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Dense Cultures of *Neisseria gonorrhoeae* in Liquid Medium

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Cultivation of *Neisseria gonorrhoeae* was effected in a conical glass culture vessel surrounded by a constant-temperature water jacket, and with facilities for stirring, aeration, and *p*H measurement and control. With the use of an aerated peptone-based medium, containing polypropylene glycol to prevent foam build-up, the yields obtained over the *p*H range from 5.8 to 7.4 were determined. The greatest yield was obtained at *p*H 6.4 when the dry weight was 1.5 g/liter. At *p*H 7.2 to 7.6, lysis was extensive.

Relatively few investigations have been made on the fractionation of the antigens and the toxin of *Neisseria gonorrhoeae*. In view of the rapidly increasing incidence of gonorrhoea in almost every country, in spite of the use of modern chemotherapeutics, there is an obvious need for intensive studies on both immunological and biochemical aspects of the organism. As a first step in this work, it is necessary to obtain relatively large quantities of bacteria, grown under reproducible conditions.

One of the difficulties is the wide diversity of growth requirements of different strains, and there have been recent attempts to widen the scope of media (14; C. E. Lankford, Bacteriol. Proc., p. 40, 1950; C. H. Parke, Ph.D. Thesis, Univ. Maryland, College Park, 1964). However, there is obviously also a need for studies of the cultivation technology required for obtaining good yields. In the studies of Tauber and Garson (16) on the endotoxin of one particular strain, 25 liters of medium divided in 1-liter lots yielded 60 to 100 mg (dry weight) of cells per liter of medium, after 6 days of incubation at 36 C. One method of increasing densities was demonstrated by Gerhardt and Hedén (11). This incorporated a biphasic culture system with a liquid medium overlay on a Nutrient Agar base. Concentrations of bacteria up to three to nine times those obtained in single-phase controls were achieved by this method, which is, however, difficult to scale up.

The following study was carried out with the aim of producing high concentrations of bacteria in liquid medium under controlled conditions, which would permit scaling up the batch size by the use of large-scale equipment or the yield per time unit by continuous operation.

MATERIALS AND METHODS

Bacteria. A laboratory strain of *N. gonorrhoeae*, designated strain III, and two freshly isolated strains, 39076 and 47693, all obtained from the National Bacteriological Laboratory, Stockholm, Sweden, were used in the preliminary studies.

For the majority of experiments, a freshly isolated strain, 80408, preserved by storage in liquid nitrogen, as described elsewhere (3), was used. The colonial appearance of all strains was of the type 4 described by Kellogg et al. (1963).

The bacteria were revived by plunging an ampoule, taken from the liquid nitrogen storage refrigerator, into a water bath at 37 C, after which the suspension could be used as a direct inoculum in liquid medium, or more commonly, to streak a number of plates.

Medium. The basic medium used in these experiments consisted of Proteose Peptone No. 3 (Difco), 30.0 g; (20.0 g for preliminary experiments with shaken flask cultures); K_2HPO_4, 3.0 g; KCl, 6.5 g; soluble starch, 10.0 g; glucose, 10.0 g; polyglycol antifoam (Dow Chemical Corp., Midland, Mich.), 0.01% (not included in preliminary experiments); and distilled water, 1,000 ml. The glucose was sterilized separately and added to the medium prior to inoculation. The *p*H was adjusted to the value specified before autoclaving and was controlled during the course of the experiments by the addition of 2 N NaOH.

Inoculum for 500-ml cultures. The inoculum was prepared by transferring bacteria from a peptone-starch-agar plate, after incubation overnight at 37 C in the presence of 5% CO_2, to 50 ml of the liquid medium of *p*H 7.2. The 250-ml Erlenmeyer flask used was fitted with a stopper having two air filters, so that the gas space could be filled with a mixture of 5% CO_2 in air, before sealing. The flask was incubated for 12 to 18 hr at 37 C on a rotary shaker table. A 25-ml amount of inoculum broth was then used for 500-ml cultures, and 5 ml was used for 100-ml culture control runs.

