JOURNAL OF BACTERIOLOGY, Apr. 1977, p. 292-296
Vol. 130, No. 1

Printed in U.S.A.

Effects of Carbon Dioxide, Urea, and Ammonia on Growth of *Ureaplasma urealyticum* (T-Strain Mycoplasma)

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Received for publication 31 August 1976

By use of a simple device for continuous CO_2 gassing of *Ureaplasma urealyticum* cultures growing in a liquid medium, we have been able to separate some of the effects of urea, CO_2, ammonia, and pH on growth. The CO_2 acted as a superior buffer in the pH range 5.7 to 6.8, which is optimal for *Ureaplasma* growth. It was, therefore, possible to observe the effect of repeated additions of urea to the culture without alkalinization of the growth medium. We found that the repeated additions of urea did not enhance *Ureaplasma* growth, and the resultant accumulation of ammonium ions (>2,000 μg/ml) did not cause more rapid death under these conditions. By abruptly changing the gaseous environment from CO_2 to N_2, it was possible to cause a rapid pH change in the culture to a value above 8.0. This resulted in a more rapid death of the organisms.

The ureaplasmas differ from the classical mycoplasmas in at least two ways: their ability to hydrolyze urea and their failure to grow in the conventional mycoplasma media to titers higher than 10^7 colony-forming units per ml. The function of the *Ureaplasma* urease and its effect on growth of the organisms have been of interest ever since it was first shown that urea promotes the growth of ureaplasmas (22) and that the organisms hydrolyze it (2, 12, 18, 21). Studies to investigate whether there is a correlation between the urease activity and enhanced *Ureaplasma* growth due to urea have so far failed to provide a clear answer (1, 7, 8, 21). Thus, it is still not clear whether urea by itself or CO_2 and ammonia (NH_3), its hydrolysis products, are responsible for the growth-promoting effect. The product of the interaction of ammonia with water at pH below 9.0 is ammonium ion (NH_4^+). It is this ion that accumulates in the *Ureaplasma* growth medium. If the intact urea molecule is the growth-promoting factor, then its depletion from the growth medium by the urease activity of the organisms would be expected to account for the cessation of *Ureaplasma* growth before titers comparable to those of classical mycoplasmas are reached. Testing this hypothesis has proven to be a difficult task, because the addition of high concentrations of urea to the medium inhibited *Ureaplasma* growth (10, 21) and because repeated additions of smaller quantities of urea resulted in the accumulation of NH_4^+, requiring compensatory pH adjustment to avoid unfavorably high pH, in addition to any possible direct toxic effects of the NH_4^+ (2). It became clear that to elucidate the role of urea in *Ureaplasma* growth, it would be essential to separate the effects of urea, CO_2, NH_4^+, and pH on *Ureaplasma* growth and study them independently. This has also been difficult to accomplish. Ford and MacDonald (2) found that in the acidic pH range, ammonia could not be removed from *Ureaplasma* cultures by aeration, and attempts to remove ammonia by precipitation as NH_4MgPO_4 were also unsuccessful. Some success with ammonia removal from growing cultures was achieved by Hendley and Allred (5) using a "vacuum flow" system which depended on growing the organisms in a shallow layer of liquid medium under a slight negative pressure. They succeeded in obtaining a slightly longer stationary phase but did not obtain the higher titers (>10^7 color change units per ml) desired. Several laboratories have been able to show that improved buffering of the growth medium improves *U. urealyticum* growth, especially on solid medium. Manchee and Taylor-Robinson (6) used *N*-2-hydroxyethylpiperazine-*N'*-2'-ethanesulfonic acid (HEPES) buffer in a solid medium and obtained colonies that were considerably larger than the usual "T" colonies (22). Likewise, Romano et al. (16) increased *Ureaplasma* colony size by incorporating L-histidine or phosphate buffer into the medium. A 5 to 15% CO_2 atmosphere has also been recommended as the best gaseous environment for *Ureaplasma* growth on agar

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(22). We have found that an atmosphere of 100% CO_2 is preferable, since it acts as a superior buffer in the acidic pH range which is optimal for *Ureaplasma* growth (14).

