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Characteristics of *Ureaplasma urealyticum* Urease

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Sonication of *Ureaplasma urealyticum* cells grown in a dialysate growth medium effectively separated the cytoplasmic fraction from the membrane fraction, with both fractions relatively free from exogenous contaminating proteins. The urease activity was associated with the cytoplasmic fraction, and the ureaplasmal urease exhibited a specific activity higher than that of crystalline jack bean urease. The enzymatic activity of the ureaplasmal enzyme was optimum at pH 7.5 and was resistant to the chelating agents EDTA and sodium citrate. Sulfhydryl-blocking agents such as $HgCl_2$ and $Pb(NO_3)_2$ inhibited the ureaplasmal urease, which was also shown to be particularly sensitive to flurofamide and, to a much lesser extent, to acetohydroxamic acid. Electrophoretic analysis of the proteins of the ureaplasmal cell fractions combined with Western immunoblot with an antiserum to the ureaplasmal urease indicated that the urease constitutes a major component of the cytoplasm and is composed of several 70-kilodalton polypeptides.

Ureaplasma species differ from all other mycoplasmas (class *Mollicutes*) by possessing urease activity (32). Urease activity has been detected in a large variety of bacteria (for references, see reference 16), but the ureaplasmas are the only organisms known to depend on urea for growth (6, 9, 29). Urea hydrolysis appears to play a major role in the energy metabolism of ureaplasmas by promoting ATP synthesis through a chemioosmotic mechanism (12, 13, 24, 25). This rather unique energy-yielding mechanism is essential for the ureaplasmas, which are known to lack the major energy-yielding (glycolytic and arginine dihydrolase) pathways established so far for other mollicutes (19). The fact that specific urease inhibitors inhibit the growth of ureaplasmas (5, 8, 14, 26) supports the key role of urease in ureaplasmal growth.

Despite the apparent importance of the ureaplasmal urease, our knowledge of the structure and properties of the enzyme is very fragmentary. Early attempts to characterize the enzyme in crude cell extracts (13, 14, 26, 31), as well as more-recent efforts directed at purification of the enzyme (3, 30), encountered great difficulties because of the extremely low yields of ureaplasmas and the high level of contamination of the harvested organisms with foreign proteins from the growth medium (21). To overcome this problem, we employed large volumes of a dialysate broth medium (9) which yielded on harvest satisfactory amounts of cells relatively free of medium contaminants. By employing sonication as an effective means of lysing the cells and separating the cytoplasm from the membrane fraction and by using a sensitive and highly reproducible method for measuring rates of urease activity, we were able to improve considerably the definition of some of the structural and functional properties of the urease from the type strain (T960, serovar VIII) of human *Ureaplasma urealyticum*.

MATERIALS AND METHODS

Organism and growth conditions. The type strain of *U. urealyticum* (T960, serovar VIII) was obtained from M. C. Shepard (Camp Lejeune, N.C.). The organism was grown in 14- to 24-liter batches of a dialysate medium (7) (pH 6.0) supplemented with 25 mM urea, 50 mM 2-(*N*-morpholino) ethanesulfonic acid (MES buffer; Sigma Chemical Co., St. Louis, Mo.), 2% dialyzed agamma globulin horse serum, 0.001% phenol red, 1 mM sodium sulfite, and 100 U of penicillin per ml (9). The cultures were incubated at 37°C for 18 to 20 h, and the organisms were collected by centrifugation at 12,000 × *g* when the pH of the culture reached a value of 6.9 to 7.1. The pellets from two of the three large batches prepared were black. The pellets were washed in the cold three times, suspended in 0.15 M NaCl containing 5 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; Sigma) (pH 7.5) and 2 mM 2-mercaptoethanol in the cold, resuspended in 5 to 10 ml of the above-described TES-buffered saline solution, and immediately stored at −70°C.

