Tetrazolium Reduction as a Measure of Metabolic Activity for Glass-Adherent Mycoplasma pneumoniae

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The reduction of tetrazolium was used to assay the metabolic capability of developing Mycoplasma pneumoniae cultures on glass. Generally, the amount of tetrazolium reduced correlates with the amount of growth as measured by protein. Until a culture enters the late phase of the growth cycle, the drop in pH of culture medium provides similar information. In this last stage of growth, protein appears to be leveling. The pH continues to fall, but tetrazolium reducing activity decreases. Thus, considering the entire M. pneumoniae growth cycle, formazan production is a more reliable measure of metabolic capability of the organisms than either protein or pH. The reduction of tetrazolium provides a quantitative means of assessing enzymatic activities of glass-adherent M. pneumoniae.

The ability of Mycoplasma pneumoniae to attach firmly to glass surfaces was first reported in 1967 (14); by utilizing this property of adhesiveness, the preparation of concentrated mycoplasma suspensions was simplified. Cultivation of mycoplasmas on glass surfaces also was used to obtain large yields of organisms for studies involving mycoplasma membranes (9, 10) and for the preparation of a M. pneumoniae vaccine (13).

A variety of mycoplasma species attach to glass and plastic containers (16). Purcell et al. (11) have cultivated eight human mycoplasma species on glass, and the ability of mycoplasmas to attach to glass seems to be a common characteristic of this genus.

In the past, a method for measuring the metabolic capability of the undisturbed mycoplasma cell mass was not available, and quantitating glass-adherent mycoplasmas (GAM) involved detachment of the organisms and assay of the number of colony- or acid-forming units. However, a 2- to 10-fold increase in assay titers was noted after exposure to ultrasound (14), and when interpreting counts of organisms detached from glass, the presence of aggregates must be considered.

Application of tetrazolium reduction to mycoplasma enzymatic studies was first investigated by Somerson and Morton (15), who found that mycoplasma colonies reduced tetrazolium compounds. Lecce and Morton (6) performed metabolic studies on mycoplasma isolates from man by using 2,3,5-triphenyl-tetrazolium chloride (TTC) reduction anaerobically as an index of the organism's ability to use various substrates as electron donors. After the isolation of M. pneumoniae, Kraybill and Crawford (4) observed that this species was the only one of human origin that, under aerobic conditions, reduced TTC in agar. The aerobic reduction of TTC by broth cultures of M. pneumoniae was used in serological studies by Senterfit and Jensen (12). More recently, Woods and Smith (18) reported a tetrazolium agar overlay technique for rapid, presumptive identification of M. pneumoniae.

In this present report, a method is presented to assay metabolic activity of mycoplasma cultures developing on glass by measuring the amount of tetrazolium reduced. We determined that a correlation exists between the amount of TTC reduced and the quantity of GAM.

MATERIALS AND METHODS

Medium. M. pneumoniae was grown in a broth medium of a formulation changed slightly from that already reported (9). The basal portion of the medium contained 7.35 g of mycoplasma broth base (Baltimore Biological Laboratories, Cockeysville, Md.)

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and 11.9 g of N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES; Calbiochem, San Diego, Calif.) in about 900 ml of demineralized water. The pH was adjusted to 7.5 with 10 N NaOH prior to autoclaving. After cooling, the following ingredients were added aseptically to the base: 50 ml of 25% yeast extract solution (Grand Island Biological Co., Grand Island, N.Y.); 30 ml of bovine serum fraction (BSF; Huntington Research Center, Baltimore, Md.); 4.8 g of powdered Eagle minimum essential medium added in 50 ml of demineralized water (EMEM; GIBCO); 10 ml of 50% glucose; 4 ml of 0.5% phenol red solution (GIBCO); 500,000 units of buffered potassium penicillin G (Chas. Pfizer and Co., Inc., Laboratories Div., New York, N.Y.). Twenty-five milliliters of the medium was dispensed per each 6-oz (Brockway) prescription bottle.

For rinsing the cell sheets before addition of the tetrasodium solution, we used medium of the same formulation, but with EMEM (Flow Laboratories, Inc., Rockville, Md.) minus phenol red and glutamine; the latter compound was added separately from a sterile solution. This change excluded color due to the phenol red.

Growth of organisms. M. pneumoniae strain CL-8 was isolated in 1969 at Children's Hospital, Columbus, Ohio, from the throat of a 6-year-old boy who had atypical pneumonia. It was identified as M. pneumoniae by immunofluorescent and complement-fixing techniques (3, 14). The organism was subcultured and cloned with passage on agar and broth media; after eight subcultures, the CL-8 strain grew as a confluent layer of organisms attached to glass. After further subculture to the 16th passage, culture fluids were discarded, and the organisms were harvested by scraping the GAM into media containing 10% BSA. After pooling, suspensions were divided into 1.2-ml samples and stored in vials at -60 C to serve as inoculum.

