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Role of Urease in the Formation of Infection Stones: Comparison of Ureases from Different Sources

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Bacterial and vegetable ureases were found to differ in certain important respects. For maximal clinical relevance, in vitro studies on the pathogenic role of urease should use whole bacterial cells of *Proteus* spp., and urease inhibitors should be assessed without preincubation of enzyme with inhibitor. Urease from *Proteus morganii* was very different from ureases of other species of *Proteus*; this factor should be taken into account when infections with *P. morganii* are being treated.

Urease (urea amidohydrolase; EC 3.5.1.5) is found in large amounts in jack beans (also known as sword beans) (*Canavalia ensiformis*), soybeans (*Glycine max*), and other members of the Leguminosae, as well as in a wide variety of human tissues (the gastric mucosa, liver, kidney, and erythrocytes) and in bacteria, yeasts, molds, plants, and molluscs (23).

Bacterial urease has been implicated in the pathogenesis of several diseases, namely hepatic coma (24), bovine pyelonephritis (22), and the formation of infection stones (12).

Inhibition of urease has been suggested as a means of preventing these clinical conditions (8, 11, 16). However, despite the fact that bacterial ureases are responsible, much of the experimental work has been done with ureases from vegetable sources (3, 7, 17), since there appears to be a tacit assumption that "urease" is a single entity.

If the properties of ureases from different sources vary markedly, the use of nonbacterial enzymes may well give rise to erroneous conclusions. To establish the possibility of such error, we compared various properties of bacterial and vegetable ureases.

MATERIALS AND METHODS

Chemicals. Acetohydroxamic acid (AHA), thiourea, and methylurea were obtained from Aldrich Chemical Co.; ethylurea was obtained from BDH; Benurestat was obtained from Norwich Pharmacal; ethyl methane sulfonate and hydroxyurea were obtained from Sigma Chemical Co.; Sephadex G-25 and G-200, blue dextran, and standard proteins for molecular weight determinations were from Pharmacia Fine Chemicals, Inc., as were materials for gel electrophoresis.

Jack bean urease, a partially purified extract (Sigma), was used at a concentration of 250 μg/ml. Soybean urease, a crude extract (BDH), was used at 10 mg/ml.

Bacterial strains. The urease-positive strain of *Escherichia coli* (E14066) was obtained from Colindale Central Public Health Laboratory, London. All other strains used were clinical isolates from the Diagnostic Microbiology Laboratory of this hospital.

Production of urease-negative mutants. Urease-negative mutants were obtained from the four species of *Proteus* (one strain each) by the method of MacLaren (19), using ethyl methane sulfonate at pH 8.

Bacterial urease. Crude bacterial urease was prepared as described previously (13). Whole-cell suspensions were made from overnight cultures in Todd-Hewitt broth (100 ml) by centrifuging, washing once in distilled water, and suspending in distilled water (10 ml).

Urease assay. Urease activity was measured with a substrate concentration of 5 mg/ml using an ammonium-sensitive electrode (13), and expressed as micromoles of urea hydrolyzed per minute per milligram of protein.

Enzyme kinetics. Michaelis constants (K_m) were determined at pH 7 in tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (5).

The concentrations of AHA and Benurestat (known inhibitors of urease [1, 14]) producing 50% inhibition of urease (I_{50}) were determined (i) directly, by adding the inhibitor solution to the substrate before the enzyme, and (ii) after preincubation (5 min) of enzyme with inhibitor before addition to the reaction vessel. I_{50} was also determined with whole-cell preparations.

The mechanism of inhibition was investigated by measuring the K_m of urease in the presence of various concentrations of inhibitor.

The optimal pH for urease activity was investigated by using substrate (5 mg/ml) in 0.2 M Tris-maleate adjusted to pH 5, 6, and 7 with 0.2 M imidazole and in 0.1 M Tris-hydrochloride at pH 7, 8, and 9.

The activation energy (E_A) was determined by using a substrate concentration of 5 mg/ml in Tris-hydrochloride buffer (pH 7). Arrenhius plots were drawn ($\log_{10} V$ versus $1/T$). E_A (joules/mole) was determined from the slope of the graph $E_{A/2.03\,R}$ where $R = 8.3\ \mathrm{J \cdot mol^{-1}\ {}^{\circ}K^{-1}}$. The temperature coefficient (Q_{10}) was calculated from enzyme activities at 20 and 30°C.

