Electron Microscope Study of Effect of Benzalkonium Chloride and Edetate Disodium on Cell Envelope of *Pseudomonas aeruginosa*

R. M. E. RICHARDS *x and R. H. CAVILL ‡

Abstract □ Electron micrographs of *Pseudomonas aeruginosa* grown in nutrient broth or broth containing subinhibitory concentrations of benzalkonium chloride indicate that benzalkonium chloride at 50 and 100 μg/ml strips off the outer cell membrane. Cells grown in the presence of edetate disodium, 50–100 μg/ml, had convoluted surfaces (wavy in cross section). Cells damaged by growing on nutrient agar containing benzalkonium (200 μg/ml), when subsequently grown for 16 hr in nutrient broth, produced cells with apparently normal outer layers. Similar cells grown on nutrient agar plus benzalkonium (500 μg/ml) when grown for 16 hr in nutrient broth had normal resistance to edetate disodium lysis, but cells grown overnight in broth plus benzalkonium (500 μg/ml) showed increased sensitivity to edetate disodium lysis. Cells grown on nutrient agar in the presence of benzalkonium (800 μg/ml) grew in broth plus benzalkonium (10 μg/ml) without stripping of their external membrane, but replicate inocula into broth plus benzalkonium (10 μg/ml) and edetate disodium (50 μg/ml) produced cells with structural damage to the outer layers of the cell envelope.

Keyphrases □ *Pseudomonas aeruginosa*—cell envelope, effect of benzalkonium chloride and edetate disodium, electron microscope study □ Benzalkonium chloride—effect on cell envelope of *Pseudomonas aeruginosa*, electron microscope study □ Edetate disodium—effect on cell envelope of *Pseudomonas aeruginosa*, electron microscopy study □ Antibacterial agents—benzalkonium chloride, effect on cell envelope of *Pseudomonas aeruginosa*, electron microscope study □ Chelating agents—edetate disodium, effect on cell envelope of *Pseudomonas aeruginosa*, electron microscope study

Surprisingly little is known about the mode of antibacterial action of quaternary ammonium compounds, despite many publications concerning these substances since their introduction (1). The normal structure of bacteria and fungi appears to be unaffected by quaternary ammonium compounds and other surfactants (2). Several studies (3, 4) found that quaternary ammonium compounds caused a release of cellular constituents, so antibacterial activity was linked, at least in part, to this effect. Evidence was presented (5) of cytological damage induced by low doses (<100 μg/ml) of quaternary ammonium compounds, and this damage was related to cell death. A high dose (900 μg/ml) stripped off the cell walls of *Escherichia coli* and *Staphylococcus aureus*. Another suggested mode of action was the splitting of lipoprotein complexes with a resultant widespread action of autolytic enzymes throughout the cell (6). Other concepts relevant to the mode of action of quaternary compounds against microorganisms were reviewed (7).

Edetate disodium reversed the resistance of *Pseudomonas aeruginosa* cells which were resistant to a quaternary ammonium compound (8). It was postulated that the *P. aeruginosa* had become impermeable to the quaternary ammonium compounds due to an increased lipid content and that the edetate disodium enabled the quaternary compound to penetrate this barrier and then reach its site of antibacterial action.

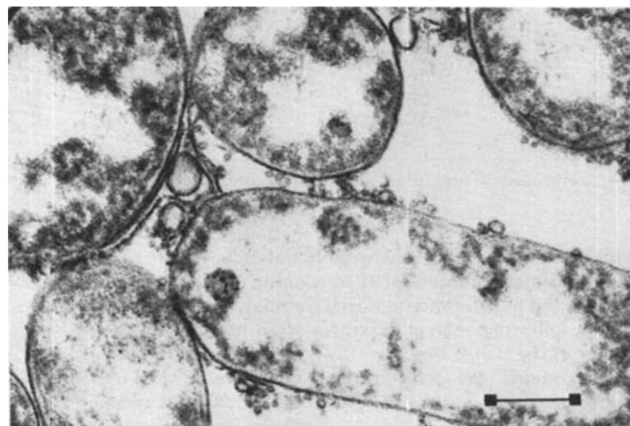

Figure 1—P. aeruginosa *cultured in broth containing benzalkonium chloride (50 μg/ml). (Bar represents 0.25 μm.)*

