

# Influence of Serum and Calcium on the Bactericidal Activity of Gentamicin and Carbenicillin on *Pseudomonas aeruginosa*

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Because calcium was found to be antagonistic in vitro to the activity of colistin and polymyxin B on *Pseudomonas aeruginosa*, the effects of calcium and serum on gentamicin and carbenicillin were also examined. Serum was antagonistic to gentamicin in antibiotic tube dilution tests on five strains of *P. aeruginosa*. Serum was not antagonistic to carbenicillin in tube dilution tests. Physiologic concentrations of calcium antagonized the activity of gentamicin but not carbenicillin. The antagonism observed with gentamicin was less than that previously seen with colistin. The antagonistic effect of calcium and serum was removed by a chelating agent. Gentamicin and carbenicillin may be more active in vivo against *P. aeruginosa* than colistin or polymyxin B.

Physiologic concentrations of calcium have been shown to be antagonistic to the bactericidal activity of colistin on *Pseudomonas aeruginosa* in vitro (5, 6). Serum obtained from volunteers after injection of therapeutic doses of colistin was highly bactericidal for *Escherichia coli* but had no activity against *P. aeruginosa* unless the calcium concentration was decreased by dilution with broth or addition of chelating agents. It was of interest to determine whether calcium also antagonized the bactericidal activities of gentamicin or carbenicillin on *P. aeruginosa*. Serum has been reported to antagonize gentamicin (15) but not carbenicillin (1, 13). Neither is highly bound to serum proteins.

## MATERIALS AND METHODS

Six strains of *P. aeruginosa* and one of *E. coli* were obtained from Kenneth J. Ryan of the Clinical Microbiology Laboratory at the University Hospital. All had been recently isolated from blood cultures of patients and all isolates were thought to have been clinically significant. An additional group of 21 strains, representing seven immunotypes, was obtained from Myron Fisher of Detroit (7). The results of studies on all strains of *P. aeruginosa* were similar. Individual strains will not be further identified.

Gentamicin sulfate was obtained from Schering Corporation and carbenicillin from Bristol Laboratories. Concentrated stock solutions were made up, distributed in small samples, and frozen at  $-65^{\circ}\text{C}$  until used.

The methods have previously been described in detail (5, 6). Serum was obtained from a few healthy adult donors and stored at  $-65^{\circ}\text{C}$  until thawed for use. Tests for antibiotic susceptibility were carried out by a twofold tube dilution technique in Mueller-Hinton broth (Baltimore Biological Laboratory) and various dilutions of human serum. Tubes were made up of 0.4 ml of antibiotic in broth, 0.4 ml of bacterial inoculum in broth, and 0.8 ml of serum diluted in broth. The bacterial inoculum contained approximately  $5 \times 10^8$  organisms. The minimal inhibitory concentration (MIC) was read after overnight incubation at  $37^{\circ}\text{C}$  as the lowest concentration of antibiotic that inhibited visible growth. All tests were run in duplicate and were read "blindly" by one of us.

Studies on the kinetics of the interaction between serum and other reagents on the concentration of antibiotics were carried out as described (5, 6). Tubes were made up of 0.4 ml of antibiotic in broth, 0.4 ml of bacterial inoculum in broth, and 0.8 ml of other reagents in broth. Antibiotic, bacteria, and other reagents were mixed at time 0 and incubation was begun at  $37^{\circ}\text{C}$  in a shaker-water bath. At intervals, samples of 0.1 ml were taken and streaked on the surface of the agar plates. The plates were incubated overnight and the colonies were counted.

Four healthy adults were given intramuscular injections of 80 mg of gentamicin sulfate after informed consent was obtained. Several weeks later the same four adults were given intramuscular injections of gentamicin sulfate (80 mg) and carbenicillin (1 g). Serum was collected before the injections and at intervals of 1, 2, 4, and 6 hr thereafter. Specimens of serum for each time interval were pooled sepa-

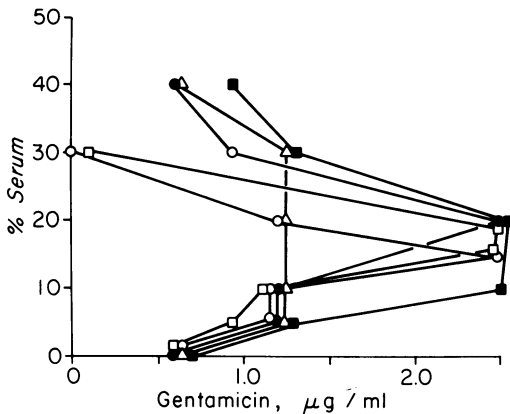


FIG. 1. Isobolograms of the activity of human serum and gentamicin on five strains of *Pseudomonas aeruginosa*.

