The influence of cultural conditions on the production of diphtheria toxin / A.F. Watson and Elsie Langstaff.

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THE INFLUENCE OF CULTURAL CONDITIONS ON THE PRODUCTION OF DIPHTHERIA TOXIN.

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I.—THE DEVELOPMENT OF CULTURAL METHODS FOR THE ROUTINE PREPARATION OF DIPHTHERIA TOXIN AND TOXOID.

A FEW years ago Hartley and Hartley (1922) described a general method for the preparation of diphtheria toxin and its results in their hands. They emphasised the importance of the production of satisfactory antigens in laboratories where the preparation of therapeutic sera is carried out on a large scale. Since these results were published certain advances in this field have been made and a general survey of the work is included in the three parts of this paper. The present part deals especially with the development of methods although, in devising these, advantage has been taken of work described in the second and third papers. These methods were made possible by the use of the Ramon (1922) flocculation test for titrating culture filtrates of *C. diphtheriæ* against antitoxin *in vitro*. Over a long period individual culture filtrates were tested and two aspects of importance in routine production were soon evident. It was found that the variation from filtrate to filtrate from the same medium autoclaved at pH = 8·0 (especially with media made by tryptic digestion of muscle) might be very great (*infra*, p. 394). In attempting to trace the cause of this variation, the importance of the heat stability of media became prominent. Although the ill effects of heat on media have been reported by many workers, the influence of autoclaving at pH = 8·0 in contributing to this variation had not been suspected by us (*infra*, p. 405).

It was decided at the beginning of the work to carry out a detailed investigation of the construction of medium by the tryptic digestion of muscle, a process which Hartley (1922) adapted to the preparation of diphtheria toxin from Douglas' (1914) original method. The media used in this work and that in the following two papers were prepared by two modifications of enzyme digestion and by dissolving 2 per cent. of commercial peptone X in beef-heart infusion.*

* The beef-heart infusion was prepared by boiling muscle and water in the proportion of 1 lb. of minced muscle to 1 litre of water. The infusion was filtered, 2 per cent. peptone and 0·5 per cent. salt added and the pH adjusted to 8·0. After filtering the medium, sterilisation, method of inoculating etc. were carried out as for digest media.

Preparation of media.

The development of the methods for preparing digest media may perhaps be made clear, if the technique which has been used for nearly two years for the preparation of graded filtrates is first described in detail. Two methods were employed.

The short period digestion (*method* 1.)—The digests were carried out in four litre glass jars. Six hundred grms. of horse muscle, minced and freed from fat and connective tissue, were placed in each jar and one litre of tap water added. To destroy any antitryptic activity of the muscle and effect solution of some nitrogenous material, the jars were given a preliminary steaming until they reached a temperature of 80° C. To each jar was then added one litre of 0·15 normal sodium carbonate. This lowers the temperature of the jars and brings the *p*H of the contents within the zone 8·4-8·8. As soon as the contents reached a temperature of 44°-45°, 40 c.c. of trypsin extract and 20 c.c. of chloroform were added to each jar, and the jars were placed in an incubator at 35° C. The digestion was allowed to proceed for 2·5 hours with occasional stirring. To each jar was then added 16 c.c. of concentrated hydrochloric acid, and the contents of the jars were bulked in a steam cauldron, taken up to 100° C. and filtered. The *p*H of the warm acid filtrates was adjusted to 8·0, the medium filtered again and allowed to remain in an alkaline state in the cold room overnight. The adjustment of the *p*H of the acid medium may be left till the following morning to avoid leaving it so long in the alkaline state. To ensure accurate volume adjustments from batch to batch, the volume of bulked medium was made up proportionally each time with glass-distilled water.

The long period digestion (*method* 2.)—The proportions of muscle, carbonate solution, trypsin etc. were the same as for method 1, which was closely followed up to the time when the enzyme was added. Instead however of leaving the enzyme to act for 2·5 hours at 35° C., the jars were kept at room temperature for 72 hours, lightly covered with parchment paper; the contents of the jars were stirred daily, after which the treatment with acid etc. was carried out as in method 1.

The distribution of medium into containers.—Following Hartley's technique, double Winchester quart bottles (capacity 5 litres) previously sterilised were used as containers, 1 litre of medium being placed in each bottle. Great care was taken to plug the bottles with cotton wool as loosely and uniformly as possible. Eighteen to thirty litres of each batch were filled out in this way and the remainder was stored after Seitz filtering into large bottles for use when its value was known (see below).

The sterilisation of the medium.—The most satisfactory method used for large amounts of media was by filtering at *p*H = 8·0 through a Seitz filter press into the previously sterilised double Winchester quart bottles. These were then steamed at 100° C. for ten minutes. Alternatively the medium was autoclaved at ca. *p*H 4·0 and the *p*H of the medium adjusted to 8·0 before Seitz filtering, after which no further heat was applied (below, p. 406). At the beginning of the work, the technique of Hartley and Hartley (1922) was adopted and sterilisation was effected by autoclaving. Steam was passed freely through the

autoclave for one hour after which the pressure was raised slowly to 10 lb. and maintained at this level for half an hour. This procedure was abandoned as soon as its deleterious effect on the medium was established.

Chemical analysis of medium.—The following estimations were carried out on a sample of the sterilised medium:—

(*a*) The *p*H was determined.

(*b*) Total nitrogen (T.N.) was estimated by Kjeldahl's method.

(*c*) Amino nitrogen (V.S.N.) was determined by Van Slyke's (1912) method.

(*d*) Proteose nitrogen was determined by Hedin's (1904) method.

Method of control.

One of the chief difficulties encountered in this particular field of investigation is the variability of the media. There would appear to be two groups of factors determining the final potency of the culture filtrates. The primary factors are those concerned with the actual production of the medium which involves the use of horse muscle over which there is no practicable control. As soon as the medium is made it has, for any one strain of *C. diphtheriæ*, a potential toxin-producing value. This may be deleteriously affected by secondary factors, *e.g.* by the heat which is applied during sterilisation, by the type of bottle in which the medium is contained, by the temperature of growth, by the ease of access of air to the culture during growth, possibly by the amount of inoculum which it receives initially and other as yet unknown factors (below, pp. 394 ff.). It frequently happened during the preliminary study of the problem that a number of experiments carried out with one consignment of muscle proved useless owing to the control medium failing to give filtrates of any value. To overcome this difficulty a method of control suggested by our colleague, Mr A. T. Glenny, was adopted. In the absence of an absolute control, a continuity of comparison was ensured by this means. The main features of this control, used chiefly with digest media made by method 2 and peptone media, are outlined below.

(*a*) *Media.*—Two volumes of medium were prepared every week on two successive days from one consignment of muscle. Enzyme extractions were carried out from large batches of pancreas. Definite days were allotted for the preparation and inoculation of media and the "harvesting" of toxins.

The extracts were prepared in quantities of 50-60 litres by the method of Cole and Onslow (1916) from fresh pig's pancreas, using absolute alcohol in the early part of the work and later methylated spirit (which serves equally well and is more economical). At room temperature or in the incubator the extract usually reaches maximum potency after 48-72 hours. At lower temperatures 4 days or more may be necessary. Considerable variation exists in the chemical analyses and the proteolytic activity of the extracts from batch to batch.

(*b*) *Method of growing cultures.*—The bottles containing the media were placed in an incubator on definite shelves, the temperature of each shelf being recorded twice daily. Each batch of medium was divided into five sub-batches, A—F (later reduced to three, A—C), each sub-batch containing a minimum of six bottles, three per shelf. Sub-batch A of the fifth medium was inoculated at the same time as sub-batch B of the fourth medium, sub-batch C of the third medium, sub-batch D of the second medium and sub-batch F of the first medium, and so on. The bottles were inoculated from "preliminary" double Winchester quart bottles containing 2-day old cultures of the bacillus, a substrain 110 of the original Park-Williams' strain (P. W. 8), one culture being used for each shelf (*i.e.* isothermal inoculation) and one loopful of film used for three bottles. The results of this method of control are shown in tables I. and II.; its value lies in the provision of a continuous control and in making available for experiment media of known potential value for toxin production.

Period of growth, film formation, etc.

Film formation was usually complete in 24 hours, and after about 7 days' growth was luxuriant particularly with the enzyme digests where festoons and heavy deposits were characteristic. Experimentally, it was found that digest medium reached its maximum at the end of 10 days. With peptone media the growth period was limited to 7 days (Hartley and Hartley, 1922). Before removing from the incubator, a smear was made and examined for purity.

The sterilisation of cultures.

The cultures were killed after growth during the early part of the work with 0·5 per cent. pure phenol. Later, when the deleterious effect of phenol was realised (see Moloney and Weld (1925), Glenny, Pope, Waddington and Wallace (1926)), toluol was used if toxin was required. When it was desired to produce toxoid by the action of formaldehyde according to the method of Glenny and Südmersen (1921) and Glenny and Hopkins (1923), the technique of Ramon was adopted and 0·5 per cent. of neutralised formalin (0·2 per cent. formaldehyde) was added directly to the culture. By this means the detoxication of the phenol-free antigen was commenced and could be continued by subsequent incubation of the filtrates.

The determination of the "strength" of culture filtrates.

All "normal" filtrates (*i.e.* those phenolised or toluolised filtrates with *p*H of more than 7·0 and those formalised filtrates of *p*H more than 6·0) were tested for potency (Lf units * per c.c.) by the method of Ramon (1922) as elaborated by Glenny and Okell (1924) and Glenny

* The Lf dose is that amount of filtrate which is equivalent to one unit of antitoxin by the flocculation test (Glenny and Okell, 1924).

and Wallace (1925). Samples were normally tested over a range of 2-24 units at two unit intervals, but differences of half a unit were on occasion estimated. A selective method of bulking filtrates according to their Lf values was adopted. By this means large volumes of graded filtrates could be obtained for immunising and experimental purposes. The lower grade filtrates were concentrated by the method of Watson and Wallace (1924).

