ON VITAMINES, AMINO-ACIDS, AND OTHER CHEMICAL FACTORS INVOLVED IN THE GROWTH OF THE MENINGOCOCCUS.¹

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THE experiments recorded in the following paper were undertaken at the suggestion of the Medical Research Committee, in order to obtain some light, if possible, on the underlying principles which determine the success or failure of a bacteriological medium when used as a menstruum for the growth of the meningococcus of cerebro-spinal fever.

I. EVIDENCE THAT THE MENINGOCOCCUS UNDER CULTIVATION *IN VITRO* NEEDS THE PRESENCE OF AN ACCESSORY GROWTH FACTOR NOT FOUND IN THE ORDINARY NUTRIENT MEDIA.

There are several media which have been used with success by different workers (1916¹¹). These differ considerably in composition, but they all have one common feature, namely, the presence in varying amount of some fluid secreted by the living organism; for instance, blood, serum, or ascites fluid. Egg media, Vedder's medium of heart broth and starch, and Gordon's pea-flour tryptic-agar have also been successful.

It will perhaps make for clearness if it is stated dogmatically at this point, that after working for some months with various media, the writer has come to the belief that an essential feature in any medium required to grow the meningococcus is the presence of bodies of the type of the accessory growth hormones, or vitamins, which have already been shown by Hopkins (1912⁸) and others to be a *sine qua non* in mammalian diet. Evidence in support of this belief is given below.

The ordinary nutrient agar (*i.e.*, heart-broth peptone-agar) does not, as a rule, support the growth of the meningococcus unless the latter is an old laboratory culture, and unless it is inoculated very thickly on to the medium. It can be transformed into a satisfactory medium

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by the addition of various substances, all of which, however, have their origin in the metabolic activities of an organism. In order to test the power of such substances to stimulate growth in cultures of the meningococcus, 5 c.c. of melted nutrient agar were put into each one of a series of test tubes and the tubes were sterilised. After the final sterilisation, but before the agar had solidified, a few drops of the fluid under examination were added aseptically to each tube. The tubes were then sloped and inoculated. In every experiment the strain of meningococcus taken was emulsified with mammalian Ringer solution, and a series of tubes was inoculated from the same platinum loop. The growths obtained in any one series are therefore strictly comparable. The emulsions used were just cloudy, but not in any way to be considered thick. In all the tables 0 signifies no growth, (+) a trace, + good growth, ++ very heavy growth.

TABLE I.—February 27, 1916.

MEDIUM.	MENINGOCOCCUS STRAIN.					
	E.	Jn.	A.	M.	Cs.	Bs.
Heart-broth peptone-agar	0	0	0	0	0	0
„ + 2 drops fresh blood	++	++	++	(+)	++	++
„ + 0.5 c.c. sterile milk	+	+	++	0	+	(+)
„ + 0.5 c.c. "cow and gate" milk	(+)	+	+	(+)	+	(+)
„ + 0.5 c.c. "true-milk" milk	+	(+)	(+)	+	0	(+)
„ + 0.5 c.c. alcoholic extract blood	++	++	++	+	++	++
„ + serum	...	++	...	...	++	...

Note.—The alcoholic extract of blood was prepared by mixing fresh blood with four times its volume of alcohol and allowing to stand for some days. The clear extract was taken down to dryness, and taken up in distilled water. The "cow and gate" milk and the "true-milk" milk are commercial preparations, made by drying ordinary cows' milk. They appear to contain the vitamine factor, although to a reduced extent.

The organisms used in the experiments recorded in Table I. were laboratory stock. These, as will be shown later, are not nearly so dependent on an abundant supply of the accessory growth hormones as the freshly isolated strains. Nevertheless, they show quite clearly the striking difference in growth obtained by adding some such substance as blood or milk. This influence cannot be due to the additional amount of protein present, since the stimulating factors can be extracted from fresh blood by 80 per cent. alcohol.

In the earlier stages of the work the basal medium used was ordinary nutrient agar made with heart broth +: peptone (Allen and Hanbury's), 1 per cent.; salt, 0.5 per cent.; agar, 2 per cent.—the whole made neutral to litmus. Later, this was abandoned in favour of a tryptic digest of heart made according to the directions of Douglas (1914⁴). Douglas' tryptic-agar is a medium which

gives very free growth of the laboratory strains of meningococcus, but cannot be used alone, for primary cultures. Finally, to test the theory of the vitamine influence, media were made from a tryptic digest of casein (Casein, Ltd., Laitproto, No. 6), by the method given by Cole and Onslow (1916²). Since Hopkins and others have shown that ordinary commercial casein frequently contains traces of the accessory growth hormones, two vitamine-free media were prepared: (1) By using a digest of Hammarsten's pure casein; (2) by using a digest of commercial casein which had previously been purified by extracting for a week with 5 or 6 changes of 80 per cent. alcohol.

