery of the toxin has never been effected. Working at low temperatures, however, removal of the toxin from the filtrates is practically complete. Subcutaneous injection of the filtrates after removal of the precipitate, usually shows the presence of same toxin in solution but the amount is practically negligible.

(c) *The stability of the toxin in the nitrogenous material precipitable from the culture filtrates of the bacillus by glacial acetic acid.*

The precipitate from culture filtrates of the bacillus is insoluble in distilled water but readily soluble in dilute alkali solution. When in caustic soda solution it may be *dried in vacuo* over sulphuric

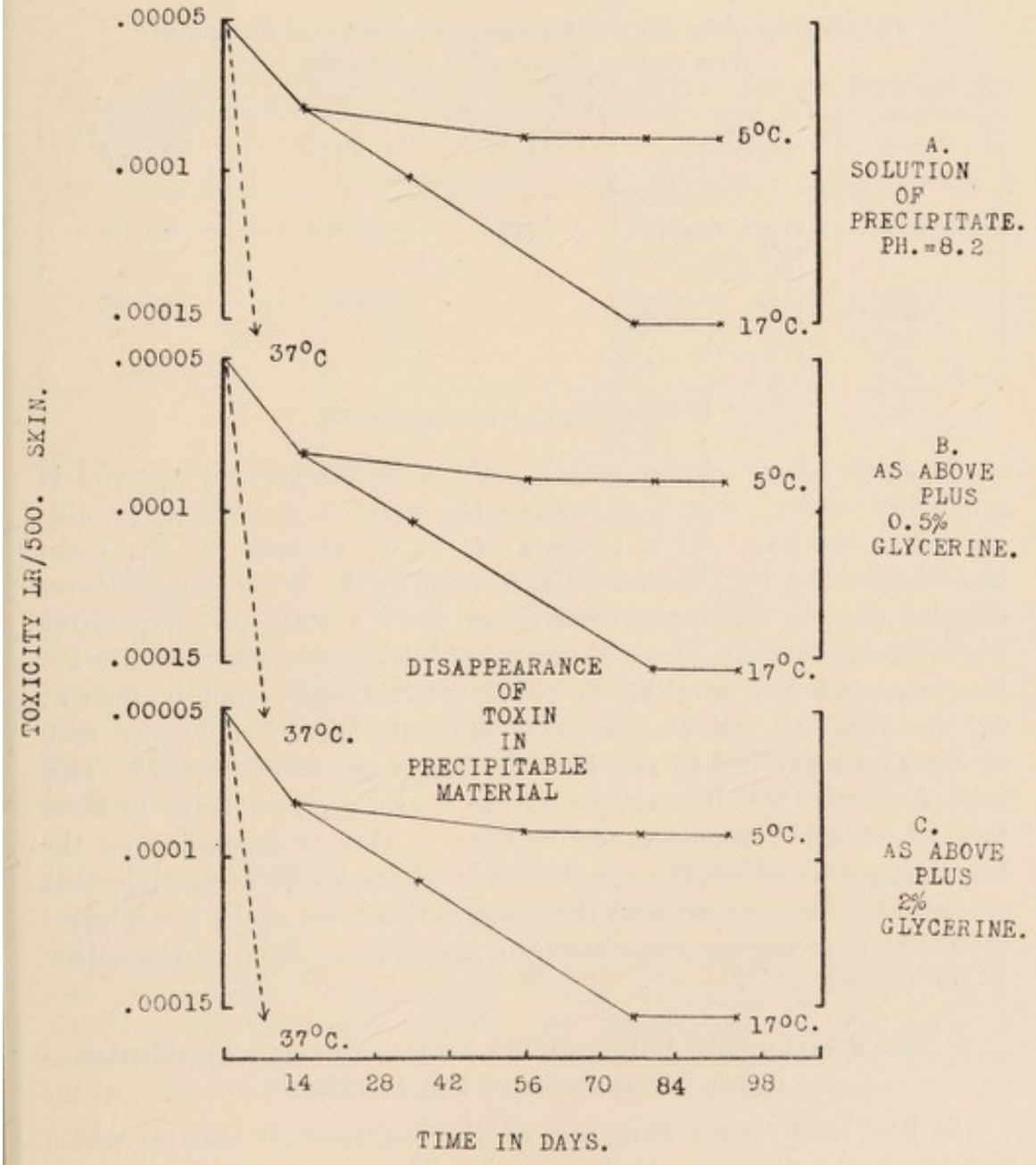

FIG. 3.

acid without detectable loss of potency. This dried product is very stable and may be kept at room temperature for several months at least without loss.

