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Title FACTORS RELATED TO RESISTANCE OF BACTERIA TO QUATERNARY AMMONIUM GERMICIDES

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This study was carried out to compare the effects of a quaternary ammonium compound (QAC) upon strains of Pseudomonas aeruginosa which were either sensitive or resistant to the germicide. The cationic QAC used was alkyldimethylbenzylammonium chloride. The resistant types were isolated from the sensitive population by selection of mutants which grew following exposure to different concentrations of germicide. A study also was made of the potentiating effect of lysozyme on the activity of QAC. An attempt was made to classify an unknown QAC-resistant bacterium isolated from sewage sludge.

Germicidal activity was measured by two methods. The Weber and Black method was used to determine the germicidal effect of QAC at a low concentration (200 ppm) during various periods of exposure time. Spectrophotometric measurements of cell lysis also were used when higher concentrations of QAC and longer exposure times were employed. Results indicated that cells exposed to low concentrations of QAC

were killed by lysis of cells occurred only upon the addition of higher concentrations acting for longer exposure times.

The potentiating effect of lysozyme on QAC activity was demonstrated using a modified Weber and Black method. It also was shown by means of a spectrophotometric procedure as well as manometric methods. The germicidal action of QAC toward both QAC-resistant and sensitive organisms was enhanced by addition of 0.025% lysozyme. Although the germicidal mechanism of QAC is still not understood, the penetration of QAC through the cell membrane appeared to be accelerated by the presence of lysozyme in the medium.

Degradation of QAC by growing cultures of the unknown sludge bacterium was demonstrated by following the decrease in QAC concentration during the time of incubation; in 35 days, 63% of added germicide at 500 ppm was apparently degraded. Since the organism was unable to utilize QAC, as a sole source of carbon or nitrogen, it appeared that QAC molecules were either firmly absorbed on the cell surface or dissipated by an unknown mechanism within or around the cell.

The unknown QAC-resistant sludge organism was classified as *Aerobacter cloacea* on the basis of results from usual bacteriological taxonomic tests.

FACTORS RELATED TO RESISTANCE OF BACTERIA TO QUATERNARY AMMONIUM GERMICIDES

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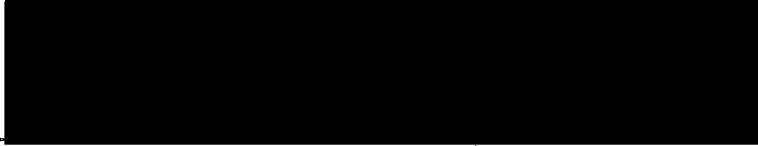

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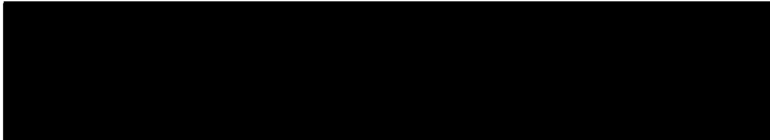

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FACTORS RELATED TO RESISTANCE OF BACTERIA TO QUATERNARY AMMONIUM GERMICIDES

INTRODUCTION

Previous studies have shown that a number of species of Pseudomonas frequently display high resistance to QACs. Comparisons between resistant and sensitive strains have been made from a number of standpoints, however there have been no experiments reported which provide a satisfactory explanation for the mechanism of action of QACs on bacteria. The extensive use of QACs in food sanitation as well as their use as disinfectants in the medical profession have provided an opportunity to observe increasing resistance of bacteria to these chemicals. The present investigation was undertaken with this background in mind, and was directed towards providing useful information to be used in future research on improved applications of QACs as germicides and detergent sanitizers.

The studies described in this thesis compare characteristics of QAC resistant and QAC sensitive strains of the same species. Research also included an evaluation of the potentiating effect of lysozyme on the germicidal activity of QAC toward QAC resistant and sensitive microorganisms. In addition the possible utilization of QAC by a QAC resistant bacterium with consequent reduction in effective concentration of QAC in the growth medium was examined.

HISTORICAL

General Information

Quaternary ammonium compounds (QACs) were first synthesized in 1903 in Germany by Einhorn and Gottler (23, p. 150-153). A more thorough study of the chemistry and bacteriology of QACs was made by Jacobs and his co-workers (29, p. 566-568; 30, p. 569-576; 31, p. 577-799) at the Rockefeller Institute. They found that the germicidal property of hexamethylenetetramine was due to the hexamethylenetetramine nucleus, but that this ability could be enhanced by incorporation into the molecule of aliphatically bound halogen. Almost twenty years elapsed before further significant contributions were made to enhance knowledge on the germicidal properties of QACs; in the year 1935, an important contribution to the present-day use of surface active QACs as germicides and antiseptics was made by Domagk who called attention to the high antibacterial activity of alkyldimethylbenzyl ammonium chloride solutions. Subsequent investigations by a number of research workers (21, p. 427-429; 17, p. 133-139; 19, p. 25-27; 20, p. 42-43; 24, p 156-167; 25, p 1-8; 28, p. 35; 38, p. 173-192; 49, p. 15-25) led to the wide spread use of QACs as bacteriocides and cleaners in various industries.

In recent years, a number of review articles concerning QACs have been published (43, p. 569-579; 35, p. 119-121; 46, p. 459-463; 37, p. 339-344). These have been prompted by the great research interest in these compounds and their wide spread use in the medical

and food sanitation fields.

Properties of QACs

The term 'QAC' refers to a class of compounds that have the general formula $(R_1R_2R_3R_4)$ X where X represents a negative ion such as halide used in the salt and R represents the various substituents attached to a nitrogen nucleus of the compound. When QACs are dissolved in water the cationic and anionic portions of the molecule ionize in the solution. It is the cation moeity which is believed primarily responsible for both the bactericidal property and the surface activity of these compounds.

While the bactericidal nature of the compound is attributed to the positively charged long chain portion, the degree of action is determined by the nature of the substituents attached to the nitrogen nucleus. Baker *et al*. (4, p. 269; 5, p. 619) found that the maximum activity was obtained when the alkyl group (R) contained 8 to 16 carbon atoms. In addition to the chemical configuration of the compounds, other factors which affect the bactericidal activity of QACs have been reported by many workers to be pH, temperature, presence of organic matter, water hardness, presence of anionic surface active agents and variation in susceptibility of microorganisms. Specific effects of each of these factors were reviewed by Lawrence (33, p. 19-37, 52 and 76-82).

In addition to bactericidal properties, QACs are able to depress the surface tension of water solutions because of their tendency to collect at interfaces and lower the free energy of molecules at

surfaces. Adam and Shute (1, p. 761-764) found that the surface tension reduction by QACs was not a function of their concentration. However, concentration influenced bactericidal activity and it appeared that the reduced surface tension alone could not explain the bactericidal action of the compounds.

For practical use, the prolonged storage of QACs under various conditions is of considerable importance; this, according to Lawrence (33, p. 52-54), has been studied by several investigators. It has been found that a 10% aqueous stock solution of alkyldimethylbenzyl ammonium chloride may be stored at room temperature for at least 10 years with no evidence of a physical, chemical or bacteriological change in properties. Furthermore, it has been observed that dilute aqueous solutions of the compounds will readily withstand autoclaving at 20 pounds pressure (121°C) for at least an hour without any noticeable change in bactericidal properties.

