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Inga Anna Schroder Albin for the M.S. in Microbiology
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To determine whether some bacteria are more susceptible than others to quaternary ammonium compounds, the following bacteria were grown on agar slants containing various concentrations of alkyl dimethyl ethyl benzyl ammonium chloride (ADEBAC), and alkyl dimethyl benzyl ammonium chloride (ADBAC): Aerobacter aerogenes 12658, Alcaligenes metalcaligenes, Brevibacterium linens, Chromobacterium lividum, Escherichia coli 198, Micrococcus caseolyticus, Microbacterium flavum, Microbacterium lacticum, Proteus vulgaris, Pseudomonas aeruginosa, Pseudomonas fluorescens MI, Pseudomonas fragi, Pseudomonas saliciperda, Pseudomonas syringae, Staphylococcus aureus 209, and Streptococcus lactis E.

Gram-negative test organisms developed higher resistance to both the ADEBAC and the ADBAC compounds. The gram-positive

bacteria were found to be more susceptible to the action of these compounds.

The higher lipid content of the gram-negative bacteria appears to play a role in protecting these organisms against the injurious effects of the quaternary ammonium compounds.

Quaternary ammonium resistant strains of the test organisms were found to contain more fat bodies per cell than the sensitive strains when stained with Sudan black B.

The ADEBAC sensitive and the ADEBAC resistant strains of the following gram-negative test organisms were used for comparative tests: A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris, P. aeruginosa, P. fluorescens MI, P. fragi, P. saliciperda, P. syringae, and S. marcescens.

It was found that the growth temperature had an effect on pigment production in the case of the ADEBAC sensitive strains of S. marcescens. The test organism failed to produce its characteristic pink pigment when incubated at 37°C., a temperature above its optimum. It is postulated that the higher growth temperature has an effect on the cells' enzymatic composition.

No differences were observed among the ADEBAC sensitive and the ADEBAC resistant strains of the test organisms with regard to motility and growth patterns on TGY agar slants and in nutrient broth.

The ADEBAC apparently had an inhibitory effect on gelatinase, nitratase, tryptophanase and various carbohydrase enzymes of the ADEBAC resistant strains.

From the results obtained in this study, it may be concluded that gram-negative bacteria are more resistant, and the gram-positive bacteria more susceptible to quaternary ammonium compounds; and that the quaternary ammonium compounds have an inhibitory effect on certain bacterial enzymes. Furthermore, quaternary ammonium germicides may alter lipid metabolism of cells as they become resistant and this change may eventually be seen as involved in the mechanism of resistance.

EFFECT OF QUATERNARY AMMONIUM COMPOUNDS ON BACTERIA: COMPARISON OF SENSITIVE AND RESISTANT STRAINS OF THE SAME SPECIES

by

INGA ANNA SCHRODER ALBIN

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Professor of Microbiology in Charge of Major

[redacted]

Chairman of Department of Microbiology

[redacted]

Dean of Graduate School

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EFFECT OF QUATERNARY AMMONIUM COMPOUNDS ON BACTERIA: COMPARISON OF SENSITIVE AND RESISTANT STRAINS OF THE SAME SPECIES

INTRODUCTION

In recent years, formulations containing quaternary ammonium compounds have become widely popular as detergent-sanitizers. The reason for this is that not only are they relatively cheap, but that they are odorless, colorless, highly stable, nontoxic, nonirritating and noncorrosive. Certain chemicals are incorporated into the formulations to counteract disadvantageous effects of such factors as hard water ions and pH on the cleansing and sanitizing actions of these compounds.

Previous studies have indicated that gram-positive bacteria are more susceptible to quaternary ammonium compounds than the gram-negative bacteria. It has been reported that quaternary ammonium compounds have an inhibitory effect on bacterial enzymes. Therefore, it appeared desirable to study the effects of quaternary ammonium compounds on gram-positive and gram-negative bacteria. Also, it seemed worthwhile to compare the quaternary ammonium sensitive and resistant strains of the test organisms with regard to their reactions to various tests. Any new knowledge gained with regards to the mode of action of the

quaternary ammonium compounds as revealed from results of these comparative studies will result in the formulation of more effective detergent-sanitizers.

HISTORICAL

According to Campbell (1958) the "synthesis of quaternary ammonium compounds was first reported in 1897. Although their antiseptic activity was recognized in 1908, little interest in their use as germicides was aroused until 1928."

Domagk (1935) reported on the bactericidal acitivity of the cationic quaternary ammonium detergent alkyl dimethyl benzyl ammonium chloride, commercially known as Zephiran. Dunn (1936, 1937), as well as Heineman (1937), also wrote about the desirable antiseptic and bacteriostatic properties of high molecular alkyl dimethyl benzyl ammonium chloride. He noted that these compounds were quite stable at different temperatures over various time periods; and that they were more rapid in their germicidal action against Bacillus subtilis at an alkaline pH than at an acid or neutral pH.

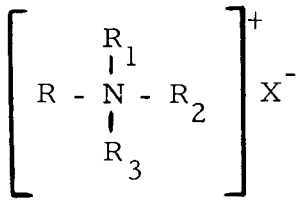
General Properties of Quaternary Ammonium Compounds

Quaternary ammonium compounds when used as disinfectants have the advantages of being odorless, colorless, highly stable, nontoxic, nonirritating and noncorrosive. They are effective over a relatively wide pH range, and are not unduly affected by the presence of organic matter. They appear to be somewhat

more effective against gram-positive organisms than against gram-negative organisms or spores. The fact that their efficiency is reduced in the presence of such hard-water ions as calcium, magnesium and iron, is one of the disadvantages of their use (Glassman, 1948, Ambruster and Ridenour, 1949, Lazarus, 1952, Reddish, 1954, Klimek, 1955, Borowsky, 1956, and Foster et al. 1957).

Quaternary ammonium compounds are surface active agents. They possess the ability to lower the surface tension of water, thus increasing its wetting properties. Surface active agents may be classified as anionic, cationic or nonionic. The anionic surface active agents carry a negative charge on the active portion of the dissociated molecule or anion. The cationic surface active agents, on the other hand, carry a positive electric charge on the cation. The nonionic surface active agents do not ionize in water (Mallman and Harley, 1950, Reddish, 1954, Borowsky, 1956, and Foster et al., 1957).

The quaternary ammonium compounds are cationic surface active agents, having the following chemical structure:



R represents a long chain alkyl group which is hydrophobic and contains 8 to 18 carbon atoms. It is attached to the hydrophilic portion of the molecule. R_1, R_2 and R_3 represent hydrogen, alkyl, aryl or heterocyclic groups of lower molecular weight. X represents an anion, usually a chloride. It is possible to produce a great many quaternary ammonium compounds, each having a different chemical composition, by substituting different organic radicals for R, R_1, R_2 and R_3. This does not mean, however, that all of the quaternary ammonium compounds thus formed are found to be of commercial use (Reddish, 1954, Foster et al., 1957, and Campbell, 1958).

The chemical composition of the quaternary ammonium compounds has been found to have a marked effect on their germicidal activity. This activity increases as the chain length of the alkyl group, R, increases. The quaternary ammonium compounds are most effective when the alkyl group consists of a chain of 12 to 16 carbon atoms. Compounds with alkyl chains of less than 12, or more than 16 carbon atoms are less effective as germicidal agents (Baker, Harrison and Miller, 1941 a, Valko and DuBois, 1944, 1945, Cucci, 1949, Davis et al., 1949, and Elliker, 1950).

Unlike most surface active agents, the quaternary ammonium

compounds, and particularly those with germicidal properties, have good wetting but poor detergent properties. In order to formulate an effective detergent-sanitizer it then becomes necessary to add to the quaternary ammonium compound an agent with good detergent properties (Borowsky, 1956).

Anionic surface active agents are not suitable for this purpose. They have an inactivating effect on the germicidal action of the cationic surface active agents, and vice versa. The nonionic surface active agents, on the other hand, are particularly suitable for this purpose. They do not ionize in water, and thus do not interfere with the germicidal acitivity of either the anionic or cationic surface active agents. More important, they possess excellent detergent qualities, i.e., good wetting, emulsifying and penetrating properties (Mallman and Harley, 1950, Reddish, 1954, Borowsky, 1956, and Foster et al., 1957).

Several factors affect the germicidal activity of quaternary ammonium compounds. These are: species of microorganisms involved, concentration, pH, temperature, rate and mode of action, and the effects of organic matter and hard water.

It has been found that gram-positive bacteria are quite susceptible to quaternary ammonium compounds, while gram-negative bacteria are more resistant. Relatively low concentrations

and exposure times will destroy thermoduric bacteria, but only inhibit the gram-positive sporeformers. Higher concentrations and longer time exposures are required to destroy bacterial spores.

The germicidal activity of quaternary ammonium compounds increases with an increase in pH, especially above pH 9.0. It also increases with a rise in temperature (Baker, Harrison and Miller, 1941 a, Cucci, 1949, Hadfield and Goetchius, 1951, Reddish, 1954, Borowsky, 1956, and Foster et al., 1957).

Quaternary ammonium compounds have pronounced germicidal properties in soft and in distilled water, and are relatively stable in the presence of organic matter. However, the presence of the hard-water metallic cations calcium, magnesium and iron impairs their germicidal action. This is due to the fact that these ions compete with the quaternary ammonium compounds for the negative sites on the cell's surface (Ridenour and Ambruster, 1948, Ambruster and Ridenour, 1949, Mueller and Seeley, 1951, Lazarus, 1952, Reddish, 1954, Borowsky, 1956, and Foster et al., 1957).

The quaternary ammonium compounds are relatively stable towards light and heat (Reddish, 1954, and Foster et al., 1957).

