Urease Activity and Antibiotic Sensitivity of Bacteria

GLEN R. GALE

Veterans Administration Hospital, and the Department of Physiology and Pharmacology, Duke University Medical Center, Durham, North Carolina

Received for publication 25 September 1965

ABSTRACT

GALE, GLEN R. (Veterans Administration Hospital, Durham, N.C.). Urease activity and antibiotic sensitivity of bacteria. J. Bacteriol. 91:499–506. 1966.—An investigation was made of the responses of certain urease-positive bacteria to various antibacterial drugs in the presence of highly specific urease inhibitors, in a test of the hypothesis proposed by other workers that inhibition of bacterial urease enhances the sensitivity of the cells to antimicrobial agents. Urease inhibitors employed were seven hydroxamic acids (HA). Six of the seven HA reduced the sensitivity of nine Proteus strains to ampicillin and methenamine mandelate. Two HA increased the sensitivity to colistin, and six HA increased the sensitivity to kanamycin. Investigation of the mechanism of action of the synergistic effect between kanamycin and HA led to the tentative conclusion that potentiation was mediated through an initial alteration of cell permeability by the aminoglycoside antibiotic which permitted accumulation of each of the six HA into the cell, at which point each interacted with pyridoxal phosphate. The single HA which failed to yield synergism with kanamycin failed to interact with pyridoxal phosphate in a nonenzymatic system; the other six HA produced alterations of the normal ultraviolet absorption spectrum of the coenzyme.

Extensive investigations by Seneca (7) and co-workers have yielded implications of a close association of antibiotic resistance with certain enzymes, particularly urease, in bacteria. Conclusions were based on the finding that drug-resistant bacteria can be made drug-susceptible, in many cases, through the action of enzyme inhibitors. In tests of organic mercurial compounds in vitro, inhibition of bacterial growth was shown to occur concomitantly with inhibition of urease.

Kobashi, Hase, and Uehara (6) have demonstrated that a number of hydroxamic acids (HA) are potent, highly specific inhibitors of urease, combining with it at its active site. The prototype of this group of compounds, acetyl hydroxamic acid, has no inhibitory action on a variety of other enzymes: α-amylase, papain, protease, β-glycerophosphatase, alcohol dehydrogenase, lactic dehydrogenase, vitamin K₃ reductase, catalase, and pyruvic carboxylase. Unlike the organic mercurial compounds used by Seneca (7), many HA are devoid of significant antimicrobial activity (unpublished data), and those which do possess such action are of limited spectra (4a, 5).

In view of the availability of this group of compounds with urease inhibitory activity but with only limited antibacterial activity, a study was made of the effects of some of these agents on the responses of certain urease-positive organisms to a number of antibacterial drugs.

MATERIALS AND METHODS

All Proteus strains were isolates of recent human origin from the bacteriology laboratory of the Durham Veterans Administration Hospital. The following HA were synthesized by Hynes Chemical Research Corp., Durham, N.C.: glycyl HA (GHA), L-alanyl HA (LAHA), D-alanyl HA (DAHA), β-alanyl HA (BAHA), isonicotinyl HA (INHA), 3-aminobutyryl HA (3ABHA), and hydroxyurea (HU). All were sterilized by filtration with Millipore filters. Antibiotic sensitivity test discs (Sensi-Discs, BBL) were used. Methenamine mandelate was supplied by...
Warner-Lambert Research Institute, Morris Plains, N.J.; ampicillin and kanamycin sulfate were from Bristol Laboratories, New York, N.Y. Tryptic Soy Medium (Difco) was used for growth and maintenance of the Proteus strains. Urea Agar (Difco) was used to demonstrate urease activity in the bacteria and inhibition of this activity by HA.

In initial tests for altered sensitivity of Proteus, Tryptic Soy Medium plates, with and without each HA, were uniformly streaked with an aqueous suspension of each organism, and sensitivity discs were then placed on the surface of each plate. After 18 hr, zones of inhibition were recorded (in millimeters) from the edge of each disc to the periphery of the inhibited area.

Measurements of growth curves were done with 500-ml flasks equipped with side arms (diameter, 19 mm; Belco Glass, Inc., Vineland, N.J.), which permitted periodic observations of turbidity in a Coleman spectrophotometer at 640 m\(\mu\). Each flask contained 50 ml of liquid medium, and incubation was at 30°C on a reciprocating shaker. Responses of Proteus strains to various concentrations of each HA, with and without kanamycin, were measured in 50-ml Erlenmeyer flasks containing a final volume of 20 ml; growth was assessed as optical density at 640 m\(\mu\) after 6 hr of incubation at 30°C on a reciprocating shaker.

