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The Value of Douglas's Medium for the Production of Diphtheria Toxin

BY

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THE VALUE OF DOUGLAS'S MEDIUM FOR THE PRODUCTION OF DIPHTHERIA TOXIN.*

By Percival Hartley.

From the Wellcome Physiological Research Laboratories, Herne Hill, London.

Shortly after the outbreak of war, it became evident that Witte's Peptone would soon be unobtainable for bacteriological work. The stocks of this material were limited, the demand for culture media increased enormously, and the discovery of a substitute became a matter of urgent necessity.

Douglas (1914 [10]) described a simple method for the preparation of nutrient broth which was widely adopted. Although evolved in the first instance as a war emergency measure, and perhaps regarded as such, the method then introduced of preparing media by the action of enzymes on proteins has been shown by numerous other workers to be so convenient and economical and to yield such excellent results, that it has found a place in the permanent routine of many bacteriological laboratories.

Douglas's method consists in the digestion of ox muscle with trypsin. The digestion is allowed to proceed for two or three hours: the mixture is then acidified with acetic acid and brought to the boil. Any undigested muscle is filtered off, salts are added to the filtrate and the reaction adjusted. The medium thus obtained is distributed into suitable containers and sterilised.

In January 1915, Harden and myself (1915 [12]), working at the Lister Institute, repeated Douglas's experiments and confirmed his results. We found that organisms grew luxuriantly on this medium and, moreover, that it was suitable for the preparation of both tetanus and diphtheria toxins, in the latter case a minimum lethal dose of 0·001 c.c. being obtained. On account of other duties in connection with the war, it was not possible to continue this work at the time. In September 1919, the subject was taken up again by one of us (P. H.), and during the last two years medium prepared by a method closely resembling that of Douglas has yielded high-grade toxin. Since the statement has frequently been made that medium prepared in the laboratory by the action of enzymes on proteins is unsuitable

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for toxin production, the results given below may be of interest to workers in this field.

Cole and Onslow (1916[5]) described a method for the preparation of a substitute for peptone and a standard nutrient medium. Casein is subjected to prolonged digestion by means of trypsin, the reaction of the broth being adjusted in the simple way described in their paper. This constituted a very real advance, and the claims made for their medium are, by general agreement, well founded. Among other advantages, this method renders the bacteriologist independent of the slaughter-house, and he is able to prepare his medium at any time and anywhere—a practical point of considerable importance during the late war. It is a curious fact, however, that although this medium will support the growth of the diphtheria bacillus, the writer has never yet succeeded in obtaining diphtheria toxin from it, even when the "tryptamine" from casein has been enriched by the addition of fresh muscle extract. Davis (1920[7]) also found that Cole and Onslow's medium failed to yield high-grade toxin. Broth made by the tryptic digestion of fibrin and a "light digestion" of casein were tested for toxin production by Bunker (1919[2]), the minimum lethal dose reaching 0·005 c.c. in the former case, and 0·01 c.c. in the latter. Robinson and Rettger (1917[19] and 1918[19]) made a comprehensive study of "opsine," a biuret-free substance introduced by Dalimier and Lancereaux (1913[6]), which is stated to be prepared by the action of pepsin, trypsin, and erepsin on certain proteins. They found that opsine was an excellent culture medium for bacteria. The diphtheria bacillus grew well, but the yield of toxin was negligible, and although largely increased by the addition of fresh meat infusion, the amount obtained even then was very small. Martin (1898[16]) described a method for the preparation of medium by the autodigestion of pigs' stomachs. He obtained toxins which killed guinea-pigs weighing 500 grms. in doses of 0·01 c.c. and when fresh veal infusion was added, toxins were obtained which killed animals of similar weight in doses of 0·002 c.c. G. Dean (1907[8]) and Penfold (1921[18]) have reported favourably on Martin's method. The writer has prepared media by the action of pepsin on minced muscle, serum albumin and egg albumin, and has found that, although toxin has been obtained by their use, the results were neither so good nor so regular as those obtained by the action of trypsin on muscle.