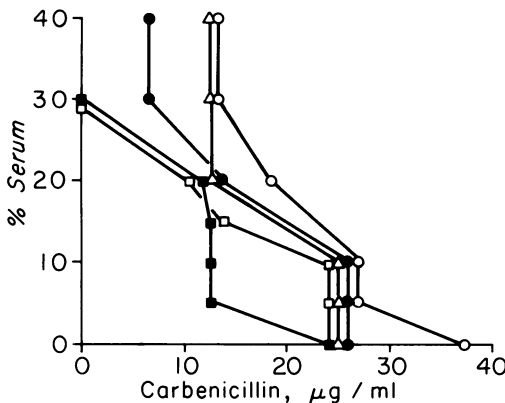


FIG. 2. Isobolograms of the activity of human serum and carbenicillin on five strains of *Pseudomonas aeruginosa*.

rately. Antibacterial activity of the serum pools was determined in tubes containing 1.8 ml of serum and 0.2 ml of bacterial inoculum in broth. The bacterial inoculum contained about  $8 \times 10^8$  viable organisms. Serum and bacterial inoculum were mixed at time 0 and incubated at 37 C in a shaker-water bath. Samples of 0.1 ml were taken at intervals for colony counts. Serum pools were tested for antibacterial activity against strains of *E. coli* and *P. aeruginosa* that had been isolated from blood cultures.

## RESULTS

Human serum was antagonistic to the bactericidal activity of gentamicin in the tube dilution test. Results of studies on five strains of *P. aeruginosa* are presented in Fig. 1 in the form of isobolograms (9, 10). Each point in the figure represents the MIC for gentamicin by the tube dilution test in the indicated concentration of serum. Results for a single strain are

connected by lines. Serum was antagonistic to the activity of gentamicin on all five strains of *P. aeruginosa*.

Results of a similar study on serum and carbenicillin are presented in Fig. 2. Serum was

TABLE 1. Influence of serum,  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and ethylenediaminetetraacetic acid (EDTA) on the bactericidal activity of gentamicin on *Pseudomonas aeruginosa*

Reagents	Colony count at 3 hr <sup>a</sup>
Controls	
Broth only	610
20% Serum	680
$10^{-3}$ M $\text{MgCl}_2$	644
$10^{-3}$ M $\text{CaCl}_2$	631
$2.5 \times 10^{-3}$ M $\text{Na}_2\text{EDTA}$	466
$2.5 \times 10^{-3}$ M $\text{MgEDTA}$	459
$2.5 \times 10^{-3}$ M $\text{CaEDTA}$	518
Gentamicin (0.63 µg/ml)	65
Gentamicin + 20% serum	683
Gentamicin + $10^{-3}$ M $\text{CaCl}_2$	485
Gentamicin + $10^{-3}$ M $\text{MgCl}_2$	143
Gentamicin + $2.5 \times 10^{-3}$ M $\text{Na}_2\text{EDTA}$	2
Gentamicin + $2.5 \times 10^{-3}$ M $\text{MgEDTA}$	21
Gentamicin + $2.5 \times 10^{-3}$ M $\text{CaEDTA}$	74
Gentamicin + 20% serum + $2.5 \times 10^{-3}$ M $\text{MgEDTA}$	112
Gentamicin + 20% serum + $2.5 \times 10^{-3}$ M $\text{CaEDTA}$	629

<sup>a</sup> Mean colony count at time 0 was  $285 \pm 31$  (Mean  $\pm$  1 SD).

