Some Characteristics of *Ureaplasma urealyticum*. Urease Activity in a Simple Buffer: Effect of Metal Ions and Sulphydryl Inhibitors

By STEPHEN L. SWANBERG,* GERALD K. MASOVER† AND
LEONARD HAYFLICK‡

*Division of Urology, Stanford University School of Medicine,
Stanford, California 94305, U.S.A.*

(Received 11 May 1978)

Urealytic activity of the cytoplasmic fraction of *Ureaplasma urealyticum* prepared by digitonin lysis was assayed in a simple buffer system (HEPES plus EDTA) by measuring the release of $^{14}\text{CO}_2$ from $[^{14}\text{C}]\text{urea}$. The K_m of this preparation agreed with our previous observations of the same activity measured in a more complex reaction mixture. The substrate concentration at which maximum velocity occurred was approximately 20 mM. The activity was sensitive to heavy metals and inhibitors which react with sulphydryl groups such as *N*-ethylmaleimide and *p*-chloromercuribenzoate. It was not inhibited by Ca^{2+} or Mg^{2+} or by the reaction products, ammonia and carbon dioxide.

INTRODUCTION

The ability to hydrolyse urea is given as the major differentiating characteristic of *Ureaplasma urealyticum* (Shepard *et al.*, 1974). Recently, this urealytic activity was studied using a crude cytoplasmic preparation from *U. urealyticum* (Masover *et al.*, 1976). The activity was shown to be soluble and not membrane-associated, a finding in agreement with those of others (Delisle, 1977; Furness & Cole, 1975; Vinther, 1976). Estimates of the pH and urea concentration optima were made, and the effects of several enzyme inhibitors were tested. However, it was apparent that a simpler reaction medium than the one used [a culture medium (Basal Medium Eagle, BME) plus EDTA] was needed so that other requirements of *U. urealyticum* urealytic activity could be studied. Preliminary experiments (Masover *et al.*, 1976) indicated that *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) was satisfactory for such a system. Therefore we have studied *U. urealyticum* urealytic activity and several of its properties in this simpler reaction mixture.

METHODS

Organism and growth media. *Ureaplasma urealyticum*, serotype VIII (formerly T-strain 960), was originally supplied by M. C. Shepard (Camp Lejeune, N.C., U.S.A.) after being cloned eight times. It was passaged in our laboratory more than 30 times in medium without added urea, as previously described (Masover & Hayflick, 1973).

The routine growth medium for T-strains has been described previously (Masover *et al.*, 1974). More recently, modified Edward medium (Razin & Rottem, 1976) was used in which 5% (v/v) unheated horse

* Present address: Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, Massachusetts 02138, U.S.A.

† Present address: Children's Hospital Medical Center, Bruce Lyon Memorial Research Laboratory, Oakland, California 94609, U.S.A.

‡ To whom correspondence should be addressed.

serum replaced the Bacto-PPLO serum fraction. Urea was added to a final concentration of approximately 10 mM and the pH was adjusted to 6.3. Some CO₂ trapping experiments were done in Basal Medium Eagle (BME). Batches of late-exponential phase ureaplasmas were harvested by centrifuging at 27000 *g* for 15 min and washed twice in 0.25 M-NaCl prior to lysis with digitonin (Rottem & Razin, 1972).

Ureaplasmas were quantified by the method previously described (Masover *et al.*, 1975) and expressed as 50 % colour change units (c.c.u.₅₀) ml⁻¹.

Digitonin lysis. Digitonin lysis of ureaplasmas was accomplished (Rottem & Razin, 1972) using 20 µg digitonin ml⁻¹ in 0.25 M-NaCl solution for most experiments. The cytoplasmic fraction was separated from the membrane fraction by centrifuging digitonin-lysed organisms at 27000 *g* for at least 60 min at 4 °C. Assuming digitonin lysis to be complete, the cell-free preparation represents the cytoplasmic contents of late-exponential phase ureaplasmas from 2 l culture dissolved in 40 ml 0.25 M-NaCl. The yield of such a preparation ranged from 4 to 20 µg protein ml⁻¹ (160 to 800 µg total), and enzyme activity varied from about 0.5 to 3.4 µmol urea hydrolysed (µg protein)⁻¹ (incubation time 1 h) in the conditions tested. The extent of membrane contamination of cytoplasmic preparations was estimated to be approximately 10 % by measuring contamination of membrane preparations with a cytoplasmic enzyme (urease) and contamination of cytoplasmic preparations with a membrane-bound enzyme (ATPase). The ratio of specific activities [i.e. activity (µg protein)⁻¹] was about 10:1 both for cytoplasmic urease:membrane urease and for membrane ATPase:cytoplasmic ATPase (Masover *et al.*, 1977b).