General results.

Table I. shows a typical sowing. Initial and final, and maximum and minimum temperatures during growth were recorded for each shelf. The four routine broths consisted of sub-batches of media made from two consignments of muscle (method 2). The experimental broths included two autoclaved modifications of one of the filtered control broths (CEE 8) and one commercial peptone broth. The ill effects of over-autoclaving media at *p*H 8·0 are seen (below, p. 405) as well as the general index of the bottle variations, especially with the autoclaved broth (below, p. 398).

TABLE I.

Method of control.

Typical sowings of experimental broths with four routine controls to ensure continuity of comparison.

		Routine control broths (method 2).								Experimental broths.					
		CE 5(D). Autoclaved broth.		CE 6(C). Autoclaved broth.		CE 7(B). Autoclaved broth.		CEE 8(A). Filtered broth.		B. 2057. Autoclaved broth.		B. 2062. Over-autoclaved broth.		B. 2064. 2 per cent. peptone broth.	
cle batch . .		55		55		57		57		57		57		...	
psin batch . .		85		85		85		85		85		85		...	
f.	Temperature.	*p*H.	Lf.	*p*H.	Lf.	*p*H.	Lf.	*p*H.	Lf.	*p*H.	Lf.	*p*H.	Lf.	*p*H.	Lf.
	35·2	6·4	4	8·3	10	8·2	4	8·2	16	...	..	8·2	4	7·9	10
	32·9 34·1	6·4	4	8·3	12	8·1	4	8·0	14	...	...	contam.	...	7·8	6
	29·7	6·4	2	8·3	10	8·2	4	8·1	16	...	...	contam.	...	7·9	12
	33·9	6·0	4	8·3	12	...	...	8·2	14	8·3	14	8·1	2	7·9	12
	31·9 33·0	6·0	4	8·3	10	contam.	...	8·2	16	8·3	16	8·2	4	7·9	8
	28·7	contam.	...	8·3	8	con. tam.	...	8·2	12	8·2	12	8·2	4	7·9	10
	30·5	...	...	...	...	8·2	10	...	...	...	...	...	...	7·8	8
	29·1 29·9	...	...	...	...	8·2	10	...	...	...	...	8·1	8	7·1	2
	28·4	...	...	...	...	8·1	12	...	...	8·0	16	8·0	8	7·1	4

Table II. shows the classification of results from eight typical preparations of filtered digest medium made by method 2. Similar records have been obtained from nearly two hundred batches of medium representing the analysis of results from about four thousand individual filtrates. These records have indicated the relative importance of several factors involved in routine production of toxin.

(i) From horizontal readings, the influence of sowing a medium at different times is shown. Change of inoculum and slight temperature differences during growth would seem to have little effect. The definite gradation in value from higher to lower obtained with many batches would point to some deterioration of medium after storage over the period tested (not exceeding 6 weeks).

(ii) From vertical readings, comparisons between batches of media made from the same consignment of muscle on different days, also between batches of media prepared from different consignments of muscle, are possible. Although the technique was never consciously changed, the value of media made at different times from the same muscle batch varied. This may of course be due to bacterial interference during digestion rather than to the personal factor. The differences between meat batches are also emphasised and shown more markedly in chart I.

(iii) From diagonal readings, we may see differences in media sown at the same time, every condition possible being the same for each batch, so that here the relative values of the media may be compared. Consecutive sowings show little variation in this relative order and these combined give a definite index to the value of the media.

TABLE II.

Method of control.

Classification of results for eight batches of media (method 2) sown in sub-batches on different occasions.

Harvesting date of "A" sub-batch.	Average Lf value per c.c. of filtrates. Sub-batches.					Sowing date of "A" sub-batch.	Medium number.	Muscle batch.	Trypsin batch.	Total N.mgrms. per 10 c.c.	Amino N.mgrms. per 10 c.c.
	A.	B.	C.	D.	F.						
12.10.25	13	14	12	8	...	2.10 25	46	77	94	25·5	6·3
12.10.25	8	4	4	2	...	2.10.25	47	77	94	24·1	5·0
19.10.25	5	5	4	2	2	9.10.25	48	78	94	21·1	4·3
19.10.25	11	12	11	...	...	9.10.25	49	78	94	25·3	6·3
26.10.25	12	10	8	8	...	16.10.25	50	79	94	19·3	6·0
26.10.25	12	15	7	..	...	16.10.25	51	79	94	24·6	6·5
2.11.25	18	16	15	15	...	23.10.25	52	80	94	19·1	4·7
2.11.25	20	...	14	14	...	23.10.25	53	80	94	26·5	7·1

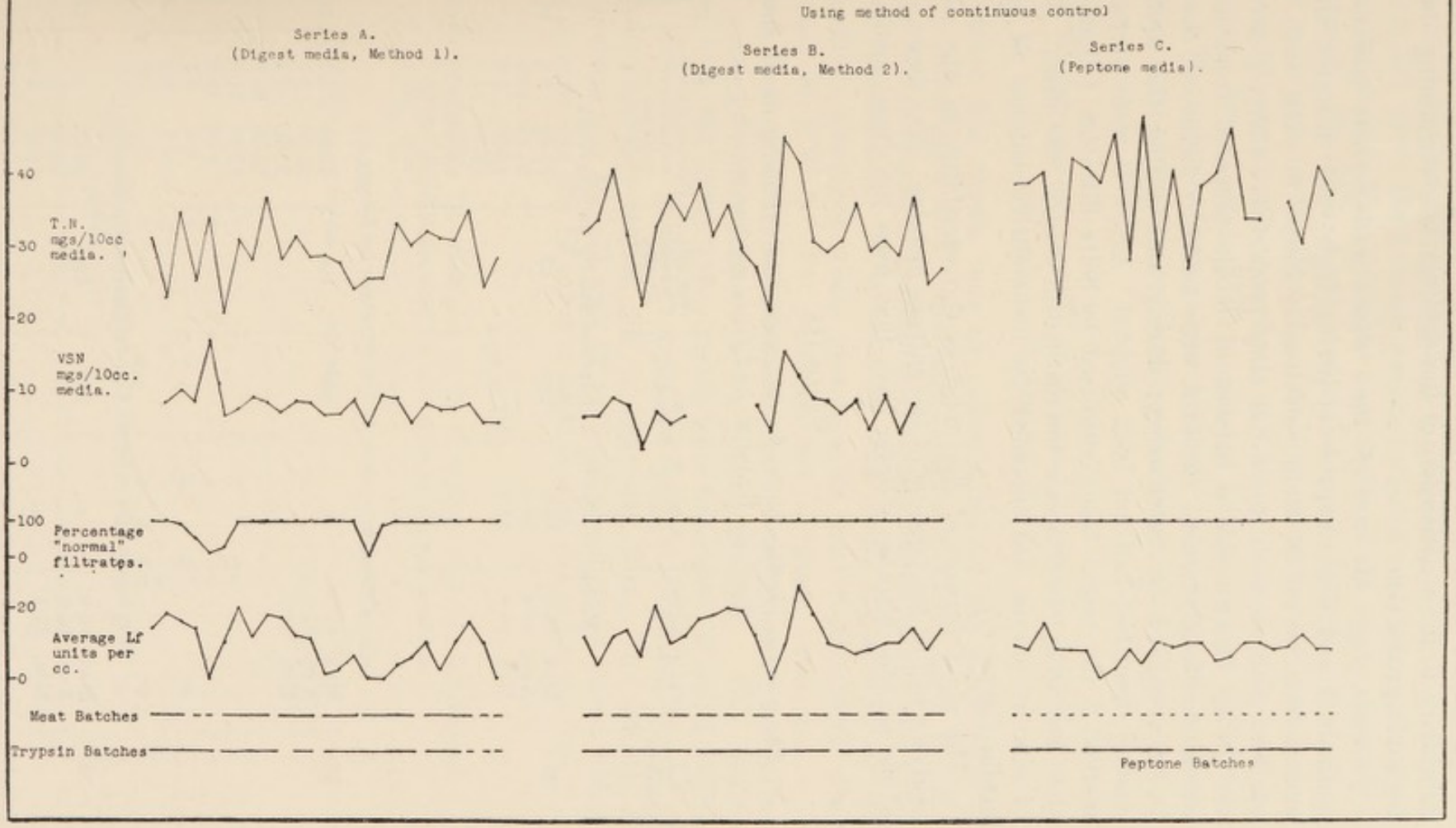

CHART I.—Variation of consecutive batches of media made by three methods.

To summarise, it would appear that all these factors are definitely subsidiary to the constitution of the medium in determining its value for toxin production.

Results from the use of these digest and peptone media.—Both methods 1 and 2 are capable of giving high value filtrates although over a long period of time variation is found to exist both in the analyses and in their toxin-inducing properties. Chart I gives the variability of consecutive batches of media prepared from different consignments of muscle. Series A were made by method 1, the media being sterilised by autoclaving; during this period the method of control described had not been adopted. Series B were prepared by method 2, the media being sterilised by Seitz filtration followed by a short steaming; in this case the method of control was used. A series of similar curves are included for consecutive batches of peptone media (series C).

The chief feature of the chart is the variability in the nitrogen analyses and in the potency of the culture filtrates. The average value of the peptone filtrates is lower than that from the digest media. The

TABLE III.

The average results obtained with consecutive batches of digest media (methods 1 and 2) and from peptone media.