A good quaternary ammonium detergent-sanitizer should provide for both cleaning and sanitization in one operation. The

formulation should contain, besides the germicide and the nonionic detergent, a sequestering agent and an alkaline salt for maximum efficiency. The sequestering agent is incorporated into the formulation in order to soften the water by the removal of hard-water ions. Since the quaternary ammonium compounds possess practically no buffering action in water, the pH of the solution is therefore, raised to an alkaline pH by using alkaline salts in the formulation (Hadfield and Goetchius, 1951, and Borowsky, 1956).

The Mode of Action of Quaternary Ammonium Compounds

The importance of determining the mode of action of the ionic surface active compounds resides in the fact that this knowledge would lead to the formulation of ever more potent disinfectants and chemotherapeutic agents. This is the reason so much work has been done in this field in the past. At the present, it is not quite clear what relationship exists between the chemical structure and the biological activity of known surface active bactericides (Newton, 1958).

Newton (1958) states that the theories put forth, so far, as explanations for the mode of action of surface active bactericides have dealt with: the inactivation of specific enzymes, the general denaturation of cell proteins, and the disorganization of a cell

permeability barrier.

Since the surface of the bacterial cell has been found to be negatively charged, it is safe to assume that the positively charged cationic surface active agents would be attracted to it. McCalla (1940), Baker, Harrison and Miller (1941 a), Valko and DuBois (1944), Valko (1946), and McQuillen (1950), share this belief.

Valko and DuBois (1944) believe that the bacteria function as ionic exchangers. They found that the destructive effects of the cationic surface agents on bacteria could be counteracted by using a high molecular anion, such as sodium dodecyl sulfate.

Baker, Harrison and Miller (1941 a, b) noted that cationic detergents inhibited bacterial respiration or acid production. These authors believe that the ability of detergents to lower the surface tension, permits them to disorganize the cell membrane, and subsequently denature the cell's proteins that are necessary for its growth and metabolism. The addition of a phospholipid before or at the same time as the detergent, was found to protect the bacterial cells from the anti-metabolic action of the detergent. Baker, Harrison and Miller (1941 c) assumed that the phospholipids were attracted to the bacterial cell in much the same manner as the detergent.

Hotchkiss (1946) demonstrated that the cell membrane of

bacteria becomes irreversibly damaged when adsorbtion of the maximum amount of detergent molecules occurs upon its surface. This results in the leakage of all the soluble nitrogen and phosphorus compounds present within the cell, and would account for the inhibition of cellular metabolism and the subsequent death of the cell.

Gale and Taylor (1947) found that the cell walls of Streptococcus feacalis became affected when the organism was treated with surface active compounds. This resulted in the release of the internal amino acids.

Mitchell and Crowe (1947) demonstrated, by means of an electron microscope, that treatment of bacterial cells with surface active agents results in the alteration of their surface structures. They also noted that certain cocci, treated with tyrocidin, will release their internal amino acids as a result of the rupture of the cell wall.

Knox et al. (1949) showed that treatment of Escherichia coli with bactericidal amounts of cationic detergents resulted in specific inhibition of certain detergent-sensitive enzymes, such as lactic acid oxidase. In the authors' opinion, this would account for the increased permeability, metabolic inhibition, and death of the treated cells.

Salton, Horne and Cosslett (1951) demonstrated, by means of an electron microscope, that treatment of bacteria such as E. coli with cetyltrimethyl ammonium bromide resulted in the stripping off of the cell envelopes. Salton (1951) found that cellular constituents were released faster when the temperature or the concentration of this same compound was increased.

Stedman, Kravitz and King (1957) studied the effect of a quaternary ammonium germicide on Serratia marcescens. They discovered that high concentrations of the compound caused leakage of the cellular material. The authors concluded that this was of more significance than the fact that low concentrations of the germicide inhibited the energy yielding reactions of the organism.

Newton (1960), and later Voss (1963), reported evidence to show that surface active compounds can combine with and modify the structure of the bacterial cell wall and membrane.

It is clear from the work that has been done so far that more research is needed in this field in order to elucidate the exact mechanism of action of the ionic surface active compounds.

Development of Resistance to Quaternary Ammonium Compounds

The development of resistance, or the ability of bacteria

to grow in the presence of ever increasing concentrations of antibacterial substances, is a gradual process. Its ease and degree of development, if any, are largely governed by the microorganism and antibacterial agent involved. Either way, occurrence of resistant bacteria usually poses certain problems where either drugs or disinfectants have been in use for the control of pathogens or contaminants. It becomes necessary to utilize, or even formulate, new chemotherapeutic or germicidal agents in order to destroy the resistant bacterial strains. These new compounds, unfortunately, often prove to be not as effective as the original ones, and the search for more potent antibacterial substances must continue.

In order to understand the mechanism by which bacterial resistance is acquired, much work has been done in this field. Abraham (1953) feels that any theory which strives to elucidate this mechanism should consider the roles played by the bacterium and the antibacterial agent. It is essential to determine whether resistance is due to a nuclear gene mutation, a cytoplasmic change; or whether the antibacterial substance is responsible for the natural selection or formation of resistant cells.

Bryson and Demerec (1950) believe that as a result of spontaneous mutations, a bacterial culture contains cells of

various genetic make-up. Either through the inhibitory or the lethal action of antibacterial substances, all the cells in a bacterial culture, except those few that are resistant, are eliminated. From this it would appear that resistant cells are present in a culture before they come in contact with these substances. This resistance becomes further enhanced through subsequent mutations that take place in the presence of antibacterial substances.

Miller and Bohnhoff (1950) noted that the most widely favored theory on bacterial resistance concerns the one which assumes that the antibacterial agent plays a role only as a selective agent, and not as an inducer of resistance. The antibacterial agent selects the resistant mutants that may be present in a bacterial culture by preventing the growth of non-resistant cells.

Eagle, Fleischman and Levy (1952) summarized their similar views on this subject as follows. A large percentage of the cells of a bacterial culture grown at low levels of an antibacterial substance is more resistant to it than the parent cells. This resistance is due to an adaptation to the antibacterial substance, or a mutation in the course of multiplication in its presence. Highly resistance cells are obtained at high levels of antibacterial substance due to either its selective action, or as a result of an adaptive process.

Chaplin (1952 a, b) presumed that resistance to quaternary

ammonium compounds is due to the production of a lipid by the bacterial cells. This lipid forms a protective surface coating capable of withstanding the disruptive surface force of the disinfectant. It would appear that the degree of resistance is a function of the amount of lipid contained within a bacterial cell. The reason gram-negative bacteria are more resistant to quaternary ammonium disinfectants than the gram-positive bacteria, may be due to the possibility that this protective substance is a lipid protein complex which occurs only in gram-negative bacteria. The author feels that "the greater the initial resistance of the species the greater is the potential for acquiring higher resistance."

MacGregor (1958) noted that Pseudomonas aeruginosa, capable of growing on tryptone-glucose-yeast extract agar containing 2,000 ppm of alkyl dimethyl benzyl ammonium chloride, could be sensitized by treatment with ethylene diaminetetraacetate. He believes that the reason for the resistance of P. aeruginosa strains to quaternary ammonium compounds resides in their impermeability to these compounds.

Saleh (1964) believes that resistance to inhibitors may be a useful taxonomic tool, especially in the case of sulfate-reducing bacteria.

MATERIALS AND METHODS

Development of Resistance to Quaternary Ammonium Compounds

Test Cultures

The bacteria used were Aerobacter aerogenes strain 12658, Alcaligenes metalcaligenes, OSU strain, Brevibacterium linens, OSU strain, Chromobacterium lividum, OSU strain, Escherichia coli, strain 198, Micrococcus caseolyticus, OSU strain, Microbacterium flavum, OSU strain, M. lacticum, OSU strain, Proteus vulgaris, OSU strain, Pseudomonas aeruginosa, OSU strain, P. fluorescens, strain MI, P. fragi, OSU strain, P. saliciperda, OSU strain, P. syringae, OSU strain, Serratia marcescens, OSU strain, Staphylococcus aureus strain 209, and Streptococcus lactis strain E.

Culture Media

Tryptone Glucose Yeast Extract Agar (TGY)

Tryptone	5.0 gm
Yeast extract	2.5 gm
Dextrose	1.0 gm
Agar	15.0 gm

For use as controls, the above ingredients were dissolved in 1 liter of distilled water, and dispensed in 10 ml amounts into test tubes. For use with quaternary ammonium solutions, the above ingredients were dissolved in 500 ml of distilled water in order to obtain double strength agar, and dispensed in 5 ml amounts into test tubes. The test tubes were capped and autoclaved at 15 pounds pressure (121^{o}C) for 20 minutes.

Lactic Agar (T_{19})

Tryptone	20.0 gm
Yeast estract	5.0 gm
Gelatin	2.5 gm
Dextrose	5.0 gm
Lactose	5.0 gm
Sucrose	5.0 gm
Sodium chloride	4.0 gm
Sodium acetate	1.5 gm
Ascorbic acid	0.5 gm
Agar	15.0 gm

The same procedure was followed as that used in the preparation of TGY agar.

Quaternary Ammonium Solutions

The two quaternary ammonium compounds used in developing bacterial resistance were Klenzade's Accoquat 1602 and Accoquat 1230. The Accoquat 1602 was alkyl dimethylethylbenzyl ammonium chloride (ADEBAC) while Accoquat 1230 was alkyldimethylbenzyl ammonium chloride (ADBAC). The concentration of these quaternary ammonium products, used as stock solutions, was 51.5 per cent.

The stock solutions were diluted with distilled water to give the desired concentration in parts per million (ppm). Each solution was prepared in double strength concentration, dispensed in 5 ml amounts into test tubes which were capped and autoclaved at 15 pounds pressure (121 ^{o}C) for 20 minutes.

Method of Developing Resistance to Quatenary Ammonium Compounds

The test organisms were grown on agar slants. Broth was not used as it was found that upon mixing with quaternary ammonium solutions, a precipitate formed. This made it impossible to determine the extent of bacterial growth in a broth-quaternary ammonium medium by means of a spectrophotometer.

