Autolysis of *Neisseria gonorrhoeae* Relation between mechanical stability and viability

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Summary

The relationship between the mechanical stability and the viability of *N. gonorrhoeae* (Type 4) in suspension was investigated. A correlation between viability and optical density recordings was often found. However, in spite of increased mechanical stability in solutions with low pH (5.2) or containing Cu^{++} or sucrose (10 per cent.), these environments were toxic to the gonococci. A viability preserving effect by Mg^{++} (4 mM), Ca^{++} (4 mM), spermine (0.5 mM), polyvinylpyrrolidone (10 per cent.), and low temperature (4°C) was demonstrated. The possibility of improving transport media for gonococci is discussed.

Introduction

Neisseria gonorrhoeae is one of the most fragile Gram-negative bacteria. This property of gonococci is of practical importance, since the organisms easily die and this leads to a falsely negative bacteriological diagnosis. During 24 hrs of storage at room temperature, 12 to 79 per cent. of positive specimens become negative in the best of various transport media evaluated (Hosty, Freear, Baker, and Holston, 1974).

It has recently been clearly demonstrated that the fragility of gonococci is due to their pronounced tendency to lyse spontaneously as soon as their growth ceases (Hebeler and Young, 1975; Elmros *et al.*, unpublished). By using optical density recordings as a measure of the mechanical stability of *N. gonorrhoeae* in suspension, we have observed several factors that influence the stability of gonococci, both negatively and positively. The aim of the present investigation was to study the relationship between mechanical stabilization and viability of gonococci. It was of particular interest to see whether conditions exerting a stabilizing influence would also have positive effects on the viability of the bacteria. Such information might be of value for the development of more effective transport media for the gonococcus.

Material and methods

Micro-organism and materials

The antibiotic-sensitive strain 82409/55 of *N. gonorrhoeae* was obtained from Dr. Alice Reyn, Copenhagen, Denmark. Colonial morphology typing was performed after growth of the bacteria on Gc Medium Base agar (Difco) with supplement B added (Kellogg, Peacock, Deacon, Brown, and Pirkle, 1963; Elmros, Hörstedt, and Winblad, 1975). Cells of colonial type 4 were used throughout this study. This type is not inclined to change colony type during culture, and does not autoagglutinate to the same degree as cells of colony types 1, 2, and 3. The latter property is important for optical density recordings and in particular for viability determinations (colony count).

The chemicals used were from Sigma Chemical Co., St. Louis, Mo., USA, and KEBO AB, Stockholm, Sweden. All glassware was regularly washed with acid and carefully rinsed with glass-distilled water. Double distilled water was used for all solutions.

Cultivation

The gonococci were cultivated on Gc Medium Base plus supplement B as solid medium. The same medium with agar omitted was used as liquid medium. The agar plates were incubated at 36°C. in air containing 6 per cent. CO_2 for 48 hrs before colony counting. Liquid cultivation was performed at 36°C. in side-arm flasks equilibrated with 10 per cent. CO_2 in air and sealed with rubber stoppers. Growth in liquid medium was followed by optical density recordings using a Klett-Summerson colorimeter with filter W66. An optical density of 100 Klett units corresponds to 3×10^8 colony forming units (CFU) of type 4 gonococci.

Viability and lysis testing

The bacteria were harvested in log phase (50-100 Klett units) by centrifugation (2,000 G., 10 min. at 4°C.) and were then re-suspended at 0°C. in the liquid to be tested. Tris-maleate buffer was used when varying pH in the experiments. In all other instances Tris-HCl (pH 7.2; 0.05 M) was used. Lysis of gonococci in a suspension was recorded as its decrease in absorption at 540 nm using a Beckman Model 25 spectrophotometer. Zero time absorption of the bacterial suspension was about 0.15. For viability measurements the bacterial suspension was diluted to 5×10^3 colony forming units (CFU) per ml. in the liquid to be tested. Sampling was done by withdrawing 0.1 ml. that was spread on an agar plate.

Results

The influence of pH on mechanical stability and viability of *N. gonorrhoeae* cells is shown in Fig. 1. It may be seen that gonococci are more stable at low pH (Fig. 1A). However, mechanical stability was not always matched by viability (Fig. 1B). At pH 5 and 6, which efficiently stabilized the cells, viability was lower than at pH 7.2, which was optimal for survival. Temperature also had an effect on non-growing gonococci (Fig. 2). Elevated temperatures promoted lysis, while at 0°C. the bacteria were stable (Fig. 2A). A similar relationship between different temperatures was observed in viability tests (Fig. 2B). Already at zero time, half or more of the bacterial population was not viable at 22°C to 39°C compared with 100 per cent. survival at 0°C. During storage, viability was gradually lost, to some extent also at 0°C. Thus, despite the stabilizing effect at 0°C, only about 20 per cent. of the bacterial population formed colonies after 90 min. at this temperature. Still, viability was much less at 22°C than at 0°C.