Cell extract preparation. The washed *U. urealyticum* cell suspension (in 5-ml aliquots) was subjected to sonication in a cell sonicator (model W-225; Heat Systems Ultrasonic Inc., Plainview, N.Y.) with the small probe at 50 duty cycles and output 5 for 4 periods of 30 s each in ice. The sonicated material was centrifuged at 36,000 × *g* for 30 min at 0°C. The clear, colorless supernatant was divided into 0.5-ml aliquots, and the pellet was resuspended in 5 ml of the TES-buffered saline. All samples were kept at −70°C. The amounts of protein in the soluble and sedimentable cell fractions were estimated by the method of Lowry et al. (11), with bovine serum albumin as the standard.

Urease activity assay. The urease activity assay followed the principle proposed by Masover et al. (13), and urease activity was determined by measuring residual unhydrolyzed [^{14}C]urea. A 1 M urea stock solution containing 1 μCi of [^{14}C]urea per ml was prepared by adding 100 μCi of [^{14}C]urea (specific activity, 0.1 mCi/mg of urea; New England Nuclear Corp., Boston, Mass.) to 100 ml of 1 M urea solution in deionized water. The reaction mixture contained 50 mM TES buffer (pH 7.5), 1 mM 2-mercaptoethanol, 10 mM [^{14}C]urea, and ureaplasma cell fractions or commercial urease preparations in a total volume of 3 ml. The reaction mixture was incubated in a 37°C water bath for 4 min prior to the addition of the substrate urea. Samples (0.5 ml) were withdrawn at 0, 2, 4, and 6 min after the addition of urea and

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transferred immediately to 0.5-ml volumes of 3.6 N H_2SO_4 in scintillation vials to stop the reaction. The vials were left open for 1 h to facilitate the removal of solubilized radioactive CO_2 resulting from urea hydrolysis. Radioactivity, representing unhydrolyzed urea, was measured following the addition of 10 ml of Aquasol scintillation solution (New England Nuclear Corp.) to the vials. Urea hydrolysis curves (residual radioactivity versus time) were constructed, and the linear part of the curves was used to calculate the initial urease activity rates, expressed as specific activity units (micromoles of urea hydrolyzed per minute per microgram of protein).

Gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (10). The material to be electrophoresed was treated with 1% (wt/vol) SDS–1 mM EDTA in 10 mM Tris-hydrochloride (pH 8.0) at 37°C for 1 h. The insoluble material was removed by centrifugation at 20,000 × *g* for 10 min at room temperature. To reduce disulfide bonds, 5% 2-mercaptoethanol was added to the SDS solubilizing solution, and the sample was boiled for 5 min immediately before loading on the gel. After completion of electrophoresis, the protein bands were detected by staining with Coomassie brilliant blue R-250.

Monospecific antiserum to urease. A polyclonal monospecific rabbit antiserum to urease was prepared by immunizing rabbits with precipitin lines excised from crossed immunoelectrophoresis slides. Briefly, precipitin peaks from serovar IV ureaplasmal antigen were developed against antiserum to the homologous serovar IV strain in crossed immunoelectrophoresis (33). A number of peaks were recognized, but one clearly defined major antigenic peak of 0.53 mobility relative to bovine albumin showed urease activity as judged by an enzyme-specific stain (4). This peak was excised from 40 slides and used as the immunogen for rabbits, following the immunization protocol of Alexander and Kenny (1) for preparing monospecific antibodies. This serum formed a single precipitin peak by crossed immunoelectrophoresis and stained for urease activity when tested against solubilized whole-organism antigens of serovars IV, V, and VIII of *U. urealyticum*.

Western immunoblot analysis. The protein bands of the SDS-polyacrylamide gels were electrophoretically transferred to Immobilon (polyvinylidene difluoride) membranes (Millipore Corp., Bedford, Mass.). Detection of the transferred protein antigens was performed by the method of Towbin et al. (34), by using the urease monospecific serum at a 1:500 dilution in phosphate-buffered saline (pH 7.4) containing 1% (wt/vol) bovine serum albumin. Antibody bound to protein bands on the Immobilon membranes was detected by treating the membranes with goat anti-rabbit immunoglobulin G conjugated to peroxidase (Bio-Rad Laboratories, Richmond, Calif.), with 4-chloro-1-naphthol as the staining reagent.