Molecular weight determination. Chromatog-

raphy on Sephadex G-200 was used. The column was calibrated with blue dextran and four proteins of known molecular weight: aldolase (158,000); catalase (232,000); ferritin (440,000); and thyroglobulin (669,000) (2).

Gel electrophoresis. Electrophoresis of freeze-dried extracts of urease was carried out by using polyacrylamide gradient gels run overnight at constant voltage (65 V). The buffer was 0.1 M Tris-hydrochloride (pH 8.5). Gels were stained either with 0.2% amido black in 7% acetic acid for detection of protein bands or with a catalytic gel stain for urease (6).

Substrate profile. Hydrolysis of hydroxyurea, ethylurea, methylurea, and thiourea by urease was investigated. The purity of these urea analogs was estimated by paper chromatography (9).

RESULTS

Specific urease activity. The mean urease activities of *Proteus mirabilis* (four strains), *P. vulgaris* (three strains), and *P. rettgeri* (three strains) were, respectively, 1.4, 1.1, and 1.1 μmol of urea hydrolyzed per min per mg of protein, but that of *P. morganii* (five strains) was considerably higher: 5.2 μmol of urea hydrolyzed per min per mg of protein.

Urease activity was undetected in two strains each of *E. coli* and *Providencia* and in the urease-negative mutants of *Proteus* (one strain from each species) by the Appareils et Procédés d'Identification system, Christensen urea broth, and our assay system.

Urease activities of whole-cell preparations were not altered by sonication (two 30-s bursts). Continued sonication of suspensions using 30-s bursts over a period of 3 min did not significantly alter enzyme activity.

Effect of urease inhibitors. (i) I_{50} values for different species. *P. morganii* urease was more resistant to AHA and Benurestat than were enzymes from the other bacterial species. This was consistent for several strains of each species. Sonicated extracts of bacterial urease (except that of *P. morganii*) were more sensitive to both urease inhibitors than were vegetable ureases (Table 1), but whole-cell preparations were less sensitive than were vegetable ureases and sonicated extracts. Preincubation of enzyme with inhibitor decreased the I_{50}. This preincubation effect was maximal at 5 min (Fig. 1).

(ii) Mechanism of action of inhibitor. The K_m of ureases from *P. mirabilis*, *P. vulgaris*, *P. morganii*, *E. coli*, jack bean, and soybean were unaffected by AHA, indicating noncompetitive inhibition. The K_m values of the ureases of *P. mirabilis*, *P. vulgaris*, and *P. morganii* were increased in the presence of Benurestat, indicating mixed-type inhibition (5) (Fig. 2 and 3).

(iii) Binding of inhibitor to enzyme. The binding of inhibitor with urease was investigated by dialysis of the extract and desalting on Sephadex G-25. The activity of ureases from *P. mirabilis*, *P. morganii*, and jack bean was measured in the absence and presence of appropriate concentrations of AHA and Benurestat. Fresh samples of enzyme extract were then mixed with the same concentration of AHA and Benurestat and dialyzed overnight against distilled water at 4°C. Controls of enzyme alone were also dialyzed. The percentage of inhibition was estimated and compared with values obtained without dialysis (Table 2).

Two separate effects were noticed. First, bind-

TABLE 1. *I_{50} of AHA and Benurestat against ureases from different sources*[a]

Source of urease	I_{50} (μg/ml)			
	AHA		Benurestat	
	Direct	Preincubated	Direct	Preincubated
P. mirabilis	140	2	7	0.066
P. vulgaris	130	0.8	11	0.01
P. morganii	3,600	6.5	920	0.5
P. rettgeri	85	1.75	7.5	0.066
Clostridium sordelli	180	13	17.6	
Staphylococcus aureus		1.08		0.47
E. coli		4		0.1
Jack bean	420	0.85	55	0.22
Soybean	400	0.98	284	0.29

[a] The bacterial ureases were sonicated extracts, except for that of *S. aureus*, which was a whole-cell preparation.