The preparation of media by the action of enzymes on proteins has been investigated by numerous workers. In many cases, the media were required, or used, for special purposes. Jordan Lloyd (1916[15]) studied the cultural requirements of the meningococcus and demonstrated the value of Douglas's and of Cole and Onslow's media. Cole and Jordan Lloyd (1917[4]) used the products of the tryptic digestion of casein for the cultivation of the gonococcus. Distaso (1917[9]) used a tryptic digest of ox and sheep serum for the study of indole production by bacteria, and Berthelot (1917[1]) digested the proteins of ox muscle with trypsin, dried the product at 45° C., and used the powder in a 3 per cent. solution as peptone. Matthews (1918[17]) used a tryptic digest of blood for the isolation of *B. influenzæ* (*Pfeiffer*). Celozzi (1918[3]) digested human placentæ with trypsin and found that the resulting solution was very suitable for the cultivation of pathogenic micro-organisms, and Stickel and Meyer (1918[22]) prepared media in the same way with equally successful results. These workers examined the products of the action of enzymes on a great variety of proteins, and showed that such products cost little to prepare, and serve as most efficient media for routine bacteriological work. Walker Hall (1918[24]) studied a number of different kinds of media, including many prepared by the action of enzymes on proteins. He compared their amino content and studied the relationship between this and bacterial growth. The value of the different media mentioned above for the production of diphtheria toxin was not investigated.

Although the general experience has been that media prepared in the laboratory by the action of enzymes on proteins have given disappointing results as far as toxin production is concerned, yet the earlier experiments of Harden and the writer, to which reference is made above, encouraged the belief that a satisfactory medium could be made provided that proper working conditions could be discovered. Other considerations which stimulated this research were those of economy and convenience. In the ordinary methods of making media for toxin production, "peptone" is dissolved in an infusion of beef or veal. The present cost of "peptone" and butcher's meat is high and makes this an expensive process. Now by the modified method of Douglas described below, the "peptone" and the essential constituents of the infusion are brought into solution together, and are both provided by the same muscle. Since it has been found possible to substitute horse flesh for veal or beef, the dual object of preparing a satisfactory medium in a convenient way at a much reduced cost has been attained.

During this work, media have been made by the action of pepsin (four different preparations) and trypsin (six different preparations) on horse muscle, serum albumin, egg albumin, minced liver tissue, minced kidney tissue, and casein. The most satisfactory results have been obtained by the action of trypsin on minced horse muscle, and of the samples of trypsin used, one made in the laboratory according to the method of Cole and Onslow has given the best and most consistent results. A preparation of this pancreatic extract made in September 1919 appeared to have lost none of its activity in November 1921, and yielded media at this latter date from which high-grade toxin was obtained (M.L.D. = 0·0018 c.c.).

An interesting fact emerged from a study of the comparative value of a number of different samples of trypsin. Included in the series was Allen and Hanbury's Liquor Trypsin Co., the preparation originally used by Douglas (1914). This proved to be very active and when the resulting broth was sown with the strain of *B. diphtheriæ* used for toxin-making in these laboratories, a heavy typical film was rapidly formed. The filtrate from this growth, however, was very acid ($P_H = 5·4$), and contained no toxin. This was repeated with the same result on two other occasions, and further experiments showed that the acid production, and consequent absence of toxin in the filtrate, was due to the fact that the Liquor Trypsin Co. contained glycerol. Other experiments were performed in which definite amounts of glycerol were added to media which had been adjusted to a suitable hydrogen-ion concentration. Growth on such media was rapid and prolific, and in a certain number of cases in which the final reaction was alkaline, the toxin produced was as great as, and sometimes even greater than, the toxin produced on the control. This, however, was not the usual result. In the majority of instances the reaction of the medium containing glycerol became acid and remained acid, and no toxin was

found in the filtrates. Experiments showed that when the buffer content of the medium was sufficiently high to maintain the reaction on the alkaline side of neutrality, the presence of small amounts of glycerol was in no way detrimental, and in some cases seemed to be beneficial. The more common result, however, was to find that the solution became acid and remained so, and consequently no toxin was found in the filtrate. On account of the uncertainty of the results obtained, the addition of glycerol to media was abandoned. The work on this subject is mentioned because the use of trypsin preparations containing glycerol may account for the failure to obtain toxin on media prepared strictly in accordance with the method of Douglas.