The pure casein was digested with trypsin according to Cole and Onslow's method, and the reaction of the broth was adjusted by their method, using phenol-sulphone-phthalein. One volume of digest was mixed with 2 volumes of a 3 per cent. solution of agar in water, and sufficient salt was added to make 0.5 per cent. In some experiments 0.1 per cent. glucose was added to the medium. The glucose is not an essential ingredient, and its presence does not materially alter the amount of growth.

It is shown in Table II. that laboratory strains of meningococcus grow on both heart-digest agar and casein-digest agar. Primary strains from the spinal fluid or the throat both need the addition of blood.

TABLE II.—April 13, 1916.

(See also Table VIII.).

MEDIUM.	MENINGOCOCCUS STRAIN.							
	Primary Cultures from Spinal Fluid.		Primary Cultures from Throat.				Laboratory Strains.	
	T.	Bn.	Ca.	M.	Ba.	Be.	L.	Re.
Douglas' tryptic-agar	0	0	0	0	0	0	+	(+)
" + fresh blood	++	++	++	++	++	++	++	++
Three days' digest of impure casein	0	0	...	...	0	0	0	0
" + fresh blood	++	++	...	...	+	+	++	++
Fourteen days' digest of casein	(+)	(+)	...	...	...	...	++	++
<i>Note.</i> —It will be shown later that the length of time during which digestion has proceeded has a decided effect on the readiness with which a strain of meningococcus grows on a blood-free medium.								

In the greater number of the experiments recorded in the tables given above, two or three drops of blood, added to a tube (5 c.c.) of medium, have served to stimulate the growth of the meningococcus markedly. In order to show whether the bacteria were using the blood proteins themselves as a food supply, or were stimulated by something in the blood to avail themselves of the peptones, etc., in the medium, a number of tubes were prepared with water-agar. This experiment was rendered even more necessary by the observation that

the bacteria grow fairly readily on a mixture of 3 volumes of defibrinated blood and 1 volume of 2 per cent. water-agar. The results are recorded below (Table III.) and show quite definitely that the blood, at the concentration used, is supplying a stimulating factor, but not the main food supply. It has already been recorded that the stimulating factor can be extracted from the blood by 80 per cent. alcohol, and it will be shown below that the blood proteins can be removed from the medium by heat without reducing its efficiency.

TABLE III.—*April 5, 1916.*

MEDIUM.	MENINGOCOCCUS STRAIN.		
	A.	Jn.	Re.
2 per cent. water-agar	0	0	0
„ + 3 drops fresh blood	0	0	0
Heart-broth peptone-agar	0	0	0
„ + 3 drops fresh blood	++	++	++

II. PROPERTIES OF THE ACCESSORY GROWTH FACTORS.

The evidence given above appears to the writer to justify the belief that blood contains an accessory factor (or factors) the addition of which to the ordinary media is essential for the growth of the meningococcus. In the early stages of the work, freshly drawn rabbit or human blood was used to supply this factor; later, defibrinated rabbit's blood was found to be satisfactory; finally, after the observation that sterilisation after adding the blood does not affect the medium unfavourably, defibrinated bullock's blood from the slaughter-house was taken into routine use.

A few experiments on the properties of this substance have been made, with the following results:—

1. *It is not readily destroyed by heat.*

TABLE IVa.—*January 29, 1916.*

MEDIUM.	MENINGOCOCCUS STRAIN.			
	A.	Jn.	Ca.	Re.
Heart-broth peptone-agar. (Control).	0	0	0	0
„ + 3 drops blood to each tube	++	++	++	4 colonies.
Blood-agar heated for one hour at 60° C.	++	++	++	3 „
„ heated for two and a half hours at 60° C.	++	++	6 colonies.	4 „
„ heated for one hour at 100° C.	++	++	++	9 „

TABLE IVB.—February 2, 1916.

MEDIUM.	MENINGOCOCCUS STRAIN.			
	A.	Jn.	Ca.	Re.
Heart-broth peptone-agar. (Control) . . .	0	0	0	0
„ + 3 drops blood	106 colonies.	51 colonies.	6 colonies.	3 colonies.
Blood-agar heated for three-quarters of an hour at 120° C.	92 „	28 „	5 „	5 „

2. *It is soluble in water and alcohol.*

This follows from Table I. given above.