A cell-free urease preparation from a strain of Proteus was obtained by ultrasonic lysis. Washed cells from 1 liter of an 18-hr culture were made to a 10% suspension in water (30 ml) and subjected to 2 min of treatment in a Sonifier (model LS-75; Branson Instruments, Inc., Stamford, Conn.). Cell debris was removed by centrifugation at 12,000 rev/min for 20 min. The supernatant solution was divided into 3.0-ml samples, freeze-dried, and stored at −20°C. Prior to estimation of urease activity by a manometric method (4), each sample was brought to 6.5 ml with 0.2 M potassium citrate (pH 5.8) and 1.0 ml was used in each Warburg vessel. The urease activity in each vessel thus represented the activity of 15.4 ml of the original culture.

RESULTS

Inhibition of Proteus urease by each HA was shown to occur both in intact cells and in a cell-free system. When incorporated into Urea Agar, 1,000 \(\mu\)g/ml completely inhibited emergence of the indicator color when one side of each culture plate was streaked with a heavy suspension of each organism and examined at 3 hr. An amount of 100 \(\mu\)g/ml of each HA reduced the color formation markedly, whereas 10 \(\mu\)g/ml of each was without detectable effect. Since bacterial urease is located intracellularly (7), some penetration of each compound into the cells appears substantiated. Figure 1 shows the effects of each HA on the cell-free urease system; the concentration of each HA which induced 50% inhibition ranged from \(4 \times 10^{-3}\) to \(5.2 \times 10^{-4}\) M for INHA.

In initial tests to determine whether synergism occurs between the urease inhibitors and antibacterial drugs, the seven HA were tested on Tryptic Soy Medium plates in conjunction with sensitivity test discs of each of eight antibacterial agents: demethylchlortetracycline, kanamycin, colistin, chloramphenicol, sulfisoxazole, methenamine mandelate, nitrofurantoin, and ampicillin. Nine Proteus isolates were employed in each determination; thus, the total number of individual tests (seven urease inhibitors \(\times\) eight antibacterial drugs \(\times\) nine organisms) was 504. Table 1 summarizes results of these semiquanti-
Fig. 2. Growth curves of three strains (500, 553, and 622) of Proteus as influenced by kanamycin, with and without each of seven hydroxamic acids. Concentrations of kanamycin: strain 500, 2 μg/ml; strains 553 and 622, 3 μg/ml; concentration of each hydroxamic acid, 200 μg/ml. Abbreviations are defined in text.

Tative tests. With each HA, with the exception of HU, there was an enhanced inhibitory zone around each kanamycin disc. Correspondingly, there was a reduction or complete annulment of inhibition of sensitive strains by both ampicillin and methenamine mandelate in the presence of all HA (except HU). The only other instance of enhanced inhibition observed was with the combination of colistin with either GHA or LAHA. All nine Proteus strains were totally insensitive
to colistin in the absence of these two HA. Activity of each of the other four antibacterial agents (sulfoxazole, chloramphenicol, nitrofurantoin, and demethylchlortetracycline) was uninfluenced by the presence in the medium of any HA. In view of the enhanced inhibition of all nine strains by kanamycin under the influence of six of the urease inhibitors, three strains were chosen for more extensive quantitative investigation.

Figure 2 shows the influence of each HA on the growth curves of three Proteus strains (designated 500, 553, and 622) with single concentrations of HA and kanamycin; concentrations of kanamycin were chosen which yielded nominal inhibition in the absence of HA. With each of the three isolates, there was moderate to marked enhancement of inhibition by kanamycin in the presence of each HA, except HU. This confirmed the results of the semiquantitative plate tests and ruled out diffusion factors as being contributory. It is noteworthy that no HA showed consistent or significant growth inhibition alone; therefore, the reduction in rate of growth of the cells appears to be a true synergism between kanamycin and each of the six HA, rather than a summation of inhibitory properties.

Tables 2 to 4 show results obtained with each of the six HA, at various concentrations, when used in conjunction with various concentrations of kanamycin against each of three Proteus strains. Data are expressed as per cent of control growth, and were obtained from single measurements of cultures during exponential growth. These experiments again demonstrate the virtually negligible effect of any HA alone on growth of the cells. Concentrations of kanamycin were chosen which, without HA, would cause not over 20% inhibition. These conditions were obtained in most experiments. Magnitude of the synergism

### TABLE 2. Effect of various concentrations of kanamycin, with and without hydroxamic acids (HA), on growth of Proteus strain 622

