APPLIED MICROBIOLOGY, Sept. 1969, p. 299-302

Vol. 18, No. 3
Printed in U.S.A.

# Resistance of *Pseudomonas* to Quaternary Ammonium Compounds

## I. Growth in Benzalkonium Chloride Solution

FRANK W. ADAIR, SAM G. GEFTIC, AND JUSTUS GELZER
*Research Department, Ciba Pharmaceutical Company, Summit, New Jersey 07901*

Received for publication 19 June 1969

Resistant cells of *Pseudomonas aeruginosa* and a waterborne *Pseudomonas* sp. (strain Z-R) were able to multiply in nitrogen-free minimal salts solution containing various concentrations of commercially prepared, ammonium acetate-buffered benzalkonium chloride (CBC), a potent antimicrobial agent. As the CBC concentration increased, growth increased until a point was reached at which the extent of growth leveled off or was completely depressed. Minimal salts solutions of pure benzalkonium chloride (PBC) containing no ammonium acetate did not support bacterial growth. When ammonium acetate was added to PBC solutions in the same concentrations found in CBC solutions, growth patterns developed that were comparable to those found with CBC. Likewise, $(NH_4)_2SO_4$ added to PBC solutions supported growth of both organisms. *P. aeruginosa* was initially resistant to CBC levels of 0.02% and it was adapted to tolerate levels as high as 0.36%. Strain Z-R was naturally resistant to 0.4% CBC. Since ammonium acetate, carried over by the CBC used in drug formulations and disinfectant solutions, has the potential to support the growth of resistant bacteria and thus make possible the risk of serious infection, it is suggested that regulations allowing the presence of ammonium acetate in CBC solution be reconsidered.

Benzalkonium chloride (a mixture of alkyldimethylbenzylammonium chlorides) is a surface-active quaternary ammonium compound. It is widely used as an antimicrobial preservative in pharmaceuticals and in clinical medicine to disinfect thermolabile material. Bacteria resistant to benzalkonium chloride may become a serious health hazard if accidentally introduced during the use of a drug or disinfectant solutions containing this antimicrobial agent. This is especially true if the particular preparation can provide a suitable substrate for growth.

During a comprehensive study on the resistance of bacteria to quaternary ammonium compounds, a strain of *Pseudomonas aeruginosa* and a water-borne *Pseudomonas* sp. were found to not only survive but also to multiply in salt solutions containing commercial benzalkonium chloride (CBC). This study explains this occurrence and discusses its practical importance.

## MATERIALS AND METHODS

**Organisms.** *P. aeruginosa* was ATCC strain 9027. The waterborne organism (hereafter referred to as strain Z-R) was originally isolated from well water; it possessed a single polar flagellum and compared exactly with the description of *P. fluorescens* (1), except that no pigment was produced.

**CBC.** CBC refers to commercially prepared benzalkonium chloride and ammonium acetate in aqueous solution meeting the requirements of U.S. Pharmacopeia (USP) XVII. Ammonium acetate is allowed as a buffer in such solutions at a level of no more than 40% of the final concentration of CBC. The CBC solutions used in these experiments contained ammonium acetate at a concentration of 38% of the weight of CBC. This type of preparation is registered under various trade names and is commonly used as an antimicrobial preservative in pharmaceutical products and as a disinfectant for medical and surgical materials.

Aqueous solutions of pure benzalkonium chloride (PBC) also met the requirements of USP XVII and differed from CBC solutions only in that they did not contain ammonium acetate.

**Media.** *P. aeruginosa* and strain Z-R were continually subcultured in a sterile minimal salts-CBC medium composed of the following (per liter of triple distilled water): NaCl, 3.31 g; $Na_2HPO_4$, 2.58 g; KCl, 2.23 g; $KH_2PO_4$, 7.42 g; and CBC, 0.1% (v/v). Sterilization was achieved by filtration through a 0.2-$\mu$m membrane (Millipore Corp., Bedford, Mass.). If increased growth yields were required, sterile glucose in a final concentration of 0.5% (w/v) was added to the minimal medium along with the following

(per liter): $CaCl_2 \cdot 2H_2O$, 0.005 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; and $(NH_4)_2SO_4$, 1.0 g. Using this glucose-expanded salts medium, PBC (0.1%, v/v) could be substituted for CBC.