Bactericidal Action of QAC

It has been demonstrated that the cationic portion of QAC is adsorbed by the bacterial cell wall and very firmly held by electrostatic attraction (39, p. 204; 16, p. 447-471). Resuggen (41, p. 31) suggested that this adsorption on the cell wall with formation of an unionized complex was responsible for the death of the cells. Dyar and Ordal (22, p. 149-167) studied the electrophoretic mobilites of several species including both gram negative and positive bacteria treated with anionic and cationic compounds. They noted that

treatment with cetylpyridinium chloride caused a decrease in the charge on the cells followed by reversal and finally stabilization of the charge. Use of anionic detergent resulted in no significant change. The reversibility of QAC adsorption on the cell surface has been shown by several investigators, suggesting that the lethal effect of QAC on bacteria may not be attributable to adsorption alone, but may be due to changes in osmotic pressure causing leakage of cell contents through the cell wall. Electronphotomicrographs of *Pseudomonas aeruginosa* cells treated with QAC support this latter possibility (33, p. 499-503). Ethylenediaminetetraacetic acid (EDTA) also was reported by McGregor and recently by Carson and Eagon (11, p. 32) to have a similar effect. Hotchkiss (27, p. 491-492) found that the combination of positively charged QAC ions with the bacterial surface takes place first, followed by damage to the cell membrane; there was subsequent release of cell nitrogen and phosphorous into the medium and eventual death. Stedman and King (45, p. 656-659) also studied the lethal effect of QACs. They stated that death of cells was caused by intracellular materials being released into the medium as a result of cell wall or membrane destruction. Recently, Ceglowski and Lear (12, p. 458) reported that the loss of nucleic acid materials by actively growing *Escherichia coli* did not appear to play a major role in the lethal effect.

Enzyme inhibition by QACs has been investigated extensively by Miller, Baker and Harrison (4, p. 249-271; 5, p. 611-620). In their studies, a large variety of synthetic detergents was examined; all

cationic compounds tested inhibited both respiration and glycolysis of gram positive and gram negative bacteria when they were diluted 1:3,000. The anionic compounds, on the other hand, showed little or no effect at the same concentration. From their results, they concluded that gram positive bacteria were more sensitive than gram negative to inhibition of metabolic functions. They suggested that the action of some detergents on bacteria may be due to: (a) disorganization of the cell membrane caused by the surface activity of the QACs and (b) an interaction between the detergents and lipoid constituents of the cell membrane as the result of denaturation of essential proteins.

The ratio of cells to inhibitor concentration also has been shown to play an important role in metabolic inhibition. For example, Knox *et al*. (32, p. 451) indicated that bactericidal action and inhibition of lactate oxidation of *Escherichia coli* depended on the detergent: bacteria ratio and not to the detergent concentration. Stedman and King (43, p. 658) in their studies of enzyme-germicide interaction, correlated the concentration of surface-germicide complex and enzyme-germicide complex to the lethal effect. They reported that the concentration of QAC required to inhibit enzymatic activity was much less than the concentration required for significant cell-surface destruction; approximately 50% enzyme inhibition was obtained with a level of QAC causing less than 2% cell surface destruction.

The metabolic inhibition of Baker's yeast by different kinds of synthetic detergents has been studied by several workers (44, p.

677-682; 2, p. 137-147; 3, p. 210-215; 7, p. 289-299). They found that these compounds, in relatively low concentration, strongly inhibited the metabolism of yeast. Armstrong, for example (2, p. 138), found that in low concentration, cationic detergents inhibited the production of acid and carbon dioxide by Baker's yeast suspensions carrying on an aerobic fermentation; anionic detergents under the same condition, however, were inactive or only slightly inhibitory. Further, the addition of inhibitory concentration of cationic detergents (2.0 x 10^{-3}M) to yeast suspensions resulted in the loss of phosphorous-containing compounds from the cells. The extent of this loss was closely related to the degree of metabolic inhibition. On the basis of these findings, he suggested that the inhibitory action of cationic detergents was largely due to their disruptive effect on the cell membrane.

Recently, Obayashi (36, p. 241) studied the germicidal action and effect of surfactants on the permeability of lactic acid bacteria; he found that the germicidal action of surfactants depended on their ability to immediately bind with the cells where they created permeability conditions unsuitable for growth. In addition, denaturation of cell protein was observed to be caused by the surfactant. Specifically, the germicidal action of cetyltrimethyl ammonium bromide was due to the fact that it bound tightly with cells, preventing growth by changing permeability and by causing release of internal substances resulting in death of the cells.

Germicidal Resistance of Bacteria

The development of resistance by bacteria to QACs has long been recognized by many workers and has been one factor preventing more widespread application as sanitizing agents in the medical and food industries. Chaplin (13, p. 373-382; 14, p. 453-458) developed a strain of *Serratia marcescens*, normally suppressed by less than 100 ppm QAC, which was able to grow in the presence of 100,000 ppm of alkyldimethylbenzyl ammonium chloride. This was accomplished by selection of survivors of disinfection, but resistance was readily lost when cells were grown on QAC-free media. He presumed that the resistance was due to the elaboration of a lipoprotein which was retained on the cell surface and was capable of withstanding the disruptive surface force of the disinfectant. In electrophoretic studies he found that a lipase labile substance was intimately associated with the surface of the normal cells but at a much greater concentration with the surface of the QAC-resistant cells. The resistant cells stained intensely with safranin, brown with iodine and yellow with picric acid while the normal cells remained colorless with these latter two reagents. Wet mounts of resistant cells were very heavily stained with Sudan Black B; normal cells were colorless. The amount of the ether-alcohol soluble fraction was 31% in resistant cells while only 5% in normal cells. Bunting (9, p. 241-250) reported that in the presence of surface-active agents, *S. marcescens* lost its ability to produce pigment; she also inferred that this was the result of selectivity rather than

mutation. Crocker (16, p. 138-144) found that a resistant strain of *E. coli* lost the ability to produce gas from lactose, and produced under-sized colonies on solid media. MacGregor and Elliker (34, p. 499-503) developed two strains of *Pseudomonas aeruginosa* capable of growing in the presence of 2,000 ppm alkyldimethylbenzyl ammonium chloride. They found that QAC resistant strains lost their resistance in the presence of EDTA. Glucose and succinate oxidation by normal and resistant strains were inhibited by ADBAC. From their findings, they suggested that EDTA may accelerate QAC action by increasing the permeability of the cells to QAC.

EXPERIMENTAL METHODS

Organisms. QAC-sensitive strain of *Pseudomonas aeruginosa* was obtained from the culture collection maintained in the Department of Microbiology, Oregon State University. A QAC-resistant strain of the same species was selected by exposing the normal wild type cells to increasing concentration of germicide on appropriate medium as described below. Colonies were subcultured from the survivors appearing on the medium containing the highest concentration of QAC. The colonies were transferred to agar slants of the same medium and with the same concentration of QAC as the medium from which they were isolated. This finally resulted in the organism showing the ability to grow in the presence of 2500 ppm (0.25%) QAC. The QAC-resistant strain was routinely cultured at 25°C on tryptone glucose yeast extract (TGY) agar slants containing 2500 ppm QAC. The culture was transferred on alternate days to maintain viability.

A QAC-resistant organism, designated bacterium A, was isolated from an activated sludge sample obtained from the domestic sewage treatment plant in Portland, Oregon. One ml of activated sludge was inoculated in a 1000 ml flask containing 400 ml of Casamino acid-glucose medium containing 500 ppm QAC. The mixture was incubated at 30°C for five days and then streaked on petri plates of TGY agar also containing 500 ppm QAC. A colony isolation was made and the streaking process repeated two more times on the same medium to obtain the pure culture. The pure culture was maintained at 30°C by transfer every other day on agar slants of TGY containing 500 ppm QAC.

A culture of Alcaligenes bookeri 9128 was obtained from the American Type Culture Collection, Washington D. C. Subculture was made by transferring the lyophilized preparation to TGY broth. The pure culture was routinely maintained on TGY agar slant, incubated at 30°C and transferred to fresh medium each week. Cultures were stored at 2 to 5°C between transfers.

Media. Tryptone glucose yeast extract (TGY) agar contained the following ingredients per liter of tap water: tryptone, 5.0 g; yeast extract, 5.0 g; dextrose, 1.0 g; K_2HPO_4, 1.0 g and agar, 15 g. The medium was adjusted to pH 7.2 before sterilization in the autoclave for 20 minutes at 121°C.

This agar was used during Weber and Black determinations, cited below, for plating suspension of cells from QAC inactivator tubes containing, in addition to the ingredients listed above, one g of Asolectin and seven ml of Tween 80 (Ruger Chemical Co., Long Island City, N. Y.) per liter of medium. The pH of this medium was adjusted to 7.2 before sterilization in the autocalve for 20 minutes at 121°C.

