Effect of Organic Mercurials and Sulfhydryl Compounds on the Urease Activity of Proteus: Inhibition by Urine and Ascorbic Acid

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Received for publication 5 May 1976

Meralluride, mercaptomerin, ethacrynic acid, and penicillamine inhibited urease activity of Proteus mirabilis. The activity of the organic mercurials and ethacrynic acid was markedly inhibited by human and dog urine. Antiurease activity could not be detected in the urine of a human and a dog given meralluride by injection. Urine from patients receiving penicillamine also failed to inhibit urease activity. Ascorbic acid inhibited, whereas dehydroascorbic acid enhanced, the activity of the mercurials, but neither agent altered the inhibitory effect of urine. The lethal effect of meralluride against Proteus occurred at the same concentration at which urease activity was inhibited, but penicillamine inhibited the enzymatic activity without affecting viability of the organism. The data suggest that these sulfhydryl-reactive compounds will not be useful against Proteus infections of the urinary tract.

The pathogenesis of Proteus infections of the urinary tract appears to be related, in part, to the generation of ammonia from urea catalyzed by bacterial urease. Several studies have demonstrated that invasion of the kidney and production of renal damage are associated with ammonia production (2, 9). In addition, the ability of Proteus to produce an alkaline urine favors the development of urinary stones and complicates the use of otherwise effective antimicrobial agents (9). A number of agents are known to interfere with urease activity, including acetohydroxamic acid and related compounds (1, 8), thiourea (2), hydroxyurea (2), suramin (14), and heavy metals such as mercury, copper, and zinc (10).

The lack of availability for clinical use of effective urease inhibitors led us to reexamine the observations of Seneca and Lattimer and their colleagues (6, 11, 12) on the ability of organic mercurials to inhibit bacterial urease. In addition, it seemed reasonable to explore the potential value of other agents with sulfhydryl activity since urease is known to be rich in sulfhydryl groups and activity might be blocked by the formation of disulfides (10). This report confirms the findings of Seneca and co-workers that organic mercurial diuretics inhibit urease activity in Proteus and also appear to have a lethal effect against the organisms. In addition, other sulfhydryl-containing agents such as penicillamine and ethacrynic acid exhibit antiurease activity. Unfortu-
Research Laboratories, Inc.), and methionine (Calbiochem). All of the compounds were diluted initially in sterile water and then added to the test medium prior to use. All values for activity are expressed as the highest effective final concentration of the compound. In individual tests, 1.0 ml of each compound to be tested was dissolved in the appropriate diluent (urea broth, trypicot soy broth, or urine), mixed with 0.1 ml of the bacterial inoculum, and incubated overnight at 37°C. When compounds were mixed, equal volumes of serial dilutions of each compound were added to each other (1 ml each), and the inoculum was added as described above.

In vitro and in vivo studies of human and dog urine. Urine was freshly obtained from four normal human subjects and one dog. In addition, a 20-kg dog was given 125 mg of meralluride intramuscularly, and serial half-hour urine specimens were collected for 4 h. A 50-year-old man, weighing 114 kg, with congestive heart failure, was given 250 mg of meralluride intramuscularly, and urine specimens were collected at hourly intervals for 4 h. Four patients with rheumatoid arthritis treated with penicillamine were studied. Urine was obtained 2 and 4 h after the last oral dose (500 mg in three and 250 mg in one patient). Urine specimens collected from the patients and the dogs were adjusted to pH 7.0, and 0.1 ml of a 1:100 dilution of an overnight culture of P. mirabilis was added to 1 ml of urine. The specimens were incubated overnight at 37°C and tested for inhibition of growth and antiusurease activity (e.g., block of a rise in pH above the initial value of the medium and of an uninoculated, sterile, control specimen).

Urease activity of acetone-killed organisms. P. mirabilis was grown overnight on a trypicot soy agar surface in bottle cultures and was harvested by a wash with 10 ml of sterile water. The fluid contained $3 \times 10^9$ colony-forming units per ml. The suspension was centrifuged, the supernatant was removed, and the sediment was washed three times with 10 ml of acetone and allowed to air dry. The sediment was then taken up in 10 ml of urea broth. All culture preparation was under sterile conditions.

