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Growth of T-Strain Mycoplasmas in Medium Without Added Urea: Effect of Trace Amounts of Urea and of a Urease Inhibitor

G. K. MASOVER, J. R. BENSON, AND L. HAYFLICK

Department of Medical Microbiology, Stanford University School of Medicine, Stanford, California 94305

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Urea is currently considered to be a requirement for the propagation of T-strain mycoplasmas. We report here the replication of T-strain 960 (ATCC 25023) in media prepared from dialyzed components with added putrescine and allantoin but without added urea, or in dialyzed medium containing small amounts of added urea. The least amount of urea which allowed growth in the medium without allantoin was above 10 μg/ml. The amount of urea estimated to contaminate the added allantoin or putrescine was 5 μg/ml or less, which is insufficient to support T-strain replication. T-strain 960 was grown in the presence of urea and the urease inhibitor acetohydroxamic acid AHA where the organisms multiplied at a slower rate in the presence of AHA than in its absence. Urea hydrolysis occurred with concomitant ammonia accumulation and pH increase in cultures with AHA added.

After the isolation and initial descriptions of T-strain mycoplasmas (18, 19), the unpredictable growth and peculiar nutritional requirements of these organisms were studied. Serial propagation was generally unsuccessful, and the number of viable organisms in broth cultures declined rapidly after 24 h (19). The rapid loss of viability suggested depletion of a required nutrient. It was found subsequently that horse sera, which contain larger amounts of urea than do other sera, were superior protein enrichments for T-strain growth and, furthermore, that dialysis or urease treatment of horse serum rendered it inactive. However, if the serum dialysate or urea was added to the medium, T-strain growth then occurred (19). It was reported later (9, 20) that urea was, in fact, the required factor, and it has since been included in T-strain media formulations as an essential component (21). Urea is indeed described occasionally as an "absolute requirement" for the growth of T-strain mycoplasmas (14), and "urease activity" is described as a requirement for T-strain multiplication (7).

T-strain ureolytic capability is currently the sole stable characteristic of these organisms which can be used to distinguish them from other members of the family *Mycoplasmataceae* in the class *Mollicutes*. Because of their uncertain properties, T-strain mycoplasmas have not yet been given a specific taxonomic designation. If urea is actually a requirement for the growth of these organisms, then T-strain mycoplasmas are unique in having such a requirement. In that case, the biological role of urea hydrolysis in T-strain mycoplasmas would be of particular interest and importance. Furthermore, if these organisms can be shown, with certainty, to have a unique urea requirement for growth, they deserve taxonomic distinction at least at the genus level according to the currently accepted criteria for assigning taxa in the class *Mollicutes* (23). If, however, urea is not a requirement for growth but simply can be utilized as a substrate, then the property of urea hydrolysis by T-strain mycoplasmas would suggest only species level distinction, even though this activity is unique among the *Mycoplasmatales*. Since the T-strain mycoplasmas are currently unnamed, it is essential to establish unequivocally whether urea is a requirement for their growth.

The evidence, until now, which argued for a T-strain urea requirement was (i) that the organisms had not been grown in media without added urea or added serum with high urea content (9, 19, 20) and (ii) that urease inhibitors (hydroxamic acids) caused either less multiplication after 18 h of incubation (7) or a reduction of numbers of organisms after 24 h of incubation (8) compared with untreated controls. The hydroxamic acids were also shown to inhibit release of CO_2 from urea during short incubations of the T-strains in separate experiments designed to avoid T-strain multiplication. These results were taken together as an

indication of the essentiality of urea hydrolysis for T-strain multiplication (7, 8).

We have reported (13a) that a T-strain mycoplasma could be serially passaged in a medium prepared from exhaustively dialyzed components with added putrescine and allantoin but with no added urea. However, we did not exclude the possibility that trace amounts of urea in the medium might be sufficient to allow for T-strain multiplication. It was expected, however, that very low levels of urea (less than 10 μg/ml) would be either depleted metabolically by T-strain ureolytic enzyme(s) or reduced to levels which were not biologically useful to the organisms.

In this paper, we extend our previous observations (13a) and show that trace or contaminant amounts of urea do not support T-strain multiplication. In addition, we show that, although a urease inhibitor, acetohydroxamic acid (AHA), reduces the rate of multiplication of T-strain 960, it does not appear to inhibit multiplication or the ureolytic activity of the organism.

MATERIALS AND METHODS

T-strains. Lyophilized T-strain 960, which had been cloned eight times, was supplied by M. Shepard (Camp Lejeune, N. C.).

Washing procedure. In some experiments, cells were suspended twice in the medium specified but without added allantoin, urea, or AHA. The preparation was centrifuged at 18,000 $\times$ *g* for 15 min at room temperature after each suspension.

Media and growth conditions. The culture medium used was that developed in these laboratories for the initial isolation of *M. pneumoniae* (10) but was modified as follows for T-strain culture and is referred to hereafter as urea medium. The fluid medium was prepared from 50 g of beefheart infusion (Difco Laboratories, Detroit, Mich.) per liter. This basal broth was supplemented with 10% unheated horse serum, 10% of a yeast extract preparation, 1% phosphate-buffered saline (Grand Island Biological Co., Grand Island, N.Y., 10$\times$ concentrate), 1,000 U of penicillin per ml, 0.002% phenol red, and 0.1% urea. The pH was adjusted to a value between 6.0 and 6.5 with 1 N HCl.

