Presence of Hydrogen Peroxide in Media Used for Cultivation of *Neisseria gonorrhoeae*

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Defined complex media used for cultivation of *Neisseria gonorrhoeae* were tested for the presence of H_2O_2 by both a spectrophotometric and a polarographic assay. H_2O_2 (35 to 165 μM) was present in all media tested. In the defined media, H_2O_2 was generated by the interaction of cysteine with other amino acids. The addition of the chelator 8-hydroxyquinoline prevented formation of detectable H_2O_2 , suggesting that metal ions were necessary. The persistence of H_2O_2 varied greatly among different media. Medium components which affected the presence of H_2O_2 were pyruvate, oxalacetate, and sodium sulfite. Sodium sulfite also generated superoxide radical. In liquid medium containing H_2O_2 , the endogenous gonococcal catalase present in an inoculum of about 2×10^7 colony-forming units/ml destroyed detectable H_2O_2 . The long lag phase which resulted from a 10-fold lower inoculum could not be shortened by the addition of exogenous catalase. Small amounts of residual H_2O_2 in agar plates of complex medium affected the viability of gonococci which had been suspended in buffer and incubated for 60 min at 37°C. Addition of pyruvate or catalase increased viable counts in medium containing H_2O_2 .

Hydrogen peroxide (H_2O_2) is generated in certain bacteriological media and suspending fluids (4, 5, 9, 21). This H_2O_2 is toxic for many catalase-negative bacteria, and the destruction of H_2O_2 increases their survival (4, 5, 9, 10, 21, 23).

The effects of H_2O_2 on aerobic, catalase-containing microorganisms have not been widely investigated. Small amounts of H_2O_2 appear to affect stressed *Staphylococcus aureus* cells (1, 3, 9, 26), because viable counts are increased when such cells are plated on medium containing catalase or pyruvate (14), both of which break down H_2O_2 .

The presence and possible effects of H_2O_2 in media used to cultivate *Neisseria gonorrhoeae* have not been previously investigated. H_2O_2 is toxic (11, 24) even though gonococci contain considerable amounts of catalase (20). While evaluating the effects of H_2O_2 and superoxide radical (O_2^-), we observed that the viability of gonococci incubated in buffer for 60 min was greater when cells were plated on medium with catalase. Therefore, we examined the plating medium, as well as other media used for cultivation of *N. gonorrhoeae*, for the presence of H_2O_2 and for its effects on gonococcal growth.

MATERIALS AND METHODS

Media. Except where noted, all media were prepared according to the instructions in the appropriate references. Minimal defined medium (MDM) of Morse and Bartenstein (18) was prepared initially by adding the components in the order listed in Table 1. After we found that the age of the amino acid solution was a variable in the amounts of H_2O_2 generated, concentrated stock solutions of each of the amino acids (except cysteine, cystine, and glutathione) were prepared. The stock solutions were stored frozen and either premixed in a pool or added individually to a mixture of deionized water, salts I, and salts II. Solutions of cysteine, cystine, and glutathione were made the same day the media were prepared. The salts and amino acids were added to deionized water so that their concentrations were the same as in the complete medium; the pH was adjusted to 7.45, and the H_2O_2 content was determined. Gonococcal genetic medium (GGM) was prepared as described (13).

Complex liquid medium (GCB) was prepared as previously described (17). The complex solid medium was GC agar (Difco Laboratories, Detroit, Mich.) with IsoVitalX enrichment (BBL Microbiology Systems, Cockeysville, Md.).

Solutions containing pyruvate, oxalacetate, or sodium sulfite were freshly prepared prior to their addition to any of the above media. Pyruvate was filter sterilized and added after the complex medium had been autoclaved. When added, the final concentrations were as follows: pyruvate, 14 mM in complex medium and MDM, but 17.9 mM in GGM; oxalacetate, 1.89 mM in GGM; sodium sulfite, 5.95 mM; and catalase, 3.8×10^2 U/ml.

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TABLE 1. Interaction of components of MDM to form H_2O_2

Additive components of MDM ^b	H_2O_2 (μM) ^a	
	2 h	24 h
Salts I	<5	<5
Salts II	<5	<5
Solution of amino acids ^c	50	42
Solution of vitamins	56	42
Biotin	43	40
Glucose	48	40
Sodium bicarbonate	42	41
Calcium chloride	54	44
Ferric nitrate	52	50

^a H_2O_2 was measured by the spectrophotometric assay after the adjustment of pH.