In another study (9), edetate disodium enhanced the activity of benzalkonium chloride, chlorhexidine acetate, and polymyxin B sulfate on growing cultures of *P. aeruginosa*. It also was demonstrated that quaternary ammonium–edetate disodium combinations were effective against *P. aeruginosa* sensitive and resistant to the quaternary ammonium compounds (10, 11).

The objective of this present investigation was to determine the usefulness of electron microscopy in evaluating the effect on cell morphology of benzalkonium chloride and edetate disodium alone and in combination. Cultures of *P. aeruginosa* growing in the presence of subinhibitory concentrations of the chemicals were used.

EXPERIMENTAL

Chemicals, Media, and Organism—Benzalkonium chloride[1] USP [alkyl (C_{14}, 50%; C_{12}, 40%; and C_{16}, 10%) dimethylbenzylammonium chlorides] and edetate disodium[2] BP (disodium ethylenediaminetetraacetate) were used. *P. aeruginosa*[3] was the test organism, nutrient broth[4] was the growth medium for liquid cultures, and nutrient agar[4] was the growth medium for solid cultures.

A spectrophotometer[5] was used to determine extinction measurements of cell suspensions at 660 nm.

Preparation of Cultures—Maintenance of stock cultures was described previously (12). One-milliliter quantities of an overnight culture of *P. aeruginosa* were used to inoculate 250-ml conical flasks containing 100 ml of nutrient broth and 100 ml of medium

[1] Rohm and Haas, New Germany, Natal, South Africa.
[2] May and Baker Ltd., Dagenham, England.
[3] NCTC 6750, National Collection of Type Cultures, Colindale, London, England.
[4] Oxoid, Oxo Ltd., London, England.
[5] Unicam SP 500, Pye Unicam Ltd., Cambridge, England.

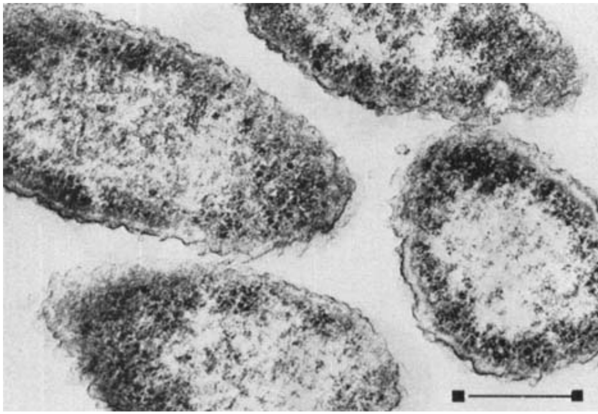

Figure 2—P. aeruginosa *cultured in broth containing edetate disodium (100 μg/ml). (Bar represents 0.25 μm.)*

containing various subinhibitory concentrations of benzalkonium chloride and/or edetate disodium. The cultures were incubated overnight (except for one set of benzalkonium-resistant cultures) at 37° in a shaking incubator[6] at 100 throws/min. Two different attempts were made to produce cultures having increased resistance to benzalkonium chloride.

On Agar—An overnight culture was spread on agar plates containing benzalkonium chloride at 100, 150, 200, and 400 μg/ml. The highest concentration showing growth was 150 μg/ml, which had three colonies. By using one of these colonies as the inoculum source, the procedure was repeated and then repeated again to obtain growth on agar containing 800 μg of benzalkonium chloride/ml.

In Broth—An overnight culture of *P. aeruginosa* was used to inoculate 100 ml of broth containing 50 μg of benzalkonium chloride/ml. The culture was incubated overnight in the shaking water bath and then used to inoculate a further 100 ml of broth containing benzalkonium (50 μg/ml). This procedure was followed six times.