Stock cultures of T-strain 960 were maintained in glass serological tubes (13 by 100 mm) with Morton-type closures and incubated at 34 to 36 C in medium without added urea (Fig. 1). For medium without added urea, the urea in the formulation just described was replaced with 0.01 M putrescine dihydrochloride, and the horse serum, which usually contains from 30 to 60 mg of urea per 100 ml, was replaced with dialyzed calf serum (Microbiological Associates, Inc., Bethesda, Md.). This medium is referred to as putrescine medium.

Titration of T-strain mycoplasmas. Titers of T-strain broth cultures were determined by a tube dilution method (15), which depends upon the ability of these organisms to produce ammonia from urea and, consequently, an alkaline shift in pH detected by the phenol red indicator. Serial 10-fold dilutions of the organisms were made in urea medium, and the greatest dilution yielding an alkaline pH shift was taken as the end point. This is expressed in color change units (CCU) and defined as the reciprocal of the logarithm (base 10) of the dilution end point.

Dialysis of media components. In addition to use of commercially dialyzed calf serum, basal broth and yeast extract were also dialyzed for some experiments. These were dialyzed at 4 C against two changes of 20 volumes of deionized water for a total of 48 h, and finally against 20 volumes of one-fourth strength Earle balanced salt solution (pH 6.5) for 24 h. The dialyzed basal broth and yeast extract, sterilized by autoclaving, were included in the complete medium as described above or in variations described below. Media prepared in this manner are referred to as dialyzed media.

Analytical methods. Ammonia was assayed by the microdiffusion-Nesslerization method of Seligson and Seligson (16). This assay is sensitive to approximately 2 μg of ammonia nitrogen and is highly reproducible.

Urea was determined by ion-exchange chromatography using an automatic amino acid analysis similar to the method of Spackman et al. (22). Samples were prepared by deproteinization with a 10% solution of sulfosalicylic acid (about 25% of the sample volume). The mixture was centrifuged and the supernate was adjusted to pH 2.0 to 2.2 using 0.3 M KOH (about 20% of the sample volume). The urea in the deproteinized samples was then separated from the other compounds present on Durrum DC-6A resin (Durrum Chemical Corp., Palo Alto, Calif.) in a column (0.9 by 31 cm bed). A 0.05 M citrate buffer containing 0.20 M lithium ion and adjusted to pH 2.80 was used as the eluant. The column temperature was maintained at 36 C. The buffer flow rate was 70 ml/h. After each analysis, the column was washed with 0.3 M LiOH. The eluted urea was developed with ninhydrin at 100 C, and the resultant complex was detected photometrically at 570 nm using a 12-mm flow cell. The recorder (Hewlett-Packard, model 7123A, San Diego, Calif.) was set to have a 1-mV range which corresponds to 0.1 full-scale absorbance. This procedure allowed for the detection of as little as 1 μg of urea.

Reagents. Putrescine (1,4-diaminobutane) dihydrochloride was purchased from Calbiochem (San Diego, Calif.). Allantoin was purchased from Sigma Chemical Co. (St. Louis, Mo.), who advised that the allantoin was free from urea and other nitrogenous compounds, as evidenced by production of only a single spot in two thin-layer chromatography systems using three detection methods. The allantoin is expected to be stable with respect to urea formation in the medium in which it was used in these studies. In addition, we undertook analyses of allantoin and putrescine for urea by ion-exchange chromatography (see below). AHA (Aldrich Chemical Co., San Leandro, Calif.) was prepared as a 100- aqueous concen-

trate and stored frozen for up to 14 days. Reagent preparations were prepared as aqueous solutions and sterilized before use by passage through a membrane filter (Millipore Corp., Bedford, Mass.) with 0.22-μm average pore diameter. Allantoin was dissolved directly in the basal broth, and the solution was sterilized by filtration before use.

RESULTS

Growth of T-strain 960 in media with trace amounts of urea. Although we were able to use putrescine in place of urea in undialyzed medium formulations, added putrescine alone did not support T-strain growth in media prepared from dialyzed components (13a). We found that urea or allantoin were necessary as well (13a). Therefore, it was of interest to determine whether residual urea, after dialysis of the medium components, or traces present as contaminants of the commercial reagents used in the dialyzed media would be sufficient to support T-strain multiplication.

T-strain 960 was passaged through more than 24 serial 10-fold dilutions in medium containing 0.033 M putrescine and dialyzed calf serum before use (Fig. 1). Cultures which were in exponential growth or early stationary phase were used as inocula for the experiments described.

The commerical allantoin was reported to be free from nitrogenous contaminants on the basis of thin-layer chromatographic analysis which is sensitive to 0.1 to 0.3% contaminants. We assayed the allantoin for urea content by the method referred to above. In analyses of different lots of allantoin, we found 2.2, 2.9, and 3.0 μg of urea per mg so that a 0.01 M allantoin medium (1.58 mg/ml) would contain a maximum of 5 μg of urea per ml due to added allantoin. Putrescine was analyzed in a similar manner but with even greater sensitivity since putrescine dihydrochloride is much more soluble than allantoin. No urea was found at a sensitivity range capable of detecting approximately 0.1 μg of urea per mg of putrescine. However, the 0.01 M putrescine medium, when assayed by the same method, was found to contain 2.9 $\pm$ 0.2 μg of urea per ml.