Synthetic medium contained 0.50 g of NH_4Cl, 0.50 g of $(NH_4)_2SO_4$, 3.0 g Na_2HPO_4, 2.0 g of KH_2PO_4, 0.01 g of $MgSO_4 7H_2O$ and distilled water to make the final volume of one liter. The medium was adjusted to pH 7.2 before sterilization in the autoclave for 20 minutes at 121°C.

All the QAC-containing media were made by mixing equal parts of double strength medium with aqueous QAC solutions which had a concentration twice that desired in the final medium.

Chemicals. Alkyldimethylbenzyl ammonium chloride (ADBAC) was obtained from Klenzade Products, Beloit, Wisconsin and used for all the experiments reported in this work. The germicide to be tested was diluted in sterile distilled water to a concentration twice that desired in the final solution. Concentrations of QAC were checked by the method reported by Furlong and Elliker (24, p. 225-234). QAC inactivator sufficient for 200 ppm QAC was made by dissolving 2.22 g of Asolectin and 15.6 ml of Tween 80 in distilled water. The mixture was heated over a Bunsen flame to exactly the boiling point at which time frequent agitation was begun along with small additions of water to prevent vigorous boiling. The solid dissolved within about two hours. The pH of the inactivator was adjusted to 7.2 before final sterilization in the autoclave for 20 minutes at 121°C.

Crystallized egg white lysozyme was obtained from Sigma Chemical Company, St. Louis, Missouri. A 0.025% solution was prepared by dissolving 25 mg of lysozyme in 100 ml of sterile phsphate buffer (0.05M) at pH 7.0. Freshly prepared enzyme solution was used for each experiment.

All media used for taxonomic studies on bacterium A were prepared according to the Manual of Microbiological Methods (10, p. 37-71).

Manometric Studies. Conventional manometric techniques as detailed by Umbreit, Burris and Stauffer (47, p. 1-16) were used for the measurement of gaseous exchange of resting cell suspensions of various organisms. Cell suspensions were prepared by washing 24-hour old

cultures from agar slopes. The harvested cells then were washed twice with sterile distilled water and finally suspended in 0.05 molar pH 7.0 phosphate buffer. The cell suspensions were standardized spectrophotometrically to an optical density of 0.50 and plated on TGY agar to determine the number of organisms. Measurement of the respiration of resting cell suspensions was expressed as microliters (μl) of oxygen uptake or carbon dioxide evolution per gram dry weight of cells. The dry weight of cells was determined by drying 2 ml of the cell suspension and weighing the resulting powder. A standard Warburg constant volume microrespirometer was used. The water bath was maintained at 30°C. Each flask contained 2 ml of cell suspension in 0.05 molar phosphate buffer at pH 7.0 and one ml of substrate; the center well contained either 0.2 ml of 20% KOH or 0.2 ml of distilled water. The concentration of glucose used was 10 micromolar (μM). All experiments were carried out in an air atmosphere. For germicidal and lysozyme-effect studies, one ml of 1,000 ppm QAC or one ml of 0.05% lysozyme was used in place of one ml of glucose. Readings were taken every 15 minutes over a period of two hours.

Effect of Lysozyme of the Germicidal Activity of QAC. Germicidal tests on the experimental organisms were conducted using the method of Weber and Black (50, p. 1406-1417) with slight modifications. QAC-sensitive organisms were grown on TGY agar slopes and the QAC-resistant organisms were grown on TGY agar slopes containing 2,500 ppm QAC for *P. aeruginosa* and 5,000 ppm for bacterium A. The growth was washed from agar slopes with sterile distilled water then resuspended in 0.05

molar pH 7.0 phosphate buffer. Number of cells was determined by plating the original cell suspension using TGY agar. Five ml of the cell suspension was pipetted into a 35 x 150 mm sterile aluminum-capped test tube which contained 5 ml of double strength QAC. For testing the potentiating effect of lysozyme on the germicidal activity of QAC, germicide and lysozyme were made up to four times the desired final concentration in buffer. Aliquots (2.5 ml) of each were mixed in a sterile tube to which 5.0 ml of washed cell suspension then was added. One ml aliquots were removed from the exposure tube at intervals of ½, 2 and 3 hours for QAC resistant organisms and at 5, 15 and 60 seconds for the QAC-sensitive organisms. The aliquots were pipetted into nine ml of inactivator. The mixture was diluted as required and plated on TGY agar containing inactivator. Plates were incubated for 48 hours at 30°C and then counted.

Spectrophotometric Studies. The effect of lysozyme on the germicidal activity of QAC was studied spectrophotometrically by following the lysis of the cells in the presence of lysozyme in QAC-containing medium. The system of Repaske (38, p. 225) was used to carry out the experiments. The test organisms were grown in CAA glucose medium for 24 hours and then harvested by centrifugation. The harvested cells were washed twice with sterile 0.85% saline. Lysis of three-ml samples suspended in 0.025 M tris aminomethane buffer, pH 8.0, containing 200 ppm QAC and 0.025% lysozyme was followed at 600 mu using a Beckman Model B spectrophotometer.

Studies on QAC Utilization by QAC-Resistant Organism. Disappearance of germicide in medium containing 500 ppm QAC was measured using the Furlong and Elliker method (26, p. 225-234) with slight modifications. The QAC resistant organism, previously grown on TGY agar slant containing 5,000 ppm QAC, was transferred into a sterile eight ounce glass bottle containing 100 ml of CAA-glucose-QAC broth. The QAC concentration of this medium was adjusted to 500 ppm. The concentration of QAC in the growing culture was titrated at various time intervals. Washings from cell aliquots harvested at these times also were tested for germicide content. Another bottle containing the same concentration of QAC without inoculum, served as a control. The concentrations of QAC in the control bottle were checked in the same manner at the same time intervals.

Taxonomic Studies. All media and methods used to identify bacterium A were adopted from the Manual of Microbiological Methods (15, p. 141-167). The classification of the organism was based on results of tests carried out which seemed appropriate from reference to Bergey's Manual of Determinative Bacteriology.

RESULTS

Effect of QAC on QAC-Resistant and Sensitive Organisms. The germicidal effect of 200 ppm QAC on QAC-resistant and sensitive organisms was carried out using the method of Weber and Black (50, p. 1406-1417); the effect of 1,000 ppm QAC on the organism was conducted by measurement of optical density changes using a Cary Model II continuous recording spectrophotometer. Table 1 shows the effect of 200 ppm QAC on a QAC-resistant and sensitive strain of *P. aeruginosa*. It may be seen that 100 percent destruction of the sensitive strain occurred within 5 minutes exposure to QAC at a concentration of 200 ppm. The resistant strain, however, was not affected within this time of exposure and furthermore, 30 minutes of exposure were required before significant destruction of this strain occurred. The effect of exposure to 200 ppm QAC on the germicide resistant bacterium A and also on *Alcaligenes bookeri* is shown in Table 2. It may be seen that the latter organism was destroyed within 60 seconds while the resistant organism was not affected within this time of exposure. As a matter of fact, exposure of bacterium A to 200 ppm ADBAC for 300 seconds had no apparent effect on the cells.

During the spectrophotometric studies, it was observed that lysis of QAC-sensitive cells occurred rapidly upon the addition of 1,000 ppm ADBAC; lysis increased with time as indicated by the decreasing optical density of the cell suspension. The decrease in optical density when ADBAC was added to the tube containing QAC-resistant cells was

Table 1. Effect of 200 ppm ADBAC on QAC-sensitive and resistant strains of P. aeruginosa

Strain	Initial count x 10^6	Percent kill during exposure period in minutes					
		1	5	15	30	120	180
		%	%	%	%	%	%
R	7	-	0	23.4	85.6	-	-
S	66	98.9	100	100	-	-	-

R represents QAC-resistant, S represents QAC-sensitive

Table 2. Effect of 200 ppm ADBAC on _A. bookeri_ (sensitive) and bacterium A (resistant)

Organism	Initial count x 10^6	Percent kill during exposure period in seconds				
		15	30	60	120	300
		%	%	%	%	%
Bacterium A	10	0	0	2	0	2
A. bookeri	8	98	99.97	100	100	100

not significant. Figure 1 shows these results. It may be seen that the optical density of the QAC-sensitive strain of P. aeruginosa decreased over two hours in a linear manner, whereas the density of the suspension of the resistant strain remained almost unchanged. At the end of two hours exposure, the optical density of the sensitive and resistant strains decreased 96% and 17% respectively.

