Sensitivity of *Pseudomonas aeruginosa* Envelope Mutants to Alkylbenzyldimethylammonium Chlorides

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Abstract □ A series of stepwise polymyxin-resistant envelope mutants of *Pseudomonas aeruginosa* was used to test the activity of a homologous series (C_{10}–C_{18}) of alkylbenzyldimethylammonium chlorides. A sterilization kinetics procedure in deionized water was devised to avoid amounts of quaternary compound above the critical micelle concentration. In all cases, there was a linear relationship between the logarithm of the rate of change of the colony count with time and the logarithm of the homolog concentration. For all strains, there was a linear relationship between alkyl chain length and the concentration required to reduce the colony count to 10% in 2 hr. The stepwise series of polymyxin-resistant strains increased in resistance to polymyxin about threefold for each step. In general, this increase resulted in a similar increase in resistance to the quaternary compound. It is proposed that death in this system may primarily be a consequence of damage to the outer membrane rather than to the cytoplasmic membrane.

Keyphrases □ Alkylbenzyldimethylammonium chlorides, various—antibacterial activity evaluated in series of *Pseudomonas aeruginosa* envelope mutants □ Antibacterial activity—various quaternary ammonium chlorides evaluated in series of *Pseudomonas aeruginosa* envelope mutants □ Quaternary ammonium chlorides, various—antibacterial activity evaluated in series of *Pseudomonas aeruginosa* envelope mutants □ Structure–activity relationships—various alkylbenzyldimethylammonium chlorides, antibacterial activity evaluated in series of *Pseudomonas aeruginosa* envelope mutants

The envelope of *Pseudomonas aeruginosa* and other Gram-negative bacteria is profoundly implicated in drug resistance (1–3). A series of stepwise polymyxin-resistant envelope mutants of *P. aeruginosa* became resistant to several other chemical agents including benzalkonium chloride (4). In the present study, this model series was used to evaluate the effectiveness of a homologous series of alkylbenzyldimethylammonium chlorides (I). A minimum inhibitory concentration (MIC) test procedure gave rise to artifacts due to colloidal association of I (5). Consequently, a test sensitive enough to avoid amounts of I above the critical micelle concentration (CMC) was developed.

In the absence of colloidal association, linear relationships were found between antipseudomonal activity and lipophilicity of the homologs of I. Thus, the universal validity of the parabolic relationship reported to exist between antibacterial activity and lipophilicity of many quaternary ammonium salts is questionable. These results are discussed in terms of the two membranes present in the envelope of the Gram-negative *P. aeruginosa*. Death in this test system may follow as a consequence of damage to the outer membrane rather than primarily being due to a disorganized cytoplasmic membrane.

Since Domagk (6) observed that alkylbenzyldimethylammonium chlorides were antibacterial, they have become accepted as effective agents in aqueous pharmaceutical formulations. The mode of action of these membrane-active agents was reviewed (7, 8); resistance to them is associated with their exclusion from the target cytoplasmic membrane (1, 9, 10). A literature review (5) suggested that many studies of benzalkonium chloride activity were carried out in a concentration range above the CMC. Consequently, some reported parabolic lipophilicity–activity relationships may be artifacts.

Table I—Results and Storage Procedure for the Selective Training of *P. aeruginosa* to Polymyxin Resistance

Culture[a]	Polymyxin, units/ml	Store
A(W)	last + ve = 7.2	A1
A1	last + ve = 18	A2
A2	last + ve = 48	A3
A3	last + ve = 180	A4
A4	last + ve = 560	A5
A5	last + ve = 2160	A6
A6	last + ve = 6300	A7

[a] Each inoculum was 10^7 cells. A(W) refers to the wild-type *P. aeruginosa* (ATCC 9027). Stores were designated as cultures twice washed by centrifugation in the simple salts medium free of polymyxin, subcultured in this medium, and kept at 20°.