Table V. illustrates this fact. The dried material was prepared by treating four litres of undialysed culture filtrate with 1 per cent. glacial

acetic acid. A precipitate was rapidly produced and settled in a short time. The supernatant liquid was siphoned off and the precipitate collected on a hard filter paper. After thoroughly washing with distilled water it was dissolved in dilute caustic soda to a P_H of 8·4. When this solution, which contained 1·4 per cent. solids, was dried *in vacuo* a product was obtained which readily dissolved in distilled water.

TABLE V.

The stability of the toxin in the nitrogenous material precipitable from culture filtrates of B. diphtheriæ.

Date.	Treatment of dried material.	Lr/500 doses per c.c.	M.L.D.	Fatal doses per gramme of material.
11·9·22	0·14 grs. dissolved in 5 c.c. alkali ($P_H = 8·4$).	10,000	* 0·00075 c.c.	47,600
11·1·23	0·14 grs. dissolved in 5 c.c. alkali ($P_H = 8·4$).	...	* 0·00075 c.c.	47,600

* Death 4/5 days: typical symptoms.

In *dilute alkali solution* at a P_H of 8·4 the precipitable material is much less stable. Fig. 3 (A) shows the rapid falling off in toxicity of such a solution. At a temperature of 37° C. most of the toxin has disappeared by the end of a fortnight. At lower temperatures deterioration or modification proceeds until a state of comparative equilibrium is set up. At 5° C. the rate of deterioration or modification is slower and the equilibrium point reached more rapidly than at 17° C. Fig. 3 (B and C) shows the complete failure of 0·5 per cent. or 2 per cent. glycerol to prevent this disappearance of "toxin." This loss of specific toxicity may be due to a change from toxin to some type of "toxoid" molecule, the velocity of change depending on the temperature to which the solutions are exposed. It is possible that further experimental work on the deterioration rates of the precipitated material under varying conditions will throw more light on the nature of the change.

3. The relationship between the amino-nitrogen in solution and the growth of the bacillus.

It has been shown that one of the fundamental changes which occurs in the Douglas medium during the growth of the bacillus diphtheriæ is a gradual increase in the nitrogen of the filtrate precipitable by glacial acetic acid. It is shown below that parallel with the production of this nitrogenous material is found a steadily increasing amino-nitrogen content of the filtrates. As soon as the toxin and precipitable nitrogen content reach a maximum, the curves representing the two changes flatten out. The production of amino-nitrogen

is common to a great number of bacilli, more especially anærobic organisms. Berman and Rettger (1918) found that the amino-nitrogen content as determined by the Sörensen formol titration of the culture filtrates of a number of strains, amongst them *B. diphtheriæ*, increased steadily with growth. Dernby (1923), with regard to this amino-nitrogen increase, draws a parallel between the activities of the diphtheria bacillus and those of trypsin.

TABLE VI.

Typical changes in the amino-nitrogen and precipitable nitrogen content of the culture filtrates during the growth of B. diphtheriæ *on the Douglas medium.*

Period of growth.	Precipitable nitrogen in filtrate.	Amino nitrogen in 10 c.c. filtrate.	Toxicity Lr/500 doses per c.c.
0 days . . .	14·4 mgrm.	11·3 mgrm.	...
2 ,, . . .	68·66 ,,	11·6 ,,	1429
4 ,, . . .	62·96 ,,	16·06 ,,	1429
7 ,, . . .	79·90 ,,	15·83 ,,	1667
10 ,, . . .	70·66 ,,	16·44 ,,	2500
15 ,, . . .	...	17·50 ,,	2000
18 ,, . . .	70·92 ,,	17·44 ,,	1667
21 ,, . . .	74·01 ,,	16·76 ,,	...
Control—			
25 days . . .	10·50 ,,	11·70 ,,	...

Table VI. shows typical amino-nitrogen changes by the Van Slyke method during and after toxin elaboration on 600 c.c. of Douglas medium. It will be seen that with this medium there is a gradual increase in amino-nitrogen as growth progresses, until a maximum is attained. The precipitable nitrogen and the toxicity of the series was also determined and the figures representing these changes are shown in the table.

