ON THE GROWTH REQUIREMENTS OF NEISSERIA GONORRHOEAE

R. GORDON GOULD, LEWIS W. KANE AND J. HOWARD MUELLER

Department of Bacteriology and Immunology,[1] *Harvard Medical School and School of Public Health Boston, Mass.*

Received for publication October 18, 1943

As Lankford, Scott, Cox and Cooke (1943) have recently pointed out, it is still very generally believed, although without valid evidence, that the gonococcus requires complex substances such as ascitic fluid, blood or serum for growth. It is well known that the gonococcus is slow-growing and is more exacting than most species of bacteria in its requirements for moisture, salt concentration, pH of the medium, incubation temperature and carbon dioxide and oxygen concentrations, but there is as yet no evidence that its actual requirements for specific growth factors are complex. Boor and Miller (1931) have grown stock strains on a liquid medium consisting of an enzymatically digested protein with added mineral salts and glucose, and Mueller and Hinton (1941) have shown that an essentially protein-free medium of casein hydrolyzate, meat infusion, starch and agar gives excellent growth of both stock and freshly isolated strains.

Specific growth requirements for variant or atypical strains have recently been reported. Lankford and Snell (1943) have found that 10–15% of strains require glutamine for primary isolation. These strains however, continuously form mutant colonies which do not require glutamine, so that this requirement probably cannot be considered as typical of the gonococcus, although it is of importance in the isolation of certain strains.

Normal strains after some weeks of daily subculturing on the Mueller-Hinton medium have a tendency to develop a requirement for glutathione, as previously reported (Gould and Mueller, in press), but here again this is a requirement of atypical strains.

The effect of cystine on the growth of the gonococcus is not well understood. Boor (1942) found that the gonococcus differed from the meningococcus in requiring cystine, but that other sulfur compounds and even sulfur itself were able to take its place, so that it may be concluded simply that the gonococcus requires sulfur in some form. This is in accord with the observations on the glutathione-dependent strains which have presumably lost the ability to synthesize glutathione from simpler compounds. The high optimal cystine concentration (0.025–0.075%) reported by Boor is at variance with the observations of others (MacLeod *et al.*, 1927) that inhibition occurs in this range. It appears that other factors such as strain differences, or interaction of cystine with some constituent of the medium may be involved.

[1] The work represented in this paper was carried out in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College and was aided by grants from the Commonwealth Fund and the United States Public Health Service.

An investigation of growth factors for the gonococcus carried on in this laboratory has had to be interrupted, and while the work is necessarily incomplete, certain facts have been quite definitely established. It has been found possible to obtain growth of several strains, including freshly isolated ones, on a medium of known composition. The existence of at least two growth factors which are not essential for growth under the conditions used, but which have a marked stimulatory action has been demonstrated and certain observations on the inhibitory action of various substances have been made.

METHOD

Plates of experimental media were streaked with a saline suspension of gonococci in such a way that well-isolated colonies were formed. After 40 hours incubation in a candle jar the diameters of 5 or more typical well isolated colonies were measured and averaged. The colony size was found to be the best criterion of the growth-promoting power of the medium. It was uniform and reproducible within about ± 10% (Gould and Mueller, in press). In general the number of colonies on the plate depended more on the size of the inoculum and the exact technique of streaking than on the nature of the medium, and was used only as a secondary criterion.

Most of the assays were carried out with two strains, one of which required glutathione and the other did not. However, as the second strain suddenly developed complete dependence on glutathione during the investigation, without exhibiting any other change in its growth requirements, glutathione was usually included in the medium and freshly isolated strains were occasionally compared with the two stock strains.

The starch casein-hydrolyzate meat-infusion medium of Mueller and Hinton was used as a basis and the attempt was made to eliminate or replace each constituent with known compounds.

Meat infusion

It was found that freshly isolated strains would grow without meat infusion although the colonies remained very small. Replacement of the meat infusion by a mixture of glucose, sodium and potassium phosphates, and a magnesium salt was found to be advantageous.

Medium A

Starch	0.15 gm.
Casein hydrolysate	0.80 gm.
NaCl	0.50 gm.
Disodium phosphate	0.28 gm.
Potassium dihydrogen phosphate	0.10 gm.
Magnesium sulfate·$7H_2O$	0.045 gm.
Agar	1.7 gm.
Water to make	100 ml.

The colony size in mm. for seven freshly isolated strains on this medium and on the same medium containing 25 ml. of double strength meat infusion per 100 ml. is given in the table.