EXPERIMENTAL

To measure the antimicrobial activities of a series of alkylbenzyldimethylammonium chlorides (I) against polymyxin-resistant *P. aeruginosa*, an initial study was performed using an MIC test procedure. Although reproducible end-points could be determined for the wild-type organism and also for organisms having low resistance to polymyxin, it was not possible to obtain end-points for the higher order polymyxin-resistant strains even when extremely high concentrations of the quaternary compound were used. In part, this result was attributed to micelle formation of these surface-active molecules interfering with the system (5), the effect being compounded by the presence of high concentrations of nutrient salts in the test environment. Therefore, a sterilization kinetics procedure was developed in which a salt-free environment existed; it was intrinsically more sensitive to the action of the compounds. Under these circumstances, artifacts in activity of I due to micelle formation were avoided.

Polymyxin B Sulfates—Freeze-dried powder[1] was reconstituted by dissolution in sterile deionized distilled water, stored at 10°, and used within 2 weeks of reconstitution.

Chemically Defined Media—Simple salts (as previously defined) giving oxygen-depleted liquid cultures (5) were used unless otherwise stated.

Organism—*P. aeruginosa* (ATCC 9027), originally obtained as a freeze-dried culture, was resuspended in nutrient broth, subcultured into the chemically defined medium, and stored at 10° on this medium solidified with agar.

Alkylbenzyldimethylammonium Chlorides[2] (I)—Four homologs having alkyl chain lengths of 10, 12, 14, and 18, with their purity as previously described (5), were studied in detail.

Selection of Resistant Variants—To produce a series of strains with a regularly changing and reproducible resistance to polymyxin, a training procedure in the chemically defined medium was used. The method involved a fixed inoculum of 10^7 cells and fixed volumes of medium containing a range of concentrations of polymyxin differing in amount by an arbitrary 20%. The inoculum consisted of an oxygen-depleted culture

[1] Donated by Dr. J. D. Gurney, Wellcome Research Laboratories, Beckenham, Kent, England.

[2] Donated by Dr. R. A. Cutler, Sterling-Winthrop Research Institute, Rensselaer, N.Y.

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Table II—Regression and Correlation Coefficients for Eq. 2[a]

	Level of Resistance to Polymyxin B Sulfate															
	Wild-Type Organism				130 units/ml				500 units/ml				1750 units/ml			
n	*a*	*b*	*N*	*r*	*a*	*b*	*N*	*r*	*a*	*b*	*N*	*r*	*a*	*b*	*N*	*r*
10	2.479	8.785	5	0.992	2.542	9.462	5	0.990	2.170	6.206	4	0.980	2.020	−4.680	5	0.985
12	2.411	9.704	6	0.987	1.665	6.408	4	0.992	1.625	5.092	5	0.973	2.521	7.415	4	0.928
14	2.449	11.244	5	0.989	2.262	10.621	5	0.995	2.295	8.912	5	0.991	1.585	4.927	5	0.984
18	0.786	2.964	6	0.933	0.990	4.335	4	0.948	1.433	6.315	5	0.992	1.734	7.149	5	0.992

[a] The *n* is the carbon number of the alkyl chain of the quaternary ammonium salt, *a* and *b* are the regression coefficients for the expression, and *N* and *r* are the number of data points used and the correlation coefficient, respectively.

of *P. aeruginosa* adjusted to 10^8 cells/ml from optical density measurements with a spectrophotometer[3]. The end-point was selected by incubating the test tubes at 37° for 7 days.

Cells from the tubes containing the highest concentration of polymyxin permitting growth were used as the inocula for the subsequent production of the next resistant step. A sample of such cells was washed twice in the growth medium after centrifugation, grown overnight, and designated as stores; such stores were kept at 20°. Stores were subcultured weekly into fresh medium, and the MIC of polymyxin against the subculture was checked monthly. If cells were needed from a store for subsequent experimentation, they were first incubated by shaking at 37° for 18 hr in the medium containing the maximum concentration of polymyxin in which the stored cells were known to show growth. For some tests, the polymyxin-resistant cells were not stored but utilized immediately subsequent to emergence.