Protein determinations. Protein was estimated by the method of Lowry *et al.* (1951) or by the 260/230 method of Kalb & Bernlohr (1975). Comparable results were obtained by both methods.

Determination of urea hydrolysis. This was determined by CO₂ trapping as described previously (Masover *et al.*, 1976) for most experiments. The procedure was later modified to enable the reaction to occur in test tubes rather than in the Erlenmeyer flasks fitted with traps for CO₂. The enzyme reaction was stopped by acidification as before and the contents of each tube were mixed vigorously twice during the hour following the reaction. This was sufficient to drive off residual CO₂ and enabled measurement of the unhydrolysed urea which remained in solution.

Liquid scintillation counting. The amount of urea hydrolysed was calculated indirectly from the amount of unhydrolysed [¹⁴C]urea remaining in the acidified reaction mixture, rather than calculating directly from the ¹⁴CO₂ in the hyamine hydroxide trap as there was a slight (< 5 %) variation in the efficiency of CO₂ trapping. Counting was done using an Isocap 300 liquid scintillation system (Nuclear-Chicago Corp., Arlington Heights, Ill., U.S.A.) with an efficiency for ¹⁴C of 85 %. Samples of the reaction mixture were added to Aquasol (New England Nuclear Corp., Boston, Mass., U.S.A.) scintillation fluid (10 ml) and counted at room temperature after 2 h in the dark. In contrast, hyamine hydroxide required 2 to 3 d to dissolve completely in the scintillation fluid.

Radioisotopes and reagents. [¹⁴C]Urea (specific activity, 54.4 mCi mmol⁻¹) and hyamine hydroxide were purchased from Amersham/Searle (Arlington Heights, Ill., U.S.A.). *N*-2-Hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), 2-(*N*-morpholino)ethanesulphonic acid hydrate (MES), sodium *p*-chloromercuribenzoate (PCMB), *N*-ethylmaleimide (NEM) and sodium iodoacetate were purchased from Calbiochem. Digitonin was obtained from ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.). Versene 100 (Na₄EDTA, chelates 100 mg CaCO₃ g⁻¹) was obtained from General Biochemicals (Chagrin Falls, Ohio, U.S.A.). Disodium (ethylenedinitrilo)tetraacetate (Na₂EDTA, chelates 250 to 300 mg CaCO₃ g⁻¹) is a 'Baker Analyzed' reagent. For all but the earliest experiments, deionized water (> 15 MΩ resistance) was obtained from a Milli-R040 Millipore system.

RESULTS

The activity of the cytoplasmic enzyme preparation in several buffer systems was compared with the activity of the preparation in BME plus 0.1 % EDTA (taken as 100) (Table 1). There was no close correlation between activity, amount of protein and c.c.u.₅₀, even when the organisms were grown in the modified Edward medium. Doubling the concentration of digitonin used during lysis did not appear to affect protein yield, whereas reducing the volume of digitonin solution to 15 % of the usual amount slightly lowered overall cytoplasmic protein yields.

Of the buffer systems tested, enzyme activity was highest in HEPES plus 0.1 % EDTA. The wide range of values for HEPES without EDTA (Table 1) may be caused by the presence of varying amounts of heavy metal ions. These are known to inhibit the activity of other ureases (Seneca *et al.*, 1972; Sumner, 1951). Millimolar concentrations of magnesium or calcium ions had little or no effect on the activity even though they were in excess of

Table 1. *Effect of buffer system and pH on U. urealyticum urease activity*

All reaction mixtures contained 4.17 mM-urea and were incubated at 37 °C for 1 h. The result for each sample was the mean from duplicate flasks. Activities were calculated as μmol urea hydrolysed $(\mu\text{g protein})^{-1}$, and are expressed as a percentage of the activity in the control [BME plus EDTA (0.1 %), pH 5].

Buffer system	pH	Activity (% of control)	Number of experiments
BME+EDTA* (0.1 %, w/v)	5	100	—
HEPES (50 mM)+NaHCO ₃ (0.893 M)	5	69	1
HEPES (50 mM)+NaHCO ₃ (0.893 M)	6	100	2
HEPES (50 mM)	6	81 (range = 52 to 111)	6
HEPES (50 mM)+EDTA (0.1 %, w/v)	6	118.5	2
BME	5	94	1
MES (50 mM)	5	39	1
MES (50 mM)	6	37	1

BME, Basal Medium Eagle; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; MES, 2-(*N*-morpholino)ethanesulphonic acid hydrate.

* The EDTA used in these experiments was the commercial preparation Versene 100. Baker's EDTA gave similar results.