^b Each sample assayed was additive; it contained the component listed plus those above it. Each sample was brought to volume, adjusted to pH 7.45, and incubated at room temperature for 2 h prior to assay for H_2O_2 . Calcium and iron, however, were added after adjustment of volume and pH, as recommended in reference 18. See reference 18 for composition of salts solutions and for concentrations of components.

^c The amino acid solution was prepared one day prior to use. See Materials and Methods for composition.

When the chelator 8-hydroxyquinoline was added to MDM, it was dissolved in ethanol and added prior to addition of amino acids. The final concentration of 8-hydroxyquinoline was 500 μM . A control showed that the ethanol had no effect on the formation or detection of H_2O_2 .

Assays for H_2O_2 . For the spectrophotometric assay, 0.3 ml of sample was mixed with 0.6 ml of a mixture of *o*-dianisidine and horseradish peroxidase (7a). The mixture was composed of 0.05 ml of 1% *o*-dianisidine in distilled water per 6.0 ml of horseradish peroxidase (0.2 mg per ml of 0.01 M potassium phosphate, pH 6.0). Absorbance values obtained at 460 nm were compared to a standard curve. H_2O_2 standards were obtained by dilution of 30% H_2O_2 ; an extinction coefficient of $43.6 \text{ M}^{-1}\text{cm}^{-1}$ at 240 nm was used to determine the actual H_2O_2 content of the standards prior to assay. However, interfering electron donors, when present, are oxidized instead of the *o*-dianisidine. Therefore, a polarographic method using catalase, which is more specific in its use of electron donors, was used to measure the H_2O_2 present in some media. In this assay, the oxygen evolved upon addition of 5 μl of catalase ($2.4 \times 10^5 \text{ U/ml}$) was measured with an oxygen probe (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Because H_2O_2 was quickly lost upon mixing, the solutions were not saturated with air prior to the assay. Values were compared to a standard curve after subtraction of any oxygen evolution obtained in the absence of catalase. A standard curve was prepared with 30% H_2O_2 diluted in 0.05 M potassium phosphate, pH 7.0. The differences in the amounts of H_2O_2 measured by the two methods are most likely due to the presence of electron donors which interfere in the spectrophotometric assay. Even though the amounts of H_2O_2 in MDM were slightly less when measured by the spectrophotometric assay than with

the polarographic assay, the former assay was used to determine the source of H_2O_2 (Tables 1 and 2) because it was easier to use.

Detection of $\text{O}_2^{\cdot-}$. The reduction of nitroblue tetrazolium (final concentration, 40 $\mu\text{g/ml}$) after the addition of sodium sulfite (final concentration, 5.95 mM) or GGM was used to detect $\text{O}_2^{\cdot-}$. The reduction of nitroblue tetrazolium was determined by its absorbance at 540 nm. The reduction, which is inhibited by superoxide dismutase, is due to $\text{O}_2^{\cdot-}$ (2).

Bacterial strains and growth conditions. *N. gonorrhoeae* strain CS-7 (17), colony type T4, was used in all experiments. After at least two nonselective transfers on defined agar medium, gonococci were inoculated to an initial density of either 2 or 20 Klett units (no. 54 filter) into 50 ml of defined medium in a 300-ml nephelometer flask. These values are equivalent to about 2×10^6 colony-forming units (CFU)/ml and 2×10^7 CFU/ml. Cultures were incubated at 37°C in a New Brunswick rotary water bath shaker at 137 rpm. Growth was followed turbidimetrically. For viability studies, gonococci growing in MDM were harvested during the late logarithmic phase by centrifugation ($3000 \times g$ for 10 min). The cell pellet was washed twice in 0.01 M potassium phosphate buffer (pH 7.0) containing 0.01% bovine serum albumin, 136 mM NaCl, 1.7 mM CaCl_2 , 0.28 mM MgCl_2 , and 1.0 mM MgSO_4 . Harvesting and washing were carried out at room temperature. The washed cells were diluted to about 10^4 CFU/ml in the same buffer. The gonococci were mixed vigorously for 5 s and plated on fresh GC agar, GC agar with pyruvate, or GC agar spread with 0.05 ml of catalase (200 U/ml).