**Growth of cultures.** Cultures were grown in 250-ml Erlenmeyer flasks containing 50 ml of medium or in test tubes (13 by 150 mm) containing 10 ml of medium. Incubation was stationary at 30 C. Growth was measured turbidimetrically by use of a Spectronic-20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.) set at 545 nm.

**Preliminary test for the selection of CBC-resistant bacteria.** Bacterial isolates (including strain Z-R) that were to be tested for natural resistance to CBC were grown in tryptone soy broth (TSB); they were then centrifuged, washed several times, and suspended in minimal salts medium. The suspensions were adjusted turbidimetrically to contain about $10^8$ cells/ml; 1-ml samples were used to inoculate tubes containing 9 ml of a minimal salts-CBC test solution. CBC was present at a final concentration of 0.02%. This concentration was selected on the basis of it being equal to or higher than the level of CBC considered to be highly effective against a wide range of gram-positive and gram-negative bacteria (3, 4, 7). After incubation for 24 hr at 30 C, 1-ml portions were withdrawn from the test solutions and transferred into 9-ml volumes of sterile TSB. If growth occurred in any of these TSB cultures, the organisms present were considered to have a degree of resistance sufficient to make them suitable for further study.

**Development of CBC resistance in P. aeruginosa.** *P. aeruginosa* was examined for its initial level of resistance to CBC by inoculating 0.1% glucose-expanded salts media containing increasing concentrations of the compound. The organism was found to grow consistently in concentrations of CBC up to 0.02%. Higher levels of resistance were successfully attained by inoculating solutions containing increasing concentrations of CBC in 0.1% glucose-salts medium with cells (at least $10^8$/ml) from the highest CBC culture showing growth. The concentration of CBC was increased in increments of 0.0025%. In this manner, the organism acquired a tolerance to 0.36% CBC. Since growth at this CBC concentration tended to be weak, cells to be used in different tests were grown routinely in 0.1% CBC or PBC.

## RESULTS

In the preliminary test for resistant isolates, strain Z-R remained viable after 24 hr in the 0.02% CBC-salts test solution, as indicated by growth upon transfer in TSB. It was noted, however, that after 72 hr the test solution itself became turbid. Examination under a phase microscope revealed actively dividing cells, suggesting that the turbidity was due to growth of the organism. Cells from this test solution were centrifuged, washed three times, and suspended in minimal salts solution. Samples of 0.1 ml (about $10^7$ cells) were used to inoculate test tubes containing 9.9 ml of minimal salts solution and increasing concentrations of three CBC preparations. Optical density readings were taken after 14 days when increases in turbidity had ceased. As is illustrated in Fig. 1A, the CBC-salts solutions supported the growth of strain Z-R (Fig. 1A), whereas no growth occurred upon replacement of CBC by PBC. However, when ammonium acetate was added to the PBC-salts solutions in the same concentrations

