Proteus vulgaris Urease: *In vitro* Inhibition by Urea Analogues. (31174)

ROLAND S. PIANOTTI, RAAM R. MOHAN, AND BENJAMIN S. SCHWARTZ
(Introduced by Henry H. Freedman)
Department of Microbiology, Warner-Lambert Research Institute, Morris Plains, N. J.

The function of urease in bacterial metabolism is not known with certainty. The enzyme is regarded as a detoxifying agent for the cell and as a catalyst whereby the bacterium derives nitrogen in the form of ammonia for the synthesis of amino acids. The association of bacterial pathogenicity and drug resistance with urease has been the subject of much uncertainty. Seneca, Peer, and Nally(1) observed that the more active producers of urease are either drug resistant or easily mutate to become drug resistant. Gupta *et al* (2) in a study of urease producing and nonproducing bacteria found no significant difference in the incidence of antibiotic-sensitive and antibiotic-resistant strains.

Since the natural substrate of this enzyme is urea, it was of interest to see what effect selected analogues of urea would have on urease activity on sonic lysates of *Proteus vulgaris* ATCC 881. Additional experiments were performed to establish whether or not any of the analogues were capable of inhibiting urease activity of intact cells or growth of *Proteus vulgaris* ATCC 881.

Materials and methods. Stock solutions and reagents. (1) The stock solutions of compounds at 4200 μg/ml were made up in M/15 Sorensen's phosphate buffer pH 7.0, or in the case of insoluble compounds, in buffer containing 5 to 10% dimethylforamide. (2) 0.042% urea solution prepared in M/15 phosphate buffer pH 7.0. (3) Berthelot phenol color reagent and alkalihypochloride reagents (3).

Preparation of crude enzyme and estimation of activity. Proteus vulgaris ATCC 881 was grown in one liter of Brain Heart Infusion Broth (Difco) for 20 hours at 37°C on a New Brunswick rotary shaker. The cells were harvested and washed twice with 0.9% sterile saline at 5°C. The washed cells were suspended in 50 ml of deionized distilled water and sonicated for 30 minutes at 8°C in a 10 KC Raytheon sonic oscillator. The sonicate was clarified by centrifugation at 6000 $\times$ *g* for 30 minutes at 8°C. The clear supernatant containing the urease was diluted to 100 ml with deionized distilled water and 10 ml aliquots were frozen and stored at —40°C until use. The protein content of the supernatant was determined according to the method of Lowry *et al*(4). The urease activity in the sonic lysate assayed in urea phosphate buffer, pH 7.0, produced 10 μg NH_3/ 1.092 mg protein/hour at 37°C.

Determination of urease inhibition using sonic lysate. The anti-urease activity of each compound listed in Tables I and II was determined in duplicate. The initial reaction mixtures contained 1.0 ml of compound (2.1 mg/ml of buffer) 2-fold serially diluted through 9 tubes of buffer plus 1.0 ml of the urease (diluted to contain 1.092 mg of protein/ml of buffer). The control (10th tube of the series) contained 1.0 ml of buffer plus 1.0 ml urease solution. Tubes were incubated for 20 minutes in a 5°C water bath. Following incubation, 0.1 ml of urea solution (5 μg/0.1 ml buffer) was added to each tube to make a final volume of 2.1 ml/tube. In this system the highest concentration of compound tested was 1000 μg/ml. A mixture of boiled enzyme plus urea served as a blank. The complete system was then incubated for 60 minutes in a 37°C water bath. The urease activity (amount of ammonia liberated from urea) was determined by the modified colorimetric method of Berthelot described by Chaney and Marbach(3).

Reversal of urease inhibition. To determine whether the antiurease activity of the compounds would be reversed in the presence of aqueous 10^{-3} and 10^{-4} M concentrations of cysteine, the test system was set up as described above. After incubating the reaction mixture containing the enzyme and the test compound for 20 minutes at 5°C, 10^{-3}

or 10^{-4} M cysteine was added to each tube. Following additional incubation for 20 minutes at 5°C, urea was added to each tube. Urease activity was determined as previously described.