By use of a simple device for continuous CO_2 gassing of cultures growing in a liquid medium, we have been able to separate some of the effects of urea, CO_2, NH_4^+, and pH on *U. urealyticum* growth and provide some answers to the questions raised above.

MATERIALS AND METHODS

Organism and growth medium. *U. urealyticum* [strain 960-(cX8)], which had been originally supplied by M. C. Shepard (Camp Lejeune, N.C.), was grown in a filtered beef heart infusion broth that was supplemented with 5% (vol/vol) horse serum, 0.01 M urea, and 0.01 M putrescine dihydrochloride (14).

Assessment of growth. A modification of the tube dilution method for the determination of color change units was used (12). A 1.0-ml sample of *Ureaplasma* broth culture was serially diluted 10-fold in urea-containing medium, and the dilutions obtained in this manner were divided into six aliquots. After 5 days of incubation at 37°C, the color change end point was calculated by the method of Reed and Muench (15) and expressed as 50% color change units per milliliter.

Control of the gaseous environment during growth. A 1- or 2-liter portion of prewarmed (37°C), filtered medium was placed in a sterile Erlenmeyer flask (3 liter). The desired inoculum of *U. urealyticum*, growing logarithmically in the same medium, was then added. The flask containing inoculated medium was sealed with a sterile rubber stopper which had been fitted with the appropriate glass tubing for a gas inlet at the bottom of the flask and had a gas vent at the top of the flask. The outside end of each of these glass tubes was attached to a filter which was comprised of a 2- to 3-inch (ca. 5 to 7 cm) length of latex rubber tubing loosely packed with glass wool. The gas inlet tube was then connected via a polyethylene coupling and latex rubber tubing to a cylinder of the desired gas (Liquid Carbonic, Chicago, Ill.). This facilitated disconnecting the flask for sampling without changing the gas flow rate. The gas flow was regulated to a slow, but constant, rate that was sufficient to maintain an atmosphere of or near 100% of the gas being introduced but slow enough to avoid excessive frothing of the medium.

Analytical methods. Ammonia analysis was accomplished by the microdiffusion and Nesslerization method of Seligson and Seligson (17). Urease activity was measured by the disappearance of radioactive urea added to the *Ureaplasma* culture. [^{14}C]Urea with a specific activity of 54.4 mCi/mmol was purchased from Amersham/Searle Corp. (Arlington Heights, Ill.). A 1 M stock solution of labeled urea was diluted in the culture medium (1:100) to give a final concentration of 0.01 M urea and 0.01 μCi [^{14}C]urea per ml. Duplicate 0.5-ml samples of the culture medium were transferred at various times to glass scintillation vials containing 0.5 ml of 3.6 N H_2SO_4 and kept at room temperature for at least 60 min to stop enzymatic activity and to facilitate the removal of solubilized radioactive CO_2. Ten milliliters of Aquasol scintillation solution (New England Nuclear Corp., Boston, Mass.) was added to each vial, and the radioactivity was determined in an Isocap 300 liquid scintillation system (Nuclear-Chicago Corp., Arlington Heights, Ill.). Samples were counted at room temperature after a 15- to 30-min equilibration in the dark.

RESULTS

Figure 1 shows a comparison of the effects of a 100% CO_2 atmosphere and a 100% N_2 atmosphere on growth and ammonia production by *U. urealyticum*. The pH of the growth medium incubated with CO_2 decreased from 6.5 to 5.7 within a few minutes. A comparison of the growth curves during the early logarithmic phase of growth shows the generation time to be about 130 min with CO_2 and about 100 min with N_2. Hence, the growth of *U. urealyticum* was somewhat slower with CO_2, probably because of the initial decrease of the pH to 5.7, which may be suboptimal for the growth of this organism. The rate and extent of ammonia production were similar for both conditions. When sufficient ammonia had accumulated in the culture that had been gassed with CO_2, the pH increased to 6.3 (Fig. 1). The amounts of ammonia produced were in excess (by about 30 μg per ml of medium) of the amount expected from hydrolysis of urea alone, confirming our previous observations (9).