Divalent metal cations and polyamines have been used to stabilize fragile envelope-defective forms of various bacteria (protoplasts, spheroplasts, L-forms) (Gonsalus and Stainer, 1960). The stabilizing effect of such compounds is probably achieved by their cross-linking of envelope components due to the formation of ionic bonds (Asbell and Eagon, 1966). All metal cations tested (Mg^{++} , Ca^{++} , Mn^{++} , Cu^{++} ; 4 mM), as well as spermine (0.5 mM) exerted a stabilizing effect on the gonococci (Figs 3A and 4A respectively), reducing the rate of lysis by 60 to 80 per cent. Cu^{++} was particularly efficient, apparently causing complete stabilization and even shrinkage of the bacteria, recorded as an increase in absorbancy (Knowles, 1971). Except for Cu^{++} , these components also had a beneficial effect on the survival of gonococci, Mg^{++} being the most protective (Figs 3B and 4B). Different concentrations of Mg^{++} were tested and 4 mM was found optimal. In contrast to the other cations, Cu^{++} was strongly toxic to the gonococci despite its efficient stabilizing effect. A

FIG. 1A Autolysis of *N. gonorrhoeae* (type 4) measured by optical density at 540 nm during incubation at different pH (5.2, 6.0, 7.2, 8.0)

FIG. 1B Viability of autolysing *N. gonorrhoeae* (type 4) measured by colony counts during incubation in tris-maleate (0.05 M) at different pH (5.2, 6.0, 7.2, 8.0)

FIG. 2A Autolysis of *N. gonorrhoeae* (type 4) measured by optical density at 540 nm during incubation in tris-HCl (0.05 M, pH 7.2) at different temperatures (0°, 22°, 37°, 39°C)

FIG. 2B Viability of autolysing *N. gonorrhoeae* (type 4) measured by colony counts during incubation in tris-HCl (0.05 M, pH 7.2) at different temperatures (0°, 22°, 37°, 39°C)

FIG. 3A Autolysis of *N. gonorrhoeae* (type 4) measured by optical density at 540 nm during incubation in tris-HCl 0.05 M pH 7.2 and containing 4 mM of different metal cations (Cu, Mn, Mg, or Ca)

FIG. 3B Viability of *N. gonorrhoeae* (type 4) measured by colony counts during incubation in tris-HCl 0.05 M pH 7.2 and containing 4 mM of different metal cations (Cu, Mn, Mg, or Ca)

FIG. 4A Autolysis of *N. gonorrhoeae* (type 4) measured by optical density at 540 nm during incubation in tris-HCl 0.05 M pH 7.2 and containing different stabilizers (PVP, sucrose, or spermine)

FIG. 4B Viability of *N. gonorrhoeae* (type 4) measured by colony counts during incubation in tris-HCl 0.05 M pH 7.2 and containing different stabilizers (PVP, sucrose, or spermine)

toxic effect was also noted when the cells were incubated in the presence of Zn^{++} (4 mM).

Fragile forms of bacteria can also be mechanically stabilized by suspending them in an environment exercising a high osmotic pressure due to molecules that cannot penetrate into the bacterial cell. High concentrations (10–20 per cent.) of sucrose (Gonsalus and Stainer, 1960) have often been used in this context. Figure 4A depicts the osmotically stabilizing effect of sucrose (10 per cent.) and the synthetic polymer polyvinylpyrrolidone (PVP, mW=40,000,

10 per cent.). Both were efficient stabilizers causing shrinkage of the bacterial cells. When their influence on viability was tested (Fig. 4B), sucrose was found to be toxic. On the other hand, the PVP had a protective effect comparable to that of spermine, Ca^{++} , and Mn^{++} .

Discussion

Dienes (1940) observed that in ageing colonies of *N. gonorrhoeae*, some cells changed into 'large bodies'. He was later able to isolate L-forms from such

colonies without the aid of penicillin (Dienes, Bandur, and Madoff, 1964). The recent paper by Hebel and Young (1975), as well as the experiments reported here, show that gonococci tend to turn into fragile forms and subsequently to lyse. This destiny appears to be true for most cells of the population as soon as their growth ceases. This is probably due to a poorly controlled action of peptidoglycan hydrolases. Such 'autolytic' enzymes exist in many, maybe all, bacteria that contain a peptidoglycan 'skeleton' in their cell envelopes, and a physiological role in cell wall expansion and cell division has been ascribed to them (Ghuysen, 1968). Directly after harvesting, nearly half of the cell population in a culture of gonococci behave like spheroplasts judging from experiments (Elmros and Burman, unpublished) during conditions, such as raised temperature and pH, that efficiently lyse protoplasts, spheroplasts, and artificial membranous bodies.

Autolysis may explain why gonococci do not always survive during the transport of specimens from patients to the laboratory and may also explain why L-forms of gonococci can be isolated from 20 per cent. of specimens from patients with untreated gonorrhoea (Gnarpe, Wallin, and Forsgren, 1972).

Except for low temperature that reduces their enzyme activity, no inhibitors of peptidoglycan hydrolases are known. As is shown here, however, lysis of gonococci can be prevented by various stabilizing environmental conditions such as low temperature, low pH, divalent cations, and high osmotic pressure. Many of these are accompanied by increased survival, while others (sucrose, low pH, and Cu^{++}) are not. The highly toxic effect of Cu^{++} on gonococci has also been reported by Fiscina, Oster, Oster, and Swanson (1973). Copper is incorporated into many intrauterine birth control devices. According to calculations by Fiscina and others (1973), the amounts of copper that leave such birth control devices may be enough to protect women

from contracting gonorrhoea, but a recent study suggests that this is not the case (Spellacy, Hiser, and Birk, 1974).

In all viability experiments reported here, the bacteria were cultured on regular agar plates, where L-forms of bacteria do not usually survive. We therefore hope that further studies to establish conditions for optimal mechanical stabilization of autolysing gonococci and above all their cultivation on special semi-solid agar substrates, allowing survival of L-forms and their conversion to cells with normal walls, will improve bacteriological diagnosis in gonorrhoea.

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