II.—COMPARATIVE CHANGES DURING THE GROWTH OF TOXIGENIC AND NON-TOXIGENIC STRAINS.

Various changes which occur during the growth of Park-Williams' No. 8 strain have been discussed with reference to their general relationship to toxin production on the Douglas medium. The investigation was then extended to the culture filtrates of various other toxigenic and non-toxigenic strains of the bacillus when grown under similar conditions on the same medium.

Source of strains.

Four toxigenic and four non-toxigenic strains were selected from a large number collected from different sources, and were all characterised by the facility with which they formed films on Douglas's medium when sown direct from Loeffler slopes. All the strains were tested for virulence to guinea-pigs by means of the intradermic and subcutaneous tests. The toxigenic strains were of course virulent and the non-toxigenic avirulent. The source and serological types (Eagleton and Baxter, 1923) of these eight strains are shown in table VII.

TABLE VII.

Source of strains.

No.	Character.	Serological type.	Source.
780	Toxigenic.	I.	Park-Williams' No. 8.
459	,,	II.	Culture from nose of carrier, 31.10.21.
55	,,	III.	Culture from throat of case of diphtheria, 4.5.21.
851	,,	IX.	Culture from throat of case of diphtheria, 27.2.22.
71	Non-toxigenic.	Atypical.	Culture from throat of carrier, 24.4.21.
655	,,	III.	Culture from throat of carrier, 30.12.21.
826	,,	Not typeable.	Culture from throat of carrier, 24.2.22.
1041	,,	,,	Culture from nose of carrier, 17.5.22.

780 was Park-Williams' No. 8 strain which is employed for the production of filtrates of high toxicity.

Method of growing cultures and collection of filtrates.

The medium used was prepared by the tryptic digestion of horse muscle (Watson and Wallace, 1923). This was distributed in ninety-six single Winchester bottles, 600 c.c. per bottle, which were autoclaved and then placed on their sides in an incubator at a temperature of 36° C. The strains were sown from overnight growths on Loeffler slopes on to small "starter" bottles containing 100 c.c. of medium. The strains after two days' growth were subcultured twice on to fresh 100 c.c. quantities of medium, after which they formed films rapidly. Each of the eight strains was then subcultured on to twelve of the large bottles containing the 600 c.c. of media. Rapid pellicle formation took place and one bottle in each series was removed at the end of varying periods up to twenty-eight days. After examination for purity of culture, 0·5 per cent. phenol was added, the bottle left at room temperature for sixteen hours and then examined for

1. toxin in solution by the intracutaneous test of Römer and Sames (1909);
2. amino-nitrogen in solution by the Van Slyke method;
3. the amount of nitrogen in the material precipitable by 1 per cent. glacial acetic acid from the culture filtrates;
4. the amount of bacterial nitrogen present in the growths produced by the bacilli;
5. the hydrogen ion concentration by the colorimetric method.

1. The changes in the toxicity of the filtrates of the toxigenic strains.

Fig. 4 shows the changes in the toxicity of the various filtrates as growth continues. It will be seen that whereas strain 780 developed a comparatively powerful toxin, the culture filtrates of the other three toxigenic strains were only slightly toxic. Maximum toxin production in the case of strain 780 was attained after ten days' growth on the 600 c.c. of medium. Three c.c. of each of the culture filtrates of the

non-toxigenic strains after the same period of growth (ten days) were injected subcutaneously into guinea-pigs without causing loss of weight or incidence of the typical symptoms. It will be seen therefore that of the eight strains one was powerfully toxic, three were only feebly toxic, whilst the remainder were completely non-toxic.