Source of chemicals. All chemicals were reagent grade. Oxalacetate, pyruvate, cysteine hydrochloride, cystine, *o*-dianisidine, catalase (catalog no. C40), and bovine serum albumin (catalog no. A4378) were obtained from Sigma Chemical Co., St. Louis, Mo. Horseradish peroxidase was obtained from either Worthington Diagnostics, Freehold, N.J., or Boehringer/Mannheim, Indianapolis, Ind. Superoxide dismutase was obtained from Boehringer/Mannheim. Hydrogen peroxide used for standards was from J. T. Baker Chemical Co., Phillipsburg, N.J.

RESULTS

Determination of H_2O_2 in defined gonococcal media. H_2O_2 was not detected when MDM was assayed immediately after preparation; however, 50 μM H_2O_2 was detected 2 h after preparation. High concentrations of H_2O_2 were maintained in MDM stored at 4°C for at least 8 days, but slow dissipation occurred over 30 days (data not shown). To determine the component(s) responsible for generating H_2O_2 , samples were assayed after the addition of each component. H_2O_2 was detected in the combination of salts I, salts II, and amino acids (Table 1). The addition of subsequent medium components did not significantly change the concentration of H_2O_2 formed by these three components. The contribution of each component was determined. The pool of amino acids was the source of the H_2O_2 , with salts I or II increasing the rate of formation (data not shown).

TABLE 2. Generation of H₂O₂ in amino acid solutions

Addition ^a	H ₂ O ₂ (μM) ^b			
	0 min	60 min	120 min	180 min
Amino acid pool ^c without cysteine	<5	22	22	22
Amino acid pool ^c without cysteine and cystine	<5	<5	<5	<5
Cystine	<5	<5	<5	<5
Cysteine and methionine	<5	<5	150	188
Methionine	<5	<5	<5	<5
Cysteine and tryptophan	<5	<5	126	136
Tryptophan	<5	<5	<5	<5
Amino acid solution ^d (4 days old)	<5	<5	50	50
Amino acid solution ^d (1 mo old)	<5	<5	<5	<5

^a The combinations of amino acids indicated were added to a mixture of deionized water, salts I, and salts II, and adjusted to pH 7.45. All amino acids were tested in the same concentrations found in MDM.

^b H₂O₂ was measured by the spectrophotometric assay at timed intervals after adjustment of pH.

^c Pools of all the amino acids found in MDM were prepared immediately prior to use from concentrated, frozen stock of individual amino acids, except for cysteine, cystine, and glutathione. When these compounds were added, individual solutions were made immediately prior to use.

^d These amino acid solutions contained all amino acids in MDM, including cysteine, cystine, and glutathione. The sterile stock solutions were stored at 4°C for various times before they were tested for their ability to generate H₂O₂ with salts I and II at pH 7.45.

Since cystine is known to produce H₂O₂ upon autoxidation (4, 7, 12, 21, 22, 25), its role in the generation of H₂O₂ in the pool of amino acids was investigated. Omission of cysteine resulted in a decreased amount of H₂O₂ (Table 2). When both cysteine and cystine were omitted, no H₂O₂ was detected (Table 2). The autoxidation of cysteine did not generate detectable H₂O₂. An interaction of cysteine with other amino acids was required. Mixtures of methionine and cysteine or tryptophan and cysteine generated high levels of H₂O₂ (Table 2). Other combinations of individual amino acids plus cysteine were not tested. The detection of H₂O₂ in stock solutions of amino acids was dependent upon age (Table 2); H₂O₂ was not detected in stock solutions older than 1 month. Since metal ions can increase the rate of cysteine autoxidation (4, 7, 12, 25), MDM was prepared in the presence of a metal ion chelator. H₂O₂ was not detected in MDM prepared with 500 μM 8-hydroxyquinoline as the initial component.

Attempts were made to prevent H₂O₂ formation or to eliminate it from MDM. Even though the deletion of cysteine and cystine prevented H₂O₂ formation, both could not be omitted because gonococci require either cysteine or cystine for growth (6). Omission of cysteine decreased the H₂O₂ formed. The small amounts formed by compensatory concentrations of cystine could no longer be detected upon addition of catalase (3.8 × 10² U/ml), sodium sulfite (5.95 mM), or pyruvate (14 mM) (data not shown).