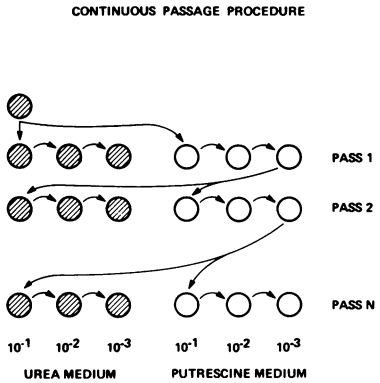

FIG. 1. *Procedure for serial T-strain culture. Each tube contained 1.8 ml of either urea medium (●) or putrescine medium (○). Both media were inoculated with 0.2 ml of a growing T-strain stock culture in urea medium, mixed thoroughly, and diluted through three 10-fold dilutions. When a color change was observed in the urea medium, the corresponding dilution in putrescine medium was transferred to the next set of culture tubes.*

It was necessary, therefore, to determine whether the trace amount of urea present in media due to allantoin plus any "background" urea in the medium without allantoin was adequate or necessary for the growth of T-strain mycoplasmas. The multiplication of T-strain 960 was monitored in experiments where reduced amounts of allantoin were added to dialyzed medium containing 0.01 M putrescine (dialyzed putrescine medium). A positive growth score was defined as either (i) an increase in titer of 0.5 CCU if it could be observed over three consecutive time points, (ii) an increase in titer of 1 CCU if it could be observed over two consecutive time points, or (iii) an increase titer of two CCU at any single time point. Growth, defined in this manner, was statistically significant ($P < 0.05$) as estimated by the Spearman estimator for serial dilution assays (11).

The amount of background urea in dialyzed medium was estimated by comparing analyses of several urea concentrations added to samples of the medium and deionized water. We found a consistant difference of 4 $\pm$ 1 μg of urea per ml of the dialyzed medium preparation which was tested.

Experimental media were inoculated with cells washed in complete medium without allantoin as described above, and the number of viable organisms was determined at approximately 12-h intervals over 79 h. For results shown in both Tables 1 and 2, initial titers, which served as points for comparison, were determined by performing multiple tube dilution assays on the initial inoculum. The initial titer represents the arithmetical mean of the determinations (expressed as $\log_{10}$). Titers at all subsequent time points were determined by a single-tube dilution assay. The lowest allantoin

concentration which allowed an unequivocal positive growth score was 790 μg/ml (5 mM), which could contain a maximum of 6.5 ± 1 μg of urea per ml as a contaminant of allantoin or other medium components (Table 1).

In subsequent experiments, allantoin was omitted from the dialyzed putrescine medium and urea was added in decreasing amounts. The urea was assayed before and after a 70-h growth period. The initial and final pHs of the medium were recorded also since previous results indicated that pH changes occurred in media containing putrescine and urea. The results (Table 2) indicated that the lowest urea concentration to give an unequivocal positive growth score was above 10 μg/ml (17, 16, and 23 μg/ml in the three experiments shown).

The final pH of the medium increased, and the maximal titer reached 6 CCU/ml only in urea concentrations above 10 μg/ml. The pH of dialyzed medium is sensitive to small additions of base such as ammonia because dialysis markedly reduces its buffer capacity. Thus, small amounts of ammonia (5 to 10 μg/ml) produce detectable pH changes. At urea concentrations below 10 μg/ml, there was no pH increase, but a decrease was found to occur after 70 h of incubation. We found a similar decrease in pH occurring in sterile incubated medium. In addition, incubated and unincubated sterile medium was assayed for urea. We found that there was no change in the amount of urea as a result of incubation. No urea remained in any of the growth tubes at levels detectable with the method described. These results suggest, therefore, that the trace amounts of urea in dialyzed putrescine medium are are insufficient to promote growth of T-strain mycoplasmas and that small amounts of urea are probably completely hydrolyzed whether the organisms increase in number or not.

Effect of the urease inhibitor, AHA. The use of urease inhibitors provides another approach to the determination of urease function in T-strains, and one such study (7) has recently been published. In that study, the organisms increased in numbers by at least 10-fold in the presence of inhibitor after only 18 h compared to a 1000-fold increase in the absence of inhibitor. However, it is possible that T-strain multiplication was not actually inhibited, but that the rate of multiplication was only reduced. Consequently, we decided to test the effects of AHA for longer time periods. AHA was chosen because it appears to have been more thoroughly studied as a urease inhibitor (2, 5, 12). Initially, four media formulations, all containing dialyzed calf serum, were tested for their ability to support T-strain multiplication in the presence of 1.8×10^{-4} M AHA. During a 60-h period, multiplication of T-strain 960 in dialyzed medium containing either 0.01 M putrescine and 0.01 M allantoin (PAL medium, Fig. 2A) or undialyzed 0.033 M putrescine

TABLE 1. *Growth of T-strain 960 in dialyzed media containing 0.01 M putrescine and reduced amounts of allantoin*

Expt	Allantoin concn		Contaminant urea[a] (μg/ml)	Initial inoculum[b] (CCU)	Titers observed at times indicated[c] (CCU)					Growth score[d]
	μg/ml	mM			9.5[e]	21.5	44.5	55	79	
1	0	0	4.0 ± 1	4	4	4	4	4	4	–
	79	0.5	4.3 ± 1	4	3	4	4	4	4	–
	158	1	4.5 ± 1	4	4	4	4	4	4	–
	790	5	6.5 ± 1	4	4	4	5	5	4	+
	1580	10	9.0 ± 1	4	5	5	6	5	4	+
2	0	0	4.0 ± 1	3	3	3	3	3	4	–
	79	0.5	4.3 ± 1	3	3	3	4	3	3	–
	158	1	4.5 ± 1	3	3	3	4	5	5	+
	790	5	6.5 ± 1	3	3	4	4	6	5	+
	1580	10	9.0 ± 1	3	4	4	5	5	4	+

[a] Contaminant urea from added allantoin (3 μg/mg) plus 4 ± 1 μg of urea per ml in medium prepared from dialyzed components.

[b] Triplicate determinations for each experiment (results identical; range = 0).