Urease activity of electrophoresed proteins. The urease activity of electrophoresed ureaplasma proteins was determined under nondenaturing conditions by a modification of the method of Senior et al. (28) as described by Mobley et al. (16). Briefly, a slab of polyacrylamide gradient gel (5 to 20%) in 400 mM Tris hydrochloride (pH 8.8)–5 mM 2-mercaptoethanol with a 4.8% stacking gel in 70 mM Tris hydrochloride (pH 6.8) was loaded with the cell extract and electrophoresed at 80 V for 24 h at 4°C. The gel was removed from the apparatus and soaked in 10 to 15 changes (about 200 ml each) of 0.02% (wt/vol) cresol red–0.1% (wt/vol) EDTA until the gel remained yellow. The solution was drained from the gel, and the gel was immersed in a 1.5% (wt/vol) urea solution. After the development of localized red bands resulting from an increase in pH caused by the enzymatic release of NH_3, the gel was photographed with slide film (Kodachrome 64; Eastman Kodak Co., Rochester, N.Y.).

Materials. Crystalline jack bean urease (type C-3; 1200 IU/mg) and a partially purified urease preparation from *Bacillus pasteurii* (type X; 100 to 200 IU/mg) were obtained from Sigma. Flurofamide (*N*-[diaminophosphinyl]-4-fluorobenzamide; lot 6692-037A; code EU-4534) was a gift of Norwich Eaton Pharmaceuticals Inc.

RESULTS

Urease activity of soluble and sedimentable cell fractions. The amount of protein in the soluble cell fraction obtained after sonication of the ureaplasma cells was approximately equivalent to that in the sedimentable fraction. Cells originating from 7 liters of culture yielded 2.70 mg of protein in the soluble fraction and 2.75 mg of protein in the sedimentable fraction. The soluble fraction exhibited a high urease activity, reaching in some batches a specific activity value of 1.15 U, which was almost 3 times as high as that of crystalline jack bean urease (Sigma) and 10 to 20 times higher than that of the *B. pasteurii* urease preparation (Sigma) when tested under the same conditions (pH 7.5). The urease activity of the sedimentable fraction was only 0.11 to 0.23 U, depending on the batch. The urease activity of the soluble fraction did not decrease significantly after 4 months at −70°C. However, preincubation at 37°C for 30 min before addition of substrate lowered the urease activity of the soluble fraction by about 20%.

Optimum pH. To ensure strong buffering capacity at a wide pH range (pH values ranging from 5.5 to 9.0), a "cocktail" made of three buffers (Sigma) MES (pK_a = 6.1), TES (pK_a = 7.5), and BICINE (*N*, *N*-bis[2-hydroxyethyl]glycine; pK_a = 8.3), each at a 50 mM concentration, was prepared. The buffer was supplemented with 1 mM 2-mercaptoethanol and adjusted to various pH levels with NaOH. The optimum pH of the ureaplasmal urease activity was 7.5 (Fig. 1). Consequently, urease activity was tested in a reaction mixture containing TES buffer (pH 7.5).

Effect of chelating agents on urease activity. To determine whether the ureaplasmal urease in the soluble fraction requires nickel for activity, as the jack bean urease and other ureases do (2, 17, 18), various concentrations (up to 40 mM) of EDTA or sodium citrate were added to the reaction mixtures 5 min prior to the addition of urea. The addition of chelating agents had no effect on the urease activity. Dialysis of the soluble fraction for 20 h in the cold against 100 mM EDTA or sodium citrate in 10 mM MES buffer (pH 7.0) containing 1 mM 2-mercaptoethanol did not affect the urease activity. Moreover, when tested at pH 5.5, the urease activity of the soluble fraction dialyzed against EDTA or citrate was about 30% higher than that of the same fraction dialyzed simultanously against buffer solution with no chelating agent. However, when tested at pH 7.0, the specific activities of all the dialyzed extracts were about the same. Addition of $NiCl_2$ to the reaction mixture up to a concentration of 40 μM had no effect on the urease activity of either the native soluble fraction or that dialyzed against EDTA or citrate.