To initiate growth, vials containing M. pneumoniae were thawed and 0.1 ml was added to medium in each prescription bottle. Bottles were incubated horizontally at 37 C (2). With incubation, distinct colonies of mycoplasmas became evident in broth and, in time, a mass of organisms covered the flat surface of the bottles. In the early stages of growth, from 24 to 60 h of incubation, colonies were sparsely scattered over the glass. Later, the mycoplasmas developed into a confluent sheet of GAM with thickening of the attached cell mass.

The pH values were determined on decanted, pooled culture fluids at various times during incubation. Preliminary work had indicated that combined pH values were representative of those obtained on separate replicate cultures (standard deviation of 15 replicate bottles equaled a 0.1 pH unit).

Protein assay. The protein concentration of the GAM was determined in the following manner. The broth medium was decanted, and the GAM was rinsed four times with 10 ml of 0.15 M phosphate-buffered saline (PBS), pH 7.3. The organisms were scraped from the glass into one ml of PBS. Protein content was determined by the procedure of Lowry et al. (7) by using crystalline bovine serum albumin (BSA) as a reference standard.

**TTC Preparation.** In initial experiments, various percentages of TTC solution were tested by using concentrations previously reported in metabolic studies (5, 15). A 0.1% concentration was optimal in our system. Also, there was greater formazan production when 0.1% glucose was incorporated into the TTC solution. Therefore, for all experiments, we used a 0.1% TTC solution in PBS containing glucose. The solution was sterilized by filtration and stored in amber-colored bottles for no longer than two days.

**Measurement of metabolic activity by TTC reduction.** This procedure involved measurement of TTC reduction by GAM and was adapted from the work of Lecce and Morton (6). Mycoplasma strain CL-8 was grown in 6-oz bottles as previously described. At intervals, culture fluids were decanted, and the GAM was rinsed once with 10 ml of medium. Twenty-five milliliters of TTC solution was added to the GAM, and cultures were incubated for 1 h aerobically at 37 C in the dark. After incubation, the red formazan was extracted from the GAM by pipetting 10 ml of ethyl acetate into each bottle and gently shaking. Ethyl acetate containing the red formazan formed a separate phase above the PBS and cell sheet. This top layer was removed by suction and, to sediment any cellular debris, centrifuged at 12,000 x g for 15 min in a Sorvall RCB-2 refrigerated centrifuge.

We assayed formazan contained in the ethyl acetate and determined optical density in a Bausch and Lomb Spectronic 20 spectrophotometer at A490 (1). These readings were used to show the metabolic activity of GAM at various stages of growth. To insure that TTC reduction was specifically indicating metabolic activity of the GAM, we used the same procedure on GAM that had been heat-killed at 60 C in a water bath for 1 h. No TTC reduction occurred with heated mycoplasmas.

**RESULTS**

**TTC reduction by GAM.** The GAM in 84-h-old cultures were examined for ability to reduce TTC by the methods just described. Reduction of the TTC was evident with the appearance of a red hue in the solution.

We determined that all of the TTC reduction occurred in the mycoplasmas attached to glass. In one set of cultures, ethyl acetate, the extractant, was added to the GAM and overlaying TTC solution; in a second set, the TTC solution was initially decanted, and the extractant was added separately to the GAM and to the decanted TTC. Bottles in the first set and in those in which extractant was added directly to the GAM showed an equal amount of reduction. No absorbance was observed with the decanted TTC solution treated with ethyl acetate, and obviously all of the reduction was associated with the GAM.

**Reaction time of the GAM with TTC.** To determine the optimal time for the GAM to react with the TTC solution, we compared
formazan production in six replicate 70-h-old (pH 7.1) and six replicate 85-h-old (pH 6.7) cultures. In both cases, cultures contained a layer of organisms attached to the glass surfaces. Formazan production was assayed at 0.5-h intervals after addition of TTC solution to the GAM (Fig. 1). After a short lag period, reduction increased linearly with time over a period of 2 h. An S-shaped curve was obtained in both sets of cultures, with a steeper slope from the 85-h-old GAM. Throughout a 3-h reaction period, the amount of TTC reduced was less with GAM from cultures taken at pH 7.1 than from those at pH 6.7. A reaction time of 1 h was chosen for subsequent experiments.

