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Title: PROPERTIES OF PSEUDOMONAS AERUGINOSA RESISTANT TO QUATERNARY AMMONIUM COMPOUND

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Pseudomonas aeruginosa cells were selected for their ability to grow in the presence of 750 ppm alkyldimethylethylbenzyl ammonium chloride (QAC). These cells were found to retain their resistance to the germicide throughout tri-weekly transfers for 7 months in tryptone glucose yeast extract (TGY) broth containing no QAC. Comparisons of the resistant and sensitive cells were made in an attempt to define the mechanism of resistance and, in addition, to provide some information as to the mode of action of QAC.

The germicidal activity of QAC solutions against both sensitive and resistant cells in TGY broth was shown to be greatly affected by the concentration of tryptone and yeast extract, but not by the amount of sugar. The pH of the broth also influenced the germicidal activity; both strains were more susceptible under slightly acid conditions. A comparison of the pH range of growth of sensitive and resistant cells demonstrated the ability of the former to grow in TGY broth at pH 4.5

while the latter could not achieve growth at pH 5.0. A study of the effects of 50 ppm QAC, buffered to various pH levels, indicated that the susceptibility of resistant cells was nearly the same as that of sensitive cells below pH 3.0, at pH 6.0 and above pH 9.0. The greatest difference between the two cell types occurred from pH 3.5 to pH 4.5 with a second peak of resistance being observed from pH 7.0 to pH 8.5.

Electron microscopy revealed many dense inclusion bodies in the resistant cells, some being near 0.2 μ in diameter. Furthermore, the resistant cells were found to be much smaller (0.35 × 1.0 μ) than the sensitive cells (0.75 × 3.0 μ). Sensitive cells possessed single polar flagella while resistant cells were completely devoid of flagella. Micrographs of sensitive cells exposed to 100 ppm QAC exhibited no visible signs of lysis and the flagella did not appear to be disrupted. In contrast, 10,000 ppm QAC caused abrupt lysis of sensitive cells. A concentration of 1,000 ppm QAC had no visible effect on resistant cells.

Broth cultures of the resistant strain displayed a distinct fruity odor. Gas chromatographic analysis showed the QAC-resistant cells, unlike the sensitive, produced large quantities of ethyl acetate and ethyl valerate. Gel electrophoresis of cell-free extracts revealed a difference in total protein and esterase patterns between the two cell types. Two bands of esterase activity were demonstrated in

sensitive cell extracts while only one band was detected in the resistant cell extracts when alpha napthyl acetate was used as the substrate.

Biochemical tests disclosed numerous differences between the two cell types, many of which appeared to be interrelated. The most significant differences were the losses in the ability of resistant cells to synthesize extra-cellular lipase and protease enzymes. Many other biochemical tests on resistant cells were negative or became positive only after prolonged incubation. Permeability studies indicated a greatly reduced rate of glucose uptake by resistant cells. Furthermore, growth curve studies indicated a slower rate of growth by resistant cells and a 15 minute longer generation time.

Properties of *Pseudomonas aeruginosa* Resistant to Quaternary Ammonium Compound

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INTRODUCTION

Quaternary ammonium compounds are the most common cationic detergents. They have assumed a prominent role in recent years in the dairy and food industries as detergent-sanitizers. The features that make QACs so appealing to these industries include their noncorrosiveness, high solubility, relatively low toxicity, high stability and their germicidal activity. In addition, they are odorless and tasteless and do not convey off flavors to the food products when residual amounts are left on the processing equipment. They have also been shown to be bacteriostatic at concentrations much less than the minimum required for bactericidal action.

It has been demonstrated that QACs are viricidal, fungicidal, and bactericidal, especially against gram-positive organisms; gram-negative bacteria are less sensitive. Pseudomonas species, however, have been shown to be readily destroyed by QACs as are thermoduric bacteria.

In spite of their wide spread use, the mechanism of action of these compounds is not fully understood. It has been reported that the principal effect is that of cytolysis. Other studies indicate denaturation of enzymes to be the predominant factor while still

others have implicated cellular lipids.

Most studies involved with the elucidation of the mode of action of QACs approach the problem by treating bacterial cells, cell-free extracts or enzyme systems with QAC and recording the effect. This has been noted as loss of activity, loss of some cellular constituent or by measuring other changes. In contrast to this, a new approach was taken in the present study in an attempt to shed new light on an old problem. An attempt was made to isolate a strain of Pseudomonas aeruginosa that would carry out normal growth processes in concentrations of QAC many times greater than that required to kill the sensitive parent strain. The fact that the resistant strain could withstand QAC while the sensitive strain could not would demonstrate the presence of a "resistance mechanism" in the resistant strain. By observing the two cell types for cytological, morphological, enzymatic and nutritional differences it was believed that this "resistance mechanism" could be identified and, in turn, lead to a greater understanding of the mechanism of action of QAC.

REVIEW OF LITERATURE

Domagk (1935), the discoverer of sulfa drugs, was one of the first workers to study the properties of alkyldimethylbenzyl ammonium chloride. He found that 100 to 1,000 ppm was an effective bactericide against nine species of pathogenic bacteria and this stimulated wide spread research on quaternary ammonium compounds. Prior to Domagk's work, there was little interest in the germicidal activity of QAC. Einhorn and Gottler (1908) published the first report on synthesis and demonstration of the germicidal activity of QAC. According to Schwarz (1953), a QAC was prepared in 1911 by R. Hunt and R. deM. Taceau of the U. S. Public Health Service by the interaction of palmitic acid with choline chloride and cetyl trimethyl ammonium bromide was prepared by A. Reychler in 1913. Jacobs and co-workers (1916) followed with a series of papers on the bactericidal properties of quaternary salts of hexamethylene-tetraamine. In 1925 (according to Price, 1952) Hartman and Kagi of the Society of Chemical Industry of Basle, Switzerland, obtained a U. S. patent for certain of their cation-active compounds which they said had therapeutic value. Their reports, which indicated that these compounds possessed germicidal activity, attracted little attention.

Applications of Quaternary Ammonium Compounds

QACs are used extensively as skin antiseptics and as sanitizing agents in eating and drinking establishments, dairies and food processing plants. In the medical field, their use has been extended to preoperative procedures, instrument sterilization, urology otology, opthalmology, oralogy, dermatology, and wound irrigation. They also play an important role in cosmetics, oils, adhesives, dyeing, tanning, ore floation, mosquito control, poultry plants, laundries, and fish hatcheries. Lawrence (1950) provides a complete review of the uses of QACs with a very extensive bibliography of compounds used up to 1949.

Designations of Quaternary Ammonium Compounds

Due to the long chemical names identifying QACs, the following abbreviations are used to refer to the various compounds cited in this thesis.

alkyldimethylethylbenzyl ammonium chloride	QAC A ADEBAC
alkyldimethylbenzyl ammonium chloride	ADBAC Roccal Zephiran Zephirol
Cetyl pyridinium chloride	CPC Ceepryn
cetyl dimethyl ammonium chloride	CDBAC

Benzethonium chloride	BC Phemerol
Cetyl trimethyl ammonium bromide	CTAB

Preparation of Quaternary Ammonium Compounds

The cationic surface-active agents are derivatives of ammonia. When all three hydrogens of ammonia are replaced by hydrocarbon groups, the resulting compound is called a tertiary amine. Tertiary amines form salts with certain simple organic chlorine compounds, consisting of a chlorine atom attached to a short hydrocarbon chain, and are known as quaternary ammonium salts. It is possible to synthesize a tertiary amine in which one of the carbon chains corresponds in length to that of a carbon chain present in a fatty acid; such is the case with quaternaries formed from oleyl chloride and asymmetrical dialkyl ethylenediamine. These surface-active quaternary ammonium salts are often called "invert soaps" because their ionization is opposite that of soap (Niven, 1955; Price, 1952). Schwarcz (1953) described the preparation of QAC as follows: the appropriate phenol is reacted with an alpha, gamma-dihalopoly-alkylene ether, employing an alkaline condensing agent. The resulting product is reacted with dimethyl amine and the tertiary amine then obtained is converted to the desired quaternary ammonium salt by reaction with an alkyl or aralkyl halide.

In industrially prepared QACs the long aliphatic group is generally a mixture ranging from C_8H_{17} to $C_{18}H_{37}$. Reck and Harwood (1953) describes their QAC production as a process of catalytic hydrogenation of naturally occuring fats. The natural source of the fat giving rise to the alkyl group can cause variations in alkyl chain length and, in turn, variations in germicidal activity among different lots of commercial products. It can also affect the quality; for example, QACs derived from tallow are superior to those derived from coconut or soybean oil.

Properties of Quaternary Ammonium Compounds

The QACs are a class of compounds commonly referred to as "cationic detergents." They are surface-active halide salts of quaternary ammonium bases which possess the ability to increase the wetting properties of water by lowering its surface tension. They have the general formula:

$$\left[\begin{array}{c} R_1 \\ R_4\text{--}N\text{--}R_2 \\ R_3 \end{array}\right]^{+} X^{-}$$

where R_1, R_2, and R_3 represent hydrogen alkyl, aryl or heterocyclic groups of low molecular weight. R_4 represents a long chain alkyl group containing 8 to 18 carbons and is the hydrophobic portion of the molecule. The X represents an anion, usually chlorine or

bromine.

The properties that give QACs their use desirability are their germicidal activity, physical and chemical stability, detergent action, effectivity over a relatively wide pH range, detergent action, and very low toxicity. In addition, they are colorless, odorless, non-corrosive, non-irritating and their residue on food-processing equipment will not cause off-flavors.

One of the methods used to express the bactericidal activity of a compound is to compare its action with that of phenol against certain bacteria under standard test conditions. The ratio of the killing power of the product compared to that of phenol is referred to as the "phenol coefficient." Price (1952) reported that QACs have been made which have phenol coefficients as high as 600 and many commercial products have phenol coefficients of 200 to 400.

Heineman (1937) discussed the physical and chemical stabilities of QAC after prolonged storage. Dilute solutions of ADBAC showed no decrease in phenol coefficient when tested 14 months after their preparation. Dilute solutions were reported by Lawrence (1950) to withstand autoclaving at 126°C (20 pounds) for at least one hour without loss of bactericidal activity. He also observed that a 10 percent solution of ADBAC was stable for 10 years.

Except in highly specialized applications, cationic-surface-active compounds are poor detergents. They are incompatible with

soaps and anionic synthetic detergents. However, QACs do have high foam value and undoubtedly act together with water to emulsify and remove dirt. This same foam value, however, has caused difficulties in aeration tanks of sewage disposal plants.

Wetting ability is determined by the lowering of interfacial tension. Adams (1938) reported that the reduction of interfacial tension by QACs was not strictly a function of their concentration. Solutions of CPC at concentrations greater than 400 ppm have a surface tension of 37 dynes per cm. More dilute solutions initially have a high surface tension which decreases to about the same level as that of stronger solutions. A dilute QAC solution of 100 ppm requires at least a week to reach its final surface tension. However, this tension may be attained immediately by the addition of an inorganic salt. Longer chain QACs attain their final surface tension more rapidly. CTAB concentrations of less than 450 ppm attained a final surface tension of 25 dynes per cm. Unexplainably, stronger solutions never dropped below 30 dynes per cm. Thomssen and McCutcheon (1949) stated that while a surface tension of 30-35 dynes per cm is good, lower values are excellent and higher values are poor.

From the preceeding discussion, it is apparent that the main role of QACs is that of a germicide, sanitizer and wetting agent.

Mode of Action of Quaternary Ammonium Compounds

One of the leading theories to explain the germicidal action of a QAC is that it disrupts the cell wall and membrane which allows leakage of vital cell constituents. Baker et al. (1941) suggested that QACs caused cell membrane disorganization and denaturation of certain proteins essential for growth and metabolism. A release of nitrogenous and phosphorus-containing substances into the surrounding medium by cells treated with QAC was observed by Hotchkiss (1946). Gale and Taylor (1946, 1947) showed that an increase in the permeability of QAC-treated cells prior to lysis was followed by a release of lysine and glutamic acid. Salton (1950) demonstrated leakage of P^{32} into the suspending fluid when Staphylococcus aureus and P. fluorescens were grown in media containing P^{32} and then treated with CTAB. A release of purines and pyrimidines from S. aureus treated with CTAB was reported by Salton and Alexander (1950).

Mitchell and Crowe (1947) provided the first electron micrographs of detergent-treated bacteria. They observed leakage of amino acids from Streptococcus faecalis following cell wall rupture. Salton et al. (1951) used electron microscopy to demonstrate the marked cytoplasmic concentration that accompanied the leakage of cell solutes. Pictures taken by Dawson et al. (1953) indicated a

similar damage to the cells of S. aureus. They postulated that CTAB splits lipoprotein complexes in the cell or cell wall which in turn enables autolytic enzymes to react more uniformly and more rapidly throughout the cell.

Salton (1950) stated that CTAB is adsorbed on the bacterial cell in a typical Langmuir isotherm which is not greatly influenced by pH between 5.2 and 8.2. Following further research he concluded (1951) that the amount of CTAB taken up by S. aureus at saturation would occupy an area 15 times the area of the cell surface and that taken up by Bacillus pumilus would be 18 times the surface area of the cell. This indicated that CTAB may be adsorbed at sites other than those available at the cell surface, especially if molecules were held in a monolayer. Adsorption sites of CTAB located in the cytoplasm were suggested by McQuillen (1950).

While formulating a cell wall stain employing CPC, Dyar (1947) noted the ability of Sudan Black B to stain the cytoplasm. He interpreted this as a penetration by the surface active agent into the cell where the hydrocarbons were subsequently stained by the dye.

Valko and Dubois (1944) stated that the initial process in the action of surface active cations was reversible adsorption by the bacteria which act essentially as cation exchangers. However, these authors also felt that the surface activity of toxic cations was not an important factor in their antibacterial action; surface-active cations

would penetrate bacteria just as basic dyes do and thus it would be incorrect to regard their adsorption as surface phenomenon. Kivela (1948) proposed that the surface-active agents are adsorbed on the surface of the bacterial cells in sufficient concentration to interfere with the osmotic balance of the organism and its surrounding menstrum and in this manner prevent the intake of nutrients. Salton (1951) reported that the cell surface of CTAB-treated cells became hydrophobic and McQuillen (1950) suggested the possibility of some detergents forming concentric shells around a bacterial cell.

Stedman et al. (1957) believed that the contribution of lysis to cell death is of secondary importance. They observed that approximately 50 percent inhibition of the glucose-oxidizing system was obtained at a QAC level which caused less than 2 percent loss of cell contents. It appeared that generalized mechanical loss of nonspecific intracellular substances was not the primary lethal effect since a 10 fold increase in germicidal concentration resulted in a 1,000 fold difference in lethal action but only a 0.3 percent change in the amount of lysis. At high germicide concentrations lysis and cell leakage may be a significant factor while at lower levels of germicide, inhibition of energy yielding reactions appears more important.

Sevag and Ross (1944) stated that the action of zephiran on yeast cells resulted from the inhibition of the cytochrome C-cytochrome oxidase system. Knox et al. (1949) reported that Escherichia

coli lacks the system of Sevag and Ross, but whatever cytochrome system is present might well be located in the lactic acid oxidase complex. They stated that any action of the QAC on this complex (as is assumed in yeast) is apparently precluded in the E. coli lactic oxidase system because of the retention of QAC sensitivity even in the presence of cyanide. It was suggested that enzyme inhibition was responsible for metabolic inhibition, cell death and increased permeability.

Roberts and Rahn (1946) studied the activity of all energy-producing enzymes of E. coli grown in the presence of growth-retarding, growth-inhibiting and lethal concentrations of CPC and ADBAC. Growth inhibition occurred at a concentration of germicide that had little effect on dehydrogenase, catalase and oxidase activities. It was proposed that bacteriostatic concentrations usually do not interfere with enzyme activity. Hence the QAC must react with some other cell constituents essential for multiplication, either a synthesis step or a part of the cell division mechanism. Lethal concentrations of QAC inactivated both the oxidase and dehydrogenase systems almost entirely but had little effect on catalase. This lethal reaction was irreversible whereas the bacteriostatic reaction was reversible.

Sykes (1939) concluded that the viability of cells is affected at concentrations lower than those which inhibit bacterial metabolism. Baker et al. (1941) found that stearyl trimethyl bromide failed to kill

Proteus vulgaris at 30 times the concentration required for 85 percent inhibition of respiration. Miller and Baker (1940) made the interesting observation that the cationic detergents used in their studies inhibited the metabolism of both gram-positive and gram-negative organisms to the same degree. They also noted that the cationic detergents which were tested almost completely inhibited the metabolism of the test organisms at a concentration of 300 ppm. Most of the compounds were equally effective at 30 ppm.

Bacterial Resistance to Quaternary Ammonium Compounds

Miller and Bohnhoff (1950) expressed the view that the most prominent theory on the resistance of bacteria to antibacterial agents was that of selectivity; that is to say, the antibacterial agent is selective for resistant organisms already present in the culture by permitting their growth while halting that of non-resistant cells. The anti-microbial agent itself, in this case, would not play the role of an inducer.

Eagle, Fleirchman, and Levy (1952) favored the theory of adaptation. They stated that a large percentage of cells grown in a low level of anti-bacterial substance is more resistant to that substance than are cells of the parent culture. This is a result of an adaptation to the antibacterial substance or to a mutation which occurred during the course of cell growth and division in its presence.

Cells grown in the presence of higher levels develop a greater degree of resistance.

Chaplin (1952b) suggested the possibility that the production of lipid by bacterial cells affords resistance to QACs. He supported this contention by showing both the loss of resistance to QACs and the loss of intense staining by Sudan Black B following exposure to the activity of lipase. In addition, there was an increase in the ether-alcohol soluble fraction and a decrease in the electrophoretic mobility of the resistant cells. The cellular lipid would neutralize disruptive surface activities of the germicide by providing a protective surface coating. This would also explain the greater resistance possessed by gram-negative bacteria which contain more lipid and lipoprotein complexes than gram-positive cells.

Dyar and Ordal (1946) "fattened" cells of Micrococcus aureus by growing them in 3 percent glycerol broth with aeration. They found that the resistance to CPC had not changed although the electrophoretic mobility was slightly higher in the control cells.

Baker et al. observed that the addition of phospholipids, cephalin and sphingomyelin to living cells gave them protection against lethal effects of synthetic cationic and anionic detergents. However, they were ineffective unless added before or at the same time as the detergent. The phospholipids possess a characteristic polar-nonpolar structure and have an affinity for the bacterial cells

which is similar to the affinity of the detergent. At very low concentration they lower surface tension. It was suggested that the protection by phospholipid might be due to an alteration in the membrane structure, thereby preventing penetration by the detergents. Earlier, Baker et al. (1941a) found no inhibition of metabolism by lecithin even though it contains a hydrophilic-hydrophobic structure and a quaternary ammonium nitrogen in the choline portion of the nitrogen.

MacGregor (1958) found that P. aeruginosa growing on TGY agar plus 2,000 ppm QAC lost its resistance when treated with ethylene diaminetetracetate. He concluded that the explanation for the QAC resistance resided in the impermeability of the resistant cells to the germicide. Chaplin (1951) found that the rate of acquisition of resistance to QAC by bacteria was not exponential, suggesting that the mechanism involved is not the development of a single enzyme system. This author (1952a) also stated that he believed that the uneven distribution of resistance of normal individual cells and the irregular rate of acquiring further resistance indicated that the resistance is a product of adaptation. Between the levels of 4,000 and 10,000 ppm it is evident that some crisis occurs before complete tolerance to the disinfectant is achieved.

Difficulties in Working with Quaternary Ammonium Compounds

Klarmann (1946) recognized the difficulties encountered in obtaining consistent results with QAC. He also reported that bacteria treated with the QAC disinfectants have a tendency to attach themselves to solid surfaces rather than to remain uniformly suspended.

McCulloch (1947) reported difficulty in working with QACs because the agglutinating effect falsely reduces bacterial counts and a coating effect of QAC on the organism may prevent multiplication on agar. Davies (1949) listed two main sources of error in research involving QACs. First, he discussed the strong bacteriostatic effect leading to the false conclusion of cell destruction. The second source was concerned with the ability of QAC to cause bacterial cell clumping.

According to Tice (1945), bactericidal efficiency tests with QACs yield results that are rarely reproducible and an attempt was made by Pressman (1946) to eliminate some of the inconsistencies encountered in testing QACs. A glass slide technique was devised by Johns (1947) largely to avoid the objections of previous techniques employed for testing sanitizing efficiencies of QACs.

Pseudomonas aeruginosa

The species name aeruginosa is derived from the Latin

adjective, aeruginosus, which means full of copper rust or verdigris, hence green. The microorganism P. aeruginosa is commonly called the blue pus organism. It was first recognized in 1872 by Schroeter and given the name Bacterium aeruginosum. In 1882 the name was changed to Bacillus pyocyaneus and in 1900 Migula introduced the name P. pyocyanea. Today it is referred to as P. aeruginosa or P. pyocyaneus (Breed et al., 1957; Burrows, 1959).

P. aeruginosa has been found in pure culture in abscesses in various parts of the body. It has appeared as the sole organism responsible for some cases of endocarditis and pneumonia. Tillotson (1966) reported it as the cause of 18.4 percent of the pneumonia cases diagnosed in the 700 bed Detroit Receiving Hospital between July 1, 1963 and June 30, 1964, where the mortality rate was 72 percent. Curtin and co-workers (1961) conducted a study of the records of 88 patients who had had 91 bouts of pseudomonas bacteremia. The prognosis was poor and 73 deaths resulted. They stated that, in general, the associated disease state was the most important single factor affecting the outcome. P. aeruginosa has also been implicated in middle ear infections, dysentery, and a fatal form of pyocyanic infection. It possesses an arsenal of weapons including hemolysin, lecithinase, lipases, proteases, elastases, gelatinase, DNAase, mucolytic enzyme, fibrinolytic enzyme, toxic extracellular slime, and pyocyanin.

Johns (1947) declared 10 seconds exposure to 100 ppm ADBAC at 20°C would result in 99.9 percent cell destruction. His data indicated E. coli to be slightly more resistant than Pseudomonas species to QAC. The hypochlorites tested were more effective. According to Curtin (1961) polymyxin B was the most effective antibiotic against this bacterium. Burrows (1959) stated that streptomycin and the tetracyclines gave variable results while penicillin and erythromycin were totally ineffective.

Range of Antimicrobial Activity of Quaternary Ammonium Compounds

Bacteria, yeast, molds and protozoa are quite susceptible to QAC while virus appear to have limited susceptibility. In general, Quisno and Foter (1946) considered gram negative organisms more resistant than gram positive organisms, but it is very difficult to draw any definite conclusions from this since there is such a tremendous variation in susceptibility of different species with respect to pH. Smith et al. (1950) proved the surface-active compounds, including QACs, were not effective in killing Mycobacterium tuberculosis, though bacteriostatic action against the tubercle bacilli was reported by Quisno and Foter (1946). Davies (1949) stated that bacillus spores exposed to 10,000 ppm QAC for 3 days were not considered killed when the recovery medium contained

inactivator but were considered killed after one hour when the recovery medium did not contain inactivator. Likewise, Kivela, Mallmann, and Churchill (1948) demonstrated that Bacillus subtilis spores apparently killed by QAC can be revived by repeated washings. The sporocidal activity of QACs against Bacillus and Clostridium spores increase with alkalinity from pH 6.4 to 9.6 and with temperature increases from 30°C to 95°C, according to Curran and Evans (1950). However, the combination of 95°C and pH 9.6 failed to kill all spores after 30 minutes in dilutions of QAC up to 1:20. Davies (1949) indicated that the virulence of pathogenic bacteria was retained after exposure to QAC.

The effect of QAC on viruses also has received some attention. A 20 ppm dilution of zephiran did not interfere with the reproduction of staphylococcal bacteriophage and (according to Maier (1939)) the phage remained capable of reproduction after 3 months contact with the QAC in a refrigerator. Hammon and Reeves (1945) demonstrated the lack of viricidal activity of 100 ppm zephiran on Western equine and St. Louis encephalomyelitis viruses. In contrast, Klein and Stevens (1945) showed complete inactivation of influenza A virus in 60 seconds by 125 ppm zephiran. They obtained similar results with phemerol and cetyl pyridinium chloride. Likewise, Klein, Kalter, and Mudd (1945) reported 250 ppm zephiran, phemerol and ceepryn completely inactivated vaccinia virus, influenza A virus, S. albus

phage and Shigella paradysenteriae phage. However, they stated that 2,000 ppm was ineffective against gamma coli phage. Kalter, Mordaunt, and Chapman (1946) took advantage of this latter fact in designing a procedure for isolation of coli phage from sewage using cationic detergents.

Dunn (1938) claimed that zephiran was effective against a number of fungi. The destruction of species of Candida, Cryptococcus, Trichophyton, and Microsporium by less than 35 ppm ceepryn was shown by Quisno and Foter (1946). Knight and Frazier (1945) mentioned the lethal action of zephiran and also a second QAC against Penicillium notatum and P. chrysogenum. The ability of 2,000 ppm zephiran to destroy heavy spore suspensions of Tr. gypseum, and Mucor sp. in less than 5 minutes was reported by Hernenian (1937). In sharp contrast to previous reports, Klarmann and Wright (1954) proclaimed QACs to be "practically devoid of fungicidal capacity in any practical dilution with respect to such important microorganisms as Tr. interdigitale and Tr. rubrum." They observed very marked fungistatic activity and called for a revision in procedures employed for fungicidal assays. It was their opinion that a recheck of the experimental results of older publications and the conclusions based on them was in order.

Fair et al. (1945) found several QACs to be effective in concentrations of 10 ppm to 35 ppm against cysts of Endamoeba

histolyticum. Ceepryn was proven by Quisno and Foter (1946) effective against Trichomonas vaginalis.

In a study on preservation of bacteriophages, vaccines and venoms of rattle snakes and cotton-mouth moccasins, titers were not altered by a 1:50,000 dilution of zephiran. Neter (1942) reported that tetanus toxin was destroyed by 100 ppm zephiran.

Quantitative Tests for Quaternary Ammonium Compounds

Three general principles have been employed in detecting cationic surface-active agents: (a) absorptiometric measurement of a colored complex, (b) precipitation of an insoluble derivative with subsequent determination of the excess of the precipitating agent or a constituent of the precipitate, and (c) diphasic titration against an anionic surfactant using a dye-transfer method to detect the end-point. In an analysis of the methods in categories (a) and (b), Cross (1965) expressed the criticism that they generally require standardization by the particular surface active agent involved, a criticism which applies to (c) to a lesser degree. Due to what he termed "limitations and sometimes doubtful stoichiometry" of existing methods, he undertook an investigation of the use of sodium tetraphenylboron to determine cationic surfactants. He found that titration with tetraphenylboron is not only satisfactory for measuring the amount of QAC present but that it also provided an indication of their structure and

permitted quantitative analysis of certain mixtures. Metcalfe et al. (1965) outlined a procedure using the tetraphenylboron reaction as a basis for direct titration of QAC. This procedure has the advantage of a one-phase system (water), requiring no buffer system and utilizing an indicator, 2'7' dichlorofluorescein, with an extremely sharp end-point. This procedure is very simple and very accurate.