Inhibitors of ureaplasmal urease. Flurofamide and, to a much less extent, acetohydroxamic acid were found to inhibit *U. uealyticum* growth (8). It was of interest, therefore, to compare the growth inhibition activities of these

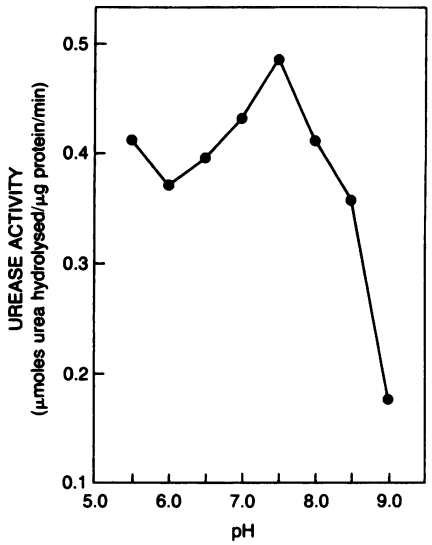

FIG. 1. Effect of pH on *U. urealyticum* urease activity. The buffer in the reaction mixture consisted of a mixture of MES, TES, and BICINE buffers, each at a 50 mM concentration, and the concentration of soluble ureaplasmal cell fraction was 14 μg of protein per reaction mixture.

compounds with their effects on ureaplasmal urease. Flurofamide was a very potent inhibitor of both the ureaplasmal and jack bean ureases, (Fig. 2). On a molar basis, flurofamide was about 100 times as active in urease inhibition as acetohydroxamic acid, another specific urease inhibitor (Table 1). Heavy metal salts, which react with sulfhydryl groups, inhibited the activity of both the ureaplasmal and jack bean ureases (Table 1). Interestingly, low concentrations of $HgCl_2$, $Pb(NO_3)_2$, and $Ag(NO_3)_2$ (results of the last not shown in Table 1) stimulated the ureaplasmal urease activity by up to 20% over that of the control but had no effect on the jack bean urease.

Inhibition of ureaplasmal urease by monospecific antiserum. To determine the effect of the monospecific antiserum

FIG. 2. Inhibition of *U. urealyticum* and jack bean ureases by flurofamide. The inhibitor was added to the reaction mixture, and the mixture was kept at 37°C for 4 min prior to the addition of the substrate.

TABLE 1. Inhibitors of urease activity

Inhibitor[a]	Concn of inhibitor (μM)	% Inhibition[b]	
		Ureaplasmal urease	Jack bean urease
Acetohydroxamic acid	10	42.3	91.7
	100	94.0	100.0
	1,000	100.0	100.0
Flurofamide	0.1	54.6	66.0
	1	100.0	92.5
	10	100.0	100.0
p-Hydroxymercuribenzoate	5	9.5	5.6
	10	4.4	13.9
	100	46.6	100.0
	1,000	100.0	100.0
Thallium acetate	100	8.7	6.8
	1,000	11.3	16.7
	10,000	50.0	30.6
$HgCl_2$	1	0[c]	0
	10	0[c]	9.1
	100	62.5	100.0
	1000	100.0	100.0
$Pb(NO_3)_2$	1	0[c]	0
	10	19.4	16.0
	100	55.7	65.9
	1,000	88.7	95.5

[a] Inhibitors were added to the reaction mixture containing the urease preparation at 37°C at 4 min prior to the addition of substrate.

[b] Data expressed as percentages of inhibition of specific urease activity in a control reaction mixture with no inhibitor.

[c] Urease activity was enhanced by up to 20% of that of the control.

to *U. urealyticum* urease on the ureaplasma soluble fraction, a mixture of these components was incubated at 37°C for 30 min before the urea substrate was added. When the incubation time was increased from 4 to 30 min, there was a decrease in the specific activity from 1.1 to 0.67 U. A 1:1,000 dilution of the monospecific serum provided the same results as when no serum was added. Lower dilutions (1:20 to 1:200) of the monospecific antiserum partially inhibited the urease activity of the soluble fraction, but complete inhibition was not observed (Fig. 3). The addition of normal rabbit serum to the reaction mixture at a final 1:20 dilution did not inhibit urease activity and even slightly enhanced it (results not shown).

