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In those laboratories in which the preparation of therapeutic sera is carried out on a large scale, the uncertainty of producing satisfactory antigens for use in the various processes is often a source of anxiety. With regard to the preparation of diphtheria toxin, it has been the experience of all bacteriologists who have worked at the subject for any length of time that periods of success and failure have alternated with bewildering frequency, in spite of the fact that, as far as could be ascertained, the same working conditions were maintained even to the minutest detail. Although many problems concerned in the preparation of diphtheria toxin still remain unsolved, yet during the past three years important progress has been made, and there are good grounds for the belief that high-grade toxin may be prepared with a greater degree of certainty now than formerly. Much of the recent work on the subject has been done in American laboratories, and in the following pages an account of our work is given.

As a result of preliminary trials and experiments, a routine method for the preparation of medium for the special purpose of toxin making was evolved. This medium yielded high-grade toxin from the first, and accordingly the method was adhered to rigidly, without conscious variation, throughout the work. The first success was obtained in November 1919 and a total number of 44 toxins, the volume of which amounted to 1012 litres, were prepared during the following six months. Preparation of toxin in large amounts ceased in May 1920, as sufficient had then been accumulated to meet the immediate requirements of the laboratory.

In Table I. the results of this six months' work are summarised.

This series contains all the toxins made during the period stated. The medium was always prepared in the same way, and strain Park Williams No. 8 was used throughout the work. Almost one-half of this toxin had a minimum lethal dose ranging from 0·002 to 0·001 c.c., and of the remainder all except 70 litres—or about 7 per cent. of the total—had a minimum lethal dose which varied from 0·005 to 0·0023 c.c.

* The substance of this paper was communicated to the Meeting of the Pathological Society of Great Britain and Ireland, at Cambridge, July 1, 1920.

TABLE I.

Showing results of diphtheria toxin production from November 1919 *to May* 1920.

Number of Toxins.	Volume of Toxins.	Minimum Lethal Dose.	Percentage of Total Volume of Toxin made.
	Litres	c.c.	
3	33	0·001	3
17	446	0·002 – 0·0011	44
13	225	0·003 – 0·0021	22
7	183	0·004 – 0·0031	18
2	55	0·005	5
1	35	0·0055	3
1	35	0·0075	3
Totals 44	1012		

By arranging the results in a slightly different way (Table II.), it is possible to compare the above figures with those obtained by Dean (1908 [4]) and Davis (1920 [3]).

TABLE II.

Comparison of the results obtained by Dean (1908) *and Davis* (1920) *and the authors.*

M.L.D. c.c.	Dean, Series 1, 1899-1902.		Dean, Series 2. 1904-1905.		Davis, 1920.		P. and O. M. Hartley.	
	No. of Toxins.	Per cent.	No. of Toxins.	Per cent.	No. of Toxins.	Per cent.	No of Toxins.	Per cent.
1/1000	0	...	0	...			3	7·0
1/800	0	...	0	...			1	2·2
1/700	0	...	1	2·6			1	2·2
1/600	0	...	1	2·6		43·0	6	14·0
1/500	2	2	8	20·5			9	20·0
1/400	14	15	9	23·1			10	23·0
1/300	17	18	7	18·0		35·5	10	23·0
1/200	17	18	7	18·0		11·3	3	7·0
1/100	21	22	1	2·6		10·2	1	2·3
>1/100	16	17	...	...		...	...	...
"other toxins"	7*	8	5†	13·0		...	...	...
Totals .	94	...	39	...	Not stated	...	44	...

* M.L.D. not accurately determined, but greater than 1/300 c.c.
† M.L.D. not accurately determined, but greater than 1/200 c.c.

Dean used a 2 per cent. solution of Witte's peptone in beef infusion, made the broth (while still hot) neutral to litmus paper and then added 7 c.c. of normal caustic soda for every litre. Davis used a 2 per cent. solution of Parke Davis Bacteriologic Peptone in beef infusion, the reaction being adjusted to $P_H = 8·0$ to 8·2. We used a method differing only in detail from that of Davis. The strain employed in the four series of results analysed in the above table was Park Williams No. 8

Method.