A procedure was developed by Furlong and Elliker (1953), based on the titration of QAC in a 1:1 mole ratio by a standard anionic surface-active agent, dicotyl sodium sulfosuccinate. This method proved very accurate in the range of 1 to 100 ppm QAC. Chin and Lach (1965) described a method based on the formation of picric acid-QAC complex in a basal medium with subsequent chloroform extraction of thecomplex; the absorbance of the chloroform phase was determined at 360 mμ. This method was reliable for concentrations as low as 10 ppm.

A number of other methods for the determination of QAC are found in the literature. Turbidometric determination of standard protein or standard anion precipitated by the QAC was proposed by Gain and Lawrence (1947) and Flanagan et al. (1948). Other methods include reaction with bromphenol (Auerbach, 1943), precipitation with potassium ferricyanide (Carkhuff and Boyd, 1954) and halide determination (Dubois, 1945). Reviews of earlier methods are provided by Lawrence (1950) and Miller and Elliker (1951).

Factors Influencing Activity of Quaternary Ammonium Compounds

Nature of Alkyl Chain

Epstein et al. (1943) concluded that the alkyl group of QACs containing a benzyl, pyridinium or trimethyl group should be a 12 to 16 carbon chain. Hoogerheide (1945) noted that as the length of the straight chain alkyl group of simple aliphatic quaternary ammonium compounds was increased from C_6 to C_{18} there was an increase in bactericidal activity. Similar observations were made by Valko and Dubois (1945). Jones et al. (1950) claimed that marked herbicidal activity of QAC was not realized until the decyl compound and that maximum activity appeared between C_{10} and C_{14}. Unsaturation in the aliphatic chain produced no detectable change in germicidal activity, according to Reck and Harwood (1953). Lawrence (1950) stated that, in general, CTAB, CTC and ADBAC were superior to more complex molecules. In working with twin chain compounds, Davis (1949) found that increasing the chain length from dihexyl dimethyl to didodecyl dimethyl increased the activity and, furthermore, the branched chain dinonyl derivative was similar in activity to the dehexyl.

Cella et al. (1952) suggested that the bactericidal activity of QACs is decreased by an increase of bulk around the nitrogen atom

and, correspondingly, activity is increased by groups which tend to increase the positive charge density of the QAC nitrogen atom or lower the critical micelle concentration.

Nature of Anion

The nature of the anion has little affect on the bactericidal properties of QACs. Shelton et al. (1946) proved there were no significant differences in the germicidal activity of chloride, bromide, iodide, nitrate, sulfate, methosulfate, acetate, benzoate, cyanide, hydrocinnamate, and fluosilicate salts of cetyl-trimethyl-ammonium. On the other hand, Hauser and Niles (1941) found the physical properties of QAC were affected by the nature of the anion. They considered surface tension to be dependent not on the cation alone, but rather that the anion determined its final degree. The chloride salt of QAC was shown to give the greatest degree of surface tension lowering, followed by bromide and then iodide.

Organic Matter

There seems to be some disagreement about the influence of organic matter on the activity of QACs. Hornung (1935) claimed that a 100 ppm (0.01%) QAC solution would have the same activity in the presence of 16 percent serum. Schneider (1935) reported only a 10 percent reduction in germicidal activity in the presence of 10

percent blood serum. Within 15 seconds in skim milk a 99 percent kill of M. pyogenes var. aureus was reported by Johns (1948).

In contrast, Neter (1942) indicated that zephiran was somewhat inhibited by infusion broth and that the inhibition was even more pronounced in the presence of human serum. Sporocidal activity was found by Curan and Evans (1950) to be greatly decreased by traces of skim milk. Quisno et al. (1946) reported that when four quaternary ammonium salts were tested in the presence of 0.2 percent agar, lethal concentrations were 3 to 6 times greater than lethal concentrations in the absence of agar.

Influence of pH

According to Baker, Harrison and Miller (1941), the inhibitory action of QAC was markedly influenced by hydrogen ion concentration. The maximum activity was exhibited in the alkaline ranges. This perhaps is the general trend but the results obtained by Soike, Miller and Elliker (1952) indicated that the bacterial species vary widely in their susceptibility with respect to pH. P. aeruginosa possessed increased resistance to QAC at pH 7 and above and was most susceptible at low pH levels; in contrast, Micrococcus caseolyticus was rapidly killed above pH 7 and was highly resistant at lower pH values. Escherichia coli exhibited peaks of resistance to QAC below pH 6 and at about pH 8, while it was highly susceptible at about pH 6

to 7. This confirmed earlier work by Salton (1950) which indicated that P. aeruginosa was more susceptible to QAC under acid conditions while M. pyogenes var. aureus was more susceptible under alkaline conditions.

Influence of Water Hardness

Numerous investigators (Lawrence, 1950) have indicated an interference in QAC activity from inorganic cations in water supplies. Mueller and Seeley (1951) observed that the interfering power of the mono-, di-, and trivalent cations was in the ratio of 1:100:10,000. They theorized that inactivation occurred because of competition with the QAC for sites on the cell surface. They arranged the following inorganic cations in decreasing order of interference: Al^{+++}, Fe^{+++}, Cu^{+++}, Zn^{++}, Ni^{++}, Mn^{++}, Ba^{++}, Fe^{++}, Mg^{++}, and Ca^{++}.

Toxicity of Quaternary Ammonium Compounds

Domagk (1935) observed little or no effect from repeated applications of 250 to 400 ppm zephiran to the conjunctiva of albino rabbits. Similarly, Maier (1939) demonstrated no effects after 10 days application of 250 ppm zephiran to the cornea of rabbits. Harshbarger (1942) reported that the incorporation of 3 percent zephiran in the food of rats resulted in no ill effects even after several months; in fact, five of seven pairs of rats showed weight gains

greater than their paired members in the control group. Based on the oral toxicity of QAC to rats, Botwright (1946) calculated that about 35 gallons of a 200 ppm solution would constitute a fatal dose for man. Accordingly, QACs possess the lowest toxicity of the known chemical germicides. Nasal toxicity for man has not been determined but Klein and Stevens (1945) revealed that mice exposed to a spray of 100,000 ppm (10 percent) zephiran for 15 minutes in a spray chamber were dead 10 minutes after their removal. Their lungs were extensively hemorrhagic, indicating rapid germicide penetration into all lobes of the lungs. They considered zephiran phemerol and cetyl pyridinium chloride to be highly toxic because one ml of a 1,000 ppm solution killed 20-gram mice. One human death ascribed to QAC was reported by Adelson (1952) as a result of accidental consumption of one ounce of a 100,000 ppm (10 percent) solution. Death resulted from asphyxia due to paralysis of the respiratory muscles and was similar to poisoning from curare.

MATERIALS AND METHODS

Bacterial Cultures

Cultures used in this study were obtained from the collection maintained in the Department of Microbiology at Oregon State University; from Lois Dickinson, Boots Drug Company in England; and from B. W. Holloway, University of Melbourne, Australia. A list of these cultures is given in Table 1. Strain QRM was used in all experiments unless stated otherwise. In future reference to cultures the letter "S" following strain numbers denotes sensitive cultures which have had no previous exposure to germicide, while the letter "R" denotes resistant cultures and the letters "RN" identify resistant cultures which have been exposed to germicide for two years and then transferred for 7 months in plain TGY broth (Table 2).

The sensitive strains were maintained in sterile (121°C for 15 min) TGY broth and the resistant strains were maintained in TGY broth containing 750 parts per million (ppm) germicide. The broth which contained germicide was termed TGY-QAC broth and, unless otherwise stated, contained 750 ppm germicide. Sensitive cultures were transferred three times a week while resistant cultures were transferred twice weekly. All cultures were incubated at 32°C.

Table 1. Pseudomonas aeruginosa strains used in present research

Strain	Source	Strain	Source
QRM	OSU collection	1C	B. W. Holloway
419	" "	2	"
9027	" "	3 met	"
C10	Lois Dickinson	271	"

Table 2. Composition of TGY Broth Medium

Ingredient	Amount per Liter
Tryptone	5.0 g
Glucose	2.5 g
Yeast Extract	1.0 g
Agar[a]	15.0 g
pH: 6.8-7.0	

[a] Added when solid medium for plating was desired.

Quaternary Ammonium Solutions

The quaternary ammonium compound Accoquat 1602 used in this study was supplied by Klenzade Corporation, a division of Economies Laboratory, Minneapolis, Minnesota. The chemical name for the compound is alkyldimethylethylbenzyl ammonium chloride and it will be referred to here as QAC (quaternary ammonium compound) or quaternary. The concentration of the stock solution was 51.5 percent. It was diluted with distilled water to the desired concentration in parts per million (ppm), and solutions were autoclaved 10 minutes under 15 pounds of pressure. Calculated

amounts were added to sterile media to give the desired concentrations.

Development of Resistance

Development of resistance to QAC was accomplished by inoculating a series of TGY broth tubes containing various concentrations of QAC. The test organisms were transferred after 7 days of incubation at 32°C. The source of inoculum for each transfer was that culture of highest QAC concentration in which growth occurred during the previous subculture.

Physical Effects of Quaternary Ammonium Compound

Effect of QAC on Distilled Water, TGY Broth and TGY Agar

Germicide concentrations ranging from 0 to 20,000 ppm were prepared in distilled water, TGY broth and TGY broth containing 30 g/l of tryptone. The solutions were mixed for 5 min and the pH determined using a Beckman Zeromatic pH meter. In a related experiment various concentrations of QAC were added to TGY agar and the agar was poured into petri dishes where the physical characteristics were then observed.

The effect of QAC on the absorbancy of TGY broth was determined by adding various concentrations of QAC, ranging from 5 to

515 ppm, to the TGY broth. The solutions were mixed for 2 min with a Vortex mixer and the absorbancy at 520 mμ was determined using a Bausch and Lomb Spectronic 20 spectrophotometer.

Effect of Bacterial Cells on the pH of QAC Solutions

Late log phase cells of P. aeruginosa QRM-S and QRM-R were washed twice with distilled water, resuspended as a very heavy slurry and a viable cell count was determined on this heavy cell suspension. Beakers were then prepared in duplicate containing 0, 100, 515, and 10,000 ppm QAC in 25 ml of Distilled water. The pH was determined for each germicide concentration. The change in pH of the QAC solutions was followed with the addition of aliquots of cell suspensions; the addition of cell suspension was followed by 1 min of mixing.

Effect of Temperature and Aeration on QAC Resistance

Eight series of test tubes were prepared containing progressively greater concentrations of QAC in 10 ml of TGY broth. Four series of tubes were inoculated with a 0.5% late log culture of QRM-S from TGY broth plus 500 ppm QAC. One series of each type of cells was placed at 37°C on a reciprocal shaker while a duplicate series remained stationary at 37°C; this was repeated at 21°C. After 7 days the tubes were assayed for growth by spotting on TGY agar plates and incubating at 32°C for 48 hr. The experiment was

repeated using P. aeruginosa strains 419, 9027, C10, 1C, 2, 3 met, and 271.

Physical Effects of QAC on Cell Suspension

Sensitive and resistant cells were grown to the late log growth phase in 250 ml of TGY broth and precipitated by centrifugation at 4,000 rpm for 10 min in the type GSA rotor of the Servall RC2 refrigerated centrifuge. The cells were resuspended in flasks containing 100 ml of sterile distilled water and 1:100 dilutions were made by transferring one ml from these flasks to a second set of flasks containing 99 ml of sterile distilled water. Sufficient QAC was added to each flask to give a final concentration of 1,250 ppm. The flasks were swirled briskly for 30 sec and allowed to stand at room temperature for observation.

Death Curves in 260 ppm QAC

P. aeruginosa QRM-S and QRM-R cells were grown to stationary phase in TGY broth and TGY broth plus 515 ppm QAC, respectively. Both cell types were precipitated by centrifugation and resuspended in 50 ml of sterile physiological saline solution. An initial plate count was made on the cell suspensions using TGY agar and the plates were incubated at 32°C for 48 hr. After removing an aliquot for plating, 50 ml of a 515 ppm QAC solution

was added to each cell suspension to give a final germicide concentration of 260 ppm. The number of viable cells was determined after 1, 5, 10, 15, 30 and 60 min.

Disc Assay Method for QAC Resistance

Petri dishes were poured with 8 ml of TGY agar. One ml of a 1:10 dilution of late log phase cells of P. aeruginosa QRM-S was added to 6 ml of TGY agar (1 percent agar) and thoroughly mixed. The seeded agar was then used to overlay the TGY agar plates, and the agar was allowed to solidify on a level surface. Plain unimpregnated discs, Bacto 1599, were soaked in various concentrations of QAC. The excess solution was absorbed on filter paper and the discs were placed flat side down on the agar surface. The plates were incubated at 32°C for 36 hr and observed for zones of inhibition around the discs.

Effect of Growth of QRM-R in the Absence of QAC

P. aeruginosa strain QRM-R was transferred tri-weekly for 7 mo in TGY broth with no QAC, and this culture was referred to as QRM-RN. The level of resistance, as well as the effect of pH on the QAC resistance, was compared between QRM R and QRM-RN. The QRM-RN culture was subjected to cultural, biochemical and cellular characterization. The results are given in other sections.

Cultural Characteristics

Colony morphology and cultural behavior of the tests organisms were determined using TGY agar plates, TGY agar slants and TGY agar plates containing various concentrations of QAC. Colonies on agar plates were examined under the low power objective of a Bausch and Lomb microscope. Pictures were taken using a 35 mm Olympus PM b camera.

Growth Curve Studies

Generation Times

P. aeruginosa strains QRM-S, QRM-R, and QRM-RN were grown to late log phase and diluted 1:100 in sterile distilled water. One ml of the dilution served as the inocula for one liter quantities of TGY broth in 2-liter stainless steel capped culture flasks. After determining the initial cell count using TGY agar, the flasks were incubated at 32°C on a rotary shaker.

Effect of QAC on the Growth Curves

Strains QRM-S and QRM-RN were grown to late log phase, diluted to 1:100 and one ml of culture was added to 1 liter of TGY broth in a 2-liter culture flask. Strain QRM-R was grown to late log phase in TGY broth plus 515 ppm QAC, diluted 1:100 and 1 ml of

dilution was used to inoculate 1 liter of TGY broth containing 515 ppm QAC in a 2-liter culture flask. The initial count was determined using TGY agar and the flasks were placed on the rotary shaker at 32°C. After 14 hours QAC was added to all three flasks to provide a final concentration of 515 ppm in the QRM-S and QRM-RN flasks and 1030 ppm in the QRM-R flasks. Counts were determined immediately before the addition of QAC and after one minute of agitation following the addition of QAC.

Effect of Low Temperature, Shaking and QAC on Growth

In an experiment similar to the previous two above, QRM-S and QRM-R cells were grown to late log phase and diluted 1:100. Again 1 ml of the dilution was used to inoculate 1 liter of TGY or TGY-QAC broth in a 2-liter culture flask. QRM-S and QRM-R were inoculated into TGY broth and placed on a reciprocal shaker at 4°C. Likewise, QRM-R was inoculated into TGY broth containing 515 ppm QAC and placed on the reciprocal shaker at 4°C. QRM-R was also inoculated into TGY broth plus 515 ppm QAC and held static at 4°C. A flask containing TGY broth plus 515 ppm QAC was inoculated with QRM-R and placed on the rotary shaker at 32°C. Plate counts were determined on TGY agar. Following 120 hr of incubation all flasks were placed at 32°C on the rotary shaker and the plate counts continued for 60 hr.

Influence of Media on Resistance

Comparison of Broth with Agar

Progressive serial concentrations of QAC were prepared in TGY agar, in TGY broth and in Hunters semisynthetic medium, as described by Stanier et al. (1966). A comparison was also made between two methods of inoculating agar slants. First, slants were inoculated by loop transfer from a previous slant; second, the surface growth of a slant culture was suspended in TGY broth and one drop of the broth suspension was used to inoculate each of the tube series of TGY-QAC agar slants. The broth tubes were inoculated with one drop of culture.

Influence of Increased Tryptone Content of TGY Broth

Tubes of TGY broth were prepared containing 0.5, 1.0, 2.0, and 3.0 percent tryptone. A serial concentration of QAC was prepared at each concentration of tryptone. Three resistant strains, QRM-R, 3 met-R and 419-R, were inoculated into the tubes. The tubes were incubated 7 days at 32°C and assayed for viable cells by spotting on TGY agar.

Influence of Glucose Concentration

TGY broths were prepared containing 0.1, 0.5 and 3.0 percent

glucose. Each broth was used in the preparation of a serial concentration of QAC. The tubes were incubated at 32°C for 7 days. They were then assayed for the presence of viable cells by spotting on TGY agar.

Resistance in Milk

A serial concentration of QAC was prepared in tubes of reconstituted skim milk containing 11 percent total solids. Identical QAC concentrations were prepared in TGY broth containing 11 percent total solids. The tubes were inoculated with late log phase cells and assayed for viable cells by spotting on TGY agar after 7 days of incubation at 32°C.

Effect of Environmental Conditions on Growth

Temperature Relationship

The ability of the test organisms to grow at 0°, 4°, 10°, 25°, 32°, 42° and 50° was established by streaking TGY agar plates with late log phase cells and incubating them at the proper temperature. The plates were observed for colony formation between one and fourteen days.

Salinity Tolerance

Tubes of TGY broth containing a total of 0, 3.0, 5.0, 6.5, 7.5 and 10.0% sodium chloride were inoculated and observed for growth.

pH Range of Growth

TGY broth adjusted to pH 4.5, 5.0, 5.7, 6.5, 7.2, 8.0, 9.0 and 10.0 with HCl or NaOH (as required), were inoculated and observed for growth.

Effect of Washing on QAC Resistance

Late log phase cells of P. aeruginosa QRM-S and QRM-R were washed 5 times in cold, sterile physiological saline solution. A series of trypticase soy broth tubes were prepared containing progressively greater concentrations of QAC. One-tenth ml of washed cell suspension was used to inoculate the tubes and the resistance was compared to that of unwashed cells inoculated into identical concentrations of QAC.

Biochemical Characteristics

Pigment Production

The ability of the test organisms to produce the water soluble phenazine pigment, pyocyanine, and the fluorescent pigment,

fluorescein, was determined by using two complex media, A and B, developed by King, Ward, and Raney (1954).

Hydrolysis of Casein and Gelatin

Casein hydrolysis was determined by inoculating bacterial masses onto TGY agar plates containing 10 percent reconstituted skim-milk. Gelatin liquefaction was determined by stab inoculation into gelatin deeps.

Aesculin Hydrolysis

The hydrolysis of aesculin was determined by observing for the appearance of a brown zone around implanted bacterial masses on the medium of Sneath (1956).

Production of Acetic Acid from Ethanol

The method of Shimwell, Carr and Rhodes (1960) was used to assay for the production of acetic acid from ethanol.

Oxidation of Calcium Lactate through Acetate to Carbonate

This was studied on the medium devised by Shimwell, Carr, and Rhodes (1960).

De-amidation of Acetamide

The method outlined by Buhlmann, Vischer, and Bruhin (1961) was employed to determine the de-amidation of acetamide.

Dihydroxyacetone Accumulation

The glycerol containing medium of Shimwell, Carr, and Rhodes (1960) was used to determine the production and accumulation of dihydroxyacetone.

Cytochrome Oxidase and Oxidase Formation

The presence of cytochrome oxidase and oxidase was tested for by the methods outlined by Gaby and Free (1958) and Kovacs (1956), respectively.

Arginine Dihydrolase

The arginine test medium was prepared and inoculated according to the method of Thornley (1960).

Gluconate Oxidation Test

Oxidation of gluconate was determined by incubating a heavy suspension of test organisms in a test tube containing one ml sterile distilled water plus 1 g of gluconate. After 24 hours at 32°C, the

presence of reducing sugar was determined by adding Benedicts solution according to the procedure of Haynes (1951).

Urease Production

The production of ammonia in a medium which contained 0.1 g yeast extract, 9.5 g Na_2HPO_4, 9.1 g KH_2PO_4, 0.01 g phenol red and 20 g urea per liter was recognized by an alkaline shift in pH and was accepted as a positive urease test.

Starch Hydrolysis

Starch hydrolysis was determined by inoculating bacterial masses on TGY agar plates containing 0.2 percent starch. After 5 days of incubation at 32°C the plates were flooded with a saturated iodine solution in 50 percent alcohol. A clear zone surrounding the bacterial growth indicated starch hydrolysis.

Nitrate Reduction

Test for nitrate reduction was accomplished using the media recommended by the Manual of Microbiological Methods (1957).

Utilization of Citrate as Sole Carbon Source

The medium of Koser (1932) was inoculated with one loopful of culture and observed for growth upon incubation at 32°C.

Indole Production

Test organisms were inoculated into broth containing 1 percent tryptone and 0.5 percent sodium chloride. After 48 hours of incubation at 32°C the Kovac's test outlined in the Manual of Microbiological Methods (1957) was used to assay for indole production.

Methyl Red and Voges-Proskauer Reaction

The basal medium for these tests was prepared as described in the Manual of Microbiological Methods (1957). The cultures were incubated at 32°C.

Lecithinase

The presence of lecithinase in the test organisms was shown by the procedure of Colwell (1964).

Catalase

Catalase was tested for by adding 3 ml of a 10 percent solution of hydrogen peroxide to 24 hour TGY agar slope cultures.

Lipolytic Activity

Inoculation of bacterial masses on to spirit blue agar (Starr, 1941) was used to determine lipolytic activity.

Proteinase Assay

The level of proteinase activity in cell free enzyme extracts of P. aeruginosa QRM-S and QRM-R was measured using Azocoll (Calbiochem, n.d.) as the substrate. Various dilutions of enzyme were made in 5 ml of buffer, then 25 mg of Azocoll were added. This mixture was incubated at 37°C with constant swirling for 15 minutes and the reaction was terminated by filtering through Whatman No. 1 filter paper with gravity flow which removed the substrate from the reaction mixture. The A_{580} was determined on the filtrate using appropriate blanks and the values then used as a measure of proteinase activity.

Protein Determination

The method of Lowry et al. (1951) was used to determine the protein concentration of enzyme preparations. The protein concentration was determined by multiplying the absorbancy of the protein assay mixture at 500 mμ by a milligram extinction ($1/E_{cm}^{mg} = 0.599$ mg^{-1} cm^{-1}). This extinction coefficient was calculated from a standard curve of various concentrations of bovine serum albumin assayed by the Lowry method. The protein concentration was expressed as mg per ml.

Lipid Studies

Lipid Staining

Smears of QRM-S, QRM-R, and QRM-RN cells were stained with Sudan Black B and observed microscopically for sudanophilic material, according to the procedure of Burdon (1946).

Presence of poly-β-hydroxybutyric Acid

Lyophilized cells of the sensitive and resistant strains were extracted with hot chloroform. The chloroform extract was filtered into 3 volumes of ether and observed for the formation of a flocculent white precipitate which according to Forsyth et al. (1958), is indicative of poly-β-hydroxybutyric acid.

Influence of Fattening on Resistance

The sensitive and resistant strains were transferred and maintained for 15 weeks in TGY and TGY-QAC broth, respectively, with three percent glycerol. Both strains were then inoculated into a series of TGY broth tubes containing progressively greater concentrations of QAC as well as 3% glycerol. After one week of incubation at 32°C the tubes were assayed for viable cells by spotting on TGY agar plates.

Total Lipid Content

P. aeruginosa QRM-S cells were grown in TGY broth for 24 hr on a rotary shaker at 32°C. P. aeruginosa QRM-R was grown in TGY broth plus 260 ppm QAC for 30 hr. The cells were removed from the broth by centrifugation, washed three times in cold sterile physiological saline solution and then lyophilized. The lyophilized cells were weighed into 15 ml Konte flasks and about 10 volumes of chloroform:methanol (2:1) were added. Water-cooled condensers were connected to the flasks and they were refluxed for 30 min in a 75°C water bath. The contents of the flasks were then transferred to centrifuge tubes. The tubes were rinsed carefully 3 times with chloroform:methanol in order to remove all the cell debris. The cell material was precipitated by centrifugation at 14,000 rpm for 30 min using the rotor of the Servall RC2 centrifuge. The solvent was then evaporated and the solvent-extracted cells were weighed and the lipid content expressed as percent of dry weight.

Effect of Possible Lipid Precursors on Resistance

P. aeruginosa strains QRM-S and QRM-R were subcultured in appropriate medium containing one of the following: acetic acid (0.2 percent), sodium acetate (0.2 percent), sodium citrate (0.2 percent), sodium pyruvate (0.1 percent), and acetone (100 or 1000

ppm). Each culture was then used to inoculate a series of tubes of TGY broth containing both progressively greater concentrations of QAC and the appropriate additive. The tubes were incubated at 32°C for 7 days and assayed for viable cells by spotting on TGY agar plates.

Effect of Treatment with Lipase

Washed cells of QRM-S, QRM-R, and QRM-RN strains were incubated for one hour in the presence of 0.1 mg/ml lipase. The cells were then used to inoculate a series of TGY broth tubes containing progressively greater amounts of QAC. The tubes were incubated 7 days at 32°C and observed for turbidity or pellicle formation. Negative tubes were spotted on TGY agar plates to check for viable cells.

Gas Chromatography

Late log cells of *P. aeruginosa* QRM-S were inoculated at the rate of 1 percent into 20 ml of TGY broth in large screw-capped test tubes and incubated on a reciprocal shaker for 24 hr at 32°C. *P. aeruginosa* QRM-R was inoculated into both TGY broth and TGY broth plus 500 ppm QAC and incubated for 48 hr under the same conditions as the sensitive strain. The cultures were placed in the refrigerator prior to analysis.

Gas-Entrainment on Column Trapping

The method used was a modification of the gas-entrainment on-column trapping technique developed by Morgan and Day (1965). Eight ml of samples were pipetted into screw capped vials (Kimble no. 60957, size no. 1) containing sufficient granular anhydrous Na_2SO_4 to saturate the aqueous portion of the sample; a few milligrams of 1-tetradecanol were added to control foaming. The original caps were replaced with ones in which two-5/32 inch holes 7/16 inch apart had been drilled, and the original liners were replaced with a 1/8 inch thick silicone rubber septum which had been cut with a no. 15 cork borer from sheet stock of Dow Corning Silastic. The vials were shaken for 15 seconds on a high speed vortex shaker.

The entrainment assembly is shown in Fig. 1. The hypodermic needles were 16 gauge without hubs. The tops of the needles were fitted with 1/16 inch Gyrolok ferrules and nuts and were attached to the Tyrolok 1/8 to 1/16 inch reducing unions which were welded to the support plate. Both needles were 22 gauge, the one on the column end was one inch long and the other one was 2 1/2 inches long. A clean, empty vial was clamped in place to shield both needles.