The effect of low concentrations of benzalkonium (50 μg/ml or less) on cells having increased resistance to benzalkonium was investigated as follows:

1. A colony growing on nutrient agar containing benzalkonium chloride (200 μg/ml) was mixed with 2 ml of broth, and 0.4-ml quantities of this suspension were used to inoculate 100 ml of plain nutrient broth and 100 ml of broth containing benzalkonium (50 μg/ml).
2. Cells growing on nutrient agar containing benzalkonium (800 μg/ml) were mixed with 5 ml of nutrient broth, and 0.2-ml quantities were used to inoculate 100-ml quantities of plain nutrient broth, broth containing benzalkonium (10 μg/ml), and broth containing benzalkonium (10 μg/ml) and edetate disodium (50 μg/ml).

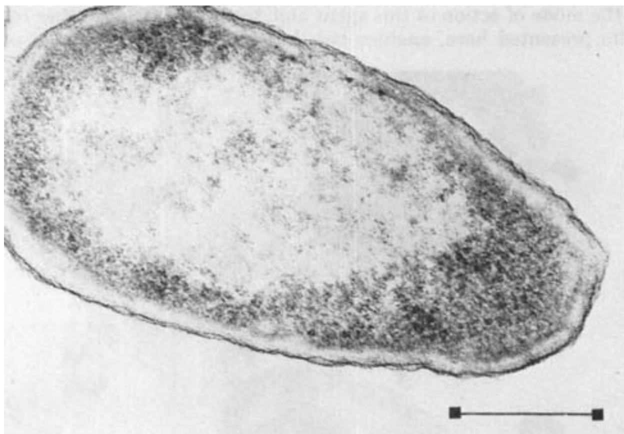

Figure 3—P. aeruginosa *cultured on agar containing benzalkonium (200 μg/ml) and then inoculated into nutrient broth and incubated for 16 hr. (Bar represents 0.25 μm.)*

[6] Baird and Tatlock, Chadwell Heath, Essex, England.

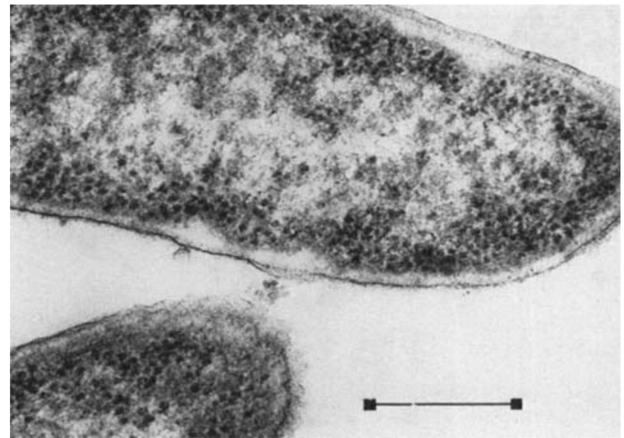

Figure 4—P. aeruginosa *cultured in nutrient broth. (Bar represents 0.25 μm.)*

These cultures were incubated for 42 hr.

Sensitivity to the lytic action of edetate disodium exhibited by normal cells and benzalkonium-treated cells was investigated as follows. Cells resistant to benzalkonium (500 μg/ml) were incubated overnight in broth with the same concentration of antibacterial and in broth without added benzalkonium. Normal cells were also used to prepare an overnight culture in nutrient broth. The resulting cultures were centrifuged and washed in an "inactivator" broth, as used by Riegelman *et al.* (13) but without the added agar. The cells were then washed in 0.5 *M* sodium chloride, suspended in 0.5 *M* sodium chloride at pH 8.2, and adjusted to a preselected extinction measurement. The lysis caused to these suspensions at 22° by adding edetate disodium (23 μg/ml) was followed at 1-min intervals up to 9 min after the addition of the chemical.