Determination of urease inhibition using intact cells. Preparation of standardized inoculum. The stock culture of *Proteus vulgaris* ATCC 881 was maintained on Trypticase Soy Agar slants (BBL). Inoculum was prepared from a 20-hour-old stationary broth culture of *Proteus vulgaris* grown at 37°C in Tryptose Phosphate Broth (Difco). The cells were harvested by centrifugation and washed in saline. Final saline suspension was adjusted to 90% light transmission at 660 mμ.

The minimum concentration (μg/ml) of each compound required to inhibit the decomposition of urea by intact cells of *Proteus vulgaris* was determined by 2-fold serial dilution in 1.0 ml volumes of Urea Broth (Difco). The standardized *Proteus vulgaris* culture described above was diluted 1:10 in Urea Broth and 0.1 ml of this dilution used as inoculum for each tube. Reactions were recorded after 4, 8, 15, and 24 hours' incubation at 37°C. A positive urease reaction was indicated by a change in color from yellow to cerise.

Determination of antimicrobial activity against Proteus vulgaris *ATCC 881.* The minimum concentration of each compound (μg/ml) required to inhibit the growth of *Proteus vulgaris* was determined by 2-fold serial tube dilution in 1.0 ml volumes of Tryptose Phosphate Broth. The standardized *Proteus vulgaris* culture described above was diluted 1:10 in Tryptose Phosphate Broth and 0.1 ml of this dilution used as inoculum for each tube. Minimum inhibitory concentration was recorded as the lowest concentration showing no visible growth after 48 hours' incubation at 37°C.

Results and discussion. The inhibition of ureolytic activity by the urea analogues evaluated is presented in Tables I and II. The activity of each compound is presented as minimum concentration (μg/ml) required for 100% and 50% inhibition of ureolytic activity in sonic lysates. Table I lists the compounds which at concentrations of less than 1000 μg/ml completely inhibit urease of sonic lysates. Compounds listed in Table II showed poor activity. The antiurease activity of compounds (Table I) was not reversed in the presence of 10^{-3} and 10^{-4} M cysteine. It was also noted that ammonia was not produced from compounds listed in Tables I and II. The following analogues of urea were not inhibitory to urease activity in sonic lysates of *Proteus vulgaris*: 2-pyrimidylurea;

TABLE I. *In vitro* Inhibition of Ureolytic Activity in Sonic Lysates of *Proteus vulgaris* ATCC 881.

Urea analogue	Minimum concentration (μg/ml) required for 100% inhibition	Minimum concentration (μg/ml) required for 50% inhibition
Thiourea	62	15– 30
Hexahydrobenzylisothiourea	500	250–500
Cyclohexylisothiourea	125	30
Allylthiourea (NC 186)	125	15– 30
N-Methylthiourea (NC 343)	62	15– 30
Acetylthiourea (NC 396)	125	62–125
Allylisothiourea · HCl (W 1675)	500	250–500
1-Nicotinoyl-2-(2-pyrazinyl)-urea (W 2280)	500	125–250
Acetohydroxamic acid (W 4904)	500	15– 30

TABLE II. *In vitro* Inhibition of Ureolytic Activity in Sonic Lysates of *Proteus vulgaris* ATCC 881.

Urea analogue	Minimum concentration (μg/ml) required for 100% inhibition	Minimum concentration (μg/ml) required for 50% inhibition
Methylisothiourea-SO_4 (W 1672)	1000	250–500
1-Nicotinoyl-2-(2-thiazolyl-urea (W 2278)	1000	250–500
1-Isonicotinoyl-3-(2-primidinyl)urea (W 2279)	1000	250–500
1-(5-methyl-2-oxo-1-pyrrolidinyl)urea (W 2608)	1000	250–500
2-5-Dimethyl-2,5-dithio-1,1'-bipseudourea (W 2691)	1000	250–500
1,3-Dicyclohexyl-1-(indol-3-ylacetyl)urea (W 3947)	1000	250–500
1-(3-Pyridyl)-3-(3-pyridylmethyl)urea (W 4942)	1000	125–250
3-Pyridylmethyl-3-pyridine-carbamate (W 5291)	>1000	62–125
1-Allyl-3-(2-pyridyl)urea (W 5292)	>1000	500
2-(2-pridyl)ethyl 3-pyridine-carbamate (W 5295)	>1000	500