[c] Single determinations at each time point.

[d] + = >1 CCU increase in titer at two or more consecutive time points or 2 CCU increase at any time point during 79 h of incubation.

[e] Hours of incubation at 34 C.

TABLE 2. *Growth of T-strain 960 in dialyzed media containing 0.01 M putrescine and reduced amounts of urea*

Expt	Initial inoculum (CCU) ±95% confidence interval[a]	Urea (μg/ml)	Growth score[b]	Titers observed at times indicated[c] (CCU)									Mean titer[d] (CCU)	Significant ($P > 0.05$) difference between 0 urea and added urea[e]
				8[f]	18	20	32	36	44	49	58	70		
1. Urea determined by dilution[g]	4.4 ± 0.5	4	−		4			5		4			4.6	−
		7	−		5			4		4			4.6	−
		10	−		4			4		5			4.6	−
		17	+		5			6		6			5.8	+
		29	+		5			5		5			5.0	−
		54	+		6			6		6			6.0	+
2. Urea assayed[h]	4.7 ± 0.8	0	−	4		4	3		5		4	4	4.3	−
		5	−	4		4	4		4		4	4	4.0	−
		10	−	3		5	4		4		4	5	4.6	−
		16	+	4		4	4		6		6	5	5.6	+
		23	+	3		5	6		5		5	5	5.4	+
3. Urea assayed	2.7 ± 0.8	0	+	2		3	3		3		5	4	4.3	−
		5	+	2		4	4		3		4	4	3.8	−
		10	+	2		2	4		4		5	5	4.6	−
		16	+	2		2	4		4		5	5	4.6	−
		23	+	3		3	4		6		5	6	5.5	+

[a] Initial inoculum represents the arithmetical mean of four determinations (experiment 1) or two determinations (experiments 2 and 3) expressed as a logarithm (base 10) according to the definition of CCU in Materials and Methods. The 95% confidence interval is determined using the Spearman estimator for serial dilution assays (11).

[b] (+) growth score = 0.5-CCU titer increase at three consecutive time points, 1-CCU titer increase at two consecutive time points, or a 2-CCU titer increase at any time point.

[c] Single determinations at each time point.

[d] Arithmetical mean of titers observed at all time points for a given urea concentration; expressed as a logarithm (base 10).

[e] *t* test in which the standard deviation for a single tube dilution assay is taken as ± 0.5 CCU, and the degrees of freedom is taken as the number of observations (time points) used to calculate the mean titer.

[f] Hours of incubation at 34 C.

[g] Medium containing 50 μg of urea per ml diluted serially with medium containing no added urea; 4 μg/ml is included in this value to account for urea expected to be present in the dialyzed medium.

[h] Amino acid analytical method described in Materials and Methods.

medium (Fig. 2B) was completely inhibited by the AHA. In media containing 0.1% urea (1.66×10^{-2} M) either with or without putrescine, the organisms multiplied in the presence of AHA, and cultures were viable for a slightly longer time than control cultures without AHA.

In subsequent experiments, the growth-inhibiting effect of 1.8×10^{-4} M AHA on T-strain 960 was tested in media containing 0.1% (1.66×10^{-2} M), 0.05% (8.33×10^{-3} M), and 0.025% (4.25×10^{-3} M) urea. In addition, the pH changes and accumulation of ammonia (NH_3) were assessed. The results of one such experiment (0.025% urea) are shown in Fig. 3. Multiplication of T-strain 960 was not inhibited by the concentration of AHA used, although the rate appeared to be reduced. In the control culture (no AHA), the pH increased sharply as the titer reached maximal levels and remained constant after maximal titer was attained. Ammonia accumulation is seen to be closely related to the increase in pH (Fig. 3). The amount of ammonia expected to accumulate if 250 μg of urea per ml (0.025%) were completely hydrolyzed is 140 μg/ml. The initial ammonia concentration in the medium in this experiment was 43 μg/ml and the maximal level was 182 μg/ml, so that the amount of ammonia generated was observed to be the amount expected from complete urea hydrolysis in the culture without AHA. The relationships between growth, pH, and ammonia levels were qualitatively and quantitatively the same when AHA was present in the medium, except that the onset of sharp

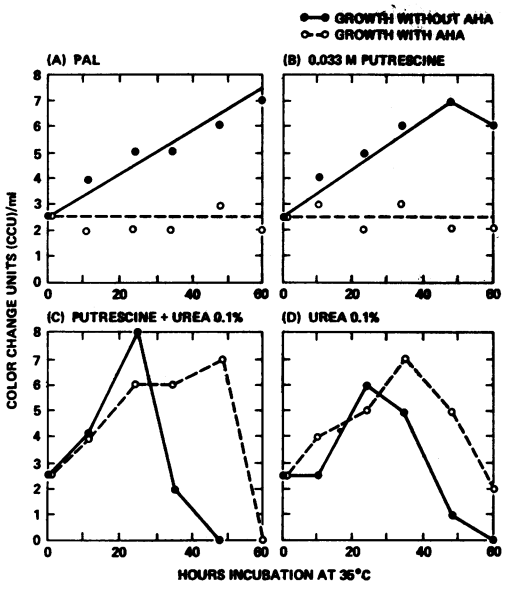

FIG. 2. *Effect of 1.8 × 10^{-4} M acetohydroxamic acid (AHA) on the growth of T-strain 960 in media with and without 0.1% added urea. A 5-ml volume of each of the media was inoculated with a 1:50 dilution of a growing culture of T-strain 960 which had been passaged through multiple, serial 10-fold dilutions in putrescine medium (0.01 M). PAL medium (A) is 0.01 M putrescine and 0.01 M allantoin in medium prepared with dialyzed components as described in Materials and Methods. All other media were prepared with undialyzed components but were supplemented with dialyzed calf serum in place of horse serum. (B) 0.033 M putrescine dihydrochloride medium, (C) 0.01 M putrescine dihydrochloride and 0.17 M urea (0.1%), (D) 0.17 M urea (0.1%). Symbols: ●, control, no AHA; ○, AHA, 1.8 × 10^{-4} M).*

increases in pH and ammonia levels were delayed for approximately 30 h.