1. *Preparation of the Infusion.*—Horse muscle, freed from fat and large vessels was minced and weighed. For each preparation of medium from thirty to sixty pounds of minced muscle were used, x pounds of muscle being soaked overnight in x litres of tap water in a jacketed cauldron (capacity 100 litres). Next morning, steam was passed through the jacketing until the temperature reached 95° C. This infusion, after cooling slightly, was filtered through paper pulp, the clear filtrate then returned to the cleaned-out cauldron and the reaction adjusted to $P_H = 8$.

2. *Preparation of Peptone Broth.*—2 per cent. Parke Davis Bacteriologic Peptone and 0·5 per cent. common salt were dissolved in the warm infusion and the reaction carefully adjusted to $P_H = 8$, the amount of alkali required being determined on a sample cooled to room temperature.

3. *Distribution of the Medium into Containers.*—Throughout this work double Winchester quart bottles (capacity 5 litres) previously sterilised, were used as containers, 1 litre of medium being placed in each. Occasionally, larger bottles (capacity 24 litres, holding 5 litres) were employed, but they are not so convenient as the former and their use is not recommended.

4. *Sterilisation of the Medium.*—Steam was passed freely through the autoclave, which accommodates about forty double Winchester quart bottles, for one hour, after which the pressure was raised slowly to 10 lbs. (time, about thirty minutes), and maintained at this level for half an hour.

5. *Chemical Analysis of the Medium.*—The bottles were placed on their sides in the incubator for two days before being inoculated. One bottle was then removed and the following estimations carried out :—(*a*) The P_H was determined ; this was usually found to be about 7·8. (*b*) Total nitrogen was determined by Kjeldahl's method. (*c*) Amino-nitrogen was determined by Sörensen's (1908 [10]) method and by Van Slyke's (1913 [12]) method. (*d*) Proteose nitrogen was determined by Hedin's (1904 [7]) method.

6. *Care of the Strain.*—Park Williams No. 8 was used throughout. This was subcultured every two days on to "starter" bottles (100 c.c. quantities of the above medium in 500 c.c. bottles). The strain was also maintained on Loeffler slopes, subcultures being made every fourteen days.

7. *Growth of the Organism, Film Formation, etc.*—Each preparation of medium was inoculated from 48-hour old "starter" bottles, a piece of pellicle being carefully floated on to the surface of the fluid in each bottle. On this medium a thin but complete pellicle was generally formed at the end of twenty-four hours, but it was not quite uniform, small white islands or patches of thicker film being scattered over the surface. After forty-eight hours the film was thicker, firmer, and more uniform, and was found to be creeping up the sides of the bottle. Frequently festoons or curtains were seen descending into the clear fluid beneath. At the end of three days these festoons had become larger, and the surface of the film crinkled and often cracked.

8. *Period of Growth and Sterilisation of the Culture.*—When litre quantities of medium were used, the bottles were removed from the incubator after seven days' growth. It is important that this time should not be exceeded, as it was found that when Parke Davis Bacteriologic Peptone was used, the toxin usually attained a maximum value about the seventh day, and there was marked deterioration on prolonged incubation at 37° C. Prior to removal from the incubator, a smear was made of the growth in each bottle and examined. 0·5 per cent. pure carbolic acid was then added, and the sterilised culture was allowed to stand for at least twenty-four hours. Filtration was effected through paper pulp. As a rule, the filtrates were sterile. Samples which failed to pass the sterility test were candled.

9. *Determination of Toxicity.*—The intracutaneous method described by

Römer and Sames (1909 [9]) and developed by Glenny and Allen (1921 [6]) in this laboratory was used. As our colleagues have shown, this method is susceptible of very great accuracy. The approximate value of the toxin having been ascertained, the minimum lethal dose was determined in the usual way, and these M.L.D. values are collected and analysed in Tables I. and II. The reaction (P_H) of each toxin was also determined.

