A SIMPLE MEDIUM FOR IDENTIFICATION AND MAINTENANCE OF THE GONOCOCCUS AND OTHER BACTERIA

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The media generally used for the cultivation of the gonococcus have certain disadvantages. Most of them are unable to maintain freshly isolated cultures in a viable state for more than a few days. They may require ingredients likely to be variable in quality (e.g., meat infusion) or not readily available (e.g., horse serum). Moreover, the incorporation of such materials as ascitic fluid (Carpenter, 1945; Mahoney, Van Slyke, Cutler, and Blum, 1946) or serum (Peizer, 1942) in fermentation media requires special procedures for sterilization or aseptic handling that may be difficult to manage in the small laboratory.

The medium here described has none of these drawbacks. It is composed of available and relatively stable materials, can be sterilized in the autoclave, and supports continued growth of freshly isolated gonococci if transferred at intervals of 10 days, or even longer for many of the strains tested. In addition, it is a reliable base medium for the performance of fermentation tests. Its chief constituent is pancreatic casein digest, which has been shown (Vera, 1944) to be useful in the identification of clostridia.

EXPERIMENTAL METHODS AND RESULTS

Pancreatic digest of casein. This was prepared by a plant-scale adaptation of the method of Leifson (1943) to meet the specifications set forth by the National Institute of Health in 1945 and now included in the United States Pharmacopoeia XIII. Of the qualifications specified, two characteristics were regarded as particularly pertinent in the present connection, namely, suitability for hydrogen sulfide production and freedom from fermentable carbohydrates. In preliminary studies with broths made from such digests of casein, several typical strains of typhoid bacilli and *Salmonella schottmuelleri* produced little or no blackening of lead acetate paper in 24 hours, and *Salmonella paratyphi* and *Shigella paradysenteriae* caused no darkening in 48 hours. In contrast, all of the test organisms readily gave positive reactions for hydrogen sulfide when they were grown in broths containing meat peptone or meat infusion. The result of this biological test is in harmony with the report of Block and Bolling (1945) that casein is deficient in available sulfur compounds, especially cystine.

In respect to fermentable carbohydrate, tests with cultures of *Escherichia*, *Aerobacter*, *Salmonella*, *Clostridium*, and cocci showed no acid production in 48 hours in casein digest broth, but showed definite acid production in broths containing meat infusion or meat peptone.

The use of cystine to supplement the digest of casein, as suggested by Boor

(1942), improved the properties of the substrate as a culture medium, but the results were not uniform. It was found, however, that the addition of both cystine and sodium sulfite provided the necessary conditions for good growth of freshly isolated as well as stock cultures of gonococci and other bacteria.

Supplemented casein digest agar. The basic semisolid medium used in the investigation had the following composition in grams per liter of distilled water:

Pancreatic digest of casein	20.0
Cystine	0.5
Sodium sulfite	0.5
Sodium chloride	5.0
Agar	3.5
Phenol red	0.017

All chemicals were cp grade and the agar was of the highest bacteriologically tested quality. The medium was prepared with and without added carbohydrate (0.5 per cent or, rarely, 1.0 per cent) and, after adjustment for a final pH of 7.3, was tubed and autoclaved at 116 to 118 C for 15 minutes. The sterile tubes were stored at room temperature, and used as needed.

Inocula of organisms suspected of being gonococci or meningococci were spread over the surface of the medium; other inoculations were made by stabbing. Cultures of *Neisseria* and of *Brucella abortus* were incubated in candle jars to supply an atmosphere enriched with carbon dioxide, unless otherwise stated.

The culture tubes were plugged with cotton. Although it was considered undesirable to disregard precautions against desiccation of the media, sterile or inoculated, it was not feasible under the experimental conditions to stopper the tubes as recommended by Carpenter and Shepard. Nevertheless, the results obtained clearly indicate the practical utility of the medium.