Sterilization Kinetics Procedure—A sterilization kinetics test system in which there can be no significant alteration of the thermodynamic activity of the quaternary ammonium compounds by the growth nutrient salts was developed. Bacteria were harvested from an agar slope, added to 25 ml of a liquid medium, and shaken overnight at 37°. A 0.5-ml aliquot of this stock culture then was added to a further 25 ml of the medium, which was shaken for 18 hr at 37°. A portion of this final culture was allowed to cool to room temperature and spun at 3000 rpm for 5 min. The cells were washed sequentially in media of strengths 100, 80, 50, and 20% and then washed twice in deionized water. This procedure avoided damage by osmotic shock (11).

The optical density of this aqueous suspension was measured, and the cell concentration was adjusted to 1.5×10^5 cells/ml with deionized water. This final suspension was slowly warmed to 37°, and 0.5 ml was added to 24.5-ml sterile aqueous concentrations of I under test such that an initial nominal cell count of 3×10^3/ml was achieved. Studies were carried out with stirred suspensions at 37°. After various times of contact of bacteria with the quaternary ammonium salt, a 0.5-ml aliquot was removed from the test system and added to 2.0 ml of polysorbate 80–lecithin broth to inactivate I (12). After a further 5 min, 0.5-ml aliquots were spread on the surface of three overdried nutrient agar plates. The plates were then incubated at 37° for approximately 16 hr, and the number of colonies was counted.

Additional experimentation was carried out to determine whether the initial transfer of the overnight culture into an eventual deionized water environment (11) and the transfer into the inactivation media significantly affected subsequent viable counts. In both cases, no effect could be observed. The initial incubation procedure ensured that cell clumping did not occur, as shown by microscopic examination. During the test procedure, cells were added to a system free of I; if the viable count of this suspension altered significantly during the time of the test, the experiment on that cell culture was discontinued.

To test that *P. aeruginosa* (ATCC 9027) remained constant in its sensitivity to the quaternary ammonium salts, an MIC procedure with dodecylbenzyldimethylammonium chloride was performed periodically. The sensitivity of the test organism remained constant. Of the series of stepwise resistant pseudomonads produced, three strains were able to grow in the medium with added polymyxin at levels of 130, 500, and 1750 units/ml. The four alkylbenzyldimethylammonium chlorides were examined at different concentration levels against the wild type and polymyxin-resistant organisms.

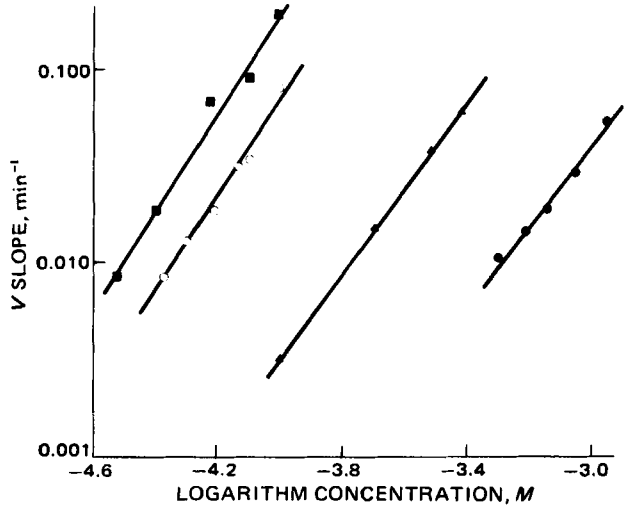

Figure 1—*Effectiveness of various concentrations of decylbenzyldimethylammonium chloride against polymyxin-resistant* P. aeruginosa. *The* V *slope refers to the rate of decrease in the viable count with time. Key:* ■, *wild-type organism;* ○, *organism resistant to 130 units of polymyxin/ml;* ▲, *organism resistant to 500 units of polymyxin/ml; and* ●, *organism resistant to 1750 units of polymyxin/ml.*

[3] Pye Unicam SP 600, Cambridge, England.

RESULTS AND DISCUSSION

By selective training of *P. aeruginosa* using aqueous solutions of polymyxin B sulfate in simple salts medium, several series of stepwise resistant pseudomonads were produced. Table I shows the results obtained for a typical series. There was an approximately threefold increase in the resistance to polymyxin with each step.