The use of double Winchester quart bottles for routine work is described, because this type of container (which has been in use in these laboratories for many years) has proved to be by far the most satisfactory. Its advantages are many. Laid upon its side, a maximum surface of medium, in relation to the volume used, is provided for film growth, and efficient aeration is secured. The bottles may be stacked in tiers in the incubator with consequent saving of space. The process of inoculation is simpler, and the risk of contamination is less with these bottles than with any ordinary type of flask, as entrance is made from the side and not from the top. Against the use of these vessels as containers, it is urged that the breakage rate is high. This is true unless the heating and cooling are carried out with care. The autoclave used is of the boiler type and is fitted with a wooden floor. In loading, care is taken that no bottle should touch its neighbour, the heating is effected slowly, and slow cooling is secured by leaving the bottles in the autoclave overnight. Adopting these precautions, the breakage rate is very small. Davis ([3]) and Bunker (1919 [1]) both use bottles as containers, though of a somewhat different type, and these authors draw attention to the advantages they possess.

The production of diphtheria toxin depends upon a number of different factors. This was emphasised by Dean and more recent workers (Davis and Ferry (1919 [2]), Bunker [1]) have insisted on the importance and interdependence of the numerous factors concerned, a view with which we are in complete accord. Of the factors concerned, the following may be mentioned:—(*a*) the strain; (*b*) the nature and quality of the peptone; (*c*) the initial reaction of the medium; (*d*) the temperature to which the medium is heated to secure sterilisation; this has an important bearing on those constituents which are supplied by the muscle; (*e*) the volume of broth inoculated—this determines, to a certain extent, the period which should be allowed for growth. There are other factors, but the importance of those mentioned has been demonstrated by experiment and confirmed repeatedly during routine work. In our experience, it is only by meticulous attention to detail throughout the whole process of toxin making that even comparatively constant results can be expected, and it is for this reason that such a full description of the method employed has been given.

Experimental.

The method described above was based upon a good deal of experimental work, a brief summary of the results of which is given below.

(a) *The Strain.*—Park Williams No. 8 proved to be the most satisfactory strain, and its toxicogenicity, as compared with other strains, is shown in Table III.

TABLE III.

Illustrating toxicogenicity of different strains of B. diphtheriæ.

Strain.	Experiment I. M.L.D.	Experiment II. M.L.D.	Experiment III. M.L.D.
	c.c.	c.c.	c.c.
Park Williams No. 8 .	0·0012	0·0012	...
78	0·0015	0·0018	...
79 G	0·0020	0·0023	...
82	0·0040	0·0040	...
Black	...	...	0·05
Swindon	...	...	0·006
Phillips	...	...	0·007
Evans	...	...	0·009

Strains 78, 79 G and 82, received from other laboratories at different times, had been maintained on artificial medium for many years. Davis carried out similar experiments and found that different cultures of the bacillus, all derived from Park Williams No. 8, exhibited decided variations in toxicogenicity, the L+ dose varying from 0·25 c.c. to more than double this value.

In Experiment III. the strains studied had been recently isolated from cases of diphtheria. The results illustrate the wide variation in toxin-producing power of different strains of the bacillus, and support the view of Davis that it is important to verify the toxicogenicity of the strain of the organism employed.

(b) *The Peptone.*—It has always been recognised that "peptone" or some constituent of the substance known by this name, is essential for the production of high-grade toxin. For many years, Witte's peptone was used almost exclusively. Latterly, many others have been available and some of these were investigated from the point of view of their value for toxin production. The toxins were tested by the intracutaneous method, the "skin test dose" or "$L_r/500$ dose"* being determined. This may be defined as the smallest volume of toxin which, when mixed with $\frac{1}{500}$ of a unit of antitoxin produces a typical positive reaction in the skin of a guinea-pig, the reading being taken about forty hours after injection.

N and P (Table IV.) were peptones prepared by the authors in the laboratory. The remainder were supplied by eight different manufacturing firms, and in five cases 2 samples, and in one case 3 samples, received at different times were studied.

The toxins obtained in the second experiment (17 samples) were less potent than those obtained in the first experiment (9 samples), but the order of value of the peptones was nearly the same in the two cases.

It is evident, therefore, that the nature and quality of the peptone used for toxin production is an important factor. These results show that some varieties of peptone yield high-grade toxin, while others under precisely the same condi-

* The convention used by Glenny and Allen (1921) was "$L_n/500$," but these authors have since discarded this convention and use "$L_r/500$," since "L_n" had been used previously in the German literature to indicate the minimal dose of toxin which, when injected, without antitoxin, intradermally into a guinea-pig, would cause a reaction. This amount of toxin is termed M.R.D.