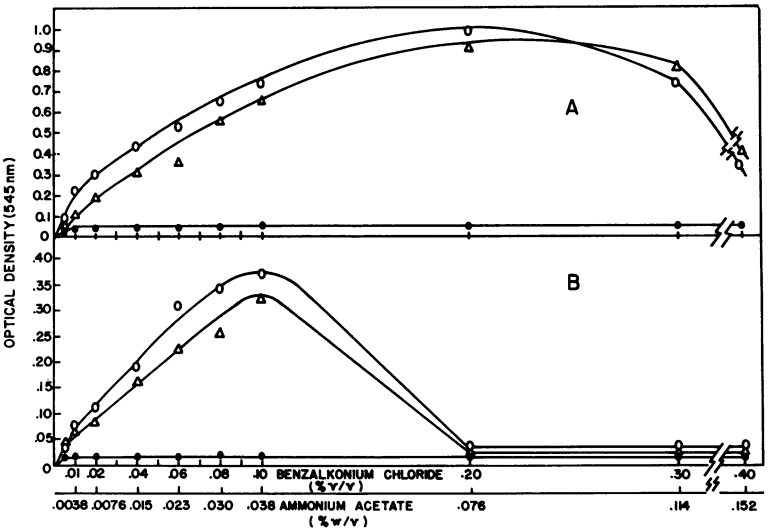


FIG. 1. *Growth of strain Z-R and P. aeruginosa in minimal salts solutions containing increasing concentrations of three types of benzalkonium chloride preparations. Symbols:* ○, *commercial benzalkonium chloride containing ammonium acetate;* ●, *pure benzalkonium chloride (PBC) containing no ammonium acetate;* △, *PBC plus added ammonium acetate. (A) Strain Z-R; (B) P. aeruginosa.*

TABLE 1. *Effect of replacing ammonium acetate with either ammonium sulfate or sodium acetate on the growth of strain Z-R and P. aeruginosa in PBC-minimal salts solution*[a]

| Addition to minimal salts solution[b] | Growth[c] | |
|---|---|---|
| | Strain Z-R | *P. aeruginosa* |
| None | − | − |
| Pure benzalkonium chloride (PBC) | − | − |
| PBC plus ammonium acetate | + | + |
| PBC plus ammonium sulfate | + | + |
| PBC plus sodium acetate | − | − |
| Ammonium acetate | + | + |
| Ammonium sulfate | − | − |
| Sodium acetate | − | − |

[a] Minimal salts solution was prepared as described in Materials and Methods.

[b] All additions were present at a final concentration of 0.1% (w/v) except for PBC which was 0.1% (v/v).

[c] Growth was read after 7 days of stationary incubation at 30 C.

found in CBC solutions, strain Z-R resumed a comparable growth pattern.

Cells of *P. aeruginosa* grown in 0.1% glucose-expanded salts solution containing 0.1% PBC were washed three times and suspended in minimal salts solution. These cells had not been adapted to PBC concentrations higher than 0.1%. The suspension (0.1 ml, $10^7$ cells) was used to inoculate minimal salts solutions (9.9 ml) containing increasing concentrations of CBC and PBC preparations. Growth was measured after 14 days when the turbidity of the cultures no longer increased. The CBC-salts solutions supported growth of this organism, whereas the PBC-salts solutions did not (Fig. 1B). As in the case of strain Z-R, upon the addition of ammonium acetate to the PBC solutions, growth patterns similar to that obtained with CBC solutions were observed.

In an attempt to gain an initial understanding of the role ammonium acetate played in the nutrition of the two organisms, the experiment shown in Table 1 was devised. It was found that $(NH_4)_2SO_4$, but not sodium acetate, could replace ammonium acetate in the PBC-minimal salts solution and still allow each organism to grow.

## DISCUSSION

The data presented indicate that low levels of ammonium acetate found in CBC solutions will support growth of benzalkonium chloride-resistant *P. aeruginosa* and strain Z-R, a waterborne *Pseudomonas* sp., whereas in solutions of PBC containing no ammonium acetate, no growth occurs. The fact that ammonium acetate could be replaced by $(NH_4)_2SO_4$ in PBC solutions suggests that a portion of the benzalkonium chloride molecule was being utilized for carbon and energy, whereas the strongly bonded nitrogen was not attacked. This, in turn, may lead to a chemical modification of the benzalkonium chloride molecule, possibly resulting in an inactivation of its antimicrobial action. Studies are currently underway in this laboratory to determine in detail the nature of these events.

The shape of the CBC growth curves in Fig. 1 may be attributed to several factors. At lower levels of CBC, growth was presumably limited due to the low levels of ammonium acetate introduced with the CBC. Assuming that the organisms can obtain carbon and energy from some part of the CBC molecule in the presence of ammonium acetate, it appears that the ammonium portion of the ammonium acetate molecule, specifically the nitrogen atom, was the sole growth-limiting factor. Thus, an increase in CBC increased the nitrogen level and growth increased proportionally.

