OBSERVATIONS ON THE GROWTH OF MENINGOCOCCI *IN VITRO* IN RELATION TO VIRULENCE.

A REPORT TO THE MEDICAL RESEARCH COUNCIL ON WORK CARRIED OUT AT THE UNIVERSITY OF CAMBRIDGE PATHOLOGICAL LABORATORY AND FIELD LABORATORIES.

By E. G. D. MURRAY and R. AYRTON.

CONTENTS.

I. Introduction.

Every bacteriologist is only too well aware of the many problems presented by the preparation of culture media for the growth of bacteria *in vitro*.

At present it is quite impossible to put forward a constructive generalisation outlining the principles of bacterial nutrition, based upon observed facts of bacterial metabolism.

A very large number of media have been described and certain of them are sufficient for the growth *in vitro* of many diverse bacteria; but the outstanding feature of our present degree of knowledge, in so far as pathogenic microbes are concerned, is the statement that each "species" behaves, to a greater or less extent, in a manner peculiar to itself.

It has long been recognised that special precautions have to be observed in order to obtain growth of certain parasitic bacteria and that the organism in question can be accustomed to grow upon what are described as the ordinary culture media in the course of a few subcultures. The question immediately arises, apart from the mere satisfaction of having obtained a culture of a given organism, whether these acclimatised bacteria may be regarded as possessing the physiological characters necessary to their parasitic existence. Our

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investigation of this problem, as applied to the meningococcus, constitutes the main subject of this paper.

Murray (1924) emphasised the important relation of medium to the determination of the Minimal Lethal Dose of a meningococcus culture, but he purposely refrained from a detailed description of the medium he used in order that it might be considered in greater detail here.

There is another question which is of considerable importance to immunologists and which receives some attention in this paper, namely, the maintenance of meningococcal cultures *in vitro* over a sufficiently long period without loss of "virulence."

II. The Estimation of the Growing Power of a Medium.

The literature abounds with so many different formulae, whereby it is recommended to make media for growing the meningococcus, that it is legitimate to suspect that a satisfactory medium yet remains to be discovered. Each entrant into this field of research finds cause for dissatisfaction in the existing formulae and proceeds to elaborate another. Still, that there are so many formulae indicates that the meningococcus can be grown quite readily and that a method is required for measuring and comparing the capacity of the various media to promote growth.

The method we have used for measuring the growing power of media during the last seven years is the following:

The surface of an agar medium, in Petri dishes, is inoculated with a chosen organism in a manner to ensure confluent growth. Convenient areas, as large as possible, are then ruled out on the glass surface of the plates and the growth over as many areas as possible is scraped off with a small sterile metal spade, made for the purpose; the growth so obtained is immediately placed in sterile weighing bottles with carefully ground stoppers and weighed. Then the stoppers are tilted and the bottles placed in a desiccator over NaOH, at reduced pressure and 37° C. and they are kept under these conditions until the weight becomes constant.

In this way the amount of moist living growth per sq. cm. surface of medium, the amount of dried bacterial protoplasm per sq. cm. and the percentage of moisture in the original growth are then easily computed. The usual precautions in weighing must be observed and the stoppers must be set in the bottles immediately the desiccator is opened because the dried bacteria are markedly hygroscopic.

We have frequently attempted to estimate, by naked eye comparison, the relative amounts of growth obtained on a number of different media, all inoculated at the same time and we have been somewhat astonished to observe how far removed from the truth our guesses were when they were compared with the actual weights of growth obtained from known areas. This in spite of a not inconsiderable experience of massive cultures of various organisms in the Army Vaccine Department during the war and elsewhere. We emphasise

this point in order to demonstrate that any naked eye method, whereby the amount of growth yielded by different media is to be estimated by looking at it *in situ*, is wholly untrustworthy. It has been our experience, that a very clear medium frequently gives a much more transparent growth of meningococcus than does some other medium not so completely freed of suspended matter, and in many such cases we have guessed that the more transparent growth was the lower yield per unit area, whereas actually the reverse was true. Thus we have come to the conclusion that the only reliable estimation of the amount of growth obtained on a medium, is one based upon actual measurement of the yield per unit area and the measure we have chosen is weight.

However carefully the various manipulations necessary to this measurement of growth are carried out, there is a considerable margin of error which is unavoidable at present. It is chiefly due to what may be called the adventitious moisture and this is determined very largely by the fluid exuded from the agar when it sets and the amount of water condensed when the plates cool. Both factors are difficult to control.

The amount of exudation moisture we regard as depending upon the firmness or rigidity of the jelly formed by the agar. It is our experience that a low concentration of agar means a moister surface when set than does a high concentration and this exudation moisture is in reality the nutritive medium and it contains a goodly proportion of solids. Other things being equal, there is a moderate degree of surface moisture most favourable to meningococcal growth and degrees of moisture varying markedly on either side of this diminish the amount of growth yielded. It may be that a too rigid jelly holds the nutrient solution too tenaciously for its ready accessibility to the micro-organism and that a too sloppy surface is approaching towards culture in a fluid medium.

The condensation moisture can be reduced by not using plates which expose a very large surface for condensation and by having the plates at a slightly warmer temperature than the medium at the time of pouring. Even so the lid and walls of the plate cool more quickly than the body of the agar and the condensation moisture is found to be very variable.

It might be suggested that the surface of the agar be dried in the usual way in the incubator but it must be remembered that agar readily forms a hard surface "skin" which is very unfavourable to meningococcal growth.

The error introduced by the inexact measurement of the area scraped can be diminished by scraping sufficiently large areas. With normal cultures the growth is removed very completely and the amount left behind is negligible compared to the possible error in the opposite direction which is introduced if care is not taken to avoid pressing on the surface and in so doing squeezing fluid out of the jelly.

In spite of these unavoidable errors the various measurements are sufficiently comparable to yield useful results and very considerable variations are revealed which, nevertheless, cannot be detected by the eye.

There is another point of importance which might easily be overlooked, if the amount of moisture the growth contains were not considered, that is the age of the culture at the time the growth is measured. If the yield of growth per unit area of the same medium is compared after 24 hours' and 48 hours' incubation, it is found that the latter shows but a small increase in mass on the former; so little in fact that it is well within the range of variation of successive subcultures on the same medium. But when the amount of moisture in growth of different ages is determined, it is found that the dried bacteria obtained from the 48-hour culture and expressed as a percentage of the moist growth, may be 1·5 to 2·5 times the percentage yielded by the 24-hour culture. Therefore it is very important that all measurements which are to be compared one with another should be made after approximately equal incubation.

The time interval we have chosen to incubate our cultures for the purpose of measuring growth is 24 hours. This choice was determined by the convenience it offered in the investigation of the growth yielded by successive subcultures on a given medium; although we use much younger cultures (14 to 16 hours) for virulence tests, because autolysis is then very much less marked than it is in older cultures, and presumably a greater proportion of the cocci are viable and there is less chance for interference by liberated endotoxin.

Even greater difficulties are encountered in the endeavour to obtain a standard inoculum for the plates in order to have really comparable measurements of growth. We are far from having succeeded in this; particularly when successive generations are studied.

III. The Variation in Growing Power exhibited by Media.

It is universally recognised that different kinds of media may give widely varying yields of growth, but perhaps it is not sufficiently realised, not only that different batches of any medium made by a described method give noticeably differing results, but that there is a considerable variation in yield of growth on any one batch.

The figures given in Table I, compiled from batches of media on which a sufficient number of observations have been made, are instructive.

The first point we wish to emphasise is that the yield of meningococcal growth per unit area, whether in terms of moist growth or dried cocci, varies enormously in different cultures on the same batch of medium and that these differences are usually not perceived by naked eye examination. It is for this reason that Murray (1924) stressed the point that it is not possible to establish a constant Minimal Lethal Dose for a strain of meningococcus in terms of agar slopes, Roux bottles, etc., as has often been attempted.

But still more important, from the point of view of the present paper, is the large difference between the figures representing the variation for any one batch of medium and the figures derived from observations of a number of batches representing the variation for the kind of medium. These variations are brought out particularly well when expressed as a percentage increase or

decrease on the arithmetical mean for the separate batches and for the kind of medium, and we claim that the divergence between the sets of figures demonstrates that it is extremely difficult to make two batches of medium exactly alike. We have taken a great deal of trouble in attempting to make media of uniform quality, but the above figures show that we have not succeeded to any great extent. Nevertheless, that we have not failed altogether is demonstrated by the figures representing the dried cocci as a percentage of the moist growth. It is clearly shown that in "Trypagar" and "*EDB/N*" the variations for the batch and those for the medium diverge widely; but on turning to "*EDB/S*" it is seen that there is a much closer correspondence between the two sets of figures. The importance of this point has been discussed already (Murray, 1924) and need not detain us here.

Table I.

Medium	Yield of moist growth per sq. cm. in mgms.				Yield of dried growth per sq. cm. in mgms.				Dry as % of moist growth			
	Maximum	Arithmetical Mean	Minimum	Maximum and Minimum as % increase or decrease on Mean	Maximum	Arithmetical Mean	Minimum	Maximum and Minimum as % increase or decrease on Mean	Maximum	Arithmetical Mean	Minimum	Maximum and Minimum as % increase or decrease on Mean
Trypagar No. 3	1·75	1·45	1·20	+20·7 −17·2	—	—	—	—	—	—	—	—
5	1·68	1·63	1·55	+ 3·1 − 4·9	0·329	0·304	0·273	+ 8·2 −10·2	22·0	19·9	17·6	+10·8 −11·3
6	1·40	1·29	1·15	+ 8·5 −10·9	0·243	0·223	0·175	+ 9·0 −21·5	18·1	16·8	14·7	+ 7·5 −12·5
batches of Trypagar aken together	1·75	1·43	1·15	+22·4 −19·6	0·329	0·258	0·175	+27·5 −32·2	22·0	19·0	14·7	+15·8 −26·3
DB/N No. 36	3·11	2·73	2·27	+13·9 −16·9	0·524	0·448	0·380	+17·0 −15·2	16·9	16·2	15·5	+ 4·3 − 4·3
37	3·40	2·85	2·06	+19·3 −16·9	0·562	0·488	0·413	+15·2 −15·4	20·1	17·3	15·8	+16·2 − 8·7
48	2·77	2·63	2·44	+ 5·3 − 7·2	0·500	0·437	0·388	+14·4 −11·2	19·0	17·3	15·1	+ 9·8 −12·7
63	2·54	2·12	1·79	+19·8 −15·6	0·488	0·415	0·355	+17·6 −14·5	22·2	19·6	18·0	+13·3 − 8·2
72, 73	2·53	2·30	1·91	+10·0 −17·0	0·460	0·406	0·349	+13·3 −14·0	19·3	17·6	15·8	+ 9·7 −10·2
4 batches of *EDB/N* aken together	3·40	2·38	1·44	+42·9 −39·5	0·562	0·432	0·227	+30·1 −47·5	22·2	18·0	15·1	+23·3 −16·1
DB/S No. 105	2·21	1·90	1·52	+16·3 −20·0	0·436	0·364	0·300	+19·8 −17·6	19·9	19·3	17·9	+ 3·1 − 7·3
110	1·74	1·63	1·54	+ 6·8 − 5·5	0·338	0·335	0·333	+ 0·9 − 0·6	21·6	20·3	19·4	+ 9·0 − 4·4
batches of *EDB/S* aken together	2·51	1·83	1·19	+37·2 −35·0	0·546	0·369	0·296	+48·0 −19·8	21·9	19·9	17·9	+10·05 −10·05
HD/V No. 196	3·8	3·2	2·6	+18·8 −18·8	0·67	0·51	0·43	+31·4 −15·7	17·7	16·1	14·8	+ 9·9 − 8·1
198	4·0	3·1	2·3	+29·0 −26·0	0·75	0·52	0·40	+44·2 −23·1	18·9	16·8	15·1	+12·5 −10·1
202	3·9	2·8	2·2	+39·3 −21·4	0·60	0·45	0·36	+33·3 −20·0	16·8	16·2	15·8	+ 3·7 − 2·5
209	2·7	2·1	2·0	+28·6 − 4·8	0·48	0·35	0·30	+37·1 −14·3	17·5	16·4	15·2	+ 6·7 − 7·3
batches of *EHD/V* aken together	4·0	2·8	2·0	+42·9 −28·6	0·75	0·45	0·30	+66·6 −33·3	18·9	16·3	14·8	+15·3 − 9·2

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It would appear that in obtaining this degree of success in making "*EDB/S*" medium, a proportion of the yield of growth per unit area had to be sacrificed and even were this necessary it would be worth while. Nevertheless, this does not entirely represent the truth, for further study of this type of medium has enabled us to make a medium, "*EDB/V*" or "*EHD/V*" from which we obtain a yield of growth even superior to "*EDB/N*" without losing the stability of the percentage yield of dried cocci characteristic of "*EDB/S.*" Other experiments now in progress seem to promise still further improvements. We do not claim that the superiority of the latest form of our medium over those previously used is entirely due to any one factor, for it is exceedingly difficult to determine the effect of varying even one constituent because its influence is often closely bound up with the general balance of the remainder. In order to realise the result of an alteration it is frequently necessary to re-examine the whole question, by determining the optimal concentration of each constituent of the medium in relation to the primary alteration of the factor under examination. To do this very thoroughly would almost be a life's work because of the infinite number of possible combinations. We shall, however, attempt, in Sections IV and V, to give an indication of the influences of the separate constituents of the medium.