In the expression $L_r/500$, "L" is used to indicate that a mixture of toxin and antitoxin is used, as in Ehrlich's conventions L_o and L_+, and "r" is used to indicate that a positive reaction is produced.

tions yield little or none. Further, different samples of peptone received from the same source at different times vary in quality. This was a disturbing fact, and led to the search for a laboratory-made peptone for the purpose of toxin-making. An account of the work on this subject by one of us is given in a subsequent paper (*vide* p. 479).

TABLE IV.

Illustrating the relative value of different peptones for the production of diphtheria toxin.

Peptone.	Toxin, "Skin Test Dose, or $L_r/500$ Dose."	
	Experiment I.	Experiment II.
	c.c.	c.c.
A1	0·1	0·1
A2	>0·1	0·1
B	0·01	0·01
C1	0·008	0·006
C2	>0·1	no growth
D	...	0·02
F1	0·001	0·002
F2	...	0·02
F3	...	>0·1
H1	0·00012	0·0006
H2	...	0·005
K1	...	0·0015
K2	0·0005	0·0008
L1	0·0004	0·0012
L2	...	0·01
N	...	0·001
P	...	0·0008

Other experiments were carried out (Table V.) to study the effect of varying the amount of peptone. Good results had been obtained by using 2 per cent. solutions in meat infusion, and the inquiry was instituted to see whether any economy might be effected by using smaller quantities. At the same time, and for similar reasons, the effect of varying the amount of meat infusion was investigated. As before, the $L_r/500$ dose was first determined and then the $L_r/50$ dose (by testing the toxins against $\frac{1}{50}$ of a unit of antitoxin).

TABLE V.

Illustrating the effect, on toxin production, of varying the amount of peptone and infusion.

Composition of the Medium.		Toxin Values.	
Infusion.	Peptone.	$L_r/500$.	$L_r/50$.
		c.c.	c.c.
Full amount	2 per cent.	0·0002	0·0018
,, ,, ...	1·5 ,,	0·00025	0·0023
,, ,, ...	1·0 ,,	0·0005	0·0048
,, ,, ...	0·5 ,,	0·002	0·018
,, ,, ...	0·2 ,,	>0·1	...
,, ,, ...	nil	>0·1	...
3/4 of full amount . . .	2 per cent.	0·0002	0·0022
1/2 ,, ,, . . .	2 ,,	0·0003	0·0028
1/4 ,, ,, . . .	2 ,,	0·00045	0·0045
1/10 ,, ,, . . .	2 ,,	0·00045	0·0048
Nil	2 ,,	>0·01	...

From the results of these experiments, it appeared that no useful purpose would be served by reducing the concentration of either peptone or infusion, and accordingly the quantities used in routine practice were as stated in the description of the method given above.

The results illustrate the well-known fact that although the diphtheria bacillus will grow either on peptone alone or on infusion alone, no toxin is formed in either case. The simultaneous presence of each is necessary for the formation of toxin. Davis and Ferry have studied this question much more thoroughly than we have done, and they have shown that certain amino-acids and "accessory factors probably of a vitamine character" are essential for toxin production. In practice, the former are supplied by the peptone and the latter by the meat infusion.

(c) *The Initial Reaction of the Medium.*—Bunker,[1] Davis and Ferry,[2] Davis[3] and Dernby and David (1921[5]) investigated the effect of varying the hydrogen-ion concentration of the medium on the potency of the resulting toxin. Bunker found that the bacillus would grow on medium, the initial reaction of which lay between P_H 5·7 and P_H 8·7, and that when growth was started it (*i.e.*, the reaction) might pass either of these limits. He regards an initial reaction of P_H 7·3 to 7·5 as the most favourable for the development of the heaviest pellicle. Dernby and David observed growth on media, the initial P_H of which varied between 6 and 8·3; they consider an initial reaction of P_H 7·2 to 7·6 as most favourable for toxin production. Davis states that potent toxin is produced only when the initial reaction falls between P_H 7·2 and 8·3 with an optimum of P_H 8·0 to 8·2.

The results of three experiments are given in Table VI. The P_H values were determined just before the flasks were inoculated: the toxins were tested by the intracutaneous method against $\frac{1}{50}$ of a unit of antitoxin, the $L_r/500$ dose having been previously ascertained.