No. of batches.	Total N. mgrms. per 10 c.c.	% Amino N. / Total N.	No. of filtrates.	Per cent. "Normal" filtrates.	Percentage filtrates containing Lf units per c.c.		Highest Lf value per c.c.
			Method 1.—Series A. (sterilisation by autoclaving).				
25	Max. 36·6	57·3	638	87	25-30	2·6	27 units.
	Aver. 29·4	28·0	...	...	20-25	13·9	
	Min. 20·4	18·1	...	...	16-20	3·4	
					12-16	18·0	
					8-12	20·6	
					6-8	12·4	
					< 6	29·1	
			Method 2.—Series B. (sterilisation by filtration).				
26	Max. 45·1	...	265	100	30-35	1·1	32 units.
	Aver. 32·1	...	...	...	25-30	2·6	
	Min. 21·2	...	...	...	20-25	10·6	
					16-20	23·0	
					12-16	21·5	
					8-12	24·9	
					6-8	7·5	
					< 6	8·8	
			Peptone media.—Series C. (sterilisation by filtration).				
23	Max. 46·9	...	237	100	25-30	...	17 units.
	Aver. 33·5	...	...	...	20-25	...	
	Min. 22·1	...	...	...	16-20	3·8	
					12-16	18·6	
					8-12	36·3	
					6-8	13·9	
					< 6	27·4	

former while showing greater nitrogen variations in the media exhibit less variations in the average value of the filtrates.

Table III. gives the average results obtained with the media dealt with in the chart. Here again the superiority of the digest methods is emphasised. The highest value obtained for any filtrates was thirty-two Lf units per c.c. (method 2). The highest value filtrate obtained by method 1 was examined in detail and found to contain 22 Lr doses, 22 Lo doses, 18 L+ doses, 1700 guinea-pig fatal doses and 27 Lf units per c.c. The value of these digest methods (especially method 2) for the preparation of very potent filtrates can therefore be appreciated.

The development of a medium adapted to the requirements of a particular substrain of P. W. 8.

Evidence has shown that the constitution of the medium is of primary importance and these digest methods would seem to offer a means of developing a medium particularly suited to any one strain after acclimatisation. It is unlikely that media constructed by the two methods described would give equally good results with other substrains of P.W. 8. For instance, five of these substrains were collected from different laboratories in Europe and America and given eight consecutive subculturings on digest medium. The culture filtrates from the eighth subculturing were tested for *p*H and Lf value in the usual way. Table IV. gives the results which are typical of several experiments carried out on the same lines. The growth of the organisms and the *p*H and Lf value of the filtrates varied widely and in no case was the Lf value so high as with our substrain 110.

TABLE IV.

Toxin production by substrains of P. W. 8 *from different laboratories after acclimatisation on one type of digest medium.*

Substrain of P.W. 8.	Source.	Growth.	Formalised filtrates		
			No.	*p*H.	Average Lf units per c.c.
79	1	+ +	3	6·0	3
780	2	+ + +	3	7·0	4
103	3	+ + +	2	6·9	12
106	4	+ +	1	7·2	0
111	5	±	2	6·0	0
110	Routine strain	+ + +	3	7·1	18

With this fact in mind, the present methods were developed with substrain 110 which at the commencement of the work was giving filtrates of little or no value with any type of medium. Investigation

of the conditions during digestion etc. revealed those factors most suitable to vary in attempting to adapt the method to any one substrain. Experiment showed that variation of any of the conditions of digest, *e.g.* ratio and *p*H of components, type of container, temperature and time of digestion, markedly affected the value of the medium obtained. It was found best to keep empirical factors such as quantities of components, containers etc. constant, while the more easily controlled conditions of temperature and time could be varied. Thus to establish method 1 a number of batches of muscle were digested at 35° C., under exactly the same conditions with one batch of enzyme, samples being removed from 1 to 8 hours at half-hourly intervals. Similarly, to establish method 2 a number of batches of muscle were digested at room temperature under exactly similar conditions with one batch of trypsin extract and samples were taken at 24-hour intervals during 4 days. In both cases, the fractions were converted into media and tested for growth and toxin production after acclimatisation with the 110 substrain of P.W. 8. The results of this procedure are illustrated in table V., the data from which were used to establish method 2.

TABLE V.

The value of batches of media made by prolonged digestion of different muscle batches at room temperature for varying time periods.

Period of digestion.	No. of batches.	Percentage of "normal" filtrates.	Percentage of filtrates with Lf units per c.c.					
			20-25.	16-20.	12-16.	8-12.	6-8	<6
0 hours .	4	50	...	...	...	5	10	85
24 ,, . .	5	75	...	4	17	29	17	33
48 ,, . .	12*	85	4	...	6	16	20	54
72 ,, . .	15*	80	0·2	1	34	29	15	20·8
96 ,, . .	7	100	...	13	8	47	7	25

* It soon became evident that these two periods were giving the best results. A larger number of muscle batches were therefore investigated to determine the better point to stop the digestion.

Correlated with the different chemical composition of the fractions there was a variation in their value for toxin production. The 72 and 96 hour digests gave the best results, and the more convenient shorter digestion was taken as a suitable basis for the construction of media. By a similar technique, the conditions of method 1 (2·5 hours digest at 35° C.) had been determined for the same substrain. In this way, by carrying out preliminary experiments with several muscle batches (muscle being an uncontrollable factor) it would appear possible to establish a method for the preparation of media especially adapted to the requirements of a particular substrain of P.W. 8.

Bacterial interference during digestion.

It has been mentioned above that volumes of medium made from one muscle batch on subsequent days frequently varied. Bacterial growth during digestion may have contributed to this variation, since the use of preservatives and frequent stirring did not entirely suppress the growth of ærobic and anærobic organisms. Attempts were therefore made to digest under sterile conditions. Mixtures of water and muscle were steamed on three successive days or autoclaved either in the alkaline or acid state (in the latter case sterile alkali was added to raise the *p*H before digestion) and digested with trypsin extract previously filtered through a Berkefeld candle, a process which does not markedly affect its activity (Cunningham, 1918). Although this technique was laborious, it was found possible to effect digestion under perfectly sterile conditions, and the resulting media showed lower variability.

Table VI. gives one example where the muscle, water and carbonate were sterilised by steaming on three successive days: filtered trypsin was added aseptically and media prepared at the end of three days, after showing the absence of anærobic and ærobic organisms. Both

TABLE VI.

Analyses and toxin production on media made by bacteria-free sterile tryptic digestion.

Number.	Batch number.		Nitrogen mgrms. 10 c.c.			Lf units per c.c. of filtrate. No.		
	Muscle.	Tryspin.	T.N.	V.S.N.	Hedin (protease).	1.	2.	3.
B. 2141 . .	90	94	39·5	7·4	11·9	8	10	8
B. 2141A .	90	94	38·6	8·6	9·8	...	12	10

the chemical analyses of the media and the potency of the culture filtrates were similar. It would seem that if bacterial growth during digestion could be excluded in the large scale production, the variability of the media from one batch of muscle would be lessened.

The variation from culture to culture on one medium.

The fact that after filling out the same batch of medium into similarly shaped containers, sterilising by autoclaving at *p*H 8·0 and inoculating from one culture, variations existed from bottle to bottle was emphasised when individual filtrates were tested. The difficulty has been overcome in the present methods by grouping the tested filtrates according to their Lf values before bulking and using for immunising purposes. Some of the possible causes of this type of variability are examined below (p. 398).

SUMMARY OF PART I.

1. The variability of three routine methods for the preparation of diphtheria toxin has been studied.

2. A method of continuous control for use in this type of work is described.

3. Evidence is given to suggest that the composition of the medium is of primary importance in determining the value of the culture filtrates.

4. The development of methods for the preparation of a routine medium by the tryptic digestion of muscle, especially adapted to any one substrain of the P.W. 8 strain of *C. diphtheriæ*, is described. Media made by such methods show very high toxin-inducing properties.

II.—THE VARIATIONS FROM CULTURE TO CULTURE OF *C. DIPHTHERIÆ* ON THE SAME MEDIUM, MORE ESPECIALLY WITH REGARD TO TOXIN PRODUCTION.

It is a well-known fact amongst workers in the field of bacteriological research that, after taking precautions to fill out equal amounts of medium into similarly shaped culture flasks of the same size, sterilising by autoclaving in the same way and inoculating with the same number of organisms of a specific toxigenic culture, variations may occur both in the rate of growth and amount of bacillary bodies produced and also in the potencies of the culture filtrates after the same time period. The causes of this variation are but little understood and the difficulty has been overcome by many investigators by taking the average result of the growth of more than one culture to settle any point. Thus Walbum (1922) when investigating the formation of the toxin of *C. diphtheriæ* states, " In spite of absolutely similar treatment, the development and toxin formation in the different flasks may not be the same: in sampling I have taken at least four flasks and mixed them, then filtered the total contents (2800 c.c.). By such a procedure, sources of error are eliminated as the uniformity of the curves shows." Much of the work on the elaboration of bacterial toxins has been done by this type of technique, primarily because of this variation from filtrate to filtrate of the same medium and also because until recently the only methods of testing involved the use of animals and were too tedious to apply to the individual filtrates constituting one large experiment. Impetus in the last few years has been given to work in this field of research by the discovery of Ramon (1922) of an easy and rapid method of testing the potency of diphtheria toxin against antitoxin *in vitro*. Advantage has been taken of this method to test individual culture filtrates during the routine production of toxin over a long period and data from nearly ten thousand separate tests have been accumulated. At a very early stage in the work it became evident that this particular type of variation from culture to culture

could on occasion be very marked. The extent of this variability and some of the contributing causes are here examined.