Each 5 ml of double strength quaternary ammonium solution was mixed with 5 ml of melted, double strength TGY or T_{19} agar.

This resulted in 10 ml of single strength agar with the desired ppm of quaternary ammonium.

All the test organisms, with the exception of S. lactis E which was grown on T_{19} agar, were grown on TGY agar slants.

A set of agar slants, each containing a different concentration of quaternary ammonium, was inoculated with each of the test organisms. The controls used in each test did not contain quaternary ammonium compounds. The inoculum used for each successive transfer was obtained from the culture which showed growth in the highest concentration of quaternary ammonium in the preceding transfer. The cultures were incubated at each test organism's optimum temperature at all times.

The cultures grown on agar only, never being exposed to quaternary ammonium compounds, were spoken of as the sensitive strains; those grown in the presence of quaternary ammonium compounds were spoken of as the resistant strains.

Method of Staining Bacterial Fat Bodies

Staining Solution

Sudan black B.....0.25 gm

Ethyl alcohol (70%).....100.00 ml

The dry Sudan black B powder was dissolved in the alcohol at room temperature.

Flat wet mounts of the resistant and sensitive strains of the test organisms were prepared by suspending a loopful of 24-48 hours old bacterial cells in a drop of stain solution. Each mount was covered with a cover slip and examined under the oil immersion lens of a microscope (Hartman, 1940).

The wet mounts of the sensitive and resistant cells stained with Sudan black B were photographed using phase contrast microscopy. A Leitz Wetzlar phase contrast microscope equipped with a 35 mm Olympus PM-b camera was used.

Tests Used to Compare the Characteristics of Resistant and Sensitive Strains

Test Cultures

The bacteria used were the sensitive and resistant (to alkyl-dimethylethylbenzyl ammonium chloride) strains of the following test organisms. *A. aerogenes* 12658, resistant to 250 ppm; *A. metalcaligenes*, resistant to 250 ppm; *E. coli* 198, resistant to 250 ppm; *P. vulgaris*, resistant to 500 ppm; *P. aeruginosa*, resistant to 2,000 ppm; *P. fluorescens* MI, resistant to 2,000 ppm; *P. fragi*, resistant to 1,000 ppm; *P. saliciperda*, resistant to 500 ppm; *P. syringae*, resistant to 500 ppm; and *S. marcescens*, resistant to 2,000 ppm.

The sensitive and the resistant cells were taken from the TGY agar slants by means of a loop, and transferred into whatever medium was to be used in a particular test. In the case of the resistant cells growing on media containing the quaternary ammonium compound, care was taken to remove cells from the surface (where the growth was heavy), to prevent germicide carry-over.

Temperature Relations

The test organisms were inoculated into TGY agar test tube slants, and the cultures incubated at 0°C, room temperature (20-25°C), 37°C, and 50°C for 24 and 48 hours.

Motility

Motility Test Medium

Tryptose	10.0 gm
Sodium chloride	5.0 gm
Agar	5.0 gm

The above ingredients were dissolved in 1 liter of distilled water, and dispensed into test tubes which were capped and autoclaved at 15 pounds pressure (121°C) for 20 minutes. The tubes were cooled in an upright position.

The medium was inoculated with the test organisms by

stabbing each agar butt through the center. The cultures were incubated at each test organism's optimum temperature for 24 and 48 hours.

A diffuse zone of growth along the line of inoculation was an indication that the test organism under consideration was motile.

TGY Agar Stroke

Slants of TGY agar were inoculated with each test organism. The cultures were incubated at each test organism's optimum temperature for 24 and 48 hours, after which time each organism's cultural characteristics on a TGY agar stroke were observed.

Nutrient Broth

Nutrient Broth

Beef extract..... 3.0 gm

Peptone.....5.0 gm

The above ingredients were dissolved in 1 liter of distilled water, and dispensed into test tubes which were capped and autoclaved at 15 pounds pressure (121 ^{o}C) for 20 minutes.

Each test organism was inoculated into a broth tube and incubated at its optimum temperature for 24 to 48 hours, after which time its cultural characteristics in broth were observed.

Gelatin Liquefaction

Nutrient Gelatin

Beef extract.....3.0 gm

Peptone.....5.0 gm

Gelatin.....120.0 gm

The above ingredients were dissolved in 1 liter of distilled water, and dispensed into test tubes which were capped and autoclaved at 15 pound pressure (121°C) for 20 minutes. The tubes were cooled in an upright position.

Each test organisms was inoculated by stabbing through the center of a gelatin butt, and incubated at its optimum temperature for 24, 48, 72, and 96 hours.

To determine whether or not the cultures that were incubated at temperatures higher than 20°C produced gelatin liquefaction, the tubes were cooled to 20°C after incubation.

Carbohydrate Fermentation

Basal Medium

Beef extract.....3.0 gm

Peptone.....5.0 gm

Sodium chloride.....5.0 gm

Carbohydrate.....5.0 gm

To the above ingredients, dissolved in 1 liter of distilled water, was added 1 ml of 1.6 percent alcoholic (95% ethanol) solution of bromthymol blue indicator. The medium was dispensed into test tubes (150 x 18 mm) containing inverted Durham tubes (75 x 10 mm) which were then capped and autoclaved at 15 pounds pressure (121° C) for 10 minutes.

Each test organism was inoculated into the different carbohydrate broths and incubated at its optimum temperature for 24 and 48 hours.

The presence or absence of acid and gas (trapped within the Durham tube) produced by each test organism was recorded after each incubation period.

The bromthymol blue indicator is yellow in an acid medium, green in a neutral medium, and blue in an alkaline medium.

Litmus Milk

Litmus Milk

Skim milk.....100.0 gm

Litmus..... 0.75 gm

The above ingredients were dissolved in 1 liter of distilled water, dispensed into test tubes which were capped, and autoclaved at 15 pounds pressure (121°C) for 15 minutes.

Each test organism was inoculated into a tube of litmus milk, and incubated at its optimum temperature for 24, 48, and 62 hours.

The changes produced in the litmus milk by the test organisms were recorded after each incubation period.

When neutral, the litmus milk is lavender in color; it becomes red with the production of acid, or blue with the production of alkalinity. When reduction of the litmus dye takes place there is either a partial or a complete fading of the color. Upon the production of acid, a hard or acid curd is formed. A rennet curd occurs in the presence of coagulation. Peptonization occurs when milk proteins are digested.

Nitrate Reduction

Nitrate Broth

Beef extract.....3.0 gm

Peptone.....5.0 gm

Potassium nitrate .....1.0 gm

The above ingredients were dissolved in 1 liter of distilled water and dispensed into test tubes which were capped and autoclaved at 15 pounds pressure (121 ^{o}C) for 20 minutes.

Each test organism was inoculated into a tube of nitrate broth, and incubated at its optimum temperature for 14, 24, and 48 hours.

At the end of each incubation period the cultures were tested for the presence of nitrites. This was done by aseptically removing 2 ml of each culture to a clean test tube and adding to it a few drops each of the sulfanilic acid and α-naphthylamine reagents. The appearance of a pink or red color in the medium attested to the presence of nitrites.

Sulfanilic Acid Reagent

Sulfanilic acid ..... 8.0 gm

Acetic acid (5 N) ..... 1.0 lt

Alpha-naphthylamine Reagent

Alpha-naphthylamine..... 5.0 gm

Acetic acid (5N)..... 1.0 lt

Indole Production

Tryptone broth was prepared by dissolving 10.0 grams of tryptone in 1 liter of distilled water. It was dispensed into test tubes which were capped, and autoclaved at 15 pounds pressure (121 ^{o}C) for 20 minutes.

Each test organism was inoculated into a tube of tryptone broth and incubated at its optimum temperature for 24 and 48 hours.

At the end of each incubation period, 1 ml of each test culture was aseptically removed to a clean test tube to which was added

a drop at a time, Kovac's reagent to form a layer atop the culture. The appearance of a cherry-red color in the surface layer was taken as a positive test for the presence of indole.

Kovac's Reagent

Para-dimethyl-aminobenzaldehyde..... 5.0 gm

Amyl alcohol.....75.0 ml

Hydrochloric acid (conc).....25.0 ml

The para-dimethyl-aminobenzaldehyde was dissolved in the alcohol. To this was added the concentrated hydrochloric acid, and the solution was thoroughly stirred.

Hydrogen Sulfide Production

Lead Acetate Agar

Peptone..... 15.0 gm

Proteose peptone .....5.0 gm

Dextrose.....1.0 gm

Lead acetate.....0.2 gm

Sodium thiosulfate..... 0.08 gm

Agar..... 15.0 gm

The above ingredients were dissolved in 1 liter of distilled water, and dispensed into test tubes which were capped and autoclaved at 15 pounds pressure (121 ^{o}C) for 20 minutes. The tubes were

slanted in such a way as to give generous agar butts.

Each test organism was inoculated by streaking the surface of the agar slant, and then stabbing the agar butt. The cultures were incubated at each test organism's optimum temperature for 24 and 48 hours.

At the end of each incubation period, the cultures were observed for surface browning and for browning along the line of inoculation, indicative of hydrogen sulfide production.

RESULTS

Development of Resistance to Quaternary Ammonium Compounds

Table 1 shows the results that were obtained in the present study when A. aerogenes 12658, A. metalcaligenes, B. linens, C. lividum, E. coli 198, M. caseolyticus, M. flavum, M. lacticum, P. vulgaris, P. aeruginosa, P. fluorescens MI, P. fragi, P. saliciperda, P. syringae, S. marcescens, S. aureus 209, and S. lactis E were grown on agar slants with and without various concentrations of ADEBAC and ADBAC.

It was found that upon the 9th transfer onto agar containing quaternary ammonium compounds the gram-negative test organisms were able to grow at much higher concentrations than the gram-positive test organisms.