In the case of strain Z-R, a point was reached above the 0.2% CBC level where the extent of growth was suppressed regardless of the ammonium acetate concentration. On the other hand, *P. aeruginosa*, which had been adapted to 0.1% benzalkonium chloride, grew optimally at this concentration but did not survive the increase to 0.2% CBC.

In the formulation of a drug, it is mandatory that as few potential microbial substrates as feasible be added. This decreases the chance of growth of resistant bacteria that may be introduced accidentally during normal use of the drug. The antimicrobial preservative should be the least probable source of bacterial substrate. In many drugs in which CBC is used routinely, the addition of ammonium acetate could be a detriment. For example, ophthalmics are usually composed of few organic compounds other than the active agent and the antimicrobial preservative, which in many instances is CBC. Under these conditions, ammonium acetate would at least enhance the growth of a resistant contaminant and could possibly be the sole supporter of growth. Indeed, contamination of ophthalmic solutions by *P. aeruginosa* and other microorganismsas resulted in numerous cases of serious eye infections (2).

The commonplace clinical practice of using CBC solutions as a means of disinfecting needles, swabs, catheters, and surgical instruments precludes survival, much less growth, of bacteria in such solutions. However, many instances of pseudomonal bacteremia, occasionally fatal, have

resulted from the use of materials stored in contaminated CBC solutions (5, 6, 7). Furthermore, stored, unused solutions of CBC (0.13%) prepared in a hospital pharmacy have been found to be highly contaminated owing to the growth of resistant bacteria (6). Again, ammonium acetate would only serve to greatly worsen these contamination problems.

Strain Z-R was naturally resistant to CBC concentrations of 0.4%. This level is 20-fold greater than the 0.02% recommended for use in pharmaceuticals (3, 7, 8) and twofold greater than the 0.2% recommended for disinfection of medical materials (9). *P. aeruginosa* was initially resistant to 0.02% CBC and was adapted to tolerate concentrations up to 0.36%. Both organisms serve to illustrate the point that commonly occurring bacteria can multiply in the presence of what is considered high concentrations of CBC if given a carbon and nitrogen source. Thus, the potential capacity of the ammonium acetate contained in CBC solutions to support growth of resistant bacteria with the possibility of serious consequences appears to overshadow any value ammonium acetate may have as a buffering agent under the conditions in which it is used. In view of this, it is important that the U.S.P. regulation allowing its presence in CBC be reconsidered.

## LITERATURE CITED

1. Breed, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, p. 105. 7th ed. The Williams & Wilkins Co., Baltimore.
2. Foster, J. H. S. 1965. Preservation of ophthalmic solutions, part I. Mfg. Chemist 36:45–50.
3. Grundy, W. E. 1968. Antimicrobial preservatives in pharmaceuticals, p. 566–574. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization and preservation. Lea and Febiger, Philadelphia.
4. Lawrence, C. A. 1968. Quaternary ammonium surface active disinfectants, p. 430–452. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization and preservation. Lea and Febiger, Philadelphia.
5. Lee, J. C., and P. J. Kialkow. 1961. Benzalkonium chloride—source of hospital infection with gram-negative bacteria. J. Amer. Med. Ass. 177:708–710.
6. Malezia, W. F., E. J. Gangaros, and A. F. Goley. 1960. Benzalkonium chloride as a source of infection. N. Engl. J. Med. 263:800–802.
7. Plotkin, S. A., and R. Austrian. 1958. Bacteremia caused by *Pseudomonas* sp. following the use of materials stored in solutions of a cationic surface active agent. Amer. J. Med. Sci. 235:621–627.
8. Russel, A. D., J. Jenkins, and I. H. Harrison. 1967. The inclusion of antimicrobial agents in pharmaceutical products, p. 1–38. *In* W. W. Umbreit (ed.), Advances in applied microbiology. Academic Press Inc., New York.
9. Spaulding, E. H. 1968. Chemical disinfection of medical and surgical materials, p. 517–531. *In* C. A. Lawrence and S. S. Block (ed.), Disinfection, sterilization and preservation. Lea and Febiger, Philadelphia.