Electron Microscopy—Broth cultures were centrifuged, and the bulk of the supernate was removed. A volume of 8% (v/v) glutaraldehyde in 0.1 *M* Sφrensen phosphate buffer (pH 7.2) equal to the volume of the remaining cultures was added, and the resulting suspensions were maintained at 4° for 1 hr. Then the suspensions were centrifuged, the supernates were removed, and 4% (v/v) glutaraldehyde in 0.1 *M* phosphate buffer was added. After 5 hr of fixation, the samples were washed overnight in three changes of phosphate buffer. This procedure was followed by postfixation for 1 hr in 1% osmium tetroxide in 0.1 *M* phosphate buffer. Samples were then dehydrated in methanol, embedded in araldite CY212, cut with glass knives on a microtome[7], stained with 5% aqueous vinyl acetate for 30 min followed by Reynold's lead citrate for 30 min, and examined on an electron microscope[8] at 50 kv.

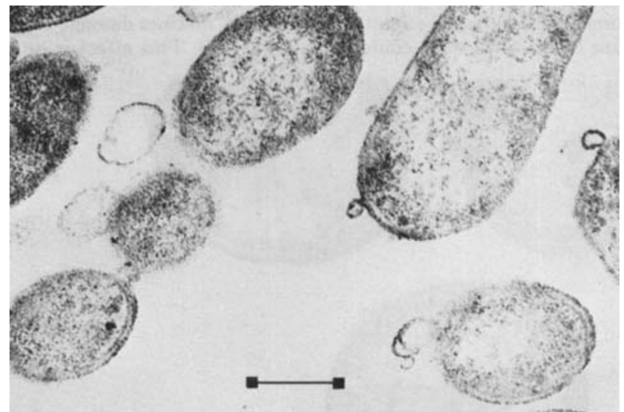

Figure 5—P. aeruginosa *cultured on agar containing benzalkonium chloride (200 μg/ml) and then inoculated into nutrient broth containing benzalkonium chloride (50 μg/ml). (Bar represents 0.25 μm.)*

[7] Reichert OMU2.
[8] Hitachi HU 11E.

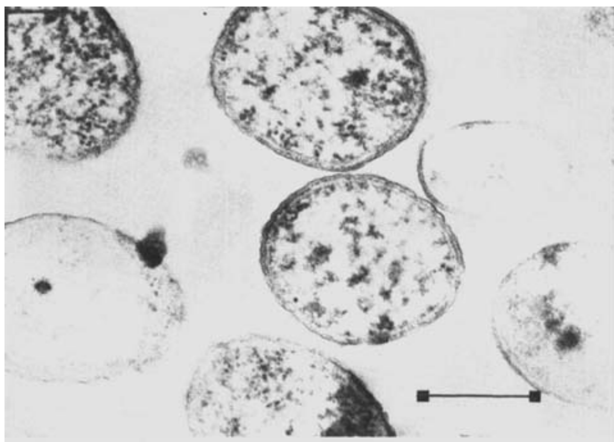

Figure 6—P. aeruginosa *cultured on agar containing benzalkonium chloride (800 μg/ml) and then inoculated into broth containing benzalkonium chloride (10 μg/ml). (Bar represents 0.25 μm.)*

Surface growth on agar was treated as follows. Small pieces of agar plus surface growth having a maximum dimension of 2 mm were immersed in 4% (v/v) glutaraldehyde in 0.1 *M* Sϕrensen buffer and fixed for 6 hr. After washing in buffer but before postfixation in 1% osmium tetroxide, the bulk of the agar was trimmed away and the resulting blocks were processed as already described for the suspensions.

RESULTS

P. aeruginosa cells grown in nutrient broth in the presence of benzalkonium chloride (50 and 100 μg/ml) or grown on solid medium in the presence of benzalkonium chloride (up to 400 μg/ml) showed widespread peeling of the outer cell membrane (Fig. 1). Cells grown in the presence of edetate disodium (50–200 μg/ml) had a distinctly wavy outer cell envelope (Fig. 2).