The column in which a U-shaped loop (3 in. deep) had been formed just beyond the column fittings was connected to the

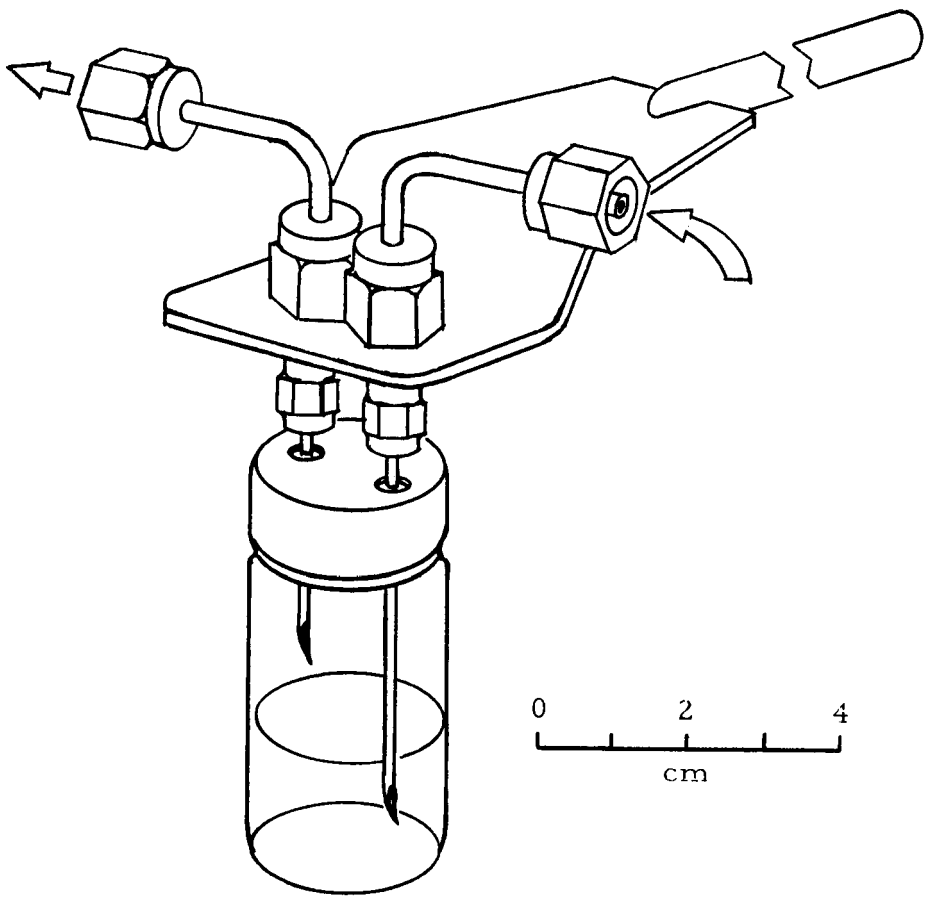

Figure 1. Details of the entrainment assembly.

entrainment apparatus. A Dewar flask containing a slurry of dry ice in 2-methoxyethanol was raised to immerse the trap-loop portion of the column. When the trap was cooled the shielding vial was removed and the septum on the sample vial was punctured by both needles, with resulting insertion to their full length. A small 50°C waterbath was raised in order to immerse the sample vial and needle assembly to the horizontal slits on the sides of the Luer-Lok fittings. Nitrogen was allowed to bubble through the sample for 5 minutes, carrying the entrained volatiles into the column trap where they were condensed on the column packing. The assembled trapping set up is shown in Fig. 2.

The waterbath was then lowered and the sample vial was pulled from the needles. In rapid succession the column was disconnected from the entrainment assembly, connected to the injection port and the oven was closed. After allowing a few moments for the oven temperature to equilibrate, the carrier gas was turned on. The chromatogram was developed and recorded in the usual manner.

In these studies a 12 ft by 1/8 inch stainless steel column packed with 60 to 80 mesh acid and alkali washed celite coated with 20% 1, 2, 3-tris (2-cyanoethoxy) propane was used. The flow rate of the nitrogen carrier gas had been adjusted to 24-30 cc per minute at a column operating temperature of 70°C. An F and M model 810 gas chromatograph was used.

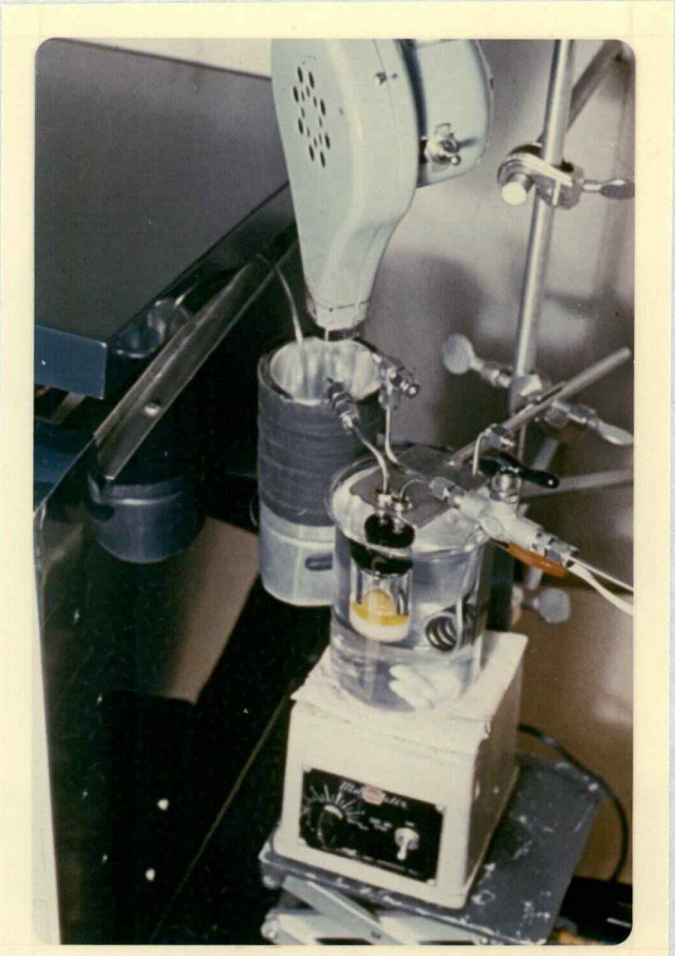

Figure 2. Assembled on-column trapping system.

Gel Electrophoresis

Gel Preparation

The apparatus used in this study, as well as the techniques employed, were described by Davis (1964).

Sample Preparation

Late log phase cells of the sensitive and resistant strains were used to inoculate two liters of TGY broth at the rate of 0.1 percent. The cultures were incubated for 48 hr on a 32°C rotary shaker. The cultures were then centrifuged at 5,000 rpm for ten minutes in the type GSA rotor of the RC2 Servall refrigerated centrifuge and resuspended in a few ml of sterile distilled water to make a heavy slurry.

Approximately 2.5 ml of cells were placed in a homogenator container and 7.5 g of glass beads were added. The container was then placed in a Braun Model MSK mechanical cell homogenizer (Bronwill 2876) for one minute. The resulting slurry of ruptured cells was centrifuged for ten minutes at 2,000 rpm and the supernatant recovered and placed in ice until it could be layered on the top of gel columns.

Two hundred μl of various proportions of large pore gel-sample mixtures were layered on top of gel columns and

photopolymerized by placing a fluorescent light at a distance of 3 in. The tubes were then placed in the electrophoresis apparatus. Electrophoresis was allowed to proceed for 30 min or until the bromphenol blue indicator band migrated to within one centimeter of the bottom. The gels were then removed from the electrophoresis tubes.

Total Protein

The protein bands were stained by placing the gels in small test tubes containing about 2 ml of fixative-stain solution. This solution was made by dissolving 1 g of Amido Schwarz (Naphthol Blue Black) in 100 ml of 7 percent acetic acid. The gels were destained by electrophoresis and transferred to small test tubes containing 7 percent acetic acid.

Esterase

The presence of aromatic esterases in sensitive and resistant cells was determined by slightly modifying the procedures of Markert and Hunter (1959) and Hunter and Maynard (1962). Following electrophoresis of ruptured cell slurries, the gels were rinsed with distilled water and placed in small test tubes containing 0.2M tris-maleate buffer, pH 5.9. Five mg of α-naphthyl acetate were added to a test tube containing 0.5 ml acetone. The tube was agitated

and then 10 mg of Fast Blue RR, dissolved in 20 ml of 0.2 M tris-maleate buffer, pH 5.9, was added. This substrate-diazonium salt solution was poured over the gels and incubated in the dark at room temperature until bands appeared. The gels were then rinsed and stored in a solution of water:7 percent acetic acid:methanol (3:3:2).

Permeability Studies

Glucose Uptake

Sensitive cells were grown in TGY broth for 14 hr and resistant cells were grown for 18 hr. A one percent inoculum was then made into TGY broth and the cultures were incubated on a rotary shaker at 32°C, the sensitive cells for 6 hr, the resistant cells for 8 hr. The cells were centrifuged and washed twice with pH 7.0 sodium phosphate buffer (0.05 M) to which 50 μg per ml chloramphenicol had been added. The cells were resuspended in flasks containing 20 ml of buffer. These flasks and an additional flask containing 3 mg glucose per ml were placed in a 37°C water bath. Once the temperature had equilibrated, 6 ml of cells were mixed with 3 ml of glucose solution. At 0, 15, 30 and 60 min a 0.4 ml aliquot was removed and diluted to 4 ml with buffer. The cells were then centrifuged and the supernatant was retained for glucose assay, according to the procedure of Dische (1955).

Effect of Toluene and DMSO on Resistance

Sensitive and resistant cells were grown to the late log growth phase, centrifuged, washed once and re-suspended in physiological saline solution containing 100 ppm of toluene. After 10 minutes of continuous shaking the toluene was allowed to rise to the surface and the underlying cell suspension was used to inoculate a series of TGY broth tubes containing increasing concentrations of QAC. Following 7 days incubation at 32°C the presence of viable cells was determined by spotting on TGY agar.

Serial concentrations of QAC were prepared in TGY broth and TGY agar, both containing 5 percent dimethyl sulfoxide. The media were inoculated with QRM-S and QRM-R cells and incubated 7 days at 32°C. The media were assayed for the presence of viable cells by visual observation. In addition the TGY broth tubes were spotted on TGY agar.

Infrared Spectroscopy

Sensitive and resistant cells were grown until the late log phase of growth in both TGY broth and TGY-QAC broth. The cells were collected by centrifugation and washed 5 times in 0.2 M potassium phosphate buffer, pH 7.0. A dense homogenous suspension of each cell type was carefully prepared. One-fourth ml of cell suspension

was pipetted onto silver chloride discs. The cells were then worked back and forth several times to obtain a uniform film. The samples were air dried in a tilted position to obtain a gradient in the film. Since the silver chloride discs are light sensitive, the films were air dried in a dark box. The discs were mounted in special holders for recording.

The instrument used in these studies was a Beckman IR5 double Beam Recording Infrared Spectrophotometer. Standardization and proper functioning of the instrument was accomplished by recording the infrared spectrum of a polystyrene film standard. The instrument was then advanced to 8.00 microns. The silver chloride disc with the gradient of dried cells was manipulated until the transmission recorded exactly 50 percent. The disc was then secured in this position and the instrument reversed to two microns and scanned through 2-16 microns. The disc was readjusted to 50 percent at 6.00 microns and rescanned.

Cytology of Sensitive and Resistant Cells

Light Microscopy

Smears of late log phase cells of P. aeruginosa strains QRM-S and QRM-R were dried on microscopic slides and gram-stained, according to the procedure of Hucker (Society of the American

Bacteriologists, 1957). The stained cells were observed under oil immersion. The cultures were also stained to determine capsule formation, according to the procedure of Hiss (Society of American Bacteriologists, 1957). The motility of young agar cultures was determined by the hanging drop technique with a phase microscope.

Electron Microscopy

Intact Cells

Test organisms used for the comparison of cell morphology were handled as follows:

Strains QRM-R, QRM-RN and QRM-S were streaked on TGY agar. After 48 hr of incubation at 32°C, the plates were tilted and several drops of sterile distilled water were dropped gently onto the bacterial growth. Copper grids with a supporting formvar film were touched to the surface of the liquid and the excess moisture withdrawn by touching the edge of the grids to a piece of filter paper. The formvar solution was made by adding 0.5 percent Formvar E (Shawinigan Products Company, 350 5th Avenue, New York, New York) to ethylene dichloride. The copper grids (Ernest F. Fullam, Inc., P. O. Box 444, Schenectady, New York) were 200 mesh.

The grids were shadowed at an angle of about 30° with an alloy of platinum:palladium (4:1) which has a density of 19.4. A white

porcelain slide with an oil droplet in the center was laid beneath the filament to permit observation of the buildup of evaporated metal. A Mikros Vacuum Evaporator VE 10 (Mikros, Inc., Portland, Oregon) was used at a vacuum of $< 0.05\mu$.

Polystyrene-latex beads (Dow Chemical Company, Bioproducts Department, Midland, Michigan) were used as an internal standard for cell size determinations. The beads had a particle diameter of 0.264 microns with a standard deviation of 0.006 microns, as determined by 577 measurements. The beads were usually added to the grids after drying of the cells on the formvar film. However some grids were prepared by mixing the latex beads with the bacteria.

Thin Sections

Test organisms were grown on the surface of TGY agar. The growth was gently removed with an inoculating loop and was mixed with 2 percent agar made in Sorensen's phosphate buffer at pH 6.8. After solidification, the agar was sliced into small blocks and fixed in 3 percent glutaraldehyde for 30 minutes. The agar block specimens were then post-fixed in 0.2 percent osmium tetroxide and dehydrated in ethyl alcohol according to the following schedule: 10 min each in 20%, 50%, and 70%, 30 min in 95% and twice for 30 min in 100%. The blocks were brought progressively into propylene oxide by three-graded ethyl alcohol-propylene oxide mixtures followed by

another 30 min in 100% propylene oxide. After the blocks were allowed to stand overnight in a 50:50 mixture of propylene oxide: araldite resin, the mixture was drained off and the agar block specimens were embedded in araldite resin contained in gelatin capsules.

Polymerization of the resin occurred after 48 hours at 60°C. Following polymerization the blocks were trimmed and sectioned with glass knives on a Porter Blum MT-1 ultramicrotome. Thin sections were picked up on formvar coated grids and doubly stained with saturated uranyl acetate and Reynold's lead citrate. Electron micrographs were taken with fine grain Kodak projector slide plates that were two by ten inches. An RCA EMU 2 D electron microscope was used and the subsequent photomicrographs were enlarged by a Durst 609 enlarger.

Dry Weight Determination

Late log phase cells of P. aeruginosa strains QRM-S and QRM-R from TGY broth and TGY broth plus 515 ppm QAC, respectively, were washed and resuspended in distilled water. A standard plate count was determined on each cell suspension on TGY agar. A one-tenth ml aliquot of each cell suspension was carefully measured into dry, weighed plancits. The plancits were dried to a constant weight in an oven at 85°C and the dry weights of the cells

were determined by the averages of the five plancits.

Nucleic Acid Studies

Preparation of Deoxyribonucleic Acid

Deoxyribonucleic acid (DNA) was isolated from sensitive and resistant cells by following the basic procedure of Marmur (1961). Four liters of TGY broth were inoculated at the rate of 0.1 percent of late log phase culture for both sensitive and resistant cultures. The cultures were incubated at 32°C for 24 hr on a rotary shaker. The cells were harvested by centrifugation at 5,000 rpm for 10 min in the type GSA rotor of a Servall RC2 refrigerated centrifuge. The cells were resuspended in 0.1 M ethylenediaminetetraacetic acid (EDTA) prepared in 0.15 M sodium chloride, pH 8.0. The cells were then centrifuged in the SS-34 rotor of the Servall RC2 at 5,000 rpm, resuspended to 25 ml in the EDTA-sodium chloride solution and 2 - 4 mg of crystalline lysozyme were added. The mixtures were held at 37°C, the resistant cells incubated for 4 hr and the sensitive cells incubated overnight.

Following incubation in the presence of lysozyme, 2 ml of a 25 percent solution of sodium lauryl sulfate was added and the flasks heated to 60°C and held for 10 min. The viscous masses were cooled in an ice bath and transferred to glass stoppered 250 ml

Erlenmeyer flasks. Sodium perchlorate, 5.0 M, was added to adjust the sodium ion concentration to 1.0 M. A volume of 24:1 chloroform-isoamyl alcohol mixture was added to deproteinize the nucleic acid solution. The mixture was shaken on a rotary shaker (160 strokes per min) at room temperature for 30 min. The resultant emulsion of protein and nucleic acid was centrifuged at 10,000 rpm for 5 min in the SS-34 rotor of the Servall RC2 centrifuge. The top layer containing crude nucleic acids was removed with a pipet. The nucleic acid was precipitated by the addition of two volumes of ice-cold 95 percent ethanol. The nucleic acid was recovered from the alcohol-nucleic acid interface by using a platinum loop. The recovered nucleic acid was then dissolved in dilute saline-citrate solution (0.015 M NaCl and 0.0015 M sodium citrate). The saline-citrate concentration was increased to 0.15 M and 0.015 M, respectively (referred to below as standard saline-citrate solution - SSC) by the addition of 1.5 M NaCl and 0.15 M sodium citrate. The process was repeated a total of five times. After the last nucleic acid precipitation and recovery, the nucleic acid was dissolved in dilute saline-citrate solution (0.015:0.0015 M) and adjusted to 0.15 M NaCl and 0.015 M sodium citrate (as before). The extracted nucleic acid was then treated with ribonuclease (0.2 percent in 0.15 NaCl, pH 5.0, Calbiochem, Los Angeles, California) at 37°C for 30 min with a final concentration of 50 μg/ml. The nucleic acid solution was treated

again with chloroform:isoamyl alcohol, precipitated and redissolved in 9 ml of dilute saline-citrate (0.015 M:0.0015 M). One ml of 5.0 M sodium acetate was added and the solution was stirred vigorously with a glass rod. The nucleic acid was precipitated by the dropwise addition of 5 ml of isopropyl alcohol to the mixing solution. The DNA was washed first in 70 percent ethanol and then in 95 percent ethanol. The purified DNA was dissolved in SSC solution at pH 7.0.

Thermal Denaturation Studies

The general procedure given by DeLey and Schell (1963) was used in an attempt to obtain thermal melting points of DNA preparation from sensitive and resistant cells.

The purified DNA was dissolved in SSC at pH 7.0 and diluted with SSC to give an absorbancy reading between 0.275 and 0.325 at 260 mμ. Three ml of each solution were placed in glass stoppered Beckman standard silica cuvettes; three ml of the SSC solution were placed in a third cuvette to serve as a blank. A cuvette holder containing the three cuvettes was then placed in the sample chamber of a Gilford Model Du spectrophotometer that was part of a Gilford Model 2000 multiple absorbance recording system.

The Gilford 2000 system was equipped with a four channel recorder, a linear thermosensor, an absorbancy read-out window,

an auxiliary offset control, a cuvette positioner and an automatic blank compensator. The sample chamber was enclosed on two sides by thermospacers which were connected to a circulating, thermostatically controlled Haake water bath. The bath circulated heated propylene glycol through the thermospacers. A second set of thermospacers, separated from the first by a plate of Bakelite insulation circulated tap water which protected the optical system of the spectrophotometer from excessive heat.

The DNA samples were equilibrated at 25°C and the absorbancy recorded. The temperature of the chamber was raised to 75°C and the samples allowed to equilibrate for about 30 minutes before further temperature increase was introduced. After equilibration at 70°C, the temperature was raised by manual adjustment of the Haake thermostat at a rate of about 0.50 degree per minute. Both the change in temperature and in absorbancy were recorded.

The recorder scale was set so that 0 and 100 percent corresponded to absorbances of 0.200 and 0.400, respectively. The blank compensator always adjusted the recorder to record the blank at 0 percent; therefore, the DNA sample absorbancy recorded had a difference absorbancy. The auxiliary offset was used to position the resultant absorbancy profile on the chart in a position which would not overlap the temperature curve. The temperature scale was calibrated so that full scale was 60° to 100°C. The chart

speed was one-fourth inch per minute, and the dwell time for recording was five seconds. The temperature was increased until a plateau in absorbancy was reached.

Attempts at Isolation of Mutants

It was determined that sensitive cells were very actively lipolytic on spirit blue agar and very proteolytic on milk agar whereas resistant cells were neither lipolytic nor actively proteolytic. Resistant and sensitive cells were then streaked on both media, and observed for colonies with opposite types of phenotypic characteristics. Thirty plates of each type of medium were streaked with each type of cell.

Phage Studies

Destruction of Phage by QAC

A comparison was made between the virucidal activity of QAC against *Streptococcus cremoris* phage 32-P and *P. aeruginosa* phage #4. *P. aeruginosa* strain IC was used in the assay. Various germicide concentrations and time exposures were used. One volume of double strength QAC was mixed with one volume of phage filtrate in TGY broth. The procedure was described by Henning *et al.* (1968) and the only modifications were the use of TGY broth, TGY agar,

and TGY-semisolid overlay. The use of QAC inactivator was incorporated in the procedure, as described by Watkins et al. (1957).

Loss of Biochemical Characteristics with Exposure of QRM-S to QAC

P. aeruginosa strain QRM-S was transferred in TGY broth containing sub-lethal concentrations of QAC until a series of resistance levels were obtained from 0 to 250 ppm. These cultures were subjected to all the biochemical tests used in these studies in order to determine a relationship between loss of character and the action of QAC.

Resistance to Chlorine and Iodine

Progressively increasing concentrations of sodium hypochlorite and iodophor were prepared in tubes of TGY broth. The tubes were inoculated at the rate of 1 percent with sensitive and resistant cells and incubated at 32°C. After 7 days incubation the tubes were spotted on TGY agar plates to assay for presence of viable cells.

RESULTS

Physical Effects of Quaternary Ammonium Compound

Effect on Media

Table 3 shows the effect of the concentration of QAC on the pH of aqueous solutions in the presence and absence of protein. It is apparent that the presence of protein neutralizes the pH effect of QAC and that this neutralization is dependent upon the amount of protein present. The pH depression of TGY broth by 20,000 ppm QAC is not sufficient in itself to be bactericidal.

Table 3. Effect of QAC concentration on the pH of distilled water and TGY broth containing 5 and 30 grams of tryptone per liter.

QAC Concentration	Distilled Water	TGY 5 g Tryptone/liter	TGY 30 g Tryptone/liter
0	6.6	6.8	6.95
50	6.0	6.8	6.9
100	5.9	6.8	6.9
200	5.5	6.75	6.9
500	5.4	6.7	6.9
1,000	4.4	6.7	6.9
2,000	4.2	6.65	6.9
5,000	3.8	6.55	6.9
10,000	3.5	6.4	6.85
20,000	3.3	6.15	6.65

The difficulty encountered in assaying for cell growth in broth cultures by spectrophotometric methods is revealed in Table 4. The addition of QAC to TGY broth caused immediate clouding which was proportional to the germicide concentration. After setting for a period of several days, a dark brown sediment appeared in the bottom of TGY broth containers which contained higher QAC levels; there was a corresponding decrease in cloudiness although complete clearing was never observed.

Table 4. Effect of QAC concentration on the absorbancy of TGY broth.

PPM QAC	A_{520}
5	0.017
25	0.060
50	0.130
100	0.180
260	0.610
410	0.750
515	1.950

Effect of Bacterial Cells on the pH of QAC Solutions

A striking difference between the pH of washed cells of QRM-R and QRM-s was discovered; the pH of resistant cells was 7.6 while that of sensitive cells was 6.7. Also, the resistant cells possessed a much greater ability to neutralize the pH effect of QAC as is evident in Table 5. In this regard, the addition of 5×10^9 sensitive cells to

25 ml of QAC solutions failed to alter the pH of a 100 ppm solution, while addition of 5×10^9 resistant cells raised the pH of a 10,000 ppm solution by 1.2 pH units. When 5×10^{11} resistant cells were added the pH of a 10,000 ppm solution was raised to pH 7.6.

Table 5. Effect of the addition of washed cells of Pseudomonas aeruginosa QRM-S and QRM-R on the pH of various concentrations of QAC in distilled water.

Cell Number	QRM-S Concentration of QAC, ppm				QRM-R Concentration of QAC, ppm			
	0	100	515	10,000	0	100	515	10,000
1×10^7	6.7	6.2	4.7	3.3	7.6	6.2	4.7	3.3
1×10^8	6.7	6.2	4.7	3.3	7.6	6.3	5.0	3.4
5×10^8	6.7	6.2	4.7	3.4	7.6	6.5	5.4	3.6
1×10^9	6.7	6.2	4.7	3.4	7.6	6.7	5.8	3.8
5×10^9	6.7	6.2	4.7	3.4	7.6	7.3	6.7	4.5
1×10^{10}	6.7	6.4	5.1	3.4	7.6	7.6	6.9	5.1
5×10^{10}	6.7	6.7	6.3	3.7	7.6	7.6	7.5	6.4
1×10^{11}	6.7	6.7	6.5	4.3	7.6	7.6	7.6	7.3
5×10^{11}	6.7	6.7	6.7	6.0	7.6	7.6	7.6	7.6

Effect on Cell Suspension

The physical effect of QAC on washed cell suspension of sensitive and resistant cells is shown in Figure 3. The sensitive cells precipitated in less than 5 min and within 60 min the entire precipitate had risen to the surface. A 1:100 dilution of sensitive cells required

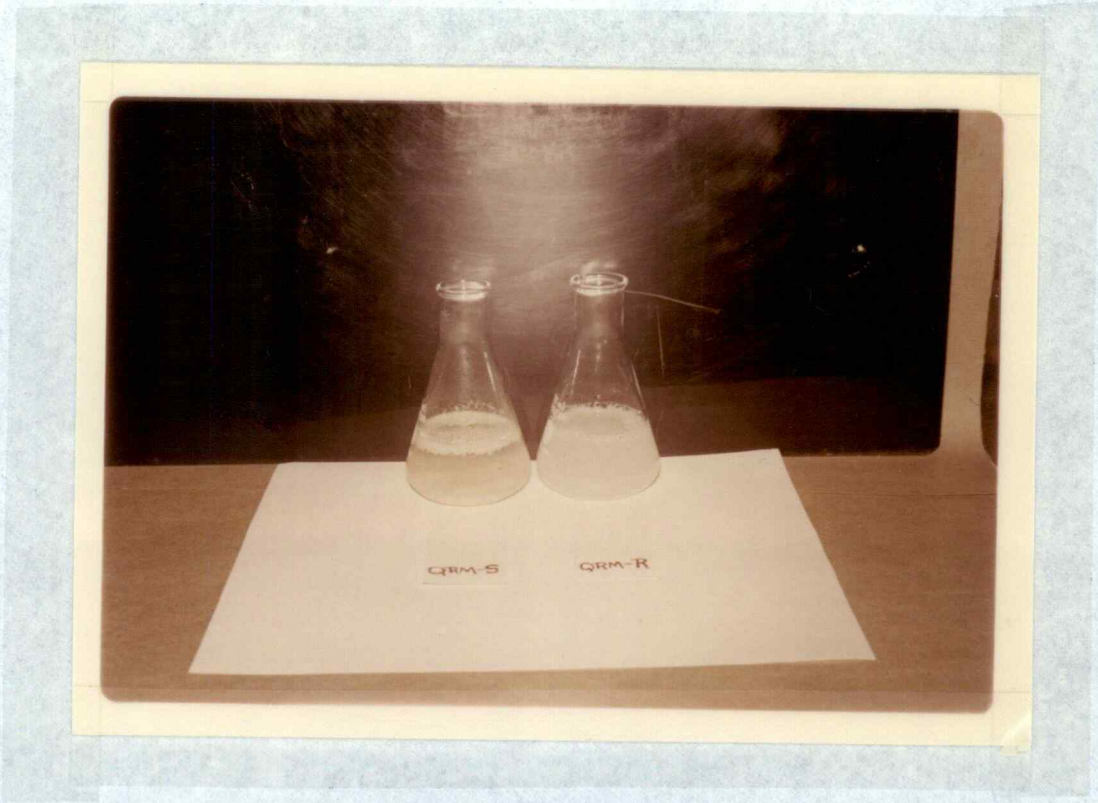

Figure 3. Effect of 1,250 ppm QAC on washed cell suspensions of Pseudomonas aeruginosa strains QRM-S and QRM-R.

several hours to precipitate. The resistant cells presented a turbid suspension after 60 min but showed no marked precipitation even after 48 hr; the same was true for the 1:100 dilution of resistant cells.