Table 2. *Effect of sulphhydryl group inhibitors on U. urealyticum urease activity*

Reaction flasks all contained HEPES (50 mM) plus EDTA (0.3 % Versene 100), pH 6, and 4.17 mM-urea and were incubated at 37 °C for 1 h. Activities are expressed as a percentage of the control which contained HEPES plus EDTA but no inhibitor.

Inhibitor added	Concn (mM)	Activity (% of control)
Iodoacetate	1	99
Iodoacetate	5	119
Iodoacetate	10	96
NEM	1	83
NEM	5	29.5
NEM	10	10
PCMB	0.005	3.4
PCMB	0.01	0.1
PCMB	0.05	2
PCMB	0.10	1
PCMB	0.50	<1

NEM, *N*-Ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

Fig. 1. Urea hydrolysis by soluble *U. urealyticum* urease activity in HEPES (50 mM) plus 0.05 % EDTA (Baker's). Complete reaction tubes were incubated for 15 min at 37 °C before injection of acid to halt hydrolysis. Results are expressed as μmol urea hydrolysed in 15 min; each point represents a mean of duplicate tubes.

the amount expected to be chelated. Similarly, ammonium ion (8.3 mM) had no effect. The effect of added EDTA was probably in chelating other inhibitory agents.

By using a higher concentration of urea and a shorter incubation time (15 min), maximum hydrolysis of urea occurred at pH 5 and pH 7, with reduced enzyme activity at pH 6. This might be explained by the observations of Delisle (1977) that ureaplasmas have several distinct urease isoenzymes. However, the characteristics of each of the purified isoenzymes would have to be studied and compared in order to interpret our observation on pH optimum as being due to isoenzyme differences.

Urease activity was inhibited by PCMB and NEM but not by iodoacetate (Table 2).

K_m for the cytoplasmic urease was determined in HEPES plus 0.05% Baker's EDTA, pH 6, at 37 °C with an incubation time of 15 min. Consistently deviant points were observed at 7.5 and 10 mM-urea (Fig. 1). Fitting the experimental data to the Lineweaver-Burk equation using linear regression by the method of least squares, the K_m was 2.17 mM, with the coefficient of determination being 0.98. This result is similar to that obtained previously (Masover *et al.*, 1976) by testing the enzyme preparation in BME plus EDTA.

DISCUSSION

The urealytic activity in a cytoplasmic fraction of digitonin-lysed *U. urealyticum* was assessed by measuring the accumulation of CO_2 , (Masover *et al.*, 1977a, b) as well as by determining the amount of unhydrolysed urea remaining in the reaction mixture. Virtually complete hydrolysis of the urea in the reaction mixture by this enzyme preparation occurred, and 91% of the radioactivity originally put into the flask as [^{14}C]urea was accounted for as $^{14}\text{CO}_2$ found in the hyamine hydroxide trap. The efficiency of CO_2 trapping in this system was 95%. Thus, the CO_2 released by urea hydrolysis was not utilized as a substrate by other enzymes. It is, therefore, difficult to relate urea hydrolysis to growth of the organisms.

Our difficulty in obtaining consistent ratios of enzyme activity to the amount of protein present are probably due to the release of cell-associated protein during digitonin lysis and to inconsistencies in the complex growth medium or to incomplete sedimentation of membrane fragments after the lysis procedure. Because of this variability of total protein to enzyme activity, all comparisons between experiments were done on the basis of activity as a percentage of a control. However, even with this limitation, it was possible to find an acceptable buffer system and to determine the effects of various factors on the activity.

Urease activity was not inhibited by EDTA suggesting that Mg^{2+} and Ca^{2+} are not required for urea hydrolysis. If so, this enzyme differs from the Mg^{2+} requiring biotin-enzyme (urea amidolyase) of *Candida utilis* (Roon & Levenberg, 1970). The complete inhibition of urease activity by PCMB and the inhibition curve generated for NEM suggest that the enzyme does possess sulphhydryl groups despite its insensitivity to iodoacetate which does not inhibit all enzymes classified as sulphhydryl group enzymes (Webb, 1966).

We have shown that the cytoplasmic urealytic activity of *U. urealyticum* can be tested in a simple buffer system which will permit more extensive characterization of the enzyme, perhaps eventually in a more purified form. Whether this function of ureaplasmas is the result of a single enzyme comparable to the ureases or other urealytic enzymes found in other organisms remains unknown, although results obtained by measuring the enzyme activity in polyacrylamide gels suggest that ureaplasmas have three distinct urease isoenzymes (Delisle, 1977). This observation might also explain our inability to show a sharp pH optimum as well as the consistently deviant points at 7.5 and 10 mM-urea (Fig. 1).

This work was supported by the Public Health Service Grant AI11805 from the National Institute of Allergy and Infectious Diseases.

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