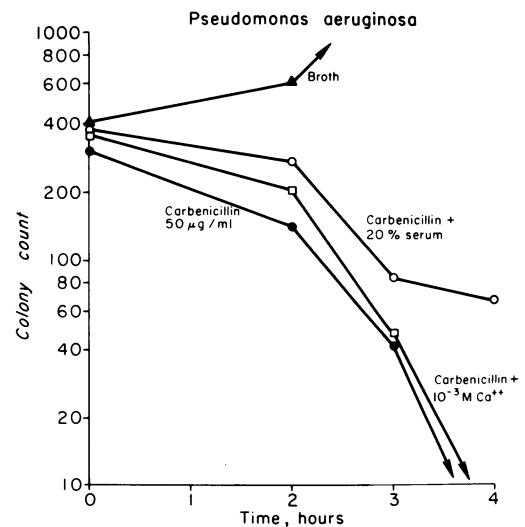


FIG. 3. Kinetics of the bactericidal activity of carbenicillin on *Pseudomonas aeruginosa* in broth alone, in broth with 20% human serum, and in broth with  $10^{-3}$  M  $\text{CaCl}_2$  added.

not antagonistic to carbenicillin.

Kinetic studies were carried out in broth on the effect of serum and other reagents on the bactericidal activity of gentamicin on *P. aeruginosa* (Table 1). Gentamicin was antagonized by the addition of serum, calcium, or magnesium. The activity of gentamicin was enhanced by the addition of disodiummethylenediaminetetraacetic acid ( $\text{Na}_2\text{EDTA}$ ) and  $\text{MgEDTA}$ . Finally, the antagonistic effect of serum on gentamicin was reversed by the addition of  $\text{MgEDTA}$ , but not  $\text{CaEDTA}$ .

Similar studies were carried out on carbenicillin (Fig. 3). The bactericidal activity of carbenicillin was slightly delayed in the presence of 20% serum but was unchanged by the addition of calcium. Neither serum nor calcium was consistently antagonistic when this experiment was repeated several times.

The antibacterial activity of serum from volunteers given gentamicin was studied. The results on *E. coli* are shown in Fig. 4. The serum specimens obtained 1 and 2 hr after the injection of gentamicin had significantly enhanced bactericidal activity as compared with the preinjection specimen. Results of studies using the same serum specimens on *P. aeruginosa* are shown in Fig. 5. The 1-hr serum pool was bactericidal. The 2- and 4-hr serum pools had less activity.

The simultaneous administration of 1 g of carbenicillin with 80 mg of gentamicin produced little change in the bactericidal activity of the serum pools on *E. coli*. The results presented in Fig. 6 should be compared with those in Fig. 4. However, the same serum specimens had considerably more activity against *P. aeruginosa* (compare Fig. 7 with Fig. 5). The bactericidal activity was much greater in the serum specimens collected 1, 2, and 4 hr after injection of the antibiotics. Also, detectable activity was present in the 6-hr specimen. The enhanced activity may well reflect the previously reported synergism between gentamicin and carbenicillin on *P. aeruginosa* (11, 14).

### DISCUSSION

The studies presented demonstrate that calcium antagonizes the activity of gentamicin, but not carbenicillin, on *P. aeruginosa* in vitro. At physiologic concentrations, calcium was less antagonistic to gentamicin than to colistin (5, 6). After injection of therapeutic doses of these agents, serum from volunteers given gentamicin was bactericidal to *P. aeruginosa*, but serum from volunteers given colistimethate

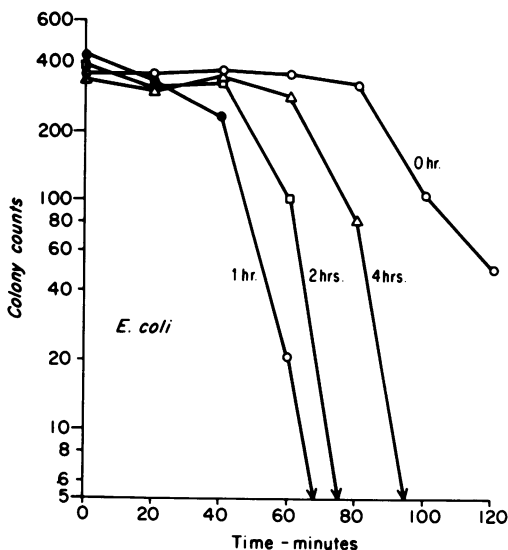


FIG. 4. Bactericidal activity of human serum pools on *Escherichia coli*. The sera were collected before and at various intervals after intramuscular injection of 80 mg of gentamicin into adult volunteers.