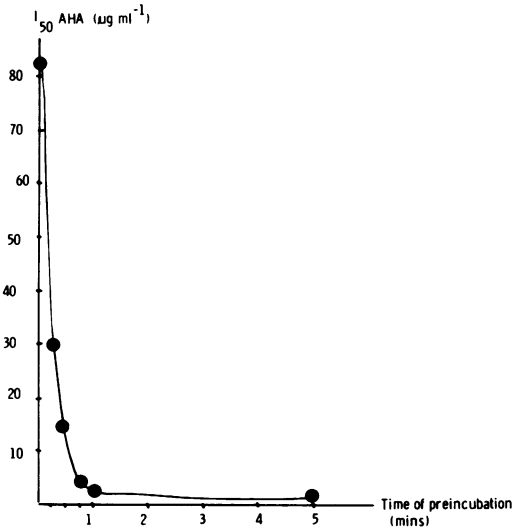

FIG. 1. *Effect of preincubation of AHA with P. mirabilis urease.*

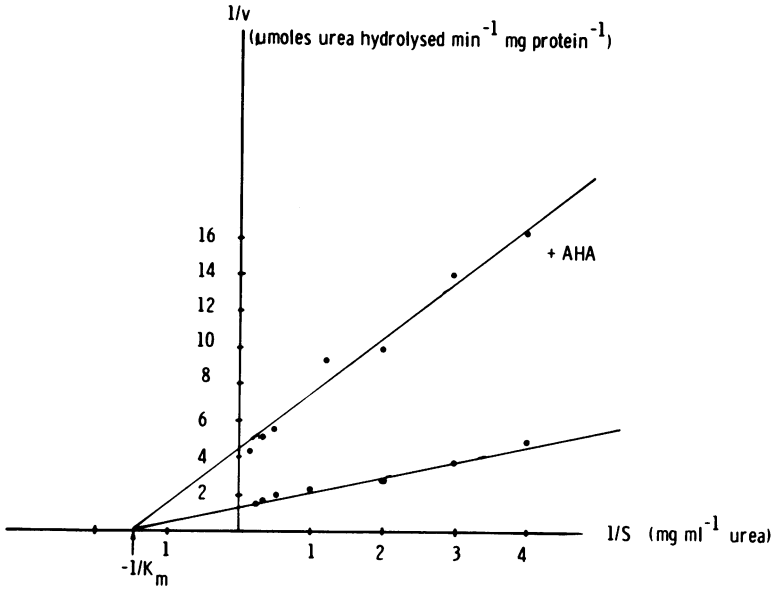

FIG. 2. *Lineweaver-Burk plot of P. mirabilis urease with and without AHA (3 μg/ml).*

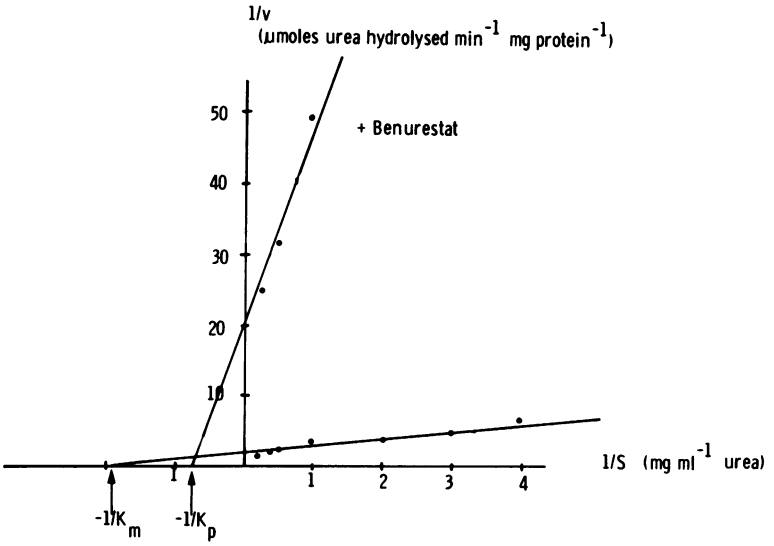

FIG. 3. *Lineweaver-Burk plot of P. mirabilis urease with and without Benurestat (0.75 μg/ml).*

ing of AHA to *P. morganii* was tighter than with other ureases, since inhibition of the enzyme by AHA was not reversed by dialysis. The inhibitor was bound to the enzyme and could not diffuse through the dialysis sac. Second, binding of Benurestat to all the enzymes was tighter than that of AHA, since there was little reversibility in inhibition after dialysis in all three ureases tested. When the urease-AHA complex was applied to a column of Sephadex G-25, 66% of the total enzyme activity was recovered from *P. mirabilis* urease, but only 39% was recovered from *P. morganii* urease. Negligible enzyme activity was lost when controls of enzyme alone were passed down the column. The strong binding of *P. morganii* urease to AHA prevented separation on the column, and hence recovery of *P. morganii* was much less than that of *P. mirabilis*.