Cells with a resistance to benzalkonium chloride (200 μg/ml in agar), when subsequently grown in nutrient broth in the absence of benzalkonium, produced cells with apparently normal outer membranes (Fig. 3). This effect is seen when the cells in Fig. 3 are compared with the cells in Fig. 4, which were grown in nutrient broth. The cells grown in the presence of benzalkonium (50 μg/ml), however, showed peeling of their outer membranes (Fig. 5).

Cells grown on agar in the presence of benzalkonium (800 μg/ml) when grown in nutrient broth containing benzalkonium chloride (10 μg/ml) produced cells that showed no peeling of their outer membrane. [Compare Fig. 6 with cells grown in nutrient broth (Fig. 7).] When grown on agar containing 800 μg of benzalkonium chloride/ml and then grown in the presence of 10 μg of benzalkonium/ml plus 50 μg of edetate disodium/ml, distinct damage to the outer layer of the cells could be seen (Fig. 8). This effect is quite

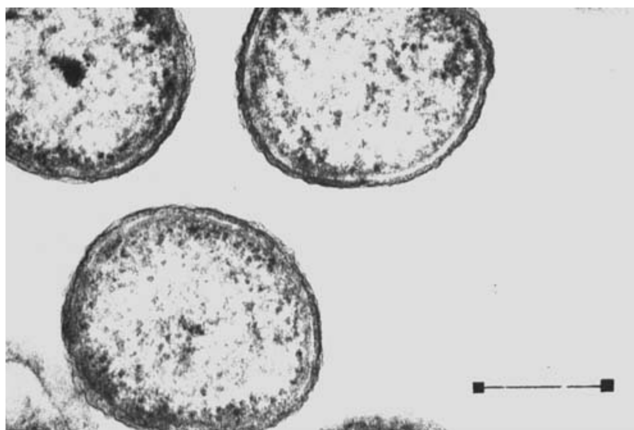

Figure 7—P. aeruginosa *cultured on agar containing benzalkonium chloride (800 μg/ml) and then inoculated into plain broth. (Bar represents 0.25 μm.)*

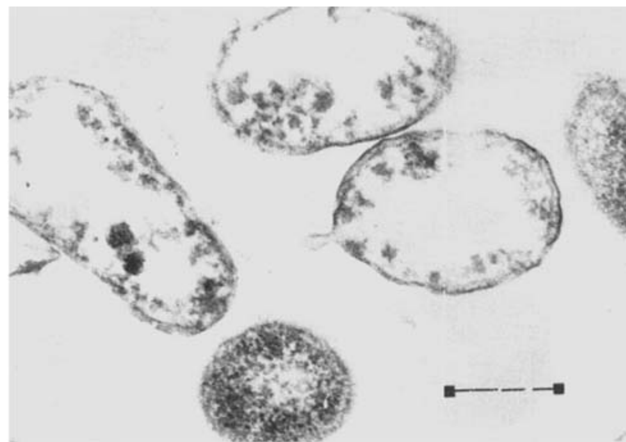

Figure 8—P. aeruginosa *cultured on agar containing benzalkonium chloride (800 μg/ml) and then inoculated into broth containing benzalkonium chloride (10 μg/ml) plus edetate disodium (50 μg/ml). (Bar represents 0.25 μm.)*

striking because these cells are able to grow in the presence of 80 times this concentration of benzalkonium. Figure 8 provides further evidence of the effectiveness of benzalkonium–edetate disodium combinations, which have been shown to be so effective against *P. aeruginosa* using different experimental techniques (8–10, 14, 15).

Repeated culturing in the presence of benzalkonium (50 μg/ml) did not produce cells resistant to the outer membrane damage caused by benzalkonium (Fig. 9).

The apparent variation in size of the cells is largely accounted for by the variation in the scale used in some of the figures. For example, the length of cells represented in all of the figures is within the 0.8–1.9-μm range.