Estimation of H₂O₂ in MDM also was determined by use of the oxygen probe. The higher values obtained by this method may be due to medium components which are oxidized by horseradish peroxidase and H₂O₂. As in the

previous determinations, pyruvate destroyed H₂O₂.

H₂O₂ was not detected in GGM when assayed by the spectrophotometric method. When assayed polarographically, 90 μM H₂O₂ was detected in freshly prepared medium. This concentration rapidly decreased to undetectable levels within 2 h. The medium alone exhibited oxygen uptake, which would minimize the amount of H₂O₂ measured with the oxygen monitor. Addition of known amounts of H₂O₂ to GGM resulted in a rapid breakdown of H₂O₂ (data not shown). Since this medium contains both pyruvate and oxalacetate, these components were omitted. However, a similar formation of H₂O₂ followed by a rapid decomposition still occurred.

Sodium sulfite is a component of GGM which might affect H₂O₂ concentration. Sulfite ion was of particular interest because of its possible effects on H₂O₂ and its ability to undergo autoxidation with the formation of O₂⁻ (16). H₂O₂ was not detected by either assay after addition of sulfite (5.95 mM, as in GGM) to known concentrations of H₂O₂ (data not shown). Oxygen uptake and O₂⁻ were detected in freshly prepared GGM or in the solutions of sulfite (data not shown). The reduction of nitroblue tetrazolium by both the medium and solutions of sodium sulfite was completely inhibited by superoxide dismutase.

Determination of H₂O₂ in complex gonococcal media. GCB was assayed for the presence of H₂O₂ by the polarographic method to confirm its absence as determined spectrophotometrically. GCB which was autoclaved and cooled at room temperature for 2 h did not contain detectable levels of H₂O₂. However, after the addition of

FIG. 1. Growth of *N. gonorrhoeae* strain CS-7 in MDM, with and without H_2O_2 . The open symbols represent growth resulting from an inoculum of about 2×10^7 CFU/ml; the shaded symbols, from a 10-fold smaller inoculum. Symbols: $\circ$ and $\bullet$, complete MDM; $\square$ and $\blacksquare$, minus cysteine with replacement cysteine; $\triangle$ and $\blacktriangle$, minus cysteine with replacement cysteine plus catalase (3.8×10^2 U/ml); $\circ$ and $\bullet$, minus cysteine with replacement cysteine plus sodium sulfite (5.95 mM).

IsoVitaleX, significant levels of H_2O_2 ($>30 \mu M$) were generated and they persisted for at least 2 h. Some medium components slowly destroyed the H_2O_2 generated. A known concentration of H_2O_2 (110 μM) added to GCB slowly disappeared; rapid degradation occurred after the addition of pyruvate (data not shown).

GC agar was expressed through clean cheese-cloth, and the liquid portion was assayed for H_2O_2 . H_2O_2 was not detected by the spectrophotometric method. However, agar plates that were assayed within 1 h after the agar had solidified contained 39 μM H_2O_2 as measured polarographically. There was a great deal of variability in the concentration of H_2O_2 in medium assayed after the day of preparation. For example, individual plates prepared from a single batch of medium and assayed 24 h after preparation contained <5 to 10 μM H_2O_2 ; if assayed 48 h after preparation, they contained <5 to 12 μM H_2O_2 . Although the assay was variable, especially at low H_2O_2 concentrations, many plates had measurable levels of H_2O_2 . GC agar containing pyruvate had no detectable H_2O_2 either on the day of preparation or on subsequent days.

Effects of H_2O_2 on gonococcal growth. The small amounts of H_2O_2 that remained in the GC

agar had a considerable effect on viability of gonococci previously incubated in buffer for 60 min at $37^\circ C$. When gonococci were plated on certain batches of GC agar, the counts obtained prior to incubation and those obtained after incubation in buffer were essentially the same; the addition of pyruvate or catalase did not increase the counts. However, when gonococci were plated on other batches of GC agar, the counts obtained prior to incubation were 60 to 70% higher than the variable counts obtained after incubation; the presence of pyruvate or catalase resulted in counts comparable to those obtained prior to incubation.