Here we would draw attention to the fact, that the average yield of growth on our *EHD/V* medium is really a very large mass for the meningococcus. The figures given for medium 209 in Tables I and II were obtained using a strain which normally gives smaller growth on any medium than any strain used to obtain the other figures in the tables.

We have shown that there is a very considerable variation in yield by different cultures on the same batch of medium. One aspect of this fact has been referred to as a periodic wave of growth by Murray (1924, p. 180), in its bearing upon the correct measurement of a dose of living cocci for injection into animals. We cannot, however, demonstrate a regular periodicity. In Table II we give the figures obtained in measuring the amount of growth yielded by media with successive subcultures at 24-hour intervals (= generations) of a strain of meningococcus. The plates were inoculated sufficiently heavily to try to be sure of confluent growth.

There is one point we wish to emphasise because it bears an important relation to the establishment of a Minimal Lethal Dose for living meningococcus cultures: that subsequent to the second generation on a medium, the yield of growth in successive subcultures at 24-hour intervals almost always exhibits more pronounced variation. The growth obtained in the first two generations may be described as being on the up-grade and when a culture is used for the inoculation of mice with purpose to determine the virulence of a strain, we consider it important to be reasonably sure that the growth is on the up-grade. For that reason we select the first, or more usually the second, generation for virulence tests. The effect of successive subculture, at intervals greater than 12 hours, on the killing power of a strain also makes it desirable

Table II.

Successive generations		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
Modified Trypagar No. 27 Strain "Netley"	M	—	1·46	1·90	1·61	1·41	2·06	1·51	1·70	5th generation growth not confluent										
	D	—	0·226	0·346	0·263	0·240	0·366	0·253	0·320											
	% D	—	15·5	8·2	16·1	17·0	18·3	16·7	8·8											
EDB/N No. 36 Strain "Netley"	M	—	2·97	3·11	2·47	2·53	2·65	3·10	2·27	6th gen. confluent growth on one plate only (scraped), the others discrete colonies										
	D	—	0·480	0·520	0·384	0·415	0·433	0·524	0·380											
	% D	—	16·1	16·7	15·5	16·4	16·3	16·9	15·5											
EDB/N No. 37 Strain "Netley"	M	—	2·92	2·06	3·12	3·40	2·76	3rd gen. one plate confluent (scraped), one discrete colonies, and one no growth												
	D	—	0·460	0·413	0·562	0·556	0·449													
	% D	—	15·7	20·1	18·0	16·4	16·3													
EDB/N No. 63 Strain "Netley"	M	—	2·08	1·89	1·97	2·03	1·96	2·06	2·43	1·79	2·49	2·01	2·41	2·38	2·54	1·85	1·87	1·84	2·44	6th gen. one plate discrete colonies not scraped
	D	—	0·420	0·356	0·386	0·376	0·436	0·413	0·464	0·358	0·485	0·406	0·437	0·488	0·455	0·363	0·375	0·355	0·484	
	% D	—	20·3	18·8	19·6	18·5	22·2	20·1	19·1	20·0	19·4	20·2	18·1	20·5	18·0	19·6	20·0	19·3	19·8	
EDB/S No. 105 Strain "Netley"	M	—	1·92	1·70	1·83	1·98	1·88	2·11	1·92	1·91	2·07	1·74	2·21							
	D	—	0·353	0·318	0·327	0·371	0·351	0·404	0·373	0·380	0·407	0·344	0·436							
	% D	—	18·4	18·7	17·9	18·8	18·7	19·2	19·5	19·9	19·7	19·8	19·7							
EHD/V No. 202 Strain 28	M	3·44	2·87	3·47	2·64	2·30	—	2·21	3·85	2·58	2·25	6th gen. all plates discrete colonies. 11th gen. no growth								
	D	0·546	0·423	0·546	0·422	0·381	—	0·356	0·603	0·423	0·380									
	% D	15·9	14·7	15·8	16·0	16·5	—	16·1	15·6	16·4	16·8									
EHD/V No. 209 Strain Pettigrew	M	2·04	2·00	2·17	2·01	2·08														
	D	0·316	0·305	0·365	0·306	0·361														
	% D	15·4	15·4	16·8	15·2	17·4														

M = Moist weight in mgms. per sq. cm. D = Dried weight in mgms. per sq. cm. % D = Dried growth as a % of moist growth.

to use as early a generation as possible; but this question will be raised again in Section VI (Table XI).

This question of marked fluctuation in the yield of growth on a given medium is one which deserves closer consideration than we have been able to give to it, and, probably, it involves physiological processes which are important to the micro-organism.

It is possible that a sufficiently even and regular growth would result if the viable state of the inoculum used for the successive generations was reasonably constant, and the fact that this condition is very difficult to realise suggests very strongly that the variation in yield of growth, on a given batch of medium, is due to the state of the organism rather than to the medium. For some time past we have made it a rule to take only discrete colonies growing at the margin of an inoculated area as the inoculum for the succeeding generation, but, although this is an improvement, it does not interfere appreciably with the usual fluctuation or even the occasional complete death of a culture.

IV. An attempted Standardisation of a Medium.

Having considered the variation in yield exhibited by media, the ground is cleared for the examination of the various phases of preparation of the medium and to discuss their relative values. It is unnecessary to recapitulate what is known of the substances found in media which successfully grow parasitic bacteria, since we have nothing to add to the broad classification of them to be found in books dealing with the nutrition of living organisms. That with which we are more intimately concerned is the manipulation of the raw materials ordinarily used for media making and we shall show that this subject still provides a wide field for research of which we have barely touched the fringe.

Broadly speaking the type of medium we are considering consists of a watery extract of fresh meat, to which is added the products of tryptic digestion of meat, inorganic salts and accessory growth substances in the form of body fluids and exudates or extracts of animal or plant tissues. This complex mixture is usually held in a jelly of agar and clarified by the coagulation in it of some albuminous substance. The reaction is adjusted to a desired degree of acidity or basicity and it is then sterilised by raising it to the thermal death-point of living matter.

It is with the details of these various manipulations that we wish to deal in this section.

(*a*) *The meat extract.*

The tissue we confine ourselves to for the present is bullocks' heart muscle and we consider it a matter of primary importance to use only freshly killed meat: we refuse any which has been dead for more than 24 hours. This is freed of fat and the larger vessels, not too finely minced and extracted with one litre of tap-water to each 500 gms. of mince at between 70° and 75° C. for three

hours. After that the temperature is raised rapidly to 100° C.; a large amount of protein is thus coagulated and this is immediately preceded by an evolution of gas (93° to 95° C.). The water lost during heating is made up by adding distilled water to the original weight of the mixture, which is then filtered. The filtrate is bright and perfectly clear and is of pinkish-yellow colour. We have made numerous observations on the weight of the meat before and after extracting and pressing and also on the volume of the fluid yielded as a finished product and we find that between 50 and 60 per cent. by weight of the meat is yielded up to the extract. The filtered extract is bottled and sterilised at 120° C. for 20 minutes when a further coagulation takes place and the pinkish tinge is lost. During the last six months we have made our extracts with *distilled* water and on autoclaving the resulting filtrate it retains more of the ruddy tinge and there appears to be considerably less secondary coagulation. We believe the substitution of distilled water for tap water to be a marked improvement for additional reasons to be discussed in the subsection dealing with the influence of certain inorganic salts.

The constancy of the product obtained in this way is shown by the figures given in Table III.

Table III.

Extract No.	Total solids gms. in 100 c.c.	Ash gms. in 100 c.c.	Total Cl_2 gms. in 100 c.c.	Total N_2 gms. in 100 c.c.	Sörensen figure (see text) in c.c. $N/10$ NaOH on 10 c.c.	c.c. $N/100$ NaOH required to raise reaction of 1·0 c.c. from pH 7·0 to pH 8·0 to phenol red	Notes
40	1·56	0·41	0·035	0·166	1·7	0·6	Tap water
132	1·76	0·33	0·035	0·136	1·5	0·5	,,
134	1·45	0·36	0·035	0·144	1·7	0·7	Distilled water
158	1·52	0·36	0·053	0·150	2·1	0·6	,,
163	1·58	0·41	0·035	0·158	1·8	0·7	,,
199	1·40	0·44	0·035	0·140	1·8	0·9	,,

The method we have elaborated for filtering the meat extract deserves mention because it is very much more rapid than filtration through paper and is quite as efficient.

A sheet of fine butter-muslin is pinned on to a wooden frame in such a way that when the frame is rested in a large funnel the bag formed by the muslin does not touch the funnel. The extract and meat are stirred up and when the main part of the coarse meat has settled, leaving the fine coagulum still in suspension, it is rapidly poured into the wetted muslin bag so as to fill it. The fine coagulum settles uniformly on to the muslin and forms the actual filter, the excess of coarse meat is scooped out with a cup and returned to the can and the filter is then left undisturbed until the filtrate runs clear; this takes less than five minutes. Meanwhile the vessel containing the extract has stood undisturbed and the meat has settled together with the fine coagulum. The supernatant fluid, together with the first runnings, is now decanted into the centre of the filter, taking care not to fill above the filter-bed on the muslin and without unduly disturbing either the filter-bed or the meat in the can.

When the bulk of the fluid has been poured off from the meat, the can is balanced on the wooden frame at an angle to allow the remaining fluid to drain away into the filter, leaving the meat behind with most of the fine coagulum entangled in it. In this way we are able to save hours of time compared with filtration through paper. Starting with 1200 gms. of meat and 2400 c.c. of water, 2000 c.c. of perfectly clear filtrate was obtained in 15 minutes, and 2650 c.c. in less than 50 minutes without pressing the meat residue.

(*b*) *The digest.*

At the commencement of the present investigations we selected "Trypagar" (Gordon, Hine and Flack, 1916) as our routine medium, but we soon observed that it was extremely difficult, if not impossible, to make two batches sufficiently alike; the variation we observed is illustrated in Table I. In a previous investigation (unpublished) it was observed that the production of toxin by dysentery bacilli varied with the batch of medium and the medium then used had one point in common with "Trypagar" in that both are made with Douglas broth as a basis. We thought that in making Douglas broth the variable which was least under our control was the degree of digestion undergone by the meat and we decided to add a digest to a meat extract in such a proportion that every batch of medium would have an identical concentration of amino-acids, as determined by titration in the presence of formaldehyde. The amino-acid concentration in different digests varied considerably, but in the finished medium the increment of amino-acids in 10 c.c. of medium, due to added digest, neutralised 2·5 c.c. $N/10$ NaOH (= approximately an $N/40$ solution). This figure was determined by experiment to give maximum growth.

In making the digest no strict precautions were taken at first to exclude contamination and the presence of micro-organisms was easily demonstrated, although growth during the time allowed was slight in the high concentration of the products of tryptic digestion which accumulated very rapidly, but anaerobes were usually sufficiently numerous to cause an unpleasant smell.

The medium made in this way we call "*EDB*/*N*" (*E* = extract, *D* = digest, *B* = Blood, and *N* = non-sterile digest) and the variation it exhibited is shown in Table I. These figures show a much improved yield of growth when compared with "Trypagar," but in other respects this medium has no particular claim to superiority. The growth when scraped off in mass had a curious flesh-pink tinge which was not seen in growth from "Trypagar," but which obtains with all our media of the *ED* type. Although we used this medium extensively and the growth it yielded per unit area was satisfactory, we shall not consider it in detail because it would unduly burden this paper to describe every step in the investigation. We propose to consider as briefly as possible only the chief points which led to the development of the technique we have provisionally adopted.

