SOME ASPECTS OF NUTRITIONAL VARIATION OF THE GONOCOCCUS

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The fundamental nutritional requirements of the gonococcus are little understood at present, and until recently few serious studies had been made of this problem. This lack of understanding has resulted in a great multiplicity of culture media for the diagnosis of gonorrhea and has been responsible for a wide variation in the results reported with different methods. A further complicating factor is the occurrence of variation in the nutritional requirements of certain strains of the gonococcus, as will be described in this study.

It is generally believed that blood, serum, ascitic fluid or similar complex animal fluids or extracts are required by the gonococcus, and such materials have formed the basis for most culture media used for diagnostic purposes. A few studies, however, indicate that the basic growth requirements of the gonococcus and the meningococcus may be far more simple than generally supposed. Thus, Boor and Miller (1931) and Miller, Hastings and Castle (1932) were successful in growing the gonococcus in a liquid medium of tryptic digest of fresh egg-white with added mineral salts and glucose. More recently Boor (1942) has substituted tryptic digest of casein and 0.05 per cent cystine for the egg-white with resultant satisfactory growth of several stock strains of the gonococcus; the meningococcus did not require the cystine addition. Mueller and Hinton (1941) have lately reported highly successful isolation of the gonococcus on a casein-hydrolysate-starch-meat-extract medium. The meat infusion or extract constituted a large bulk of the medium, however, and the authors were unable to replace this unknown ingredient with any of the commonly recognized growth accessories (Mueller and Hinton, 1942). Surprisingly simple, however, is the medium successfully employed by Frantz (1942) for the cultivation of 14 of 15 strains of meningococcus; glutamic acid, cystine, mineral salts and glucose sufficed to support growth comparable to that obtained in more complex media.

In addition to the effect of blood and serum, the stimulating action of certain other materials on growth of the gonococcus has been observed. Thus, Vedder in 1915 pointed out the stimulatory effect of starch, and Pelouze (1931) and Glass and Kennett (1939) showed this effect to be due to the colloidal character of starch. McCleod and associates (1927) demonstrated the inhibitory action of certain amino acids, notably cystine and tryptophane, and suggested that blood colloids exert a protective function against such toxic effects. Mueller and Hinton (1941) emphasized the toxicity of cystine for the gonococcus and the protective action of starch.

Thus, it is apparent that an exact knowledge of the growth requirements of the gonococcus is far from its final solution.

EXPERIMENTAL STUDIES

(1) *Description of variant types.* In this laboratory, as in many others throughout the country, Proteose no. 3-hemoglobin agar (Bacto) is used for the routine cultural diagnosis of gonorrhea. In general, it is very productive as compared with smear results (Lankford, 1941) and growth of the gonococcus is usually good. It has been noted often, however, that certain strains produce small colonies, some of which are scarcely visible despite use of the oxidase reagents. Recently a satellite effect was observed in which a number of gonococcus colonies were clustered about a few colonies of a non-pigmented staphylococcus, whereas no colonies, or only extremely minute ones, were found on other areas of the plate. Tests with pure cultures proved that whereas this strain grew well on whole blood or serum agar, it would not produce detectable growth on the Proteose no. 3 agar with desiccated hemoglobin or starch, except in the presence of the white staphylococcus or certain other bacteria. In figure 1 a plate of Proteose no. 3-hemoglobin agar has received a heavy seeding of this strain of gonococcus and three spot inoculations of the white staphylococcus; the size of the satellite colonies gradually diminishes and they finally disappear toward the periphery of the plate. On blood agar (fig. 2), the stimulatory effect is reversed, and a wide zone of inhibition is evident.

Since isolation of this strain, many similar cases in the past were recalled, and a search has been made for additional ones. It was a surprise to find that 10 to 15 per cent of all strains isolated in the past few months are of this type. It must be emphasized that strains of this type grow only in the presence of certain contaminants or on areas where considerable mucus or pus has been deposited, and, in such instances, the colonies are often so minute that it becomes difficult to detect them even with the aid of the oxidase reagents. Certainly, many such strains must fail to produce any detectable growth. Therefore, if diagnostic laboratories are to continue to use the popular Proteose no. 3-hemoglobin agar, the deficient nutrilite, if such it be, must be supplied in some form.