Strain #	3	4	5	6	7	8	9
Basal medium	1.6	1.6	1.0	1.4	0.7	1.3	1.1
Basal medium + meat infusion	3.0	2.9	2.2	3.3	1.5	2.8	2.5

Each strain gave colonies approximately twice as large with meat infusion as without, but nevertheless grew fairly well on the simplified medium. No freshly isolated strains have been encountered which failed to grow on this medium. Meat infusion thus contains a factor (or group of factors) which is not essential for growth but which causes a marked stimulation in growth.

No one of a large number of vitamins, bacterial growth factors or other substances tested were able to replace the meat infusion factor, either singly or in various combinations. Yeast infusion and extract, Liebig's meat extract, and similar preparations were able to replace it partially but none of them gave quite as large colonies as meat infusion.

The factor was found to be heat stable and dialyzable; it was not precipitated by alcohol or acetone or by barium hydroxide and it was stable to heating at pH 1.2 at 100 degrees for one hour. Methods of fractionation such as precipitation with lead acetate, mercuric acetate, barium hydroxide and alcohol, or adsorption on bone charcoal followed by elution with aqueous amyl alcohol usually resulted in preparations that had only a part of the original activity. The evidence indicated that more than one factor might be involved and it is quite possible that a metabolite rather than a growth factor is concerned. Incidentally, it was regularly observed that plates containing meat infusion tended to become slightly acid during the first stage of growth and then gradually became alkaline, whereas in the absence of meat infusion the acid reaction persisted.

Casein hydrolyzate

By replacing the casein hydrolyzate in the above basal medium with mixtures of pure amino acids it was found possible to obtain growth on the following medium.

Medium B

Glutathione	0.0015 gm.
1+ Glutamic acid	0.25 gm.
1+ Histidine	0.05 gm.
Glucose	0.15 gm.
Starch	0.15 gm.
Disodium phosphate	0.28 gm.
Potassium dihydrogen phosphate	0.10 gm.
Magnesium sulfate·$7H_2O$	0.045 gm.
Ferrous sulfate·$7H_2O$	0.0005 gm.
Sodium chloride	0.50 gm.
Agar	1.7 gm.
Water to make	100 ml.

Most of the assays on this medium were done with a stock strain dependent on glutathione but five other strains, some of them freshly isolated, were also used. The colonies were fairly well distributed over the plate but were quite small, averaging from 0.4 to 0.6 mm. in diameter. Omission of any one of these constituents with the exception of magnesium sulfate and ferrous sulfate gave no isolated colonies.

A mixture of the amino acids known to be present in casein (excluding tryptophane and cystine) in the same proportions as in casein, gave little if any effect with some strains but a definite one with others. All the strains gave much larger colonies when casein hydrolysate was added. The effects of the addition of various amino acid mixtures to the above basal medium B are given for strain #1 in the table.

Basal medium B	0.4
+ a mixture of all the amino acids known to be present in casein (excluding cystine and tryptophane)	0.6
+ Smaco "vitamin-free" casein hydrolyzate	0.8
+ commercial casein, H_2SO_4 hydrolyzate	1.1–1.2

The casein hydrolyzate factor appeared to be distinct from that present in meat infusion since only a combination of both gave maximum colony size.

The casein hydrolysate factor was not extracted by butyl alcohol. After fractionation of the "residue" fraction by barium hydroxide and alcohol into the dicarboxylic acid and the diamino acid fractions, both were found to be active. Further separation of the diamino acid fraction by the silver-barium method split the activity between the histidine, arginine and lysine fractions.

None of the vitamins, purines or mineral salts which might be expected to be present in casein hydrolysate was effective, nor were any of the 18 available amino acids.

Although methionine did not produce a marked increase in colony size it did appear to produce colonies showing a better oxidase test and should perhaps be included. Occasionally hypoxanthine appeared to stimulate growth but its effect was not constant enough to justify inclusion.

It is of interest that several amino acids were definitely inhibitory when added to this medium. Glycine completely suppressed the formation of colonies of strain #1 at a level of only 5 mg. %. Tryptophane and tyrosine were both slightly inhibitory at 50 mg. % and cystine was completely inhibitory for this strain. Inhibition by tryptophane and cystine was noted by MacLeod (1927).

In addition, adenine was extremely inhibitory in this medium; 0.5 mg. % of adenine sulfate completely suppressed growth and the effect was detectable down to 0.025 mg. %. However, in the presence of other purines or of casein hydrolysate it had no effect even in much higher concentration.

The medium is even simpler, with the exception of the inclusion of glutathione, than one described by Lankford *et al.* (1943) as giving no growth of any strains tested. The discrepancy may be due to differences in the technique of inoculation, and this aspect of the problem calls for further work. The procedure followed in these experiments has consisted in suspending one loop of growth from

the surface of a 24-hour growth on a starch agar slant in 0.5 ml. saline. A loop full of the suspension is so streaked that practically all of the inoculum remains on a small area at the top of the plate, and the amount distributed over the rest of the plate is far too small to be estimated. Growth on the thickly streaked area was disregarded for most purposes and only the formation of isolated colonies was regarded as significant.