3. *It is very readily adsorbed from solution.*

An account has already been published (1916⁹) of a method of preparing a transparent, colourless, blood-agar that has had the blood proteins precipitated by heat, but nevertheless contains the vitamine factor, and it was pointed out that the medium must on no account be filtered by the routine methods once the blood has been added. An account of the observations that led up to the devising of the technique will be given here.

The first observation that suggested that the vitamine factor was capable of being adsorbed out of solution was made during an early and unsuccessful attempt to prepare a transparent blood-agar. A fourteen days' digest of casein was taken, and the reaction adjusted to $P_H = 7.35$ by using phenol-sulphone-phthalein as indicator. To every 100 c.c. of this broth was added 5 c.c. of defibrinated bullock's blood, and the mixture was boiled and filtered. An agar medium

TABLE V.—June 24, 1916.

MEDIUM.	MENINGOCOCCUS STRAIN.					
	Bl.	By.	G.	Pr.	Ha.	Ha Spinal Fluid.
Tryptic heart-agar + blood	+++	+++	++	+++	+++	+++
„ coagulated and decanted	+++	+++	++	+++	+++	+++
„ coagulated and filtered	0	0	0	0	0	0
Tryptic casein-agar + blood, decanted	+++	++	(+)	+	+	++
„ filtered	0	0	0	0	0	0

was made by mixing 1 part of the filtrate with 2 parts of 3 per cent. water-agar. The resulting medium was in no way better than an ordinary tryptic casein-agar free from blood. A second attempt was made by adding the agar to the broth first, and then the blood. The mixture was again boiled and filtered, and gave a medium considerably improved but conspicuously less satisfactory than that which was obtained by adding the blood to the melted agar in the culture tubes, heating, and allowing the resulting clot to remain in the bottom of tube. It therefore seemed possible that in the first case the vitamine factor had been adsorbed and carried down by the coagulated proteins ;

and in the second, had been protected by adsorption on to the agar particles. In both cases the possibility became apparent that part of the vitamine might have been lost in the filter paper. To test this possibility a tryptic heart-agar and a tryptic casein-agar were melted, mixed with 5 per cent. of defibrinated blood and steamed. A portion of each medium was then cleared by decanting, the remainder was filtered through paper pulp on a Buchner funnel. The media were sown with five strains in early subculture and also from an infected spinal fluid. See Table V.

A final test was made by taking heart-agar as above. Part of this was cleared by centrifuging, part by passing through paper pulp on a Buchner funnel. The remainder was allowed to stand till nearly clear, the clear fluid was strained through glass-wool. Of the centrifuged portion part was tubed for inoculation, the rest was poured through a Chardin filter paper, and then tubed. Inoculation gave the following results:—

TABLE VI.—*July 5, 1916.*

MEDIUM.	MENINGOCOCCUS STRAIN.			
	Hs Spinal Fluid.	Pr.	G.	Bl.
Tryptic heart-agar + 5 per cent. blood, coagulated by heat. Filtered on Bucher funnel . . .	0	0	0	0
„ Centrifuged	+	++	++	++
„ Centrifuged and filtered through Chardin paper	0	0	0	0
„ Strained through glass-wool	+	++	++	++
Tryptic casein digest + blood. Coagulated and strained through glass-wool	+	++	++	++

The evidence therefore seems to suggest that the vitamine factor is a substance that can be adsorbed by protein (as already suggested by Hopkins, 1912⁶) and by filter paper. This makes the filtering of a vitamine medium by the routine methods an impossibility. Such a medium can be cleared by the centrifuge or by decantation. The latter tends to leave specks in the medium, but these can be removed by straining through a plug of glass-wool, either in a common funnel by gravitation, or on a Buchner funnel under slightly reduced pressure. The glass-wool does not appear to adsorb the vitamins.

4. *It gives a considerable viability to Meningococcus grown in vitro, the conditions regarding moisture being satisfactory.*

Strain J was used. A blood-nutrient-agar slope was inoculated on 10th December 1915. It was placed in the incubator in a screw-top bottle over 24 per cent. sulphuric acid. Successful subcultures were taken on 11th, 12th, 13th, 14th, 18th, and 20th December 1915; 7th, 13th, and 21st January 1916; 2nd February 1916; and 5th April 1916. The last culture taken, therefore, was on the 117th day. This prolonged life of the strain *in vitro* is similar to the results found by Elser and Huntton (1909⁵), using ascitic-agar slants as the culture medium.

Blood media contrast strongly with the pea-flour tryptic-agar as regards viability. In the following experiment ten strains were subcultured on to the latter on 18th January 1916. Subcultures from these on to blood-nutrient-agar slopes were made on 19th January. Further subcultures from both sets of tubes were made to test the viability on the two media. The subcultures were made on the dates given on to blood-agar slopes. When these were successful they are marked in the table with a +. In testing to see whether a culture was living, subcultures were made by inoculating as much as possible of the solid growth on to a blood-agar slope.