With higher urea concentrations, less of a delay in the alkaline pH shift was observed in the cultures containing AHA, but maximal titers were similar in all cases. The maximal pH and ammonia levels reflected the initial urea concentration, and amounts of ammonia accumulated were within 10% of the amounts expected if all of the urea were completely hydrolyzed.

The results of experiments which tested various concentrations of AHA in cultures of T-strain 960 grown in 0.05% urea medium are shown in Fig. 4. The relationships between growth and pH are similar to those shown in Fig. 3. There were reduced growth rates with higher AHA concentrations and an increasing delay of the pH change with increasing concentrations of AHA. The pH of the cultures containing 14.4 × 10^{-4} M AHA required 200 h of incubation to reach levels comparable to the other cultures tested.

The stability of AHA in the culture medium was tested by preincubation of sterile medium with and without AHA for various periods up to 96 h before inoculation with T-strain 960. The pH changes and titer increases of the media were similar for all preincubation periods, and

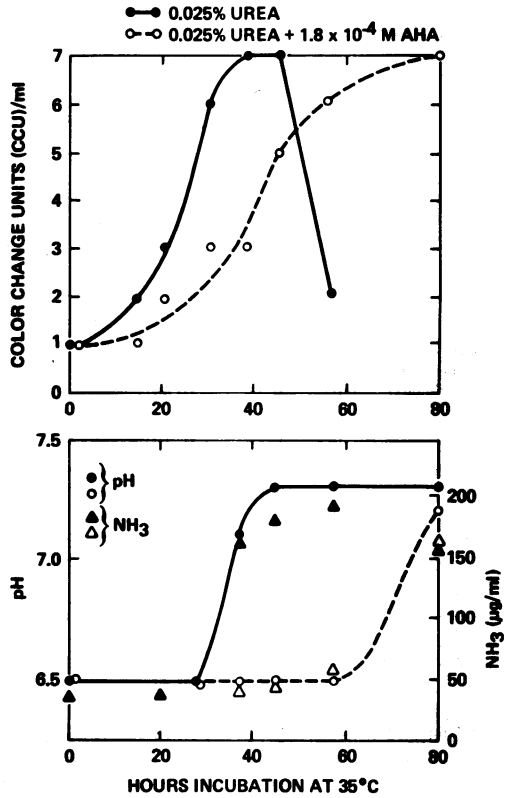

FIG. 3. *Effect of 1.8 × 10^{-4} M acetohydroxamic acid (AHA) on growth on T-strain 960 and on pH and ammonia changes in medium containing 0.025% urea. The urea medium was prepared with 10% dialyzed calf serum in place of horse serum and was divided into two parts. AHA was added to one part as a 100× aqueous concentrate of the desired final concentration. Five-milliliter quantities of media were inoculated from T-960 stocks which had been growing in 0.01 M putrescine medium, and washed as described in Materials and Methods. Media were inoculated with a 1:1000 dilution of cells washed as described in Materials and Methods. Solid symbols (●, ▲) represent control (no AHA) cultures. Open symbols (○, △) represent cultures containing 1.8 × 10^{-4} M AHA.*

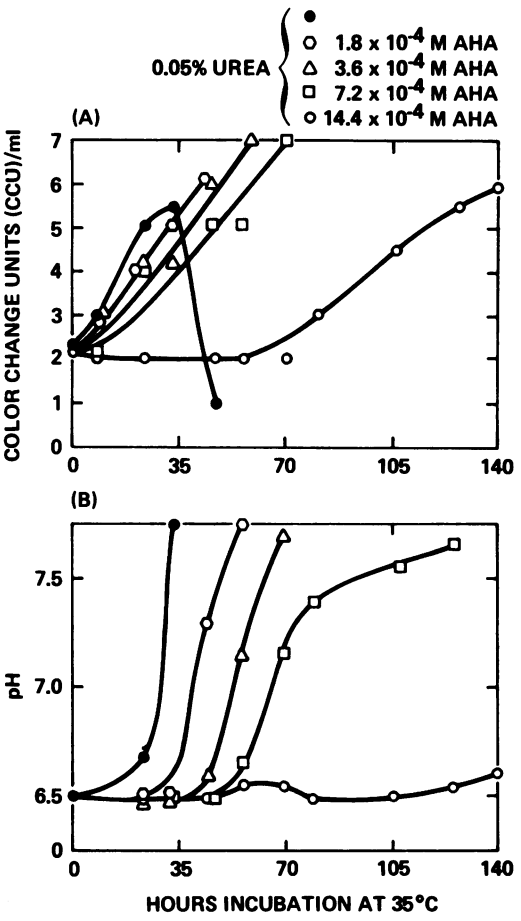

FIG. 4. *Effect of various concentrations of acetohydroxamic acid on growth of T-960 in 0.05% urea medium. Media was prepared as described in Fig. 3. Samples (10 ml) of medium with and without AHA were inoculated with a 1:1000 dilution of T-strain 960 growing in 0.01 M putrescine medium. Three replicate cultures with AHA and two cultures without AHA were inoculated for each of the AHA concentrations tested. (A) Color change units (CCU) were determined in from one to three of the replicate cultures for each point. Each point is the arithmetical mean for the number of determinations made. In all points which reflect multiple determinations, replicate cultures were within 1 CCU of each other for a given time point. (B) pH was measured in all cultures at each point. The range in any set of three measurements did not exceed 0.25 pH units (one set of measurements at 127 h). The range for all other pH measurements was 0.15 pH units or less.*

the pH change in the cultures containing AHA were delayed approximately 24 h compared with the control cultures. Thus, the effect of the AHA was not influenced by incubation in sterile growth medium over a time period similar to that in the other experiments described, nor was it altered by storage of complete medium containing 1.8×10^{-4} M AHA and 0.25% urea at 4 C for 3 months.