(2) *Distribution and properties of the deficient nutrilite.* A limited survey was begun to find a suitable source of the deficient substance. Whole blood, plasma or serum, tissue extracts, and yeast and liver extracts were found capable of replacing the staphylococcus factor, and in fact promoted luxuriant growth of these strains. Freshly prepared liver and yeast extract proved to be the most potent sources, producing maximum stimulation in dilutions up to 1 part in 200–400 parts of agar, and detectable effects up to 1 to 5,000. Blood or plasma had a maximum effect at 1/50–1/100 dilution. For these tests a rather crude assay method was employed; one unit was designated as that quantity of extract required to produce detectable growth with a surface seeding per ml. of agar of a 1/10,000 dilution of a suspension of a 24-hour culture.

Studies with the various extracts proved the nutrilite to be destroyed completely by autoclaving. At 100°C. approximately 40 per cent is inactivated in

10 minutes, 70 per cent in 30 minutes, and 80–90% after one hour. Fractional sterilization of the extracts at 100°C. was accomplished, but since a considerable portion of the active substance is lost, this method was discarded. In our studies extracts sterilized by Seitz filtration have been employed, but toluene-preservation was found to be generally satisfactory and, of course, much less troublesome. It is necessary, however, to perform sterility tests before the toluene-preserved material is put to routine use. It is suggested, therefore, that any laboratory employing Proteose no. 3-hemoglobin agar, or similar autoclaved media, add 1–2 ml. of toluene-preserved liver or yeast extract to each 100 ml. of melted agar just prior to pouring plates.

Attempts to concentrate the thermolabile factor are underway, and a few preliminary observations may be noted. It is dialyzable and diffuses readily through agar. It is soluble in ethyl alcohol and acetone, by which means proteins may be precipitated from the extracts. It is not precipitated by mercuric acetate and is not adsorbed by activated charcoal from acid solution. Assay of the concentrates indicates a relatively high activity; approximately 2 micrograms of dried organic material per ml. of agar will produce detectable stimulation.

Using a Proteose no. 3 starch agar base, on which these deficient strains are unable to grow, various commonly used growth accessories were added singly and mixed in an effort to substitute a known compound. None of the compounds tested were active. They were: cozymase, cocarboxylase, calcium pantothenate, pyridoxin, thiamin hydrochloride, riboflavin, nicotinic acid, biotin concentrate, a folic acid preparation, p-aminobenzoic acid, inositol, a yeast nucleic acid preparation, uracil, adenine sulfate, guanine hydrochloride, thymine, xanthin, choline hydrochloride, taurine, glutathione, and asparagine. Asparagine and guanine were inhibitory at a concentration of 1 mgm. per cent.

(3) *Growth on casein-hydrolysate medium.* These studies were repeated with a relatively simple base medium having the following composition:

Acid-hydrolyzed, "Vitamin-free" Casein, SMACO, (Darco absorbed) 5%		100 ml.
Tryptophane		100 mgm.
Glucose, c. p. anhydrous		3 grams
Starch, soluble, "purified"		10 grams
Agar (Bacto granular)		20 grams
Mineral solution A		20 ml.
K_2HPO_4	20 grams	
KH_2PO_4	20 grams	
Water	200 ml.	
Mineral solution B		20 ml.
NaCl	20 grams	
$MgSO_4 \cdot 7H_2O$	0.5 grams	
$FeSO_4$	0.04 grams	
$MnCl_2$	0.01 grams	
Water	200 ml.	
Water to make		1000 ml.

pH 7.4–7.6

No growth of any strain was obtained on the base medium, but 1 part of liver extract in 200 parts of agar permitted growth of all strains tested comparable to that on Proteose no. 3 starch agar with the liver extract addition. If the same quantity of autoclaved liver extract were added, only the so-called "normal" strains would develop. This thermostable factor, which appears to be required by all strains of the gonococcus, was not replaceable by any of the pure compounds listed above.