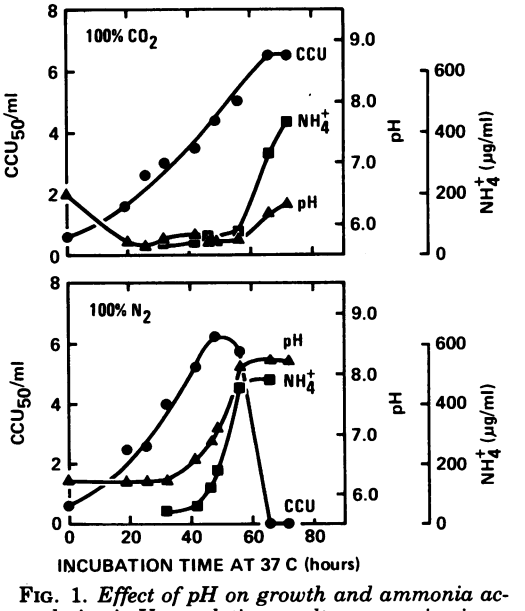

FIG. 1. *Effect of pH on growth and ammonia accumulation in U. urealyticum cultures growing in an atmosphere of 100% CO_2 (top) or 100% N_2 (bottom). CCU, color change units.*

Figure 2 shows the results of an experiment in which urea depletion rather than ammonia accumulation was measured in a culture incubated with CO_2. The higher initial inoculum used in this experiment as compared to that in Fig. 1 enabled the observation of the complete growth curve within a period of 3 days. The pH of the medium, which initially decreased to 5.7, increased to pH 6.3 and remained stable once all the urea was hydrolyzed. The maximum titer of viable organisms obtained in this experiment (about 5×10^7 50% color change units per ml) represents the highest titer attained in our experiments with CO_2. The growth curve in Fig. 2 shows a stationary phase of about 8 h followed by a decline phase in which the organisms had an exponential death rate. In other experiments, the stationary phase extended from 8 h up to 14 h.

Figure 3 shows the results of repeated additions of urea on *U. urealyticum* growth in a CO_2 atmosphere. One of the cultures in this experiment did not receive any supplementary urea; another culture received supplementary urea to a final concentration of 0.01 M at the end of the logarithmic phase of growth, i.e., at the time when the urea originally present in the medium was completely depleted (see Fig. 2). A third culture received five supplements, 0.01 M each, of urea at various time intervals during the stationary and decline phases of growth. It can be clearly seen in Fig. 3 that the growth curves were virtually the same whether or not supplementary urea had been added. Although it is not shown in Fig. 3, the supplementary urea was completely hydrolyzed within 3 h after each addition. NH_4^+ accumulated in the expected quantities. The amount of ammonia that accumulated in the culture receiving five supplements of urea exceeded 2,600 μg/ml. The pH in the flasks with one or no urea supplements remained at or near 6.3, whereas the pH in the flask receiving five urea supplements reached pH 6.8.

The experiment shown in Fig. 4 was designed to assess the effect of pH on *U. urealyticum* growth. Transfer of the culture from CO_2 to N_2, just before it reached the peak of its logarithmic phase of growth, caused a pH shift to the alkaline side (pH > 8.0) within 3 h. As can be seen in the figure, the culture under N_2 maintained its highest titer for approximately 8 h and then died more rapidly than the culture incubated under CO_2. The culture incubated under CO_2 also reached a titer about fivefold higher than that of the culture transferred to an N_2 atmosphere.