TABLE VII.

PEA-FLOUR TRYPTIC-AGAR TUBES.	MENINGOCOCCUS STRAIN.									
	Bt.	Bg.	Ba.	Be.	Ch.	Ct.	E.	Hr.	M.	Sr.
Tubes inoculated, January 18, 1916 .	...	...	...	...	...	...	...	...	...	...
Subcultured, " 19, " .	+	+	+	0	+	+	+	+	+	+
" " 21, " .	+	(+)	(+)	...	0	+	+	+	+	+
" " 22, " .	0	0	0	...	0	0	0	0	+	+
" " 24, " .	...	...	...	...	...	...	...	...	+	+
" " 31, " .	...	...	...	...	...	...	...	...	+	+
" February 21, " .	...	...	...	...	...	...	...	...	0	0
BLOOD-AGAR SLOPES.										
Tubes inoculated, January 19, 1916 .	...	...	...	...	...	...	...	...	...	...
Subcultured, " 21, " .	...	...	...	...	...	...	...	...	...	...
" " 22, " .	...	...	...	...	...	...	...	...	...	...
" " 24, " .	+	+	+	...	+	+	+	+	+	+
" " 31, " .	+	+	+	...	+	+	+	+	+	+
" February 21, " .	+	+	+	...	0	+	+	0	+	0

Though the presence of the stimulating factors of blood undoubtedly gives long life *in vitro*, it will be shown below that the factors are not necessary to prolong the life of subcultures once these are growing freely on media made from well-digested protein. The discussion of this point will be resumed later.

III. CHANGES IN THE METABOLISM OF THE MENINGOCOCCUS AS A RESULT OF SUBCULTURE.

A striking feature in testing the various media under examination is that as a strain passes through various stages of subculture, it becomes less and less dependent on an abundant vitamine supply. In common parlance, it becomes "easier" to cultivate in the laboratory. This is, of course, no new observation; it has been recorded innumerable times of all the more "difficult" pathogenic bacteria. It seems, however, to the biochemist who has intruded into the realms of

TABLE VIII.A.—*Meningococcus Strain, Rt.*

MEDIUM.	NO. OF CULTURE IN VITRO.								
	May 18, 1916.	May 19.	May 20.	May 21.		May 22.	May 23.	May 24.	May 25.
	Primary Culture from Spinal Fluid.	1st Sub-culture from Blood Medium.	2nd Sub-culture from Blood Medium.	3rd Subculture from Blood Medium.		4th Sub-culture from Casein Digest.	5th Sub-culture from Casein Digest.	6th Sub-culture from Casein Digest. Inoculated thickly from Loop.	7th Sub-culture from Casein Digest.
				Inoculated thickly from Loop.	Inoculated thinly from Emulsion.				
Tryptic digest heart	0	0	0	0	0	(+)	(+)	++	++
" + fresh blood	++	++	++	++	++	++	++	...	...
Tryptic digest of commercial casein. .	0	0	0	++	0	+	+	+	++
" + blood	++	++	...	...	...	...	...	...	...
Tryptic digest of Hammarsten's casein .	0	0	0	0	0	0	0	(+)	0
" + blood	++	++	...	..	...	...	...	...	...
Tryptic digest of extracted casein . .	0	0	0	(+)	0	(+)	0	(+)	+
" + blood	++	++	...	...	...	...	...	...	...

TABLE VIIIb.—*Meningococcus Strain, JI.*
(Primary Culture and First Subculture on Pea-flour Tryptic-Agar).

MEDIUM.	NO. OF CULTURE IN VITRO.									
	May 18.	May 19.	May 20.	May 21.		May 22.	May 23.	May 24.	May 25.	
	3rd Sub-culture from Pea-flour Tryptic-Agar.	4th Sub-culture from Blood Medium.	5th Sub-culture from Heart Digest.	6th Subculture from Blood Medium.		7th Sub-culture from Heart Digest.	8th Sub-culture from Casein Digest.	9th Sub-culture from Casein Digest. Inoculated thickly from Loop.	10th Sub-culture from Casein Digest.	
Tryptic digest heart . . .	...	+	(+)	++	++	++	+	++	++	
" + blood . . .	...	++	++	...	++	...	...	...	...	
Tryptic digest commercial casein . .	0	(+)	0	++	+	++	+	++	++	
" + blood . . .	++	++	...	...	...	...	...	...	...	
Tryptic digest of Hammarsten's casein .	0	0	0	0	0	(+)	0	+	0	
" + blood .. .	++	++	...	...	...	...	...	...	...	
Tryptic digest of extracted casein . .	0	0	0	0	(+)	+	(+)	+	++	
" + blood . . .	+	+	...	...	...	...	...	...	...	