**RESULTS**

Effect of various compounds in inhibiting pH change by P. mirabilis in urea broth. Meralluride blocked a change in pH in urea broth (i.e., it maintained the pH at 7.0 compared to 9.2 observed with uninhibited growth of Proteus) at concentrations ranging from 5 to 80 $\mu$g/ml depending on an inoculum size of $10^4$ to $10^5$ viable cell units per ml. All pH end points paralleled the bactericidal concentrations; i.e., the drug simultaneously killed the organisms and interfered with ammonia production. Meralluride exhibited the same effect with two other strains of P. mirabilis and P. rettgeri. Mercaptopemerin was less active against the test strain of Proteus, requiring 50 to 400 $\mu$g/ml depending on inoculum size. Ethacrynic acid was bactericidal and blocked urease activity when tested in one experiment at 32 $\mu$g/ml and in another at 125 $\mu$g/ml. Penicillamine differed from the other compounds in being able to inhibit urease activity at concentrations of 400 to 500 $\mu$g/ml but failed to inhibit growth of the organism. A representative experiment is presented in Table 1.

**Effect of meralluride in inhibiting bacterial urease.** In view of the observation that the mercurial compounds killed the Proteus strain at the same concentration as that which inhibited a rise in pH in urea broth, it was important to determine whether the effect of the drug was directed primarily against the organism rather than urease activity. To test this point, meralluride was added, in serial dilution, to a preparation of acetone-killed organisms in urea broth and incubated overnight at 37°C together with control samples, which did not contain the drug. Urease activity was inhibited completely (i.e., no change of pH from 7.0 to 9.2) by drug concentrations of 40 $\mu$g or more per ml. In contrast, the pH of control tubes containing no mercurial rose to 9.2 after overnight incubation. In a parallel experiment, 40 $\mu$g of drug per ml sterilized a urea broth culture of about $10^5$ viable P. mirabilis per ml.

**Inhibitory effect of urine on activity of sulphydryl-reactive compounds.** Urine specimens were obtained from four normal subjects. Osmalalities varied from 232 to 1,078 with a mean of 641 mosmol/kg. The pH of each specimen was adjusted to 7.0 (pH of 5.5 or less inhibits this strain of Proteus). Sixteen- to 64-fold more meralluride was required to inhibit the organism in urine than in broth. In addition, the urine of one subject was diluted serially in urea broth and tested for activity of meralluride in urea broth and human urine.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Minimum conc that blocks pH rise ($\mu$g/ml) Urea broth</th>
<th>Minimum bactericidal concn ($\mu$g/ml) Urea broth</th>
<th>Minimum conc that blocks pH rise ($\mu$g/ml) Urine*</th>
<th>Minimum bactericidal concn ($\mu$g/ml) Urine*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meralluride</td>
<td>5</td>
<td>80-160</td>
<td>5</td>
<td>80-160</td>
</tr>
<tr>
<td>Mercaptopomerin</td>
<td>100</td>
<td>1,600-6,300</td>
<td>100</td>
<td>1,600-6,300</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>32</td>
<td>500</td>
<td>32</td>
<td>500</td>
</tr>
<tr>
<td>Penicillamine</td>
<td>500</td>
<td>1,000</td>
<td>2,000</td>
<td>1,000</td>
</tr>
</tbody>
</table>

a Inoculum size, about $10^4$ colony-forming units per ml. Diuretic and sulphydryl compounds showing no activity at 500 to 1,000 $\mu$g/ml include cysteine, cystine, methionine, furosemide, and acetazolamide.

b Depending upon subject.
against the *Proteus* strain. Even when the urine was diluted 1:32, it required twofold more drug to inhibit the organism than in urea broth. A similar inhibitory effect of urine was observed with mercaptomerin and ethacrylic acid. Urine was also inhibitory to penicillamine, reducing activity at least twofold (Table 1).

Urine from a dog (pH 7.8) completely inhibited the activity of 6,300 μg of meralluride or mercaptomerin per ml. Undiluted specimens of urine collected in serial half-hour or sequential hourly volumes over a 4-h period from a dog and from a human given 125 and 250 mg of meralluride intramuscularly, respectively, failed to inhibit the growth of *Proteus* or prevent a rise in urinary pH. Urine from four patients receiving 250 to 500 mg of penicillamine orally was also ineffective.