The effect of two concentrations of AHA was also tested in medium containing 0.25% urea and, in addition to growth, pH, and ammonia levels, depletion of urea was also monitored (Fig. 5). Ammonia and pH vary with multiplication and AHA concentration as discussed previously (Fig. 3 and 4), but also correspond

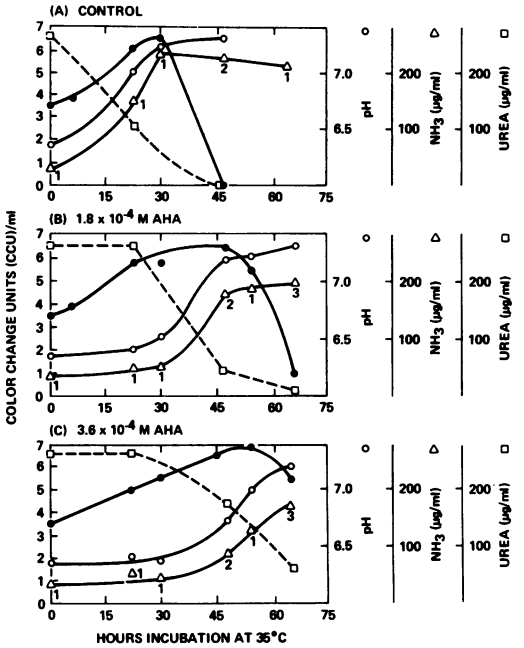

FIG. 5. *Effect of two concentrations of acetohydroxamic acid (AHA) on T-strain 960 titer, pH of growth medium, and ammonia and urea concentration in 0.025% urea medium. Media were prepared and inoculated as described in Fig. 4. Three replicate cultures were prepared for each AHA concentration (control = 0 AHA, 1.8×10^{-4} M AHA, and 3.6×10^{-4} M AHA). Each time point for growth (CCU, ●) and pH (○) represents the arithmetical mean for a single determination on each of the three replicate cultures. The range for CCU at all times points was 1 CCU, and the range for pH measurements did not exceed 0.1 pH unit. The ammonia (△) was determined in from one to three of the replicate cultures (number of measurements denoted by the number under the open triangles). Urea (□) was determined by the amino acid analytical method on one to three replicate cultures from each set of three. The range of values in all cases where more than one determination was made is within 10% of the mean.*

with urea depletion. It can be seen that, during the first 22 h of incubation in the presence of both concentrations of AHA, no depletion of urea could be detected, and there was no increase in pH or ammonia levels in the media. However, there was a 1 to 3 CCU increase in titer above the initial titer of 3.5 CCU/ml. The reproducibility of the urea analysis with urea concentration of 250 μg/ml is ± 10 μg/ml. Thus, it was possible that small amounts of urea were depleted but not detected.

The experiment was, therefore, repeated and urea depletion was monitored at shorter time intervals (Fig. 6). Since the initial inoculum for this experiment is less than 2 CCU/ml, it can be compared directly with the experiment shown in Fig. 3. Growth curves and pH curves are seen to be almost identical. Urea depletion and pH increase correspond and relate to the titers of the cultures. When a higher titer (about 5 CCU/ml) is reached, the rate of urea depletion becomes maximal and is not altered by the presence of AHA. The urea depletion curves appear to be sigmoidal for both control and AHA cultures, suggesting that AHA is not affecting T-strain urease but might be slowing growth by some other mechanism, and that the number of organisms present at a given time controls the rate of urea depletion.

The experiments represented by Fig. 3 were initiated with washed cells. The results are comparable to those obtained with unwashed cells (Fig. 4, 5, and 6). Thus, neither the washing procedure nor the presence of approximately 10^{-5} M putrescine added to experimental cultures via unwashed inocula after the AHA effect on T-strain 960.

DISCUSSION

The possibility that trace amounts of urea may be necessary for T-strain growth will be difficult to exclude absolutely until a chemically defined growth medium is developed. However, we believe that earlier reports (7, 9, 20) that urea is required for growth are open to question. This belief is based on the evidence described below.

Dialyzed putrescine medium containing 0.5 mM allantoin and total contaminant urea of 4.3 ± 1 μg/ml did not support the growth of T-strain 960 (Table 1), although growth occurred in the dialyzed putrescine medium containing 5 mM allantoin and 6.5 ± 1 μg of contaminant urea per ml.