Effect of Temperature and Aeration on QAC Resistance

The aeration and incubation temperature of cultures was found to have both negative and positive effects on resistance, depending upon the strain used. The strain itself appeared to be more important than either aeration or temperature increases from 21°C to 37°C. It was evident, however, when exposed to QAC strain QRM was the most resistant of all the strains used.

Disc Assay Method for QAC Resistance

There were no indications of zones of inhibition even with discs soaked in 5,150 ppm QAC.

Death Curves in 260 ppm QAC

A comparison of the affect of 260 ppm QAC on the viability of sensitive and resistant cells is presented in Figure 4. The difference in effectiveness of QAC on the two cell types is dramatic. The viable cell concentration of QRM-R was hardly affected by the germicide while >99.99 percent of sensitive cells were destroyed within one minute of exposure.

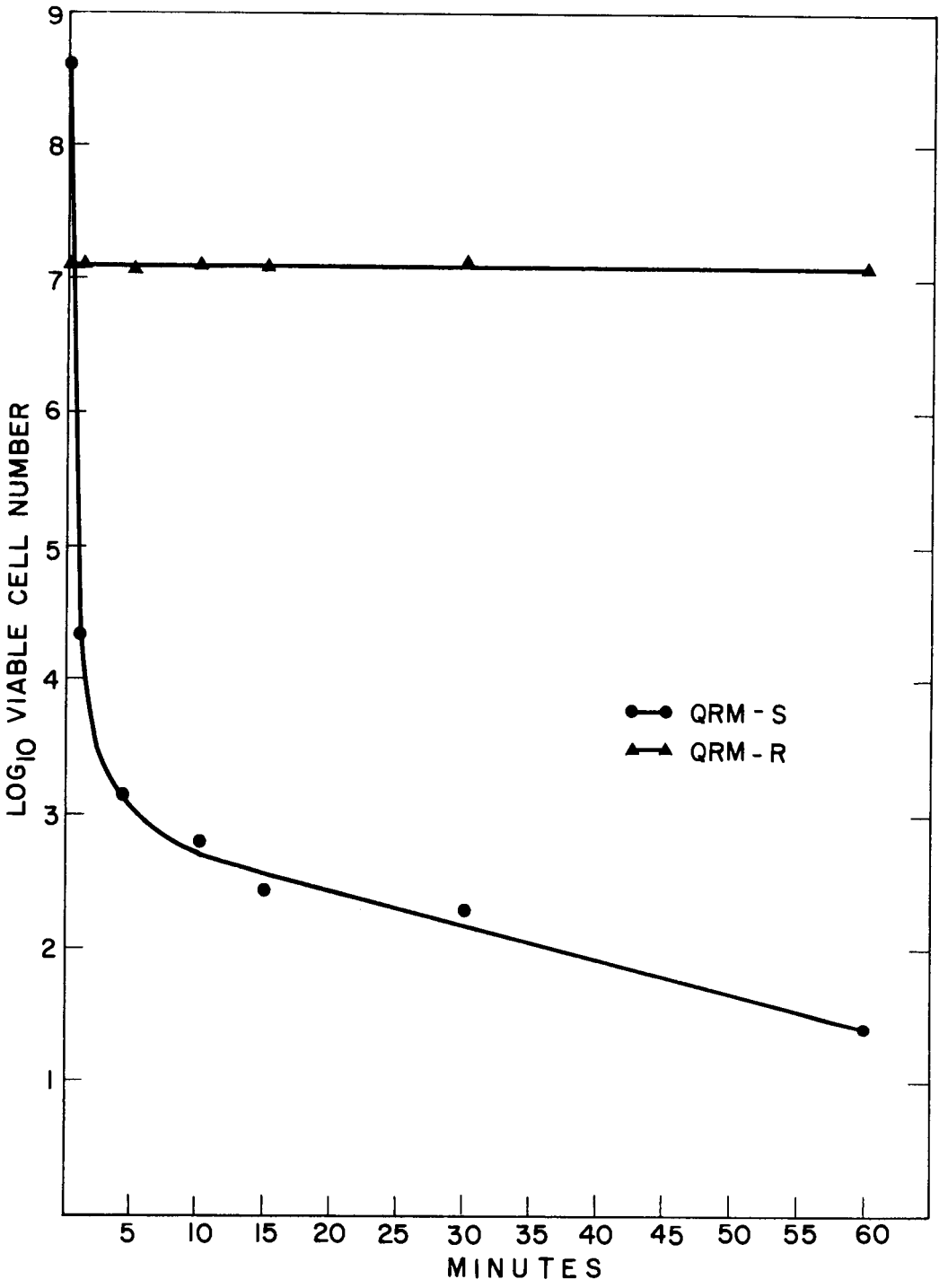

Figure 4. Effect of exposure of QRM-S and QRM-R cells of Pseudomonas aeruginosa to 260 ppm QAC for the times indicated.

Resistance of QRM-R After Growth in the Absence of QAC

After 7 months of growth in the absence of QAC, the resistance level of QRM-RN in TGY broth at pH 6.8 was determined to be 760 ppm, equal to that of QRM-R. There appeared, however, to be a difference in the effect of pH on these cultures; QRM-R was resistant to 310 ppm at pH 8.0 and 25 ppm at pH 9.0, whereas QRM-RN was resistant to 515 ppm at pH 8.0 and 200 ppm at pH 9.0.

Cultural Characteristics

The colony morphology of QRM-R, QRM-RN and QRM-S strains of P. aeruginosa on TGY agar was nearly identical. All were 2 to 5 mm in diameter, flat, glistening, gray and transluscent. The colony diameters of QRM-R on TGY broth plus 1,000 ppm QAC ranged from 1 to 4 mm although most were 1 to 2 mm in diameter. The colonies of QRM-R appeared less moist then either of the other strains. On TGY agar plus 5,000 ppm QAC the colonies of QRM-R were pinpoint.

Microscopic examination of colonies of sensitive and resistant cultures on TGY agar and TGY agar plus QAC revealed a marked difference in colony morphology. As can be seen in Figure 5, the resistant cells revealed a tendency to concentrate either cell masses or cell products toward the center of the colony. Sensitive cells

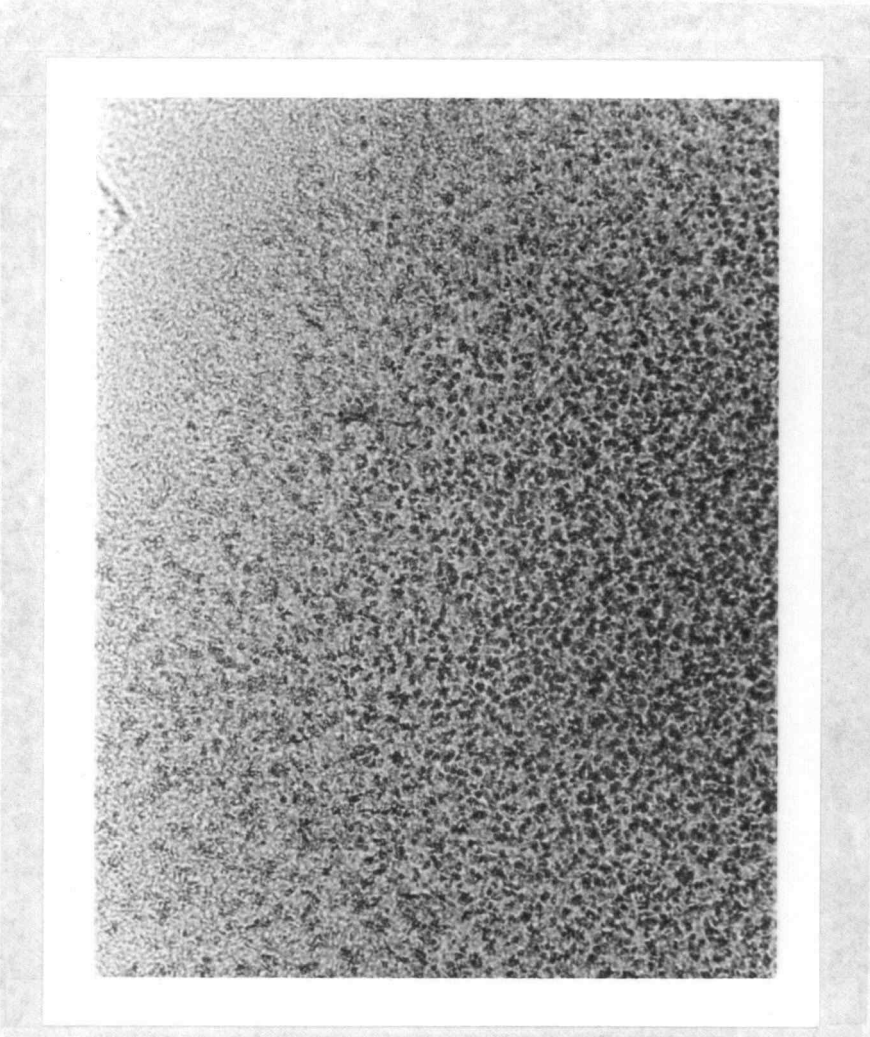

Figure 5. Colony of Pseudomonas aeruginosa QRM-R (X100) on TGY agar showing a concentration of dense areas toward the center.

(Figure 6), on the other hand, revealed no such phenomenon on TGY agar but appeared rather uniformly granular. However, colonies of sensitive cells on TGY agar containing 500 ppm QAC (Figure 7) showed a tendency toward a central concentration of dense materials that resembled the macroscopic appearance of Aspergillus niger.

Growth Curve Studies

Figure 8 illustrates the growth patterns of sensitive and resistant cells in TGY broth. The sensitive cells had a generation time of 42.8 min while the generation time of resistant cells was 58.4 min, 25 percent longer than that of sensitive cells. The growth of resistant cells in the absence of QAC for 7 mo (QRM-RN cells) did not affect the growth rate of the cells, as also can be seen in the figure. Their generation time was 57.7 min.

Sensitive and resistant cells in TGY broth cultures did not increase in number during a 125-hr incubation period on a shaker at 4°C (Figure 9). The resistant cells in TGY-QAC broth similarly treated showed a marked decrease in viable cells with >99.9 percent destruction in 125 hr. Another resistant culture, treated in the same manner except that it was shaken, exhibited a similar pattern of cell death. In contrast, a resistant culture in TGY-QAC broth which was incubated at 32°C on a rotary shaker attained a viable cell count in excess of 1×10^9 in 75 hr.

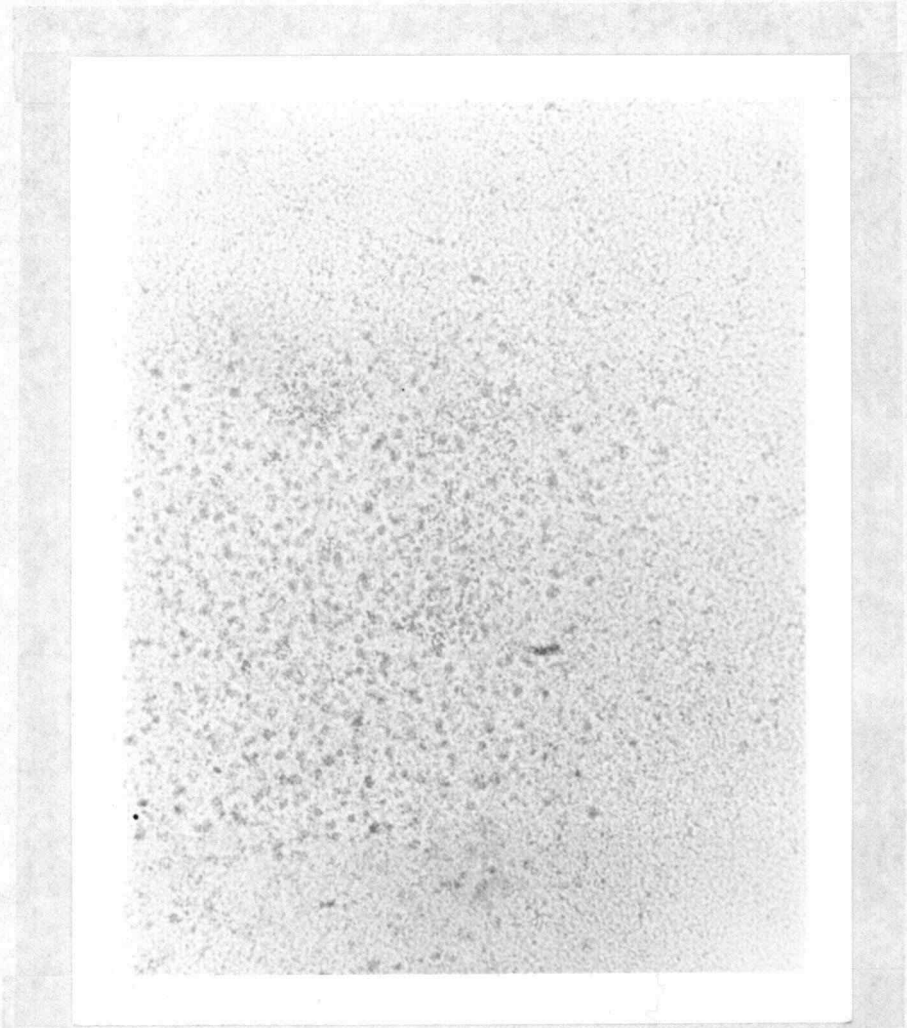

Figure 6. Colony of Pseudomonas aeruginosa QRM-S (X100) on TGY agar showing a homogeneous granular consistency.

Figure 7. Colony of Pseudomonas aeruginosa QRM-S cultured on TGY agar containing 500 ppm QAC showing a granular peripheral zone and small dense areas toward the center.

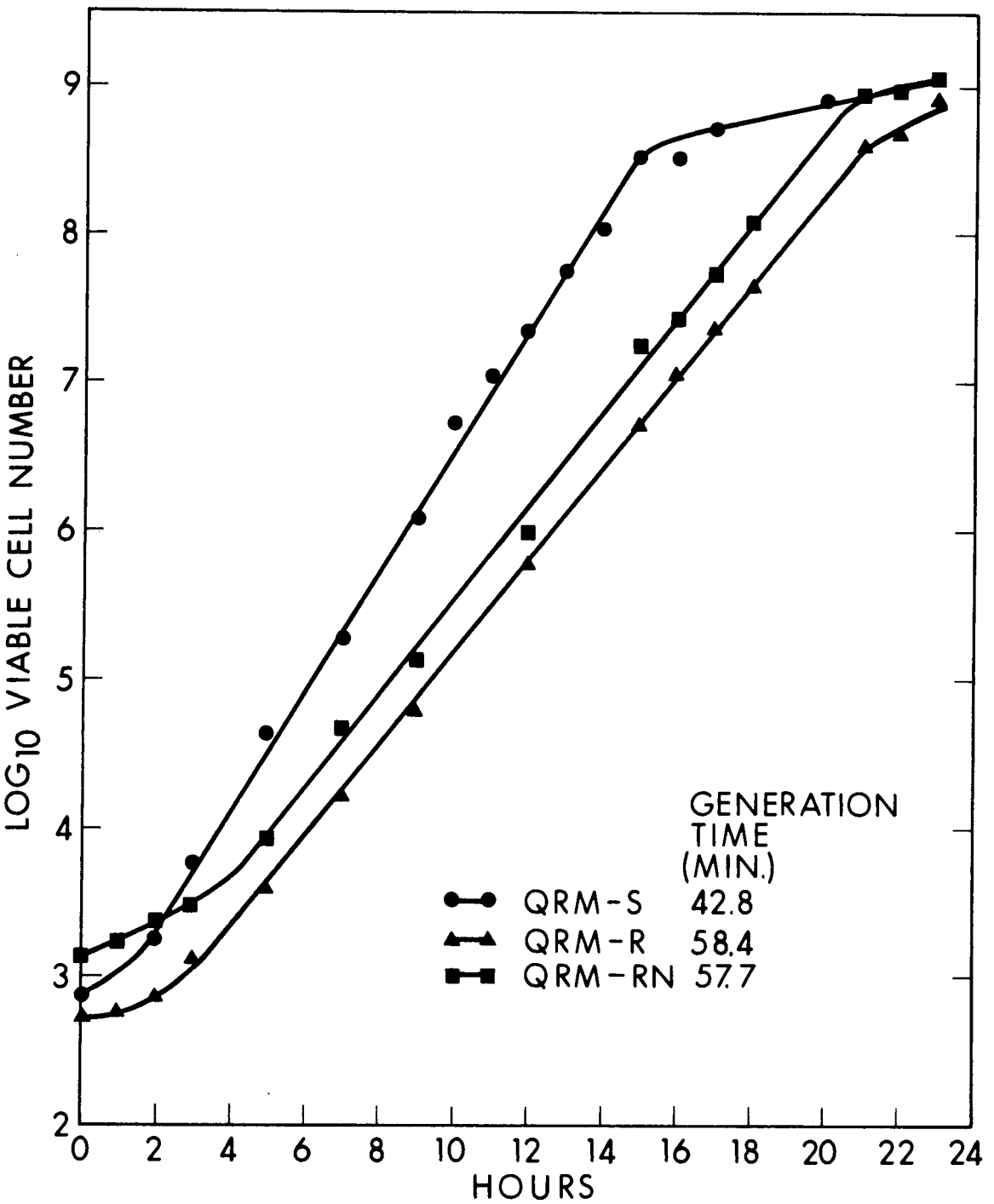

Figure 8. Growth curves of Pseudomonas aeruginosa strains QRM-S, QRM-R and QRM-RN in TGY broth incubated at 32°C on a rotary shaker.

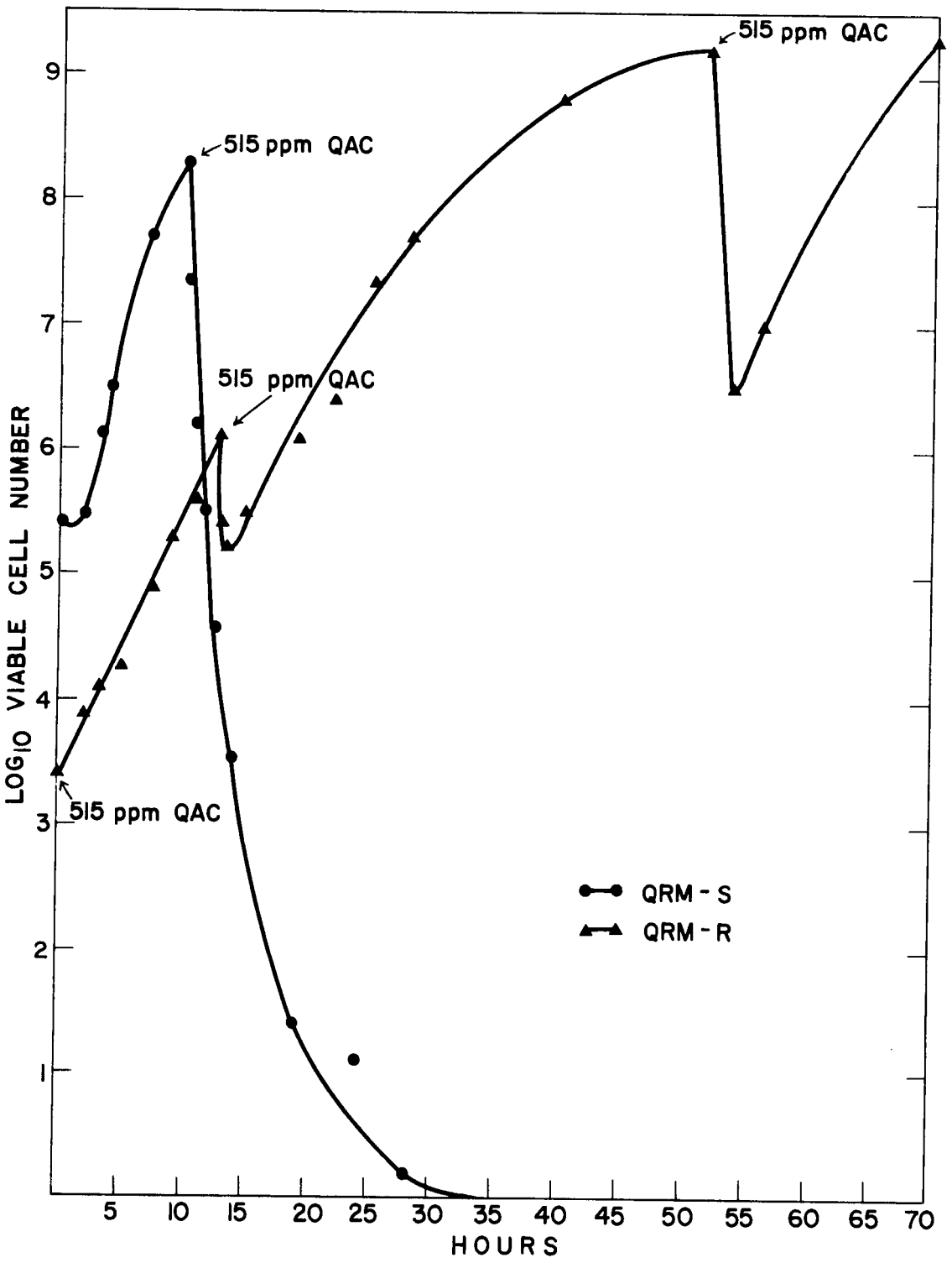

Figure 9. Effect of addition of 515 ppm QAC on the growth curves of Pseudomonas strains QRM-S and QRM-R.

The transfer of the culture flasks from 4°C to the rotary shaker at 32°C resulted in an almost immediate increase in viable cell counts (Figure 10). It can still be seen that the sensitive cells had a much shorter generation time.

Effect of Media on QAC Resistance

Resistance in Broth and Agar

In earlier stages of these studies cultures were maintained on TGY agar slants and transferred by loop inoculation from slant to slant. Figure 11 compares this method of determining QAC resistance with three other methods. It is evident that the effect of QAC on QRM-S cells is greatly diminished on agar. Loop inoculation, which transfers a greater mass of cells, resulted in growth of sensitive cells in the presence of >5,000 ppm QAC. This was decreased to 500 ppm when a one drop broth inoculum was used. Similarly, resistant cells, transferred by loop inoculation from one slant culture to another, grew in the presence of 7,500 ppm, while those from one drop broth inoculation on TGY agar slants failed to grow in concentrations greater than 750 ppm. Results with strain QRM-RN were identical with those obtained for strain QRM-R.

Resistance of QRM-S to QAC was decreased in TGY broth and in a semi-synthetic broth to 30 ppm and 10 ppm, respectively.

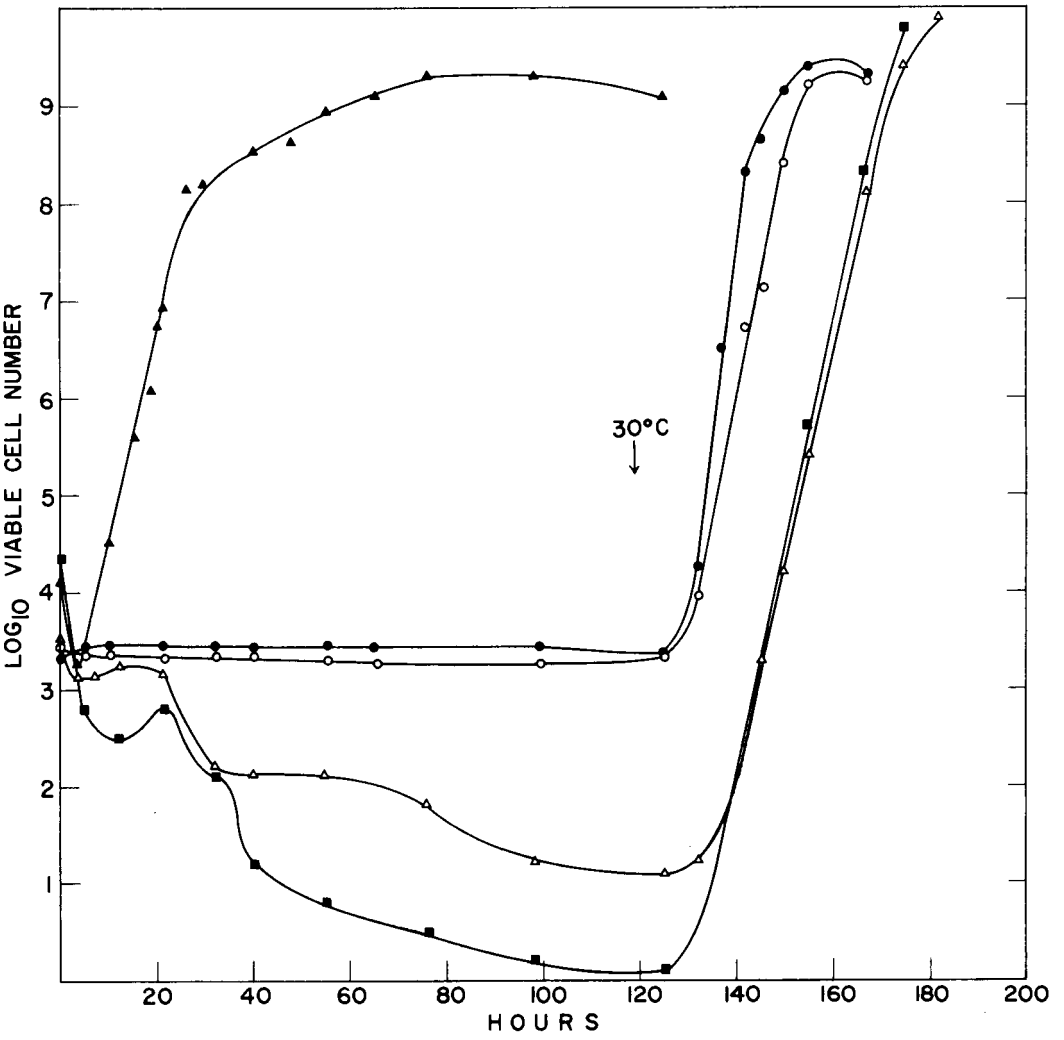

Figure 10. Effect of temperature, aeration and QAC on the growth of sensitive and resistant strains of Pseudomonas aeruginosa in TGY broth.

