GROWTH REQUIREMENTS OF THE MENINGOCOCCUS

IVAN D. FRANTZ, JR.

Department of Bacteriology and Immunology, Harvard Medical School, Boston[1]

Received for publication September 16, 1941

The meningococcus has been considered one of the more fastidious microörganisms with respect to growth requirements. Mueller and Hinton (1941) have shown, however, that excellent growth of this organism, as well as of the gonococcus, may be obtained on an agar medium containing only "double strength" meat infusion, casein hydrolysate, and starch paste. The purpose of the present investigation was to determine the essential factors in these mixtures.

EXPERIMENTAL

The organism used for the experiments was a type I meningococcus, isolated from a case of meningitis in Halifax, Nova Scotia. After approximately three transplants outside the human body, the organisms were emulsified in skimmed milk, sealed in small glass tubes, and quickly frozen in alcohol and solid carbon dioxide. The tubes were kept in a dry ice refrigerator. A fresh tube was used each day, so that the culture used for inoculation could be kept as constant as possible.

Experiments were carried out with liquid rather than solid media. The volume of medium in each tube for most of the experiments was 5 ml. The tubes were incubated in candle jars, in a slanted position. Slanting the tubes, to expose a maximum surface, was found to be very helpful. At first the organisms grow near the surface. Shaking the tubes after a few hours' incubation will make the growth more diffuse, and is another useful expedient. Use of an atmosphere containing carbon dioxide is quite important.

For estimation of the amount of growth, an inexpensive homemade photoelectric turbidimeter was used. Because of errors

[1] Aided by a grant from the Commonwealth Fund.

due to slight color in some of the media, and also uncertainty concerning the importance of variations in physical properties of the different suspensions, final conclusions were based on determination of bacterial nitrogen, by Pregl's micro-Kjeldahl method (Pregl, 1930; Mueller, 1935). Actually, for many experiments visual comparison gives almost as much information as the more refined methods.

The inoculum used was 0.1 ml. of a barely visible turbid suspension in water of a six-hour culture of the organisms studied, grown on the starch-agar medium mentioned above.

First it was learned that good growth could be obtained in a liquid medium containing only casein hydrolysate and double strength meat infusion. Starch proved to be unnecessary. Optimum growth occurred with 200 ml. of meat infusion, and casein hydrolysate corresponding to 5.5 grams of casein, in a total volume of 1 liter.

The meat infusion gave a positive test for protein, but it could be completely replaced with a dialysate which appeared to be protein free. Before attempts at further fractionation of the meat infusion, it seemed expedient to try to replace it with substances possibly present in it and known to be important in the nutrition of other organisms. Accordingly, mixtures of various salts and growth factors, including calcium pantothenate, nicotinic acid, choline, vitamin B_6, flavin, potassium, magnesium, phosphate, and glucose were tried. Replacement of the meat infusion was effected with a solution of potassium chloride, magnesium chloride, sodium phosphate, and glucose. Heavier growth was obtained with this combination than with the original cruder medium. The vitamins tried failed to enhance definitely the growth in any dilution.

These results left casein hydrolysate as the only remaining unknown. Accordingly, a mixture of 18 of the amino acids, in the proportion in which they occur in casein, was made, in an effort to replace the casein hydrolysate. It was felt that this very useful hydrolysate might contain small amounts of growth factors essential for the meningococcus, in addition to its amino acid content. Such did not prove to be the case, however, since the

amino acid mixture was equally satisfactory. The list of amino acids was eventually shortened to eight without detrimental effect, as follows:

For 1 liter of medium

Glycine	16 mgm.	*l*-Cystine	12 mgm.
dl-Alanine	70 mgm.	*l*-Oxyproline	8 mgm.
l-Serine	20 mgm.	*d*-Glutamic acid	1.3 grams
dl-Phenylalanine	150 mgm.	*d*-Arginine	200 mgm.

Finally, this list was further shortened to *d*-glutamic acid, 1.2 grams, and *l*-cystine, 12 mgm., by the addition of ammonium chloride.

The final medium has the following composition in grams per liter:

d-Glutamic acid	1.3	NaCl	6.0
l-Cystine	0.012	NH_4Cl	1.25
$NaH_2PO_4 \cdot H_2O$	2.5	$MgSO_4 \cdot 7H_2O$	0.6
KCl	0.09	Glucose	5.0

Considerable variation in the various concentrations is allowable, but the amounts given resulted in maximum growth. Omission of any one of them results in complete failure of growth, or its great delay. The high concentration of phosphate was used because of its effectiveness as a buffer. The pH was adjusted to 7.3, which seemed to be the optimum. The tubes were autoclaved for 10 minutes at 10 pounds pressure. Magnesium was added with a sterile pipette to the individual tubes after they were autoclaved and cooled, to avoid precipitation of magnesium phosphate. This inconvenience may be circumvented by substitution of sodium glycerophosphate for the sodium phosphate. If glucose is also added after autoclaving, to prevent slight caramelization which occasionally occurs, care must be taken to shake the tubes thoroughly before incubation.

If a young culture is used for inoculation, growth is visible in the tubes after 6 hours, and increases for 48 hours or more. After 48 hours the bacterial nitrogen is usually about 0.40 mgm. in 5 ml. of medium. Similar values were obtained with 11 other type I strains, 2 type II strains, and two strains classified as

atypical, all in early generations and all from cases of meningitis or carriers of meningococci in Halifax. Only one strain tried, a type I, failed to grow.

DISCUSSION

In view of the commonly held opinion that the meningococcus is difficult to cultivate, the simplicity of this medium is surprising. The medium works poorly, however, when agar is added and plates are poured. In view of the excellence of the starch medium in solid form, more work will be needed to learn the cause of this difficulty. Furthermore, primary isolation of the organism on the medium has not been attempted.

Although many of the amino acids used were synthetic, the two finally found to be essential were of natural origin. Study of these from the standpoint of the possible presence of impurities essential for growth is indicated.

SUMMARY

Fourteen of fifteen strains of meningococci in early generations have been cultivated on a liquid medium containing two amino acids, glucose, and inorganic salts.

I wish to thank Dr. J. Howard Mueller for his constant advice, without which this work would not have been possible, and Miss Jane Hinton, for her technical assistance and helpful suggestions. I am also greatly indebted to Dr. Leo Rane, of the Massachusetts Antitoxin and Vaccine Laboratory, for advice, numerous materials, and many ideas acquired during a month spent in his laboratory. I understand that Dr. Rane, independently and simultaneously, has also cultivated a strain of meningococci, after only one transfer outside the human body, on a medium differing only in details from the one described above. Ideas for many of these experiments came from the pneumococcus medium of Rane and Subbarow.

REFERENCES

MUELLER, J. HOWARD 1935 Studies on cultural requirements of bacteria. IV. Quantitative estimation of bacterial growth. J. Bact., 29, 383–387.

MUELLER, J. H., AND HINTON, J. 1941 A protein-free medium for primary isolation of the gonococcus and meningococcus. Proc. Soc. Exptl. Biol. Med., In press.

PREGL, F. 1930 Quantitative Organic Microanalysis. P. Blakiston's Son and Co., Philadelphia.

RANE, L., AND SUBBAROW, Y. 1940 Nutritional requirements of the pneumococcus. I. Growth factors for types I, II, V, VII, VIII. J. Bact., **40**, 695–704.