Effect of Staining the Test Organisms with Sudan Black B

It was found that when the quaternary ammonium sensitive and resistant strains of the above test organisms were stained with Sudan black B, the resistance strains possessed more lipid than did the sensitive strains. The fat bodies stained a deep black in a clear cytoplasm.

Figure 1 a and b show the appearance of the ADEBAC sensitive

Table 1. Degree of resistance developed to ADEBAC and ADBAC in 17 different bacteria subcultured through nine transfers.

Test Organism	Gram Reaction	Temperature of Incubation	Control Grown on	Degree of Resistance Acquired to PPM of ADEBAC	Degree of Resistance Acquired to PPM of ADBAC
A. aerogenes 12658	-	30°C	TGY agar	250	250
A. metalcaligenes	-	20°C	TGY agar	250	250
B. linens	+	20°C	TGY agar	50	50
C. lividum	-	20°C	TGY agar	250	250
E. coli 198	-	37°C	TGY agar	250	500
M. caseolyticus	+	20°C	TGY agar	4	4
M. flavum	+	30°C	TGY agar	2	2
M. lacticum	+	30°C	TGY agar	4	4
P. vulgaris	-	37°C	TGY agar	500	500
P. aeruginosa	-	30°C	TGY agar	2,000	2,000
P. fluorescens MI	-	20°C	TGY agar	2,000	1,000
P. fragi	-	20°C	TGY agar	1,000	1,000
P. saliciperda	-	20°C	TGY agar	500	250
P. syringae	-	20°C	TGY agar	500	500
S. marcescens	-	30°C	TGY agar	2,000	2,000
S. aureus 209	+	37°C	TGY agar	10	10
S. lactis E	+	30°C	T_{19} agar	2	2

Figure 1 a. Appearance of the ADEBAC sensitive strain of Pseudomonas aeruginosa following staining with Sudan black B and photographed under phase contrast microscopy.

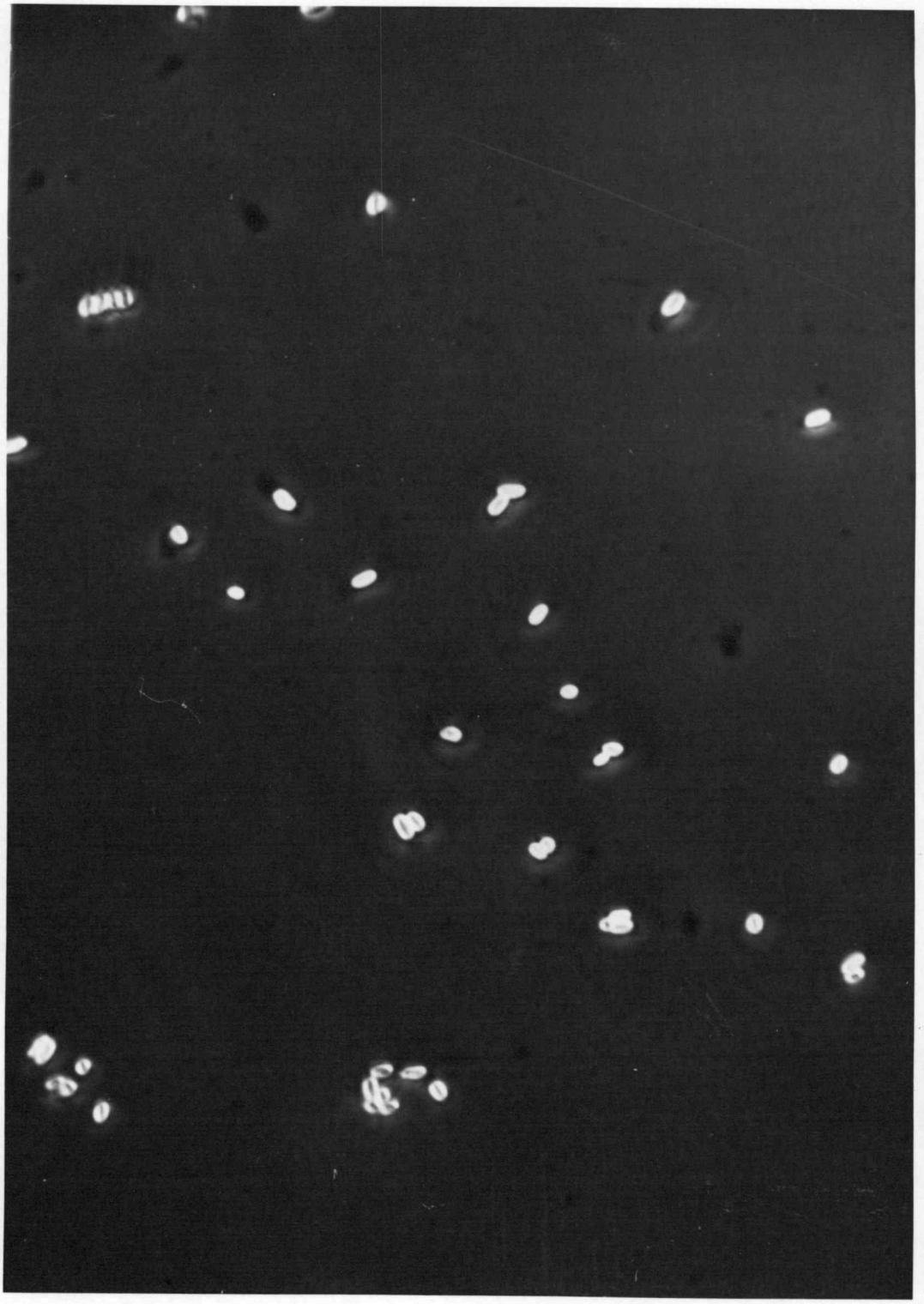
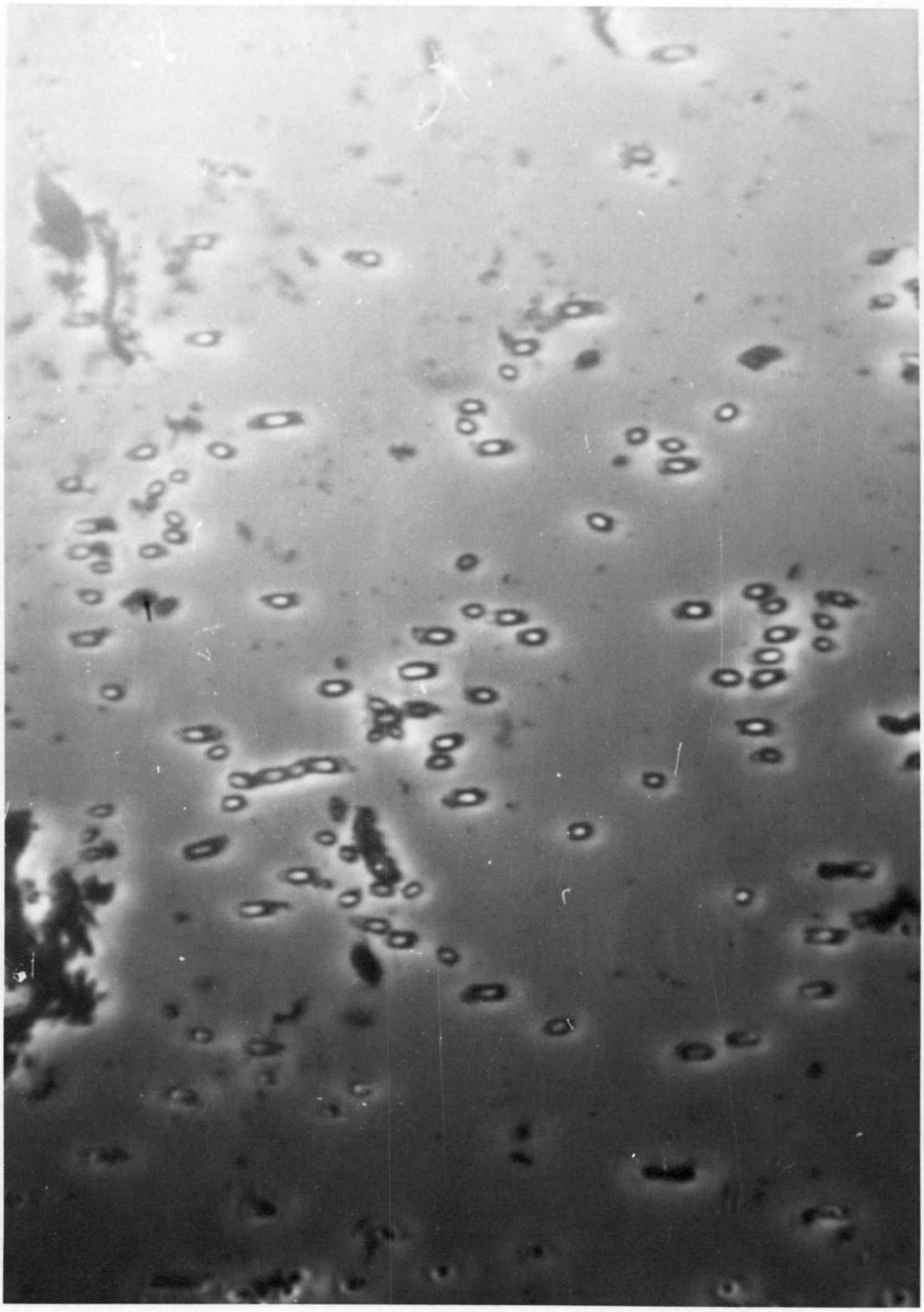


Figure 1 b. Appearance of the ADEBAC sensitive strain of Pseudomonas aeruginosa following staining with Sudan black B and photographed under phase contrast microscopy.



strain of Pseudomonas aeruginosa stained with Sudan black B and photographed under phase contrast microscopy. Figures 2 a and b show the appearance of the ADEBAC resistant strain of P. aeruginosa stained with Sudan black B and photographed under phase contrast microscopy. Figure 3 shows the appearance of the ADEBAC sensitive strain of P. fluorescens MI stained with Sudan black B, and photographed under phase contrast microscopy. Figure 4 shows the appearance of the ADEBAC resistant strain of P. fluorescens MI stained with Sudan black B, and photographed under phase contrast microscopy.