The resistance of benzalkonium-damaged cells to the lytic action of edetate disodium was shown to be less than the resistance of normal cells (Table I).

DISCUSSION

The results indicate that electron micrographs of sections of *P. aeruginosa* cells cultured in the presence of subinhibitory concentrations of benzalkonium chloride and edetate disodium give valuable insights into the effect of these agents on the outer layers of the cell envelope. The ordinary light microscope reveals no change in cellular morphology after treating bacteria and fungi with quaternary ammonium compounds (2). (This finding was confirmed in this present study.) Therefore, the electron micrograph of the benzalkonium-affected cells that show pronounced stripping of the outer cell membrane (Fig. 1) gives a more detailed understanding of the mode of action of this agent and, together with the other results presented here, enables new interpretations to be made of

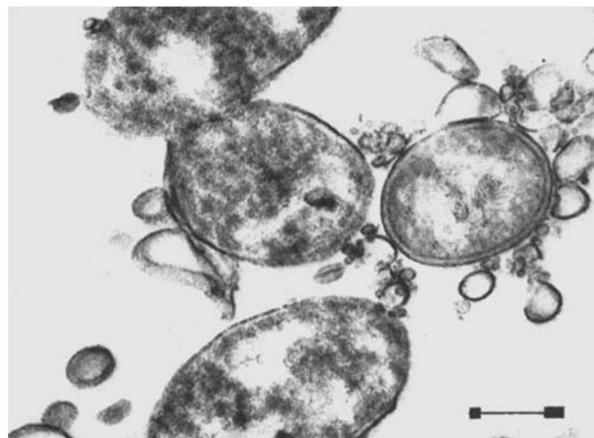

Figure 9—P. aeruginosa *cultured six times in broth containing benzalkonium chloride (50 μg/ml). (Bar represents 0.25 μm.)*

Table I—Effect of Benzalkonium Chloride on the Resistance of *P. aeruginosa* to the Lytic Action of Edetate Disodium[a]

Time after Addition of Edetate Disodium (23 µg/ml), min	Normal Cells Grown Overnight in Broth[b]	Cells Resistant to Benzalkonium (500 µg/ml)	
		Grown Overnight in Broth[c]	Grown Overnight in Broth plus Benzalkonium[d] (500 µg/ml)
0	0.290	0.288	0.288
1	0.270	0.269	0.268
2	0.270	0.269	0.262
3	0.269	0.269	0.252
4	0.269	0.263	0.245
5	0.269	0.263	0.240
6	0.264	0.263	0.231
7	0.264	0.261	0.228
8	0.261	0.259	0.219
9	0.260	0.259	0.215

[a] All cells were washed once in "inactivator" and once in 0.5 *M* sodium chloride before resuspending in 0.5 *M* sodium chloride at pH 8.2. Extinction was measured at 660 nm. [b] Reduction in extinction after 9 min = 10.3%. [c] Reduction in extinction after 9 min = 10.1%. [d] Reduction in extinction after 9 min = 25.4%.

some previous experimental findings with benzalkonium and edetate disodium. The fact that the cells retain their shape after the outer membrane is removed appears to indicate that the peptidoglycan layer remains intact. (Some results[9] with *P. aeruginosa* suspensions show that lysozyme causes considerable lysis to benzalkonium-treated cells.)

The average sterilization time for five separate duplicate determinations of benzalkonium chloride (100 µg/ml), inoculated with *P. aeruginosa* (10^7 cells/ml), was 15 min[9]. However, the same strain of *P. aeruginosa* grows in the presence of 200 µg of benzalkonium/ml in nutrient broth (10). It was thought that, in this latter situation, benzalkonium was having no effect because it was unable to penetrate the bacterial cell. In general, this concept has been held since MacGregor and Elliker (8) found that edetate disodium apparently enabled the quaternary ammonium compounds to penetrate to their sites of activity in *P. aeruginosa* cells having an increased resistance to quaternary ammonium compounds. This concept is seemingly supported by a similar situation with edetate disodium and lysozyme. Edetate disodium renders Gram-negative bacteria sensitive to the action of lysozyme, and it has been suggested that edetate disodium increases the permeability of the outer layers of the cell, allowing lysozyme to penetrate to its substrate (peptidoglycan) (16, 17).