QRM-S (●–●) and QRM-R (O–O) - shaker at 4°C
QRM-R (Δ–Δ) - shaker at 4°C with 500 ppm QAC added
QRM-R (Δ–Δ) - shaker at 32°C with 500 ppm QAC added
QRM-R (■–■) - stationary at 4°C with 500 ppm QAC added

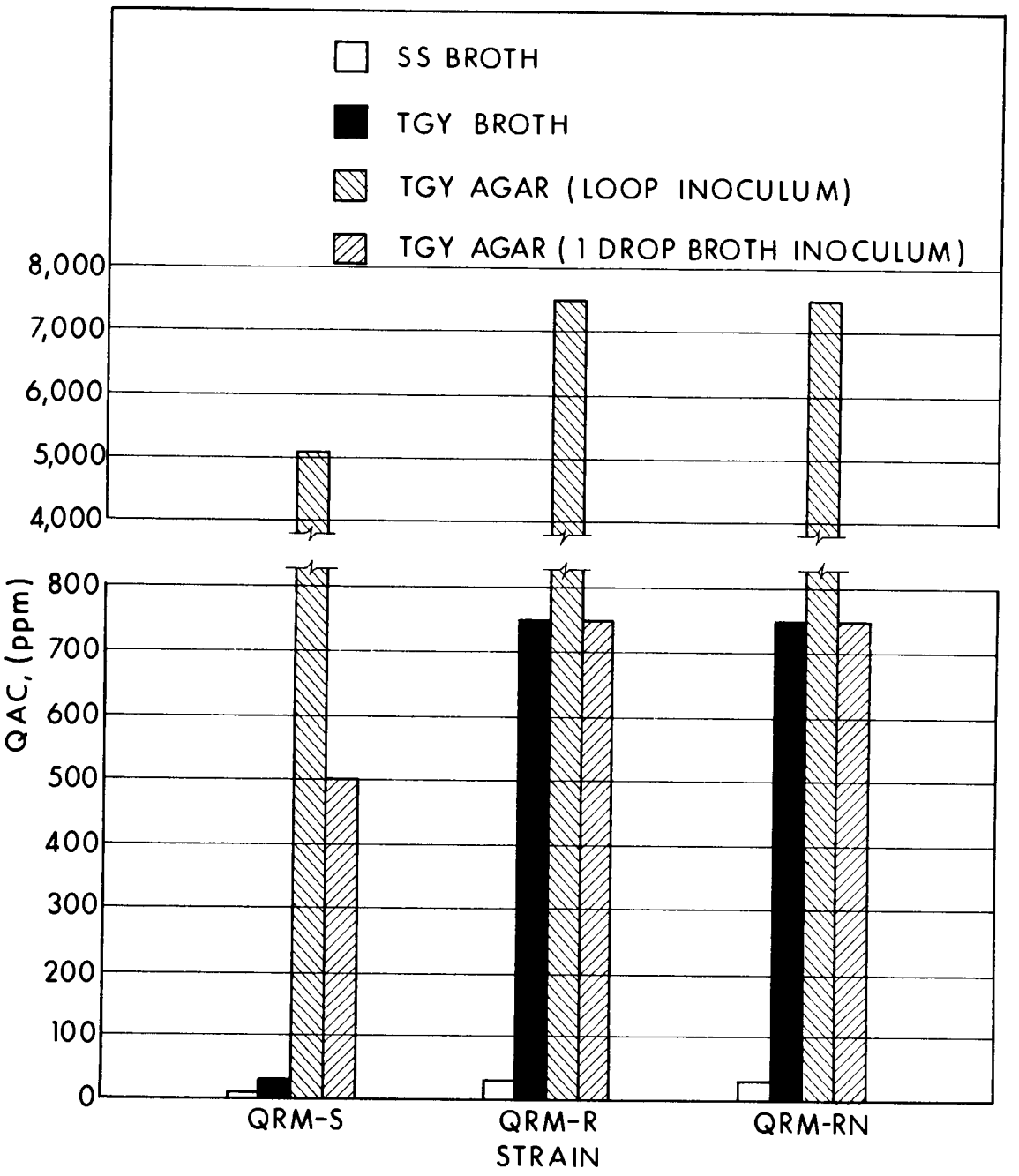

Figure 11. Effect of the medium on QAC tolerance of Pseudomonas aeruginosa strains QRM-S, QRM-R and QRM-RN.

Strains QRM-R and QRM-RN maintained a resistance level of 750 ppm in TGY broth. Although they were only tolerant of 40 ppm in semi-synthetic broth, it was still four times the concentration that sensitive cells could tolerate.

Concentration of Ingredients

The level of QAC resistance was determined for 3 different strains of P. aeruginosa in TGY broth containing different concentrations of tryptone. The ability of the three strains to grow in greater concentrations of QAC when more protein is present in the broth is illustrated in Figure 12. A six fold increase in tryptone concentration (from 5 to 30 g/l) resulted in an apparent ten fold increase in QAC resistance.

Increasing the glucose concentration of TGY broth from 0.1 percent to 3.0 percent had no effect on the resistance of sensitive or resistant cells to QAC.

Resistance in Milk

Sensitive and resistant cells were much more resistant to QAC in milk than they were in TGY broth. In milk culture both cell types were able to grow in the highest QAC concentration used, 3,600 ppm. In TGY broth the sensitive cells failed to grow in concentrations exceeding 37 ppm while resistant cells grew in 750 ppm.

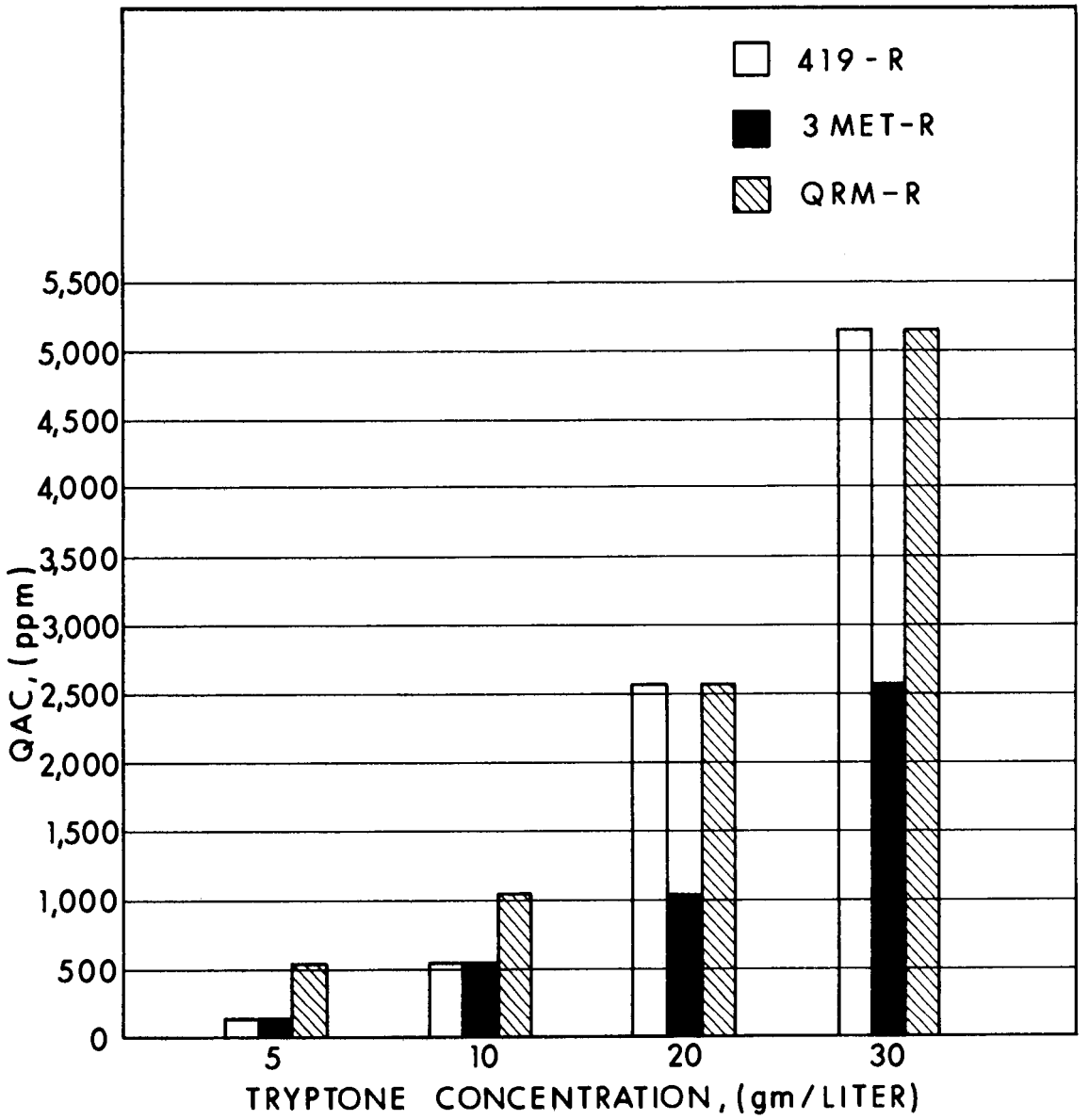

Figure 12. Effect of tryptone on the QAC tolerance of three resistant strains of Pseudomonas aeruginosa.

Effect of Environmental Conditions on Growth

Although the formation of colonies on TGY agar plates was much more rapid for QRM-S at 25°C and 32°C, the contrary was true at 42°C. Strain QRM-R and QRM-RN produced a heavier, more rapid growth than did QRM-S at the higher temperature. No strain produced colonies at 4°C or at 50°C (Table 6).

Table 6. Comparison of cultural features of Pseudomonas aeruginosa strains QRM-S, QRM-R and QRM-RN.

Feature		QRM-S	QRM-R	QRM-RN
Growth temperature				
	50°	0	0	0
	42°	+	+	+
	32°	+	+	+
	25°	+	+	+
	4°	0	0	0
	0°	0	0	0
Saline tolerance				
	0%	+	+	+
	3%	+	+	+
	5%	+	+	+
	6.5%	0	0	0
	7.5%	0	0	0
	10.0%	0	0	0
pH range of growth				
	4.5	+	0	0
	5.0	+	0	+
	5.7	+	+	+
	6.5	+	+	+
	7.2	+	+	+
	8.0	+	+	+
	9.0	+	+	+

0 = no growth
+ = growth

Table 6 indicates no difference in salinity tolerance of QRM-S, QRM-R and QRM-RN strains. There is a difference, however, in the time required to produce a positive test. QRM-S was positive in 5 percent NaCl in 24 hr while 8 days were required for QRM-R and QRM-RN. No strain grew in concentrations greater than 5 percent.

Sensitive cells were able to grow at all pH levels from 4.5 to 9.0. Neither QRM-R nor QRM-RN grew at pH 4.5 and QRM-R failed to grow at pH 5.0.

Resistance of Washed Cells

Both washed and unwashed cells of the resistant strain were capable of growing in TGY broth containing 2,575 ppm QAC which was the highest test concentration used. Unwashed cells of the sensitive strain were inhibited by concentrations greater than 50 ppm; in striking contrast, the same cells subjected to 5 washings were able to grow in 1,025 ppm QAC. It had been noted that these washed cells were slightly viscous and subsequent treatment with DNAase eliminated the viscosity.

Biochemical Characteristics

A summation of the results of the biochemical tests conducted on the test organisms is given in Table 7. Some of the most important

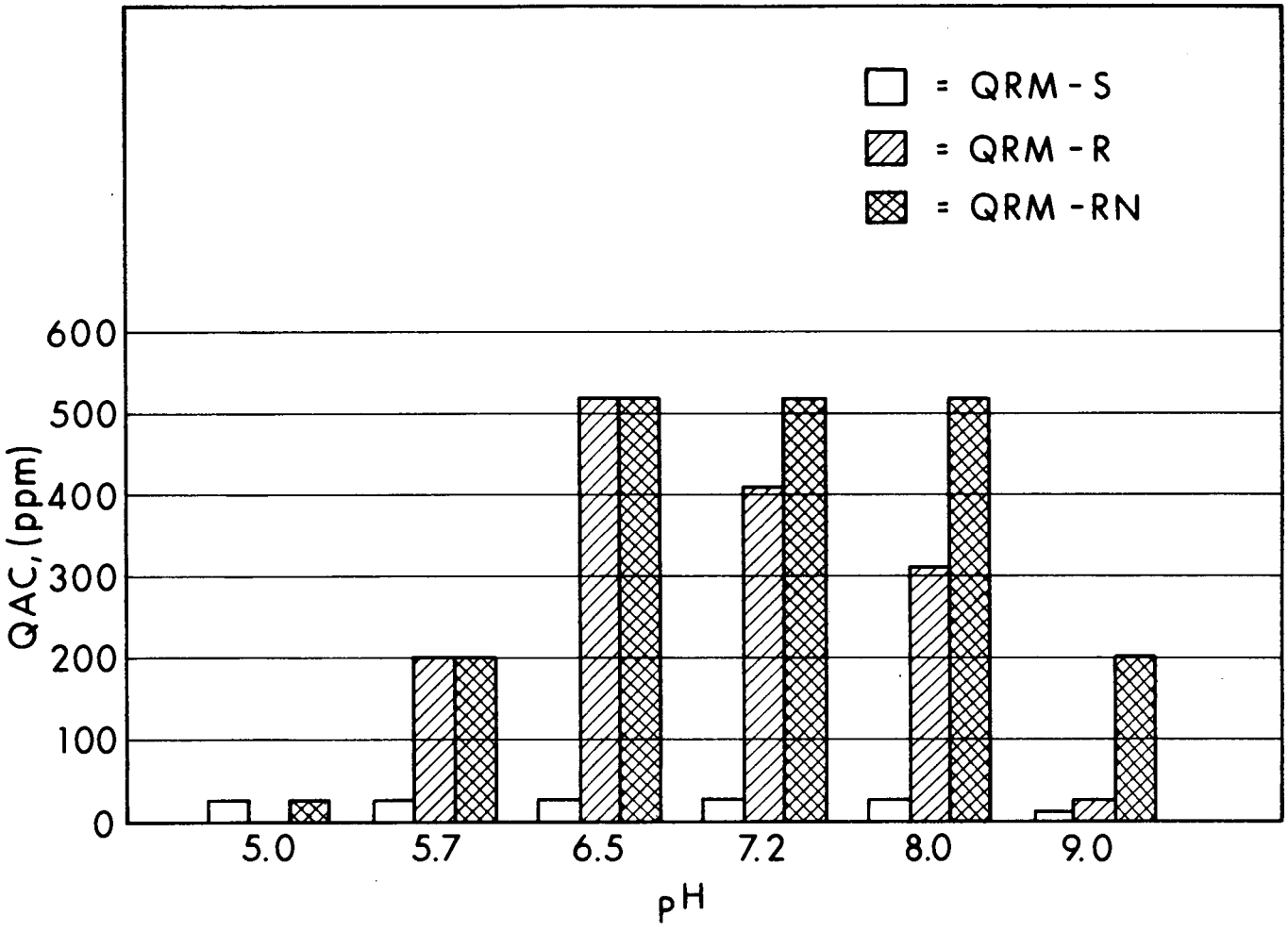

Figure 13. Effect of pH on the growth of Pseudomonas aeruginosa strains QRM-S, QRM-R and QRM-RN in TGY broth containing various concentrations of QAC.

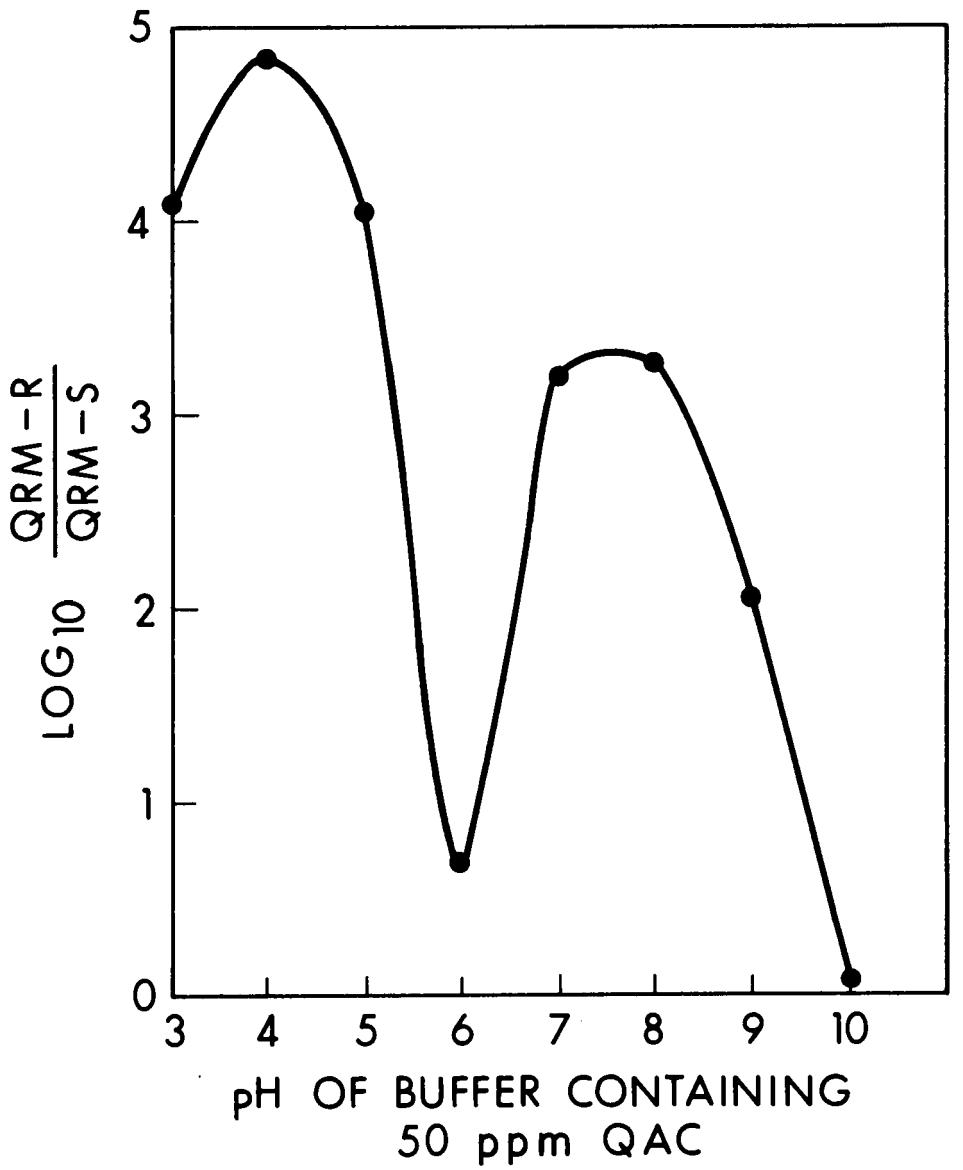

Figure 14. Comparison of the effect of pH on QAC resistance of sensitive and resistant strains of Pseudomonas aeruginosa.

differences were displayed in the test for pigment production, lipase activity, proteinase activity, gluconate oxidation and lecithinase.

Table 7. Biochemical characteristics of Pseudomonas aeruginosa strains QRM-S, QRM-R, and QRM-RN.

Character	QRM-S	QRM-R	QRM-RN
Pigment production			
Pyocyanin	4 (green)	0	4 (blue)
Fluorescein	4	0	0
Hydrolysis of			
Milk	4	0 (14 da)	2 (14 da)
Gelatin	4	2 (14 da)	2 (7 da)
Aesculin hydrolysis	0	0	0
Acetic acid from ethanol	0	0	0
Oxidation of calcium lactate	4	4	4
De-amidation of acetamide	4 (2 da)	0 (4 da)	2 (2 da)
Dehydroxyacetone accumulation	0	0	0
Cytochrome oxidase	4	4	4
Oxidase	4	4	4
Arginine dihydrolase	4	4 (slow)	4 (slow)
Gluconate oxidation	4	4 (slow)	4 (slow)
Urease	0	0	0
Starch hydrolysis	0	0	0
Nitrate reduction	4	0	2
Indole production	4	0	2
Methyl red	4	4 (slow)	4 (slow)
Voges-Proskauer	0	0	0
Catalase	4	4	4
Lipase	4	0	0
Proteinase	4	2	--
Kosers citrate	4	2	2

4 = strong positive; 2 = weak positive; 0 = negative

The sensitive cells produced profuse amounts of both pyocyanin and fluorescein pigments. This was true also when sub-lethal concentrations of QAC were added to the media. QRM-R cells failed to

produce either pigment, even in the absence of QAC. QRM-RN did not produce fluorescein but did produce pyocyanin. This pyocyanin pigment was predominantly blue, while the pigment of the parent sensitive strain was green.

Figure 15 depicts the differences in lipase activity. The lack of lipase activity in the resistant organism is evident from the absence of the blue precipitate found in the sensitive culture. The lack of lecithinase activity of the resistant cells could be a related characteristic.

The proteinase activity of cell sonicates is shown in Figure 16. The activity of this enzyme is much greater in the sensitive cells than it is in the resistant cells and correlates well with the slower gelatin liquefaction by resistant cells. Figure 17 demonstrates the failure of resistant cells to proteolyze milk on milk agar plates.

Many of the biochemical tests on the resistant strain resulted in a positive test only after prolonged incubation; examples of this are growth in litmus milk, gluconate oxidation, acetamide hydrolysis and arginine dihydrolase activity.

Lipid Studies

Lipid Staining

A preponderance of sudanophilic material was found in both

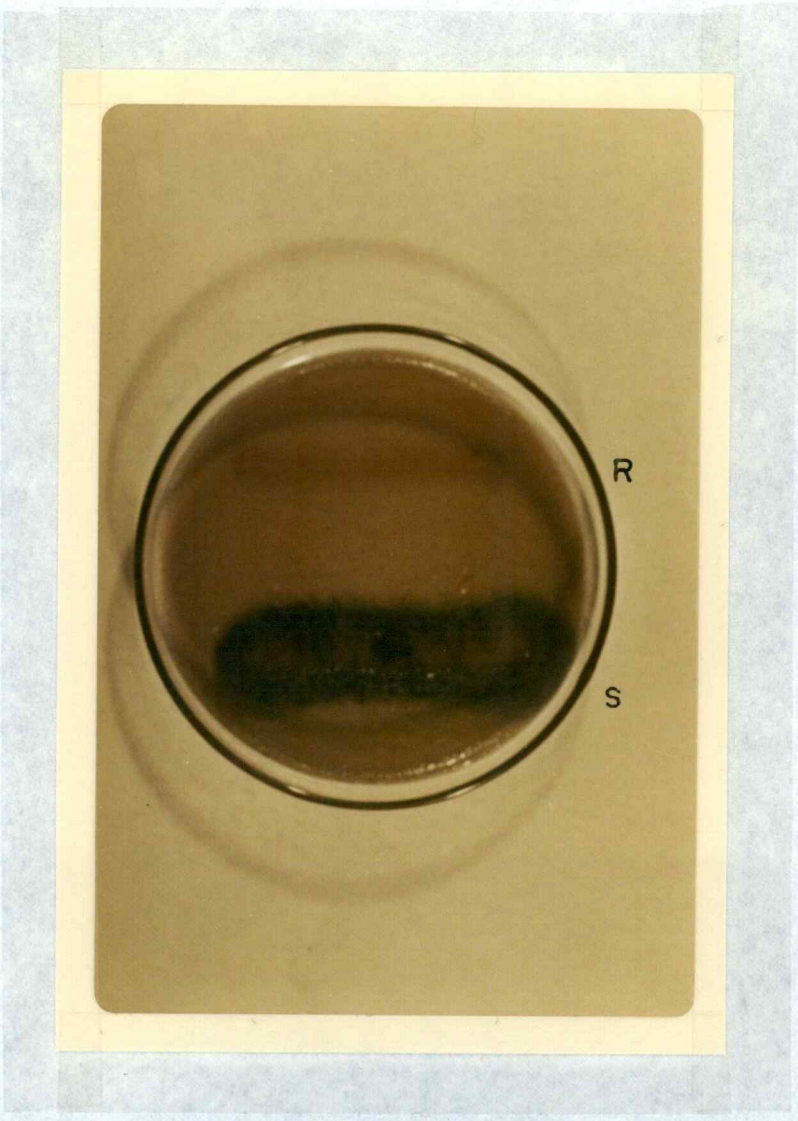

Figure 15. Comparison of the lipolytic activity on Spirit Blue agar after 14 days. (S) Sensitive strain and (R) resistant strain of Pseudomonas aeruginosa.

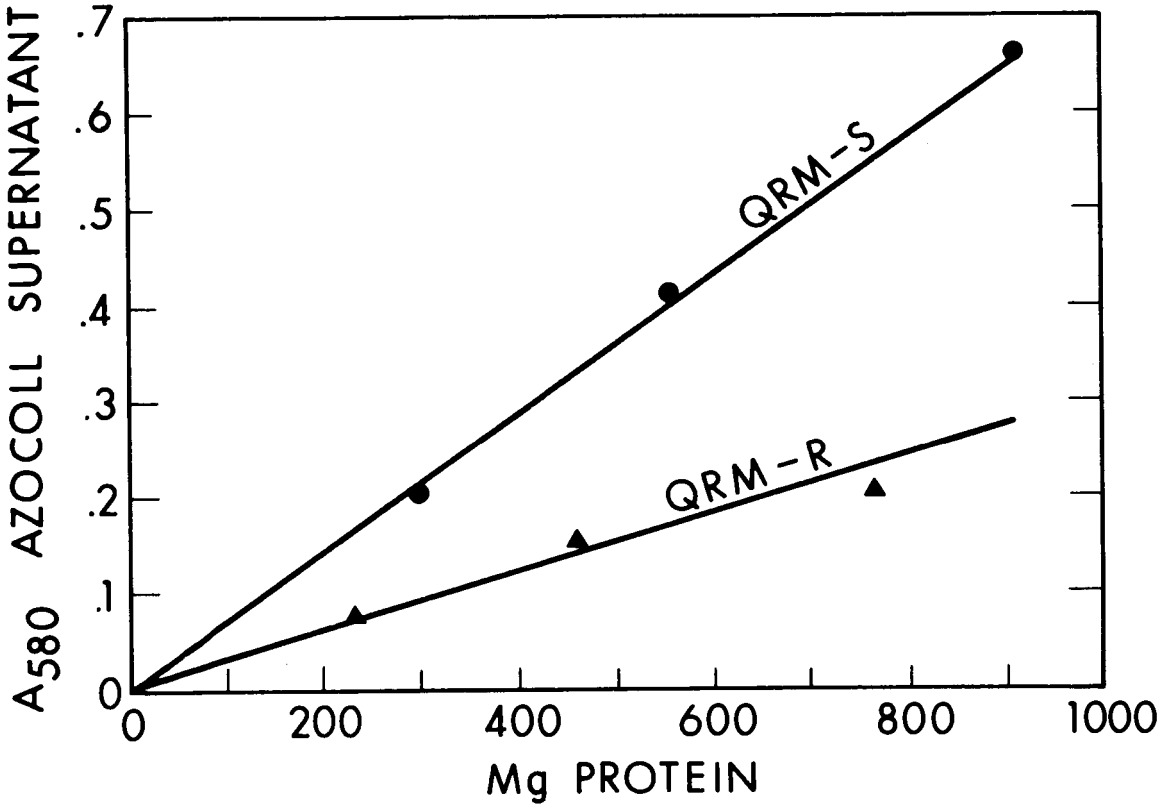

Figure 16. Comparison of the proteolytic activity of sonicates of sensitive and resistant strains of Pseudomonas aeruginosa.