Tests Used to Compare the Properties of Sensitive and Resistant Strains

Table 2 shows the results obtained when the test organisms were incubated at 0°C, room temperature (20-25°C), 37°C and 50°C for 48 hours.

It was found that the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, E. coli 198, P. vulgaris, and P. aeruginosa did not markedly differ in their ability to grow at a given temperature. The ADEBAC resistant strains of A. metalcaligenes, P. fluorescens MI, P. fragi, P. saliciperda, P. syringae and S. marcescens, on the other hand, showed weak growth at 37°C as compared to the good growth exhibited at this same

Figure 2 a. Appearance of the ADEBAC resistant strain of P. aeruginosa following staining with Sudan black B, and photographed under phase contrast microscopy.

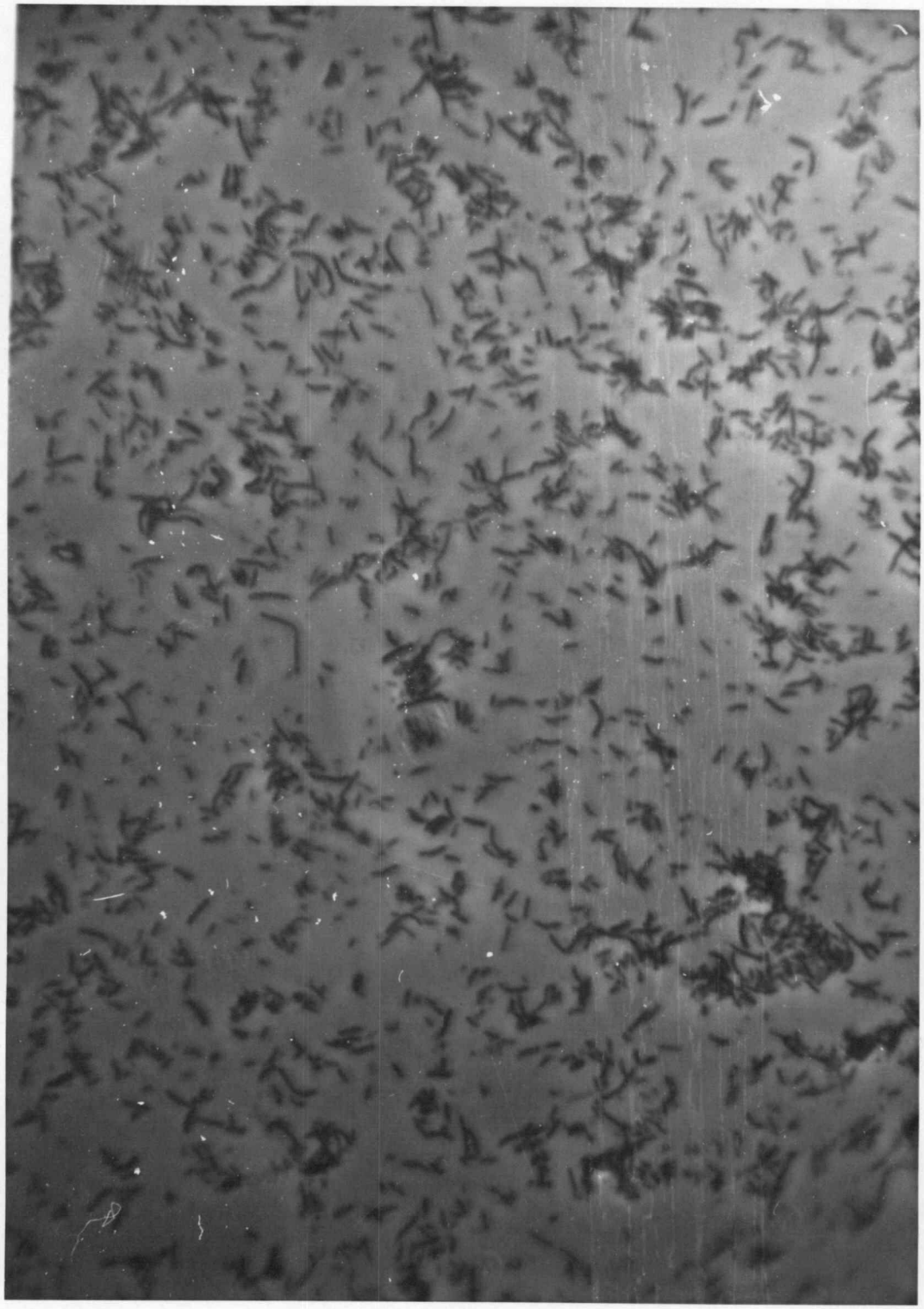


Figure 2 b. Appearance of the ADEBAC resistant strain of P. aeruginosa following staining with Sudan black B, and photographed under phase contrast microscopy.

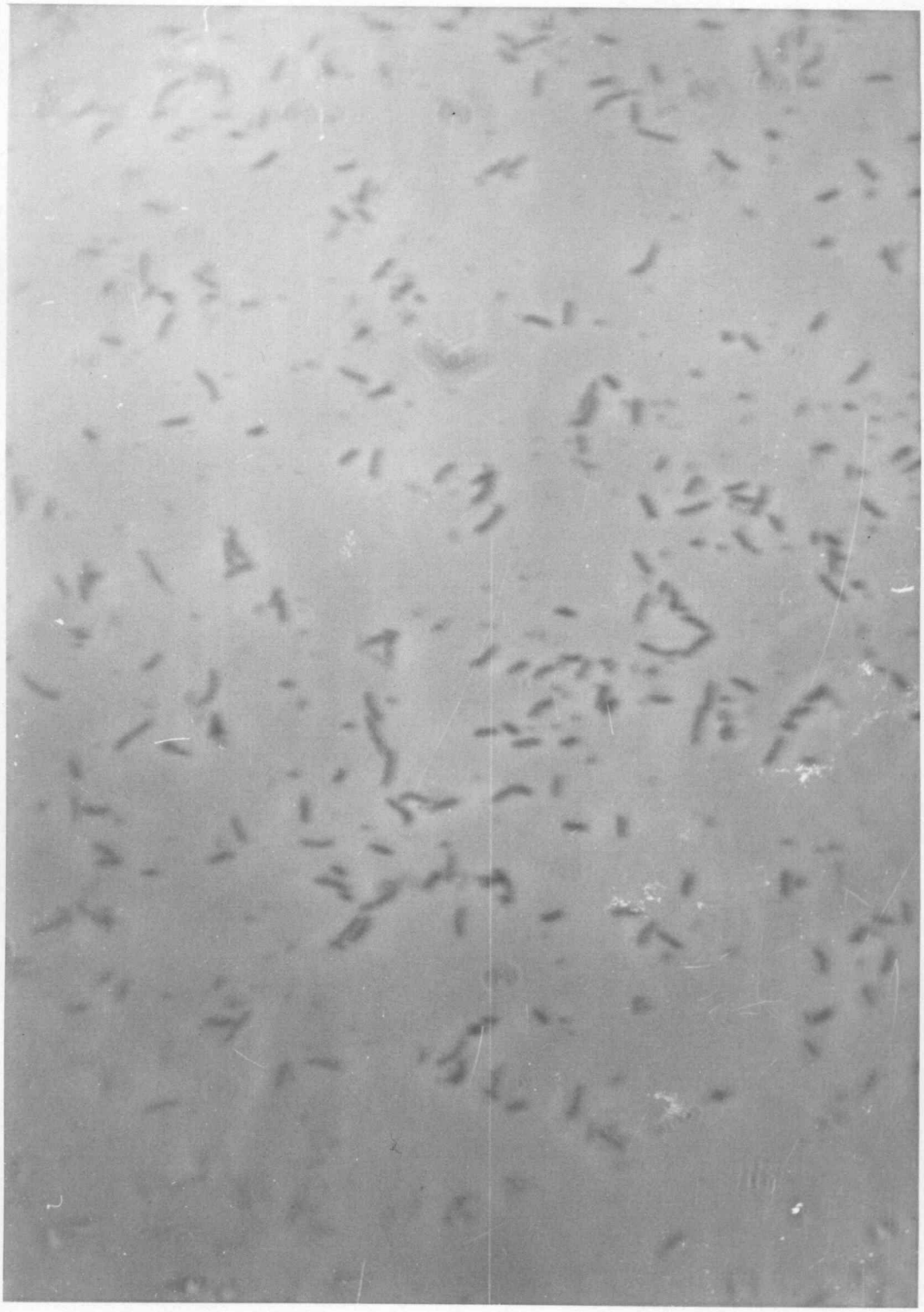


Figure 3. Appearance of the ADEBAC sensitive strain of P. fluorescens MI following staining with Sudan black B, and photographed under phase contrast microscopy.