Effect of Lysozyme on the Activity of QAC. It has been demonstrated that gram-negative organisms are resistant to the action of lysozyme at physiological hydrogen ion concentrations. However, the lysis of these cells by lysozyme has been shown to occur upon addition of Versene (EDTA) (40, p. 225-232). By replacement of Versene with low concentrations of tetradecyldimethylbenzyl ammonium chloride, the lysis of gram negative cells by lysozyme also was demonstrated by Ceglowski and Lear (12, p. 458-462). In the present study an investigation of the combined action of 0.025% lysozyme and 200 ppm QAC on sensitive and resistant bacterial cells was carried out. Table 3 shows the percentage of QAC-resistant organisms destroyed after exposure periods of 5, 15 and 30 minutes. Due to the highly resistant nature of the organisms tested, the exposure time was extended from the 15 to 60 seconds usually used in Weber and Black determinations to the 30-minute period shown in the table. Lysozyme alone, it may be seen, had almost no effect on both organisms tested. However, the addition of 0.025% lysozyme to the medium containing 200 ppm QAC increased lethality to 100% for P. aeruginosa and to 98% for bacterium A at the end of 30 minutes of exposure time. Attempts also were made to measure a

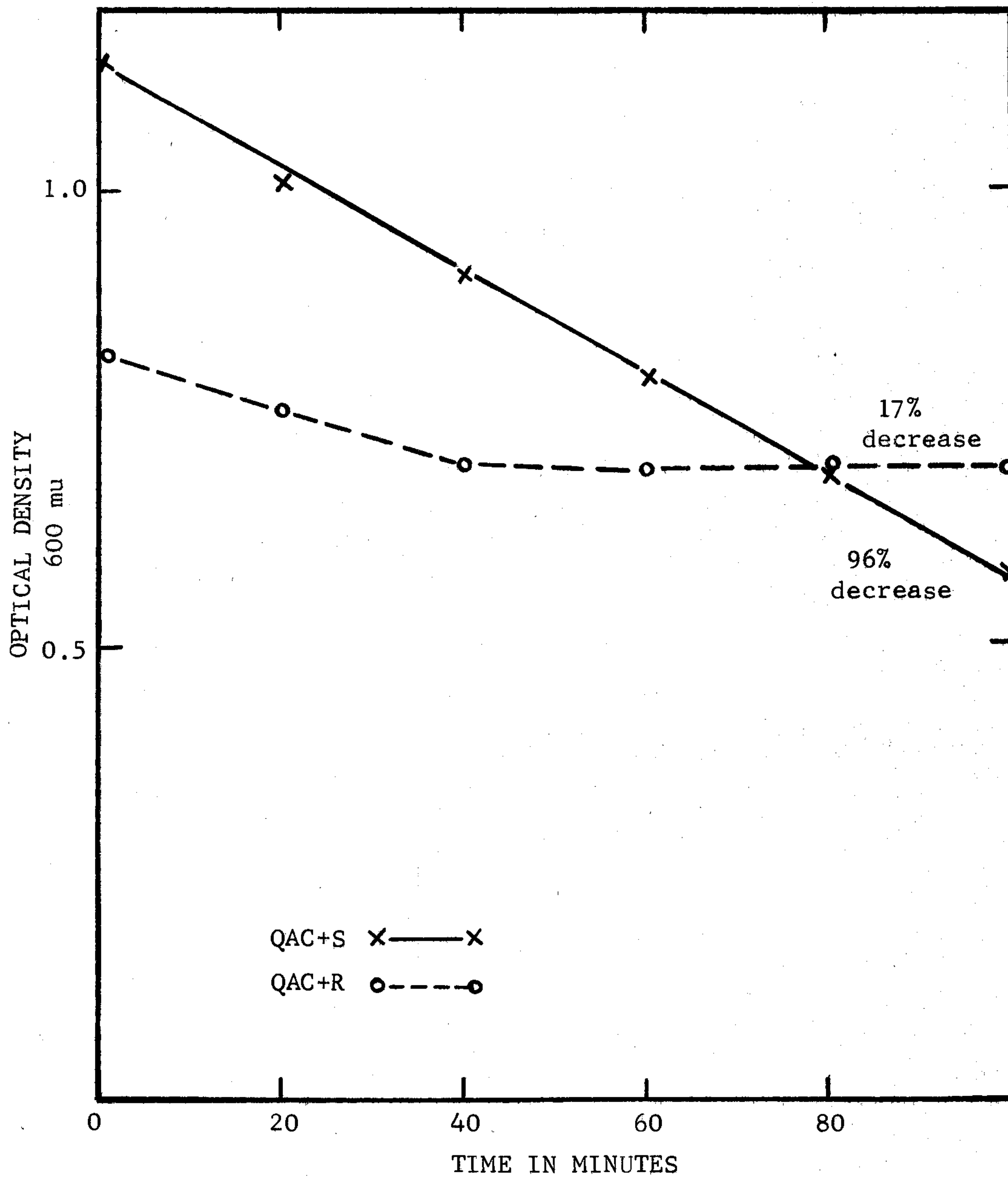

Figure 1. Effect of 1000 ppm ADBAC on QAC-sensitive and resistant cells of Pseudomonas aeruginosa as measured spectrophotometrically immediately after addition of germicide to 11 hour CAA-amino acid broth culture.

Table 3. The effect of lysozyme on the germicidal activity of QAC against QAC-resistant P. aeruginosa and bacterium A

Organism	Addition	Percent kill during exposure period in minutes		
		5	15	30
		%	%	%
P. aeruginosa	Lysozyme	0	0	5
	QAC	0	23.4	85.6
	Lysozyme + QAC	40.3	68.9	100
Bacterium A	Lysozyme	0	2	5
	QAC	8	19	40
	Lysozyme + QAC	7	25	97.9

Plate counts from which lethality was calculated were made in triplicate and averaged.
Concentration of lysozyme was 0.025%.
Concentration of QAC was 200 ppm as ADBAC.

potentiating effect on the action of QAC upon sensitive organisms. As shown in Table 4, the activity of the QAC alone was so rapid that it was not possible to observe any lysozyme effect.

The amounts of oxygen uptake by QAC resistant and sensitive organisms are shown in Table 5. The results indicate that the oxygen uptake by both resistant and sensitive organisms was depressed by the combined action of lysozyme and QAC. This respiratory inhibition by the action of lysozyme and QAC was greater than that obtained by action of 200 ppm QAC alone. A lower respiration rate of the QAC-resistant cells in the presence or absence of substrate also may be seen in Table 5. Also in case of the resistant organisms, respiration is not stimulated by glucose and may even be repressed. The addition of lysozyme as a substitute for the substrate, however, caused an apparent stimulation of the rate oxygen uptake by the QAC-resistant strain of *P. aeruginosa*.

Spectrophotometric studies confirmed the potentiating effect of lysozyme on QAC activity; Table 6 indicates the lysis of QAC-resistant and sensitive organisms was increased by addition of lysozyme to QAC.

Degradation of ADBAC by Bacterium A. The biodegradation of anionic detergents by bacteria has recently been found by Payne and Feisal (37, p. 339-344). In view of this, attempts were made to determine whether or not bacterium A could use ADBAC as a source of carbon or nitrogen. Figure 2 shows that the organism failed to grow in a culture medium which had no carbon or nitrogen source added other than ADBAC. However, as also seen in the figure, addition of glucose

Table 4. The effect of lysozyme on the germicidal activity of QAC against QAC-sensitive organism _P. aeruginosa_ and _A. bookeri_

Organism	Addition	Percent kill in the exposure period in seconds		
		15	60	300
		%	%	%
P. aeruginosa	Lysozyme	0	0	0
	QAC	92.5	98.9	100
	Lysozyme + QAC	93.2	95.8	100
A. bookeri	Lysozyme	0	0	0
	QAC	95.5	99.98	100
	Lysozyme + QAC	96.8	100	100

Concentration of lysozyme was 0.025%.
Concentration of QAC was 200 ppm as ADBAC.