(4) *Pleomorphism of variant strains.* Growth of the "deficient" strains on a medium containing a sub-optimal quantity of the thermolabile growth accessory results in characteristic and extensive morphological aberrations. Microscopically, these strains present a picture of large, distorted and swollen cells, dumbbell shapes, "hollow" and "ring" cells, and other pleomorphic, atypical forms (fig. 6). So characteristic is this picture that such strains may be detected almost invariably from a gram stain of those colonies which develop on the deficient medium. On the other hand, a person unacquainted with pleomorphic phenomena might well fail to suspect that such organisms are actually gonococci. If these strains are transplanted to a medium containing an optimal quantity of the thermolabile nutrilite, the morphology is quite typical, however. Furthermore, in smears from exudates the intracellular and extracellular gonococci of this type are normal in every respect.

(5) *Production of "normal" mutants.* All of the deficient strains isolated thus far undergo variation to produce "normal" mutants capable of synthesizing the thermolabile factor. If a heavy inoculum of one of the original deficient types is plated on Proteose no. 3 hemoglobin (or starch) agar, one or more colonies develop, which, on continued transplant on the same medium, grow luxuriantly and are in all respects similar to the "normal" type. Using the technic of obtaining mutants as developed by Lewis (1934) in his studies of coli-mutabile, it was found that approximately 1 cell in every 50,000 originals was a spontaneous mutation capable of synthesis of the thermolabile factor. To date all attempts to reverse the process and produce deficient types from variant and normal strains have failed, but this phenomenon has been observed *in vivo* in a hospitalized patient.

(6) *Other variants.* In addition to the deviation in nutritional requirements described above, several other comparable variations have been observed, but less thoroughly studied. For example, the "VM" strain (fig. 4) was produced from an original deficient variety. Although growth of this variant occurred on unsupplemented Proteose no. 3 hemoglobin (or starch) agar, the colonies were no larger than 0.1 to 0.4 mm. in diameter and were quite adherent to the agar. The cellular morphology, however, was quite typical and the addition of autoclaved liver or yeast extract sufficed to produce colonies of normal size. The relative instability of this strain was evident by the continual production of "normal" mutants; indeed, it was difficult to maintain in this intermediate phase.

(7) *Clinical studies.* In order to test the practical value of the addition of unautoclaved extracts to Proteose no. 3 hemoglobin agar, a parallel series of

cultures were taken in the out-clinic of the John Sealy Hospital. Although an insufficient number of cultures for statistical evaluation has been obtained to date, the superiority of the medium with liver or yeast extract supplement seems evident. A 12 per cent higher yield of positive cultures, the majority of which were of the "deficient" variety, was obtained with the medium containing the added extract.

At the present time no correlation of the occurrence of the deficient-type gonococcus with any particular condition of the patient has been observed. While it is true that such strains have been found with considerable frequency in sulfonamide-resistant cases, this character seems to be an independent variable, according to preliminary *in vitro* studies of sulfonamide resistance.

SUMMARY

1. A type of gonococcus has been described which does not grow on autoclaved culture media except in the presence of certain other bacteria or with the addition of fresh extracts of liver, yeast, or blood or similar substances.

2. These strains occur with considerable frequency, so that failure to detect them constitutes a serious defect in cultural diagnosis.

3. These deficient strains grow luxuriantly if 1/200 filtered or toluene-preserved liver extract is added to the sterile, autoclaved medium.

4. Strains of gonococcus requiring the thermolabile factor present in the extracts produce spontaneous mutations capable of its synthesis.

5. The factor involved is relatively simple, dialyzable, alcohol-soluble, and is destroyed by autoclaving. It is not replaceable by a number of known pure nutrilites.

6. All strains of the gonococcus tested grow well on casein-hydrolysate-starch agar with 1/200 liver extract. Only the "normal" strains grow if the extract is autoclaved.

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FIG. 1. SATELLITE COLONIES OF GONOCOCCUS ON PLATE OF PROTEOSE NO. 3 HEMOGLOBIN AGAR

Spot inoculations of non-pigmented staphylococcus.

All colonies of gonococcus shown were developed with dimethyl-paraphenylenediamine monohydrochloride.