At this stage we thought that some degree of control over the concentration

of amino-acids in the medium was the all-important factor. However, in attempting to determine the virulence of strains of meningococcus grown on this medium, we obtained ample evidence of the inconstancy which has been so commonly emphasised as characteristic of that organism. But it must be remembered that at that time we had not recognised the more important details of technique which it is essential to observe in order to determine the virulence of a culture and to ensure the successful repetition of an experiment. We were contented for the time being with the idea that maximal growth indicated a good medium and assumed that such a culture was healthy and probably possessed the physiological characters of the disease-producing meningococcus.

Nevertheless we were uneasy about what might be the influence of the slight but mixed infection of the digest on the efficiency of our medium and so proceeded to digest the meat with such strict precautions that contamination was completely avoided. Such sterile digests were used to make the medium called "*EDB/S*" (*S* = sterile) in Table I, maintaining the same increment of amino-acids. At first we were greatly disappointed with this medium because of the poor yield of growth per unit area compared with *EDB/N*, but an examination of the properties of the growth caused us to abandon *EDB/N* as a bad medium; but we have learned since that it was the general adjustment of the medium which was at fault and not the digest (see Section V, Table VIII). Two of our reasons are illustrated in Table I: firstly, the moisture represented as a percentage of the living growth is much less variable than the media used up to this time; secondly, the yield of dried growth per unit area is not greatly inferior to *EDB/N*. A third reason, to which we attached considerable importance, must be stated briefly here although it will be dealt with in greater detail later. Batches of both *EDB/N* and *EDB/S* then in use were inoculated from the same culture of a given strain of meningococcus and it was found that the growth from the former failed to kill mice and rats whereas the growth from the latter killed quite regularly. This result was confirmed with certain other batches of these media. Although the interpretation of these results is a matter of difficulty, it appeared to be evident to us that there was a difference in the physiological state of the bacteria as grown on these media and that the mass of growth yielded by a medium was less important than the killing power of a culture.

At this time the general treatment, the relative concentrations of meat, water and trypsin, and also the reaction and time of digestion were as near as we could make them alike for the different digests, but the contaminated digests always showed a very much higher concentration of "amino-acids," in terms of c.c. *N*/10 NaOH in the presence of formaldehyde, than the sterile digests did. This is probably due to an enterokinase-like substance supplied by the contaminating bacteria (see Richet and Richet, 1921, p. 1060).

However, since the amino-acid increment due to digest in the finished medium was the same in both *EDB/N* and *EDB/S*, the property it is desirable

to control is definitely independent of the general amino-acid figure, determined by titration in the presence of formaldehyde. This is indicated by both growth and killing power (see Section V).

In order to investigate this matter further numerous experiments were made to determine approximately the conditions necessary to allow the sterile digests to contain an equally high concentration of amino-acids as the contaminated digests, and guided by the results of this investigation we have adopted the following method of making our digest:

Immediately after the extract has been drained away the residual meat mince is suspended in as many litres of *N*/100 HCl as there were kilos of original fresh meat before extraction, and put into a flask fitted up so that samples can be withdrawn and sterile fluids added when required. This is then autoclaved for 30 minutes at 130° C. to ensure sterility; it is advisable to heat large volumes of material to 100° C. in a water-bath or steamer before putting them into the autoclave. When cooled sufficiently the flask is placed in an incubator at 37° C. and left overnight in order to make certain that the large bulk of material reaches the same temperature throughout and to test for sterility.

The pancreatic extract is sterilised by filtering it through a Pasteur-Chamberland candle "*F*" and is added in the proportion of 20 c.c. to each litre of *N*/100 HCl used. It is essential to add the trypsin to the acid in order that it may be activated (see Richet and Richet, 1921, p. 1060). We have found by experiment that the amount of digestion which takes place in a given time, other things being equal, is very much greater when the pancreatic extract is added to an acid substrate than when it is added directly to one of *p*H 8·0 (the region of optimal reaction for tryptic digestion).

We find that trypsin can be activated quite as well by treating it with *N*/100 HCl before adding it to the meat suspended in an alkaline medium. Adding it to an alkaline medium containing 0·5 per cent. $CaCl_2$ (see Richet and Richet, 1921, p. 1060) is also effective.

The routine we have adopted is to add the trypsin to the meat suspended in *N*/100 HCl and after 5 hours' contact to adjust the reaction to *p*H 8·0 as described subsequently and allow digestion to proceed at 37° C. Originally the reaction was adjusted by adding 10*N* NaOH, and sufficient was added at once to maintain the alkalinity over the desired time; this was found to be 15 c.c. per litre. As this raised the alkalinity at the commencement slightly more than is desirable we now add the equivalent quantity of Na_2CO_3 (= 0·8 per cent. anhydrous Na_2CO_3). We have not found our trouble sufficiently rewarded when we added the necessary alkali at short intervals during the course of digestion, and it increases the risk of contamination. Digestion is allowed to proceed for two to three weeks and during that time somewhere in the region of 10 per cent. of the meat is digested.

That the degree of digestion is sufficient for our purpose is indicated by titrating the amino-acids in the presence of neutralised formalin using phenol-

phthalein as indicator and expressing the result in c.c. $N/10$ NaOH required to neutralise the amino-acids in 10 c.c. of the filtered digest: this we call its Sörensen figure and for reasons to be discussed later we require our digest to have a Sörensen figure not less than 20. The digest is usually very dark coloured and when it is undiluted it is extremely difficult to see the end-point of the indicator. Therefore, in making our titration, 5·0 c.c. of the digest is made up to 25·0 c.c. with distilled water, boiled and rapidly cooled and titrated to the first faint change of the phenolphthalein with $N/10$ NaOH; a similar sample is titrated to the same point and kept as the control colour; 5·0 c.c. of freshly neutralised formalin (40 per cent. $C.H_2O$) is now added and the mixture titrated to match the control. This second increment of $N/10$ NaOH represents the Sörensen figure for 5 c.c. of the digest. We always check the result by adding excess of alkali and allow the mixture to stand for ten minutes, then titrate back with $N/10$ HCl. We have found repeatedly that amounts of formalin up to 4 c.c. give an increasing Sörensen figure and that amounts over 5 c.c. do not alter the result. We usually find it necessary to redistil commercial formalin to obtain a satisfactory solution for this titration, otherwise it discolours on neutralisation.

(c) *The concentration of amino-acids.*

We stated above that whatever the concentration of amino-acids in the digest might be, we only added sufficient to increase the Sörensen figure of the extract by a definite amount which had been determined by experiment. This procedure served a useful purpose by indicating two important points: (1) That the yield of growth per unit area is not dependent on the general concentration of amino-acids in the medium as represented by the Sörensen figure. This point is amply demonstrated by the figures given in Table I where the media described as *EDB/N* and *EDB/S* have the same Sörensen figure and only differ in that the latter contains an actually greater percentage of digest. (2) That, as has been mentioned already, and will be discussed in detail later, the killing power of a given strain of meningococcus has been observed to vary constantly with the medium on which it is grown. In the instance already mentioned the two media had the same Sörensen figure.

Since we are satisfied that a constant concentration of amino-acids in the finished medium, as expressed by the Sörensen figure, does not contribute to the stabilisation of the culture either in respect of yield of growth or killing power and as sufficient evidence to support this conclusion is to be found in Table I and Section V of this paper, no useful purpose would be served by detailing experiments.

It has been our experience that the amount of substances in the digest which are essential to produce the most efficient medium bear some relation directly proportional to the degree of digestion indicated by the Sörensen figure of the digest. We do not consider digestion to have proceeded sufficiently far for our purpose until a Sörensen figure of not less than 20 has been reached.

(*d*) *The added inorganic salts.*

Among the characters used for the recognition of meningococcus colonies in cultures from the nasopharynx are the consistency of the growth and the ease with which it emulsifies. It is said that it "picks up like paint"; it ought to be soft and not stick to the medium, neither ought it string out like mucilage nor be friable; and it ought to emulsify readily and evenly.

In working with pure meningococcal cultures isolated from cerebro-spinal fluids and grown on the many varying media we have made for experimental purposes, we have frequently obtained growths which exhibit those very characters the typical meningococcus is supposed not to possess. Quite commonly the growth is very slimy and difficult to remove from the surface of agar media and is very obstinate to emulsify in physiological saline. When picked up it strings out into an almost elastic thread and maintains that character when placed in saline, giving a very imperfect emulsion even after prolonged shaking. On the other hand, the growth may pick up quite readily but have a matt, granular appearance and when scraped into a heap the surface appears to be dry and wrinkled. This second type of growth gives a flocculent emulsion which very rapidly settles out of suspension. These are extremes which might be called the "Sticky" and "Granular" and can be produced by any given strain on two different media and be associated in each case with a very good yield per unit area. Somewhere between the two occurs the "characteristic" smooth, moist, flesh-coloured growth, which emulsifies easily and smoothly.

Microscopically no morphological distinction can be drawn between the cocci constituting the mass of growth exhibiting these various characters.

Possibly these observations explain certain disagreements in the literature.

Before entering into the details of our observations, as far as we have carried them, it is as well to draw attention to the remark of Nicolle, Debains and Jouan (1918, p. 151) that: "If it is desired thoroughly to know the macroscopical appearance of meningococci, it is necessary to examine them in a mass of several grammes."

There are two influences exercised by these physical characters exhibited by meningococcal cultures: The first is their influence upon the measurement of the growing power of a medium and on this the "granular" type of growth has no effect, but the "sticky" kind is difficult to scrape up cleanly and without the risk of picking up pieces of medium or unduly squeezing moisture out of the agar. Although it only introduces a marked error in the case of extreme stickiness, it has the disadvantage of being difficult to manipulate. The second influence depends upon the imperfect emulsion they produce and has more profound effects upon general experimental work, such as inoculating animals or measuring quantities by a dilution method, etc., and this is particularly marked in the case of the rapidly sedimenting emulsion resulting from the "granular" kind of growth.

We first endeavoured to discover whether these physical characters of the growth were associated in any way with the frequently observed capsulated appearance of the cocci and the virulence of the culture, but we failed to find any such association. Since the virulence of a culture remained unaffected by them it was desirable to eliminate the extreme variations, which were a source of trouble in the general experimental work and we turned to the effect of varying the concentration of salts in the media. Although we do not pretend to have made a complete investigation of the influence of salts, we have obtained certain interesting results and to some extent we have succeeded in eliminating the extreme slimy and granular types of growth.

All the experiments now to be considered were made with media of the *EDB/S* type.

In the first place there were occasions when we accidentally omitted to add NaCl and $CaCl_2$ to the medium used for general work and confirmed their absence by titration subsequent to absolute failure to obtain growth on those batches, although other batches made with the same materials were satisfactory. These defective media gave the usual growth when we added the salts in the concentrations we then used (0·5 per cent. NaCl, 0·0125 per cent. $CaCl_2$). At this time we made our extracts with tap water. On one occasion we reserved a portion of the medium which gave no growth without and good growth with salts, added 0·0125 per cent. $CaCl_2$ and varied the NaCl concentration with the following results:

(1) 0·25 per cent. NaCl gave growth which was not sticky and scraped well.

(2) 0·50 per cent. NaCl gave growth which was moderately sticky but scraped well.

(3) 0·75 per cent. NaCl gave growth which was sticky and scraped badly.

(4) 1·00 per cent. NaCl gave growth which was very sticky and would not scrape at all.

Medium (3) gave very nearly half the yield per sq. cm. given by media (1) and (2) which were approximately equal. This very definitely demonstrates that sticky growth can be due to a too high concentration of NaCl. Subsequent experiments showed that in the presence of 0·0125 per cent. $CaCl_2$, when the extract has been made with tap water, the range of concentration of NaCl varying between 0·1 per cent. and 0·3 per cent. gave smooth moist growth and that stickiness commenced to appear with 0·4 per cent. and increased with higher concentrations of this salt. No lower concentration than 0·1 per cent. was tried at this time.

Without entering deeply into the question, we selected 0·25 per cent. as the concentration of NaCl giving the most desirable form of growth for our purposes and then proceeded to test the effect of varying the concentration of other salts.

Media made with tap water extracts and containing 0·25 per cent. NaCl without additional $CaCl_2$ invariably gave sticky growth and the following

two experiments (Table IV) are instructive in showing that stickiness can be sufficiently inhibited by adding an appropriate amount of $CaCl_2$ and that an excess only increases this defect in the presence of NaCl.