DISCUSSION

By use of a simple apparatus for controlling the gaseous environment of *U. urealyticum* cultures in liquid medium, it was possible to produce an atmosphere of essentially 100% CO_2. The CO_2 which was constantly bubbling through the growth medium acted as a superior buffer in the pH range of 5.7 to 6.8, the range which was found to be optimal for *Ureaplasma* growth (14, 20, 22). Hence, the use of CO_2 enabled us to examine the effects of urea and its hydrolysis products on *Ureaplasma* growth under conditions where pH changes were minimal. It was not necessary to add any reagent other than CO_2 to the culture to accomplish this. The system had the additional advantage that the CO_2 could be removed and replaced by another gas which had no buffering capacity, such as N_2, so that the effect of ammonia and pH on growth could be readily tested, again without the addition of any reagent to the culture medium. In this manner, our system ena-

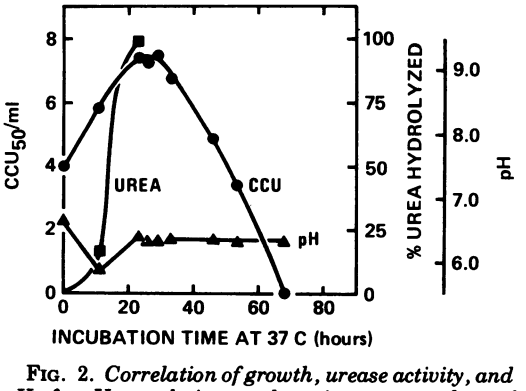

FIG. 2. *Correlation of growth, urease activity, and pH of an U. urealyticum culture in an atmosphere of 100% CO_2. The medium contained 0.01 M [^{14}C]urea. CCU, color change units.*

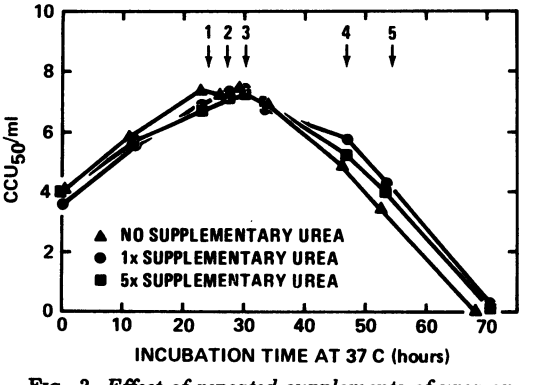

FIG. 3. *Effect of repeated supplements of urea on U. urealyticum growth in an atmosphere of 100% CO_2. The arrows indicate the time and number of urea supplements. CCU, color change units.*

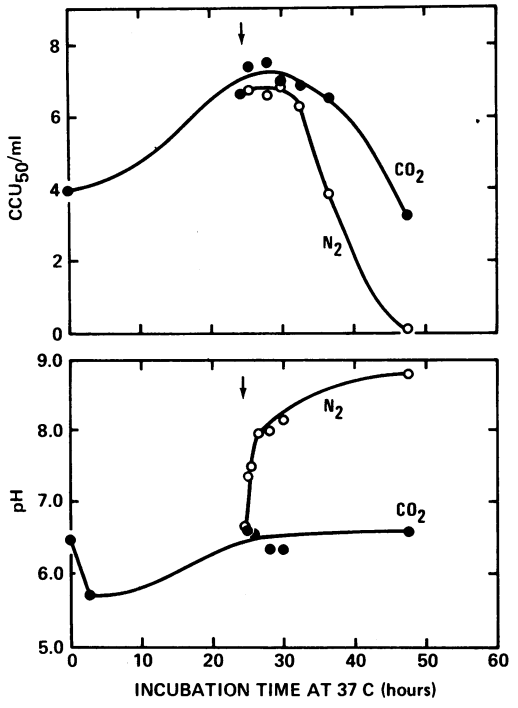

FIG. 4. *Changes in growth (top) and pH (bottom) on transfer of a U. urealyticum culture from a CO_2 to an N_2 atmosphere. A U. urealyticum culture growing under 100% CO_2 was divided into two equal parts at the time marked by the arrow; one part was kept under CO_2 (●), and the other was transferred to a N_2 atmosphere* (○). *CCU, color change units.*

bled us to differentiate between the effects of urea, NH_4^+, CO_2, and pH on *U. urealyticum* growth.