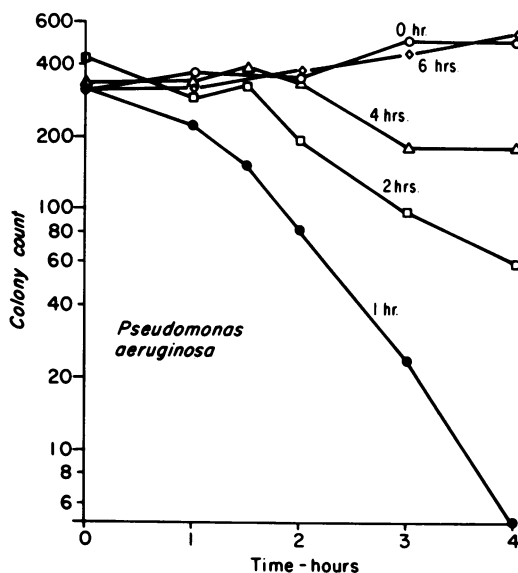


FIG. 5. Bactericidal activity of human serum pools on *Pseudomonas aeruginosa*. Sera were collected before and at intervals after injection of 80 mg of gentamicin.

was not (5). On the basis of these in vitro studies, it might be predicted that gentamicin would be more active in vivo than colistin. On the other hand, gentamicin may be less active in vivo than one would predict on the basis of

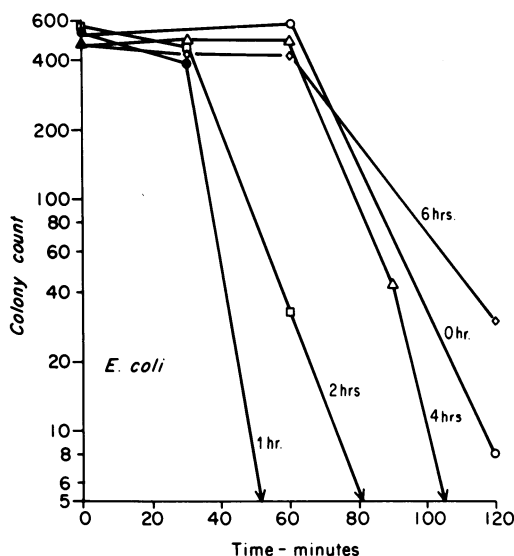


FIG. 6. Bactericidal activity of human serum pools on *Escherichia coli*. Volunteers were given 80 mg of gentamicin and 1 g of carbenicillin.

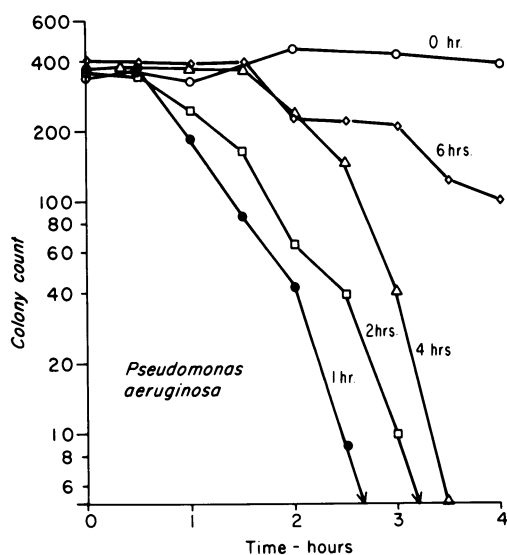


FIG. 7. Bactericidal activity of human serum pools on *Pseudomonas aeruginosa*. Volunteers were given 80 mg of gentamicin and 1 g of carbenicillin.

standard in vitro tests which do not take into account the antagonism by calcium.

It is difficult to determine whether the antagonism observed in vitro has any significance in experimental animals or man. Few studies have been reported comparing gentamicin, colistimethate, and carbenicillin in experimental *Pseudomonas* infections.

Hepding found that gentamicin was more effective than sodium colistimethate or polymyxin B in the treatment of experimental *Pseudomonas* infections of mice (8). A small study on one strain of *P. aeruginosa* concluded that polymyxin B and gentamicin had similar efficacy in experimental *Pseudomonas* infections (2). Controlled studies in man apparently have not been reported.