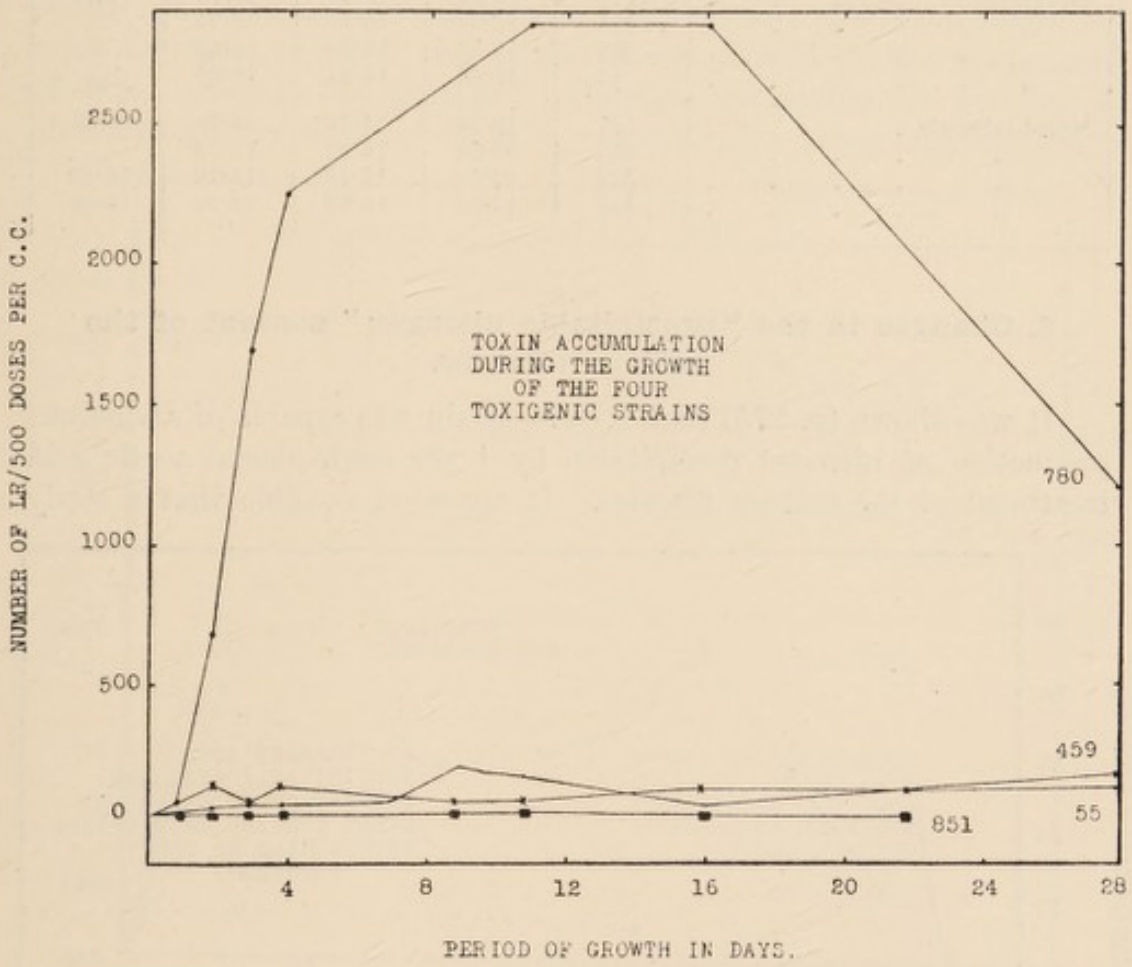

FIG. 4.

2. The changes in the amino-nitrogen content of the filtrates.

It has been shown (p. 280) that there is a steady increase in the amount of amino-nitrogen in the culture filtrates as growth of the powerfully toxigenic strain of the bacillus proceeds and toxin accumulates in solution.

Table VIII. shows changes in the amino-nitrogen content of the filtrates of the eight strains during twenty-eight days' growth under similar conditions on the same media. It will be seen that there is a general tendency for the amino-nitrogen content to increase with growth, and that the most powerfully toxigenic strain is somewhat more vigorous in its production of this type of nitrogen, but there is no sharp difference in the behaviour of the toxigenic strains from that of the non-toxigenic strains.

TABLE VIII.