bacteriology, that the "difficult" bacteria have been so classed because in the early history of cultivation *in vitro*, they did not grow freely on the media then commonly employed. It seems highly likely that the success of the more modern media depends on an abundant vitamine supply. The preceding tables (pp. 120, 121) show the stages passed through by the meningococcus in laboratory cultures. It is readily seen that the organism becomes increasingly capable of sustaining its life on the amino-acids present in the medium, and decreasingly dependent on the stimulating power of fresh blood. The latter finally becomes superfluous, though the fact that while even in old cultures which grow freely on Douglas' tryptic-agar and Cole and Onslow's digest of impure casein-agar, growth is not obtained easily on agar made from a digest of pure protein, suggests very strongly that a minimal quantity of the stimulating factors are always essential to the medium. Indeed, until a hundred colonies can be obtained for every hundred germs sown, there is always the possibility that some of the germs sown die off and release a vitamine supply to the survivors. This is undoubtedly what happens when successful cultures are obtained by a thick sowing on to ordinary nutrient agar.

The adaptive change in metabolism shown by the freshly isolated meningococcus when grown *in vitro* suggests the possibility that the stimulating action of the accessory growth hormones may be due to a property of increasing the velocity of the chemical changes involved in metabolism. Since the meningococcus grows after several subcultures on heart-broth agar of three hours' digestion, it seemed possible that evidence might be obtained on this point by studying the comparative rates of the adaptive change on trypsin digests which differed among themselves only in their degree of digestion. Accordingly, a digest was made as follows:—

60 grms. casein dissolved in 600 c.c. 1 per cent. sodium carbonate.

12 c.c. pancreatic extract was added plus 15 c.c. chloroform.

Digestion was allowed to proceed at 37° C.

100 c.c.	withdrawn after 1 day.
100 "	" " " 3 days.
100 "	" " " 5 "
100 "	" " " 7 "
100 "	" " " 9 "

The broth was neutralised to $P_H = 7.35$, using phenol-sulphone-phthalein as indicator, and was mixed with agar as described above.

Inoculation with a fresh strain of meningococcus gave the following results (Tables IXA. and IXB.). It should be noted (see also Tables VIIIA. and VIIIB.) that the number of subcultures through which a strain has to pass before it grows freely on blood-free media varies with the strain; also that by sowing a solid mass of bacteria direct from a loop, they can be induced to grow on the blood-free media at almost any stage.

TABLE IXA.—*Meningococcus Strain, Ha.*

MEDIUM.	June 20.	June 21.		June 22.	June 23.	June 24.	June 25.	June 26.	June 27.	June 28.	June 29.
	Primary Culture from Spinal Fluid.	Inoculated from Loop.	Inoculated from Emulsion.	2nd Subculture.	3rd Subculture.	4th Subculture.	5th Subculture.	6th Subculture.	7th Subculture.	8th Subculture.	9th Subculture.
		1st Subculture.									
Casein digest-agar, 1 day .	0	+	0	0	0	0	0	0	0	+	+
" " 3 days .	0	(+)	0	0	0	0	0	0	0	(+)	+
" " 5 " .	0	(+)	0	0	0	0	0	0	0	0	+
" " 7 " .	0	+	0	0	0	0	0	0	+	+	+
" " 9 " .	0	+	0	0	0	0	0	0	0	+	+
Tryptic heart-agar .	0	(+)	0	0	0	0	0	0	0	+	...
" " + blood .	+	...	+	+	+	+	+	+	+	+	...

TABLE IXB.—*Meningococcus Strain, Bl.*
(Primary, 1st, and 2nd Subculture on Pea-flour Tryptic-Agar.)

MEDIUM.	June 17-20.		June 21.		June 22.	June 23.	June 24.	June 25.	June 26.	June 27.
	3rd-6th Subculture.		Inoculated from Loop.	Inoculated from Emulsion.	8th Subculture.	9th Subculture.	10th Subculture.	11th Subculture.	12th Subculture.	13th Subculture.
			7th Subculture.							
			+	+						
Casein digest-agar, 1 day	0	0	+	+	0	0	+	0	0	+
" " 3 days	0	0	0	0	0	0	0	+	+	+
" " 5 "	0	0	(+)	0	0	0	0	(+)	+	+
" " 7 "	0	0	(+)	0	0	+	+	+	+	+
" " 9 "	0	0	0	0	0	+	+	+	+	+
Tryptic heart-agar	0	0	0	0	0	0	0	0	0	0
" " + blood	+	+	...	+	+	+	+	0 (!)	+	+

A few experiments on the viability of subcultures on blood-free media are given in Table X.