Polypeptide composition and urease detection in *U. urealyticum* cell fractions. The polypeptide profiles of the soluble and sedimentable fractions prepared for electrophoresis by solubilization in SDS at 37°C in the absence of 2-mercaptoethanol differed markedly from each other (Fig. 4A). A major double band (about 210 kilodaltons [kDa]) was particularly prominent in the soluble fraction. The molecular weight of this double band was assessed by comparison with high-molecular-weight markers (Pharmacia, Inc., Piscataway, N.J.) electrophoresed on a polyacrylamide gradient gel (5 to 20%) (data not shown). Preparation of the cell fractions for electrophoresis by boiling in SDS in the presence of 2-mercaptoethanol resulted in the disappearance of the high-molecular-weight double band and a significant increase in the intensity of a 70-kDa band (Fig. 4B).

Western immunoblots of the above-described gels with the anti-*U. urealyticum* urease antiserum (Fig. 5) showed most

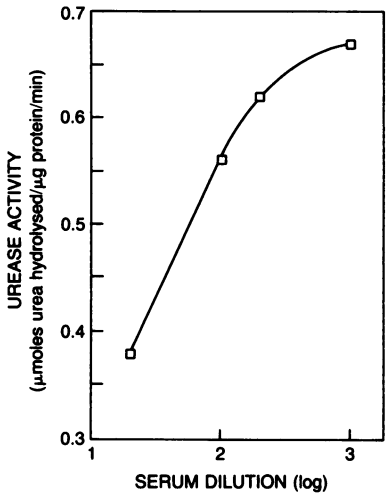

FIG. 3. Inhibition of *U. urealyticum* urease by a specific antiserum. Various dilutions of the antiserum were added to the reaction mixture containing the ureaplasma soluble fraction (3.9 μg of protein), and the reaction mixture was incubated at 37°C for 30 min before the addition of [^{14}C]urea.

intensive staining of the high-molecular-weight (210-kDa) bands in the soluble fraction and, to a lesser extent, in the sedimentable fraction. The soluble fraction contained in addition a reactive band of 70 kDa and a weaker band of 17 kDa, while the sedimentable fraction contained a 45-kDa band reacting with the antiserum. Immunoblots of the gels with material solubilized by SDS in the presence of 2-mercaptoethanol revealed in the soluble fraction the 70-kDa band and in the sedimentable fraction the 45-kDa band only.

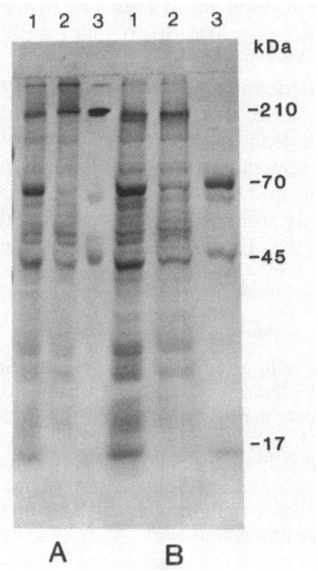

FIG. 4. SDS-polyacrylamide gel electrophoresis of *U. urealyticum* cells (lanes 1), sedimentable cell fractions (lanes 2), and soluble cell fractions (lanes 3). (A) The material prepared for electrophoresis was solubilized in SDS at 37°C for 1 h in the absence of 2-mercaptoethanol. (B) The material was solubilized by boiling for 5 min in SDS containing 2-mercaptoethanol. A total of 25 μg of proteins was loaded per lane. The molecular masses indicated on the right were determined by the migration of molecular weight standards.