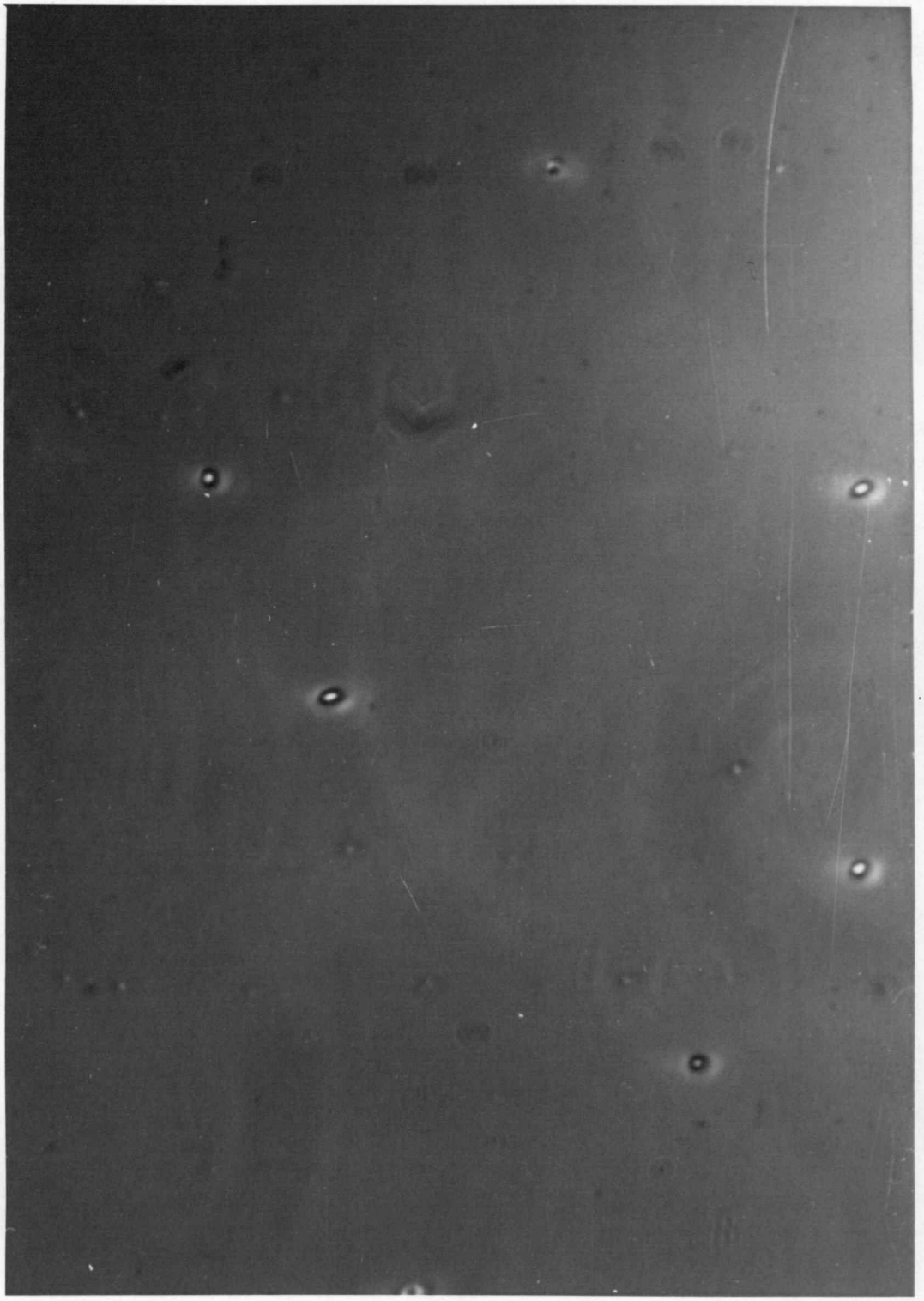


Figure 4. Appearance of the ADEBAC resistant strain of *P. fluorescens* MI following staining with Sudan black B, and photographed under phase contrast microscopy.

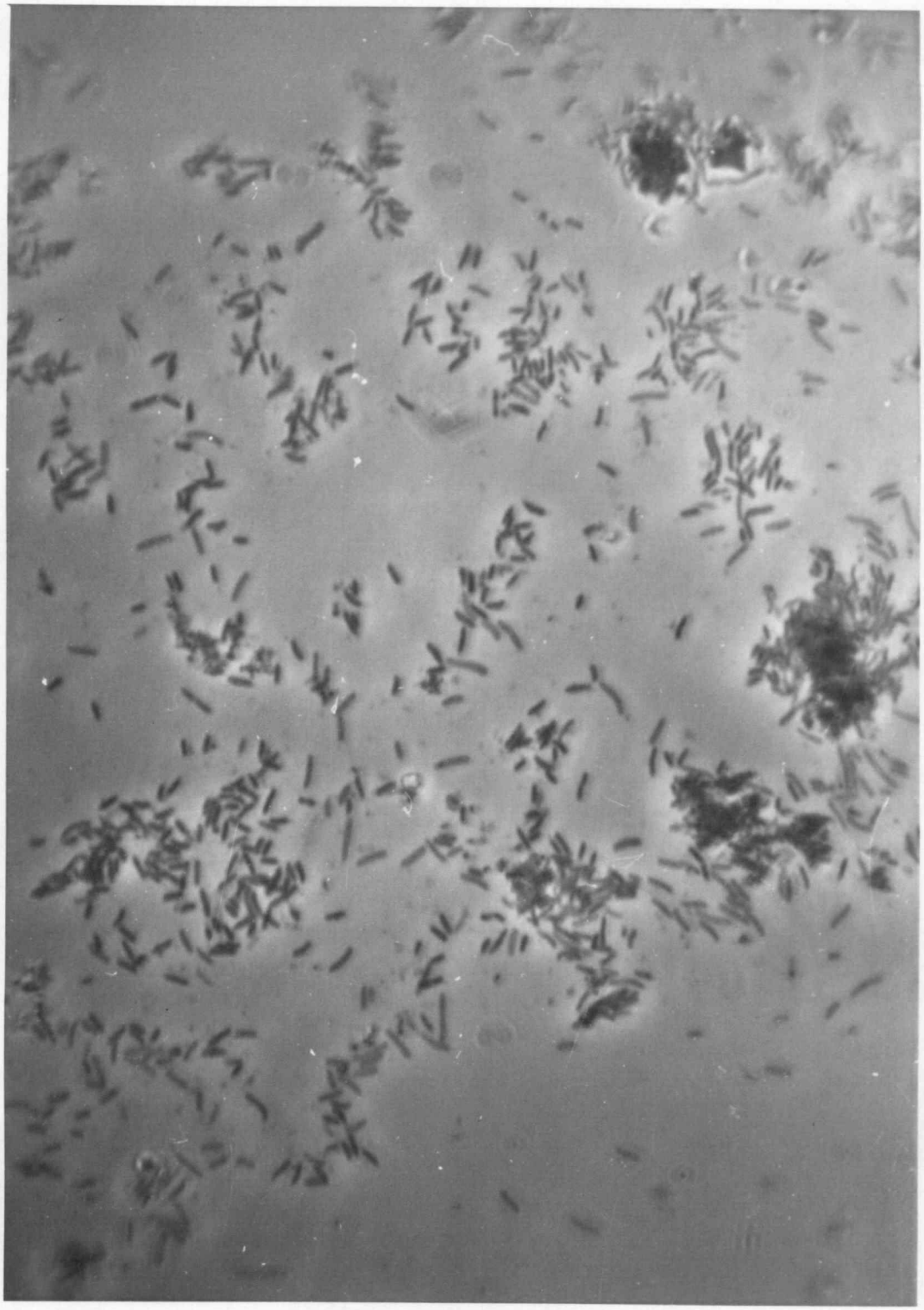


Table 2. Effect of temperature on the growth of ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Developed Resistance to PPM of ADEBAC	Sensitive Strain								Resistant Strain							
		0°C		Room Temp.		37°C		50°C		0°C		Room Temp.		37°C		50°C	
		24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.	24 Hrs.	48 Hrs.
A. aerogenes 12658	250	(+)*	(+)	+	+	+	+	-	-	(+)	(+)	+	+	+	+	-	-
A. metalcaligenes	250	-	-	+	+	+	+	-	-	-	-	+	+	(+)	(+)	-	-
E. coli 198	250	(+)	(+)	+	+	+	+	-	-	(+)	(+)	+	+	(+)	+	-	-
P. vulgaris	500	-	-	+	+	+	+	-	-	-	-	+	+	(+)	+	-	-
P. aeruginosa	2,000	(+)	(+)	+	+	+	+	-	-	(+)	(+)	+	+	(+)	+	-	-
P. fluorescens MI	2,000	+	+	+	+	+	+	-	-	+	+	+	+	(+)	(+)	-	-
P. fragi	1,000	+	+	+	+	+	+	-	-	+	+	+	+	(+)	(+)	-	-
P. saliciperda	500	(+)	(+)	+	+	+	+	-	-	(+)	(+)	+	+	(+)	(+)	-	-
P. syringae	500	(+)	(+)	+	+	+	+	-	-	(+)	(+)	+	+	(+)	(+)	-	-
S. marcescens	2,000	-	-	+	+	+NP	+NP	-	-	-	-	+	+	-	-	-	-

* + Growth
- No Growth
(+) Weak Growth
NP No Pigment Produced

temperature by the ADEBAC sensitive strains.

Motility

The experimental results, listed in Table 3, show that as far as motility is concerned, there was no difference exhibited among the ADEBAC sensitive and the ADEBAC resistant strains of the test organisms.

TGY Stroke

The results, Table 4, show that ADEBAC apparently had no effect on surface growth properties of the resistant strains of the test organisms. No marked differences were observed between the sensitive and resistant strains grown on TGY agar slants.

Nutrient Broth

Table 5 shows that the growth pattern in nutrient broth was the same for both the sensitive and the resistant strains.

Gelatin Liquefaction

Table 6 shows the results obtained when the test organisms were tested for gelatin liquefaction.