While far from optimal, this medium should be of value as a basis for the assay of growth-stimulating factors for the gonococcus.

Starch

The effect of starch is presumably of a physico-chemical nature since Mueller and Hinton found that treatment of starch with salivary juice completely destroyed its activity. Glass and Kennet (1939) had previously shown that various forms of particulate carbon had a similar protective action.

Evidence has been obtained showing that the effect of starch is to neutralize in some way an inhibitory effect of agar or of some substance often associated with agar.

1. Medium A gave no growth without starch using Merck agar but good growth was obtained in a liquid medium of the same composition except for the omission of both agar and starch.

2. Several substances including gastric mucin and the insoluble fraction of whole yeast autolyzate were capable of completely replacing starch and bone charcoal or partially replacing it.

3. Several strains grew as well without starch as in its presence on two different samples of Bacto-agar but required starch on Merck agar. However, some strains do require starch in the presence of Bacto-agar. Both the Mueller-Hinton medium and medium A were used in these experiments.

This inhibitory effect of agar has quite possibly contributed in large part to the prevalent belief that the gonococcus is difficult to cultivate.

Glucose

If the glucose and phosphate components of medium A were mixed, adjusted to pH 7.4 and autoclaved at 15 lbs. for 30 minutes, the resulting medium was found to inhibit the growth of both of two strains tested. Addition of meat infusion protected against this effect. Glucose autoclaved in water solution was not inhibitory nor did the mild autoclaving of the whole medium produce inhibition. Although only preliminary experiments were completed the effect appeared to be similar to that reported by Stanier (1942) for the cytophaga.

Cystine

All of the strains dependent on glutathione were inhibited by cystine as discussed elsewhere (Gould and Mueller, in press). No isolated colonies were formed, but in the area where the inoculum was heavy a thicker layer of growth was obtained in the presence of cystine. Most of the experiments were done using a cystine concentration of 0.05% but changes in the concentration did not

alter the effect in a qualitative sense. Freshly isolated strains which did not require glutathione were in general not inhibited by cystine. In fact some stimulation of growth was observed which consisted in a thicker colony rather than in a larger diameter and could not be assayed quantitatively by this method. This effect varied greatly among different strains.

In conclusion, these results are being reported with the hope that they may be of use to others in the field. The points to be emphasized are 1) the importance of a quantitative method of assay, 2) the use of as many different strains as practicable and in particular of freshly isolated strains, 3) the fact that the effect of any substance on growth of the gonococcus depends on the other components of the medium.

SUMMARY

1. Growth of several strains of gonococcus has been obtained on a medium consisting of glutamic acid, histidine, glucose, starch, glutathione, magnesium and iron salts, phosphates, sodium chloride, and agar.

2. Casein hydrolyzate and meat infusion contain unknown factors which greatly stimulate the growth on this medium but which are not essential.

3. Evidence has been obtained showing that the function of starch in the growth of the gonococcus is as a protection against the inhibitory effect of certain samples of agar.

REFERENCES

BOOR, A. K. 1942 A difference in metabolic requirements of meningococcus and gonococcus. Proc. Soc. Exptl. Biol. Med., **50**, 22–25.

BOOR, A. K., AND MILLER, C. P. 1931 Growth requirements of gonococcus in a liquid nonprotein medium. Arch. Path., **12**, 137.

GLASS, V., AND KENNET, S. J. 1939 The effect of various forms of particulate carbon on the growth of the gonococcus and meningococcus. J. Path. Bact., **49**, 125–133.

GOULD, R. G., AND J. H. MUELLER. In press.

LANKFORD, C. E., SCOTT, V., COX, M. F., AND COOKE, W. R. 1943 Some aspects of nutritional variation of the gonococcus. J. Bact., **45**, 321–326.

LANKFORD, C. E., AND SNELL, E. E. 1943 Glutamine as a growth factor for certain strains of *Neisseria gonorrhoeae*. J. Bact., **45**, 410–411.

MCLEOD, J. W., WHEATLEY, B., AND PHELON, H. V. 1927 On some of the unexplained difficulties met with in cultivating the gonococcus: The part played by amino acids. Brit. J. Exptl. Path., **8**, 25–37.

MUELLER, J. H., AND HINTON, J. 1941 A protein-free medium for primary isolation of the gonococcus and meningococcus. Proc. Soc. Exptl. Biol. Med., **48**, 330–333.

STANIER, R. V. 1942 The cytophaga group: A contribution to the biology of myxobacteria. Bact. Revs., **6**, 143–196.