Type.	No.	Milligrams Van Slyke nitrogen per 10 c.c. filtrate.			
		0 days.	9 days.	16 days.	28 days.
Toxigenic	780	13·54	18·59	16·71	16·79
,, ...	459	13·54	16·73	16·25	...
,, ...	851	13·54	14·84	16·42	...
,, ...	55	13·54	16·21	16·09	...
Non-toxigenic	71	13·54	14·80	15·05	15·85
,, ...	655	13·54	16·72	15·73	...
,, ...	1041	13·54	14·78	15·79	16·30
,, ...	826	13·54	15·59	15·99	15·28

3. Changes in the "precipitable nitrogen" content of the various filtrates.

It was shown (p. 275) that P.-W. 8 strain was capable of a vigorous production of nitrogen precipitable by 1 per cent. glacial acetic acid treatment of the culture filtrates. It appeared possible that a study

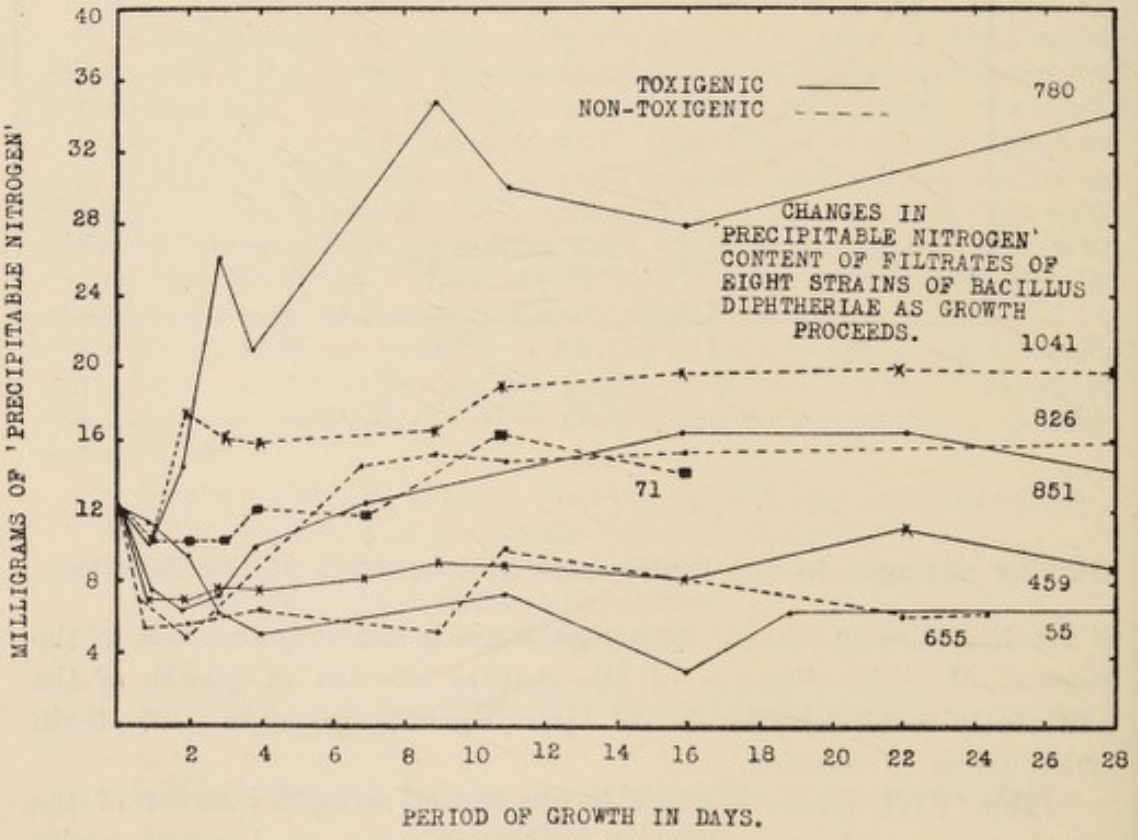

FIG. 5.

of this "precipitable nitrogen" content of the filtrates of the eight strains might bring to light some essential difference between toxigenic and non-toxigenic strains of the bacillus.

Fig. 5 shows the changes in amount of precipitable nitrogen which takes place during the growth of the various strains. It will be

seen that in the case of the most powerfully toxigenic strain the usual rapid increase of precipitable nitrogen takes place during the first eight or ten days after which no further accumulation occurs. Slight changes are recorded in the cases of the other culture filtrates, but there is no sharp contrast between the behaviour of the feebly toxigenic strains and the non-toxigenic ones. No marked difference seems to exist between toxigenic and non-toxigenic strains in the production of this "precipitable nitrogen," although the ability to produce it in comparatively large amounts is possessed by a powerfully toxigenic