Fermentation studies with Neisseria spp. Thirty-five strains of gonococci were transferred from chocolate agar slants, which were primary subcultures from diagnostic plates, to the basic medium and to the basic medium with glucose or maltose added. All of the cultures in the glucose medium developed an acid reaction within 24 hours. The plain and maltose cultures showed an alkaline change of the indicator at that time. This alkaline shift has been found to be characteristic of all strains thus far examined.

Transfers were made directly from 172 colonies on chocolate agar diagnostic plates[1] into maltose medium; 16 to 20 hours later, growth and alkalinity were visible in 150 tubes, and within 48 hours an additional 17 strains had grown. During the course of the experiments an occasional transfer failed to grow, but cultures were obtained from all plates having oxidase-positive colonies of gram-negative cocci. Glucose was fermented by all of the strains.

A series of 58 colonies from diagnostic plates was transferred into the casein digest medium with and without cystine and sulfite. All of the strains grew in

[1] The plates were obtained from the Bureau of Laboratories of the Baltimore City Health Department, through the courtesy of T. C. Buck, Jr., Assistant Director.

the presence of the sulfur compounds, but only 20 developed in their absence. Of 30 stock cultures also studied, 12 were able to grow in the absence of the sulfur compounds.

Twenty-eight strains of meningococci produced typical fermentation reactions in 16 to 24 hours in the supplemented casein digest medium in the presence of added carbohydrates. When tested in the medium without cystine and sulfite, the cocci grew, though more slowly, thereby delaying the appearance of a color change of the phenol red.

Maintenance of Neisseria. In the absence of fermentable carbohydrate the gonococci remain viable for prolonged periods in this medium. By transfer at 10- to 14-day intervals, 251 strains have been maintained for at least 3 months, and some of them for over 3 years; 155 of the group were stored at 37 C, and the remainder were held at room temperature. Young cultures of gonococci from all strains, including those maintained as long as 3 years on this medium, showed the cellular morphology typical of the species.

Transplants from a limited number of cultures made even 3, 4, or 5 weeks after inoculation usually grew out, although after such long storage the original cultures had darkened and dried considerably. The poorest results were obtained from a series of 58 strains held for a month, at the end of which time only 46 (79 per cent) gave positive subcultures.

Transfers made from cultures in the glucose medium after incubation for 2 days frequently failed to grow. However, some gonococci may survive longer in the fermented medium, because 25 of 58 yielded subcultures after 4 weeks of incubation and 2 of another group of 14 strains were still viable after 5 weeks.

The 28 strains of meningococci were grown and stored at 37 C. They were maintained without difficulty by subculturing at 3- or 4-week intervals.

Cultivation of other organisms. In addition to *Neisseria* cultures, 10 freshly isolated strains of *Brucella*, 10 of *Corynebacterium diphtheriae*, and numerous streptococci gave conventional fermentation reactions in the medium containing appropriate carbohydrates. The brucellae and one pneumococcus strain required 2 days of incubation, and the other organisms grew in 1 day. In the absence of the sulfur compounds, the diphtheria bacilli required 8 to 24 hours longer to develop definite reactions. In the medium without fermentable carbohydrate, 4 strains of pneumococci, 2 beta streptococci, 9 *B. abortus*, 1 *Brucella suis*, and 10 cultures of diphtheria bacilli have been maintained at room temperature for more than 2 years by monthly transfers.

Carbon dioxide requirement. There was no significant difference apparent in cultures grown at the same time upon the casein digest medium with and without reinforcement of the atmosphere with carbon dioxide. However, all of the cultures so tested, which included many gonococci, and all of the meningococcus and brucella strains previously mentioned, had been transferred at least twice after isolation. An experiment was therefore performed to determine whether freshly isolated gonococci would grow in the supplemented casein digest medium without addition of carbon dioxide to the atmosphere. Eighty-five colonies from diagnostic plates were inoculated into the maltose medium, transfers were then made