Table 5. Effect of lysozyme on oxygen uptake by QAC-resistant and sensitive organisms in the presence or absence of ADBAC

Organism	Microliters of oxygen uptake per gram of dried cells in two hours				
	Endogenous	Glucose	Substitute for glucose		
			Lysozyme	QAC	Lysozyme + QAC
_P_. _aeruginosa_ (R)	749	687	1030	661	611
_P_. _aeruginosa_ (S)	3,721	12,656	3,147	2,656	1,577
Bacterium A	417	316	50	106	0
_A_. _bookeri_	1483	1517	1346	202	41

Concentration of lysozyme was 0.025%
Concentration of QAC was 200 ppm as ADBAC

Table 6. The effect of lysozyme on the germicidal activity of QAC on QAC resistant and sensitive organisms as measured spectrophotometrically

Organism	Destruction of cell as expressed by percentage of turbidity change in 20 minutes			
	Buffer	Lysozyme	QAC	Lysozyme + QAC
	%	%	%	%
P. aeruginosa (R)	-3.6	-3.9	-35.5	-22.6*
P. aeruginosa (S)	-3.7	-3.8	-52.0	-66.7
Bacterium A	+8.7	-17.9	-11.5	-51.9
A. bookeri	-4.1	-9.0	-56.0	-76.6

*Exposure time was 10 minutes in this case.
Each cuvette contained 3 ml sample; 0.025% lysozyme, 200 ppm QAC and 0.25 M Tris buffer at pH 8.0

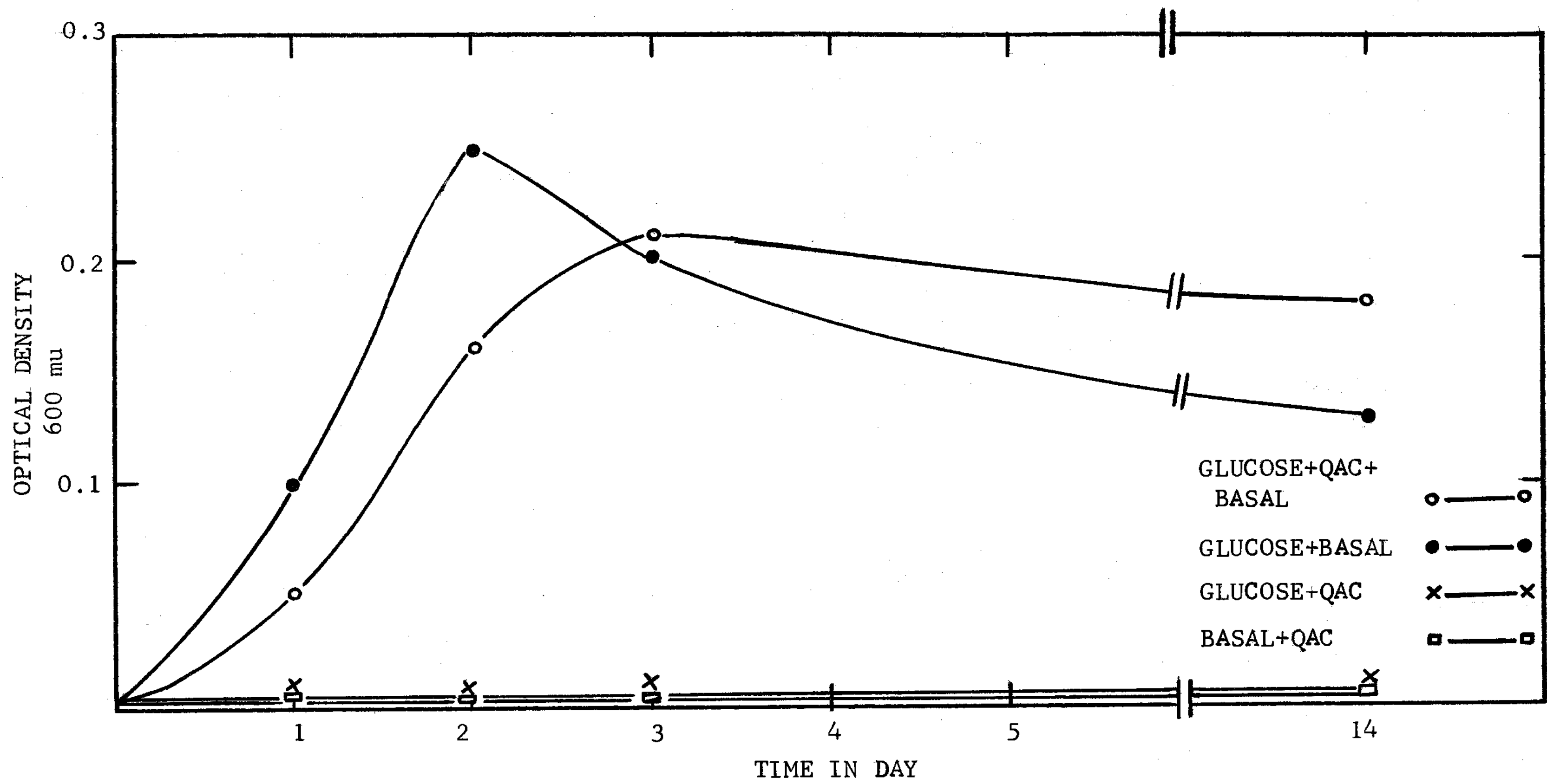

Figure 2. Growth of bacterium A at 30°C in basal medium in the presence (500 ppm) and absence of ADBAC.

to the medium resulted in growth which did not differ significantly from growth in glucose medium not containing ADBAC. Additional experiments indicated that bacterium A may have the ability to degrade QAC during growth in a glucose medium. Figure 3 shows the disappearance of QAC with time during growth of bacterium A; bacterial population increased with the disappearance of QAC from the culture medium. There was 63% loss of the ADBAC originally present at the end of 35 days of incubation while the concentration in the control revealed almost no change.

Possible degradation of QAC by bacterium A also was suggested by determination of the foaming properties of glucose-QAC medium before and after growth of the organism. Figure 4 shows the loss in foaming properties as a result of the growth of the resistant cells.

Identification of Bacterium A. The unknown QAC-resistant organism isolated from sewage sludge was found to grow nearly as well in medium containing 500 ppm ADBAC as in medium lacking the QAC. Figure 5 shows the growth curves of the organism in CAA-glucose broth at three different concentrations of QAC. ADBAC at 500 ppm gave only slight retardation of the growth while 10,000 ppm of QAC caused about 50% inhibition. It is well known that most gram negative organisms are killed or greatly inhibited by QAC at concentrations of 200 ppm unless resistant mutants are selected by continuous cultivation in media of increasing amounts of QAC. The ability, therefore, of bacterium A to grow in the presence of 10,000 ppm QAC with only one previous exposure to medium containing QAC (1500 ppm) suggested that the population