Apparatus. The bacteria were grown in an aerated

and agitated fermentor (Biotec, Stockholm, Sweden) of the type described in detail by Holmström and Hedén (12). The growth vessel (Fig. 1) consisted of an inverted, cone-shaped glass chamber with a water jacket through which water at 37 C was pumped. At the upper end of the vessel, there were four small ground glass joints for *p*H measurement, stirrer, alkali addition, and air supply. The large central ground-glass joint housed a connection for the outgoing air filter and also a sampling tube.

The *p*H measurement was effected by a Radiometer Titrator Type TT1 (Radiometer, Copenhagen, Denmark). For *p*H control, a peristaltic pump which can be seen in Fig. 1 was actuated by the Titrator, and alkali was introduced via a capillary tube in the vessel below the culture surface.

The stirrer was a small stainless-steel impeller with the shaft mounted in a glass tube and driven by a variable-speed motor, via a magnetic coupling, totally enclosed in glass to eliminate the possibility of infection through bearing seals.

The CO_2-air gas mixture was fed through a sintered-glass sparger into the medium in the vicinity of the stirrer. Samples of the culture broth were taken by a syringe through a rubber ampoule stopper.

Growth measurement. Bacterial growth was estimated during the course of cultivation by means of optical density measurements made on an Eel "Spectra" absorptiometer (Evans Electro-selenium Ltd., Harlow, Essex, England) at a wavelength of 580 mμ. Because of large size variations in the bacteria at certain *p*H levels, the values obtained did not always correlate with the dry weight, so final yields were always determined by the latter method. The purity of the cultures was checked both by Gram staining and by examination of the colonial appearance after incubation on peptone-starch-agar plates for 24 hr at 37 C. In case of doubt, the oxidase reaction with a 1% aqueous solution of tetramethyl-*p*-phenylenediamine was also used.

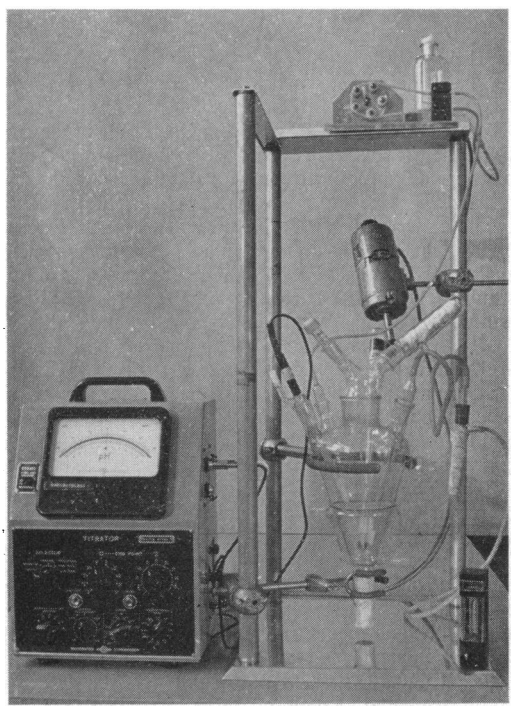

FIG. 1. *Cultivation equipment, showing vessel, magnetically driven stirrer, pH controller, and pH regulation pump.*

RESULTS

Preliminary experiments. Initially, cultures were made in shaken Erlenmeyer flasks to determine whether the yield of bacteria could be increased by incorporating growth factors, or by the addition of water-soluble compounds of high molecular weight, to absorb possible toxic metabolites.

The standard technique in these experiments was to grow the bacteria in 100 ml of medium contained in 250-ml flasks having sealed "Quickfit" tops, with tubes ending in air filters to enable the gas space to be filled with a mixture of 10% CO_2 in air. The bacteria were then grown on a rotary shaker table at 112 rev/min at 37 C.