It was found that both the ADEBAC sensitive and the ADEBAC

Table 3. Comparison of motility of ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	-*	-	-	-
A. metalcaligenes	20°C	250	-	-	-	-
E. coli 198	37°C	250	+	+	+	+
P. vulgaris	37°C	500	+	+	+	+
P. aeruginosa	30°C	2,000	+	+	+	+
P. fluorescens MI	20°C	2,000	+	+	+	+
P. fragi	20°C	1,000	+	+	+	+
P. saliciperda	20°C	500	+	+	+	+
P. syringae	20°C	500	+	+	+	+
S. marcescens	30°C	2,000	+	+	+	+

* + Motile
- Non-Motile

Table 4. Comparison of the cultural characteristics of ADEBAC sensitive and ADEBAC resistant bacterial strains of various species on TGY agar slants.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	Spreading cream, moist glistening	Spreading cream, moist glistening	Spreading cream, moist glistening	Spreading cream, moist glistening
A. metalcaligenes	20°C	250	Filiform cream, moist viscid growth	Filiform cream, moist viscid growth	Filiform cream, moist viscid growth	Filiform cream, moist viscid growth
E. coli 198	37°C	250	Spreading white, moist glistening	Spreading white, moist glistening	Spreading white, moist glistening	Spreading white, moist glistening
P. vulgaris	37°C	500	Spreading gray, moist	Spreading gray, moist	Spreading gray, moist	Spreading gray, moist
P. aeruginosa	30°C	2,000	Spreading white, thin glistening	Spreading white, thin glistening	Spreading white, thin glistening	Spreading white, thin glistening
P. fluorescens MI	20°C	2,000	Filiform yellowish, moist glistening	Filiform yellowish, moist glistening	Filiform yellowish, moist glistening	Filiform yellowish, moist glistening
P. fragi	20°C	1,000	Spreading cream, moist glistening	Spreading cream, moist glistening	Spreading cream, moist glistening	Spreading cream, moist glistening
P. saliciperda	20°C	500	Filiform white, moist glistening	Filiform white, moist glistening	Filiform white, moist glistening	Filiform white, moist glistening
P. syringae	20°C	500	Filiform cream, moist glistening	Filiform cream, moist glistening	Filiform cream, moist glistening	Filiform cream, moist glistening
S. marcescens	30°C	2,000	Filiform pink, moist glistening	Filiform pink, moist glistening	Filiform pink, moist glistening	Filiform pink, moist glistening

Table 5. Comparison of the cultural properties of ADEBAC sensitive and ADEBAC resistant bacterial strains of various species in nutrient broth.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	Some T*	T	Some T	T
			No P	P	No P	P
			No S	S	No S	S
A. metalcaligenes	20°C	250	T	T	Some T	T
			No P	No P	P	No P
			S	S	S	S
E. coli 198	37°C	250	Some T	T	No T	Some T
			No P	No P	No P	No P
			S	S	Some S	Some S
P. vulgaris	37°C	500	Some T	Some T	Some T	Some T
			No P	No P	No P	No P
			Some S	Some S	Some S	Some S
P. aeruginosa	30°C	2,000	Some T	Some T	No T	Some T
			P	P	P	P
			S	S	S	S
P. fluorescens MI	20°C	2,000	Some T	Some T	No T	No T
			No P	No P	No P	No P
			S	S	S	S
P. fragi	20°C	1,000	Some T	Some T	No T	No T
			No P	No P	No P	No P
			Some S	Some S	Some S	Some S
P. saliciperda	20°C	500	No T	No T	No T	No T
			No P	No P	No P	No P
			Some S	Some S	Some S	Some S
P. syringae	20°C	500	Some T	Some T	No T	No T
			No P	No P	No P	No P
			Some S	Some S	Some S	Some S
S. marcescens	30°C	2,000	T	T	No T	T
			No P	No P	No P	No P
			S (pink)	S (pink)	S (pink)	S (pink)

* T Turbidity
P Pellicle
S Sediment

Table 6. Gelatin liquefaction by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain				Resistant Strain			
			24 Hours	48 Hours	72 Hours	96 Hours	24 Hours	48 Hours	72 Hours	96 Hours
A. aerogenes 12658	30°C	250	-*	(+)	+	+	-	-	-	-
A. metalcaligenes	20°C	250	-	(+)	+	+	-	-	-	-
E. coli 198	37°C	250	(+)	+	+	+	-	-	-	-
P. vulgaris	37°C	500	(+)	+	+	+	-	-	-	-
P. aeruginosa	30°C	2,000	(+)	(+)	+	+	-	-	-	-
F. fluorescens MI	20°C	2,000	(+)	(+)	+	+	(+)	(+)	+	+
P. fragi	20°C	1,000	-	-	-	-	-	-	-	-
P. saliciperda	20°C	500	(+)	(+)	+	+	-	-	-	-
P. syringae	20°C	500	(+)	(+)	+	+	(+)	(+)	+	+
S. marcescens	30°C	2,000	-	(+)	+	+	-	(+)	+	+

* + Gelatin Liquefied
- Gelatin Not Liquefied
(+) Weak Gelatin Liquefaction

resistant strains of P. fluorescens MI, P. syringae and S. marcescens liquified gelatin. Neither the sensitive nor the resistant strains of P. fragi liquefied gelatin.

The sensitive strains of A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris, P. aeruginosa and P. saliciperda liquefied gelatin, but the resistant strains did not.

Carbohydrate Fermentation

Tables 7, 8, 9, and 10 show the results of carbohydrate fermentation obtained in the present study.

It was found that the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, and also those of E. coli 198, fermented glucose, sucrose, lactose, and maltose, with subsequent production of acid and gas, equally well.

The sensitive strains of A. metalcaligenes produced acid and gas from glucose and maltose, but only acid from sucrose. An alkaline reaction was observed in maltose. The resistant strains had no effect on glucose, sucrose or lactose. Acid was produced from maltose.

Both the sensitive and the resistant strains of P. vulgaris produced acid and gas from glucose, sucrose and maltose. Apart from an alkaline reaction produced by the sensitive strains, the sensitive

Table 7. Glucose fermentation by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	AG*	AG	AG	AG
A. metalcaligenes	20°C	250	A	AG	NC	NC
E. coli 198	37°C	250	AG	AG	AG	AG
P. vulgaris	37°C	500	AG	AG	AG	AG
P. aeruginosa	30°C	2,000	AG	AG	A	AG
P. fluorescens MI	20°C	2,000	AG	AG	NC	NC
P. fragi	20°C	1,000	AG	AG	NC	NC
P. saliciperda	20°C	500	A	A	NC	NC
P. syringae	20°C	500	A	AG	NC	NC
S. marcescens	30°C	2,000	A	A	A	A

* A Acid Formation
AG Acid and Gas Formation
NC No Change

Table 8. Sucrose fermentation by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	AG*	AG	AG	AG
A. metalcaligenes	20°C	250	A	A	NC	NC
E. coli 198	37°C	250	AG	AG	NC	A
P. vulgaris	37°C	500	A	AG	AG	AG
P. aeruginosa	30°C	2,000	A	A	NC	AL
P. fluorescens MI	20°C	2,000	A	A	NC	NC
P. fragi	20°C	1,000	NC	A	NC	AL
P. saliciperda	20°C	500	A	A	NC	NC
P. syringae	20°C	500	A	A	NC	NC
S. marcescens	30°C	2,000	A	A	A	A

* A Acid Formation
AG Acid and Gas Formation
AL Alkaline Reaction
NC No Change

Table 9. Lactose fermentation by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	AG*	AG	AG	AG
A. metalcaligenes	20°C	250	NC	AL	NC	NC
E. coli 198	37°C	250	AG	AG	AG	AG
P. vulgaris	37°C	500	NC	AL	NC	NC
P. aeruginosa	30°C	2,000	AL	AL	NC	AL
P. fluorescens MI	20°C	2,000	AG	AG	NC	AL
P. fragi	20°C	1,000	NC	AG	AL	AL
P. saliciperda	20°C	500	NC	AL	NC	AL
P. syringae	20°C	500	NC	AL	NC	NC
S. marcescens	30°C	2,000	A	AG	NC	AG

* A Acid Formation
AG Acid and Gas Formation
AL Alkaline Reaction
NC No Change

Table 10. Maltose fermentation by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	AG*	AG	AG	AG
A. metalcaligenes	20°C	250	A	AG	NC	A
E. coli 198	37°C	250	AG	AG	AG	AG
P. vulgaris	37°C	500	AG	AG	AG	AG
P. aeruginosa	30°C	2,000	AG	AG	NC	AL
P. fluorescens MI	20°C	2,000	AG	AG	NC	AL
P. fragi	20°C	1,000	AG	AG	NC	AL
P. saliciperda	20°C	500	A	A	NC	NC
P. syringae	20°C	500	A	A	NC	NC
S. marcescens	30°C	2,000	A	A	A	A

* A Acid Formation
AG Acid and Gas Formation
AL Alkaline Reaction
NC No Change

and the resistant strains had no effect on the lactose.

The sensitive strains of P. aeruginosa produced acid and gas from glucose and maltose, and only acid from sucrose. Lactose was not fermented; an alkaline reaction was obtained. The resistant strains produced acid and gas from glucose only; alkaline reactions were obtained in the case of the other carbohydrates.

Acid and gas were produced by the sensitive strains of P. fluorescens MI from glucose, lactose and maltose. Only acid was produced from sucrose. The resistant strains, on the other hand, had no effect on any of these carbohydrates.

The sensitive strains of P. fragi produced acid and gas from glucose, lactose and maltose; and only acid from sucrose. The resistant strains had no effect on these carbohydrates other than to give alkaline reactions in sucrose, lactose and maltose.

Acid was produced by the sensitive strains of P. saliciperda in glucose, sucrose and maltose. The resistant strains had no effect on any of the carbohydrates.

Identical results were obtained in the case of P. syringae as in that of P. saliciperda, with the additional production of gas in glucose by the sensitive strain.

Acid was produced in glucose, sucrose and maltose by both the sensitive and the resistant strains of S. marcescens. Acid and

gas were produced in lactose by both the sensitive and the resistant strains.

Litmus Milk

The results of Table 11 show that there were no marked differences between the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, P. aeruginosa, P. fluorescens MI, P. syringae, and S. marcescens. The ADEBAC resistant strains of A. metalcaligenes, E. coli 198, P. vulgaris, P. fragi, and P. saliciperda differed as shown in their action on litmus milk from that of the ADEBAC sensitive strains of these test organisms.