<table>
<thead>
<tr>
<th>Conc of HA</th>
<th>DAHA</th>
<th>BAHA</th>
<th>GHA</th>
<th>INHA</th>
<th>3ABHA</th>
<th>LAHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/ml</td>
<td>0</td>
<td>100</td>
<td>103</td>
<td>103</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>103</td>
<td>115</td>
<td>103</td>
<td>88</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>119</td>
<td>111</td>
<td>99</td>
<td>97</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>106</td>
<td>99</td>
<td>97</td>
<td>91</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>102</td>
<td>105</td>
<td>74</td>
<td>100</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>113</td>
<td>100</td>
<td>67</td>
<td>96</td>
<td>92</td>
</tr>
</tbody>
</table>

* See footnote to Table 1 for abbreviations of acids.
† Concentration (micrograms per milliliter) of kanamycin.
‡ Figures indicate per cent of control growth of Proteus 622 in the presence of kanamycin in the concentrations designated, in combination with six hydroxamic acids, each at the concentration designated.

### TABLE 3. Effect of various concentrations of kanamycin, with and without hydroxamic acids (HA), on growth of Proteus strain 553

<table>
<thead>
<tr>
<th>Conc of HA</th>
<th>DAHA</th>
<th>BAHA</th>
<th>GHA</th>
<th>INHA</th>
<th>3ABHA</th>
<th>LAHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg/ml</td>
<td>0</td>
<td>100</td>
<td>74</td>
<td>59</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>92</td>
<td>91</td>
<td>50</td>
<td>116</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>97</td>
<td>90</td>
<td>44</td>
<td>112</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>104</td>
<td>84</td>
<td>36</td>
<td>112</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>102</td>
<td>84</td>
<td>31</td>
<td>107</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>96</td>
<td>73</td>
<td>32</td>
<td>103</td>
<td>95</td>
</tr>
</tbody>
</table>

* See footnote to Table 1 for abbreviations of acids.
† Concentration (micrograms per milliliter) of kanamycin.
‡ Figures indicate per cent of control growth of Proteus 553 in the presence of kanamycin in the concentrations designated, in combination with six hydroxamic acids, each at the concentration designated.
TABLE 4. Effect of various concentrations of kanamycin, with and without hydroxamic acids (HA), on growth of Proteus strain 300*

<table>
<thead>
<tr>
<th>Concen of HA</th>
<th>DAHA</th>
<th>BAHA</th>
<th>GHA</th>
<th>INHA</th>
<th>3ABHA</th>
<th>LAHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>100</td>
<td>75</td>
<td>86</td>
<td>68</td>
<td>95</td>
<td>90</td>
<td>87</td>
</tr>
<tr>
<td>200</td>
<td>83</td>
<td>71</td>
<td>72</td>
<td>95</td>
<td>85</td>
<td>65</td>
</tr>
<tr>
<td>300</td>
<td>86</td>
<td>70</td>
<td>53</td>
<td>99</td>
<td>84</td>
<td>44</td>
</tr>
<tr>
<td>400</td>
<td>86</td>
<td>76</td>
<td>43</td>
<td>98</td>
<td>81</td>
<td>30</td>
</tr>
<tr>
<td>500</td>
<td>90</td>
<td>75</td>
<td>38</td>
<td>93</td>
<td>82</td>
<td>21</td>
</tr>
</tbody>
</table>

* See footnote to Table 1 for abbreviations of acids.
† Concentration (micrograms per milliliter) of kanamycin.
‡ Figures indicate per cent of control growth of Proteus 300 in the presence of kanamycin in the concentrations designated, in combination with six hydroxamic acids, each at the concentration designated.

was rather large in each experiment. As an example, 4 µg/ml of kanamycin alone inhibited strain 622 an average of about 11% in six experiments (Table 2). This was increased to up to 80% with 500 µg/ml of LAHA. No experiment failed to show a clear enhancement of inhibition. Data on HU are not included in Tables 2 to 4, because values obtained with up to 500 µg/ml of HU in the presence of kanamycin did not differ appreciably from values obtained with kanamycin alone.

These data, although not completely in accord with the suggestions of Seneca (7) regarding a direct relationship between urease activity and drug susceptibility, unequivocably demonstrate significant potentiation in vitro of inhibition of Proteus by kanamycin in the presence of certain urease inhibitors. Consistent inability to demonstrate such an enhancement with kanamycin plus HU, however, casts some doubt that the potentiation is due to the urease inhibitory activity per se of the HA, since HU was shown to be inhibitory to the cell-free urease preparation as well as to the enzyme in intact cells. Regarding the mechanism of potentiation, it appeared of interest to inspect other properties of the HA in an effort to determine whether HU differs in some way from the other six HA.