Optimal pH. Figure 4 shows the relationships between pH and urease activities. *P. morganii* urease was equally active at pH 5, 6, and 7 and was more active at pH 4 and than 9. The other ureases had narrow optimal ranges and were

more active at pH 9 than 5. Altering the pH between pH 5 and 9 did not affect the degree of inhibition produced by AHA or Benurestat.

Effect of mercuric chloride. All ureases were completely inhibited by micromolar $HgCl_2$.

Gel electrophoresis. The electrophoretic mobility of the urease of *P. morganii* was less than that of *P. mirabilis* and *P. vulgaris* ureases. Several bands of urease activity were detected by electrophoresis of jack bean, *P. mirabilis*, and *P. vulgaris* ureases. Only one distinct band of urease activity was obtained from *P. morganii* urease.

Substrate profile. Hydroxyurea (5 mg/ml) was hydrolyzed by ureases from jack bean, *P. mirabilis*, and *P. morganii* 340, 59, and 100 times slower, respectively, than urea at the same concentration. Thiourea was not a substrate at 5 mg/ml. Ureases of jack bean, *P. mirabilis*, *P. morganii*, *P. vulgaris*, and *P. rettgeri* did not hydrolyze methylurea (5 ml/ml) or ethylurea (5 mg/ml). Apparent hydrolysis observed could be accounted for entirely by residual urea (0.4 and 0.2%, respectively) contained in these compounds (demonstrated by paper chromatography). Hydroxyurea was shown to be pure by the same technique.

Methylurea and ethylurea were not urease inhibitors at 200 μg/ml. Hydroxyurea was an inhibitor of both bacterial and vegetable ureases. With 5 min of preincubation of enzyme with inhibitor, the I_{50} of hydroxyurea with jack bean urease was 25 μg/ml; with *P. morganii* urease it was 125 μg/ml; and with *P. mirabilis* it was 2.5 μg/ml.

Other properties of urease. Table 3 gives values of K_m, E_A, Q_{10}, and molecular weight for the various ureases.

TABLE 2. *Binding effect of urease inhibitors*

Urease inhibitor	Source of urease	Concn of inhibitor (μg/ml)	% Inhibition	
			Predialysis	Postdialysis
AHA	*P. mirabilis*	3	56	23
	P. morganii	6	58	77
	Jack bean	2	74	0
Benurestat	*P. mirabilis*	6	92	42
	P. morganii	6	95	85
	Jack bean	0.5	87	82

TABLE 3. *Properties of bacterial and vegetable ureases*

Source of urease	K_m for urea (M)	E_A (joules/mol)	Q_{10}	Mol wt
P. mirabilis	0.0099	19.4	1.15	560,000
P. vulgaris	0.0093	35.6	1.03	>800,000
P. morganii	0.00024	56.58	1.48	669,000
P. rettgeri	0.0016	23.5	1.2	>800,000
E. coli	0.0015	33.3	1.24	
Jack bean	0.0016	41	1.5	560,000
Soybean	0.0012	12.9	1.1	

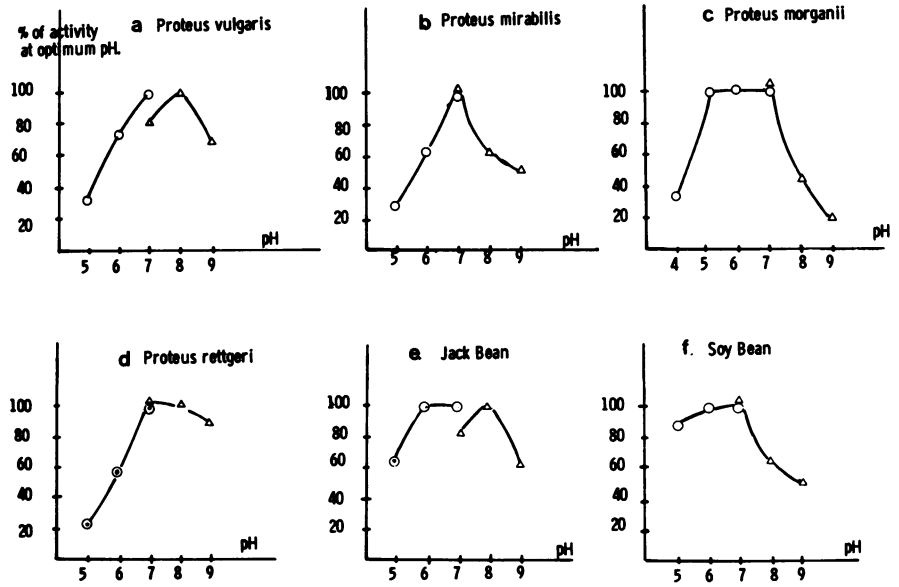

FIG. 4. *Optimum pH for different ureases. Symbols: ○, Tris-maleate-imidazole; △, Tris-hydrochloride.*