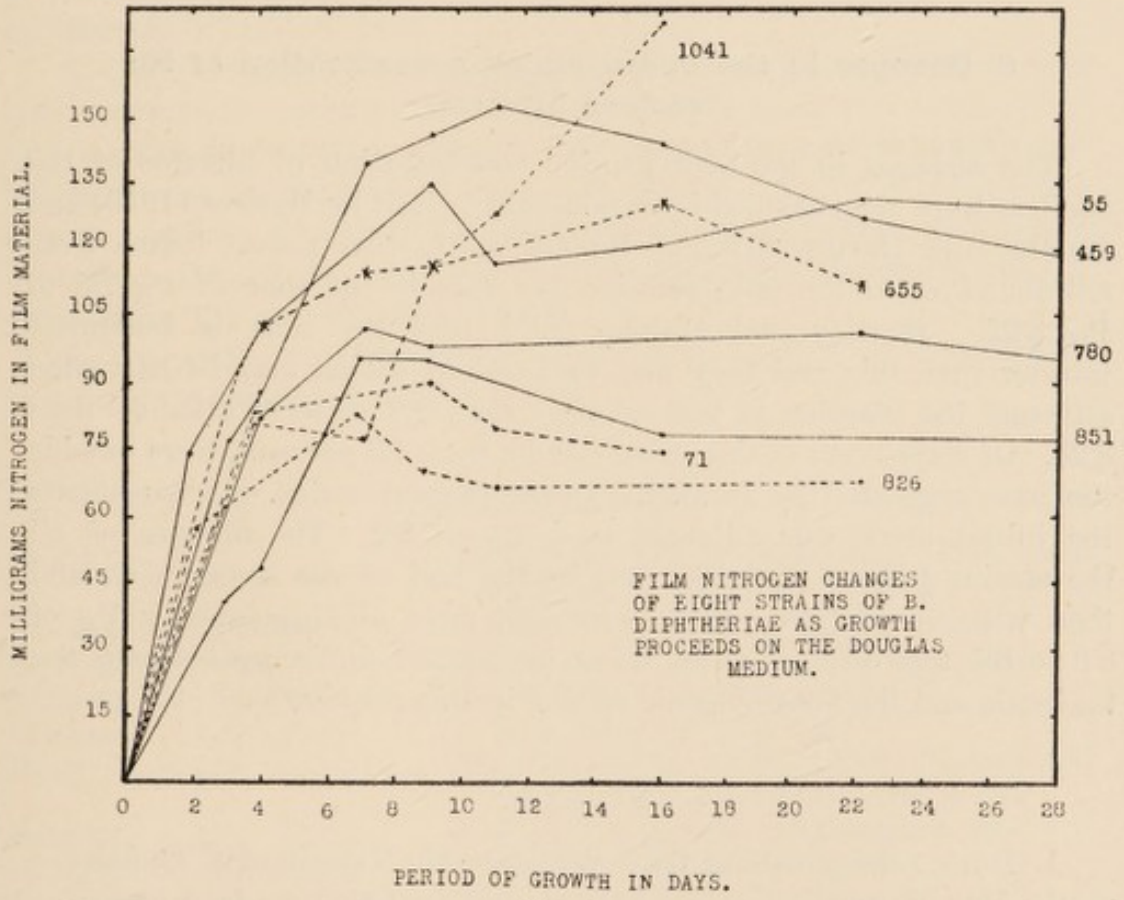

Fig. 6.

strain rather than a feebly toxigenic or non-toxigenic one. With this fact must be correlated in some way its greater toxin-producing capacity, since it has been shown above (p. 276) that the accumulation of this precipitable nitrogen may be taken advantage of for the isolation of a highly toxic material from the culture filtrates of the bacillus.

4. The changes in the "bacterial nitrogen" of the cultures.

In order to obtain some quantitative measure of the rates and amounts of growth of the various strains, the quantities of bacterial nitrogen were determined after varying time periods by the technique already described (p. 273).

Fig. 6 shows the changes which take place during the growth

of the eight strains. There is a general tendency for the curves to reach a maximum between the seventh and tenth days, although one strain (1041) appears to occupy an anomalous position. Further, strain 780, which has been shown above to be unique in its production of toxin and "precipitable nitrogen," occupies a central position. It is evident that the production of precipitable nitrogen which, in the case of toxigenic strains, contains most of the toxin of the filtrates is not a function purely of the growth of the bacillus, but is rather in some way peculiar to the strain itself.