In a previous report (Gale, Can. J. Microbiol., in press), it was shown that two HA with antimycobacterial properties were antagonized by pyridoxal phosphate (PyP), and formed complexes with PyP as evidenced by alterations in the ultraviolet absorption spectrum of the coenzyme. To determine whether the synergism herein described is antagonized by PyP, culture flasks were prepared containing graded concen-

**FIG. 3. Antagonism by pyridoxal phosphate (PyP, 1,260 µg/ml) of the synergism between L-alanyl hydroxamic acid (LAHA, 500 µg/ml) and kanamycin (K) at concentrations indicated on abscissa. Coenzyme and hydroxamic acid were equimolar.**
trations of kanamycin, with and without LAHA and LAHA plus PyP. Figure 3 shows reversal of the synergism by PyP.

As a consequence of this observation, the effect of each HA on the ultraviolet absorption spectrum of PyP was determined after mixing aqueous solutions of each acid. In addition, two hydroxamates of carboxyl nonamino acids, propionyl HA (PHA) and butyryl HA (BHA), were included in the scans, as well as hydroxylamine. Figure 4 (A, B, C) shows that each HA tested, with the singular exception of HU, produced an aberration of the normal spectrum of PyP. Changes recorded ranged from marked elevation of the optical density of the control curve with INHA and PHA to production of new absorption maxima with DAHA, LAHA, BHA, 3ABHA, and GHA. It thus appears that the capacity of each HA to synergize the action of kanamycin may be a correlate of the capacity o

![Figure 4](http://jb.asm.org)
each to alter the absorption spectrum of PyP, and the ability of any HA to complex with PyP is not dependent upon the presence of a primary amino group in the molecule. In addition, kanamycin itself apparently interacts with PyP, as shown in Fig. 4D.

Fitzgerald and Bernheim (3) described an antagonism by urea of the bacteriostatic action of streptomycin on a mycobacterium and on Escherichia coli. Since streptomycin and kanamycin are closely related structurally and have a similar action on peptide synthesis at the ribosomal level (1), it was considered of interest to determine whether exogenous urea in any way influences the action of kanamycin on Proteus. With the use of three strains of the organism and procedures substantially the same as those used to obtain the data in Tables 2 to 4, it was found that the presence of urea at concentrations up to 2,000 μg/ml in no way altered the response of the cells to kanamycin. Therefore, no reciprocal relationship exists between synergism of kanamycin by urease inhibitors on the one hand, and antagonism of kanamycin by stimulating the enzyme to possibly greater velocity through added substrate. Besides this apparent difference in streptomycin and kanamycin, preliminary experiments with the three Proteus strains showed that the action of streptomycin was in no way influenced by the presence of urease inhibitors.

Experiments designed to demonstrate the antagonism by urease inhibitors of the action of ampicillin and methenamine mandelate confirmed the results of the plate tests and showed a considerable reversal of the action of ampicillin by LAHA (Fig. 5). The mechanism of this antagonism has not been investigated. Experiments with methenamine mandelate and LAHA similarly revealed that antagonism of the former by the latter was not due simply to an effect on rate of diffusion of methenamine mandelate in the plate tests. However, the three strains tested were not markedly susceptible to methenamine mandelate in liquid shake culture, even after adjusting the pH of the medium to 6.5. A typical experiment showed that 600 μg/ml conferred 20% inhibition, and this value was reduced to 5% in the presence of 200 μg/ml of LAHA.

Tests employing seven strains of Pseudomonas showed that none of the six HA which were synergistic with kanamycin altered in any way the responses of the organisms to the eight antimicrobial drugs. The seventh, HU, is inhibitory to Pseudomonas (5).

**DISCUSSION**

The foregoing data indicate that, within the limits of the seven HA and eight antibacterial drugs tested against nine strains of Proteus, no definite and consistent correlation exists between inhibition of bacterial urease per se and enhanced sensitivity to antimicrobial agents, as has been proposed by Seneca (7). Indeed, investigations regarding the mechanism of action of kanamycin (1, 2) at its presumably principal site of action at the ribosomal level have not implicated urea or urease as being a contributory factor.

In view of the fact that the six HA which enhance the action of kanamycin are the same compounds which interact with PyP in a nonenzymatic system, and the potentiated antimicrobial activity is antagonized by PyP, the actual mechanism of antibacterial action would appear to be mediated by HA rather than by kanamycin. The known property of aminoglycoside antibiotics of inducing marked changes in cell permeability, thereby promoting a possibly greater accumulation of the six HA in the cell, is not incompatible with such a hypothesis.

Experiments have been initiated to determine whether certain HA which possess antimycobacterial properties (4a) show an increased activity against mycobacteria when combined with kanamycin.

**ACKNOWLEDGMENTS**

This investigation was supported by Public Health Service grant GM-09389 from the Division of General Medical Sciences.
The technical assistance of Marceile B. Riddick and Helen H. McLain is gratefully acknowledged.

LITERATURE CITED