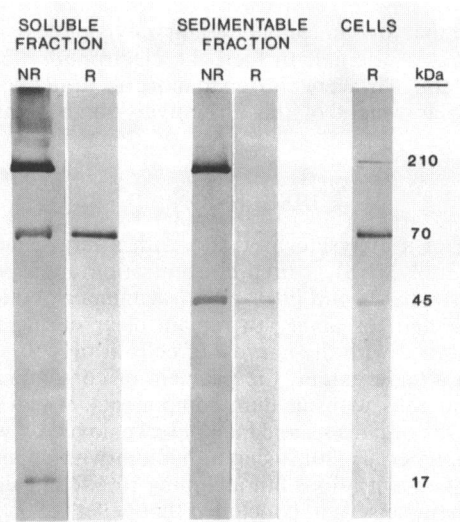

FIG. 5. Immunoblots of the soluble and sedimentable fractions and of whole *U. urealyticum* cells prepared for electrophoresis by solubilization in SDS at 37°C for 1 h in the absence of 2-mercaptoethanol (nonreducing conditions; NR) or by boiling in SDS containing 2-mercaptoethanol for 5 min (reducing conditions; R). A total of 10 μg of proteins was loaded per lane. The antiserum used was the monospecific antiserum to urease.

Whole *U. urealyticum* cells solubilized and electrophoresed under the same conditions showed a major reactive band of 70 kDa and minor bands of 210 and 45 kDa (Fig. 5).

Under nondenaturing conditions, electrophoresis of the soluble fraction enabled the detection of urease activity, which was limited to a single band (Fig. 6A). The apparent molecular weights of the ureases were estimated by using a standard linear curve obtained with the four largest protein markers (Fig. 6A, lane 1). The molecular masses were 120 kDa for the *B. pasteurii* urease, 220 and 400 kDa for the two major bands of the jack bean urease, and 245 kDa for the ureaplasmal urease. The electrophoretic analysis of the cytoplasmic fraction of *U. ureaplasma* (Fig. 6A, lane 4) revealed, by Coomassie staining, only the band corresponding to the urease. The apparent purity was due to the fact that minor components were not detected under these con-

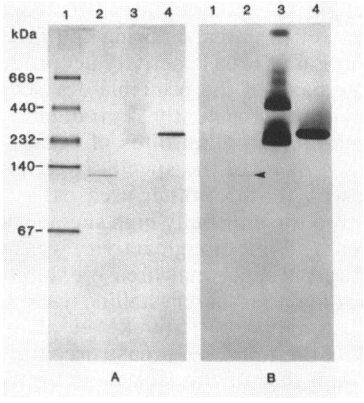

FIG. 6. Electrophoretic analysis under nondenaturing conditions of molecular weight markers (lanes 1), *B. pasteurii* (lanes 2) and jack bean (lanes 3) ureases, and the soluble fraction of *U. urealyticum* (lanes 4). (A) Coomassie blue staining; (B) urease-active bands following staining with cresol red. The arrow indicates the faint band of *B. pasteurii* urease.

ditions and that low-molecular-weight polypeptides probably migrated out of the gel. In fact, the electrophoretic conditions (80 V for 24 h) were chosen to more accurately estimate the molecular weight of these relatively large urease proteins.

DISCUSSION

The use of a dialysate broth (7) with a large amount of buffer (50 mM MES), a urea concentration appropriate to maximal growth (25 mM [9]), and 2% agamma globulin horse serum (selected for ability to remain clear during incubation), combined with the harvest of cells at pH 7.0, circumvented, to a large extent, the problem of contamination of ureaplasmal cells with medium components, which cosediment with the organisms and cannot be removed by washing (27). Precious et al. (20), using a medium with similar urea and buffer concentrations but including 10% fetal calf serum rather than horse serum, concluded that pellets of organisms purified through a sucrose gradient show minimal contamination with medium components. In our experiments, we found that the amount of protein in the soluble cytoplasmic fraction was about equivalent to that in the sedimentable fraction consisting of membranes and medium contaminants. A mycoplasma membrane fraction usually contains 30 to 40% of the total cell protein, the rest being cytoplasmic proteins (22). Hence, the amount of contamination with foreign medium proteins of the cell pellets we prepared was relatively low. In comparison, Masover et al. (14), while working with ureaplasmas cultivated in a conventional ureaplasma medium supplemented with 10% horse serum, obtained a membrane fraction containing over 25 times as much protein as the soluble fraction. Significant contamination of the ureaplasma cell pellet with growth medium proteins was also acknowledged in the more-recent studies of Eng et al. (3) and Stemke et al. (30).