FIG. 2. SAME STRAIN ON RABBIT BLOOD AGAR SHOWING ZONE OF INHIBITION

FIG. 3. ARTIFICIAL SATELLITE PHENOMENON

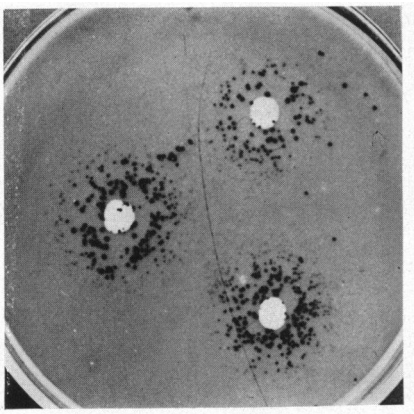
Agar plug contains yeast extract, which diffuses for some distance into the unsupplemented Proteose no. 3-starch agar.

FIG. 4. "VM" VARIANT ON LOWER STREAK COMPARED WITH "NORMAL" MUTANT ABOVE. PROTEOSE NO. 3 HEMOGLOBIN AGAR

FIG. 5. MORPHOLOGY OF "NORMAL" MUTANT FROM PROTEOSE NO. 3-HEMOGLOBIN AGAR. BASIC FUCHSIN STAIN

FIG. 6. PLEMORPHISM OF SAME ORIGINAL "DEFICIENT" STRAIN FROM PROTEOSE NO. 3 HEMOGLOBIN AGAR CONTAINING SUB-OPTIMAL QUANTITY OF THERMOLABILE FACTOR

Magnification the same as in figure 5. Basic fuchsin stain



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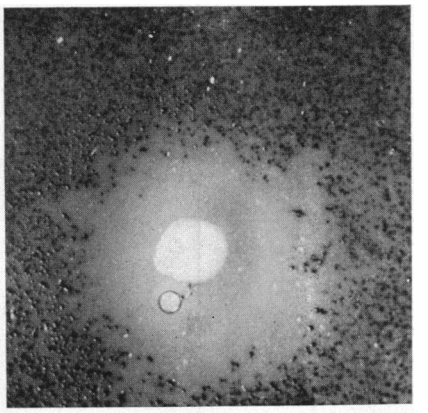

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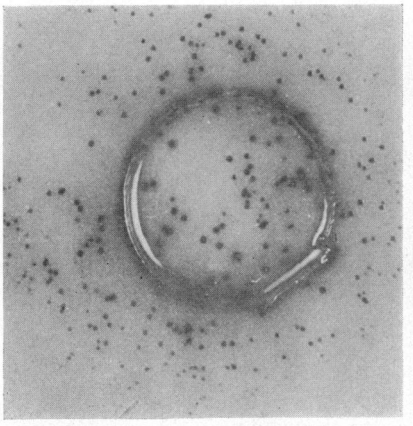

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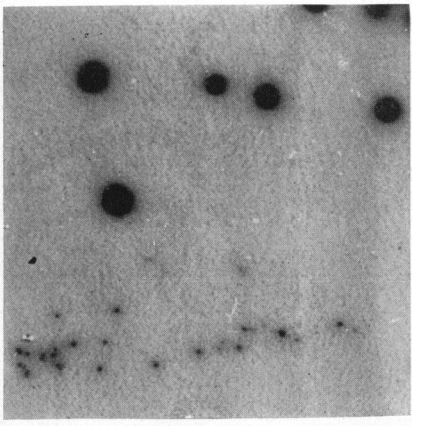

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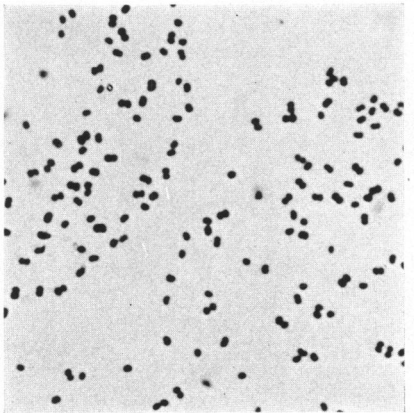

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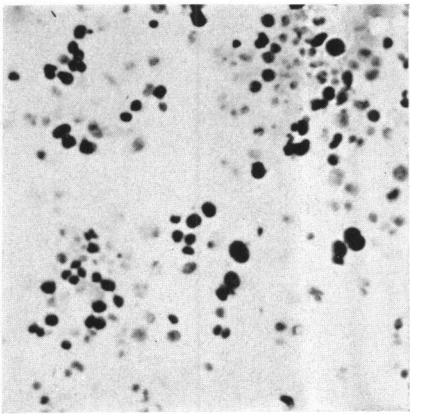

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