The same medium with allantoin replaced by urea supported growth only when more than 10 μg/ml was added (Table 2). This level of urea was determined by analysis of the medium for some experiments, and therefore traces of urea present initially in the medium are included in

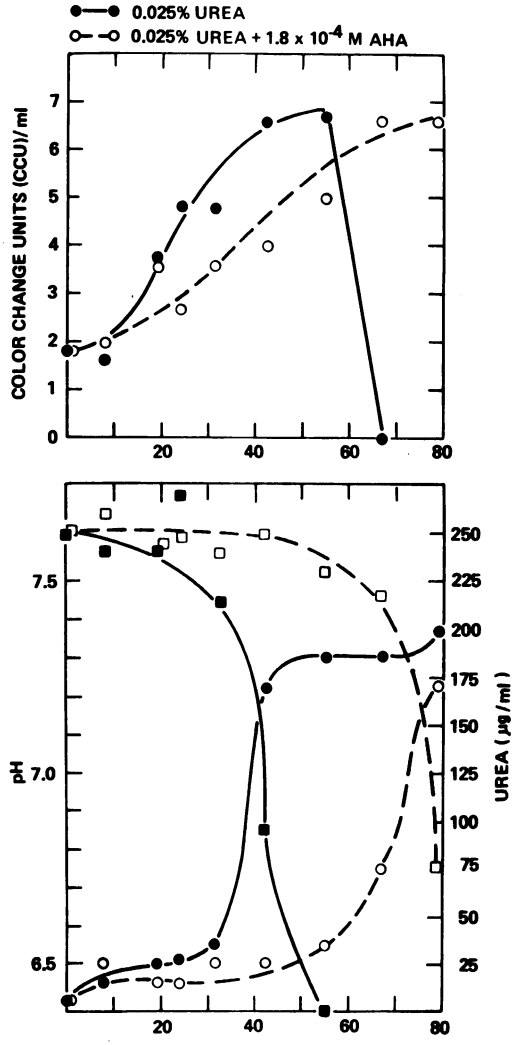

FIG. 6. *Effect of 1.8 × 10^{-4} M acetohydroxamic acid (AHA) on T-strain 960 titer, pH of growth medium, and urea depletion in 0.025% urea medium. Media were prepared and inoculated as described in Fig. 4. Three replicates were prepared for control (no AHA) and AHA cultures. Each time point for growth or pH without AHA (●) or with AHA (○) represents the arithmetical mean for a single determination on each of the three replicate cultures. The range for CCU for most time points was 1 CCU (2 CCU at 19 h with AHA), and the range for pH measurements did not exceed 0.1 pH unit among the replicate cultures at a given time point. Urea (■, control; □, AHA) was determined by the amino acid analytical method on one of the replicate cultures from each set of three.*

the amounts shown (Table 2). There were no significant differences found in the amounts of urea in fresh and incubated samples of sterile medium at the minimal initial urea concentrations found to give a positive growth score (Table 2: 16, 17, and 23 μg of urea per ml). We interpret these data to mean that trace amounts of urea in a medium prepared from dialyzed components are not sufficient to support T-strain multiplication.

Shepard (personal communication) also found that an agar medium prepared with dialyzed horse serum and containing residual urea at a final concentration of 8 μg/ml did not support the growth of several T-strain isolates. Thus, his result is compatible with ours and is interpreted to mean that the trace amounts of urea would not be expected to support T-strain multiplication.

A 5-CCU increase in titer was observed during 60 h of incubation in dialyzed (Fig. 2A) or undialyzed (Fig. 2B) media containing no added urea and without AHA. Since the trace urea expected to be present in the dialyzed or undialyzed medium is not sufficient to allow T-strain multiplication, some other factors are apparently required. It is possible that some medium component is metabolically converted to urea which is then utilized by the T-strains. If that is true, however, urea would not be considered a requirement for growth but its precursor would.

When AHA was included in these same media (Fig. 2A and 2B), titers were not altered. Thus, multiplication of T-strain 960 was inhibited by a urease inhibitor in media without added urea, but was not inhibited by the urease inhibitor in media with added urea (Fig. 2C, 2D, 3-6). This suggests that the AHA effect on T-strain 960 might be unrelated to inhibition of T-strain urea hydrolysis. The hydroxamic acids have been shown to have no growth-inhibiting effect on a number of bacterial species both with and without urease activity, but they do inhibit bacterial ureases (6). High concentrations (0.1%, wt/vol) of sorbic hydroxamic acid are effective inhibitors of fungi which are not ureolytic (3), and hydroxamic acids also play a role in iron chelation in the respiration of some organisms (1). The iron levels of growth media are, in turn, of considerable importance for the growth of some fungi (4). Thus, there are other possible explanations for the observed hydroxamic acid effect on T-strain mycoplasmas which do not involve urease or an inhibition of multiplication, and these remain to be explored.

In any case, the effect of AHA on growing cultures of T-strains cannot yet be said to be an inhibition of the enzyme urease, as has been shown to occur when crystalline enzyme is used (2, 5, 12), because it is not known whether (i) T-strain mycoplasmas have this same enzyme, (ii) the inhibitor enters the cells, (iii) incorporation of the inhibitor by the organisms is required to produce an effect, or (iv) if the inhibitor is metabolized or altered in any way on prolonged incubation with the T-strains. Nevertheless the AHA, or a derivative, has the effect of slowing the rate multiplication of T-strain 960 in urea medium and, therefore, of delaying the alkaline pH shift, ammonia accumulation, and urea depletion in T-strain 960 cultures (Fig. 3-6).

In Fig. 3, 5, and 6, which test comparable conditions, we showed that the pH increase, ammonia accumulation, or urea depletion had the same maximal rate whether AHA was present in the medium or not. Also, the rapid change in these indicators of urea hydrolysis occurred only after the titer of the cultures reached 5 CCU/ml. When AHA was present, these titers were reached after a longer incubation period. Thus, there was an apparent delay in urea depletion and in other variables measured in the culture milieu.