Table IV.

Experiment	Medium No.	% NaCl	% $CaCl_2$	Character of growth	Regrowth in 9 hours over area scraped in morning
I	1	0·250	0	Sticky, scrapes badly, emulsifies easily	—
	2	0	0·0125	Smooth, scrapes well, emulsifies easily	—
	3	0·250	0·0050	Sticky, scrapes well, emulsifies easily	—
	4	0·250	0·0200	Slightly sticky, scrapes well, slightly stringy emulsion	—
	5	0·250	0·0400	Sticky, scrapes fairly well, stringy emulsion	—
II	A	0·250	0	Very sticky, scrapes badly	Grown well
	B	0	0·010	Smooth, scrapes well	Not grown
	C	0·250	0·005	Sticky, scrapes fairly well	Grown well
	D	0·250	0·010	Smooth, scrapes well	Not grown

It appears from these experiments that in the presence of 0·25 per cent. NaCl the concentration of $CaCl_2$ required to give smooth moist growth is between 0·01 and 0·02 per cent. and probably nearer the smaller figure. A result which we have confirmed using media made with distilled water extracts.

At the time of doing Exp. II (Table IV) we had become interested in the question of regrowth of the meningococcus over the area from which the growth had been scraped cleanly and in the appearance of secondary colonies superimposed on previously existing growth. The latter phenomenon had occurred very rarely and usually only after prolonged incubation on *EDB/N* media and was the rule with *EDB/S* media, when such media exhibited a difference in virulence in the cultures. We were inclined on that account to stress the importance of sterile digests (see Section VI) and it is interesting, therefore, to notice the inhibition of regrowth by 0·01 per cent. $CaCl_2$ in Table IV.

Since we attach importance to regrowth as a criterion of a good medium, this effect of $CaCl_2$ caused us to try the effect of adding KCl to the medium; because of the high concentration of potassium salts described in actively growing malignant growths and the high concentration of those salts in egg-yolk; egg medium being one on which the meningococcus lives a very considerable time.

To an *EDB/S* medium containing 0·25 per cent. NaCl and 0·01 per cent. $CaCl_2$ we added KCl in varying proportions with the following results:

(1) No KCl gave smooth growth and very slight regrowth.

(2) 0·01 per cent. KCl gave slightly granular growth and very pronounced regrowth.

(3) 0·015 per cent. KCl gave markedly granular growth and marked regrowth.

(4) 0·02 per cent. KCl gave very pronounced granular growth and slight regrowth.

The regrowth in this experiment was noted after 24 hours' re-incubation,

while in the $CaCl_2$ experiment (Table IV) it was noted after only 9 hours' re-incubation. This experiment is interesting not only because it suggests that the potassium salt has some influence on the regrowth of the culture over an area upon which it has already grown, but because the salt also appears to influence the production of the granular type of growth when both sodium and calcium salts are present. Further, when the NaCl and $CaCl_2$ are present in the proportion named above the optimal concentration of KCl is identical for both regrowth and the least granular type of growth.

In another experiment the medium was made with distilled water extract and the salts were added in varying proportions, but in no medium were more than two salts present. As far as possible all the other conditions were maintained alike for the different media and the results obtained are shown in Table V.

Table V.

Medium No.	NaCl %	$CaCl_2$ %	KCl %	Mean weight in mgms. of dried growth per sq. cm.	Mean dry growth as a % of the mean moist growth	Physical character of growth	Regrowth 24 hours after scraping
1	0	0	0	0·525	17·1	Smooth	None
2	0·25	0	0	0·373	18·7	Very sticky	,,
3	0·25	0·005	0	0·375	19·4	Sticky	Very slight
4	0·25	0·01	0	0·563	19·8	Smooth	,,
5	0·25	0·02	0	0·445	19·6	,,	,,
6	0·25	0	0·01	0·511	19·3	Very sticky	Fair
7	0·25	0	0·02	0·431	19·7	,,	Very good
8	0·25	0	0·04	0·448	19·0	,,	,,

There are two points to be noted in comparing this with other experiments previously described: firstly, that the media were made with a distilled water extract; and secondly, that freshly laked blood was added as the accessory growth factor, which, as will be shown later, in common with fresh ascites fluid stimulates growth under otherwise unfavourable conditions, and probably accounts for growth being obtained on medium No. 1. This experiment suggests:

(*a*) That the addition of NaCl causes stickiness which is counteracted by an optimal proportion of $CaCl_2$ and which is enhanced by adding KCl without $CaCl_2$.

(*b*) That the addition of KCl stimulates regrowth over the area from which growth has once been removed.

(*c*) That KCl in the presence of NaCl and the absence of $CaCl_2$ does not cause the growth to be granular.

We have not followed the question of the influence of inorganic salts as far as we would have liked, because it was not the primary object of our quest. We were mainly concerned with an attempt to render the conditions of growth such that the extremes of "Sticky" and "Granular" growth did not appear; for no other reason than that those extremes made our work more than ordinarily difficult. To some degree we have been successful, for, since adopting 0·25 per cent. NaCl, 0·01 per cent. $CaCl_2$ and 0·01 to 0·02 per cent. KCl as the

concentration of those salts to be added to a medium made with distilled water extract, we have not experienced extremes of sticky or granular growth in other than experimental media to which excessively high concentrations of digest have been added.

Nevertheless, our investigation does not supply a complete explanation, for we possess one strain of meningococcus which is habitually slightly sticky on our ordinary media, though never unmanageably so and we shall show that there are other influences than inorganic salts tending to cause stickiness.

It is expedient to be aware of the concentration of NaCl in the digest within reasonable limits. With all media made from one particular digest we were troubled with sticky growth, until we checked the concentration of NaCl it contained as the result of neutralising the alkali which had been added in the course of digestion. On taking this into account and adding proportionally less NaCl to the finished medium, the growth obtained was of the desired consistency.

One other point deserves mention: the growth may be smooth and satisfactory when 12 to 16 hours old and quite noticeably sticky at 24 hours. In any case stickiness increases with age.

The killing power of the various strains used in these experiments was known and the growth yielded by these media, with varying concentrations of salts, gave no indication of variation in "virulence" for mice.

(*e*) *The reaction.*

It would be misleading were we to state that the reaction of our medium is adjusted to a definite *p*H, since we have not taken into account the salt and protein errors and the titration has not been done at a constant temperature. At present we are content to adjust the reaction by a standard method whereby we do obtain fairly constant results, because our titrations have but slight variations which are well within the relatively wide range of tolerance of the bacteria to changes in the reaction of their environment and because comparatively large quantities of alkali or acid are necessary to produce a marked change in the reaction of the medium. The colorimetric method is employed, with phenol red as the indicator and we match our medium to standard Sörensen solutions of phosphates, superimposing a blank to compensate for the natural colour of the medium.

The medium is heated in a water-bath to melt it and drive off the CO_2 and 1 c.c. in a cordite tube is diluted with 4 c.c. of boiling distilled water. Two such tubes are prepared and to one of them the indicator is added in the same concentration as it is added to the tubes of Sörensen buffer solutions; the other tube is kept as the colour control. The diluted medium is made to match the buffer solution having a $p\text{H} = 7{\cdot}2$ and then it is boiled and cooled rapidly under the tap without shaking, before the final reading is made.

After the calculated amount of NaOH has been added to the bulk of the medium, we are careful to take a sample to check the reaction. Agar media

which have been titrated when diluted to prevent too rapid setting and to the bulk of which the calculated amount of alkali has been added, frequently are considerably on the acid side of the desired reaction. We have not investigated this point, nor do we offer any explanation, but we suggest that the omission to check the final reaction of the bulk of the medium has led to the statement having been made that ordinary media become increasingly acid with autoclaving. We have frequently autoclaved our finished media four and five times without the reaction changing in the process. On the other hand, if the desired reaction of the medium is more alkaline than that we require, then there is certainly a fall in the *p*H owing to the precipitation of phosphates, but it is not necessary to autoclave it in order to observe this change. When we have previously partially removed the phosphates from our media, we have usually found that the calculated quantity of alkali is sufficient to adjust the reaction to the desired *p*H. Liquid media (broth) do not behave in this peculiar way in our experience and the calculated quantity of alkali required to adjust the reaction to "*p*H 7·2" has always been corect, but, besides being free from agar, these media have not been diluted for purposes of titration.

The reaction we have selected is that which we found by experiment to yield maximum growth per unit area and it is certainly rather more acid than the optimal reaction generally claimed for the meningococcus.

(*f*) *The clarification.*

A perfectly transparent agar medium is so much a convenience as almost to be a necessity and is always preferable to another equally good in all other respects but this. When the extract and digest are clear to start with, there is little more needed to render the finished medium sufficiently transparent for almost all purposes, since the haziness due to the agar is but slight. But owing to the extract and digest being markedly acid the necessary adjustment of the reaction occasions a flocculent precipitate of phosphates to form, which if distributed through the medium interferes with its transparency. Although this precipitate readily deposits and the clear medium can be decanted off, an economy of time and medium can be effected by first filtering through ordinary surgical lint, which holds back the bulk of the precipitate as well as all the fine dirt in the agar. Were it not essential to add accessory growth factors to obtain cultures of freshly isolated or virulent strains of meningococcus, there would be no need to filter the medium at all. For a considerable time we met this need by adding freshly drawn horse blood which was coagulated by heat in the presence of the agar; this coagulum was removed most satisfactorily by filtration through lint, the medium held in the lint and clot was wrung out by hand, and the finished product was glass clear. The removal of this fine coagulum by filtration through paper is an extremely slow process and usually results in the loss of a considerable quantity of the medium.

Later, when freshly drawn horse blood was no longer available, we evolved

a process whereby the accessory growth factors are derived from the heart muscle used for making extracts and digests. The method will be described presently, but it is necessary to state here that an extremely fine coagulum is produced which is not removed easily by filtration through lint. However, we have found it to be more easily removed if it is produced and filtered in the presence of the glutinous phosphate precipitate, with which it seems to become entangled. We obtain this condition by adjusting the reaction of the medium to match the Sörensen solution of *p*H 7·6 (in the manner already described) before coagulation takes place. At this degree of alkalinity a copious precipitate of phosphates is produced which facilitates the removal of the fine protein coagulum by filtration through lint. The production of the phosphate precipitate reduces the alkalinity of the medium to some extent; so that the reaction now is usually that which we require for our finished product, namely *p*H 7·2. The filtrate obtained in this way is quite sufficiently clear, but usually a slight haziness remains; this can be removed by filtration through paper or silted up lint if desired, but if some specially clear medium is required it is easily obtained by decanting the top layer which has cleared itself by sedimentation. That only a fraction of the phosphates in the medium is removed by this process is demonstrated by making the medium much more alkaline subsequently (*p*H 8·0 or 9·0), when a heavy precipitate is produced immediately.

But a word of warning is necessary since we have observed, when we have purposely poured into a plate a sample of medium containing a copious precipitate of phosphates and other matter, in such a way that the precipitate is not distributed evenly, that then the growth of meningococcus is much thicker over the dense precipitate than it is over the clear portions of the plate. In fact, when the accessory growth factors are derived from the heart muscle, there is no doubt that we get better results when the medium is slightly cloudy than we do when it is glass clear. When, however, the accessory growth factors are obtained by coagulating blood in the medium with heat, perfectly cleared medium is quite as efficient as that which is cloudy.

(*g*) *The sterilisation.*

We have not been able to detect any alteration in the finished medium, nor in the individual ingredients after repeated autoclaving at 120° C. for 20 minutes, as tested by the yield of growth and the killing power of the meningococcus. When the bulk of material to be sterilised in this way is large, we take the precaution of raising it to 100° C. in a water-bath or steamer before autoclaving it, otherwise the desired temperature is not reached and sterilisation is incomplete. To test the temperature to which the material has been subjected we use pure chemical compounds of known melting point, enclosed in sealed glass tubes.

The statement has frequently been made, that, subsequent to autoclaving a medium, the reaction is more acid than it was to start with. We are em-

phatically of the opinion that this is not the case with media which are not markedly alkaline and do not contain sugars. There are two conditions which we consider explain such observations, but we do not know to what degree they are interdependent: firstly, that the calculated quantity of alkali has been added to the bulk of the medium and the reaction has not been checked prior to autoclaving; secondly, that the formation of a copious precipitate of phosphates has separated in a markedly alkaline medium and in so doing reduced the alkalinity. In any case the second eventuality ought to be safeguarded against by resorting to the obvious expedient of checking the reaction before sterilisation, and, if necessary, removing the excess of phosphates.