TABLE X.—*Meningococcus*.

MEDIUM.	STRAIN Hs—ORIGINAL CULTURE, June 28.					
	SUBCULTURED					
	July 1.	July 4.	July 7.	July 13.	July 21.	July 28.
Casein digest-agar, 1 day .	+	+	+	+	+	+
„ 3 days .	+	+	+	+	+	+
„ 5 „ .	+	+	+	+	0	0
„ 7 „ .	+	+	+	+	+	+
„ 9 „ .	+	+	+	+	+	+
Tryptic heart-agar .	0	0	...	...	...	...
„ + blood .	+	+	+	+	+	0
	STRAIN B1—ORIGINAL CULTURE, June 30.					
	SUBCULTURED					
	July 2.	July 4.	July 18.	July 21.	July 28.	August 4.
Casein digest-agar, 1 day .	+	+	+	0	...	...
„ 3 days .	+	+	+	+	+	+
„ 5 „ .	+	+	+	+	+	+
„ 7 „ .	+	+	+	+	+	+
„ 9 „ .	+	+	+	+	+	+
Tryptic heart-agar .	+	+	+	+	+	0
„ + blood .	+	+	+	0	...	...

IV. FOOD REQUIREMENTS OF THE MENINGOCOCCUS. RELATION OF THE VITAMINE SUPPLY TO THE AMINO-ACID SUPPLY.

Two features appear to the writer to stand out strongly after surveying the work that has been published to date on the artificial culture of the meningococcus. The first is the importance of the vitamine factor. If the media employed by various workers are classified, they fall under four main headings—(1) Blood media; (2) serum media, including media made with ascites or hydrocele fluid; (3) Dorset's egg medium (1913³); (4) vegetable media, containing starch, legumin, etc. (Gordon, 1916⁷, and Vedder, 1915¹³). Any of the first three groups of fluids may be assumed to contain vitamins. In the case of the blood media, I have given evidence in this paper that the meningococci are not feeding on the blood itself (in the concentration employed), nor are they in any way benefited by the extra proteins added, since these can be removed without reducing the efficiency of the medium. The simplest deduction to be drawn, therefore, is that the growth produced is due to stimulation by the accessory growth

hormones, or vitamines. There remain to be considered Vedder's starch medium, and Gordon's pea-flour tryptic-agar. Vedder's starch medium probably contains vitamines adherent to the starch grains. Commercial corn-flour is the form of starch employed, and it ought to be possible to test this point by using starch that has been rigorously extracted with 80 per cent. alcohol. In Gordon's medium, again, the possibility of vitamine influence cannot be excluded. He ascribes the influence of the saline pea-extract to the addition of protein in the form of legumin, but Hopkins and others have shown that proteins, unless specially purified, always carry down a greater or less amount of vitamine as adsorbed impurities. It is therefore possible that Gordon is dealing with a vitamine influence, and it is certain that the stimulation of growth is not due to additional protein. The accessory growth hormone, or vitamine, theory cannot be regarded as finally proved until those elusive bodies have been isolated, purified, and added as such. It is hoped that the observation made during the course of the present work, that the stimulating factors in a clear, optically homogeneous blood-agar can be removed to a great extent and with amazing rapidity by simply pouring the melted medium through filter paper, may prove of practical assistance in the attempts to isolate them. It is the writer's belief that the vitamine influence is not only qualitative but quantitative. The fact that laboratory strains of meningococcus can be made to grow on almost any medium, if sown thickly enough, supports this idea. Foster and Gaskell (1916⁸), in their book on "Cerebro-Spinal Fever" (p. 150), state that "for purposes of subculture it is essential to sow thickly." If this is their experience, I think the cause lies with the medium and not with the meningococcus, since in all the experiments recorded in this paper (except where otherwise stated) the tubes have been sown from an emulsion in Ringer's solution that might be described as "just cloudy." Such a dilution when sown from a loop on to a blood medium gives in twenty-four hours a growth over the whole surface of inoculation, so thick that the colonies are confluent and cannot be counted. An emulsion so dilute that the transparency of the Ringer is barely affected, gives in the same time from one loopful about a hundred well-grown isolated colonies on the surface of the slope.