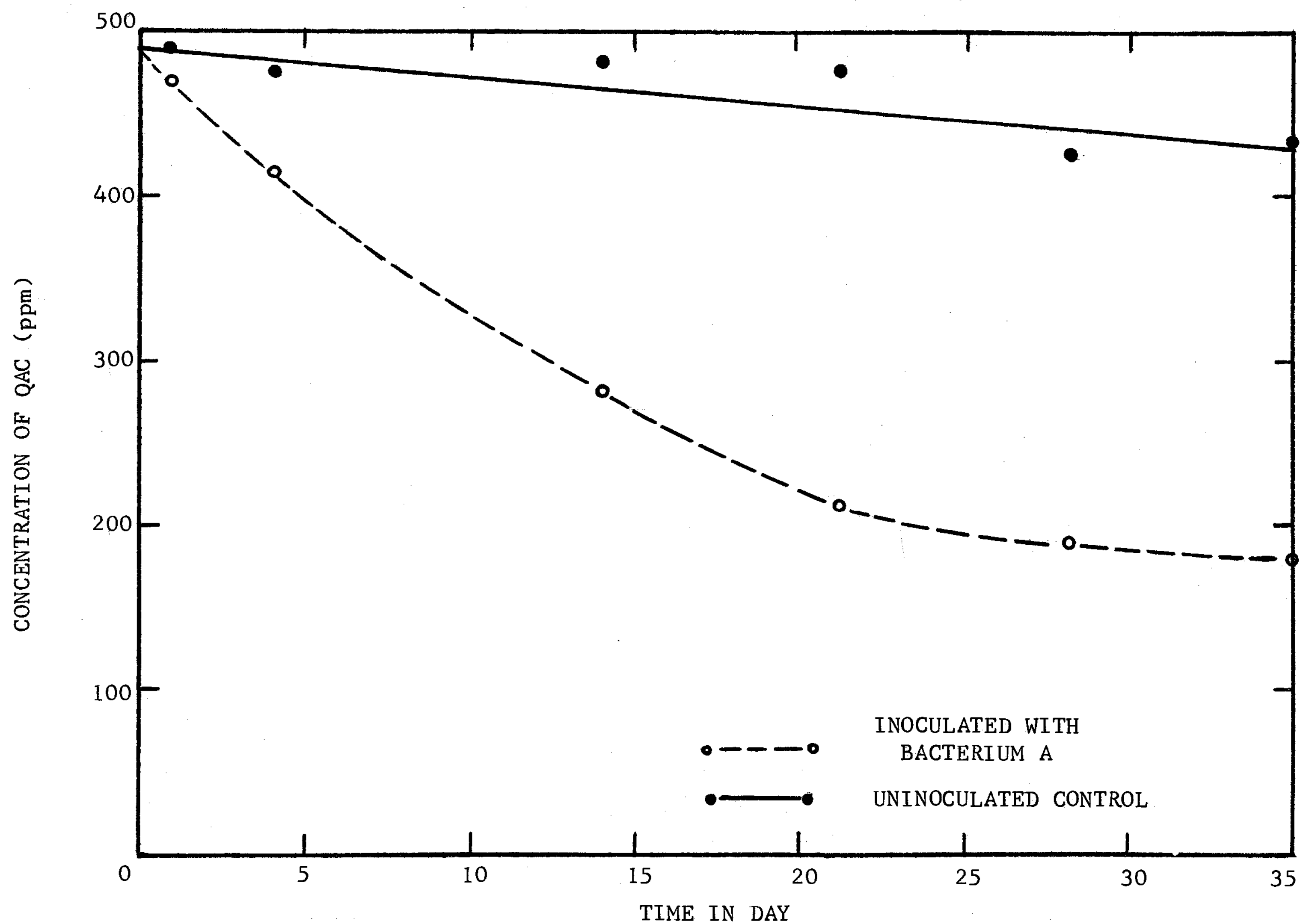

Figure 3. Apparent degradation of QAC by bacterium A during growth in a CAA-glucose medium at 30°C.

The uninoculated control (left) and the inoculated culture before shaking.

The same two flasks after growth of the organisms for 35 days and after identical shaking.

Figure 4. The foaming properties of QAC before and after incubation with QAC resistant organism bacterium A

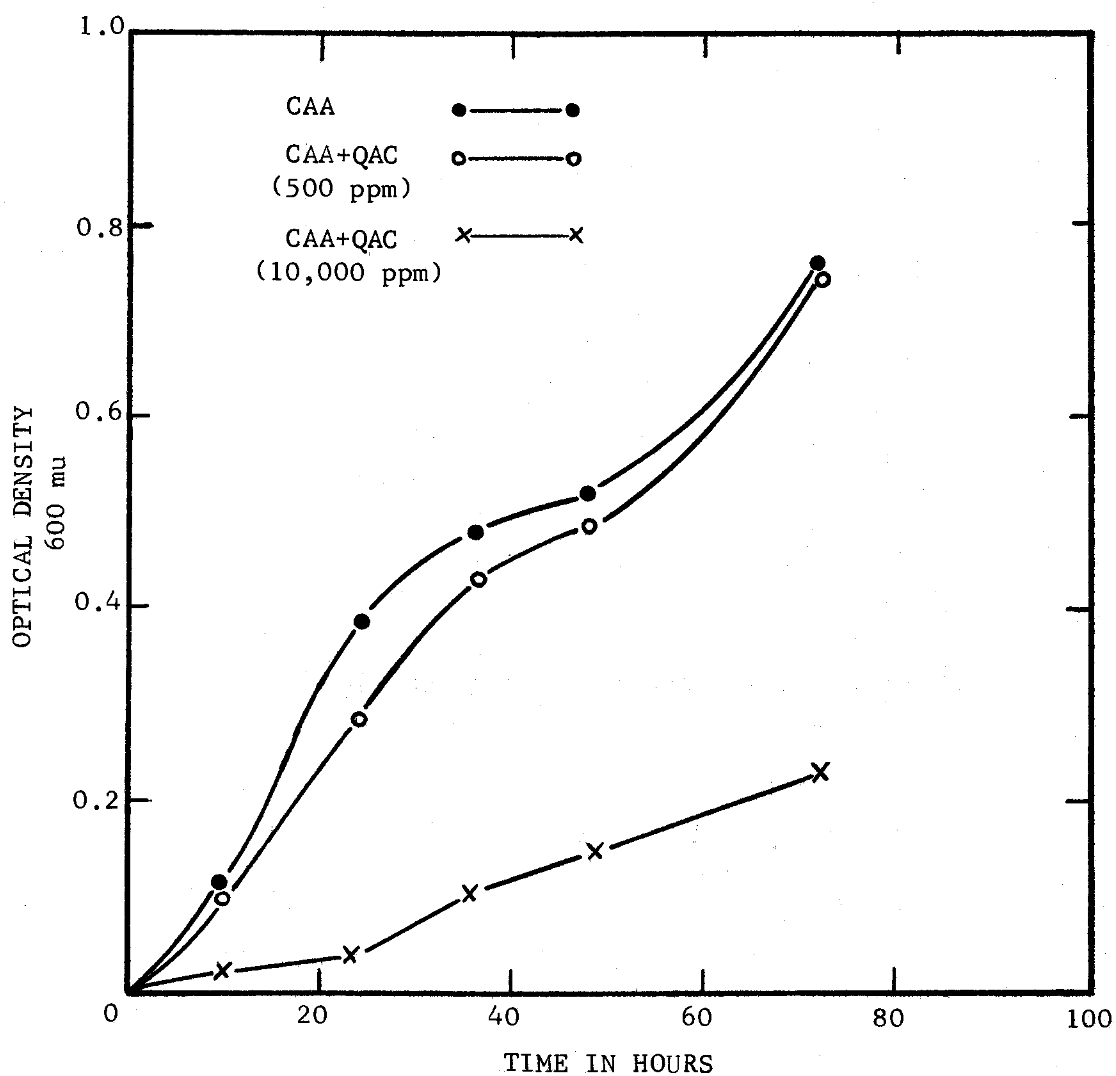

Figure 5. Growth of bacteria A in CAA-glucose broth containing no QAC (open circles), 500 ppm QAC (closed circle) or 10,000 ppm QAC (triangle).

contained a large number of resistant cells. It was of interest, therefore, to determine the genus and species identification of the organism and so taxonomic studies were carried out.

Identification of bacterium A was made using standard microbiological procedures as were determined necessary by reference to Bergey's Manual of Determinative Bacteriology (43, Bergey's Manual). Important characteristics determined for the organism were as follows:

A. Source: Raw sewage sludge

B. Cultural characters:

Vegetative cells: rods, 0.5 by 1.0 to 2.0 microns, single, motile by means of peritrichous flagella, no spores, and gram negative.

Agar colonies: yellowish white, opaque, circular; a rhizoid form was observed in the presence of QAC.

Agar stroke: growth abundant, smooth annd spreading.

Broth: no surface growth, turbid and viscous sediment.

Potato slant: light yellow pigment produced, potato became brown after 4 to 5 days.

C. Physiological characters:

Gelatin stab: gelatin completely liquefied in 48 hours at 25°C.