We have not been able to discover that repeated autoclaving produces any deterioration of the stimulus to growth due to the accessory growth factors which are essential to successful cultivation of the meningococcus.

(*h*) *The accessory growth factors*

It is well known that in primary culture the meningococcus requires these accessory substances and probably this is the principal reason why it is universally understood that special media are necessary to grow it. The absolute need for special provision of these accessory factors decreases with the time a given strain has been kept in culture and the meningococcus can readily be educated to grow on "ordinary media," as is a common practice in laboratories. But two outstanding features of the life of this organism *in vitro* definitely prevents any application of this practice to the work we are doing at present: firstly, freshly isolated strains or those which have been raised in virulence will not grow readily on ordinary media; and, secondly, the repeated subculture necessary to accustom the organism to such media immediately deprives the cultures of their power to kill mice. Thus it is essential, apart from any other properties the medium may possess, to provide the required accessory growth factors.

It is not our purpose to consider these substances with any intention to classify them, but the manner in which we used the fluids containing them falls naturally into two categories:

(1) Their addition to the medium immediately before use and after sterilisation.

(2) Their addition as an integral part of the preparation of the medium before sterilisation.

The first method requires that the substances be added to the otherwise finished medium with strict sterile precautions and at the same time exercising minute supervision over other conditions, in order that the supposedly delicate accessory growth factors are not subjected to treatment which either destroys or removes them: such as autoclaving or adsorption by large surfaces provided by filter paper or finely divided precipitates. The second method allows of the accessory growth substances, in the presence of the other constituents of the medium, to be subjected to the usual processes of sterilisation, filtration, etc., without interfering with their function. Obviously this has great advantages.

The substances we have tried by the first method are: ascites fluid, horse serum, laked horse blood, formol serum and extracts of red corpuscles. The substances we have tried by the second method are: Gordon's extract of pea-flour, freshly drawn horse blood, extracts of red corpuscles, formol serum and our own method of using extracts of heart muscle.

So much for the categoric treatment of the subject and we shall now consider each of the individual substances by their effect upon growth.

Ascites fluid, when freshly drawn and added immediately before use to agar media which have been cooled to 45° C., is undoubtedly superior to any other substance we have tried. In our experience, it enables media which are otherwise absolutely unsuitable for meningococcal growth to give a remarkable yield per unit area. Nevertheless, in addition to the difficulty of maintaining an adequate supply and contrary to the invariable statement of the case, ascites fluid does not keep well exposed to the air in vessels plugged with wool. We have not tried hermetically sealed vessels. Using the same batch of medium, or batches made as nearly alike as we know how, to which was added 5 per cent. of ascites fluid immediately before pouring the plates, we obtained profuse, smooth, creamy growth when the fluid was freshly drawn. As the fluid aged (10 days) the growth tended to become sticky, until, at last, it became unmanageable, reaching such a degree of stickiness that it could not be removed from the surface of the agar (30 days). As the sliminess of the growth increased the yield appeared to become less. We were inclined, at first, to blame every process and ingredient used in making the medium except the ascites fluid, and we spent a considerable time investigating the effect of various alterations, without improving matters until we examined the ascites fluid. Ascites fluid which had been kept in vaccine bottles with wool plugs for 30 days was very alkaline, well beyond *p*H 8·0; when it was 50 days old, 5 c.c. of it required 0·7 c.c. *N*/10 HCl to adjust its reaction to *p*H 7·2, in spite of the fact that the fluid appears to be comparatively slightly buffered. When the reaction of 5 c.c. of this fluid containing phenol red was adjusted to *p*H 7·2 and left standing in a boro-silicate glass tube for 15 hours exposed to the air, the upper layer of the fluid became very much more alkaline than the lower layer.

A hundred c.c. of ascites fluid was placed in a wool-plugged flask and the reaction was adjusted to *p*H 7·2 with full sterile precautions; it was then left in a cool dark cupboard for 6 days, when the rection was found to be *p*H 8·0 and 5 c.c. required 0·3 c.c. *N*/10 HCl to restore the reaction to *p*H 7·2. Every precaution was taken to reduce the absorption of alkali from the glass. Two litres of the same fluid were stored in the cupboard in a Winchester quart bottle with a rubber bung and only exposed to the air through a piece of galss tubing of 4 mm. diameter tightly plugged with wool; after 50 days it was more acid than *p*H 6·6 and 5 c.c. required 0·2 c.c. *N*/10 NaOH to adjust its reaction to *p*H 7·2.

The growth obtained on good media when 5 per cent. of the fluid which

had become alkaline was added, was too sticky to remove from the surface of the agar; neutralisation of the fluid (to *p*H 7·2) before using it gave smooth creamy growth once more.

It appears, therefore, that exposure to the air quite rapidly causes a change in ascites fluid, which renders it more than ordinarily difficult to use and for that reason we abandoned it.

Horse serum and laked horse blood. The serum was obtained in the ordinary way by allowing sterile blood to clot. The laked blood was obtained by drawing horse blood into an equal volume of sterile distilled water, it haemolysed immediately, subsequently a clot separated and the haemoglobin stained serum was used to supply the accessory growth factors. Both these substances were added to the medium immediately before use in quantities varying between 1 and 5 per cent. and they stimulated growth nearly, but not quite, as well as human ascites fluid.

We discontinued their use because they exhibited a similar change to that described in ascites fluid and with the same effect, without presenting any other property of particular advantage.

Formol serum was prepared as described by Nicolle, Debains and Jouan (1918). Legroux (1920), using horse serum. It was added to our media immediately before use in the quantity directed and our results were less satisfactory than those obtained with ordinary serum or laked blood. This substance also became alkaline when exposed to air and again after neutralising.

It is a curious fact that formol serum prepared as directed does not clot when autoclaved, it becomes more opalescent and a smell of H_2S is evolved; and that whole serum to which 2 per cent. of formalin has been added does not coagulate when heated at 100° C., or even when boiled, but when added to medium containing 2 per cent. of agar and our usual salts coagulation takes place on steaming or autoclaving.

Extracts of red corpuscles, prepared in the manner described by Agulhon and Legroux (1918), Legroux and Mesnard (1920), when added to media immediately before use gave results slightly inferior to those yielded by our ordinary media, to which the accessory growth factors were added before sterilisation. The only remark we have to add is that the addition of the extract before autoclaving only slightly reduced its efficiency. These extracts were designed to replace fresh blood in media on which it was required to grow Pfeiffer's bacillus.

Gordon's extract of pea-flour was used in preparing "Trypagar" as described by Gordon, Hine and Flack (1916). We did attempt a few experiments in which this extract was added to our media, but with little success as the results were always inferior to our standard method.

When this substance was used by Gordon and others, when the medium was supplied on a large scale during the epidemic of cerebro-spinal fever in the British Army, it was always accompanied by horse serum or laked rabbit blood, to be added to the medium before use. This fact made us less interested in it

than we would have been had it unfailingly grown the meningococcus in primary culture without the additional substances.

Freshly drawn horse blood. The addition of fresh blood to media is a well-established method of promoting the growth of delicate organisms and it is usual either to add it to the melted and cooled medium, or to smear the surface with blood, but each method suffers from inconveniences we wish to avoid. The coagulation of blood in the medium by heat has had its advocates. While certain of them are content with an opaque medium rather resembling a slab of chocolate, others have removed the coagulated protein by specialised methods with the idea of retaining the accessory growth factors, which otherwise, they claim, are destroyed or removed (Lloyd, 1916). The method we advocate, which has given results only surpassed by *freshly drawn* ascites fluid and perhaps the method we shall presently describe, depends upon the coagulation of fresh blood in the presence of agar and the subsequent removal of the clot, without interfering with the accessory growth factors required by the meningococcus in any degree we can detect. At the same time the method has this advantage, that no elaborate nor troublesome technique is required either for the removal of the coagulum or the sterilisation of the finished medium. When all the ingredients of the medium have been added and the melted agar has been cooled to 50°—55° C., 7 per cent. of freshly drawn horse blood is added, with stirring to make a homogeneous mixture, which is then gradually raised to 100° C. in the steamer. By keeping it at this temperature for 30 minutes a considerable shrinkage of the coagulum is produced. The agar is then allowed to cool and set and so is *left overnight*. We consider it to be important to let the agar set in the presence of the coagulated blood, as we have tried on many occasions the effect of removing the coagulum immediately after heating at 100° C. and without preliminary setting of the agar, but the result has always been most unsatisfactory. It is possible that the alteration of the physical state of the agar during setting plays some part in the process. The next morning the agar is melted, decanted off from the coagulum which will have contracted further and strained through surgical lint in a hot-water funnel; finally the clot is tipped into the filter and after draining the remaining medium is gently wrung out by twisting up the lint. After the reaction of the filtrate has been adjusted to *p*H 7·2, it is distributed as required and sterilised in the autoclave at 120° C. for 20 minutes. The resulting medium is glass clear, although there may be a small flocculent deposit which rapidly settles out, and the virulent meningococcus grows readily in primary culture. The removal of the coagulum is facilitated by carrying out the process in straight-sided enamelled pails, within which are placed closely fitting muslin bags, held in position by strips of cane. When coagulation has taken place the strips of cane are removed and the bag is gently drawn to one side; the bag holds back the clot while the medium is decanted and eventually, after draining, the bag and contained clot are wrung out into the lint filter.

We wish to comment on a few points arising out of this method:

The quantity of blood used is the maximum which we found gave us a manageable coagulum; greater quantities were inconvenient and did not seem to possess any advantage. Better results seem to have been obtained when the reaction of the medium in which the blood was coagulated had been adjusted to *p*H 7·0—7·2, rather than when it was definitely acid or alkaline.

We tried various methods of separating the coagulum, such as filtration through glass wool, paper and lint, also simple decantation, but we were unable to detect any difference in the growing power of the product. Filtration through lint possesses the great advantage of being a rapid process with very little loss of material.

Sterilisation by steaming at 100° C. on three successive days showed no advantage over autoclaving. In fact we find that this medium stands several autoclavings on different occasions at 120° C. for 20 minutes, without appearing to change in any way other than becoming clearer.

In the course of our work it has frequently been necessary to make a series of media, in small amounts, in which some one constituent varied. This was greatly simplified by the fact that the coagulation of blood in a solution of agar in extract, with the subsequent addition of the other constituents, was quite as effective as coagulating the blood in the otherwise finished medium.

We call this form of medium "*EB* agar" (Extract, Blood) and "*EDB* agar" when the digest has been added.

Fresh heart muscle. Under certain conditions it is difficult to get a sufficient quantity of freshly drawn blood to make large amounts of medium in the manner described and it occurred to us to try to use the heart muscle from which we make our extract and digest. But before proceeding we wish to repeat that we only use freshly killed meat.

Attention has already been drawn to the copious coagulum we observed in making our ordinary extract, when it was raised to 100° C., but we wish now to emphasise another closely related point. When the meat has been extracted at 70° to 75° C. for three hours the fluid is a rich tawny red colour, which changes when heated at 100° C. to a pinkish yellow, with the separation of the coagulum and on autoclaving a further change of colour to a bright yellow takes place, with the separation of a fine whitish precipitate. At each stage the fluid has a bright, crystal clear appearance.

We therefore filter the extract immediately after its three hours at 70° to 75° C., in the manner described under the method of making the extract, and into this filtrate we stir an equal volume of a 4 per cent. solution of agar in ordinary extract cooled to 70° C. The whole is then raised to 100° C. and treated strictly in the manner described for coagulating blood in the medium, with the exception that the muslin bags are dispensed with because there is not so much coagulum to remove. The resulting filtrate is not so clear as when blood has been used but its transparency can be much improved by entangling the fine coagulum in a precipitate of phosphates, as described under methods of

clearing medium. But we find it advisable not to clear the medium thoroughly as definitely better results are obtained when it is slightly cloudy.

This preparation, which we call "*EH* agar" forms the basis of our medium and it keeps well stored in this form after autoclaving. To it we add the desired quantities of digests and salts and adjust the reaction, to make the finished medium which we speak of as "*EHD* agar" (Extract, Heart, Digest). This medium is no whit inferior to "*EDB* agar," in fact we are rather inclined to judge it as better. The accessory growth factors in this form are resistant to autoclaving and we strongly recommend the method for making media for general purposes.