Another factor which was instrumental in our successful separation of the cytoplasmic fraction was the choice of sonication as the means for cell lysis. Sonication was found (23) to be more effective for cell lysis than either digitonin or osmotic lysis as used by Masover et al. (13, 14). Sonication probably is also more effective than the freezing-and-thawing procedure employed by Eng et al. (3) and Stemke et al. (30). The addition of 2-mercaptoethanol to the wash medium in which organisms were suspended was also an advantage, because the compound protected the essential sulfhydryl groups of the urease during cell fractionation.

The determination of urease activity according to residual [^{14}C]urea, on the basis of the principle devised by Masover et al. (13), provided consistently reproducible results, enabling the calculation of initial rates of urease activity and meaningful comparisons of the specific activity of the ureaplasmal urease with those of other ureases.

Our data point to the unusually high specific activity of the ureaplasmal urease. Even though activity was measured in a crude cell fraction, ureaplasmal urease exhibited a specific activity higher than that of crystalline jack bean urease. Although the estimation by Stemke et al. (30) that the specific activity of their purified urease preparation is at least 90-fold higher than that of the jack bean urease is open to question because of the uncertainties concerning the protein content of their ureaplasmal urease preparation, our data also indicate the extremely high potency of the ureaplasmal urease. Recently, Precious et al. (20) have indicated that the specific activity of the ureaplasmal urease was approximately 80 times that of crystalline jack bean urease.

The potent urease activity of the soluble cytoplasmic fraction enabled an effective reevaluation of several of the ureaplasmal urease properties reported previously. Thus, we have shown that the ureaplasmal urease has a definite optimum pH at about 7.5, supporting the finding of Eng et al. (3) and in contrast to the findings of Masover et al. (14) and Romano et al. (26), who reported an acid pH optimum.

Of special interest is our finding that the ureaplasmal urease, as well as the jack bean urease, is extremely susceptible to flurofamide, a potent inhibitor of the urease activity of members of the *Proteeae* tribe (15). Kenny (8) found that flurofamide is 1,000 times more active than acetohydroxamic acid in ureaplasma growth inhibition. Our data show that flurofamide inhibits the ureaplasmal urease at a concentration about 1,000 times lower than that found to inhibit ureaplasma growth (8). This difference may be attributed to the need of the inhibitor to cross the cell membrane barrier in growth inhibition experiments. The possibility that binding to serum proteins of the growth medium decreased the effectiveness of flurofamide was not supported by experiments in which dialyzed horse serum was added to the urease reaction mixture. On a molar basis, flurofamide was about 100 times as active as acetohydroxamic acid in inhibiting the urease activity. These findings correspond with the much weaker growth inhibition effect of the latter compound (8).

Urease activity in ureaplasmas is localized in the cytoplasm (13, 23), as was found in other bacteria (for references, see reference 16). The urease constitutes a major protein component of the ureaplasma cytoplasm (Fig. 4). Identification of the 70-kDa protein as the major, and possibly the only, subunit of the ureaplasmal urease was indicated by electrophoresis of the cytoplasmic fraction boiled in SDS in the presence of 2-mercaptoethanol. This treatment resulted in the complete disappearance of the high-molecular-weight double band (210 kDa) accompanied by an increase in the intensity of the 70-kDa band (Fig. 4), which was the only band found in this preparation to react with the anti-urease antiserum (Fig. 5).

In conclusion, despite the satisfactory cytoplasmic fraction we prepared, the minute amounts of material available may defy purification of the urease by conventional procedures. Another approach to overcome this problem may be based on cloning and expression of the ureaplasmal urease gene, in the way recently achieved for the *Providencia stuartii* urease (16). Experiments in this direction are now under way.

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