Besides the vitamine factor, the meningococcus needs for its growth a certain proportion of the products of protein hydrolysis. It is interesting to note here that Bainbridge (1911¹) and Sperry and Rettger (1915¹⁰) have shown that a large number of non-pathogenic bacteria, including the typical "putrefaction organisms," cannot grow in a synthetic medium in which a pure protein forms the only nitrogen supply; whereas, if a trace (0.1 to 0.2 per cent.) of commercial "peptone" is added to the medium, they can attack not only the peptones but also the higher proteins of the medium. Rettger, Berman, and Sturges (1916¹²) have shown that the value of a commercial

"peptone" depends on its content of free amino-acids, and that in the absence of the latter, even the putrefactive organisms are unable to develop the proteolytic enzymes which are necessary for them to attack such relatively simple bodies as the peptones and proteoses. Meningococcus grows well in the presence of additional vitamins, on a medium in which proteolytic decomposition has not proceeded to a great extent, *e.g.*, heart broth that has been trypsinised for three hours. After a certain number of subcultures, it will grow on the tryptic heart-agar without further additions. This medium almost certainly cannot be regarded as vitamin free. Meningococcus grows very freely in primary culture on a tryptic digest of casein (ten to fourteen days' digestion) if blood is added. On one occasion I have obtained a primary culture direct from the spinal fluid (strains T and Bn), on to the simple tryptic casein-agar, though the resulting growth was considerably less than that obtained by inoculating a blood-agar slope on the same occasion. After a number of subcultures, varying in number from one to twelve or more, the meningococcus grows with great freedom on the digested casein medium without any addition of body fluids. This medium contains free amino-acids in great quantity. When subculturing any strains and following their history, it is generally found that they will grow much sooner on the digested casein-agar (ten days' digest), than on the digested heart-agar (three hours' digest). I attribute this to the greater proportion of free amino-acids in the former medium. The evidence given in Table X. also suggests that the longer the casein has been digested, the fewer subcultures have to be passed through before the meningococcus can use it unadulterated as a medium for growth. These facts seem to me to point to a correlation between the amount of amino-acid essential to a medium and the amount of vitamin present, and suggest that the more there is of the former the less there need be of the latter. This possible relationship, at present unproven, and the slow adaptive change exhibited by the strains under laboratory conditions, whereby finally they are able to dispense very largely with their vitamin supply, suggest that the stimulating factors which are undoubtedly present in body fluids may act as catalysers, quickening the reaction velocity of the proteolytic metabolism, and that the failure of growth, in their absence, may be attributed, not to the impossibility of a certain reaction being able to take place, but to the fact that it cannot occur with sufficient rapidity to supply the needs of the growing organism.

VIABILITY OF THE CULTURES.

Besides the conditions of successful subculture, there remains to be discussed the question of the viability of the cultures on the various media under discussion. Briefly it may be stated as follows: On tryptic heart-agar it is frequently short, a few days; on tryptic heart-

agar, or ordinary nutrient agar, with added blood, it is long, three weeks to six months or more; on well-digested casein-agar, it is long. These facts, therefore, could be claimed in support of the catalyses theory of vitamine action; in the tryptic heart digest there is probably a low content of free amino-acid, sufficient to start the culture, but insufficient to maintain it. In the same medium, with added blood, the stimulating factors in the blood so increase the proteolytic powers of the organism that it can manufacture the amino-acids that it needs. In the thoroughly broken-down casein medium there is a sufficient quantity of free amino-acid present to maintain the culture over a considerable period of time.

It is perhaps well to emphasise at this point the very potent influence of moisture on the viability of cultures. All the culture tubes used by me in my researches have been kept persistently in a moist incubator. In a dry incubator, at 37° C., cultures on blood slopes die in a few days; by adopting the simple precaution of placing a beaker of water in the incubator, the life of the cultures is prolonged to three weeks, or even longer; by sealing into closed vessels, the viability can be prolonged to several months. Gaskell uses Vedder's starch medium in the form of stabs for maintaining the life of his cultures. In these, the surface of the medium is a concave meniscus, and any moisture exuding from the main bulk of the medium would tend to collect in the hollow. He finds that if the stab sets obliquely, or if the agar cracks in the tube, then the cultures die off very rapidly. Finally, it seems justifiable to attribute the long life of cultures sealed into tubes of Dorset's egg medium to the fact that they are kept on a vitamine-rich medium under conditions of constant moisture.

SUMMARY.

1. The primary cultivation of the meningococcus *in vitro* is only possible in the presence of certain accessory growth factors (the so-called vitamines) present in blood, serum, milk, and other animal fluids, and probably present also in vegetable tissues.

2. These accessory factors are bodies which are moderately heat stable. They are soluble in water and alcohol. They are rapidly adsorbed from solution by filter paper, but do not appear to be adsorbed by glass-wool.

3. There is a relationship of the inverse order between the amount of amino-acid present in the medium and the amount of vitamine required to stimulate the growth of laboratory strains, and it is therefore suggested that the action of the accessory growth factors is to increase the reaction velocity of the proteolytic metabolism of the meningococcus.