It is best to avoid autoclaving "*EH* agar" more frequently than is absolutely necessary as the finely suspended matter tends to agglomerate and settle out as a reddish deposit, leaving the agar perfectly clear and the growth obtained on this very transparent medium is erratic and in the case of highly virulent strains it may be uncertain. In this respect "*EHD* agar" differs from "*EDB* agar" which is unaffected by thorough filtration or the clearing effect of repeated autoclaving.

If, however, the deposit is added to part of a plate of perfectly clear "*EHD* agar," satisfactory growth occurs on that part of the surface overlying the deposit; and if the fine precipitate is evenly distributed through the medium before use, perfectly satisfactory growth is obtained over the whole surface. For this reason we are content at present with the slightly cloudy medium obtained by filtration through lint and do not clear it by means of the phosphate precipitate or any other method.

(*i*) *The influence of agar-agar.*

Certain influences of agar have been discussed (Section II) but there are a few other points worth considering. Agar in "powder" or other "specially prepared" form is less satisfactory than plain "bleached fibre" (China grass); principally because the prepared agar is much less constant in the character of the jelly furnished by different batches.

Although treatment with acid probably increases the imbibition of the agar, we have not observed any particular advantage exhibited by agar so treated.

There is, however, one disadvantage in washed agar used wet, that it absorbs and holds about eight times its dry weight of water in spite of squeezing it as hard as it will allow; when the concentration of dry agar used in the medium is 2 per cent., this water dilutes the extract used by 16 per cent., and in order to avoid this in experimental media we used to dry the agar after treating it with acid (0·01 per cent. H_2SO_4) and washing until acid free, on the lines indicated by Cunningham (1919). We have, however, abandoned this process in favour of four washings with distilled water, wringing the agar as dry as possible in a clean cloth between each.

Up to the present we have not investigated the influence of agar on the growth of the meningococcus, although we have noted some observations

which tend to show that it is not the inert substance it is usually supposed to be.

The concentration of agar we favour in our medium at present is 2 per cent. of dry fibre, principally because this gives a sufficiently firm jelly, enabling us to scrape the growth off its surface without risk of taking up pieces of agar which would falsify our weighings. Nevertheless a more ready growth is obtained on lower concentrations and the work of Jenkins (1921) on the gonococcus is instructive; although we are not inclined to confine the activity of agar to concern moisture only.

In our opinion the influence of agar is one of the difficult factors to control in making media and concerning which very little is known.

(*j*) *Summary of the method of making "EHD agar."*

(1) *The extract.* Ox heart muscle, which has been killed not more than 24 hours, is freed of fat, minced, but not too finely and suspended in twice its weight of distilled water in an open can in a steamer and its temperature raised gradually to 70°–75° C., with occasional stirring to prevent the surface layer getting too hot. This temperature is controlled by a thermometer in the mixture. After 3 hours the temperature is allowed to rise to 100° C. for 15 minutes to get a firm coagulum. It is then filtered through its own meat, as described under (*a*) of this section. *Do not filter through lint or paper.* This furnishes the extract, which can either be autoclaved and stored, or used immediately to make a 4 per cent. solution of agar.

(2) *The "EH agar."* Proceed as in making the extract until the end of the three hours' heating at 70° to 75° C., then filter through the meat. To this filtrate is added an equal volume of the 4 per cent. solution of agar in extract after cooling it to 70° C.; this mixture is returned to the steamer and raised to 100° C. for one hour. It is then *left to set* and stand overnight. The next morning it is melted and strained through lint. This filtrate is the "*EH* agar," which can be autoclaved and stored if not required immediately.

(3) *The finished "EHD agar."* To the required amount of melted "*EH* agar" are added: 0·25 per cent. NaCl, 0·02 per cent. KCl, 0·01 per cent. $CaCl_2$ and the desired concentration of digest (Section V). The reaction is adjusted to *p*H 7·2 and the medium distributed as required and autoclaved at 120° C. for 20 minutes.

(4) *The digest.* The residual meat and fine coagulum filtered off from the extracts are suspended in a quantity of *N*/100 HCl equal to the weight of original raw meat. This bulk is raised to 100° C. and then autoclaved at 130° C. (25 lbs. to the sq. in.) for 30 minutes, then cooled to 37° C., incubated 24 hours and its sterility assured. Then 2 per cent. of sterile pancreate extract is added and left in contact with the HCl for 5 to 15 hours to activate the trypsin, after which 0·8 per cent. anhydrous Na_2CO_3 is added in the form of a sterile 32 per cent. solution. This strength of Na_2CO_3 readily dissolves at 37° C. Digestion is allowed to proceed until a "Sörensen figure" of not less than 20 is produced

(see (*b*) of this section). When this result has been obtained, 2 per cent. of 10*N* HCl is added and the digest autoclaved and while still hot filtered through paper in a hot funnel. The acidified digest filters quite as rapidly as it is slow when alkaline. At least one advantage of strict sterile precautions is that the digest retains a pleasant meat-like smell throughout the process, both in the alkaline and the acid condition. Any objectionable smell or traces of H_2S detected shows that contamination has occurred. The filtrate is put into clip-top milk bottles and autoclaved at 120° C. for 20 minutes, and repeated autoclaving has no apparent deleterious effect.

V. The Influence of the Constitution of the Medium on Virulence.

Attention has already been drawn to our observation that the killing power of a given strain of meningococcus was greater or less according to the medium on which it was grown (Section IV (*b*)) and that we were then inclined to attribute the alteration in the physiological state of the parasite, resulting in a lowered virulence, to the contamination of our digest by certain other organisms during digestion. We have not been able to demonstrate that such contamination has this definite effect.

Nevertheless this primary observation induced us to investigate the relation of growth to medium, not so much from the point of view of the mass yielded by the medium as the physiological state of the growth, measured by its capacity to kill mice inoculated intraperitoneally.

This line of research has yielded what we venture to think are important results and we propose to consider them in some detail.

The killing power of a culture is estimated by determining the Minimal Lethal Dose in the way described by Murray (1924, p. 177), and, in our attempt to determine what constitutes a good medium, although we regard variation in killing power as more important, we have not neglected to observe the mass of growth yielded. At the outset we discontinued the use of contaminated digests and confined our attention to those made with strict sterile precautions, with the intention of reverting to our first observation by means of controlled growth, of known organisms, in digests which proved to give good results.

As has been stated, our early sterile digests had a much lower "Sörensen figure" than did the contaminated ones and our first endeavour was to produce sterile digests with a concentration of amino-acids quite as high as that which the contaminated digests appeared to possess. This was particularly desirable as we thought that the general amino-acid concentration, indicated by the "Sörensen figure," might afford a means of standardising media both from the point of view of virulence and growth.

The following experiment bears directly upon these ideas and the results are very instructive. We prepared three solutions:

(*a*) In 3000 c.c. of extract we dissolved 2 per cent. of washed and dried agar, 0·5 per cent. NaCl, 0·125 per cent. $CaCl_2$ and adjusted the reaction to

*p*H 7·2. This solution was then filtered through lint which had been washed with boiling distilled water.

(*b*) 250 c.c. of digest No. 109, with a Sörensen figure of 32·0 c.c. N/10 NaOH on 10 c.c. of digest, was treated like the extract in (*a*).

(*c*) 650 c.c. of digest No. 118, with a Sörensen figure of 13·1 c.c. N/10 NaOH on 10 c.c. of digest, was also treated like the extract in (*a*).

These three solutions were then mixed in the proportions shown in Table VI to make media *A*, *B* and *C*. The reaction was checked with the interesting result that it needed readjusting in each of the *B* and *C* media. These were then distributed in 100 c.c. volumes in marked flasks and autoclaved at 120° C. for 20 minutes. When the media were used they were melted and cooled to 50° C. and 5 per cent. of ascites fluid was added, three plates of each were poured, all of which were inoculated consecutively from a growth of a fairly virulent strain, first generation from an egg culture on a medium known to give good results. A sixteen hours' growth on the various media was inoculated intraperitoneally into mice (= 2nd generation from the egg culture = 1st generation on the special media), two mice were used for each dose. The result of this experiment is shown in Table VI.

In considering these results it must be observed: Firstly, that the accessory growth factors were supplied by ascites fluid and for that reason growth was obtained on medium *A*; also that the ascites fluid used was then beginning to influence the sticky character of the growth (Section IV (*h*)). Secondly, that the concentration of NaCl added may be considered to be high (Section IV (*d*)), and that we did not take into account the salt content of the digests. Bearing these points in mind, the physical character of the growth is capable of interpretation. With regard to the mass of growth yielded by the various media, it is evident that the addition of digest not only increases the yield compared with medium *A*, but that there is some suggestion of an optimal range of concentration, which is more marked in the *C* media than the *B*. Further, the difference between the *B* and *C* groups of media bears no marked relation to the "Sörensen figure" increment due to the digest. At the same time there appears to be a definite decrease in the percentage of moisture in the growth yielded by the high concentrations of digest compared with the low, but this may be due to the contained salts (see Table V).

The outstanding feature of the experiment is the behaviour of the cultures towards mice. This shows definitely that there is an optimal range of concentration of digest, which alone affords the meningococcus such conditions that allow of its development of something essential to its manifestation of a parasitic existence. Furthermore, this "virulence range" appears to be independent of the increment of the "Sörensen figure" due to the digest, and, except in the case of the *C* media, it does not show any marked relationship to the mass of growth yielded. On the grounds of deficiency, it is not surprising that the effective range of the digest should exhibit a low limit, but it could not have been expected that an upper limit would be found; this suggests the

presence of an inhibition factor overpowering the effect of the presence of a sufficiency of the necessary substances. Yet this upper limit is definitely marked in both the *B* and *C* media.

Table VI.

Medium No.	% concentration in (*a*) of (*b*)	% concentration in (*a*) of (*c*)	Increment of Sörensen figure in c.c. *N*/10 NaOH on 10 c.c. due to added digest	Generation	Growth in mgms. per sq. cm. of 5 successive generations on each medium: Moist	Growth in mgms. per sq. cm. of 5 successive generations on each medium: Dry	Dry growth as a % of moist	Character of growth	Killing power of the first generation on each medium (2 mice to each dose): Dose in mgms. of living culture per 20 gms. mouse	Killing power of the first generation on each medium (2 mice to each dose): Result
A	0	0	0	1	1·02	0·18	17·5	All slightly sticky	8	Both lived
				2	0·80	0·13	16·7			
				3	0·54	0·09	16·7		4	,,
				4	0·60	0·10	17·2			
				5	0·95	0·17	18·2		2	,,
B 1	2	0	0·64	1	1·35	0·25	18·2	All slightly sticky	8	Both died
				2	1·23	0·20	16·2			
				3	1·23	0·21	17·3		4	,,
				4	1·16	0·20	17·7			
				5	1·28	0·26	20·0		2	One died
B 2	8	0	2·56	1	1·32	0·25	18·7	Sticky	8	Both died
				2	1·61	0·31	18·9			
				3	1·50	0·28	18·6		4	,,
				4	1·53	0·28	18·8			
				5	1·46	0·30	20·5		2	One died
B 3	32	0	10·24	1	1·43	0·29	20·3	Very sticky (2nd gen. too sticky to scrape properly)	8	Both died
				2	(0·9)	(0·15)	16·9			
				3	1·37	0·25	18·2		4	One died
				4	1·00	0·20	20·2			
				5	1·55	0·31	20·0		2	Both lived
C 1	0	5	0·65	1	0·99	0·20	20·1	Sticky	8	Both died
				2	0·81	0·13	16·4			
				3	1·36	0·26	19·2		4	Both lived
				4	1·28	0·24	18·6			
				5	1·71	0·32	18·4		2	,,
C 2	0	20	2·60	1	1·58	0·31	19·8	Very sticky (5th gen. too sticky to scrape properly)	8	Both died
				2	1·55	0·33	21·1			
				3	1·13	0·23	19·9		4	,,
				4	1·35	0·30	22·2			
				5	(0·8)	(0·15)	19·2		2	,,
C 3	0	80	10·40	1	—	—	—	Sticky	8	One died
				2	1·10	0·25	22·7			
				3	0·74	0·19	25·5		4	Both lived
				4	0·87	0·22	25·0			
				5	0·70	0·19	26·6		2	,,

In each of eight experiments performed, using different digests, we obtained results strictly like those detailed above. Experiments of this nature present many difficulties when working with the meningococcus, besides involving the use of a considerable number of mice and it is not always possible to insure that all the factors contributing to success will be in working order at the desired moment. In our experience the "virulence range" of a digest is difficult to determine if the strain used for the test is of a very high order of virulence, as the range may then appear to be unnaturally extended. In practice we

prefer to employ and get best results with a strain 2 mgms. of which kills 20 gms. of mouse within 14 to 48 hours, when grown on the medium of optimal concentration of digest. A strain exhibiting a Minimal Lethal Dose of 4 mgms. is often useful, but one with a Minimal Lethal Dose of 1 mgm. kills when grown on media which are not the best. We believe that the medium giving best results with an organism of failing virulence, probably is also the best for the very virulent.