into glucose medium, and both sets of tubes were incubated in an ordinary incubator. The cultures grew out promptly and characteristic alkaline or acid reactions were visible in 18 hours. This evidence, which indicates that freshly isolated gonococci grown in this medium do not require special provision for extraneous carbon dioxide, is in direct contrast to results obtained with other media. Chocolate agar plate cultures made in duplicate with numerous strains of gonococci (including 20 of the same series) and incubated simultaneously showed little or no growth in 18 hours and rarely had good growth after incubation for 48 hours when no provision was made for reinforcement of the atmosphere with carbon dioxide; control plates, on the other hand, which were incubated in candle jars, regularly showed fair to good growth after 18 hours and always had typical colonies after 48 hours of incubation. Similarly, broth cultures repeatedly failed to develop when tubes were incubated in air, but showed definite turbidity when kept in candle jars for 18 to 48 hours.

DISCUSSION

Pancreatic digest of casein in a medium containing both cystine and sodium sulfite has been found capable of supporting the growth of 267 strains of freshly isolated gonococci. This medium, on omission of the sulfur compounds, failed to support the growth of an appreciable proportion (60 to 65 per cent) of the strains tested. The findings indicate the importance of the sulfur compounds as components of the proposed medium, which otherwise is characterized by a low sulfur and cystine content. These observations are in general agreement not only with those of Boor but also with the results reported by Welton, Stokinger, and Carpenter (1944), who found cystine necessary for the growth of stock strains in a defined medium; by Lankford (1944), who incorporated cystine in isolation media to increase colony size; and by Landy and Gerstung (1945), who employed a casein (acid?) hydrolyzate for studying sulfonamide resistance. The apparent discrepancy of the present results with the evidence of cystine inhibition of certain glutathione-requiring stock strains of gonococci, obtained by Gould (1944) and by Gould, Kane, and Mueller (1944), may perhaps be explained by the alteration of metabolism noted during their investigations, or may be, at least in part, due to a presumably higher cystine content, especially when a meat infusion base was employed. Inhibitory action toward gonococci by large amounts of cystine (about 0.09 per cent) was described by McLeod, Wheatley, and Phelon (1927), and they also noted possible stimulation of growth on meat-extract, blood agar medium when lower percentages of the amino acid were used.

The maintenance of gonococci was readily accomplished in the supplemented casein digest medium, especially in the absence of fermentable carbohydrate. Although no attempt was made to establish the exact duration of viability of large numbers of strains in this medium, all of 251 cultures could be carried indefinitely when transferred at intervals of 10 to 14 days; the majority of tested strains remained viable for several weeks without retransfer. The absence of fermentable carbohydrate and consequent acid formation only in part account for the prolonged viability of the gonococcus cultures in this medium, since, al-

though many 2-day cultures did not yield subcultures, a surprising number survived storage for some weeks. Whatever the factors may be that favor survival or longevity of cultures, it seems clear that freshly isolated gram-negative cocci do not have an inherent tendency to grow and die quickly, and that the viability of a given population may be considerably lengthened under suitable environmental conditions.

Pancreatic digest of casein as a basic nutrient material has several practical advantages. A medium suitable for cultivation of gonococci can be prepared very simply, without the addition of tissue fluids. Its freedom from thermolabile components makes it possible to sterilize the whole medium by autoclaving. It does not require the addition of tryptophane and vitamins, as is the case when the casein has been hydrolyzed with acid, because these substances are retained during enzymatic hydrolysis of the protein. Its freedom from fermentable carbohydrates permits its use as a base in the performance of fermentation tests.

Although tested mainly with gonococci, the supplemented casein digest medium consistently displayed the same desirable characteristics, when used in comparative studies with a limited number of other bacteria.

SUMMARY

A simple autoclaved semisolid medium containing pancreatic digest of casein, cystine, and sodium sulfite provided a suitable substrate for the cultivation of freshly isolated gonococci. For the development of cultures in this medium, incubation in an atmosphere reinforced with carbon dioxide was not obligatory.

Gram-negative and gram-positive cocci, brucellae, and diphtheria bacilli could be maintained indefinitely by relatively infrequent transfers.

Accurate fermentation reactions with appropriate carbohydrates were obtained promptly with freshly isolated strains of *Neisseria* and other organisms.

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