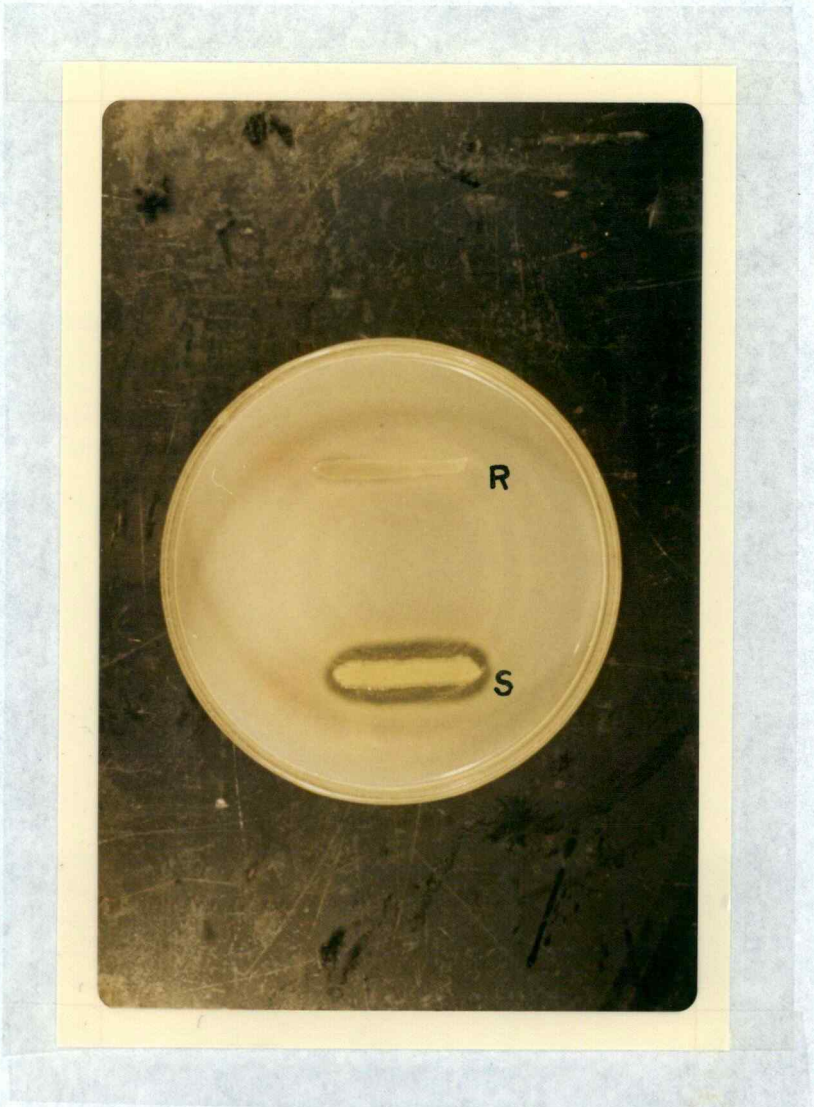

Figure 17. Comparison of the proteolytic activity of QAC-sensitive (S) and QAC-resistant (R) strains of Pseudomonas aeruginosa on milk agar.

QRM-R and QRM-RN strains of P. aeruginosa. The sensitive parent organism did not display such an accumulation of lipids.

Presence of Poly-β-hydroxybutyric Acid

Hot chloroform extractions of QRM-S, QRM-R, and QRM-RN cells indicated that there was no concentration of poly-β-hydroxybutyric acid in the cells.

Influence of "Fattening" on Resistance

It has been shown that growth in media containing 3 percent glycerol greatly enhances the lipid content of cells. Growth of sensitive and resistant strains in the appropriate medium containing 3 percent glycerol for 15 weeks did not affect their susceptibility to QAC.

Total Lipid Content

Total lipid determinations were repeated four times and the average total lipid was calculated. P. aeruginosa QRM-S cells contained 8.4 percent total lipid while QRM-R cells contained 12.5 percent. That is to say, resistant cells contain 48% more lipid than sensitive cells.

Effect of Possible Lipid Precursors on Resistance

The addition of acetic acid, sodium acetate, sodium pyruvate, sodium citrate and acetone had no effect on the resistance of either P. aeruginosa QRM-S or QRM-R to QAC. It was noted that the resistant culture in broth containing either 100 ppm or 1,000 ppm acetone did not produce a fruity odor.

Gas Chromatography

The detection of a fruity odor produced by resistant cells and the apparent absence of the odor in sensitive cells led to GLC analysis of the head space of both cultures. From the results compiled in Table 8, a number of conclusions can be drawn. First, it appears that sensitive and resistant cells used acetaldehyde and propanal and that both produced ethanol. Also, only sensitive cells produced 2-butanone. Third, the fruity ethyl esters, ethyl acetate and ethyl valerate, are produced extensively by resistant cells and only traces are elaborated by sensitive cells.

Gel Electrophoresis

Total Protein

Total protein bands from sensitive and resistant cells were not identical; there were differences in some of the early bonds and also

in some of the later bands.

Table 8. Gas chromatographic head space analysis of TGY broth cultures of Pseudomonas aeruginosa QRM-S and QRM-R.

	cm^2 Peak Area			
T_R (cm)	TGY Broth	QRM-S	QRM-R	Peak Identity
4.5	---	2.7	1.2	?
5.2	24.0	0.6	3.5	acetaldehyde
9.2	10.5	---	---	propanaldehyde
12.8	17.6	2.3	48.9	ethyl acetate
13.8	356.0*	---	---	QAC Index
14.8	---	---	60.5	?
15.3	---	53.5	24.7	ethanol
16.8	53.6	---	10.8	?
18.2	---	---	---	ethyl propionate
19.6	6.5	---	---	
23.3	---	---	---	ethyl butyrate
24.0	---	4.9	---	2-butanol
26.5	---	---	6.7	?
29.6	---	1.9	82.8	ethyl valerate
30.8	---	---	16.4	?
32.4	---	5.25	---	?

*only in TGY-QAC broth

Esterase Activity

As shown in Figure 18, 2 esterase bands were found in gels prepared from slurries of ruptured sensitive cells. Only one band was present in resistant cell slurries.

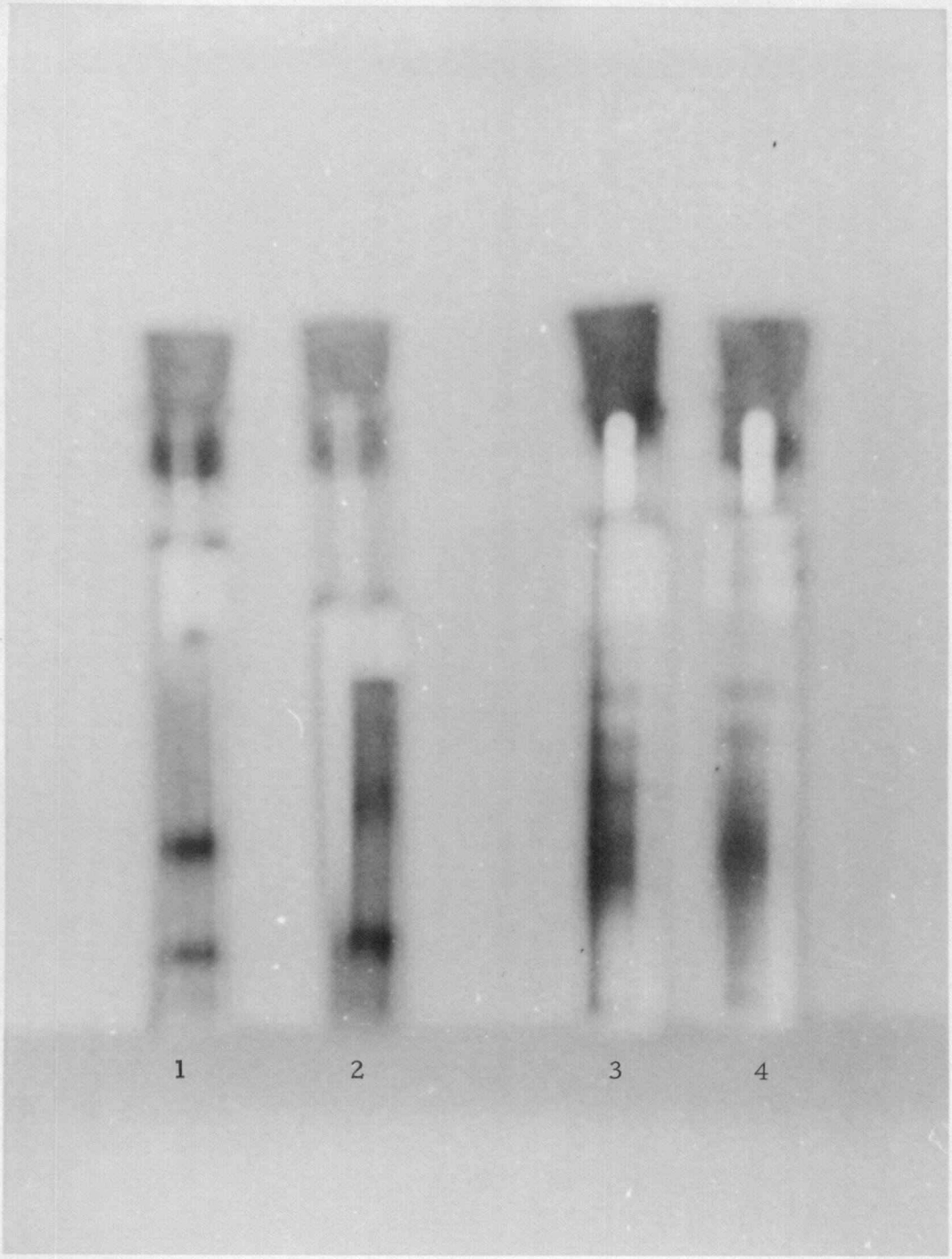

Figure 18. Gel electrophoresis. Pseudomonas aeruginosa QRM-S (1) revealed two esterase bands while only one band was observed in QRM-R (2). Gels 3 and 4 represent the total protein bands of QRM-S and QRM-R, respectively.

Permeability Studies

The rate of glucose uptake by resistant cells was much slower than the absorption by sensitive cells. It can be seen in Figure 19 that the amount of glucose absorbed in 5 min by sensitive cells exceeded that taken up in 60 min by resistant cells.

Attempts to alter cell surface or cell membrane characteristics with toluene or DMSO had no effect on the QAC susceptibility of either sensitive or resistant cells.

Infrared Spectroscopy

Infrared spectroscopy revealed little difference in the pattern of P. aeruginosa strains QRM-S and QRM-R.

Cytology of Sensitive and Resistant Cells

Microscopy

Using light microscopy, the sensitive and resistant cells were both small gram-negative rods but the resistant cells appeared to be a little smaller than the sensitive cells. Both cells possessed a capsule although that of the resistant cells was more pronounced. Observation of the cultures by the hanging drop technique revealed that the sensitive cells were motile while the resistant cells were not.

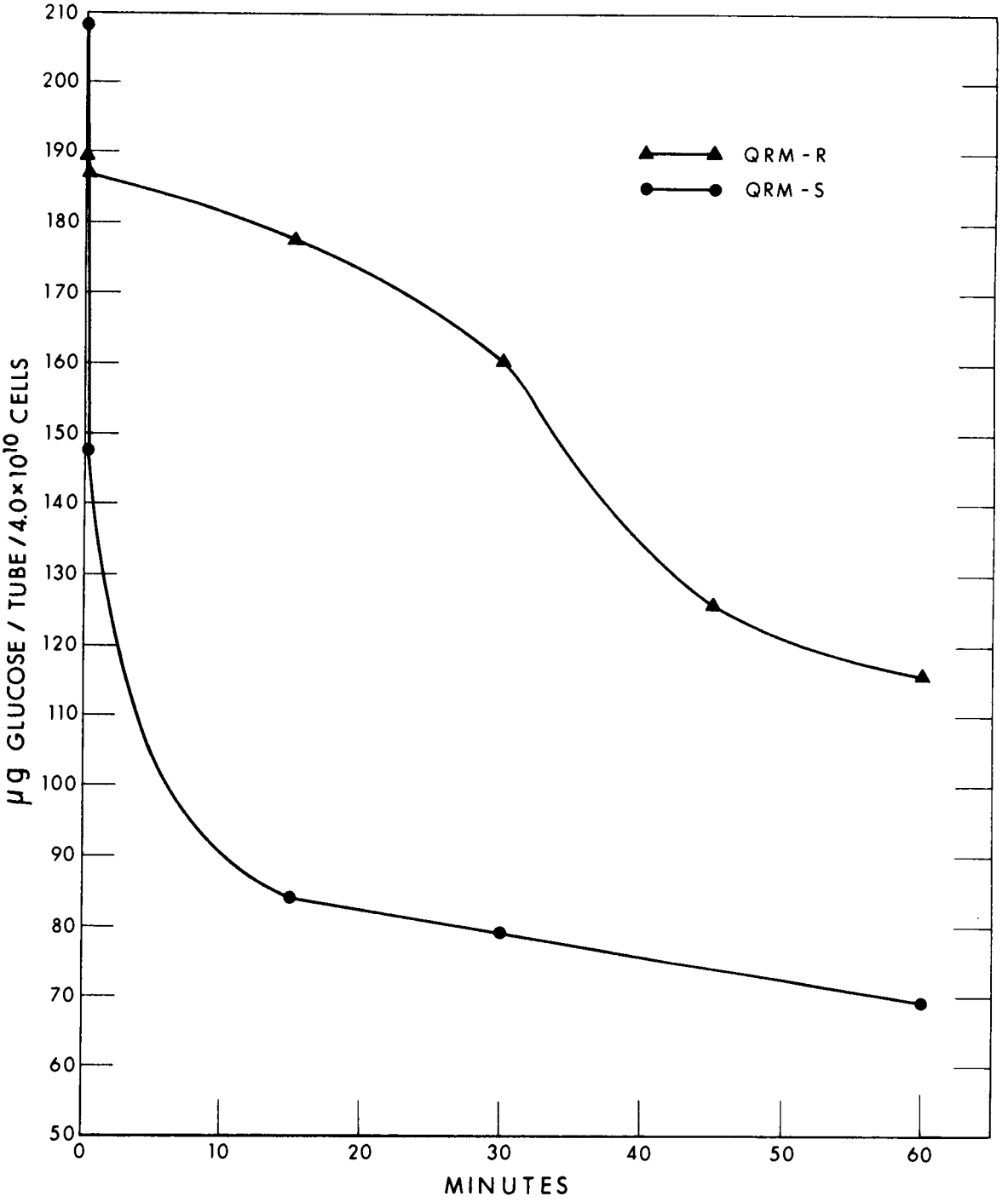

Figure 19. Glucose uptake by resting cells of QAC-sensitive and QAC-resistant strains of Pseudomonas aeruginosa.

Electron micrographs verified the presence of polar flagella on the sensitive cells (Figures 20, 21) and the absence of flagella from resistant cells (Figure 22). With the use of 0.264 μ latex beads as an internal standard, the average size of QRM-S cells was calculated to be 0.75 × 3.0 μ and the average size of QRM-R cells was 0.35 × 1.0 μ. It was interesting to note that QRM-RN cells were 0.6 × 2.5 μ, which is nearer the size of the sensitive cells.

In addition, dense areas were observed within the resistant cells (Figures 22, 23, 24), but not in the sensitive cells. These dense areas or bodies remained discrete after being released by cell lysis (Figure 25). Figure 26 shows a dense inclusion body with a 0.264 μ latex ball immediately adjacent to it. The size of the inclusion body, in this case, is about 0.17 × 0.20 μ. There is some indication of crystalline structure, although this can not be definitely stated at this time.

Electron micrographs (Figure 27) of thin sections of QRM-S cells revealed no dense areas corresponding to the inclusion bodies of the resistant cells. Due to the presence of QAC, initial attempts to obtain thin sections of QRM-R cells met with failure.

The effect of QAC on the cells of sensitive and resistant strains is shown in Figures 28, 29 and 30. It was noted that the flagella of sensitive cells appeared to be undisturbed by the presence of 100 ppm QAC and there was no apparent cell lysis. However, the

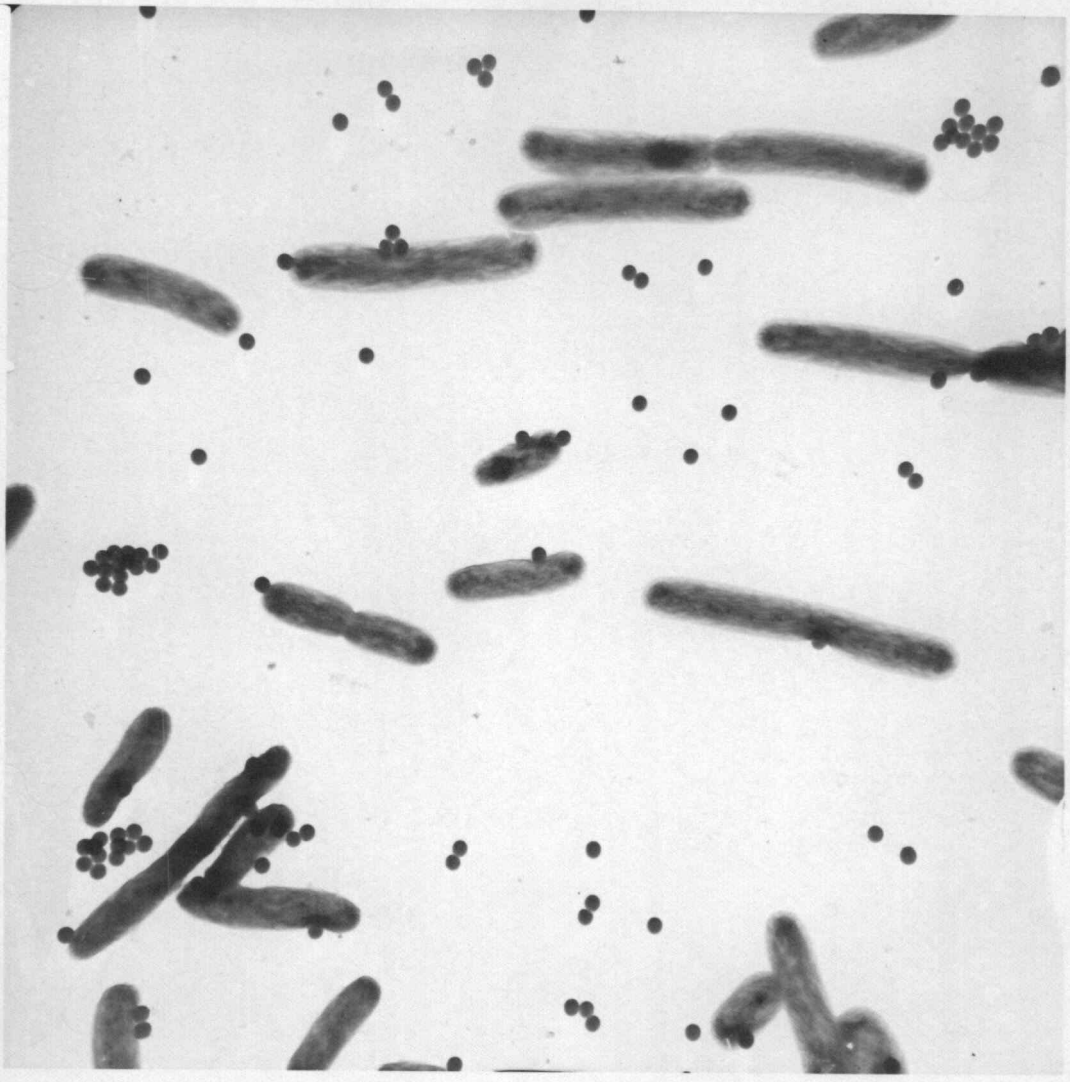

Figure 20. Electron micrograph of Pseudomonas aeruginosa QRM-S cells showing single polar flagella. Note internal standard latex beads 0.264μ in diameter. Platinum:Paladium shadow cast. Magnification approximately ×6,000.

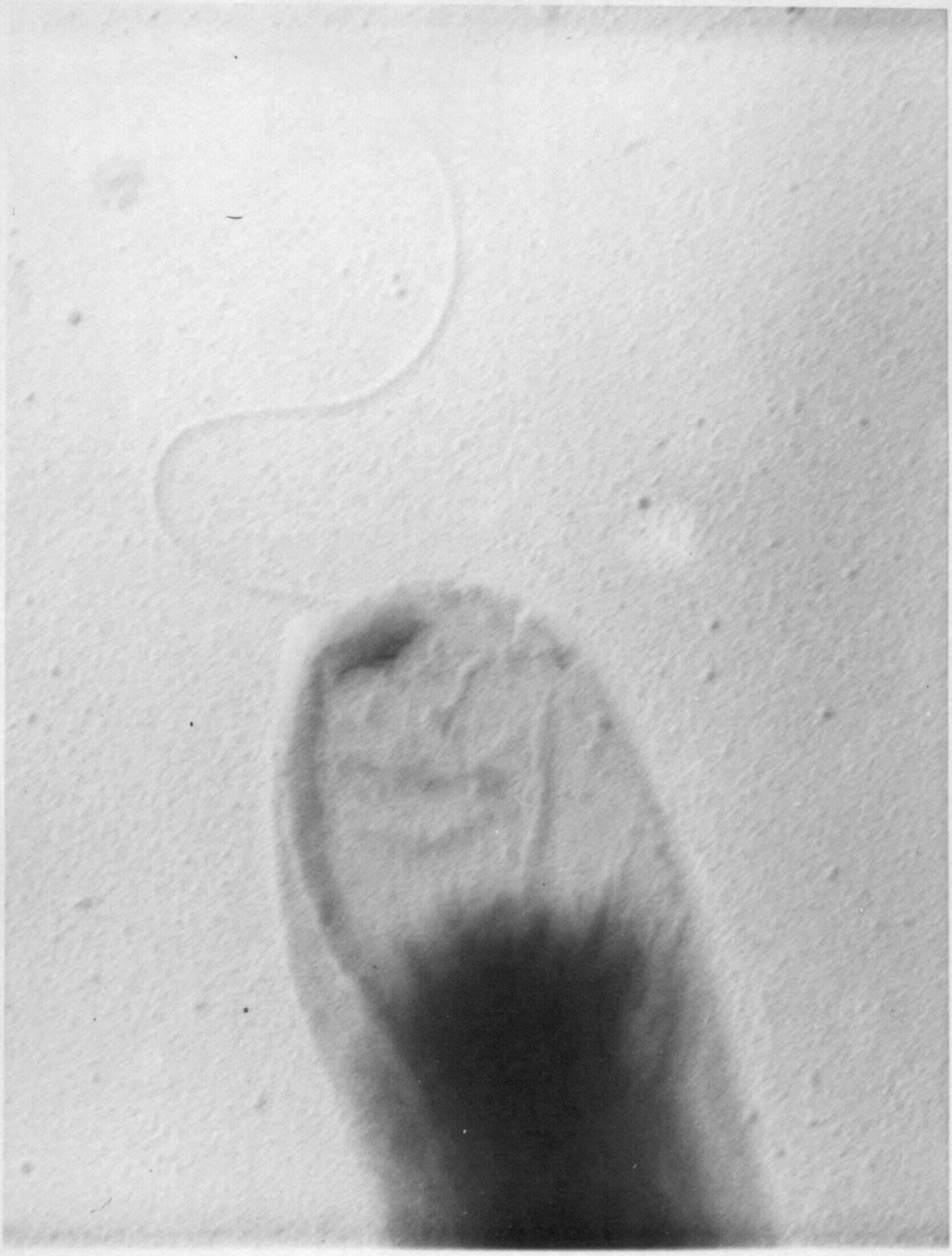

Figure 21. Electron micrograph of Pseudomonas aeruginosa QRM-S cell. Note polar flagella and shrinkage of cytoplasm from the cell wall. Platinum:Paladium shadow-cast. Magnification approximately ×65,000.