The present study, however, indicates that, at the concentrations tested, the growing of cells in the presence of benzalkonium has a more drastic effect on the external layers of the cell than does growing in the presence of edetate disodium. Therefore, when edetate disodium is added to cultures of *P. aeruginosa* multiplying in the presence of benzalkonium, it appears that the edetate disodium is able to gain access to cell structures (possibly including the cytoplasmic membrane) on which it has a more potent effect than it has on the normal intact cell. This event would explain the results of Adair *et al.* (11), who found that, although a benzalkonium-sensitive strain of *P. aeruginosa* was able to grow in the presence of 10,000 µg of edetate disodium/ml, a benzalkonium-resistant strain did not show growth after 2 days in broth containing edetate disodium (10 µg/ml). However, after the benzalkonium-resistant strain was grown for 16 hr in the absence of benzalkonium, it regained the ability to grow in the presence of edetate disodium (10,000 µg/ml). Figure 3 shows that a culture of *P. aeruginosa* resistant to benzalkonium, when grown for 16 hr in the absence of the antibacterial, regained its outer membrane as per normal cells (Fig. 4).

Cells grown in the presence of benzalkonium (500 µg/ml) were more susceptible to the lytic action of 23 µg of edetate disodium/ml than were cells that had been grown in the presence of benzalkonium (500 µg/ml) and then grown overnight in the absence of the antibacterial (Table I). These latter cells showed the same level of resistance to 23 µg of edetate disodium/ml as normal cells grown for the same length of time.

The sensitivity of the cells grown in the presence of benzalkonium was not due to adsorption of the antibacterial on the cell surface, because the cells had been washed with inactivator medium to eliminate this possibility. Nor is it thought that cellular autolysis is induced by benzalkonium, which causes the outer membrane to peel off as a secondary effect, causing increased sensitivity to edetate disodium. This is not thought to be the case because cell suspensions of *P. aeruginosa* are immediately more susceptible to lysis by a benzalkonium–lysozyme combination than they are to benzalkonium alone[9].

Therefore, it appears that the outer membrane acts as a barrier to edetate disodium, and this would support the statement that "the outer membrane provides the basis of the barrier layer of the gram-negative cell wall" (18).

MacGregor and Elliker (8) also found that a strain of *P. aeruginosa* resistant to a quaternary ammonium compound was moré sensitive to the action of edetate disodium (100 µg/ml) than normal cells, and other workers found that edetate disodium did not exhibit any pseudomonicidal activity against normal *P. aeruginosa* (15). Of relevance too are the findings of Brown (19) and Richards (10) that edetate disodium combined with either phenylmercuric nitrate or chlorobutanol did not exhibit greater antipseudomonal activity than phenylmercuric nitrate or chlorobutanol as single substances. This finding could result from the two antibacterials not requiring assistance in penetrating the outer membrane and not having an action on the outer membrane that would assist the penetration of edetate disodium to internal sites of activity. (Neither phenylmercuric nitrate nor chlorobutanol at concentrations of 5 and 1000 µg/ml, respectively, markedly strip the outer membrane of *P. aeruginosa*[9].)

None of the procedures used to develop cells having an increased resistance to benzalkonium appeared to result in cells with external membranes resistant to the stripping action of the antibacterial agent. Whether or not the *P. aeruginosa* survives such damage may depend on the tonicity and/or viscosity of the system. The results presented here, however, indicate that the resistance of *P. aeruginosa* to benzalkonium is at a location or locations internal to the external membrane, most likely at the cytoplasmic membrane.