The media used were those described above :—tryptic digest media, methods 1 and 2, and peptone media. Most of the media had been previously tested for toxin production by the methods described and the possibility of carrying out experiments with media of no value for toxin production was therefore avoided. Care was taken to bulk the media before filling out into culture bottles, to plug them loosely and uniformly, and where groups were being compared to inoculate from the same culture on to the experimental media which were maintained in isothermal positions during growth. The substrain "110" of P.W. 8 was used throughout.

The variation from culture to culture on the same medium is, in our experience, more marked in media made by the tryptic digestion of muscle than in other types although the average strength of the culture filtrates from the former is relatively high. As examples we give two extreme cases of variation that were encountered with these types of media before the method of continuous control was adopted. In each case one large volume of medium was mixed, adjusted to *p*H 8·0 and then filled out into double Winchester quart bottles, one litre in each, and the bottles autoclaved together.

Case I. Thirty-four bottles of medium prepared by method 1, adjusted to *p*H 8·0 and autoclaved at the same time, were placed in the incubator as close together as possible. They were inoculated with the substrain 110 and left for 10 days. The temperatures of the cultures were taken at the end of growth ("harvesting temperatures"). Owing to slight variations in the temperature of the incubator, these "harvesting temperatures" were not exactly the true temperatures of growth. At the end of growth, the cultures were treated with 0·5 per cent. pure phenol, filtered after 24 hours and the filtrates tested for *p*H and potency (Lf units per c.c.). Table VII. shows the results obtained. Of the 34 filtrates, 17 had an average value of 24 units per c.c. while 13 had a value of less than 2 units per c.c. The extreme values were 27 and less than 2 units. Only one filtrate contained 27 units per c.c.

TABLE VII.

*An extreme case of variation in p*H *and Lf value from bottle to bottle in one batch of medium prepared by method* 1 *(sterilised by autoclaving at p*H 8·0).

Number of bottles of medium (1868).	Filtrates.		Lf units per c.c.			Temperatures of cultures at time of harvesting.
	*p*H.	Number of bottles.	Max.	Average.	Min.	
34	7·7 to 8·4	17	27	24	18	33° to 35°
	7·2 to 7·7	4	10	8	6	35° ,, 36°
	<7·2	13	...	<2	...	36° ,, 37°

Case II. Eighteen bottles of medium prepared by method 2 and adjusted to *p*H 8·0 were autoclaved together and placed in the incubator. They were inoculated in three sub-batches using the method of control described (above p. 385) *i.e.* every possible factor being rigorously controlled. In all bottles except one, the culture filtrates were acid at the end of growth and contained no appreciable Lf units (4 or less units per c.c.). In the exceptional bottle the *p*H was 8·0 and the filtrate contained 19 units per c.c.

TABLE VIII.

*An extreme case of variation in p*H *and Lf values from bottle to bottle in one batch of medium prepared by method* 2 *(sterilised by autoclaving at p*H 8·0).

Medium.	Filtrates.	Shelf.	*p*H. Sub-batch.			Lf units per c.c. Sub-batch.		
			1.	2.	3.	1.	2.	3.
CE 5	(1)	3	A	A	A	...	4	2
,,	(2)	3	A	A	A	...	...	...
,,	(3)	3	A	A	A	...	...	4
,,	(4)	2	A	8·0	A	...	19	4
,,	(5)	2	A	A	A	...	...	2
,,	(6)	2	A	A	A	...	...	2

A = *p*H < 7·0.

The normal variations with media autoclaved at pH = 8·0.

The results given above illustrate extreme cases of variation. A general impression of the normal variations will be obtained from a scrutiny of some of the tables given already and will perhaps be made more clear from table IX.

Using the method of control described, a batch of digest medium made by method 2 and a batch of peptone medium were investigated over a period of some weeks. The following data are included in table IX. :—

(i.) The arrangement of the cultures in the incubator.

(ii.) The maximum and minimum temperatures during growth for each shelf of the incubator.

(iii.) The *p*H and Lf value of the individual culture filtrates at the end of growth. The cultures were treated with 0·5 per cent. phenol in all cases except the A and B sub-batches of the peptone media: formalin (0·5 per cent.) was added to these and the *p*H was correspondingly lower.

(iv.) The extent of the "bottle variation" of the filtrates (last column). Although the growth and *p*H of the cultures at the end of growth were approximately the same for individual filtrates from the same medium, the *in vitro* measurements of the potency showed variable results. Thus as with the digest medium, although the mean value was 12-14 units per c.c., three filtrates contained only 2 units per c.c. and one filtrate 18 units per c.c. In the case of the peptone medium, the mean value was 8-10 units per c.c. although three filtrates contained 4 units per c.c. and one 12 units per c.c.

TABLE IX.

A. *Media made by tryptic digestion of horse muscle (method 2).*
(Total N. 34·7 mgrms. per c.c., of which 24·6 per cent. was amino N).

Temperature of growth.		Bottle.	*p*H. Sub-batches.					Lf (units per c.c.). Sub-batches.					"Bottle" variation.	
Max.	Min.		A.	B.	C.	D.	F.	A.	B.	C.	D.	F.	Lf units per c.c.	Number of bottles.
35·5	29·7	1	8·2	8·2	8·3	8·2	8·2	12	14	10	16	14		
		2	8·2	8·2	8·3	8·2	8·2	2	10	12	10	12		
		3	8·2	8·3	8·3	8·2	8·2	12	2	10	12	14	2	3
													4	1
33·9	28·7	4	8·2	8·2	8·3	8·2	8·2	12	14	12	16	14	8	2
		5	8·2	8·2	8·3	8·2	8·2	14	18	10	14	12	10	5
		6	8·2	8·2	8·3	8·2	8·2	8	12	8	14	16	12	13
													14	13
33·0	29·6	7	8·0	8·2	...	8·2	8·2	2	12	...	12	14	16	3
		8	8·0	8·3	...	8·1	8·2	NF	12	...	14	14	18	1
		9	8·0	8·2	...	8·1	8·2	12	14	...	4	14		

Average per cent. variability (see table XIII.) = 88 per cent.

B. *Media made from 2 per cent. peptone X in bullock's heart extract.*
(Total N. 38·8 mgrms. per c.c., of which 17·4 per cent. was amino N).

			A.	B.	C.	A.	B.	C.		
36·0	30·0	1	7·2	7·4	8·3	10	6	6		
		2	7·2	7·3	8·3	8	8	12		
		3	c.	c.	8·3	c.	c.	8	4	3
									6	5
34·6	29·4	4	7·2	7·3	...	10	8	...	8	6
		5	7·1	7·3	8·3	10	8	6	10	6
		6	7·2	7·3	8·3	10	6	4	12	1
34·1	28·7	7	7·2	7·4	...	10	4	...		
		8	7·2	7·3	...	8	6	...		
		9	7·2	7·3	...	10	4	...		

Average per cent. variability = 67 per cent.

Media sown as sub-batches at weekly intervals.
NF = no flocculation with anti-toxin.
c = contaminated cultures.
A and B sub-batches of the peptone media were treated with formalin instead of phenol.

This experiment was carried out in the early spring months and at this period of the year the temperatures of the incubator show comparatively greater variations than at other periods owing to sympathetic response to relatively wide outside fluctuations: there was for example a variation of as much as 6° C. on one shelf during the course of the experiment. The average values of the filtrates on each of the three shelves were approximately the same. This is in accordance with the general impression received in the early part of the work that, provided the temperature is 36° C. or below, slight temperature differences scarcely affect the potencies of the filtrates. Above 36° C., however, these differences become significant.

Variations of the filtrates as shown by chemical analyses.

Correlated with the variations in the Lf values of the culture filtrates from one medium, variations are found in the hydrogen ion concentration. In extreme cases the variation in *p*H may be marked (see table VIII.). Differences can frequently be shown in such analyses as amino nitrogen determinations. It has been shown by Watson and Wallace (1924) that there is an increase in this type of nitrogen during growth of the bacilli. If a series of cultures be made similarly, analyses of the filtrates after any one period often show small but significant differences.

The factors contributing to this type of variation.

It has been shown that one batch of medium filled out into bottles and inoculated with *C. diphtheriæ* may give culture filtrates containing different amounts of toxin and may show pronounced differences in hydrogen ion concentration and amino nitrogen after growth. Some of the factors possibly contributing to this type of "bottle variation" may now be discussed.

(i.) *The chemical cleanliness of the culture flasks.*—While so little is known of the influence of foreign substances on the growth and metabolism of bacteria, it may be that the presence of a minute amount of some substance will produce a variation in the potency of the filtrates from the same medium. An instance where a small amount of potassium nitrate left in the culture flask inhibited toxin production may be mentioned. A trace of soap will retard growth and toxin production. Fat and grease are also to be avoided since they prevent film formation. In addition to bacteriological sterility, the chemical cleanliness of the culture flask is essential.

(ii.) *Uneven heating effects during autoclaving.*—In the preceding paper, the method adopted for the routine sterilisation of media was by filtering through a Seitz press followed by a short steaming, and it is shown below (p. 406) that this technique usually preserves the value of the media, whereas sterilisation by autoclaving at *p*H 8·0 decreases it. Autoclaving at *p*H 8·0 also seems to increase the variability from filtrate to filtrate of cultures made from the medium. Thus in one experiment, seven batches of digest media prepared by method 2 and adjusted to *p*H 8·0 were each divided into two parts, one of which was autoclaved and the other filtered and steamed. The highest and lowest Lf values of the culture filtrates from each sub-batch of autoclaved and filtered medium were then determined. The difference between these values as a percentage of the highest value was taken as the index of "bottle variation" for the particular sub-batch. This is more pronounced with the autoclaved than with the filtered media (table X.).

TABLE X.