The following compounds were added to the basic medium to determine whether they assisted in the growth of the three strains: dextran (D 500, Pharmacia, Uppsala, Sweden), starch, dextrin, glycogen, serum, and gelatin. Strains III and 47693 were found to grow as well, or in some instances better, without an addition of any kind. Strain 39076 was not helped by any of the compounds tested, except soluble starch at a concentration of 1%, which gave 5% better growth.

Various additions to the starch-containing medium were made when the freshly isolated strains were grown. The redox potential was lowered by the addition of 0.1% sodium thioglycolate, but this reduced growth. Trace quantities of $MgSO_4$ (0.04%), $MnSO_4$ (0.002%), $FeCl_3$ (0.0004%), and $CoCl_2$ (0.002%) gave no significant differences in yields, so the mineral content of the peptone was obviously adequate and was not limiting the yield obtained.

The laboratory strain was found to have no requirement for CO_2, but strain 39076 gave practically no growth unless CO_2 was added to the atmosphere, in this case at a concentration of 10%. For strain 47693, 5% or 10% CO_2 in air gave similar growth. If a CO_2-nitrogen gas mixture was used, none of the strains grew.

Yeast extract was tested in 0.1 to 0.5% quantities as a replacement for CO_2 with both fresh isolates, since Tauber and Garson (1957) reported that yeast extract could perform this function. Difco yeast extract, both as manufactured and

TABLE 1. *Growth of strain III in 250-ml flasks with and without baffles*

Vol of medium	Type of flask	Max oxygen transfer rate (mmoles per liter per hr)	Optical density
ml			
50	Normal	25	0.51
50	Baffled	68	0.62
100	Normal	14	0.32
100	Baffled	31	0.40
150	Normal	8	0.19

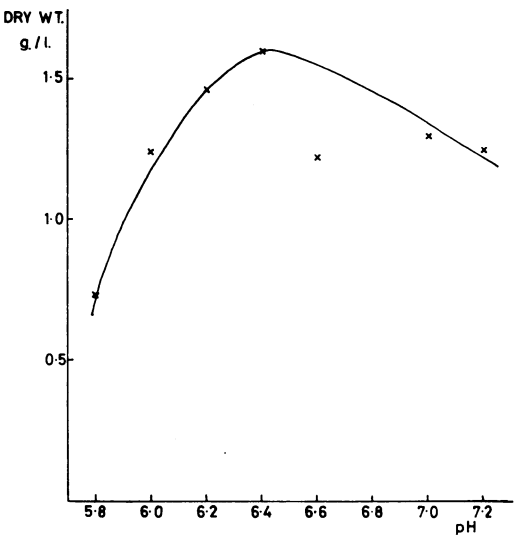

FIG. 2. *Yield of Neisseria gonorrhoeae strain 80408 at various pH values.*

as purified (1), however, prevented growth completely in these experiments, and it was concluded that some inhibitors were present.

Strain III, which had no CO_2 requirement, was grown in flasks with cotton plugs to maintain sterility. Some flasks had indented sides to act as baffles, increasing the gas transfer rate, which was measured according to the method of Cooper et al. (6). Table 1 shows the optical density obtained under different conditions when growth was measured 13 hr after identical inoculation.

The results in Table 1 show that increased aeration, achieved either by increasing the surface area to volume ratio, or by using greater agitation, produced much more rapid growth.

These experiments showed the importance of adequate aeration, and, together with the observation that the *p*H dropped to 5.8 during cultivation, suggested that a stirred fermentor with *p*H control would offer the best method of obtaining dense cultures of *N. gonorrhoeae*.

Effect of aeration and agitation on growth in pH controlled fermentor. Since the literature (15, 17) reports a *p*H value of 7.2 or higher as being suitable for the growth of *N. gonorrhoeae*, initial experiments in the fermentor were made at this value.