With the sterilization kinetics procedure, death-time curves were obtained for all homologs. The relationship between viable count and time was exponential (5) and given by:

$$V_t/V_0 = e^{-kt} \qquad \text{(Eq. 1)}$$

where V_0 and V_t refer to the number of viable bacteria in the reaction vessel at time zero and time t, respectively, and k is a constant for the

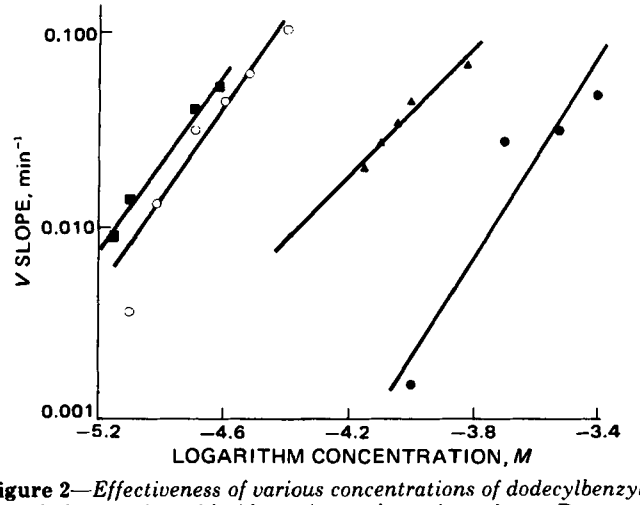

Figure 2—*Effectiveness of various concentrations of dodecylbenzyldimethylammonium chloride against polymyxin-resistant* P. aeruginosa. *Key: same as Fig. 1.*

Table III—Computed Molarity of Quaternary Homologs Required to Reduce the Viable Count to 10% of the Original in 2 hr [a]

	Level of Resistance to Polymyxin B Sulfate			
n	Wild Type	130 units/ml	500 units/ml	1750 units/ml
10	4.139×10^{-5}	2.884×10^{-5}	1.523×10^{-4}	4.506×10^{-4}
12	1.298×10^{-5}	7.998×10^{-6}	3.871×10^{-5}	1.713×10^{-4}
14	3.625×10^{-6}	2.429×10^{-6}	1.625×10^{-5}	3.804×10^{-5}
18	3.861×10^{-7}	3.337×10^{-7}	1.382×10^{-6}	4.772×10^{-7}

[a] See footnote to Table II.

system depending on the sensitivity of the organism and the concentration of quaternary salt used.

The log of the rates of change of log viable count with time, log (d log V/dt) for each quaternary at each concentration level, and against all organisms was derived using least-squares regression according to the expression:

$$\log (d \log V/dt) = a \log (\text{quaternary concentration}) + b \quad \text{(Eq. 2)}$$

The end-point of activity for I in the sterilization kinetics test was that concentration of quaternary producing a reduction in viable count to 10% of the original in 2 hr. All derived regression and correlation coefficients are given in Table II. All correlations obtained were significant at the $\alpha = 0.001$ level.

Figures 1–4 show those relationships existing between antimicrobial effectiveness of the four homologs against each organism studied and at each concentration of quaternary. In all cases, there was a linear relationship between the logarithm of the concentration and the logarithm of the rates of change of colony count with time. Table III gives the computed 2-hr end-point for all compounds against all organisms. The dose–response curves (Figs. 1–4) were generally parallel for each quaternary compound, suggesting a similar mode of action against each resistant strain (5).

Figure 5 demonstrates the relationship between the chain length of the I homologs and the concentrations calculated to produce the required end-point. There was a straight-line relationship between alkyl chain length and activity.

Relationships between the levels of resistance to polymyxin and the levels of resistance to the quaternaries were presented graphically. Figure 6 shows the results for tetradecylbenzyldimethylammonium chloride, using a linear–linear scale. Figure 7 shows the comparative resistance levels of all homologs toward all organisms studied, using the 2-hr end-point and analysis on a log–log basis. In all cases, the strain trained to be resistant to a maximum of 130 units of polymyxin/ml was the most sensitive to the quaternary compounds. An increase in polymyxin resistance above this value, however, increased resistance to quaternary action. The increase in sensitivity of the 130-units/ml strain over the wild type is shown more markedly on the log–log plot due to scaling factors. The pattern of sensitivity of this series to both drugs is a consequence of changes in envelope structure (3, 4, 13).