The contribution of the heat-of-autoclaving factor to the bottle variation of filtrates prepared by method 2.

Number of bottles tested.		Lf units per c.c.				Percentage variability.	
		Highest.		Lowest.			
Autoclaved.	Filtered.	Autoclaved.	Filtered.	Autoclaved.	Filtered.	Autoclaved.	Filtered.
5	5	15	22	8	20	47	9
5	5	10	22	6	18	40	18
6	6	14	22	2	16	86	27
6	6	8	14	4	10	50	29
3	3	16	16	12	12	25	25
3	3	8	14	6	14	25	0
4	6	10	14	6	8	40	43
		Average percentage variability . .				45	22

Table XI. shows results obtained with various batches of peptone media (made by dissolving 2 per cent. of peptone X in beef heart extract). Each volume of medium was divided into two sub-batches and treated as before. In this series there is not such a large difference between the variability of the autoclaved and filtered media. This may perhaps be correlated with the fact that although the digest media usually give higher toxin values than "peptone" media, the relative effect of the heat of autoclaving on the latter is less marked (below p. 407).

TABLE XI.

The contribution of the heat-of-autoclaving factor to the bottle variation (X peptone media).

Number of bottles tested.		Lf units per c.c.				Percentage variability.	
		Highest.		Lowest.			
Autoclaved.	Filtered.	Autoclaved.	Filtered.	Autoclaved.	Filtered.	Autoclaved.	Filtered
5	4	13	16	10	12	23	25
6	8	7	7	4	4	43	43
18	8	8	12	4	6	50	50
12	12	10	14	6	9	40	36
15	12	16	17	8	12	50	29
		Average percentage variability . .				41	37

This "bottle variation" is then definitely increased, especially with digest media, by autoclaving at *p*H 8·0. As soon as sterilisation in this way was discontinued and other methods which required the application of less heat adopted, smaller variations among individual filtrates were found, although the complete elimination of variability was by no means effected. In investigating the other possible

contributory factors media which had been sterilised by filtration and short steaming were used exclusively.

(iii.) *Variations in the amount of inoculum.* The routine adopted during the present work has been to inoculate three bottles of medium from one loopful of film. The number of organisms introduced into each bottle has therefore been only approximately the same. Moloney and Hanna (1921) found that medium inoculated with a large number of organisms gave less toxin than the same medium inoculated with a smaller number. We have been unable up to the present to confirm this with the media under review and have been unable to show that slight variations in the amount of inoculum exert very much influence on the degree of bottle variation. For instance, bottles of medium inoculated with an equal number of drops of an emulsion of bacilli showed a similar degree of variation to bottles inoculated with a loopful of film. The inoculum factor is probably of minor importance.

(iv.) *The varying tightness of the plugs in the culture flasks.* Sierakowski and Zadjel (1924) have shown that hermetically sealed cultures of *B. coli, C. diphtheriæ*, etc., irrespective of the original *p*H, ultimately approximate to *p*H 6·8 and these results are attributed to the retention in the culture of carbon dioxide formed by the bacteria. Under these conditions of acidity, the failure of the bacillus to elaborate toxin can be appreciated (Bunker, 1919). Table XII shows the results of a series of experiments where the cultures were grown on media prepared by the prolonged digestion of horse muscle and sterilised by filtration (method 2), all the conditions being kept constant except the type of plug in the culture flask. The following points emerged from these experiments:—

1. "Bottle variation" was eliminated or reduced when the plugs were all of the same sort.
2. The most satisfactory plugs were loosely inserted cotton wool or phenolised longcloth (finely meshed muslin).
3. The employment of tight parchment-covered cotton wool plugs or rubber stoppers introduced a variation and toxin production was markedly inhibited.
4. Removal of the carbon dioxide over the cultures by aeration during growth from flasks with rubber stoppers resulted in better growth and toxin production.

Abt and Loiseau (1925) have demonstrated the production of CO_2 during the growth of *C. diphtheriæ* on Martin's broth. They estimated that more than half of this is freed during growth and the remainder in about equal quantities is dissolved in the medium or combined as bicarbonates. There is probably a tendency for the mixture of carbon dioxide and air over the culture to reach an equilibrium after a short period of growth. When this equilibrium is attained in a stoppered flask no further release of carbon dioxide from the culture can take

TABLE XII.

The growth and toxin production of C. diphtheriæ *in bottles with and without free access of air.*

Media No.	B. 2223.			B. 2226.			B. 2227.			CES 69 C.			B. 2190.		
	Growth.	*p*H.	Lf.	Growth.	*p*H.	Lf.	Growth.	*p*H.	Lf.	Growth.	*p*H.	Lf.	Growth.	*p*H.	Lf.
Cotton wool plug	+ + +	8·0	13	+ + + +	8·6	11	+ + + +	8·4	14	+ + +	7·2	6	+ + +	6·4	12
	+ + +	7·9	13	+ + + +	8·5	9	+ + + +	8·4	14	+ + +	7·2	6	+ + +	6·4	12
	+ + +	8·0	13	...	...	...	+ + + +	8·5	14	+ + +	7·2	4	+	...	...
Phenolised longcloth	+ + +	8·0	13	+ + + +	8·5	11	+ + + +	8·0	14	...	...	...	...	...	...
	+ + +	7·9	14	+ + + +	8·6	10	+ + + +	8·3	14	...	...	...	...	...	...
	+ + +	8·0	14	+ + + +	8·6	9	+ + + +	8·2	14	...	...	...	...	...	...
Tight cotton wool and parchment	+ + +	7·2	8	...	...	...	...	...	...	...	...	...	+ +	6·1	2
	+ + +	7·3	8	...	...	...	...	...	...	...	...	...	+ +	6·0	4
	+ + +	7·3	8	...	...	...	...	...	...	...	...	...	...	...	...
Rubber stoppers	+	7·0	4	...	...	...	+	7·0	4	+ +	5·8	2	+	5·5	0
	+	7·0	4	...	...	...	+	6·9	4	+ +	5·7	2	+	5·5	0
	...	...	...	...	...	...	...	...	...	+ +	5·8	2	...	...	...
Rubber stoppers; CO_2 removed from culture flasks after 5 days	...	...	...	...	...	...	+ + +	7·0	6	...	...	...	...	...	...
	...	...	...	...	...	...	+ + +	7·0	10	...	...	...	...	...	...
	...	...	...	...	...	...	+ + +	7·0	8	...	...	...	...	...	...
Rubber stoppers + capillary tubes	...	...	...	+ + +	6·9	7	+ + +	6·9	3	...	...	...	...	...	...
	...	...	...	+ + +	6·7	3	+ + +	6·9	8	...	...	...	...	...	...
	...	...	...	+ + +	7·0	6	...	...	...	...	...	...	...	...	...

place. Under such circumstances the culture medium remains acid and contains little toxin. If however this equilibrium is disturbed either artificially by the removal of carbon dioxide or by natural diffusion methods (*e.g.* by providing the flask with a loosely inserted plug), then the retention of gas by the culture is prevented and the further production of toxin proceeds. Uniform plugging of the culture flask is essential if the "bottle variation" is to be reduced, and for accurate work it is possible that one layer of finely meshed muslin maintained in position with a rubber band will effect this with the least chance of variability.

(v.) *Slight differences in the temperature of growth.*—Much information has been obtained during the course of the present work on the influence of the temperature of growth on toxin production on these types of media, and it has been found that in some temperature zones very slight differences may affect the toxin production, whereas at other temperatures such differences produce negligible effects. For a period of some months during the study of two types of media (methods 1 and 2) prepared by the tryptic digestion of muscle the actual temperatures of the cultures grown on the three shelves of the incubator were taken at the end of growth ("harvesting temperatures"). Chart II series 1 represents diagrammatically the average value of culture filtrates prepared from media made by method 1. The limits of the "harvesting temperatures" for each shelf during the experiment are given. There is a somewhat steeper slope between the values for the top and middle shelves than between the middle and bottom. A considerable variation was introduced therefore by growing cultures on these three shelves. As soon as this fact was realised the general temperature of the incubator was lowered about 3° by adjustment of the temperature capsule of the regulator. Chart II (series 2) shows observations of a similar kind made during work on a second variation of tryptic digest media (method 2) which were made after the use of media made by method 1 had been discontinued. In this lower temperature zone, the average values for the three shelves were similar and no such differences as existed before the general temperature was lowered could be traced.

While growth in isothermal positions is of importance if the "bottle variation" is to be eliminated, there is a wide zone below approximately 36° where filtrates of similar potency may be found after the same period of growth. In a temperature zone embracing temperatures immediately above and below this critical temperature, differences in the potency of the filtrates may be traced.