Correlation of TTC reduction and pH decline with age of the culture. We determined the amount of TTC reduction as related to the cultural age of the GAM. At times ranging from 40 to 93 h, mycoplasma cultures were assayed for TTC reduction (Fig. 2). Some ability of GAM to reduce TTC was observed in cultures during the first 60 h of growth, concomitant with the increasing formation of a confluent layer of organisms; this period will be referred to as phase I. The pH of the culture medium dropped slowly during phase I. From 60 to 75 h of incubation, after confluency was obtained (phase II), the ability to reduce TTC increased rapidly and was correlated with a decline in pH. The TTC reducing activity reached maximum values around 70 to 75 h, or within a culture medium pH range of 6.7 to 6.9. Below this range, there was a rapid decrease in the organism’s ability to reduce TTC; however, the pH continued to drop at a steady rate.

To summarize this experiment, a direct correlation was found during phase II between the amount of TTC reduction and the age of the culture. As a consequence, there also is a direct relationship between the amount of TTC reduction and the pH of the decanted culture fluids. Beyond phase II, pH does not indicate the organism's metabolic capability.

Correlation of TTC reduction with mycoplasma growth. To determine if a correlation existed between TTC reduction and mycoplasma growth, we measured the increase of GAM in terms of total protein. Two sets of mycoplasma cultures, each consisting of 4 to 8 replicate cultures, were used. Samples were taken between 24 and 90 h. One set was used to assay protein content of GAM, the other for TTC reducing capability.

At 24 h, growth of GAM was sparse; there was essentially no TTC reducing ability, and protein measured less than 100 μg of glass-adherent material. Both TTC reducing activity and protein increased slowly during phase I (Fig. 3). By the beginning of phase II, about 65 h, TTC reducing activity and protein rose rapidly, concomitant with the appearance of a confluent mass of GAM. The increase in TTC reducing activity remained linear during phase II and was paralleled by a rapid rise in protein, the
latter serving as a measure of the quantity of mycoplasma growth on glass. In phase II, the pH dropped steadily and faster than in phase I. After approximately 80 h of incubation, both the amount of the TTC reduction and protein appeared to be leveling, but the pH of the culture continued to drop steadily. In a second experiment, we employed GAM only in the growth period from 65 to 90 h (Fig. 4). Results were similar to those reported above except that TTC reduction reached a peak before 90 h. These two experiments showed that TTC reducing activity correlated with the amount of growth as measured by both protein content and pH reduction.

DISCUSSION

Prior to our work, TTC reduction was not employed to measure the metabolic activity of GAM. Other investigators have used a change in pH of broth cultures as evidence of growth and active metabolism of the organism (17). The growth of M. pneumoniae is accompanied by acid production resulting from glucose fermentation, and the incorporation of phenol red into the medium permits easy observation of this resultant pH change. Thus a decline in pH with color change in the medium is associated with increasing growth of M. pneumoniae. From our data, a decreasing pH is an indication of this growth increase, but only before cultures have entered the latter phase of the growth cycle. Until that point, there is a correlation between the level to which the pH has declined and the amount of TTC that will be reduced by the GAM. This correlation exists because both the TTC reduction and pH decline serve as measures of actively metabolizing cells. In the late phase of the growth cycle, the pH continued to decrease and was not paralleled by increased TTC reducing activity of GAM. In this declining phase, the ability to reduce TTC is a more reliable measure of metabolic activity than that afforded by pH. Apparently, acidity limits the organism's metabolic capabilities. In time, there is also a loss of antigenicity (8). It has been shown (8) that as the pH of the culture declines, viability measured in terms of acid-forming units decreases rapidly until no organisms are detected at a pH below 5.

Except for the late phase of the culture, the amount of TTC reduction also correlated with growth as measured by protein content of GAM; the greater the protein concentration of GAM, the more TTC was reduced. After prolonged incubation, tetrazolium reducing capability declined, whereas protein values remained constant or increased. These findings suggest that
TTC reduction measured GAM for their metabolic capabilities and, as could be anticipated, the protein determinations served to measure the total mass of organisms on glass, regardless of their metabolic state.

Thus, if a large mass of GAM is desired, perhaps for chemical analyses or for specific antigenic components, incubation of cultures can be prolonged. If actively metabolizing GAM are needed, such as for enzymatic studies, TTC reducing activity can indicate the best time for harvest. For a compromise, a high yield of metabolically capable organisms, both tetrazolium reduction and pH should be considered.

The reduction of TTC provides a quantitative means of assessing the enzymatic activities of glass-adherent M. pneumoniae. Considering the entire growth cycle, tetrazolium reduction is a more reliable measure of the metabolic capability of GAM than are either protein or pH.

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