Portions of the plots (Fig. 7) having positive slopes show that approximately four times the amount of each quaternary was necessary for each step in resistance to each strain of *P. aeruginosa*, although this value varied both between chain lengths and between polymyxin resistance levels. The value of this increase in quaternary concentration necessary to be effective against higher order polymyxin-resistant strains is useful in comparing pseudomonal resistance to both chemical types. The three resistant organisms were between 3.5 and 3.8 times more resistant to polymyxin than their next step variant, which is similar in magnitude to the value obtained when considering the alkylbenzyldimethylammonium chlorides.

The rate at which low molecular weight organic nonelectrolytes diffuse across biological membranes correlates with their lipid–water partition coefficient within limits. Lien *et al.* (14) studied a large range of mainly membrane-active antibacterial agents for activity against Gram-positive and Gram-negative bacteria. The ideal lipophilic character (log P_0) for a set of congeneric drugs was much higher for Gram-positive bacteria (about 6) than for Gram-negative bacteria (about 4) (14). This difference was attributed to the relatively lipid-rich wall of Gram-negative bacteria that retained lipophilic drugs and hindered penetration. However, colloidal association may have contributed to the interpretation of these

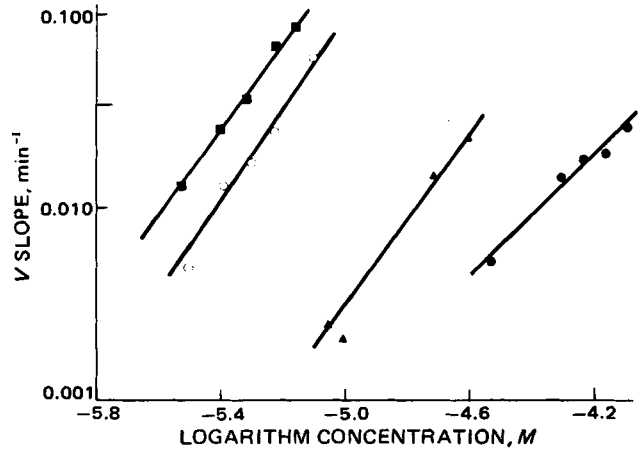

Figure 3—*Effectiveness of various concentrations of tetradecylbenzyldimethylammonium chloride against polymyxin-resistant* P. aeruginosa. *Key: same as Fig. 1.*

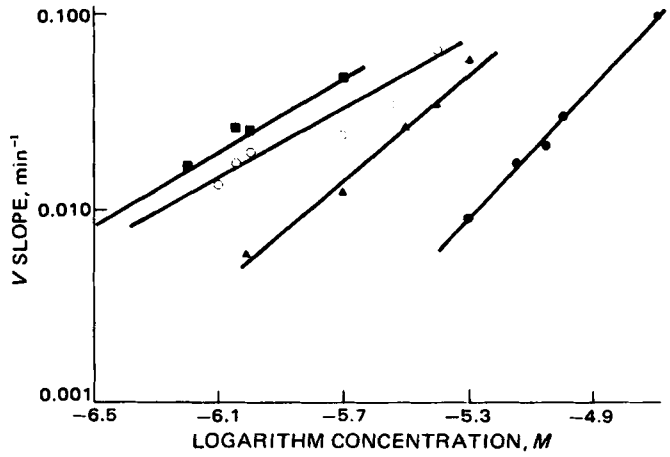

Figure 4—*Effectiveness of various concentrations of octadecylbenzyldimethylammonium chloride against polymyxin-resistant* P. aeruginosa. *Key: same as Fig. 1.*