Our results, combined with those of others, enable us to reach several conclusions concerning the role of urea and the effects of its hydrolysis products on *U. urealyticum* growth. The exhaustion of the urea supply in the medium does not seem to be responsible for the limited growth of *U. urealyticum*. The repeated additions of urea to the growth medium did not increase the titer of viable organisms (Fig. 3). This corroborates the findings of Ford and MacDonald (2) that repeated additions of small quantities of urea to the growth medium did not improve *U. urealyticum* growth, even when the pH of the medium was kept below 7.0. However, they were using conventional batch cultures, so that observations could be only made during a limited time period (8 h), whereas in our system we were able to test the effect of repeated urea additions throughout the entire growth cycle of the culture.

Another conclusion is that the CO_2 liberated by urea hydrolysis cannot be regarded as the growth-promoting factor provided by urea, since it did not replace urea in the growth of *U. urealyticum* (14) and the radioactivity incorporated from [^{14}C]urea by growing *U. urealyticum* cells was negligible (1, 13). The improvement of *U. urealyticum* growth due to CO_2 can be accounted for by its excellent buffering capacity in the acidic pH range which is optimal for *U. urealyticum* growth. It is of interest that, although CO_2 is one of the hydrolysis products of urea, it did not inhibit the *U. urealyticum* urease activity, even when present in great excess. Neither did it cause any toxic effect on *U. urealyticum* growth.

Regarding the ammonia that had been liberated together with CO_2 on hydrolysis of urea, previous results indicated that like CO_2, NH_4^+ cannot replace urea in *U. urealyticum* growth (8), and it does not inhibit urease activity (11). However, the accumulation of NH_4^+ from urea hydrolysis may inhibit *U. urealyticum* growth. The question is whether this inhibitory effect is direct, due to the toxicity of the ammonium ion itself, or indirect, due to the effect of ammonia on the pH of the medium. Our results show that when the pH is kept in the acidic range by CO_2, the NH_4^+ accumulated does not affect growth, even at concentrations higher than 2,000 μg/ml. Hence, the accumulation of NH_4^+ per se does not appear to inhibit growth as long as the medium is kept in the acidic pH range. However, the accumulation of NH_4^+ in the absence of a CO_2 atmosphere might indirectly inhibit growth by increasing the pH to a level above the range suitable for *U. urealyticum* growth. Increasing the pH of the medium to values higher than 7.5 by NaOH has been shown to inhibit *Ureaplasma* growth (2, 8, 14, 20). Thus, it appears that alkalinization of the medium in the absence of ammonia or ammonium ions is sufficient to inhibit *Ureaplasma* growth (see reference 8). The ability to extend the growth cycle of *U. urealyticum* by pH adjustment alone has been recently reported by Windsor and Trigwell (23). Yet the removal of NH_3 or NH_4^+ from *Ureaplasma* cultures by dialysis (9) or vacuum (5) or by an ion-exchange resin (23) was found to improve *U. urealyticum* growth, as reflected by higher titers of viable organisms (9) or by prolonged stationary phase (5, 23).

Conflicting results have been reported on the effect of exogenous ammonium ions added to *Ureaplasma* cultures. Thus, Furness (3) did not detect any growth inhibition of *U. urealyticum* by NH_4^+ concentrations as high as 1,000 μg/ml, when the pH of the culture was acidic, whereas Ford and MacDonald (2) found some inhibition of growth by high concentrations of NH_4^+, even

at an acidic pH. The addition of 400 μg of NH_4^+ per ml to cultures without added urea was also found to inhibit *Ureaplasma* growth at an acidic pH (8). In our opinion, it is probable that the effects of exogenous NH_4^+ on the cells differ from the effects of the ammonia produced within the cells by the intracytoplasmic urease (11). Hence, it would be premature to derive conclusions about toxicity of NH_3 or NH_4^+, as this undoubtedly depends on the experimental conditions employed and perhaps on factors as yet unknown to us.

Nevertheless, our data indicate that the NH_4^+ which accumulates in the medium as a result of urea hydrolysis within the cells is not toxic when the pH of the medium is kept at an acidic level with excess CO_2.

ACKNOWLEDGMENT

This work was supported by Public Health Service grant AI-11805 from the National Institute of Allergy and Infectious Diseases.

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