1-(2-pyrimidyl)-3-phenylthiourea; urea nitrate; (N^4-acetylsulfanilamido)urea (W 807); α-bromohexahydrobenzylurea (W 1207); 1-nicotinoyl-3-(2-pyrimidinyl)urea (W 2277); guanylurea sulfate (W 2495); [2-(5-acetylindol-3-yl)ethyl]urea (W 3447); 1-[2-(5-acetylindol-3-yl)ethyl]-3-butylurea (W 3522); 1-[3,4-dihydro-3-oxo-4-(p-tolyl)-2-quinoxalinecarbonyl]urea (W 4232); benzyloxyurea (W 4759); 1,1′-vinylenebis[3-benzyloxyurea] (W 4853); 1,1′-(2,6-pyridinediyl)bis[3-allylurea] (W 4948); chloroacetylurea (W 5055); 1-allyl-3-(3-pyridyl)urea (W 5293); allyl-2-pyridinecarbamate (W 5297). With the exception of chloroacetylurea, none of the compounds at 1000 μg/ml inhibited the growth of the test organism in Tryptose Phosphate or Urea Broth. Chloroacetylurea was bacteriostatic at 125 μg/ml.

The degree of inhibition of urease in sonic lysates varies markedly with the point of attachment and degree of substitution on the carboxamide, thiocarboxamide, or hydroxamide moiety. However, no definite sequential structure and activity correlation was evident. Thiourea activity was marked. Thiourea has been shown to specifically inhibit the urease activity of intact cells and cell-free extracts of *Corynebacterium renale*(5). Substitution on the sulfur atom of the thiourea molecule tended to diminish activity. However, N-methyl and N-acetyl substituted thiourea were as active as thiourea. Comparing the activities of compounds against urease with their chemical structures, it is apparent that none of the aryl substituted urea analogues tested had activity comparable with thiourea.

The *in vitro* capacity of a compound to inhibit urease activity of cell lysates does not appear to be associated with an ability to inhibit growth, or to inhibit ureolytic activity of intact cells. With the exception of acetohydroxamic acid, each compound tested failed to inhibit ureolytic activity of intact cells present in urea broth.

Acetohydroxamic acid at 75 μg/ml is reported to suppress ureolysis in urea broth cultures of *Proteus mirabilis*(6). The suppression is, however, overcome within the third and fourth hours of incubation and at the end of 12 to 18 hours of incubation the extent of ureolysis equals that of control cultures. These observations suggest that acetohydroxamic acid probably acts as a substrate competitor, but not as an inhibitor of urease synthesis. In our experiments, 1000 μg of acetohydroxamic acid per ml completely inhibited ureolysis by intact cells in urea broth without concomitant growth inhibition.

Summary. Thirty-five analogues of urea have been evaluated for their ability to inhibit urease activity and growth of *Proteus vulgaris* ATCC 881. Enzyme activity was determined by the method of Chaney and Marbach. The ureolytic activity in a sonic lysate of *Proteus vulgaris* was markedly inhibited by thiourea, N-methylthiourea and N-acetylthiourea. The inhibition was not reversed by 10^{-3} and 10^{-4} M cysteine. None of the aryl substituted urea analogues had activity comparable to that of thiourea. Acetohydroxamic acid (1000 μg/ml) inhibited ureolytic activity of intact cells in Urea Broth (Difco) without concurrent growth inhibition. Chloroacetylurea inhibited the growth of the test organism (125 μg/ml), but failed to inhibit urease in Urea Broth or in sonic lysates. The capacity to inhibit urease activity in cell lysate does not appear to be associated with an ability to inhibit growth, or to inhibit ureolytic activity of intact cells.

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