TABLE VI.

Illustrating the effect of varying the initial reaction of the medium on toxin production.

Experiment I.		Experiment II.		Experiment III.	
Initial Reaction of Medium.	Toxin, $L_r/50$.	Initial Reaction of Medium.	Toxin, $L_r/50$.	Initial Reaction of Medium.	Toxin, $L_r/50$.
P_H.	c.c.	P_H.	c.c.	P_H.	c.c.
9·2	>0·1	...	...	9·35	no growth
8·7	0·025	8·5	0·003	8·9	0·0028
8·2	0·0058	8·2	0·0018	8·4	0·0024
7·8	0·0048	7·8	0·0015	8·0	0·0020
7·35	0·0075	7·3	0·0018	7·6	0·0020
6·8	0·012	6·6	0·0018	7·0	0·0020
6·4	>0·1	6·4	0·002	6·6	0·0022
6·1	>0·1	6·1	>0·1	6·1	0·01

In Experiments I. and II., the strongest toxin was obtained from the sample of medium the initial reaction of which was $P_H = 7·8$. In Experiment III., however, the toxins obtained from the samples of media, the P_H of which was 8·0, 7·6 and 7·0 respectively, were of equal value and even when the range was wider, there was little difference in potency. The differences were small in some cases, but could be measured by the intracutaneous method of testing.

(d) *The Infusion.*—Throughout this work, the infusion basis has been made from horse flesh, and in this respect our method differs from those employed by

other workers. The use of horse flesh was introduced in these laboratories during the war as a measure of economy, and in our work has yielded such satisfactory results that we have confined ourselves to its use and have not studied infusion made from beef or veal. Neither has it been found necessary to remove the fermentable substances occurring in muscle extract by a preliminary treatment with *B. coli*, as advocated by Theobald Smith (1899 [11]) and practised by Bunker, nor with yeast (Dernby and David). Davis [3] could find no advantages of veal over beef; he obtained equally satisfactory results with fresh meat or meat from cold storage, and regards the preliminary treatment with *B. coli* as entirely superfluous. This worker's experience is based upon the production of thousands of gallons of toxin, the L_+ dose of 90 per cent. of which was 0·5 c.c. or less. The method described in this paper resembles that of Davis very closely, and although we can only speak of the preparation of rather more than a thousand litres, yet the whole of this was of value for immunising purposes and most of it of high-grade quality. It is probable that the importance of the infusion lies in those constituents which Davis and Ferry regard as "accessory factors, probably of a vitamine character," and these occur in beef, veal, and horse flesh. Our attention to the significance of some such constituents of the infusion—substances relatively sensitive to heat—was first aroused when we failed to reproduce our early small-scale laboratory results when working on a large scale in the media kitchen. Investigation showed that media, which had been prepared in exact accordance with the method worked out in the laboratory, were being sterilised by heating for one hour at a pressure of 22·5 lbs. (129° C.). On such media the organism grew well, but practically no toxin was formed. Excessive heating was discontinued, and the very next sample of medium, sterilised by heating at 10 lbs. pressure for thirty minutes, yielded toxin the minimum lethal dose of which was 0·001 c.c.

Table VII. records a typical experiment carried out to investigate the action of heat on media destined for toxin-making. Equal volumes (2 litres) were used and the toxins were tested by the intracutaneous method.

TABLE VII.

Illustrating the effect of heat on media used for toxin preparation.

Sterilisation.	Toxin, $L_T/500$.	Other Toxin Tests.
	c.c.	c.c.
1. Heated at 100° C. for one hour on three successive days . . .	0·0002	...
2. Heated at 10 lbs. pressure for thirty minutes	0·0001	M.L.D. = 0·001. L_+ dose = between 0·08 and 0·1.
3. Heated at 100° C. for one hour on three successive days; then heated at 10 lbs. pressure for thirty minutes	0·0003	...
4. Heated at 22·5 lbs. pressure for one hour	>0·1	...

Thus, prolonged heating at a high temperature completely ruined an otherwise perfectly satisfactory medium. It may be mentioned that growth and film formation were practically the same in all four cases.