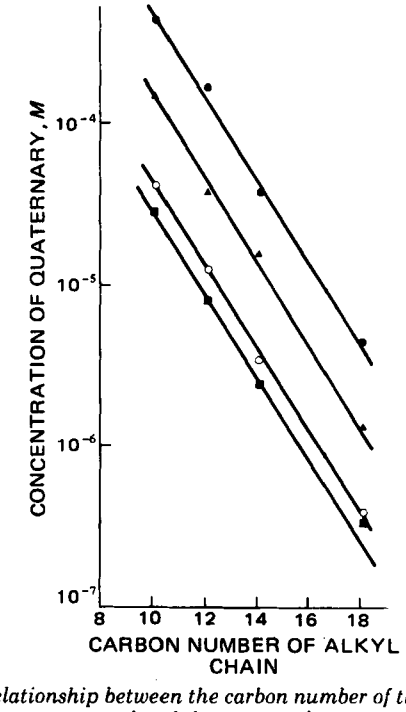

Figure 5—*Relationship between the carbon number of the alkyl chain of the quaternary compound and the computed concentrations required to reduce the viable count of different polymyxin-resistant strains to 10% of the original in 2 hr. Key: same as Fig. 1.*

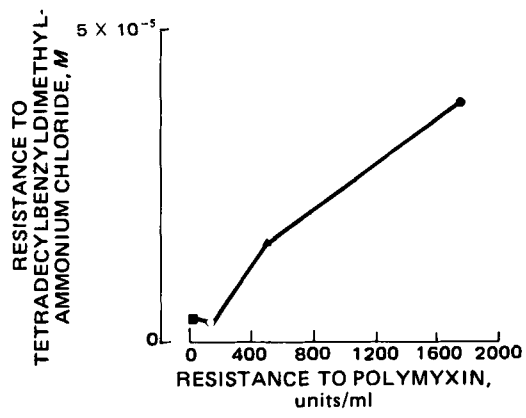

Figure 6—*Relationship between levels of resistance to polymyxin and levels of resistance to tetradecylbenzyldimethylammonium chloride for the various pseudomonads studied. Key: same as Fig. 1.*

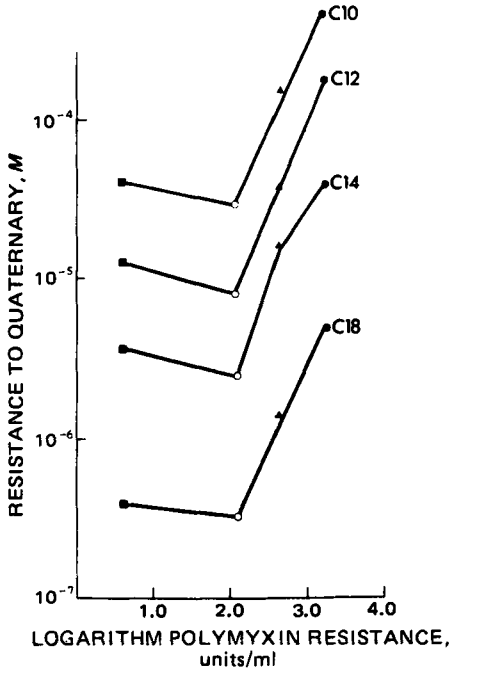

Figure 7—*Composite diagram showing relationships between levels of resistance to polymyxin and to all homologs studied. Key: same as Fig. 1.*

data (5). Furthermore, the outer membrane of Gram-negative bacteria is not a simple lipid barrier (1, 2) and may possibly have water-filled pores lined with protein as an aid to penetration by hydrophilic molecules (3, 15).

The linear relationship between carbon number and antibacterial activity (Fig. 5) is interesting. Probably the differential possibilities of uptake by hydrophobic outer membrane sites, diffusion through outer membrane pores (I, mol. wt. 276–388), and micelle formation in the periplasmic space result in a nonlinear carbon number–activity relationship for a primary effect on the cytoplasmic membrane. The results are consistent with the hypothesis that bacterial death was a consequence of quaternary action primarily at the outer membrane. Under these circumstances, increasing lipophilicity would be associated with increased outer membrane disruption without the drawback of retention by a prior lipid site. These effects tend to be enhanced by this hypotonic test system. A similar lethal outer membrane effect was proposed for polymyxin action (16).

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