The gas flow rate was 0.2 to 0.5 liters per min, and the agitator speed varied from 500 rev/min, giving just liquid mixing, with very little entrainment of bubbles, to a speed of approximately 2,000 rev/min, which gave an almost uniform bubble distribution throughout the vessel, the bubbles themselves having extremely small dimensions.

Dry-weight determinations were made on the termination of growth, as observed from optical-density measurements. Within the range of air-flow rates and agitation used, there was little difference in the yield of bacteria. This was of the order of 1 g/liter $\pm$ 0.2 g, achieved in 10 to 14 hr, whereas control flasks took 36 hr to reach their maximal growth of 0.1 to 0.3 g/liter. In an experiment where the air flow was stopped in the logarithmic-growth phase, the growth curve was halted immediately.

Effect of pH on growth. The yield of bacteria in *p*H controlled cultures at various *p*H levels was measured. All experiments were made with a 0.5 liters per min of CO_2-air gas mixture, and with an agitator speed of approximately 1,000 rev/min. The results are shown in Fig. 2. The maximal yield of 1.6 g/liter (dry weight) was obtained at *p*H 6.4. Occasional experiments gave even higher figures, 2 g/liter being reached on one occasion. Below *p*H 6.2 there was a marked drop in yield, and at *p*H 7.0 and above there was a considerable increase in lysis. It proved impossible to cultivate the bacteria at *p*H 7.4, because the lysis caused foaming to such an extent that the antifoam action was overcome.

Morphological variation. The bacteria were examined by phase-contrast microscopy during the course of growth. At *p*H 7.2, it was noted that certain of the bacteria, instead of retaining the characteristic gonococcus morphology, became rounded and highly swollen—similar in appearance to spheroplasts. At the end of the cultivation, they could account for as many as 5 to 10% of the total population. Between *p*H 6.4 and 6.8, the production of these large forms was reduced to negligible proportions, but at lower *p*H levels their proportion was again somewhat increased.

Photographs of these large forms are shown together with normal gonococci in Fig. 3. On some occasions, they appeared to be segmented as in Fig. 4.