5. Changes in the hydrogen-ion concentration of the various filtrates.

The changes in the hydrogen-ion concentration of filtrates of the bacillus have been studied in considerable detail by Bunker (1919) and Hartley and Hartley (1922). Bunker states that potent toxins were not found when the final reaction lay outside the zone of P_H 7·8 to P_H 8·25. Hartley and Hartley hold that the zone is somewhat broader than this, and they find that potent toxins may be harvested although the reaction is less alkaline than 7·8 or more alkaline than 8·25. Of sixty-eight toxins examined by them 50 per cent. were outside the zone suggested by Bunker. In the present series of experiments the initial broth was adjusted to a P_H of 8·0. The filtrates of all the strains became acid (P_H 6·8) by the end of the second day, and then without exception became steadily more alkaline until a P_H of 8·0 to 8·4 was reached, there being no marked difference between the toxigenic and the non-toxigenic strains in this respect.

Summary.

1. During the growth of Park-Williams' No. 8 strain of *B. diphtheriæ* on the Douglas medium at least three important changes take place:—

(1) The nitrogenous material precipitable by glacial acetic acid increases to a maximum and then slightly decreases.
(2) There is a steady increase in the amino-nitrogen content of the culture filtrates as growth proceeds and toxin accumulates in solution.
(3) The weight of the bacilli (or the amount of nitrogen in this material) increases to a maximum and then slightly decreases.

The curves representing these changes reach a maximum at approximately the same time as the toxicity curve of the culture filtrate.

2. The nitrogenous material precipitated by glacial acetic acid from the filtrates gives a highly toxic solution when dissolved in dilute

alkali to a P_H of 8·4, and if the precipitation is carried out at low temperature contains most of the toxin of the filtrate.

3. The deterioration rates of the toxin of the precipitated material have been studied. It is quite stable if dried *in vacuo* over sulphuric acid immediately after precipitation and re-solution. If, however, the material is left at room temperature in dilute alkali solution, deterioration (or modification) proceeds until a state of comparative equilibrium is set up. At 5° C. the rate is slower and the equilibrium point reached more rapidly than at 17°. At 37° C. the rate of deterioration or modification is very rapid. The presence of glycerol has no influence on these deterioration rates.

4. Comparing this highly toxigenic with three feebly toxigenic and four non-toxigenic strains, no difference could be made out in respect of the increase in bacterial nitrogen, the increase in amino-nitrogen, and the hydrogen-ion changes in relation to growth. The highly toxigenic strain, however, produced far more nitrogen precipitable with acetic acid than the others. In this respect the feebly toxigenic did not differ from the non-toxigenic strains.

5. The precipitate produced by adding acetic acid to culture filtrates is intimately associated with the toxin.

REFERENCES.

Banzhaf	Pathogenic Micro-organisms, Park and Williams, *New York*, 1920, p. 175.
Berman and Rettger . . .	*J. Bact.*, 1918, vol. iii., p. 369.
Brieger and Boer	*Deutch. med. Woch.*, 1896, vol. xxii. p. 783.
Brieger and Fränkel . . .	*Berlin klin. Woch.*, 1890, p. 241.
Bunker.	*J. Bact.*, 1919, vol. iv. p. 379.
Dean	The Bacteriology of Diphtheria, Nuttall and Graham Smith, *Cambridge*, 1908, p. 489.
Dernby.	*C. R. Soc. Biol.*, 1923, vol. lxxxviii. p. 109.
Eagleton and Baxter. . .	*J. Hygiene*, 1923, vol. xxii. p. 107.
Glenny and Allen	This *Journal*, 1921, vol. xxiv. p. 61.
Glenny and Walpole . . .	*Biochem. J.*, 1915, vol. ix. p. 298.
Hartley	This *Journal*, 1922, vol. xxv. p. 479.
Hartley and Hartley . .	This *Journal*, 1922, vol. xxv. p. 468.
Römer and Sames	*Zeitschr. f. Immunitätsforsch.*, 1909, Orig. iii. p. 344.
Von Groer	*Biochem. Zeitschr.*, 1923, vol. cxxxviii., p. 13.
Watson and Wallace . . .	This *Journal*, 1923, vol. xxvi. p. 447.

PRINTED BY OLIVER AND BOYD, EDINBURGH