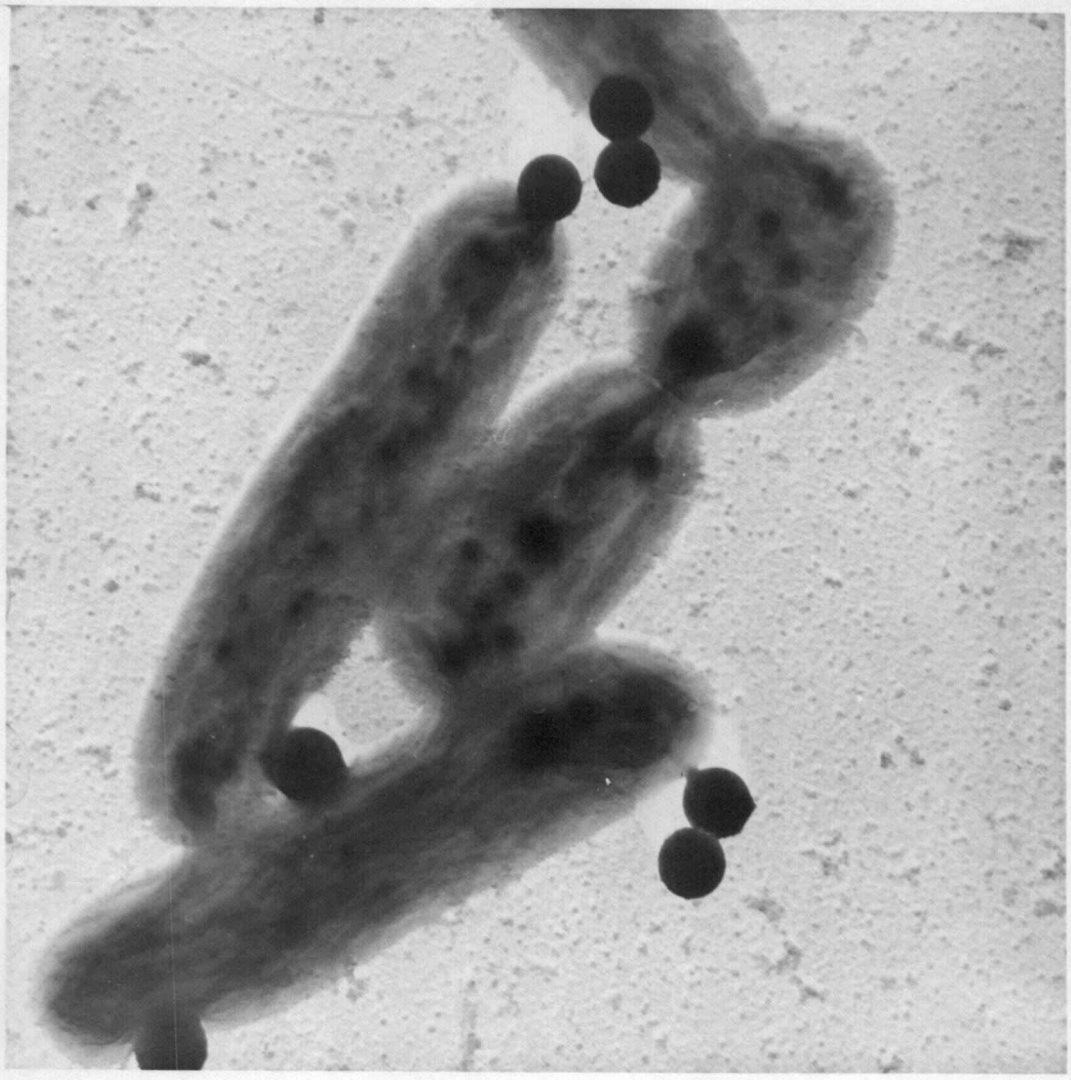

Figure 22. Electron micrograph of Pseudomonas aeruginosa QRM-R cells showing no flagellation. Note the presence of dense inclusion bodies. Magnification approximately ×20,000.

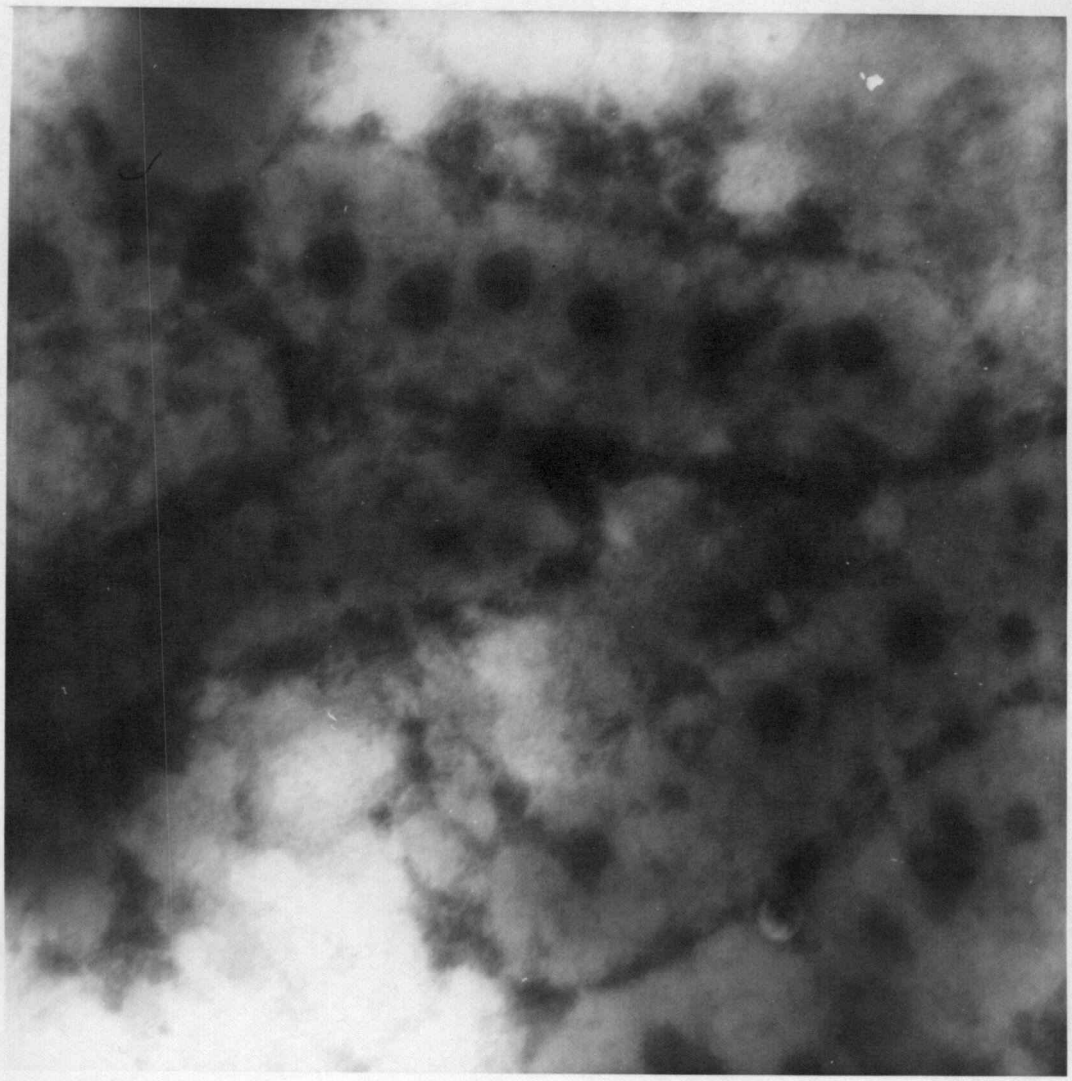

Figure 23. Electron micrograph of unshadowed, unstained cells of Pseudomonas aeruginosa QRM-R revealing the presence of dense inclusion bodies. Magnification approximately ×25,000.

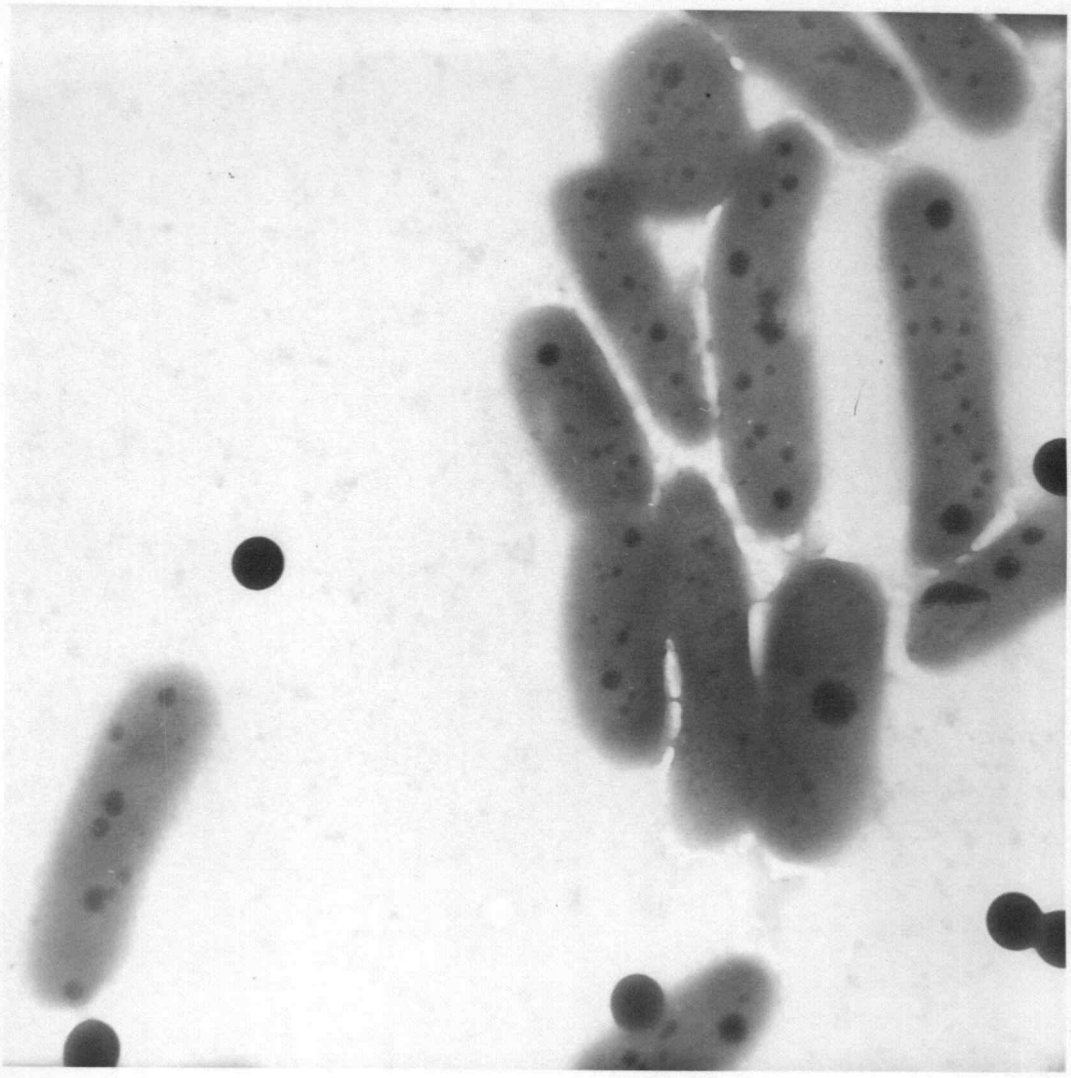

Figure 24. Underexposed electron micrograph of Pseudomonas aeruginosa QRM-R showing the variation in number and size of the inclusion bodies. Magnification approximately ×12,000.

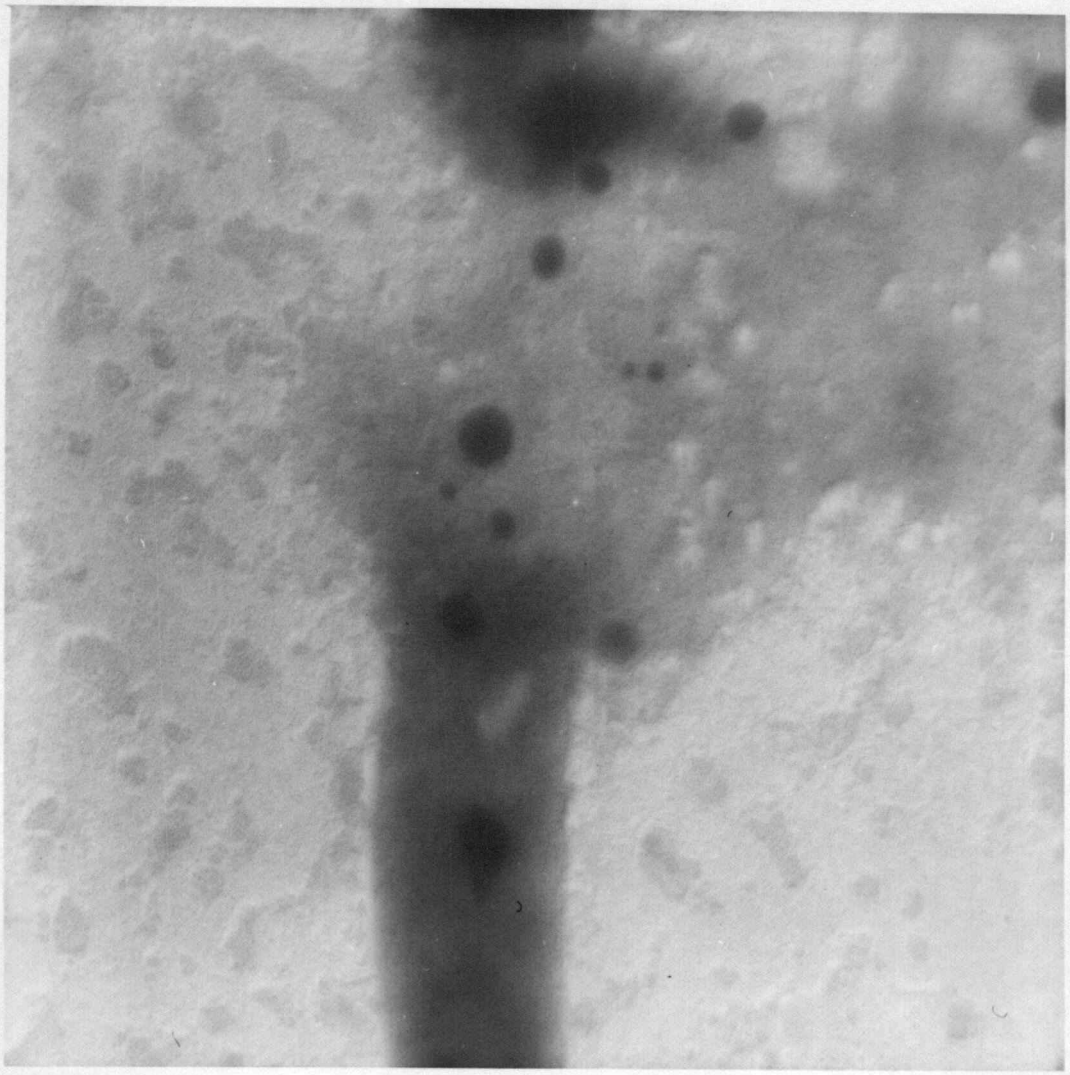

Figure 25. Electron micrograph of dense inclusion bodies that have been released from a lysed QRM-R cell. Magnification approximately ×18,000.

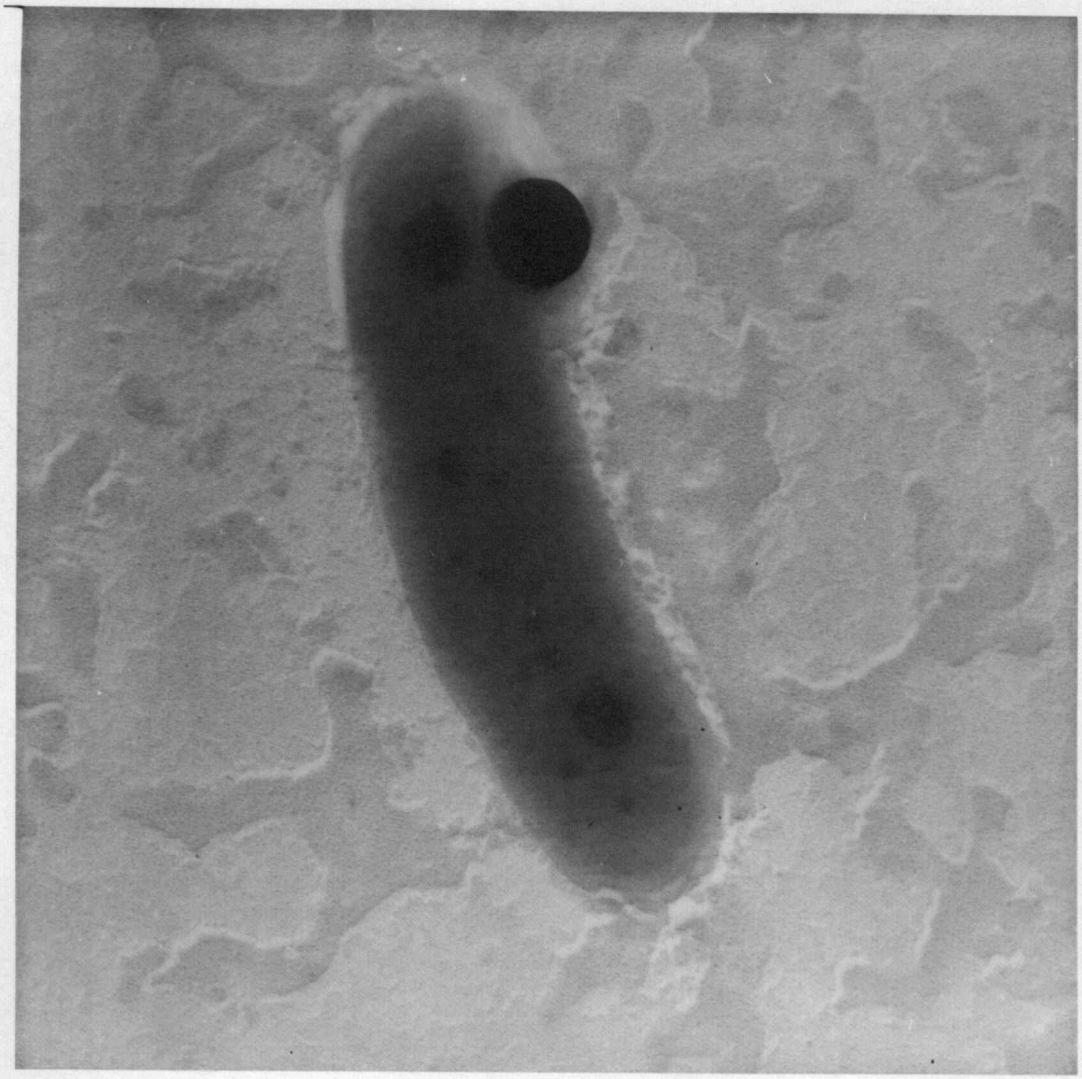

Figure 26. Electron micrograph of a resistant cell showing dense inclusion bodies and a 0.264μ latex ball as an internal sizing standard. Magnification approximately ×40,000.

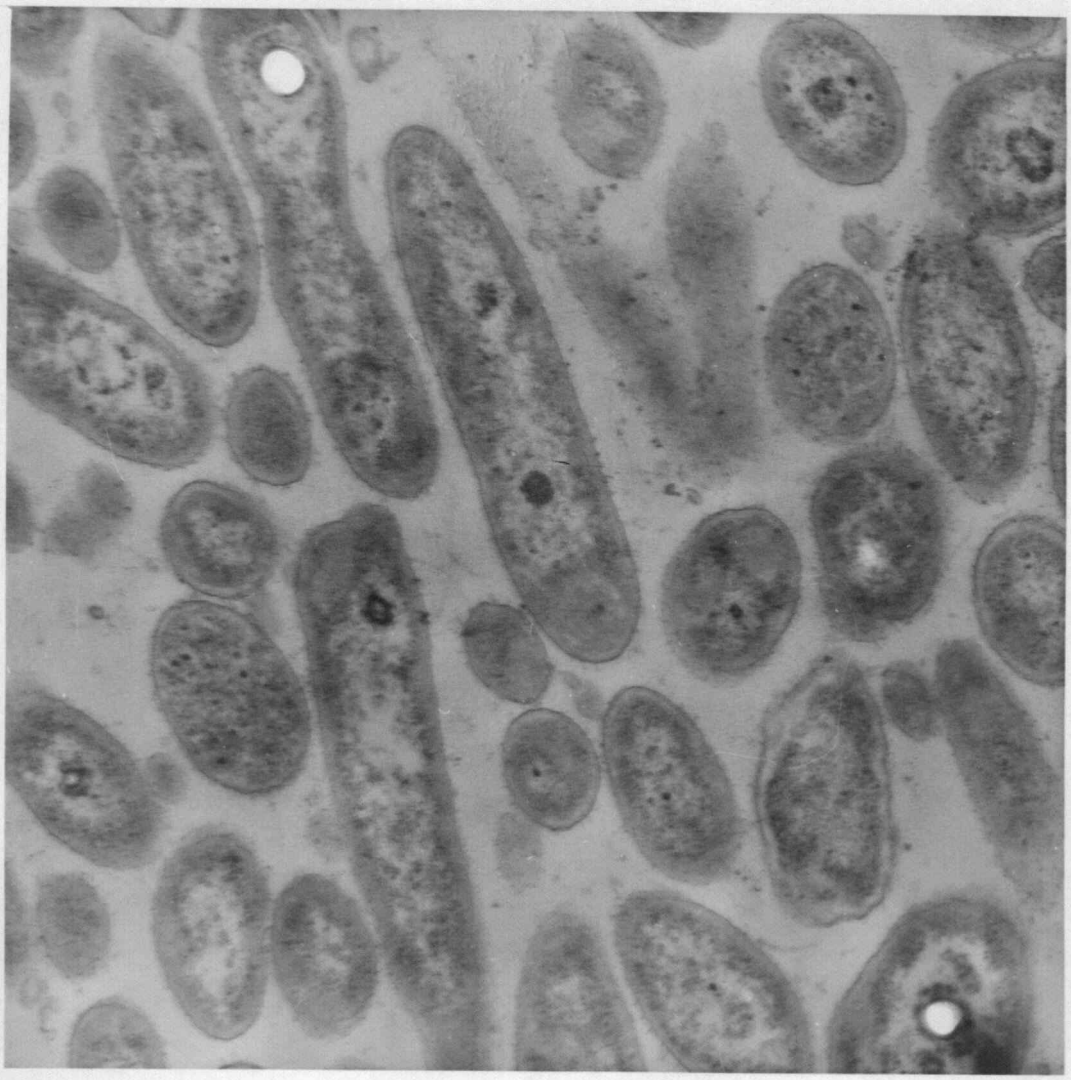

Figure 27. Thin section of cells of Pseudomonas aeruginosa QRM-S. Note the absence of dense inclusion bodies. Magnification approximately ×15,000.

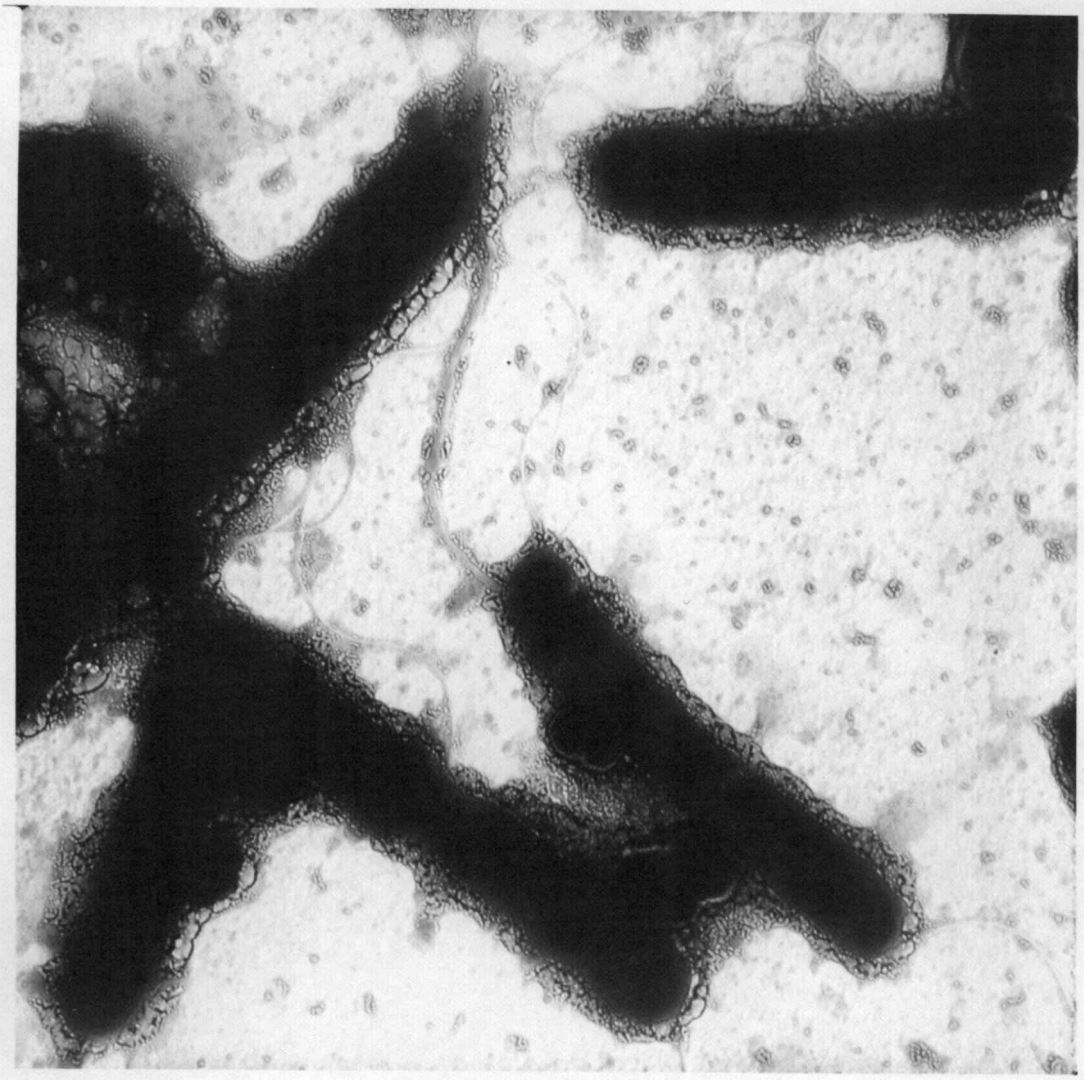

Figure 28. Effect of 100 ppm QAC on cells of Pseudomonas aeruginosa strain QRM-S. No cell lysis was apparent and the flagella were not disrupted. ×18,000.