It was found necessary to modify the original process in other particulars. Thus, in order to destroy the antitryptic power of the muscle, Douglas heats the minced tissue in a solution faintly alkaline to litmus. Experiment showed that better results were obtained if this preliminary heating were conducted in the faintly acid solution formed when muscle is mixed with water. Further, in the original method, digestion for two or three hours is recommended, and no antiseptic is added. It was found necessary to limit, as far as possible, the proteolytic activity of putrefactive organisms, and accordingly digestion was carried out in the presence of chloroform. Prior to the introduction of this modification the results were uncertain and variable. It was found that when putrefaction occurred during digestion, the resulting media proved to be of little value for toxin-making. It is probable that certain protein cleavage products are necessary for the elaboration of toxin, and such may be provided by the action of the enzyme on the muscle, whereas the mixture resulting from the growth of the putrefactive organisms may be quite unsuitable. It is difficult to prevent putrefaction when working on a large scale. After many trials, six hours' digestion in the presence of chloroform was adopted as a routine. Numerous attempts to prepare media by more prolonged digestion yielded inferior results.

Method.

The different stages of the process having been subjected to experimental study, the following routine technique was adopted. The figures given are for small quantities of media.

150 grms. of minced horse muscle are mixed with 250 c.c. of tap water and heated to 80° C. in a steamer. 250 c.c. of 0·8 per cent. sodium carbonate solution (the anhydrous salt) are then added, and the mixture cooled to 45° C., after which 5 c.c. of chloroform and 5 c.c. of Cole and Onslow's pancreatic extract are added. The mixture is incubated at 37° C. for six hours, the vessel being shaken at frequent intervals. 40 c.c. of normal hydrochloric acid are then added, and the mixture heated in a steamer for half an hour, then cooled and filtered. The reaction of the filtrate is adjusted to $P_H = 8$, and the medium distributed into containers. For the sterilisation of small quantities (100 c.c. medium in half-litre bottles) free steam is passed through the autoclave for one hour, then the

pressure is raised slowly to 10 lbs. and the steam turned off. For larger quantities (1 litre of medium in double Winchester quart bottles) the same method of sterilisation is adopted, except that the pressure is maintained at 10 lbs. for half an hour.

The amount of pancreatic extract required varies with different preparations. Two other samples gave good results when used in the proportion of 4 c.c. and 2·5 c.c. respectively. Good results have also been obtained with "pancreatin" (used in the proportion of 1 grm. with the quantities given above), but experience has demonstrated the superiority of Cole and Onslow's pancreatic extract. It is convenient to make this in large quantities, using as many as a dozen pigs' pancreas for the purpose. As Cole and Onslow point out, filtration is very slow, but the filtrate can be collected every twenty-four hours and acidified; the final product is very stable. The experience in this laboratory has been that the slight trouble involved in the preparation of the pancreatic extract has been amply repaid by the results obtained by its use.

Observations.

Reaction Change during the Growth of *B. diphtheriæ* on Douglas's Medium.

Two series of observations were made, a small and a large sample of the same medium being used. The strain used was Park Williams No. 8. Small samples were removed at intervals and the reaction determined. The results are given in Table I.

Table I.

Time of Incubation.	P_H.	
	100 c.c. Sample.	1000 c.c. Sample.
0 days (before inoculation) . .	7·9	7·95
1 ,, (after inoculation) . .	7·3	6·9
2 ,, ,, ,, . .	7·95	7·05
3 ,, ,, ,, . .	8·1	7·4
4 ,, ,, ,, . .	8·25	7·65
5 ,, ,, ,, . .	8·3	7·75
7 ,, ,, ,, . .	8·4	8·1
8 ,, ,, ,, . .	8·4	8·1
10 ,, ,, ,, . .	8·4	8·15
12 ,, ,, ,, . .	8·4	8·2

The reaction changes are typical. As with ordinary 2 per cent. peptone broths, the acid phase passes quickly when a small volume of medium is used, the P_H returning to the initial value in two days. With litre quantities the interval is five to seven days, and the degree of alkalinity reached is not so high.

Toxin Production after Various Periods of Incubation.

Two experiments (Table II.) were carried out to ascertain, if possible, the period of growth required to secure the best results, and to determine whether, during prolonged incubation, the toxin deteriorates in value.

In the first experiment, 21 litres of broth were distributed into twenty-one double Winchester quart bottles, and all inoculated at the same time. Bottles were withdrawn on the 8th, 10th, 12th, 14th, 16th, and 21st days respectively, and toxicity tests carried out.

In the second experiment, 30 litres of broth were dealt with similarly and bottles removed on the 6th, 11th, and 14th days respectively.

The strain used was Park Williams No. 8 and the toxicity of the samples was determined by the intracutaneous method (Römer and Sames (1909 [20])); (Glenny and Allen (1921 [11])).