DISCUSSION

The enzyme activities in whole-cell and sonicated preparations were similar, indicating no permeability barrier to urea through the cell wall. Urease activities in several strains of *P. morganii* were consistently higher than those of the other three species of *Proteus* and were also not inducible, unlike the ureases in the other species of *Proteus* (22a). The significance of inducibility of urease in relation to the formation of infection stones has been discussed (22a). A potentially important source of error arising from work on the effects of urease inhibitors on vegetable extracts is that inducibility is not taken into account.

AHA and Benurestat had differential inhibitory activity against the various ureases. Much of the work on urease inhibition has been carried out with jack bean urease. However, it is clear that since infection stone formation is caused by urease-producing bacteria, results obtained by using bacterial rather than vegetable enzymes would be more likely to reflect the situation occurring in vivo.

P. morganii urease was more resistant to urease inhibitors than were the ureases of the other *Proteus* spp. This result is relevant to a consideration of appropriate dosages of urease inhibitors. Binding of both inhibitors to *P. morganii* urease appeared to be much tighter than binding to the other ureases.

I_{50} values were greater with whole cells than with sonicated extracts, indicating a permeability barrier against AHA and Benurestat at the cell wall. Work with whole cells provides a situation closer to that found in vivo. However, most work on bacterial ureases is with the sonicated extract (21).

Preincubation of enzyme with inhibitor reduced the I_{50}. This suggests that the inhibitor binds progressively to the enzyme, altering the conformation of the active site of the enzyme to impede substrate binding. However, this situation would not occur in vivo, because both urease inhibitor and urea are present simultaneously. Despite this, most workers have determined the I_{50} after preincubation (3, 7, 17), thus introducing a further source of error. Our results indicate that whole bacterial cells should be used and that inhibitory activity should be assessed without preincubation.

AHA is a noncompetitive inhibitor. Therapeutically this is useful since its effect will not be reduced by large amounts of substrate (urea) in the urine. Benurestat exhibits mixed-type inhibition, is more active than AHA, and binds more strongly to ureases. Thus, the mechanisms of inhibition of Benurestat and AHA are different.

The optimal pH for urease activity was similar to that reported in the literature (18). All ureases except that of *P. morganii* showed greater activity at pH 9 than 5. Thus, acidification of the urine would be a useful addition to therapy; unfortunately, this is difficult (4), especially in the presence of infection with *Proteus* spp.

We found, contrary to other reports (17), that changing the pH had little effect on urease inhibition. This is encouraging since an alkaline urine would not reduce the effectiveness of the urease inhibitor.

Hydroxyurea, unlike other analogs tested, was found to be both a substrate and an inhibitor of bacterial and vegetable ureases. This agrees with previous results (9).

As sulfhydryl enzymes (15), all ureases were inhibited by micromolar $HgCl_2$.

The K_m values of jack bean and *P. mirabilis* ureases were similar to those previously reported (18; J. A. Anderson, F. Kopko, A. J. Seidler, and E. G. Noble, Fed. Proc. **28**:764, 1969). The K_m of *P. morganii* urease differed considerably from that of the other species of *Proteus*. However, since the concentration of urea in urine far exceeded the K_m, all enzymes would be working at maximal velocity in vivo.

Values for E_A were similar to those found by Sizer (25). Unlike Magana-Plaza et al. (20), we found no discontinuities in Arrenhius plots. Values of Q_{10} were of the order expected for bacterial species (5).

The molecular weights obtained were higher than those reported in the literature (26). This may have been due to differences in techniques used and to association of the enzyme into polymeric forms on the column or aggregation of the enzymes before application to the column. This could explain the diversity of molecular weights obtained for the different ureases. Several authors (10; Anderson et al., Fed. Proc. **28**: 764, 1969) have shown bacterial urease to have molecular weights less than that of jack bean urease. Our results indicate no such difference.

The urease from *P. morganii* has properties which differ markedly from those of the ureases of the other three *Proteus* species. It has greater activity, lower K_m, and a different optimal pH, is noninducible, and is more resistant to urease inhibitors, but binds more tightly to them. The mobility of ureases in gel electrophoresis studies suggests further differences. These properties should be borne in mind when laboratory models of infections with *P. morganii* are being investigated.

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