Davis [3] points out the detrimental effect of over-heating, and recommends that the sterilisation period of the medium should be as short as possible. Martin (1898 [8]) recommends sterilisation of his medium by filtration. Davis also points out that re-sterilisation is detrimental. On one occasion during this work, a doubt (which subsequently proved to be unfounded) was felt as to the sterility of

a particular preparation of medium ; a few bottles were retained as controls and the rest heated again at 15 lbs. pressure for thirty minutes. All the bottles were inoculated at the same time from the same "starter" bottle and the toxins tested. The control (autoclaved once, at 10 lbs. for thirty minutes) yielded toxin the minimum lethal dose of which was 0·0045 c.c.; the remainder (autoclaved twice (1) at 10 lbs. for thirty minutes, and (2) at 15 lbs. for thirty minutes) gave toxin the minimum lethal dose of which was 0·05 c.c.

Numerous other experiments have been carried out, but the results at present do not furnish a complete explanation of the effect of heat on media. Prolonged heating is detrimental and from the point of view of toxin production may be disastrous. Throughout the whole process of preparing media for toxin production, the ingredients should be subjected to as little heat as possible.

The results (Table I.), though satisfactory from the point of view of practical immunisation (according to present-day standards) yet show considerable variation. Thus, the minimum lethal dose varies from 0·001 c.c. to 0·0075 c.c. On theoretical grounds, since the whole of this toxin was prepared in the same way (that is, under conditions which, as far as one could judge or one could make them, were constant), it was reasonable to expect greater constancy in the results than was actually obtained. Dean, whose experience was very wide, writing in 1907, stated that a uniform high toxicity was not attainable by the use of any of the methods known at that time. It is possible to prepare toxin to-day which is of higher value than that formerly produced, but the results are still very variable (Table II.). At present, one can only speculate as to the cause of this. In the first place, the quality of the horse flesh supplied is beyond laboratory control and different consignments may yield different amounts of those muscle extractives which play such an important part in toxin production. Another possible cause may arise from the fact that when toxin is produced on this medium, a maximum potency is attained, after which deterioration occurs. This maximum is reached usually, but not invariably, on the seventh day. It is possible, therefore, that some toxins were gathered before the maximum potency had been reached, and others after deterioration had begun (see following paper with special reference to the growth of strain Park Williams No. 8 on media containing Parke Davis Bacteriologic Peptone). Finally, the recent work of Warden (1921 [13]) may ultimately throw light, not only on such variations as are referred to above, but on the whole process of toxin formation. According to Warden, toxin formation is the result of the interaction of certain fatty acids and other constituents of the medium, and he emphasises the colloidal nature of the solutions used in toxin-making. The preparation of colloidal solutions of constant composition and properties is very difficult, and if Warden's claims prove to be well founded, this difficulty may well explain the widely different results which have been obtained, not only by different workers, but by the same investigators striving, above all other things, to secure constancy of working conditions.

REFERENCES.

1. Bunker . . . . . *Journ. of Bacteriol.*, 1919, vol. iv. p. 379.
2. Davis and Ferry . . . *Ibid.*, 1919, vol. iv. p. 217.
3. Davis . . . . . *Ibid.*, 1920, vol. v. p. 477.
4. Dean . . . . . "The Bacteriology of Diphtheria," Nuttall and Graham-Smith, Cambridge, 1908, p. 449.
5. Dernby and David . . *Journ. Path. and Bacteriol.*, 1921, vol. xxiv. p. 150.
6. Glenny and Allen . . *Ibid.*, 1921, vol. xxiv. p. 61.
7. Hedin . . . . . *Journ. Physiol.*, 1904, vol. xxx. p. 155.
8. Martin . . . . . *Ann. de l'Inst. Past.*, 1898, xii. 26.
9. Römer and Sames . . . *Zeitschr. für Immunitätsforsch.*, 1909, iii. 344.
10. Sörensen . . . . . *Biochem. Zeitsch.*, 1908, vii. 45.
11. Theobald Smith . . . . *Journ. Exp. Med.*, 1899, vol. iv. p. 373.
12. Van Slyke . . . . . *Journ. Biochem.*, 1913, vol. xvi. p. 121.
13. Warden, Connell, and Holly *Journ. of Bacteriol.*, 1921, vol. vi. p. 103.

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