TABLE II.

Time of Incubation.	Toxin Value, L_f/500 Dose.	
	Experiment I.	Experiment II.
	c.c.	c.c.
6 days	...	0·0003
8 ,, ...	0·0002	...
10 ,, ...	0·0002	...
11 ,, ...	...	0·0002
12 ,, ...	0·0002	...
14 ,, ...	0·0003	0·0002
16 ,, ...	0·0002	...
21 ,, ...	0·0002	...

At the end of each experiment the whole brew was mixed, filtered, and the minimum lethal dose was determined. In Experiment I. (total volume, 21 litres) the value was 0·0018 c.c.; in Experiment II. (total volume 30 litres) the value was 0·0022 c.c.

The earliest date on which maximum toxicity was attained was not accurately determined, but is probably about the end of a week. It was found that no decline in value occurred even after prolonged incubation. This is a point of some practical importance. Bunker working with media containing Witte Peptone showed that there is a point at which toxin development is at a maximum after which potency is lost. P. Hartley and O. M. Hartley (1922 [13]) found that media containing Parke Davis Bacteriologic Peptone exhibited the same phenomenon, decline in value after the maximum had been reached being very marked in some cases, particularly when small quantities of media were used. The medium described in this paper appears to be free from this defect.

Chemical Analyses of Douglas's Medium.

During the course of this work, certain chemical analyses were carried out in order to follow the action of the enzyme on the proteins studied and with the object of comparing the composition of the different preparations of media.

A sample of the finished sterilised broth was retained for this purpose. The reaction was ascertained; total nitrogen was determined by Kjeldahl's method, amino nitrogen by Van Slyke's method (1913 [23]), also by Sörensen's method (1908 [21]), and proteose nitrogen by Hedin's method (1903 [14]). The reaction became a

little more acid on autoclaving, the P_H falling from 8 to 7·9 or 7·8. The nitrogen content varied with the particular preparation of enzyme used and with the duration of digestion. When Cole and Onslow's pancreatic extract was allowed to act on horse muscle for six hours, the final broth contained from 3·5 to 4 mgrms. of nitrogen per cubic centimetre. Usually from 18 to 22 per cent. of this occurred in the amino form, and about the same quantity was precipitable by Hedin's tannic acid solution. When the time of digestion was more prolonged, or the amount of enzyme used relatively large, more nitrogen was brought into solution, the percentage of amino nitrogen being slightly increased and the percentage of proteose nitrogen decreased.

Results.

The results obtained by the use of the method described above are summarised in Table III.

Table III.

Number of Toxins.	Total Volume of Toxins. c.c.	M.L.D. c.c.	
1	600	0·0010	Small quantities made in the laboratory.
1	600	0·0012	
7	5,700	0·0015	
3	1,800	0·0018	
2	1,200	0·0020	
2	1,200	0·0025	
1	600	0·0030	
1	600	0·0035	
1	600	0·0038	
1	600	0·0040	
1	15,000	0·0015	Large quantities made in the media kitchen.
1	21,000	0·0018	
1	17,000	0·0020	
1	30,000	0·0022	
2	48,000	0·0030	
1	15,000	0·0035	
1	12,000	0·0050	
1	50,000	0·0060	
29	221,500		

Summary and Conclusions.

1. Media prepared by a method very similar to, and based upon, that of Douglas, have yielded diphtheria toxin on twenty-nine occasions, the total volume obtained being 221·5 litres.

2. All of this toxin was of use for immunising purposes, about 72 per cent. having a minimum lethal dose of 0·0035 c.c. or less, and 28 per cent. having a minimum lethal dose which lay between 0·002 c.c. and 0·001 c.c. These results compare favourably with those obtained from media prepared by other methods.

3. The use of horse flesh in place of beef or veal is an economy, the cost of materials being somewhat less than one-third of the outlay involved in making 2 per cent. peptone broth made also with horse-flesh infusion.

4. The method is convenient since the horse muscle provides at the same time the protein cleavage products and the constituents of the meat infusion, both of which are essential for the elaboration of toxin.

5. The choice of the trypsin preparation is important. Samples containing glycerol should not be used. The value of Cole and Onslow's pancreatic extract has been demonstrated.

6. Reaction changes in this medium during the growth of *B. diphtheriæ* are typical.

7. Toxin produced in this medium is stable, no deterioration being observed during prolonged incubation.

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