Part of the study of Anderes *et al.* (20) compared the percentages of cell dry weight of total, free, and bound lipids from *P. aeruginosa* grown at 37° in the absence and presence of a quaternary ammonium compound. These investigators found no increase in the amount of lipids produced by the resistant cells grown at 25°. In fact, a reduction of lipids occurred in the resistant cells at 37°; this result appears to rule out the resistance to the quaternary ammonium being due to a nonspecific blanketing action of lipids, a phenomenon that has been implicated in certain situations (21).

It can be concluded that part of the mechanism of action of benzalkonium chloride in subinhibitory concentrations is to damage severely the external membrane of *P. aeruginosa*. Edetate disodium also damages the outer layers of the cell envelope and, when used in combination with benzalkonium chloride, appears to have a potent effect on sites internal to the external membrane of *P. aeruginosa* cells.

[9] R. M. E. Richards, unpublished results.

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Oil–Water Distribution of *p*-Alkylpyridines

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Abstract □ The distribution of a homologous series of *p*-alkylpyridines between water and six organic solvents with varying degrees of polarity was investigated. The distribution coefficients were considered as reflections of the strength of net interactions involved in the solvents. The order was chloroform > octanol > carbon tetrachloride > butyl ether > hexadecane > octane. The effect of the methylene group upon the distribution coefficients differed little among the six solvents. The relative constancy was attributed to the predominance of the dispersion forces in the incremental effect.

Keyphrases □ Alkylpyridines—distribution between water and six organic solvents, distribution and partition coefficients □ Distribution coefficients—*p*-alkylpyridines, water and six organic solvents, effect of methylene group □ Partition coefficients—*p*-alkylpyridines, water and six organic solvents □ Pyridines, *p*-alkyl—distribution and partition coefficients, water and six organic solvents

The distribution behavior of solutes between two immiscible phases is a phenomenon important to numerous pharmaceutical and biological situations. The partition principle has been utilized in various chromatographic and extraction techniques common to many analytical pharmaceutical chemistry procedures. In biopharmaceutics, the pH–partition principle is basic to the theories and concepts of drug absorption, tissue distribution, renal reabsorption, and other membrane transport situations important to bioavailability and therapeutics. The partitioning of organic compounds between water and various oil phases has been studied to correlate biological activities (1–10).

Earlier systematic investigations of partition coefficients employed several alcohols and ethyl ether as the oil phases (11, 12); in general, the partition coefficients of different solutes differed considerably less in the butanol–water system than in the ether–water system. Depending on the nature of the solute, the following linear relationship was observed between the partition coefficient obtained from the ether system and that obtained from the butanol system (11, 12):

$$\log (k_{\text{butanol}}) = A \log (k_{\text{ether}}) + B \qquad \text{(Eq. 1)}$$

where the k's are the partition coefficients, and A and B are constants. Later, this relationship was shown to hold between other solvent pairs (13, 14) when the partition coefficients of a number of barbiturates between water and various organic solvents were studied.

The partitioning of compounds in several alkyl homologous series between several organic solvents and semiaqueous solutions has been studied (15). A linear relationship was found between the logarithm of the partition coefficient and the number of carbons in the chain:

$$\log (k) = A + Bn \qquad \text{(Eq. 2)}$$

where A and B are constants, and n equals the number of carbons. The equation did not hold for the lower terms of the homologous series ($n < 3$ or 4). The deviation was ascribed to the inductive effect in which the interactions between the parent group and the adjacent CH_2 groups changed the physical properties of these groups.

Equation 2 implies that $\log (k)$ is additive; *i.e.*, $\log (k)$ is equal to the sums of the contribution from each group in the molecule. Using octanol and water as the partitioning phases, Hansch and Anderson (16, 17) defined a substituent constant (π) as follows:

$$\pi_x = \log (k_x) - \log (k_o) \qquad \text{(Eq. 3)}$$

where k_o is the partition coefficient of a parent compound between octanol and water, and k_x is that for a derivative. Tables of π values for various functional groups have been compiled (16–19). The π_x for the CH_2 group has a value of 0.5.

The primary purpose of this research was to con-