The highest "Sörensen figure" we have met with in our sterile digests was one of 49; the lowest observed effective concentration of this digest, as determined by virulence, was 1·8 per cent., a concentration of 0·54 per cent. gave a good yield of growth but of markedly reduced virulence; the highest effective concentration observed was 15 per cent., but higher concentrations were not tried for virulence, but 21 per cent. gave a reduced yield of growth.

The digests exhibiting a "Sörensen figure" of not less than 20 had a wider effective "virulence range," particularly noticeable in that it extended to much lower concentrations of digest in the medium, than those other digests whose "Sörensen figure" was in the region of 12 to 15. It is for this reason that we consider digestion sufficiently advanced for our purpose, only when the "Sörensen figure" has a value of not less than 20 (see Section IV (*c*)).

One other experiment may be quoted in detail, as it illustrates our general experience of the relation of growth to virulence in the type of experiments now under consideration. In this case the basis of the medium was "*EB* agar" (Section IV (*h*)) and the salt concentrations were 0·25 per cent. NaCl, 0·01 per cent. $CaCl_2$ and 0·02 per cent. KCl (Section IV (*d*)). The concentrations of digest and general results are shown in Table VII.

Table VII.

Medium	% concentration of digest 150	Increment to *Sörensen figure* due to the digest in c.c. *N*/10 NaOH to 10 c.c. of medium	*Arithmetical mean of* Moist growth in mgms. per sq. cm.	Dried growth in mgms. per sq. cm.	Dried growth as a % of moist growth	M.L.D. in mgms. per 20 gms. of mouse (1st gen. on medium)
A	1	0·24	2·8	0·49	18·6	8
B	2	0·48	3·2	0·57	17·8	2
C	4	0·96	3·2	0·58	18·5	2
D	8	1·92	3·0	0·55	18·1	4 (±2)
E	16	3·84	2 plates were contaminated, 1 used for mice. Further generations not continued			8

It is evident, that although a marked difference in killing power exists between the growths obtained on the various media, the differences in the yield of growth per unit area of medium are relatively slight and certainly would not be appreciated by inspection. For reasons such as the result of this experiment, we do not believe that the yield of growth is necessarily a reliable index of a good medium for highly specialised parasitic bacteria. The variation in the percentage of moisture, which is the chief source of error in carefully measured doses of weighed growth, cannot be held accountable for the differences in killing power of the cultures.

As the result of this enquiry, we were naturally forced to the conclusion that the standardisation of a medium for the meningococcus, is a much more difficult and delicate matter than our previous experience and the literature of the subject had led us to believe.

By taking into consideration the "virulence range" of the digest and by adjusting the various other factors discussed in Section IV, we evolved our "*EDB/V*" and "*EHD/V*" medium (*V* = virulence). The yield of growth and degree of constancy of this medium has been discussed in Section III (Table I), where it is shown to be the best medium we know at present.

Although we have shown quite definitely that this "virulence range" exists, we have no idea of its underlying cause; and we have not succeeded in devising a sufficiently delicate test whereby to determine the optimal point in the range. Our present practice, in making the medium, is to use an amount of digest a little higher than the lowest effective concentration determined by the killing power of the cultures on mice. This requires that each digest has to be titrated by experiments using the Minimal Lethal Dose of a suitable culture as the indicator, and, although it may be considered a troublesome necessity, we are convinced that it is well worth while. In order to minimise the labour we make amounts of digest varying between two and six litres, and, since the required concentration is only in the region of 2 per cent. of digest, these large volumes make a considerable quantity of effective medium.

We may now reconsider the question raised concerning the effect of contamination of the digest in the course of its manufacture. We took a sterile digest, the "virulence range" of which we had determined, neutralised and contaminated a portion of it with *B. subtilis*. That organism would only grow with great difficulty in the concentrated digest, although abundant, typical growth was obtained in dilutions. In time, however, sufficient growth had taken place for a deposit of fluffy balls of bacilli to accumulate at the bottom of the vessel, but no surface film was formed, though abundant typical growth resulted with subculture on to ordinary media. This purposely contaminated digest was then filtered through a sterile Pasteur-Chamberland candle "*F*" and the filtrate was used in varying concentrations to make media. So far as we could discover, the "virulence range" had not been altered by the contamination. The experiment was repeated with the same result. But two things must not be lost sight of: firstly, the degree of contamination was comparatively slight for a freely growing organism like *B. subtilis*; and, secondly, our original digests were contaminated by a varying mixture of organisms, including anaerobes.

Before proceeding further with this type of experiment we thought fit to examine some of the old "non-sterile" digests we had kept. Varying amounts of seven different digests, which had been used for making "*EDB/N*" medium, were pooled; in a portion of this we dissolved 2 per cent. of dry agar and this solution was mixed with *EH* agar in the proportions shown in Table VIII. In adding the salts allowance was made for the NaCl content of the digest and

the final concentrations were 0·25 per cent. NaCl, 0·01 per cent. $CaCl_2$ and 0·02 per cent. KCl. The media were inoculated with a strain of good killing power, from a culture first generation from egg (M.L.D. = 2·0 mgm. of growth on *EHD/V* for 20 gms. of mouse).

Table VIII.

Medium	% concentration of pooled "non-sterile" digest	Increment of Sörensen figure due to digest	Growth in mgms. per sq. cm.		Dried growth as a % of moist	M.L.D. in mgms. per 20 gms. of mouse (= 1st gen. on these media = 2nd gen. from stock egg)
			Moist	Dried		
A	0·5	0·18	2·2	0·32	14·8	4
B	2	0·71	2·5	0·38	15·4	2
C	8	2·85	3·0	0·49	16·6	2
D	32	11·39	2·1	0·40	19·2	4

From the result of this experiment (shown in Table VIII) it is quite evident that the pooled digest has a "virulence range," in spite of having been contaminated during digestion. But we now regret that we did not examine each of the digests separately, for it is probable that they had individual differences.

Our original conception that contamination altered the digest, to an extent which interfered with the virulence of the cultures grown on it, is shown by the above experiment to be without foundation.

The difference we originally observed between certain "contaminated" and "sterile" digests, in regard to the virulence of the growths they respectively yielded, is readily explained by the fact that the media were then made by adding the amount of digest required to give a definite increment in the Sörensen figure. This method we have since shown to be unreliable as the Sörensen figure bears no relation to the "virulence range" of the digest; this is amply demonstrated in Tables VI, VII and VIII. Even though contamination of the material during the course of digestion does not decrease its value, we strongly advise the adoption of strict sterile precautions, if only to avoid the very objectionable smell which often results from contamination. Sterile digests have, in fact, a very pleasant and appetising smell.

VI. The relation of Medium to Viability and Maintenance of Virulence.

In the foregoing sections we have discussed the influence of the constitution of the medium upon physiological characters of the meningococcus which can be measured in terms of reproduction *in vitro* and adaptation to a parasitic existence. In the present section we wish to consider briefly a few scattered observations on the duration of the life and the virulence of cultures of that organism *in vitro*.

It is generally agreed that the life of a culture of meningococcus on the ordinarily used media is short and it is very commonly stated that the culture may die within 48 hours. Various media have been considered satisfactory because stock cultures remained alive for one or two months, and it is quite

likely that the strains used had been subjected to subculture before the test was performed. In our experience, before we had investigated the facts detailed in the preceding sections, Dorsett's egg medium maintained the life of meningococcal cultures better than the other media we had tried. A large proportion of cultures on egg could be recovered when they were a year old and only an occasional one died in less than six months, but absolute certainty only prevailed with monthly subculture. In any case direct subculture from an egg slope a month or more old on to agar media was often a matter of difficulty, it frequently failed to give any growth and quite commonly only a few scattered colonies resulted, even with quite heavy inoculation. Subculture from egg to egg gave better results, but, even so, the appearance of only a few scattered colonies was a far too frequent occurrence; and these scattered colonies commonly had to be subcultured daily for a few generations before vigorous growth obtained. Thus it became our usual practice, when growth of a particular strain living on egg was required on agar medium, to resort to an intermediate young subculture on egg.

When we first observed the difference in virulence of cultures grown on particular samples of *EDB/N* and *EDB/S* media, we were examining the influence of repeated subculture on the growth of the meningococcus. Two series of subcultures were being run at the same time: one at 12 and the other at 24-hourly intervals. In the 24-hourly series there was a marked initial lag with each subculture, but in the 12-hourly series growth appeared more and more rapidly with successive subculture, until, after a few generations, 4 hours' incubation yielded a considerable growth. In the course of 36 days the *EDB/N* medium with which we had started came to an end and we proceeded with a batch of *EDB/S*; these were the same batches of media on which we had noticed the difference in virulence, but we did not at first observe any marked difference in the growth as these generations were carried out on slopes, in test-tubes plugged with wool in the ordinary way. We had previously noticed that cultures which were kept at 37° C. for 14 to 30 days, occasionally gave rise to scattered colonies superimposed on the old growth, so these various cultures were all kept for a month to watch for this secondary growth, without any precautions to prevent drying of the medium. Out of 108 cultures grown on *EDB/N* (No. 86) (71 from the 12-hour and 37 from the 24-hour series) only 4 showed secondary growth; whereas of 37 cultures grown on *EDB/S* (No. 88) (25 of the 12-hour and 12 of the 24-hour series) every one gave good secondary growth.

In view of the experiments described in Section V, the outstanding feature of this observation depends upon the fact that growth yielded by *EDB/S*, No. 88, was virulent, whereas the same strain grown on *EDB/N*, No. 86, failed to kill mice. Backed by our observation on virulence in relation to these media, secondary growth immediately became a character of importance in our eyes, and caused us to think of the possibility of making a medium which would maintain a culture alive for a considerable time without loss of virulence.

At this time we thought these important differences might be due to the digest having been contaminated or not during its preparation and we resolved to test the viability of cultures on *EDB/N* and *EDB/S* media, with the result that in this case the non-sterile proved to be the better, but still fell far short of egg for this purpose, as is shown in Table IX.

Table IX.

Medium kept at 37° C. with waxed plugs	Subcultured from	Number of cultures	Number shown to be alive 9 months later, by subculture on *EHD/V* medium
EDB/N No. 72	Bacteriolysis Expt. 50, XVII	24	14
EDB/S No. 138	Bacteriolysis Expt. 50, XVII	26	7
Egg	*EDB/N* 72	18	18
Egg	*EDB/S* 138	16	15

Media 72 and 138 showed an identical increment in the "Sörensen figure" due to added digest; but there is considerable evidence that the concentration of digest 122 used for making No. 138 and other media was not favourable for virulence, and some evidence that No. 72 was a better medium from this point of view. No very precise information on this point is available because we had not yet recognised the principle of the "virulence range."

The recognition of the "virulence range" was immediately applied in the form of our early *EDB/V* medium and when this was used we observed that there was a rapid regrowth of the meningococcus over the area from which the original growth had been cleanly removed. This observation was followed by the experiments relating to inorganic salts in the medium (Section IV (*d*)) and the relation of regrowth of the culture to potassium salts was consequently noticed.

Previously we had tried a medium described by Wadsworth (1903), a weak agar jelly containing about 75 per cent. of serum or ascites fluid, in which the pneumococcus maintained its virulence at a constant level for several weeks (Wadsworth and Kirkbridge, 1918); some cultures of meningococcus died quite soon and others survived for a long time in this medium. But we were struck by the advantages of the weak agar jelly and the benefit of being able to dispense with waxed plugs. We therefore made *EDB/V* medium, containing the required amount of digest and salts but only 0·5 per cent. of agar; this medium we call "*F*." In order to have present the accessory growth factors required by the meningococcus, our practice is to dilute "*EB*" or "*EH* agar" with extract to which we have added the required amount of digest and salts, and, after adjusting the reaction, to distribute it in wool-plugged tubes under a layer of liquid paraffin and autoclave it.