**Effect of ascorbic and dehydroascorbic acids on the inhibitory effect of meralluride against *Proteus***. Since the oxidation-reduction of sulfhydryl groups is known to affect their biological activity, ascorbic acid, a reducing compound, and dehydroascorbic acid, an oxidant, were examined for their ability to alter the action of meralluride on urease activity. The results of box titration experiments of the effect of ascorbic and dehydroascorbic acids on the action of meralluride against *Proteus* in urea broth are shown in Fig. 1. Ascorbic acid at 2,500 μg/ml had no effect against the organism, but dehydroascorbic acid was inhibitory at concentrations of 625 μg or more per ml. Ascorbic acid demonstrated a marked inhibitory effect on the activity of the mercurial at concentrations as low as 79 μg/ml, whereas dehydroascorbic acid markedly enhanced the activity at 20 μg or more per ml. Addition of dehydroascorbic acid to human urine obtained from two healthy volunteers, however, failed to enhance the antibacterial activity of the mercurial.

**DISCUSSION**

Ureases obtained from a wide variety of species have been shown to be rich in sulfhydryl groups and to be inhibited by heavy metals such as mercury, copper, and zinc (10). The observations of Seneca and co-workers (6, 11, 12) on the activity of mercurial diuretics on inhibition of urease activity of *Proteus* and the critical role of ammonia production in urinary infections produced by this group of bacteria stimulated us to reevaluate their potential use.

The current results document the activity of some organic mercurial diuretics and sulfhydryl-reactive compounds such as ethacrylic acid and penicillamine against *Proteus*. The mercurials appear to kill *Proteus* at the same concentration at which they inhibit the urease activity of nonviable organisms, but these probably are unrelated effects since urease is not required for cell growth (1, 2, 8, 9). Penicillamine, as for acetohydroxamic acids (1, 8), inhibited urease activity without affecting bacterial growth. Unfortunately, the organic mercurials and ethacrylic acid show little promise for clinical use in inhibiting urease formation in urinary infections since their activity is inhibited markedly by urine. This effect did not appear to be related directly to osmolality, since it was observed in human urines widely varying in concentration and even in diluted urine. The nature of the urinary inhibitory substance is unknown. It may be due to interference by disulfides or other products of sulfhydryl metabolism reacting with the mercurials. Cysteine, for example, is known to reverse the inhibition of urease by mercurials (11).

Ascorbic acid inhibited, whereas dehydroascorbic acid augmented, the activity of meralluride against *Proteus*. These compounds were studied because of the known reactivity of sulfhydryl groups to conditions of oxidation and reduction (4, 5). The mechanism by which ascorbic acid inhibited the action of the mercurial is not entirely clear since the system contained whole, growing bacteria rather than
purified urease. One possibility is that ascorbic acid tends to interfere with binding of the enzyme to mercury by maintaining the sulfhydryl groups in the reduced state. The activity of copper in inhibiting urease was studied by Mapson (7). He demonstrated that ascorbic acid enhanced inhibition of urease by copper by reducing Cu²⁺ to Cu⁺ ions. In contrast, antiurease activity of Hg⁰ salts was decreased by ascorbic acid, in accord with the present findings. That diets are rich in ascorbic acid, which is excreted readily into the urine, may account, in part, for the inhibitory effect of urine against mercurials.

The major excretory product of common organic mercurials is the cysteine complex (13) rather than the free drug. The activity of the complex against urease was not studied; however, the urine of a dog and a human subject given meralluride by injection failed to inhibit Proteus either because of inactivation of the mercurial by urine or because of the formation of the complex, or both.

This study supports, in part, the demonstration by Seneca and co-workers (6, 12) that organic mercurials inhibit Proteus and its urease activity. Inactivation by urine, however, may explain the minimal efficacy of mercurials in treatment of urinary tract infections reported by this group.

Penicillamine, although active in vitro, requires relatively large amounts to inhibit urease and does not affect bacterial growth, and its activity is decreased in urine. The drug is excreted rapidly in the urine as a mixture of free penicillamine and penicillamine-cysteine disulfide. About 50% of the dose is excreted in the first 7 h; 73% is excreted by 24 h (3). No antiurease activity could be detected in urine of four patients receiving the drug.

Although these results make it unlikely that the sulfhydryl-active compounds examined in this study will prove to be useful in treatment of Proteus infections of the urinary tract, it is still possible that they may be effective in renal tissue, where Proteus produces damage, and that other related compounds will be found to be more active.

LITERATURE CITED