Litmus milk: slightly acid with pH 6.8, coagulation before reduction, slow peptonization.

Carbohydrate fermentation: glucose, lactose and sucrose fer- fermented anaerobically with

production of acid but no gas in 48 hours.

Indole: not produced

Nitrites: produced from nitrate

Starch: not hydrolized

Lipase: slightly positive

Methyl red: negative

Voges-Proskauer: positive

Citrate: used as sole carbon source

Hyrogen sulfide: not produced

Growth requirements: good growth on ordinary laboratory media, optimum temperature 30°C, ranged from 25°C to 37°C, poor at 10°C, no growth at 50°C.

Using this information, the organism was classified as _Aerobacter cloacae_ in the following manner:

Cells contain no photosynthetic pigment and reproduced by binary fission:

Class II. _Schizomycetes_.

Rod shaped, single, rigid cells, motile by peritrichous flagella.

Order IV. _Eubacteriales_.

Gram negative, straight rods, glucose fermented anaerobically with production of acid, frequently found in alimentary tract of vertebrates.

Family IV. Enterobacteriaceae.

Lactose fermented anaerobically, prodigiosin not produced, not parasitic on plants:

Tribe I. Escherichieae.

Alginic acid not decomposed, lactose fermented within 48 hours, acetoin produced, methyl red test negative, citric acid used as sole source of carbon, not encapsulated, isolated from fecal containing material:

Genus II. Aerobacter.

Glycerol fermented, with the production of no visible gas, gelatin liquefied:

A. cloacae .

Figure 6 shows the morphological appearance of the colonies of the organism as they appeared when grown 24 hours in the presence (200 ppm ADBAC) or absence of QAC. The striking rhizoid colonial growth induced by QAC is evident.

Effect of QAC on Pigmentation in Serratia marcescens. In addition to P. aeruginosa, Serratia marcescens also was used in preliminary experiments. In agreement with the work done by Bunting (9, p. 241-250), cultures of S. marcescens were found unable to produce red pigment in the presence of low concentrations of QAC (100 ppm) on TGY agar plates. Figure 7 shows the appearance of 24-hour old colonies on TGY agar plates in the presence and absence of 100 ppm QAC. Bunting concluded that the absence of pigmentation of S. marcescens in the presence of low concentration of detergents (0.05%)

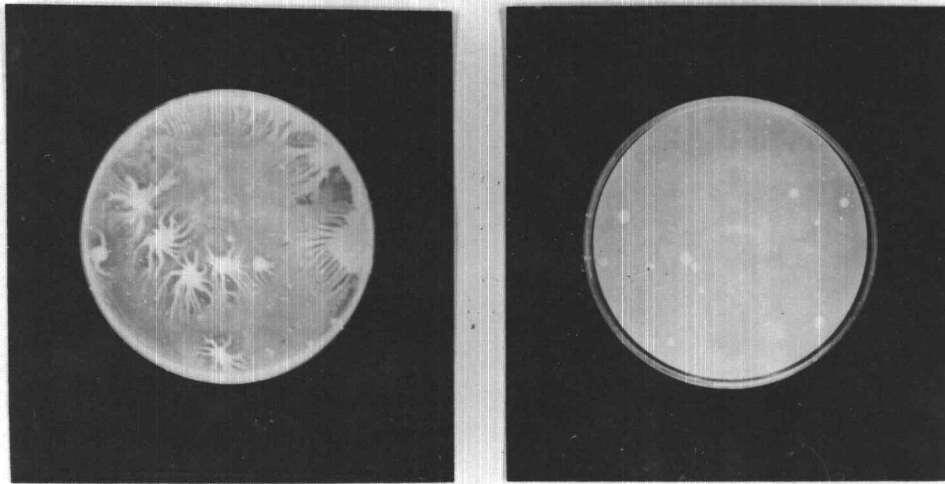

Figure 6. Rhizoid appearance of colonies of resistant organism when cultivated on TGY agar containing 220 ppm ADBAC (left) or on normal TGY agar (right)

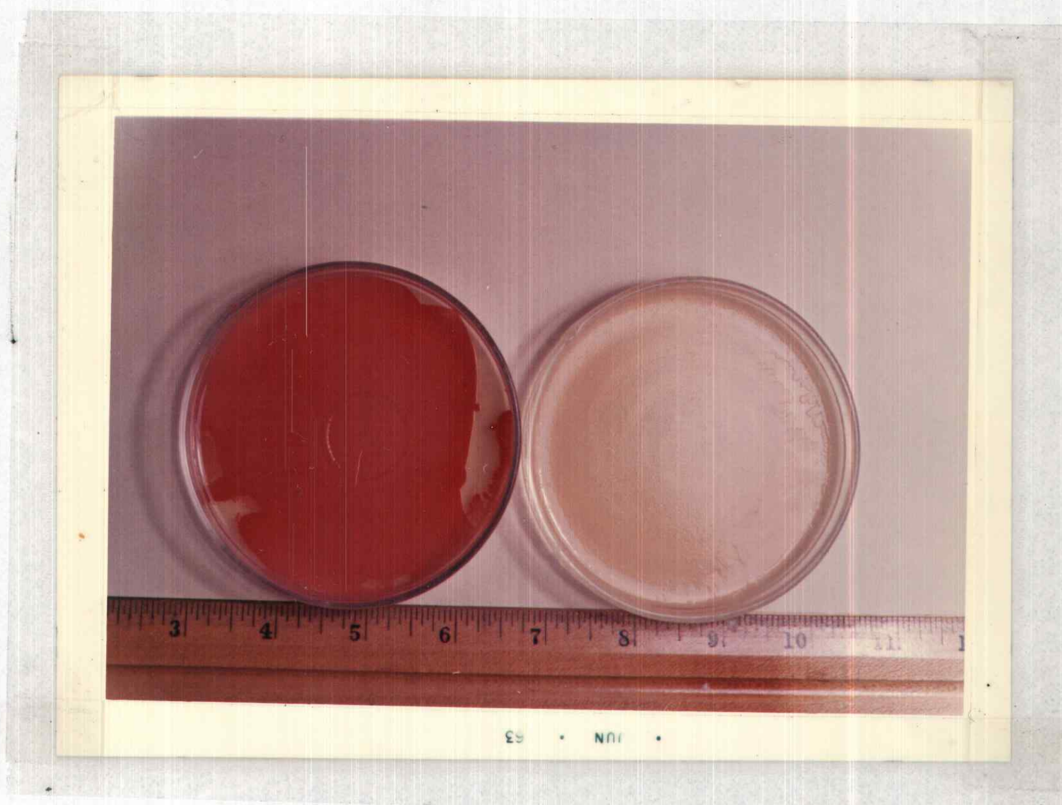

Fig. 7. Effect of QAC (100 ppm ADBAC) on pigmentation of <u>Serratia</u> <u>marcescens</u>. Plate at left shows growth on TGY agar without QAC while plate at right shows growth on TGY containing QAC. Plates were incubated for 24 hours at 30°C.

was due to the selective action of the detergent. Since the strain of *S. marcescens* used in the present study was highly susceptible to ADBAC, no further attempt was made to develop a resistant strain.

DISCUSSION

The data presented in this thesis are preliminary in nature and might be regarded as a small contribution to a long term research program to be continued by other workers on the mechanism of action of QACs on microorganisms. Several experimental approaches for future research are suggested by the results.

The ability of *P. aeruginosa* to mutate to QAC resistance was established by selection of survivors exposed to high concentrations of germicide. However, resistance was found to be lost by only a few transfers on TGY medium containing no QAC. This suggests that reversion to wild type occurs readily and that the sensitive parent types may grow better or have shorter generation times than the mutants. QAC-resistance of *P. aeruginosa* was demonstrated by various methods in this study. Table 1 and Figure 1 show, by different means, the striking difference in activity of QAC against QAC-resistant and sensitive strains of this organism. The activity indicated by percent kill in Table 1 appears greater than that shown by degree of lysis in Figure 1, even though the concentration of QAC in the latter case was five times greater than the former. The data in Table 1 represent the actual number of cells killed by QAC and the results in Figure 1 express lysis as the percentage decrease in optical density of a cell suspension over a period of time. The methods cannot be equated and thus it is not surprising that the apparent QAC action shown in Table 1 is greater than that shown in Figure 1, since cells killed by QAC may not necessarily have been lysed. From the two types of data it

therefore appears that destruction of both sensitive and resistant cells by QAC involves death which may or may not be followed by lysis of the cell.