(vi.) *The presence of undetected contaminating organisms in the cultures.*—The variable morphology of diphtheria bacilli has long been recognised. Yarisawa (1926) has recently discussed the coccoid form of the bacillus which has frequently been found with the P.W. 8 strain used during the present work but no definite evidence that this

affects the toxin production has been obtained. Instances have been found however where, of three cultures sown from the same "starter" bottle on the same medium, one or more have developed this "coccoid" form while the smears from the other cultures were normal. After one sub-culturing on Lœffler slopes these coccoid forms resume the normal morphology of P.W. 8. The difficulty of reading such smears is enhanced by these occurrences and the possibility of small contaminating organisms being overlooked on mere microscopic

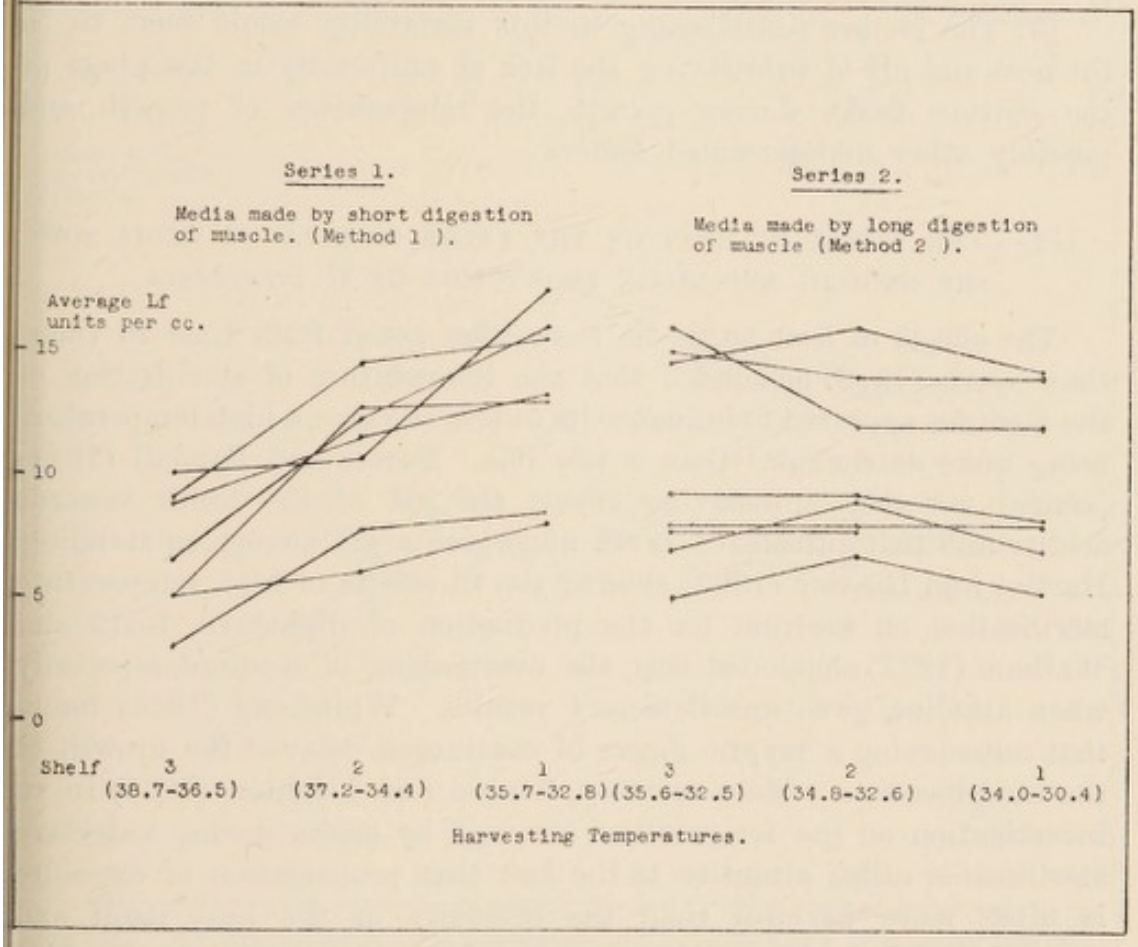

CHART II.—Effect of temperature of growth of *C. diphtheriæ* on the efficiency of media.

examination cannot be altogether excluded. The technique adopted during the investigation of sowing all the media on one shelf from a single culture (above p. 386) enabled the person reading the smears to see at a glance the distribution of the contaminating organisms. Any abnormal Ramon tests were immediately checked by re-submitting the smear from the bottle to further examination. So many cases have occurred where, with preliminary care to ensure purity of culture, variations were found in filtrates from cultures which microscopically were identical that it would seem that only rarely can the variation from bottle to bottle be attributed to the presence of undetected contaminating organisms.

SUMMARY OF PART II.

(1) Variations exist in the growth of the bacillus and in the chemical analyses and potency of the filtrates when culture flasks containing similar amounts of the same medium adjusted to *p*H 8·0 and autoclaved are inoculated with P.W. 8 strain of *C. diphtheriæ*.

(2) These variations with some media may be considerable. Extreme cases are reported where acid filtrates having practically no flocculating value with antitoxin were found alongside alkaline filtrates (*p*H 8·0) with a very high flocculating value.

(3) The factors contributing to this variability would seem to be the heat and *p*H of autoclaving, the lack of uniformity in the plugs of the culture flasks during growth, the temperature of growth and possibly other undetermined factors.

III.—THE EFFECT OF HEAT ON THE VALUE OF CULTURE MEDIA FOR THE GROWTH AND TOXIN PRODUCTION OF *C. DIPHTHERIÆ*.

The effects of heat on media have been noted from time to time; thus Norris (1918) concluded that the temperature of sterilisation of the medium appeared to influence its nutritive value, a high temperature being more detrimental than a low one. Foster and Randall (1921) pointed out that autoclaving affects the *p*H of the broth towards acidity and that autoclaved broth undergoes a *p*H change on standing. Hartley and Hartley (1922) showed the ill effects of high temperature sterilisation on medium for the production of diphtheria toxin and Walbum (1922) concluded that the overheating of medium, especially when alkaline, gave unsatisfactory results. Whitehead (1924) found that autoclaving a tryptic digest of caseinogen delayed the growth of the streptococcus. More recently, Benton and Leighton (1925) in an investigation on the temperature attained by media during autoclave sterilisation called attention to the fact that prolongation of exposure is often more harmful than the intensity of the heat itself and concluded that discrepancies frequently occur owing to irregular heat penetration of material being autoclaved.

The media used during the course of the work were those described above. The general technique for comparing the value of groups of media forming one experiment may be given. Each group was composed of at least three double Winchester quart bottles containing one litre of medium. The groups were arranged in isothermal positions in the incubator and inoculated from the same culture of the bacillus (substrain 110 of P. W. 8). The experiments were controlled by the method of continuous control (above, p. 385). The cultures were loosely plugged with cotton wool and grown for ten days in tryptic digest media and seven days in peptone media. Frequent observations of pellicle formation, appearance of film etc. were made during the period of growth, at the end of which the cultures were treated with formalin (if toxoid was required) or with phenol or toluol (if toxin was required). After twenty-four hours a small sample of each culture filtrate was tested for *p*H and specific principle (Lf units per c.c.) by the method of Ramon (1922) as elaborated

by Glenny and Okell (1924) and Glenny and Wallace (1925). If estimation of the amount of nitrogen in the bacterial bodies (Watson and Wallace, 1924) was required, the whole culture was filtered, washed with physiological saline and subjected to a Kjeldahl determination.

The effect of autoclaving media at pH 8·0.

Following the technique of Hartley and Hartley (1922) media were, during the preliminary part of the work, adjusted to pH 8·0 and then autoclaved, steam being passed through the autoclave for one

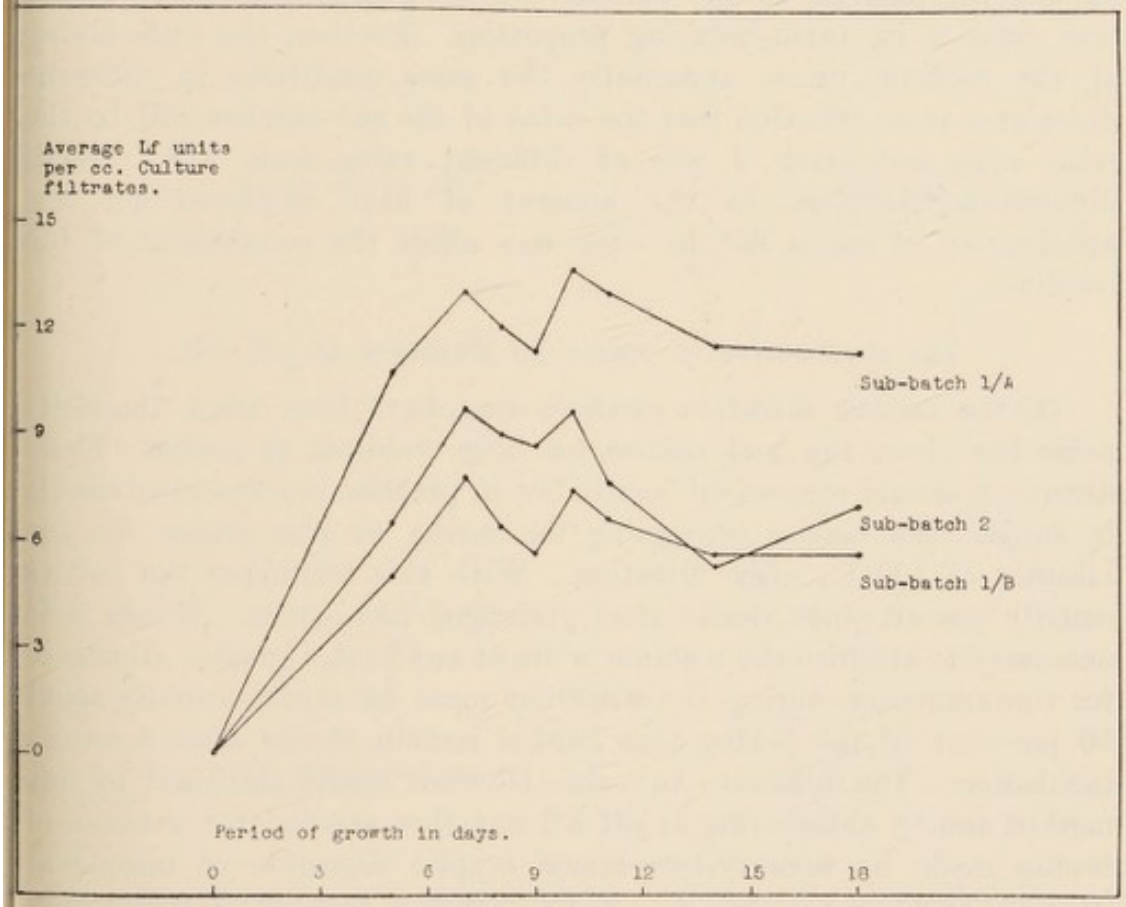

CHART III.—The differences in toxin production on the same batch of medium autoclaved apparently under the same conditions.

hour, the pressure raised slowly to 10 lb. and maintained at this level for thirty minutes. An example of the magnitude of the variation that may be introduced by such a technique when applied to digest media was shown in an experiment designed primarily to determine the period of maximum toxin production on medium made by method 1. Three sub-batches 1/A, 1/B and 2 from one large volume of medium were autoclaved separately. The bottles of 1/A were removed immediately the autoclaving was completed. Batch 1/B was then placed in the same autoclave, *i.e.* the autoclave was warm at the commencement. At the end of sterilisation the door of the autoclave was opened but the bottles were left in overnight—the cooling period being much longer than in the case of the first batch.