We next determined whether the large amounts of H_2O_2 present in MDM affected either the growth of *N. gonorrhoeae* or the size of the inoculum necessary to initiate growth. MDM was assayed for H_2O_2 by the spectrophotometric method after preparation of the medium, after incubation of the medium in CO_2 , immediately before and after inoculation, and throughout growth. After inoculation of the medium with about 2×10^7 CFU/ml, H_2O_2 could no longer be detected (data not shown). Growth of gonococci in MDM containing catalase was similar to growth in MDM which contained H_2O_2 prior to inoculation (Fig. 1). MDM containing sulfite did not support growth (Fig. 1). Cultures inoculated with a 10-fold smaller inoculum had a longer lag phase; the lag phase of cultures inoculated into MDM containing catalase was not shorter than that of those inoculated into MDM containing H_2O_2 (Fig. 1).

DISCUSSION

Large amounts of H_2O_2 (25 to 165 μM) were generated by interaction of the components of all media tested. The formation of H_2O_2 in MDM was dependent upon the interaction of cysteine with other amino acids. The presence of metal ions appeared essential since the chelator 8-hydroxyquinoline completely prevented generation of detectable H_2O_2 in MDM. Thus, the formation of H_2O_2 may be due, at least in part, to the interaction of cysteine with metal ions contaminating the other amino acids. Metal ions increase the autoxidation rate of cysteine (4, 7, 12, 25), but the concomitant detection of H_2O_2 is usually difficult since these metal ions may also break down H_2O_2 . The presence of H_2O_2 in the mixture of cysteine, other amino acids, and salt solutions of MDM may reflect the relative stability of this compound in this particular mixture. Since all media tested contained cysteine and cystine, the source of H_2O_2 in all media may be the same, i.e., the interaction of cysteine, other amino acids, and metal ions or the interaction of cysteine with metal ions alone.

The persistence of the H₂O₂ varied greatly between media. GGM contained pyruvate, oxalacetate, and sodium sulfite, all three of which affect the detectable levels of H₂O₂. Pyruvate (14) and oxalacetate, or the pyruvate resulting from its spontaneous decomposition (19), decompose H₂O₂; sodium sulfite reduces H₂O₂ or interferes with both assays. Sodium sulfite, however, does not appear to be a desirable addition, since it generates O₂⁻ (15). Although the direct effects of O₂⁻ on medium components or gonococci, or both, are not known, addition of sodium sulfite to MDM resulted in a medium which did not support growth of strain CS-7. GCB and GC agar contained a component(s) which slowly decreased the H₂O₂ concentration upon storage. The variation in the levels of H₂O₂ between batches of GC agar probably reflects an uncontrolled aspect in the preparation or storage of the medium. Factors which could influence either the amount of H₂O₂ generated or its stability include the length of autoclaving (9, 23), the amount of mixing prior to pouring plates, the temperature of the medium upon addition of supplements containing cysteine, and variations in temperature and UV light during storage.

Other variables affected the stability of H₂O₂ in liquid defined medium. In MDM, the concentration of H₂O₂ was dependent upon the age of the amino acid pool; in GGM, the age of the stock solution containing sodium sulfite affected the amount of O₂⁻ generated (data not shown). The relationship of age to the effectiveness of pyruvate and oxalacetate in destroying H₂O₂ was not tested. Since the stability of pyruvate in solution is dependent upon its purity (26) and since oxalacetate undergoes spontaneous decomposition (19), the age of stock solutions may also influence the stability of H₂O₂.

The effects of H₂O₂ on growth in MDM did not appear significant because washed inocula apparently contained enough lysed cells so that the gonococcal catalase rapidly destroyed the H₂O₂. However, low concentrations of H₂O₂ had a considerable effect on viable counts when cells were plated on GC agar. Isolated organisms may be susceptible to the low concentrations of H₂O₂ found in some agar plates. Susceptibility of gonococci to low concentrations of H₂O₂ may be characteristic of stressed cells only. Counts of gonococci obtained prior to incubation in buffer were consistent and were not dependent upon the batch of GC agar. Only after incubation for 60 min at 37°C was viability enhanced by the presence of catalase or pyruvate. What may constitute a stress factor in the physiological milieu is not known. Thus, the presence of H₂O₂ in freshly prepared medium used for the isolation of *N. gonorrhoeae* may reduce the recovery of this organism from clinical specimens.

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