There might be three stages involved in the death of a cell upon the addition of QAC to the medium: 1. Large amounts of cations are adsorbed and accumulate on the cell surface to form a thick zone; this accumulation prevents entrance of nutrients into the cell and growth of cells is inhibited. 2. The toxic cation layer outside the cell alters the permeability barrier allowing the surface active germicide to disrupt the cell wall and cell membrane; inactivation of cellular enzymes may also occur at this time. 3. Lysis of the cell occurs followed by the release of cellular materials into the medium. The ability of the QAC-resistant strain to survive this lytic activity (Figure 1) implies that the cell wall or cell membrane structure has become altered in some manner.

The QAC-resistant bacterium isolated from sewage sludge exhibited a high degree of resistance to the germicide; the organism grew well in the presence of QAC at levels as high as 10,000 ppm. Since both cultural and physiological characters of a given organism may be changed when it is grown in QAC-containing medium where development of resistance occurs (12, p. 453-458), bacterium A could have been a known species which had undergone some change. A preliminary taxonomic study undertaken early in this work indicated the organism may be a strain of _A. bookeri_. This organism was therefore obtained from the American Type Culture Collection and used for comparison to

the sludge organism. In addition to testing the QAC-resistance of this organism using both Black and Weber (50, p. 1406-1411) and spectrophotometric methods, the potentiating effect on the action of QAC by lysozyme was also investigated.

The activity of QAC against all organisms used in this study was enhanced by adding 0.025% lysozyme; the effect however was more easily demonstrated using the resistant strains. Action of lysozyme alone had little or no effect on the organisms as may be seen from the data in Tables 3, 4, 5 and 6. The potentiating effect of lysozyme may also be seen in these tables. The ability of lysozyme to break down the polysaccharides in the bacterial cell wall by hydrolyzing the 1-4β linkages of the glucosamine-muramic acid-polypeptide complex has been known for some time (8, p. 1783-1784). The lysis by lysozyme, however, requires that the enzyme can readily associate itself with the substrate. This occurs easily in many gram positive bacteria, especially Micrococcus lysodeikticus, but gram negative bacteria resist this association through an external lipid layer. The potentiating effect of lysozyme on QAC activity therefore suggests that a synergism exists between these two materials. Perhaps the QAC can penetrate the lipid layer, exposing the lysozyme substrate to the enzyme. This in turn allows the QAC to penetrate the cell and accelerate the lysis rate.

Data in Table 5 provide evidence supporting the idea that organisms resistant to QAC may establish permeability barriers which prevent the entry not only of germicide but other compounds. From the table

it may be seen that both sensitive organisms (P. aeruginosa (S) and A. bookeri) had higher rates of respiration in the presence of glucose than did the resistant cells. The endogenous respiration rates indicate further that this may be the case since the lower activity by the resistant cells may reflect their inability to store reserve materials again because of reduced permeability.

Dissipation of QAC by growing cultures of bacterium A was evident during the incubation period. Figure 3 shows that there was about 36.7% of the original concentration of QAC recoverable in the medium at the end of 35 days of incubation. The total amount of germicide dissipated (63.3%) appeared not to be due to adsorption by the cells as shown by the fact that the QAC could not be recovered from four successive washings of the cell mass. This suggested that the organisms could obtain needed carbon, nitrogen or energy from the QAC during growth. However, subsequent experiments showed that this organism was unable to utilize QAC in the medium as the sole source of carbon or nitrogen (Figure 4). It should be pointed out, however, that QAC molecules firmly adsorbed on the cell surface might prevent the total recovery of QAC despite repeated washings of the cell mass. Further research to explain the nature of the apparent degradation of QAC by the resistant bacterium therefore is warranted.

Since foaming is a well known indicator characteristic of surface active agents, the reduction of foaming ability also may reveal a decrease of QAC concentration during microbial growth. Results (Figure 4) showed that this apparently was the case for bacterium A,

though an alternative, that surface tension reducing agents were produced during growth, was not tested.

It was interesting that during this work an organism which was so highly resistant to QAC could be isolated with such relative ease. This suggests that this particular bacterium may be widely distributed in nature or that there are many types capable of mutating to QAC resistance. At any rate, it was important to attempt to classify this unknown organism. Gram and flagella stains were the tests upon which were based the placement of the unknown organism in the Order *Eubacteriales*. At the family level, the organism presumably belonged to the *Enterobacteriaceae* by virtue of its ability to ferment glucose anaerobically. Although a few organisms in the family *Achromobacteraceae* also ferment glucose anaerobically, they are restricted in habitat to fresh or salt water. Isolation of the unknown from sewage sludge suggested it was of intestinal origin and a member of the *Enterobacteriaceae*. The organism was able to ferment lactose anaerobically but did not produce prodigiosin, a pigment usually found in Tribe *Serratiaae*, thus the organism apparently was a member of either the *Escherichieae* or *Erwinieae* tribe. All of the seventeen species in the Tribe *Ewinieae* are plant pathogens and it seemed unlikely that bacterium A was a member of this group. Thus it was placed in the *Escherichieae* tribe and in the genus *Aerobacter* from results of IMViC tests, lactose fermentation and the inability to decompose alginic acid in neutral medium. The differentiation of the two species in the genus *Aerobacter* can be made by glycerol fermentation and gelatin liquefication. Bacterium A fermented glycerol within 48 hours

and liquefied gelatin, indicating that the organism is, or at least closely related to, Aerobacter cloacae. It should be emphasized however, that this is a tentative conclusion based on the assumption that the organism is of intestinal origin and not pathogenic for plants.

As was pointed out, results of this research suggest some logical experiments to test further the causes of apparent altered permeability in QAC resistant cells. One of these would involve a comparative study of the amount and type of lipid material in sensitive and resistant cells. Another would be to compare the fragility of protoplasts prepared from sensitive and resistant cells to QAC. A study may be made on the effect of QAC on isolated enzymes from sensitive and resistant cells. Attempts might also be made to alter QAC resistance by growing cells in media rich in different types of lipid precusors. The rate of incorporation of C-14 labeled acetate by sensitive and resistant cells may also reveal differences pointing to involvement of lipids in the mechanism of QAC resistance. Also it may be possible to show differences between sensitive and resistant cells in sensitivity to fat solvents and in ability to support bacteriophage absorption and replication. Finally it may be possible to observe in ultra thin sections under electron microscopy the nature of changes initiated in sensitive and resistant cells by QAC.

SUMMARY

A significant difference in activity of QAC against QAC-sensitive and resistant strains of _P. aeruginosa_ was demonstrated. In the presence of 200 ppm of QAC, viable cell counts revealed that 100% destruction of the sensitive strain occurred within five minutes exposure whereas the resistant strain was not affected under these conditions. The lysis of sensitive and resistant strains was observed when a high concentration of QAC (1000 ppm) was used. However, lysis of the sensitive strain was much more pronounced than that of resistant strain. The resistant strain also demonstrated less respiratory activity in the presence of glucose.

The germicidal activity of QAC was enhanced by adding lysozyme (0.025%) to the medium. Lysozyme alone had no effect on the organisms tested. Results indicated that the penetration of QAC through the cell wall and membrane was accelerated by the presence of lysozyme.

QAC in a growing culture of an unknown resistant bacterium isolated from sewage sludge was degraded 63% during 35 days of incubation. Results showed that the organism was unable to utilize QAC as sole source of carbon or nitrogen and therefore two suggestions were offered to account for the apparent breakdown: 1. QAC molecules were firmly adsorbed to the cell surface, 2. QAC was dissipated by an unknown mechanism within or around the cell.

The unknown QAC-resistant bacterium isolated from sewage sludge was classified as _Aerobacter cloacae_.

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