Batch 2 was sterilised at the same time in a second autoclave under the same conditions as the 1/A batch. The growth of the bacillus on the groups of medium for the same period was, to the eye, similar for the three batches. The amounts of toxin however were quite different for the three batches, as will be seen from chart III. The points on the curves were obtained by taking the average value of at least five filtrates for each point. It will be seen that the medium which had been placed in the cold autoclave and removed immediately the sterilisation was completed gave the highest value filtrates, whereas by leaving the medium in the autoclave overnight considerable damage was done to its toxin-inducing properties. Further, the autoclaving of the medium under apparently the same conditions in different autoclaves is no criterion that the value of the sub-batches will be the same since sub-batch 2 was of different value from 1/A. Small differences, therefore, in the amount of heat employed for the sterilisation of media can in some way affect the metabolism of the bacillus.

The sterilisation of media by filtration at pH 8·0.

Of the various filtration methods that have been tried, the Seitz press has given the best results for large volumes of media. These filtrations should remove all bacilli, but in practice it is found advisable to subject the bottles containing the media to free steam for ten minutes at 100° C., after filtration. With this technique the bottles usually remain quite sterile after prolonged incubation. Where it is necessary to sterilise the medium without any heat, a greater allowance for contamination during the filtration must be made—usually about 50 per cent. of the bottles thus treated remain sterile after a week's incubation. The difference in value between media sterilised by this method and by autoclaving at pH 8·0 was then studied for a series of broths made by seventy-two hours' tryptic digestion of muscle at room temperature (method 2). Eight different batches of media were each divided into two parts before sterilisation. One part was autoclaved as described above and the other part was filtered through a Seitz press and steamed for ten minutes at 100° C. The value of the media was then tested with P. W. 8 strain in the usual way. The results are shown in chart IV (series A).

The cultures were grown in isothermal positions in the incubator and after ten days were filtered individually and tested for Lf value. Each point on the curve represents the average value of at least five of these individual filtrates. The average Lf value of the eight batches of autoclaved media was nine units per cubic centimetre. That of the filtered steamed media was sixteen units per cubic centimetre. In one case the percentage increase in the value of the filtered over the autoclaved medium was 120. That sterilisation by autoclaving at pH 8·0 may also affect peptone media deleteriously

although to a less extent than tryptic digest media is shown in series B, chart IV. One half of each batch was sterilised by autoclaving and the other half by filtration through the Seitz press and steaming as before. In one exceptional case the autoclaved medium was better than the filtered. It will be seen from the chart that the average value of the digest media is of a higher order than that of the peptone media and that the difference between autoclaved and filtered digest media is more pronounced than between similar types of peptone

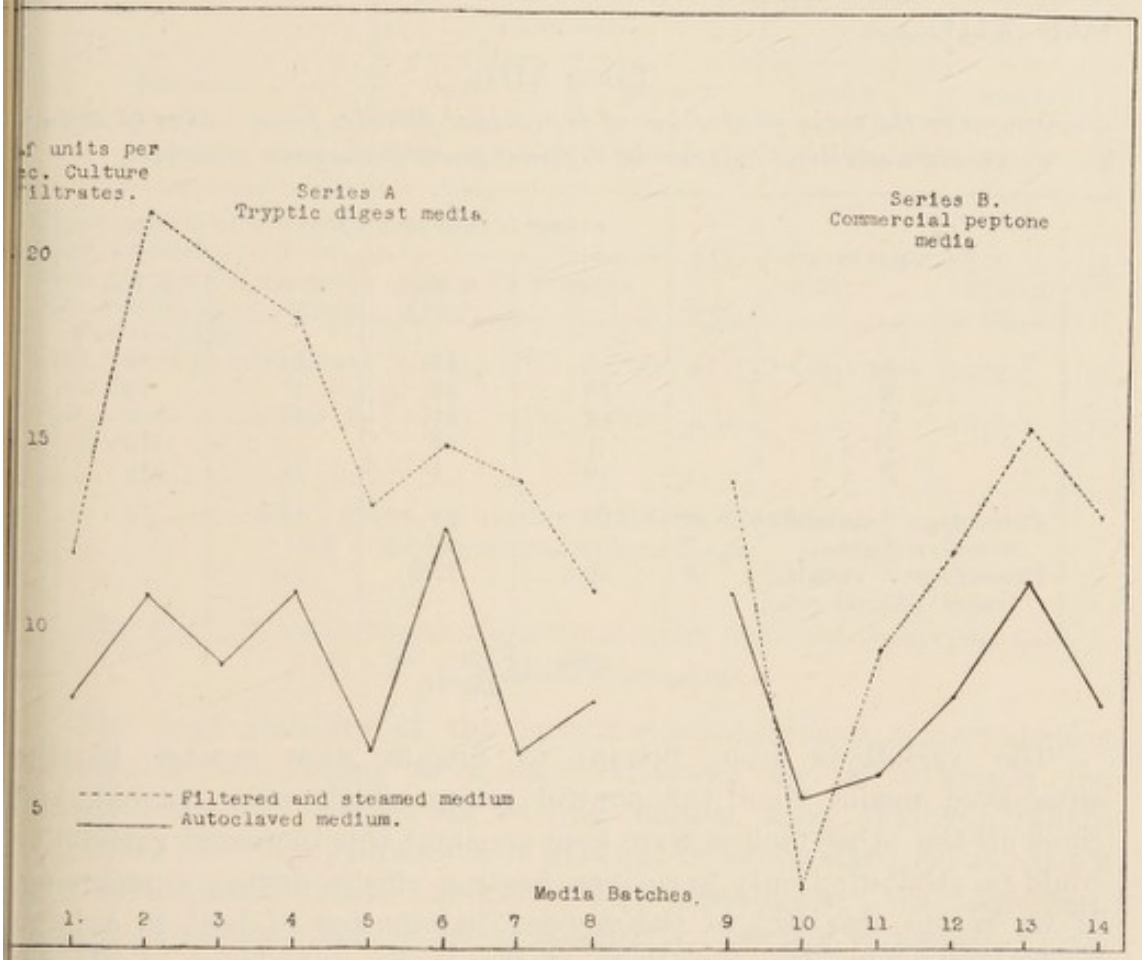

CHART IV.—The relative values of culture media sterilised by (*a*) filtration and (*b*) autoclaving for the production of diphtheria toxin.

media. The average difference in the groups of autoclaved and filtered digest media was seven units per cubic centimetre whereas similar groups of peptone media gave an average four-unit difference. It may be that the heat-labile substances are damaged during the preparation of the commercial "peptones" themselves to a greater extent than those of the digest media. This would explain the relatively greater heat stability of the peptone media and possibly contribute to their lower average value for diphtheria toxin production.

Autoclaving may also increase the variability from filtrate to filtrate of the same medium. Even though a batch of medium is sterilised in one autoclave at the same time the possibility of uneven

heating effects must not be excluded. Although this cannot be demonstrated with every batch of medium, cases have been encountered during the course of the work. Five bottles of digest medium from a large number of preparations were autoclaved in fixed parts of the same autoclave. They were then sown with *C. diphtheriæ* from the same culture in isothermal positions in the incubator. At the end of ten days' growth the cultures were carefully examined for purity and the filtrates tested for their flocculation value with antitoxin. The results of four of these experiments are collected in table XIII.

TABLE XIII.

Variations in the toxin production of individual filtrates from bottles of culture medium sterilised together in different parts of the same autoclave.

Position in autoclave.	Average Lf units per c.c. after 10 days' growth.			
	B. 2231.	B. 2238.	B. 2261.	B. 2265.
A . . .	2	11	14	9
B . . .	10	12	17	10
C . . .	8	14	17	9
D . . .	1	13	...	10
F . . .	10	4	14	10
Percentage "variability"* of autoclaved group	90	71	18·3	10
Percentage "variability" of control filtered group	10·5	10·5	?0	...

* "Variability" $= \frac{(\text{Max. Lf} - \text{Min. Lf}) \times 100}{\text{Max. Lf.}}$

The variations from filtrate to filtrate were greater in the autoclaved media than the control group which were filtered, and since all the other factors were kept constant this increased variability could be attributed only to uneven heating effects during autoclaving. If we accept this fact of the uneven distribution of heat in a large loaded autoclave, the variations of the media autoclaved at *p*H 8·0 can be understood. At this *p*H few media remain quite clear after autoclaving and the precipitation which occurs no doubt contributes to the variation.

*The sterilisation of media at p*H 8·0 *by methods involving increasing amounts of heat.*

The gradual destruction of the toxin-inducing powers of media adjusted to *p*H 8·0 by increasing heat as involved in different methods of sterilisation is shown in table XIV.