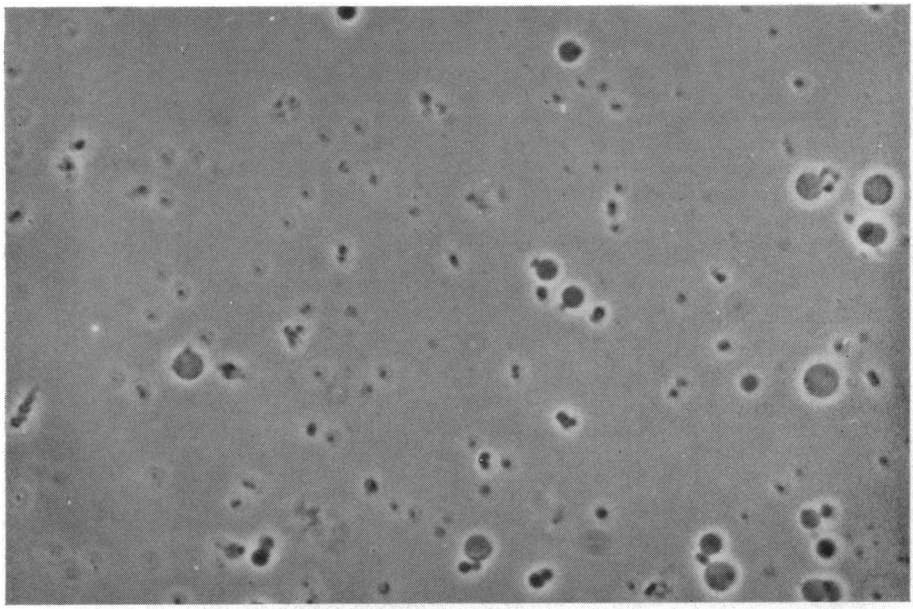

FIG. 3. *Large forms of Neisseria gonorrhoeae.*

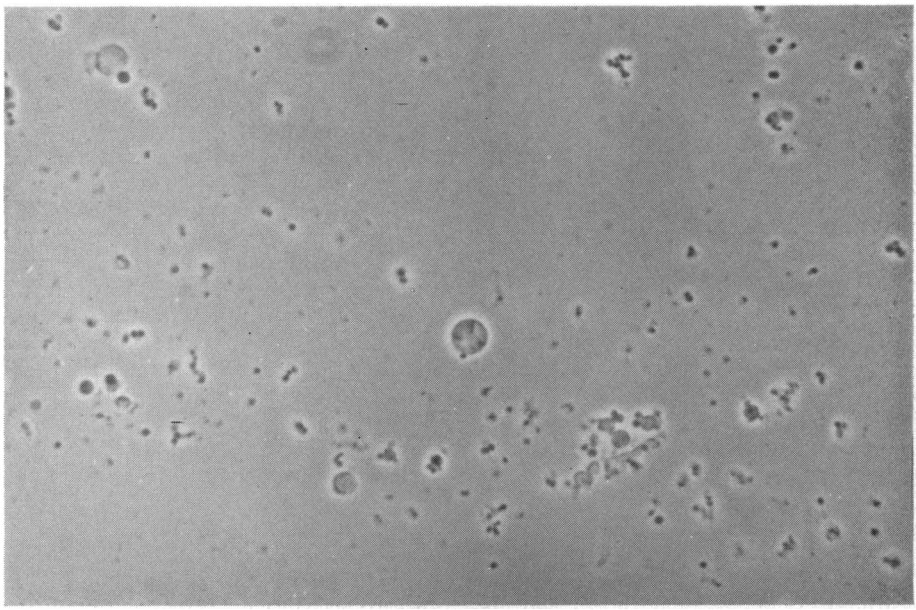

FIG. 4. *Segmented large cell of Neisseria gonorrhoeae.*

Complement-fixation properties. A complement-fixation test on the bacteria from a culture grown at *p*H 6.4 showed this strain to have good complement-fixing properties. This fact, which is of course relevant for the production of complement fixation antigen in large quantities, is dealt with in more detail in a separate paper on continuous culture of gonococci (4).

DISCUSSION

The first reports of the production of large forms by *N. gonorrhoeae* were by Dienes (7) and

by Brown and Hayes (5) who worked with bacteria grown on agar plates. The bacteria were observed to be swollen, and occasionally a secondary growth of L forms was observed on their disintegration. The large forms were considered by Dienes to be the precursors of L forms, and were more thoroughly observed in *Streptobacillus moniliformis* (8). Dienes reported in this case that the phenomenon disappeared after a few transfers, remaining only in cultures preserved by refrigeration in a CO_2 icebox (8). Since the gonococci in this study were preserved in the frozen state, there could be some connection between the two observations, but it seems more likely that it is a response to cultural conditions, as is shown by these studies.

In a recent publication, Dienes et al. (10) reported that examination of many strains yielded several which produced large bodies on the agar plates. By a staining technique, it was possible to observe that these cells were deteriorating after 2 to 3 days of incubation at 30 C, but on the following day a secondary growth of small cocci with normal morphology reappeared. It was noted that the secondary growth started from the large bodies and not from remaining normal cocci, since they sometimes appeared inside the otherwise empty large bodies.

An attempt was made in the present studies to separate the large bodies from normal diplococci by the addition of penicillin (100 units/ml) during growth; many of the diplococci were not lysed but became rounded like L forms. The large cells did not appear affected by the penicillin. Dienes (10) reported somewhat similar results with bacteria cultivated on agar plates.

The large cells could be of significance in the failure of treatment of gonorrhoea with penicillin, since Barile et al. (2) have published a photograph of bacteria which resembled L forms, isolated from a patient with a resistant strain.

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