We have not had this medium in use sufficiently long to be able to discuss its properties fully, but we may say that it promises well, for the following reasons: The meningococcus grows readily in it in primary culture from cerebro-spinal fluid and a profuse growth is obtained on subculture on to our ordinary medium (*EHD/V*), even when the culture is five months old. The

Minimal Lethal Dose of this culture was 2 mgm. for 20 gms. of mouse when put into "*F*" medium and it showed no alteration in killing power in four months; after five months it killed more often than not in a 2 mgm. dose (slightly irregular) and with absolute certainty in a 4 mgm. dose for 20 gms. of mouse. This is a considerable improvement on our experience with egg medium. Furthermore we have never worked with such virulent strains as those isolated on "*F*" medium.

During the last three years we received seventeen cultures of freshly isolated strains of meningococcus from private friends and as the result of an appeal by the Secretary of the Medical Research Council and the Principal Medical Officer of the Ministry of Health. These were grown on various media of which we know nothing, nor do we know how often they had been sub-cultured before we received them, but the relation of the Minimal Lethal Dose of the cultures to the medium on which they were sent to us is set out in Table X and has certain points of interest.

Table X.

Minimal Lethal Dose for 20 gms. of mouse	>8 mgms.	8 mgms.	4 mgms.	2 mgms.	Totals on each medium
Dorsett's egg	2	2	0	3	7
Inspissated serum	6	1	0	0	7
Trypagar	0	0	0	1	1
Unknown agar	0	0	0	1	1
EDB/N	0	0	1	0	1
Totals of each M.L.D.	8	3	1	5	17

The striking feature of these results is that no strain received on inspissated serum was virulent; but the other figures are difficult to interpret without further investigation of the problem.

We are greatly indebted to Professor H. R. Dean for his personal appeal to a wide circle of workers, asking them to collect primary cultures on our media and they have kindly consented to help us in this direction. During the last three months we have received ten suitable cultures on "*F*," egg and serum; each set having been inoculated direct from the same sample of cerebro-spinal fluid. Up to the present all the cultures on "*F*," nearly all on egg, and certain of the serum cultures have been virulent. Of these two were received from the same source as the serum cultures given in Table X.

It is yet too early to discuss these results; but at present it appears that the *primary culture* may be virulent even when the medium is not the most suitable, although on subculture its virulence may be lost. Of the virulent cultures shown in Table X, we know that the one we received on "Trypagar" was inoculated direct from the cerebro-spinal fluid.

There is one other aspect of the maintenance of virulence *in vitro* which deserves consideration. It is well known that many parasitic bacteria lose their virulence if frequently subcultured at intervals of 24 hours and that the meningococcus is particularly apt to behave in this way. It is especially interesting, therefore, to notice the relation of killing power to successive

subculture at 24-hourly intervals on *EHD/V* medium and the results of two such experiments are given in Table XI.

Table XI.

Experiment	Generations at 24-hourly intervals	Dose of living meningococci per 20 gms. of mouse (2 mice were inoculated with each dose) 8 mgms.	4 mgms.	2 mgms.	1 mgm.	0·5 mgm.
A	1	+ +	+ +	+ +		
	2		+ +	+ +	+ +	
	4		+ +	(+)(+)	(+)(+)	(+) 0
	5	+ +	+ (+)	+ +		
B	1		+ +	+ +	+ +	
	2		+ +	+ +	+ 0	+ 0
	3	+ +	+ +	+ (+)	+ 0	
	4	+ +	(+) 0	(+)(+)		
	5	+ +	+ +	+ (+)		
	6	+ +	+ +	+ (+)		

+ = one mouse died in under 48 hours.
(+) = one mouse died between 48 and 86 hours.
0 = one mouse definitely survived.
Blank space = dose not tried.

These experiments suggest that it may yet be possible to produce a medium and a method of using it, which will allow of repeated subculture without loss of virulence. The fluctuations in the time the mice took to die seem to be greater than compared with the fluctuations in yield of growth shown in Table II (Section III), for here again the rise and fall may be irregular after the first two generations. It is conceivable that this fluctuation in killing power and yield of growth, may be determined by the proportion of dead cocci contained in a given mass of growth; this interpretation is particularly suggested by the fact that the mass representing one Minimal Lethal Dose of killed cocci, when the organisms are entire, is very many times greater than that representing one Minimal Lethal Dose of the given living meningococcus culture.

We readily admit that the observations contained in this section are most incomplete, but, at least, they indicate that much may yet be done by a thorough investigation of the influence of medium on the maintenance of viability and virulence of cultures *in vitro*.

VII. Discussion.

Dopter (1921, p. 416) remarks, that the immunisation of animals against the meningococcus is a very delicate process which presents many technical difficulties and this observation is emphasised by his discussion of the methods advocated by eminent authorities. There appears to be no doubt that the immunisation of horses, with the object of producing a potent therapeutic anti-meningococcal serum, is by no means accomplished with any degree of certainty. Flexner, Dopter, Gordon, Nicolle and others, have produced unassailable evidence that a highly potent therapeutic serum can be produced occasionally, but it is quite evident that failures have been a common experience. Similar failures appeared to us to call for a close study of the characters

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of meningococcal antigens and the present paper deals with part of this investigation.

A study of the literature of anti-meningococcal serum, reveals no convincing evidence of any character which might serve as a guide in the production of a successful serum. The virulence of the cultures used as antigens does not appear to have been investigated and the explanation of this undoubtedly lies in the fact that it is generally admitted that the attempted titration of meningococcal virulence has resulted in failure.

In this paper we have discussed in detail some of the inherent difficulties presented by the cultivation of the meningococcus *in vitro*, and, although we cannot claim to have made an entirely satisfactory medium, it will be admitted that we have established that the constitution of the medium exercises an important influence on the "virulence" of the culture.

We have no evidence to show how important it may or may not be, to use only highly virulent meningococcal cultures as antigens for the production of potent therapeutic serums. Those concerned with the production of serums commonly express as their opinion, that it is desirable to use only freshly isolated strains, but judging by their behaviour towards mice, the freshly isolated strains shown in Table X of this paper are very different organisms from those we are obtaining in primary culture on our "*F*" medium. Thus, "freshly isolated strain" becomes a term of no exact meaning without qualifying it by describing the properties of the medium. Possibly "freshly isolated" strains are more likely to represent correctly the prevalent agglutinable types.

A few months ago we started to immunise horses in terms of the degree of virulence of cultures (titrated in mice) and it is a matter for regret that this part of our investigation had to be abandoned, through circumstances not under our control, just at a time when we appeared to have mastered some of the chief difficulties in the manipulation of meningococcal virulence.

The work of Cotoni, Truche and Raphael (1922), although dealing with the pneumococcus, bears on this question with considerable weight. In discussing the protective power of active immunisation with vaccines and the production of potent protective serums, they repeatedly emphasise that satisfactory results have been obtained only when very virulent cultures were used. They even go so far as to say (p. 82) "It is impossible to obtain an active serum with an avirulent or slightly virulent pneumococcus" and (p. 78) "To prepare a multivalent serum it appears to us to be an absolute necessity to use a very virulent pneumococcus."

There is no doubt that the position of anti-meningococcal therapeutic serum still is most unsatisfactory. The identification of agglutinating types and the application of this knowledge was undoubtedly a step forward on the evidence of Gordon, Nicolle, Netter, but the production of a potent serum of any type is not a certainty. The use of virulent cultures as antigens may prove to be merely "clutching at a straw," but whether this is the case or not can only be known when the method has been tried. In any case it is

important to remember that a fundamental principle of Pasteur's active immunisation was to use successive doses of increasing virulence, and that we can make no claim to greater success to-day than he achieved.

The evidence we have brought forward is a step towards making it possible to test whether the virulence of a meningococcal culture bears any relation to its antigenic capacity. But for the present we must content ourselves with agreeing with Nicolle and Césari (1924, p. 76) that this information is most desirable.

We suggest that the experiments described in this paper show that the general question of culture media needs further investigation. There are certainly components in media which influence to a profound degree the physiological state of the micro-organisms grown on them. Since writing this paper we have read the interesting work of Felton and Dougherty (1924) who show that the virulence of a strain of pneumococcus can be enormously increased by repeated subculture in milk at intervals of 2 to 8 hours (p. 141), and that although similar subculture in meat extract or ordinary broth results only in lowering the virulence, an increased amount of "Peptone" will allow of it being maintained (p. 164). This work raises the hope that with further experiment it may be possible to maintain, or even increase, meningococcal virulence by suitable cultural methods.

It still remains an open question, whether the cultures we are accustomed to use may be considered to be normal healthy organisms and representative of their kind. That is to say, we do not know whether the physiological state of the organisms in our cultures *in vitro* is identical with that of those actively causing disease in their natural host. Up to the present we have not been able to detect a difference between naturally virulent primary cultures and those in which the virulence has been raised by the method described by Murray (1924, p. 194), but in both cases we are dealing with cultures.

In this respect it is interesting to note that we have seen, on several occasions, a definite early purulent meningitis in mice that have been inoculated intraperitoneally, with cultures grown on our medium standardised by virulence tests. Microscopically the scanty pus was quite typical of the disease and the meningococcus was recovered in culture. One of our most striking instances of meningitis in a mouse resulted from a culture whose virulence had been raised by the *in vitro* method. But since we have not made a systematic investigation of the point, no definite conclusion can be drawn.

The special advisory committee upon bacteriological studies of Cerebrospinal Fever during the epidemic of 1915 (Medical Research Committee, 1916, p. 20), in discussing culture media for the growth of the meningococcus, paraphrase Gordon (1916) as follows:

"The requirements of a good routine medium for the purpose have been stated as follows:

1. The meningococcus must grow on it readily and with certainty.

2. It must be easily and cheaply made and must not involve ingredients now difficult to procure in this country.

3. It must be of such a nature that it can be stored and sent out in bulk from a central laboratory.

4. It should preferably be transparent.

5. The viability of the meningoccccus on it should be as prolonged as possible."

Our *EDB/V* or *EHD/V* medium fulfils all these requirements quite as well and certain of them better than any of the many media we have tried. Furthermore, the evidence we have brought forward in this paper allows us to add another very important requirement: That the medium must allow the meningococcus cultures grown on it to develop and maintain the physiological characters contributing to virulence. We might also add requirements relating to the physical characters of the growth: particularly percentage of adventitious moisture.

We are only too well aware of the tentative nature of many of our observations and that a fuller investigation of many points would add to the value of our paper; but work of this nature could easily be prolonged for an indefinite time and still remain incomplete. It will be admitted, perhaps, that we have at least recognised a problem requiring solution and taken a step in a direction from which useful results may be forthcoming.

In conclusion we wish to thank those who have kindly sent us cultures and all who have promised to do so should they get cases. We are particularly indebted to Professor H. R. Dean and Dr Duncan Forbes, who have kindly made personal appeals for cultures to be sent to us on our own media.

Finally we wish to thank J. Bain and E. Pleasance for their painstaking and willing assistance, which has contributed so largely to the success of the work.

VIII. Conclusions.

(1) That it is extremely difficult to make any two batches of a given medium sufficiently alike to obtain identical cultural results with the meningococcus.

(2) That this is largely due to our insufficient knowledge of

(*a*) the raw materials required, and

(*b*) the relative concentrations of the ingredients necessary, to afford the optimal conditions required by the organism to develop their natural physiological state essential to a successful parasitic existence.

(3) That the yield and physical characters of the growth are insufficient criteria whereby to judge a given medium as good or bad, since the killing power of a culture appears to be to a certain degree independent of these. That the present state of our knowledge requires that several characters be examined simultaneously in judging a medium for the meningococcus; such as:

(*a*) the alacrity with which growth takes place,

(*b*) the yield of growth,

(*c*) the physical characters of the growth,

(*d*) the viability of the culture,

(*e*) the virulence, and

(*f*) the maintenance of virulence with age and subculture.

(4) With the kind of medium considered in this paper, a good deal of truth is expressed by saying:

(*a*) that the virulence of the culture is chiefly affected by substances contributed by the digest;

(*b*) that the added inorganic salts and possibly the physical state of the agar, contribute largely to the physical characters of the growth; although other factors are also concerned;

(*c*) that the yield and viability of the culture is determined by all the factors being correctly balanced.

(Elimination of by-products has not been discussed.)

(5) That there is an optimal range of concentration for tryptic digest of heart muscle, over which virulent cultures of the meningococcus are obtained.

(6) That media made with due consideration of the "virulence range" of the digest used, are favourable to the viability and maintenance of virulence of the culture.

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