4. The meningococcus, after isolation from the body, gradually develops a change in its metabolism of such a nature that it becomes

increasingly independent of a vitamine supply in the medium. Old laboratory strains need no additional vitamine supply if the medium contains an abundant supply of free amino-acids.

5. The main food requirements of the meningococcus are the products obtained by the hydrolysis of a protein, namely, amino-acids.

6. Cultures of the meningococcus must be kept permanently in a moist atmosphere, in order to retain their vitality.

APPENDIX I.

Key to Strains referred to in the Text.

All the strains of meningococcus used in the work which has been summarised in the present paper have been supplied to me from the 1st Eastern General Hospital, Cambridge, and I should like to offer my thanks to Captain Gaskell, R.A.M.C., for his invariable kindness and courtesy, and for much help always readily extended.

A key to the strains is given below. The dates given refer to the first isolation only.

TABLE XI.

STRAIN.	DATE OF ISOLATION.	SOURCE.	REMARKS.
A . . .	March, 1915.	Lumbar puncture.	Chronic case.
Be . . .	January 6, 1916.	Throat.	Chronic carrier.
Bg . . .	" "	"	" "
Bl . . .	June, 1916.	"	Temporary carrier.
Bn . . .	March, 1916.	Lumbar puncture.	Acute case.
Bs . . .	January 6, 1916.	Throat.	Chronic carrier.
Bt . . .	" "	"	Temporary carrier.
By . . .	June, 1916.	"	" "
Ch . . .	January 6, 1916.	"	" "
Ct . . .	" "	"	Chronic carrier.
Cs . . .	" "	Lumbar puncture.	Acute case.
E . . .	" "	Throat	Chronic carrier.
G . . .	June, 1916.	Lumbar puncture.	Acute case.
Hr . . .	January 6, 1916.	Throat.	Temporary carrier.
Ha . . .	June, 1916.	Lumbar puncture.	Mild summer case.
Jl . . .	May, 1916.	" "	Chronic case and carrier.
Jn . . .	" 1915.	" "	" "
M . . .	January 6, 1915.	Throat.	Chronic carrier.
Pr . . .	June, 1916.	Lumbar puncture.	Acute case.
Re . . .	January 1, 1916.	" "	Chronic case.
Rt . . .	May, 1916.	" "	Acute case.
Sr . . .	January 6, 1916.	Throat.	Chronic carrier.
T . . .	March, 1916.	Lumbar puncture.	Acute case.

APPENDIX II.

Preparation of a Transparent Blood-Agar.

1. As a basal medium either a tryptic digest of heart (Douglas), or a tryptic digest of casein (Cole and Onslow), may be taken. Both give good results with the meningococcus. On the whole, I have obtained better growth in primary cultures by using the heart broth, but the casein broth has the theoretical advantage of approaching more nearly to a synthetic medium, and the practical advantage of being very easily prepared and standardised.

2. Adjust the reaction of the broth to be faintly alkaline ($P_H=7.35$), using phenol-sulphone-phthalein as the indicator.

3. To every 100 c.c. of broth add 2 grms. of powdered agar and place in the steamer till the agar is dissolved.

4. Filter the melted medium through filter paper. This will lower the temperature.

5. To the melted medium add 5 per cent. defibrinated bullock's blood. The temperature should be about 65° to 75° C.

6. Replace the blood-agar in the steamer for about three-quarters of an hour.

7. Centrifuge the medium to remove the clot. It is advisable to heat up the centrifuge vessels beforehand.

8. Pour off the clear medium, tube and sterilise at 100° C., according to the usual routine method.

The medium can be cleared by an alternative method. After adding the blood (stage 5):—

6a. Transfer the medium to an Erlenmeyer flask and replace in the steamer for three-quarters of an hour.

7a. Remove the flask from the steamer and allow it to stand and set. By this time the clot will usually have settled as a compact mass at the bottom of the flask. It is useless to attempt to strain the medium until the clot has become quite compact. If necessary the medium can be steamed a second time.

8a. Place the flask in the steamer until the medium has melted.

9a. Strain through a layer of glass-wool about $\frac{1}{8}$ inch deep in a Buchner funnel. A litre of medium can be cleared in a few minutes if the straining is done under slightly reduced pressure.

10a. Tube and sterilise at 100° C.

This medium is considerably better than pea-flour tryptic-agar for both primary and secondary strains of the meningococcus, and has also been used successfully for the isolation and cultivation of *Micrococcus cattharalis*, the pneumococcus, staphylococci, and streptococci, *B. Influenziæ*, *B. diphtheriæ*, *B. xerosi*.

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