Chelation by EDTA blocked the antagonistic effect of calcium in serum. It has been shown previously that EDTA may directly attack organisms by chelating cations in the cell wall (3, 4, 12). It may be possible to enhance the activity of gentamicin or colistin in the treatment of local infections by the simultaneous administration of a chelating agent. This inference supports the observation of Wilson that EDTA increased the effectiveness of gentamicin in the treatment of experimental *Pseudomonas keratitis* in rabbits (16). His experimental rationale was that part of the tissue damage resulting from *Pseudomonas* infections was caused by a Mg-dependent proteolytic enzyme and that chelation would inactivate the enzyme. The demonstration here that calcium antagonizes gentamicin provides a second mechanism by which chelation may enhance the activity of gentamicin in *Pseudomonas* infections.

#### ACKNOWLEDGMENTS

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#### LITERATURE CITED

1. Acred, P., D. M. Brown, E. T. Knudsen, G. N. Rolinson, and R. Sutherland. 1967. New semisynthetic penicillin active against *Pseudomonas aeruginosa*. *Nature (London)* **215**:25-30.
2. Acred, P., P. A. Hunter, and G. N. Rolinson. 1970. Carbenicillin: evaluation of antibacterial activity in vivo. *Proc. Sixth Int. Congr. Chemotherapy*, vol. 1, p. 305-308.
3. Asbell, M. A., and R. G. Eagon. 1966. Role of multivalent cations in the organization, structure, and assembly of the cell wall of *Pseudomonas* organisms. *J. Bacteriol.* **92**:380-387.
4. Brown, M. R. W., and J. Melling. 1969. Loss of sensitivity to EDTA by *Pseudomonas aeruginosa* given under conditions of Mg-limitation. *J. Gen. Microbiol.* **54**:439-444.
5. Davis, S. D., A. Iannetta, and R. J. Wedgwood. 1971. Paradoxical synergism and antagonism between serum and the antibacterial activity of colistin. *J. Infect. Dis.* **123**:392-398.
6. Davis, S. D., A. Iannetta, and R. J. Wedgwood. 1971. Activity of colistin against *Pseudomonas aeruginosa*. Inhibition by calcium. *J. Infect. Dis.* **124**:610-612.
7. Fisher, M. W., H. B. Devling, and F. J. Ghabasik. 1969. New immunotype schema for *Pseudomonas aerugi-*

- nosa* based on protective antigens. *J. Bacteriol.* **98**:835-836.
8. Hepding, L. 1967. Zur Chemotherapie experimenteller *Pseudomonas*-Infektionen. Proc. Fifth Int. Congr. Chemotherapy, vol. 1, p. 215-220.
  9. Lacey, B. W. 1958. Mechanisms of chemotherapeutic synergy. Symp. Soc. Gen. Microbiol. **8**:247-287.
  10. Loewe, S. 1953. The problem of synergism and antagonism of combined drugs. *Arzneimittel-forschung* **3**:285-290.
  11. Mouton, R. P., and B. Holtrigter. 1969. Carbenicillin and gentamicin in antibiotic combinations in vitro. *Chemotherapy* **14**:371-383.
  12. Reybrouch, G., and H. van de Voorde, 1969. Effects of ethylenediaminetetraacetic on the germicidal action of disinfectants against *Pseudomonas aeruginosa*. *Acta Clin. Belg.* **24**:32-41.
  13. Smith, C. B., and M. Finland. 1968. Carbenicillin: activity in vitro and absorption and excretion in normal young men. *Appl. Microbiol.* **16**:1753-1760.
  14. Sonne, M., and E. Jawetz. 1969. Combined action of carbenicillin and gentamicin on *Pseudomonas aeruginosa* in vitro. *Appl. Microbiol.* **17**:893-896.
  15. Rubenis, M., M. Kozig, and G. G. Jackson. 1964. Laboratory studies on gentamicin. *Antimicrob. Ag. Chemother.* 1963, p. 153-156.
  16. Wilson, L. A. 1970. Chelation in experimental *Pseudomonas keratitis*. *Brit. J. Ophthalmol.* **54**:587-593.