Our results indicate that the urease inhibitor, AHA, does not inhibit T-strain 960 multiplication or its ability to hydrolyze urea, and suggest that the organisms are metabolically able to circumvent the utilization of exogenously added urea. Our previous results (13a) suggest that agmatine will replace urea in T-strain growth media, and results reported here and previously (13a) suggest that putrescine and allantoin will replace urea also.

These data provide evidence against a urea requirement for T-strain mycoplasmas, and this evidence is at least as compelling as any advanced in support of such a requirement.

It is important to differentiate clearly between T-strain capacity to hydrolyze urea (urease activity) and a "requirement for urea for growth." It is also necessary to differentiate between a requirement for urea for growth and a "requirement for urea hydrolysis for T-strain multiplication" since these statements need not necessarily be synonymous. Urea can be considered to be a requirement for growth only if it is essential in the metabolism of the organism but not provided metabolically. It may well be a requirement for growth, and yet its hydrolysis may be unrelated to T-strain multiplication. Alternatively, urea hydrolysis could conceivably be required for T-strain multiplication but,

if the urea is supplied metabolically, it would not be considered as a nutritional requirement. In the light of our findings, there is currently no compelling evidence to support either possibility. Since there is no other known organism (free living or parasitic) which has an absolute requirement for urea for growth, this must be unequivocally established before it can be accepted as a characteristic of T-strain mycoplasmas.

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LITERATURE CITED

1. Akers, H. A., and J. B. Neelands. 1973. A hydroxamic acid present in *Rhodotorula pilimanae* cultures grown at low pH and its metabolic relation to rhodotorulic acid. Biochemistry **12**:1006-1010.
2. Blakeley, R. L., J. A. Hinds, H. E. Kunze, E. C. Webb, and B. Zerner. 1969. Jack bean urease (EC 3.5.1.5.). Demonstration of a carbamoyl-transfer reaction and inhibition by hydroxamic acids. Biochemistry **8**:1991-2000.
3. Dudman, W. F. 1963. Sorbic hydroxamic acid, an antifungal agent effective over a wide pH range. Appl. Microbiol. **11**:362-364.
4. Elin, R. J., and S. M. Wolf. 1973. Effect of pH and iron concentration on growth of *Candida albicans* in human serum. J. Infect. Dis. **127**:705-708.
5. Fishbein, W. N., and P. N. Carbone. 1965. Urease catalysis. II. Inhibition of the enzyme by hydroxyurea, hydroxylamine, and acetohydroxamic acid. J. Biol. Chem. **240**:2407-2414.
6. Fishbein, W. N., P. N. Carbone, and H. D. Hochstein. 1965. Acetohydroxamate: bacterial urease inhibitor with therapeutic potential in hyperammonaemic states. Nature (London) **208**:46-48.
7. Ford, D. K. 1972. Inhibition of growth of T-strain mycoplasma by hydroxamic acids and by aurothiomalate. Antimicrob. Ag. Chemother. **2**:340-343.
8. Ford, D. K. 1973. T-strain mycoplasmas and genital chalamydiae: inhibition of T-strain urease by hydroxamic acids. J. Infect. Dis. **127**(Suppl.):82-83.
9. Ford, D. K., and J. MacDonald. 1967. Influence of urea on the growth of T-strain mycoplasmas. J. Bacteriol. **93**:1509-1512.
10. Hayflick, L. 1965. Cell cultures and mycoplasmas. Tex. Rep. Biol. Med. **23**(Suppl. 1):285-303.
11. Johnson, E. A., and B. W. Brown, Jr. 1961. The Spearman estimator for serial dilution assays. Biometrics **17**:79-88.
12. Kobashi, K., J. Hase, and K. Urehara. 1962. Specific inhibition of urease by hydroxamic acids. Biochim. Biophys. Acta **65**:380-383.
13. Lynn, K. R. 1967. Some properties and purifications of urease. Biochim. Biophys. Acta **146**:205-218.

13a. Masover, G. K., and L. Hayflick. 1973. The growth of T-strain mycoplasmas in medium without added urea. Ann. N.Y. Acad. Sci. **225**:118-130.

14. Purcell, R. H., R. M. Chanock, and D. Taylor-Robinson. 1969. Serology of the mycoplasmas of man p. 221-264. *In* L. Hayflick (ed.), The mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.
15. Purcell, R. H., D. Taylor-Robinson, D. Wong, and R. M. Chanock. 1966. Color test for the measurement of antibody to T-strain mycoplasmas. J. Bacteriol. **92**:6-12.
16. Seligson, D., and H. Seligson. 1951. A microdiffusion method for the determination of nitrogen liberated as ammonia. J. Lab. Clin. Med. **38**:324-330.
17. Shepard, M. C. 1954. The recovery of pleuropneumonia-like organisms from negro men with and without nongonococcal urethritis. Amer. J. Gonor. Vener. Dis. **38**:113-124.
18. Shepard, M. C. 1956. T-form colonies of pleuropneumonia-like organisms. J. Bacteriol. **71**:362-369.
19. Shepard, M. C. 1969. Fundamental biology of the T-strains p. 49-65. *In* L. Hayflick (ed.), The mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.
20. Shepard, M. C., and C. D. Lunceford. 1967. Occurrence of urease in T-strains of mycoplasma. J. Bacteriol. **93**:1513-1520.
21. Shepard, M. C., and C. D. Lunceford. 1970. Urease color test medium U-9 for the detection and identification of "T" mycoplasma in clinical material. Appl. Microbiol. **20**:539-543.
22. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. **30**:1190-1206.
23. Wittler, R. G. 1973. Heterogeneity of the members of the class *Mollicutes*. J. Infect. Dis. **127**(Suppl.):55-57.