There is a grading in the value of the filtrates, the over-autoclaved media being of little or no value. Heat may affect the growth-promoting powers of the media as well as the toxin-inducing properties, though this is not always the case. Growth-promoting "substances"

must be clearly distinguished from toxin-inducing "substances," and this is well shown in some cases of broth autoclaved at high temperature at *p*H 8·0 where growth may be almost normal and yet little or no toxin can be detected in the culture filtrates.

TABLE XIV.

The average Lf units per cubic centimetre of culture filtrates of C. diphtheriæ *prepared from media sterilised by different methods involving gradually increasing amounts of heat.*

Sterilisation.	Digest media.				Peptone media.			
	Method 1.		Method 2.		Method 1.		Method 2.	
	1955.	CEE 8.	B. 2224.	B. 2232.	BH 1.	B. 2239.	B. 2249.	B. 2253.
Candling . . .	5·9 (26)	...	...	...	...	...	...	...
Seitz filtered . . .	...	...	14 (2)	8 (2)	...	9 (2)	14 (5)	6·5 (3)
Seitz filtered and steamed	...	16 (6)	...	...	9·3 (6)	...	15 (2)	6·0 (5)
Steamed on three successive occasions	2 (9)	...	6 (4)	6 (3)	...	...	16 (5)	5·5 (2)
Autoclaved 30 minutes at 10 lb.	0 (58)	12 (8)	NF (4)	4 (4)	7·6 (6)	0 (3)	14 (8)	4 (4)
Autoclaved 60 minutes at 10 lb.	0 (13)	4 (9)	NF (3)	0 (3)	...	...	10 (8)	1 (3)

( ) shows the number of filtrates examined.
NF = no flocculation with antitoxin.

The effect of autoclaving digest media at different hydrogen ion concentrations.

The heat stability of the growth-promoting and toxin-inducing substances is affected to a marked extent by the hydrogen ion concentration of the media. Using animal tests Walbum (1922) concluded that toxin formation is greatest in broth sterilised at *p*H 6·3 and subsequently made alkaline before inoculation. We have been able to investigate this effect by a slightly different technique. Four batches of digest media were each divided into a series of sub-batches and each sub-batch adjusted to a definite hydrogen ion concentration. The sub-batches were then sterilised by autoclaving, in two the minimum autoclaving to produce sterility being given (20 minutes at 115° C.) and in the others high temperature sterilisation (30 minutes at 126° C.) being employed. After autoclaving the *p*H was readjusted to 8·0 and the various sub-batches carefully filtered through a Seitz press into culture bottles which were incubated without further treatment. A proportion of the bottles were not sterile and these were discarded. The remainder were inoculated with P.W. 8 strain of *C. diphtheriæ* and the results of the experiment are collected in table XV.

These results throw light on several points. It is evident that the acid and alkaline "precipitation" zones of culture filtrates (Watson and Langstaff, 1926) are also normally a feature of media, heavy

precipitates being formed at *p*H 4·0 to 5·0 and ca. 10·0. Further, the bacillus can grow and elaborate toxin without the acid precipitate. The hydrogen ion concentration at which digest media are most stable varies within narrow limits but usually within the zone 4·0 to 6·0. In

TABLE XV.

*Growth and toxin production on digest media adjusted to p*H 8·0 *after subjecting to minimum and high temperature autoclave sterilisation at various hydrogen ion concentrations.*

*p*H of autoclaving.	Precipitate after autoclaving.	No. of filtrates.	Growth.	Lf units per c.c.		Analysis of typical isothermal culture (toluolised).		
				Av.	Max.	Bacterial N.	M.R.Ds.*	Lf.
GROUP I.—*Minimum temperature sterilisation*: 20 *minutes at* 115° *C.* (10 lb. pressure).								
Series 60 *A*—						mgrms.	c.c.	
5·0	+ + +	4	+ + + + +	12·0	14	160	1/160,000	12
6·0	+	4	+ + + + +	13·0	14	...	1/200,000	14
7·0	...	6	+ + +	4·0	6	133	1/80,000	4
8·0	+	3	+ + +	5·0	6	157	1/80,000	5
10·0	+ + +	4	+ + +	0·0	0	117	1/300	0
Series 60 *B*—								
4·0	+ + + +	4	+ + + + +	8·25	10	...	1/120,000	10
5·0	+ + +	2	+ + + +	4·5	5	...	1/70,000	5
6·0	+	4	+ + +	6·0	7	...	1/50,000	2
7·0	...	2	+ + +	0·0	0	...	1/4,000	0
8·0	+	5	+ + +	0·0	0	...	1/500	0
10·0	+ + + +	5	+ + +	0·0	?1	...	1/200	?1
Control (Seitz filtered at *p*H 8·0)	...	6	+ + + +	8·7	12	...	1/150,000	...
GROUP II.—*High temperature sterilisation*: 30 *minutes at* 126° *C.* (20 lb. pressure).								
Series 80—								
4·0	...	4	+ + + +	8·8	12	...	...	...
6·0	...	2	+ + +	6·5	7	...	...	...
8·0	+ +	2	+ +	0·0	0	...	...	...
10·0	+ + +	2	+	0·0	0	...	...	...
Control (Seitz filtered at *p*H 8·0)	...	3	+ + +	8·7	9	...	...	...
Series 80 *A*—								
4·0	+	2	+ + + +	13·0	14	...	...	...
5·0	...	4	+ + +	11·5	12	...	...	...
6·0	+	5	+ + +	12·0	15	...	...	...
8·0	+	4	+ + +	N.F.	...	...	...	...
10·0	+ + +	4	+	0·0	...	...	...	...
Control (Seitz filtered at *p*H 8·0)	...	5	+ + +	10·0	11	...	...	...

N.F. = No flocculation with antitoxin.

* = The smallest amount of toxin which when introduced intracutaneously into a guinea-pig causes a typical reaction.

this zone it is safe to autoclave many media even for periods of 30 minutes at as high a temperature as 126° C. without injury to either growth-promoting or toxin-inducing substances: with greater alkalinity there is usually destruction of some essential substances and at

*p*H 10·0 this may be severe. Peptone media show the same general behaviour but the results are not always so clear cut: the greatest destruction however takes place at *p*H 10·0. Although the racemisation of the protein or other food substance may to a great extent account for this, it frequently happens that the precipitate produced in the alkaline zone (*p*H 8·0 to 10·0) will, if returned to the filtered autoclaved medium, restore in part its growth-promoting and toxin-inducing properties, as the following experiment shows. Eight litres of peptone media were sterilised by steaming on three successive days at *p*H 10·0 and the precipitate collected and suspended in 250 c.c. of water, the *p*H was adjusted (with dilute acid) to *p*H 8·0 and approximately a quarter of this was then added to each of three of the six bottles containing the precipitate-free medium (readjusted before Seitz filtering to *p*H 8·0). The results on growth and toxin production on these media are collected in table XVI.

TABLE XVI.

The precipitated growth-promoting and toxin-inducing substances of medium autoclaved at *p*H 10·0.

Series 2304. Filtrate.	A. Precipitate-free media.		B. Precipitate-free media + precipitate.		C. Control. Autoclaved *p*H 4·0.	
	Growth.	Lf. units per c.c.	Growth.	Lf. units per c.c.	Growth.	Lf. units per c.c.
1	+	N.F.	+ + +	6	+ + + +	14
2	+	N.F.	+ + +	4	+ + + +	16
3	+	N.F.	+ + +	4	+ + + +	14

N.F. = no flocculation with antitoxin.

The growth-promoting properties were to a large extent restored and some toxin was found in solution. One of the constant constituents of this precipitate seems to be inorganic phosphate and it may be that the disturbance of the phosphate level is one of the factors prejudicing the value of media autoclaved at *p*H 10·0. This however will require confirmation. We have never encountered a broth which did not suffer some injury from autoclaving at *p*H 10·0 although the degree of injury varies with different media. It is apparent that some destruction may be caused by autoclaving at any *p*H above 4 to 6 and it is difficult to reconcile with this the practice of some workers (*e.g.* Abt and Loiseau, 1922) of disregarding this point unless their particular type of medium is exceptional in its behaviour on heating in the alkaline state.

These facts have all been determined for broths completed except for sterilisation. The initial stages of media making also involve heating and probably the most satisfactory culture media both for

promoting growth and inducing toxin production can be made by limiting the amount and duration of heat at all stages including sterilisation, and, where it is necessary to heat at all, ensuring that the *p*H of the mixture is within the limits 4 to 6.

Summary of part III.

1. An important factor influencing the value of culture media for the growth and toxin production of *C. diphtheriæ* is the amount of heat to which they have been subjected during preparation and sterilisation.

2. The growth-promoting "substances" are to be distinguished from the toxin-inducing "substances."

3. The heat stability of the toxin-inducing substances is influenced by the reaction of the media. They are very labile to heat at *p*H 10·0 and suffer some injury in many cases by moderate autoclaving at any zone above *p*H 4 to 6. In this zone they are more stable. At *p*H 8·0 the destruction is usually greater the more the medium is heated during sterilisation: at high temperatures complete destruction may occur.

4. The precipitate produced by heating media at *p*H 10·0 usually possesses growth-promoting and toxin-inducing properties.

5. Equal quantities of the same medium (*p*H 8·0) filled out into similarly shaped culture flasks and sterilised together in the same autoclave may show different values for toxin production. This is attributed to uneven penetration of heat in different parts of the same autoclave.

6. The toxin-inducing and the growth-promoting substances of media adjusted to *p*H 8·0 are preserved if sterilisation is carried out by filtration through a Seitz press followed by a short steaming.

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