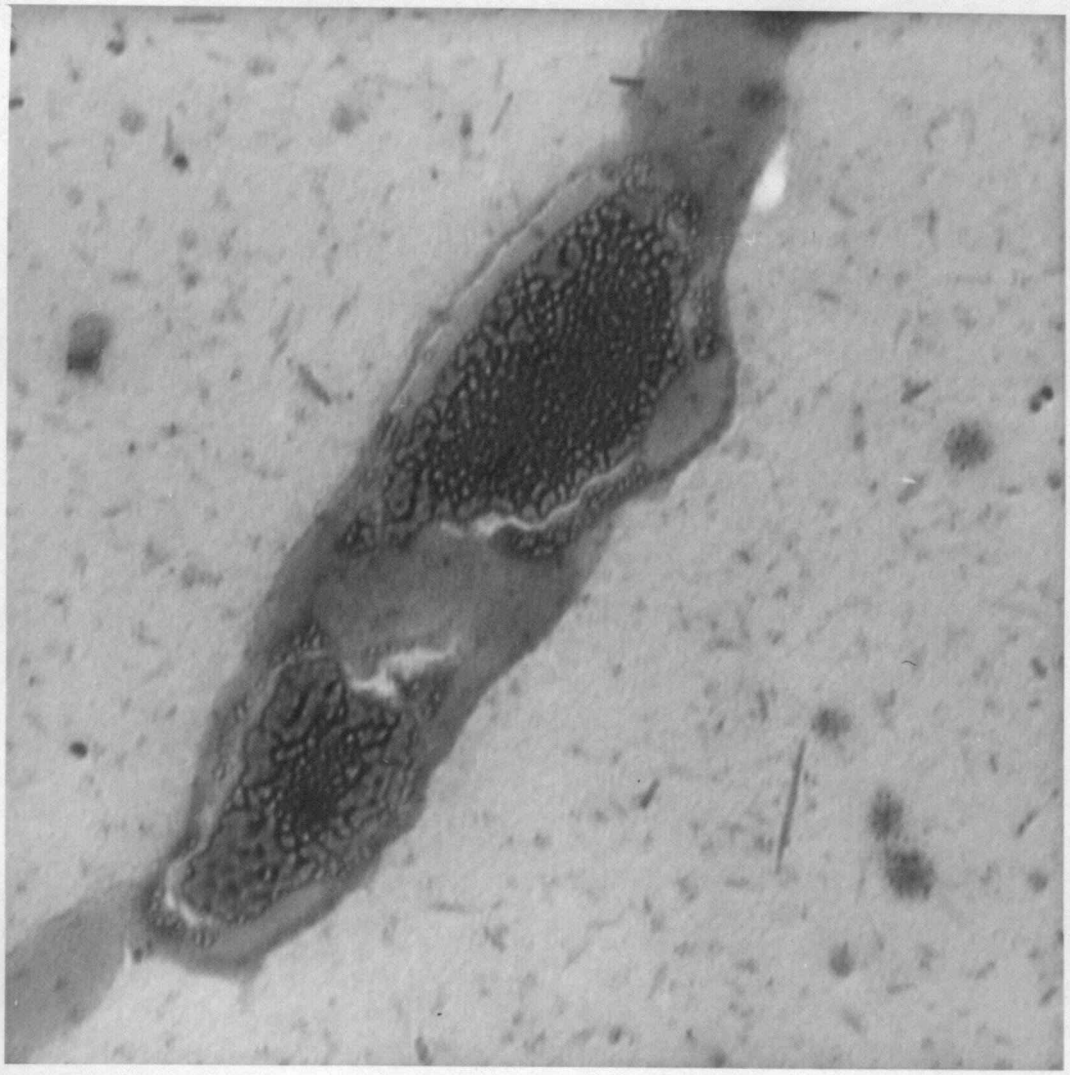

Figure 29. Effects of 10,000 ppm QAC on Pseudomonas aeruginosa QRM-S. Apparent cell lysis can be seen. Magnification approximately ×30,000.

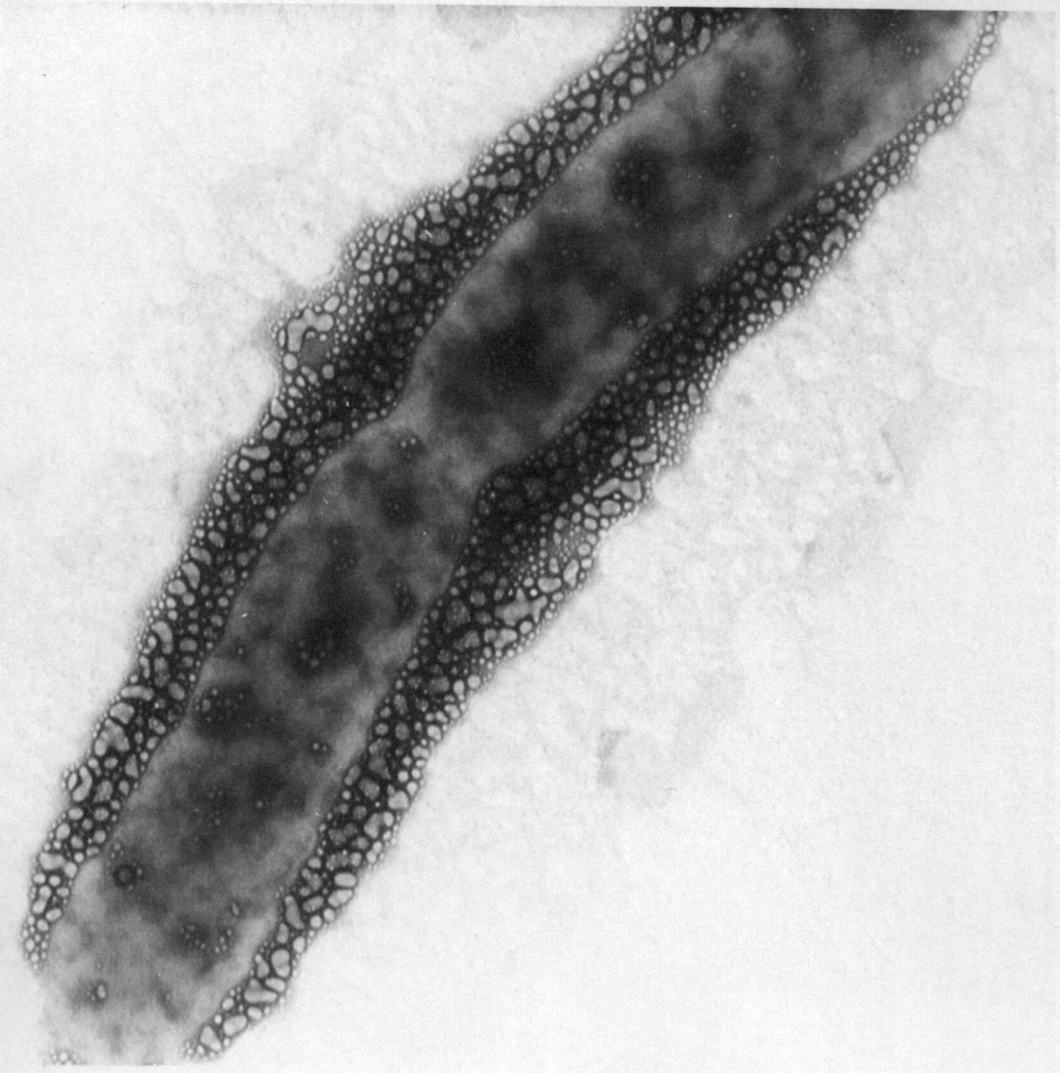

Figure 30. Effect of 1,000 ppm QAC on Pseudomonas aeruginosa strain QRM-R. There appears to be no signs of cell lysis. Magnification approximately ×30,000.

presence of 10,000 ppm QAC resulted in a violent lysis of some of the sensitive cells. Not all cells were lysed even at this concentration. The resistant cells appeared to be unaffected by 1,000 ppm QAC. It was interesting to note the coating affect of the QAC and the presence of bubble-like structures around treated cells.

Dry Weight Determinations

The average of five dry weight determinations of QRM-S and QRM-R cells showed the dry weight of a sensitive cell to be 3.05×10^{-13} g. The resistant cells had a density of 2.54×10^{-13} g.

Nucleic Acid Studies

Thermal Denaturation Studies

A true T_M value was not obtained. The proximity of the T_M value to the boiling point of the SSC solution caused difficulties in recording absorbancy changes within the range of primary interest. It can be safely stated that the T_M is between 96° and 100°C for the DNA of both sensitive and resistant cells. This is assumed to be near 98°C which is the T_M value ascribed to P. aeruginosa by Mandel (1966).

Attempts at Isolation of Mutants

Observation of approximately 20,000 colonies of QRM-R on spirit blue agar did not reveal a single lipolytic colony, nor did a similar search on milk agar reveal a proteolytic colony. Likewise, streaking of QRM-S on spirit blue agar and milk agar plates failed to detect non-lipolytic and non-proteolytic colonies.

Phage Studies

P. aeruginosa phage #4 was not completely destroyed by 30 min exposure to 10,300 ppm QAC. In contrast, no plaque forming units of S. cremoris phage 32-P could be detected after 60 sec exposure to 130 ppm QAC.

Attempts were made to determine the burst size of QRM-S and QRM-R cells using phage PC. The results varied widely and could not be duplicated. However, it was determined that both the resistant and the sensitive cells were susceptible to lysis by the same phage.

Characteristics of Sensitive Cells Exposed to Sub-Lethal QAC Concentrations

Several weeks were required to build the resistance level of QRM-S cells to 250 ppm. The biochemical characteristics of QRM-S cultures possessing levels of resistance from 25 to 250 ppm did not

vary from that of control QRM-S cells. This was true whether the biochemical test was done in sub-lethal concentrations of QAC or in the absence of QAC.

Resistance to Chlorine and Iodine

There was no apparent difference in the susceptibility of sensitive and resistant cells to sodium hypochlorite or to iodophor. Both cell types grew in the presence of 315 ppm chlorine and 33 ppm iodine.

DISCUSSION

Studies on the mechanism of germicidal activity of quaternary ammonium compounds have been plagued by inconsistency and a general inability to duplicate experimental results reported in the literature. In view of this, the first part of this study was devoted to understanding the chemical and physical properties of QAC and to the development of a reliable assay system for measurement of resistance of bacteria to the germicide.

It was observed that the addition of QAC to TGY broth or agar resulted in a marked turbidity which was dependent upon the amount of QAC added. This turbidity was not observed in synthetic media and was attributed to the interaction of the QAC and the protein in the TGY broth or agar. This interaction was shown to decrease the effective concentration of QAC either partially or completely, depending on protein:QAC ratio.

The cationic nature of QAC suggested that its antimicrobial powers depended on the lowering of pH, especially in unbuffered or weakly buffered solutions. In this regard, it was shown that at concentrations of 100, 515, and 10,000 ppm, the QAC solutions had pH values of 6.2, 4.7, and 3.3, respectively; therefore, it would appear that the solution with a strength of 515 ppm QAC at pH 4.7 could cause death of P. aeruginosa cells in an unbuffered suspension

just by a pH effect. The use of buffered solutions then would perhaps invalidate conclusions drawn about the effectiveness of QAC germicides under natural conditions where the pH is unknown.

However, it is essential to control this factor in laboratory studies designed to reveal information about mechanism of action. In interpreting results, it also must be kept in mind that it has been shown that salt concentration, type of salt, ionization and pH all affect the activity of QAC and possibly the integrity of the cell membrane and cell wall structures. Thus many factors must be considered in evaluating data that might be used to support any hypothesis on the mechanism of action of QAC.

In addition to the above, it must be realized that mixing of bacterial cells and QAC will also affect the concentration of germicide in the same manner as the addition of protein; it can not be assumed that QAC acts simultaneously and equally on all exposed cells. Rather, it is more likely that the QAC bound by some cells lessens the concentration effective against remaining cells. Consequently the last surviving cells may actually be resisting a QAC concentration many-fold less than the original concentration.

In as much as QAC is bound by protein, it was reasonable to assume that the composition of the test medium would greatly affect the level of QAC required to prevent growth. It was subsequently demonstrated that increases in the tryptone content of TGY broth

from the standard 5 to 20 and 30 g per liter caused 3 fold and 10 fold increases, respectively, in the resistance of QRM-R. Increases in yeast extract content also resulted in apparent increases in resistance, although not as great as that observed for tryptone. Similarly, QRM-S cells could grow in reconstituted non-fat dry milk (11%) with 3,600 ppm QAC added. No affect was observed when the glucose concentration of TGY broth was elevated from 0.1 percent to 3 percent.

The recognition of the importance of protein, pH, salts, and ions, on the effective germicidal concentration of QAC requires that any claim of QAC resistance be accompanied by a careful description of the conditions under which the resistance was achieved.

In the first phases of this work QAC resistance was studied using TGY agar as the experimental medium and the medium for maintaining cultures. It was noted initially that sensitive cells grew on TGY agar containing QAC concentrations in excess of 5,000 ppm; this was not, however, regarded as true resistance. Rather, it was believed that two factors were involved in allowing the sensitive cells to grow on the agar surface. First, the bottom portion of the cell mass comprising the original inoculum provided protection for the surviving cells in the upper part of the inoculum by neutralizing the effect of the QAC. Secondly, it seemed likely that the QAC was more tightly bound in an agar medium and that less was free to act

against the cells.

A more nearly accurate concept of true resistance was obtained using a semi-synthetic broth. The resistant strain grew in the presence of 40 ppm QAC while the sensitive strain grew in the presence of 10 ppm. However, this semi-synthetic broth was not used as the principal assay medium in subsequent experiments, for it was felt that experimental variation would be excessive under these conditions. Any errors or variations in diluting, pipetting, etc. would have a more pronounced effect when dealing with small concentrations. Therefore, TGY broth was selected as the culturing and test medium. The resistant strain was able to grow in the presence of 750 ppm QAC while the sensitive cells grew only in TGY broth containing less than 35 ppm QAC.

It was realized that cell growth in a broth containing 750 ppm QAC did not fully express what was happening to the cell mass comprising the original inoculum. That is to say, the number and fate of QRM-R cells from the original inoculum that gave rise to growth and pellicle formation in TGY broth was not known. The question was then asked "Are all QRM-R cells equally resistant?" This led to a comparison of death curves of sensitive and resistant cells when exposed to QAC.

This exposure of washed cell suspensions of sensitive and resistant strains to 260 ppm QAC revealed a significant difference

between the two cell types; about 99.999 percent of the sensitive cells were killed within two minutes of exposure. In striking contrast, after sixty minutes the number of resistant cells had not been detectably deminished. The reality of this difference was firmly established when ten times more sensitive than resistant cells were used in order to eliminate the effect of cell numbers or total bacterial protein as a factor favoring resistance. This indicated a true difference in the physical, chemical or metabolic nature of the two cell types.

It was recognized early in this study that the resistant culture did not exhibit pellicle formation in TGY broth with the same rapidity as the sensitive cells. Growth curve studies were then undertaken to disclose any difference in the generation times. In addition to QRM-S and QRM-R a resistant strain, QRM-RN, that had been maintained in the absence of QAC for several weeks, was included. The generation times of QRM-R and QRM-RN were about 15 minutes longer than that of sensitive cells. This indicated that a difference may exist in permeability or metabolic rate between the two types of cells.

In related experiments the effects of various factors on the growth curves of sensitive and resistant cells were determined. The addition of QAC to cultures of resistant cells resulted in an "apparent" decrease in cell count followed by a rapid increase in count back to

the previous level. The term "apparent" is used in referring to the decrease in cell counts of resistant cells exposed to QAC because it is not known whether this decrease truly represented cell death. The decline followed by a rapid population increase could represent aggregation of the bacterial cells followed by a gradual disaggregation. A possible alternative explanation is that the decrease in cell numbers represents the bacteriostatic action of the QAC followed by a gradual decrease of inhibition giving an appearance of rapid growth.

The interrelationship of temperature, aeration, and QAC concentration on the growth curves of sensitive and resistant cells was also investigated. It was noted that 515 ppm QAC alone did not cause a lasting decline in the resistant cell population and neither did incubation at 4°C. However, the combination of 515 ppm QAC and an incubation temperature of 4°C resulted in a reduction of cell count after 125 hours in excess of 99 percent. The explanation for this reduction is not yet apparent, but it appears to be a coupling of the effects of low temperature and the activity of QAC. This could be related to the decreased rate of growth as a result of enzymic sluggishness at low temperatures combined with inactivation of certain surface or membrane oriented enzymes. There may also be a change in the charged groups of the cell envelope or a change in the degree of unsaturation of the fatty acids, both of which would alter the permeability of the cells to extracellular nutrients and possibly

disrupt or retard normal cellular metabolism.

Observations of colonies of both sensitive and resistant cells formed on TGY agar and TGY agar containing QAC revealed interesting differences. Dense areas associated with colonies of resistant cells could be seen when observed microscopically. It is possible that the dense areas were due to a combination of cell aggregation and destruction by the action of QAC. This, however, seems to be ruled out by the fact that the resistant cells exhibited the phenomenon in the absence of QAC. It seems more probable that the dense areas resulted from a buildup of cellular reserves. It is not intended to proclaim this as the mechanism of QAC resistance although it might well play an active role. It also was observed that the sensitive cells exhibited a similar colony morphology when exposed to a sub-lethal concentration of QAC.

A study of cell morphology revealed resistant cells to be much smaller (1.0 X 0.35 μ) than the sensitive cells (3.0 X 0.75 μ). The size difference could be a consequence of the surface activity of the QAC. This might best be represented by the fact that resistant cells transferred several weeks in the absence of QAC regain a size, 2.5 X 0.6 μ, more nearly like that of the sensitive cells. The absence of flagella from the resistant cells in the presence of QAC could be similarly explained. However, the failure of the resistant strain, grown in the absence of QAC for several weeks, to synthesize

flagella can not be attributed solely to the surface activity of the QAC. There must be some changes induced at the genetic level which lead to permanent alterations that are retained generation after generation in the absence of QAC.

A striking and unexplained difference was observed in the electron micrographs of the sensitive and resistant cells. Dense intracellular bodies ranging from one to many per cell were observed in the resistant cells grown in the presence or absence of QAC. These bodies were discrete and did not lose their integrity when released from lysed cells. They give some indication of being either crystalline or membrane enclosed. Tests for the presence of poly-β-hydroxybutyric acid were negative.

The fact that the resistant cells grew slower and had a 25 percent longer generation time could not be attributed to the direct effect of QAC. Resistant cultures that had been maintained for several months in the absence of QAC exhibited a similar retarded growth rate. Perhaps an alteration to a less efficient metabolism could account for the differences in growth rates. In accordance with this line of thought, the resistant cultures displayed no signs of proteolytic activity of milk agar plates after one week while very large zones of proteolysis were caused by the sensitive cells in only 48 hr. Measurement of proteolytic activity of sonicates of the two strains revealed that both cultures possessed proteases. However, the

specific activity was much higher in sonicates of the sensitive strain. From this it would appear that extracellular or cell surface enzymes may be deficient in the resistant strain, causing the organisms to grow slower.

It was also considered that permeability differences existed between the two cell types. To investigate this, glucose uptake by resting cells of both strains was followed as a function of time. A striking difference in the rates of glucose uptake was found. The amount taken up in 5 minutes by sensitive cells exceeded the amount taken up by resistant cells after one hr. This difference might be explained by the difference in size of the QRM-S and QRM-R cells. It is likely that this is a partial explanation, but differences in cell permeability also must be involved. Perhaps the resistant cell has an altered cell membrane or cell wall with a different net charge as well as an altered structure. In addition cell membrane oriented enzymes involved in transport systems could be altered or destroyed in the resistant cells.

A pronounced fruity aroma was associated with broth cultures of the resistant strain. This aroma was much more noticeable from cultures grown on a shaker or sparged with air than in the stationary cultures. The sensitive culture had the usual displeasing odor of P. aeruginosa with no traces of fruitiness. Gas chromatographic analysis of cultures revealed the presence of ethyl acetate and ethyl

valerate in much greater concentrations in the resistant culture. Several minor and unidentified peaks indicated additional differences in the metabolic activity of the two strains. Since resistant cells also contained about 48 percent more total lipid than sensitive cells, the detection of ethyl esters of fatty acids in the resistant culture focused more attention on the involvement of lipids and lipid metabolism in QAC resistance.

Experiments on lipid involvement led to gel electrophoresis of cell-free extracts of both sensitive and resistant cells. The prepared gels were specifically stained for esterase activity bands. The sensitive cell extracts were found to possess two distinct esterase bands; resistant cell extracts displayed only one esterase band, corresponding to the leading band of the sensitive cells. In addition, incubation of cultures on spirit blue agar revealed the inability of the resistant strain to hydrolyze fat, a reaction readily performed by the sensitive cells. From this it would appear that the resistant cells have an impaired lipid catabolism, at least to the extent that they are unable to hydrolyze certain esters which consequently accumulate in the medium. Since the esters detected are quite volatile, it is unlikely that they are important structural components of resistant cells causing the reduced permeability observed. However, this does not exclude the possibility that higher molecular weight esters or other altered lipoidal substances are

structural components in the cell.

The question might be raised: Is QRM-R actually P. aeruginosa or a contaminant introduced during transfers required to produce a resistant strain? This must be considered since it was not possible to classify the emerging bacterium using biochemical tests and observing electron micrographs for polar flagella. Nevertheless, a great deal of confidence can be placed in the identity of the resistant organism as a mutant of the parent sensitive strain. Thermal melting determinations of the DNA isolated from both QRM-R and QRM-S indicated that both strains had T_m values around 98°C. In addition, P. aeruginosa phage 4 was capable of causing lysis in both the sensitive and resistant strains.

There are too many traits retained by resistant cells after subculturing for several months in the absence of QAC to attribute the differences between sensitive and resistant strains to purely adaptive alterations or phenotypic changes in the cells. It is quite possible that one major change in cellular metabolism could account for a great number of the differences observed here. It is known that the resistant strain has undergone some alteration in its lipid metabolism and this seems to be the key to the resistance to QAC. If this is the case it would be beneficial to consider more deeply what could be occurring in the resistant cells.

It is reasonable to suppose that sub-lethal concentrations of

QAC have some physical and chemical effect on sensitive cells. Being a surface active cationic detergent, the QAC would have some affinity for cellular lipid. If this were the sole effect of QAC one would not expect retention of resistant traits after sub-culture in the absence of QAC or after washing the cells. However, it is quite probable that the QAC penetrates the cell envelope and acts on some target area inside the cell. In the case of the organisms used in this study, at least, it is likely that the target area is the pathway of lipid synthesis or the region of DNA responsible for the coding for enzymes controlling lipid metabolism. The latter seems more likely because of the apparent stability of resistant characteristics in the resistant cells after several months of sub-culturing in the absence of QAC. As a result of this genetic alteration, cells could be produced that still store fatty acids in the form of esters, but would lack sufficient esterase activity for the rapid breakdown of these esters. Therefore, esters would pile up and some would be released into the medium resulting in a fruity odor. In addition, the type of fatty acid produced by the cell might be altered. Perhaps more unsaturated fatty acids would be incorporated into the cell membrane and cell wall structures. This could result in an alteration in the charge of the cell. Possibly due to this charge or due to the physical change in the envelope structure, caused by substituted fatty acids, the membrane could become less permeable.

With some consideration of the extracellular nature of the lipase and protease systems, it can then be reasoned that membrane-associated synthesis of enzymes would be affected greatly by changes in the membrane. Perhaps enzyme synthesis itself would be affected or, possibly, release of the enzyme into the cellular environment would be hindered. In either case, this decrease in ability to perform extracellular fat and protein breakdown would affect the rate of growth of the cell simply by limiting the carbon and nitrogen supplies going into the cell.

The thought should not be discarded entirely that the pile-up of esters of fatty acids in the bacterial cells might result in some feedback or repression effects on the metabolic pathways of the organism. This might result, also, in decreased lipase and protease activities and a slower rate of growth.

SUMMARY

The development of drug resistance in bacteria is a common phenomenon and causes a multitude of problems in chemotherapy and sanitation programs. When normal sensitive strains of Pseudomonas aeruginosa were maintained in TGY broth containing sub-lethal concentrations of QAC strains were developed that possessed high levels of resistance to QAC. This provided an opportunity to study both the mechanism of resistance and the mode of action of QAC. One sensitive strain (QRM-S) and one resistant strain (QRM-R) were selected for detailed study.

The cultural behavior of both cell types was similar in many respects. The type of colony formation on TGY agar plates and the characteristic growth on TGY agar slants were quite similar visually. Also, both cell types formed pellicles on TGY broth. The resistant cells did grow more slowly and, in broth, produced a distinct fruity odor. Inoculation of gelatin deeps with sensitive cells was followed by rapid gelatin hydrolysis while resistant cells displayed a greatly retarded gelatin hydrolyzing ability. Other biochemical tests revealed the resistant strain had lost several biochemical characteristics and were very slow in producing positive reactions in other tests. Milk agar showed no signs of proteolysis by QRM-R after 14 days. This presented the thought that resistant

cells might grow more slowly because of reduced proteolytic activity. Assay of cell sonicates for protease activity showed resistant cells to be truly deficient as compared to sensitive cells.

Growth curve studies of both strains disclosed a 15 minute longer generation time for resistant cells in TGY broth. This could relate back to the decreased proteolytic activity of resistant cells. However, an additional factor exists which may be equally important. A permeability study involving the uptake of glucose demonstrated a significant difference, with the resistant cells being much less permeable.

A study of lipid involvement indicated some major differences between the sensitive and resistant cells. The resistant strain exhibited intense staining by Sudan Black B while sensitive cells did not. Solvent extraction of lyophilized cells showed a 48 percent greater lipid content in resistant cells. In addition, the resistant cells failed to produce sufficient extra-cellular lipase to be detectable on Spirit Blue Agar after two weeks. Gel electrophoresis of cell-free extracts of both cell types revealed two bands of esterase activity with the sensitive cells while only one was obtained with the resistant cells. In light of this, the fruity odor characterizing the resistant culture became of major interest. Head space analysis of cultures of both strains using gas chromatography detected the presence of ethyl acetate and ethyl valerate in the resistant culture

and not in the sensitive culture.

Since "normal" sensitive cells demonstrated proteolytic and lipolytic activity and "normal" resistant cells did not, a search for revertants or back mutations was conducted by plating both cell types on Spirit Blue Agar and milk agar and observing for opposite colony types. Examination of several thousand colonies did not reveal any opposite cell types in either the sensitive or resistant cell populations. This indicated that the changes in cell characteristics accompanying the development of resistance were rather stable changes.

Comparison of sensitive and resistant cells by electron microscopy showed that the resistant cells were considerably smaller and lack the polar flagella which characterize the sensitive strain. Furthermore, resistant cells were found to contain dense inclusion bodies that were crystalline or membrane enclosed.

Growth of resistant cells in the absence of QAC did not meet with any pronounced decrease in QAC resistance. It is apparent that resistance does not require continual growth in QAC. The acquisition of resistance appeared to result from definite chemical changes within the bacterial cell after exposure to QAC. There does not appear to be a single step selection of mutants or resistant cell types from the normal cell population. It is quite evident from Table 9 that a great number of differences exist between the sensitive and resistant

strains. Perhaps many of these differences have no bearing on the resistance to QAC, but are secondary effects of other changes that have occurred. It seems quite likely that the key to resistance involves the lipid metabolism of the organism.

Table 9. Summary of the differences existing between the QAC sensitive and QAC resistant strains of Pseudomonas aeruginosa.

Character	QRM-S	QRM-R	QRM-RN
Growth in 500 ppm QAC	0	+	+
Monotrichous flagellation	+	0	0
Cell size			
Length	3.0 μ	1.0 μ	2.5 μ
Width	0.75 μ	0.35 μ	0.6 μ
Dry weight	3.05×10^{-13} g	2.54×10^{-13} g	g
Inclusion bodies	0	+	+
Lipase	+	0	0
2 esterase bands	+	0	0
Fruity odor	0	+	+
Head space analysis			
Ethyl acetate	0	+	+
Ethyl valerate	0	+	+
Sudan black staining	0	+	+
Percent total lipid	8.4	14.2	
Generation time	42.8 min	58.4 min	57.7 min
Glucose permeability	+	±	
Gluconate oxidation	+	±	±
Nitrate reduction	+	0	0
Growth at 42°C	±	+	+
Growth in 5% NaCl	+	±	±
pH of washed cell suspension	6.7	7.6	
Proteolysis	+	±	
Colony morphology	Homogenous	Dk. center	Dk. center

+ = positive; ± = slow or slight; 0 = none

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