Nitrate Reduction

Table 12 shows the results obtained when the test organisms were tested for nitrate reduction.

It was found that both the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris and P. fluorescens MI reduced nitrates. The sensitive and the resistant strains of P. fragi and S. marcescens did not reduce the nitrates. The sensitive strains of P. aeruginosa, P. saliciperda, and P. syringae reduced nitrates, but the resistant strains did not.

Table 11. Effect of ADEBAC sensitive and ADEBAC resistant bacterial strains of various species on litmus milk.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADBAC	Sensitive Strain			Resistant Strain		
			24 Hours	48 Hours	62 Hours	24 Hours	48 Hours	62 Hours
A. aerogenes 12658	30°C	250	Total reduction	Reduction acid coag. gas	Reduction acid coag. gas	Total reduction	Reduction acid coag. some gas	Reduction acid coag. gas
A. metalcaligenes	20°C	250	NC*	Total reduction	Total reduction	NC	NC	NC
E. coli 198	37°C	250	Total red. acid coag. gas	Total red. acid coag. gas	Total red. acid coag. gas	Total reduction	Total reduction	Total reduction
P. vulgaris	37°C	500	Slightly acid slight red.	Reduction coagulation	Reduction coagulation	Slight reduction	Reduction	Total reduction
P. aeruginosa	30°C	2,000	Total reduction	Reduction coagulation	Reduction coagulation	NC	Reduction coagulation	Reduction coagulation
P. fluorescens MI	20°C	2,000	NC	Peptonization	Peptonization	NC	NC	Peptonization
P. fragi	20°C	1,000	NC	NC	NC	NC	NC	NC
P. saliciperda	20°C	500	NC	No coagulation peptonization	No coagulation peptonization	NC	NC	NC
P. syringae	20°C	500	NC	NC	Total reduction no coagulation	NC	NC	Total reduction no coagulation
S. marcescens	30°C	2,000	NC	Pink surface growth reduction	Pink surface growth acid coag. reduction	NC	Reduction	Pink surface growth acid coag. reduction

* NC No Change

Table 12. Nitrate reduction by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain			Resistant Strain		
			14 Hours	24 Hours	48 Hours	14 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	+*	+	+	+	+	+
A. metalcaligenes	20°C	250	+	+	+	+	+	+
E. coli 198	37°C	250	+	+	+	+	+	+
P. vulgaris	37°C	500	+	+	+	+	+	+
P. aeruginosa	30°C	2,000	+	+	-	-	-	-
P. fluorescens MI	20°C	2,000	+	+	-	+	-	-
P. fragi	20°C	1,000	-	-	-	-	-	-
P. saliciperda	20°C	500	+	+	+	-	-	-
P. syringae	20°C	500	+	+	+	-	-	-
S. marcescens	30°C	2,000	-	-	-	-	-	-

* + Nitrate Reduction
- No Nitrate Reduction

Indole Production

Table 13 shows the results that were obtained in the present study when the test organisms were tested for the production of indole.

It was found that both the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658 and S. marcescens did not produce indole. On the other hand, both the sensitive and the resistant strains of E. coli 198 and P. vulgaris produced indole.

The sensitive strains of P. aeruginosa, P. fluorescens MI, P. fragi, P. saliciperda and P. syringae produced indole, while the resistant strains of these organisms did not.

Hydrogen Sulfide Production

Table 14 shows the results obtained when the test organisms were tested for hydrogen sulfide production.

The ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, E. coli 198, and P. vulgaris produced hydrogen sulfide. Both the sensitive and the resistant strains of P. aeruginosa, P. fragi, and P. saliciperda did not produce hydrogen sulfide.

The sensitive strains of A. metalcaligenes did not produce hydrogen sulfide, while the resistant strains did. The sensitive

Table 13. Indole production by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	-*	-	-	-
A. metalcaligenes	20°C	250	+	+	-	-
E. coli 198	37°C	250	+	+	+	+
P. vulgaris	37°C	500	+	+	-	+
P. aeruginosa	30°C	2,000	-	+	-	-
P. fluorescens MI	20°C	2,000	-	+	-	-
P. fragi	20°C	1,000	-	+	-	-
P. saliciperda	20°C	500	-	+	-	-
P. syringae	20°C	500	-	+	-	-
S. marcescens	30°C	2,000	-	-	-	-

* + Indole Produced
\- Indole Not Produced

Table 14. Hydrogen sulfide production by ADEBAC sensitive and ADEBAC resistant bacterial strains of various species.

Test Organism	Temperature of Incubation	Developed Resistance to PPM of ADEBAC	Sensitive Strain		Resistant Strain	
			24 Hours	48 Hours	24 Hours	48 Hours
A. aerogenes 12658	30°C	250	-*	+	-	+
A. metalcaligenes	20°C	250	-	-	-	+
E. coli 198	37°C	250	-	+	-	+
P. vulgaris	37°C	500	+	+	-	+
P. aeruginosa	30°C	2,000	-[1]	-[1]	-[1]	-[1]
P. fluorescens MI	20°C	2,000	-	+[2]	-	-[2]
P. fragi	20°C	1,000	-	-	-	-
P. saliciperda	20°C	500	-	-[2]	-	-[2]
P. syringae	20°C	500	-	+[2]	-	-[2]
S. marcescens	30°C	2,000	-	+	-	-

*[1]Brilliant Blue-Green Pigment was Produced

[2]Fluorescent Pigment Produced

\+ Hydrogen Sulfide Produced

\- Hydrogen Sulfide Not Produced

strains of P. fluorescens MI, P. syringae and S. marcescens produced hydrogen sulfide, while the resistant strains did not.

DISCUSSION

It was pointed out in the review of the literature dealing with the development of bacterial resistance to quaternary ammonium compounds that gram-negative bacteria are less susceptible to the inhibitory action of these substances than are the gram-positive organisms. It is believed that this is due to the fact that gram-negative bacteria possess a surface coating of lipid that protects them against the deleterious effects of quaternary ammonium compounds. The gram-positive bacteria possess much smaller amounts of lipid than do the gram-negative bacteria and are, therefore, more susceptible.

The results obtained in this study appear to bear this out. The gram-negative test organisms: A. aerogenes 12658, A. metalcaligenes, C. lividum, E. coli 198, P. vulgaris, P. aeruginosa, P. flourescens MI, P. fragi, P. saliciperda, P. syringae, and S. marcescens were able to grow at much higher concentrations of ADEBAC and ADBAC than were the gram-positive test organisms: B. linens, M. caseolyticus, M. flavum, M. lacticum, S. aureus 209, and S. lactis E.

The fact that the quaternary ammonium resistant bacteria posses more lipid material than the sensitive bacteria was confirmed by staining with Sudan black B.

The resistant cells were more uniformly stained than the sensitive cells which were refractile to the stain. This was probably due to the difference in the amount of lipid each type of cell possessed.

The photographs did not quite capture the detail observed by the author. The resistant cells, following staining with Sudan black B were observed to be somewhat granular in nature, probably due to the presence of the stained fat bodies.

Each test organism developed practically the same degree of resistance to ADEBAC as it did to ADBAC.

The ADEBAC sensitive and the ADEBAC resistant strains of the following ten gram-negative bacteria were used in carrying out biochemical and other tests: A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris, P. aeruginosa, P. fluorescens MI P. fragi, P. saliciperda, P. syringae, and S. marcescens.

As far as motility and growth on TGY agar slants and in nutrient broth are concerned, no marked differences were observed among the ADEBAC sensitive and the ADEBAC resistant strains of the test organisms.

Since most bacteria are capable of growing over a relatively narrow temperature range, temperature is probably the most important environmental factor that affects bacterial growth. Depending on their temperature requirements for optimum growth and

multiplication, bacteria may be broadly classified as thermophiles, mesophiles, or psychrophiles. The thermophiles are bacteria with optimum temperatures above 45°C., the mesophiles are those with optimum temperatures between 20 and 45°C, while the psychrophiles are those with optimum temperatures below 20°C.

Gunsalus and Stanier (1962) reported that as the growth temperature of mesophiles is increased, their total lipid fraction per cell decreases. The thermophiles, on the other hand, appear to be stable in this respect. These authors state that the enzymatic composition of bacterial cells fluctuates with changes in the growth temperature. A change in the cell's enzymatic composition appears to have far-reaching effects. Pigment production and biochemical test reactions become affected. The latter give weak or even negative test reactions as the growth temperature is raised to the maximum. Cellular nutrition is also affected by changes in enzymatic composition. The nutritional demands, depending on each case, may either rise or diminish with changes in the growth temperature.

In the present study, no marked differences in the growth patterns of the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, E. coli 198, P. vulgaris, and P. aeruginosa were observed. Where the ADEBAC sensitive strains of A. metalcaligenes, P. fluorescens MI, P. fragi, P. saliciperda, P. syringae,

and S. marcescens showed good growth, the ADEBAC resistant strains of these test organisms exhibited weak growth at 37°C. This would appear to bear out Gunsalus and Stanier's (1962) observation that a rise in the growth temperature affects the cell's lipid content. As has been pointed out in the review of the literature dealing with the mode of action of quaternary ammonium compounds, it is believed that the gram-negative bacteria's surface lipid coat acts as a barrier against these compounds. If this be true, then any reduction in the cell's lipid content would result in an increased permeability to quaternary ammonium compounds. This would then account for the weaker growth observed at 37°C.

It was observed, in the present study, that the ADEBAC sensitive strains of S. marcescens which produced good growth at 37°C failed to produce their characteristic pink pigment at that temperature. The above authors mentioned that lack of pigment production may be related to a decrease in the cell's enzyme composition, as a result of a change in the growth temperature.

Certain bacteria possess an extracellular enzyme, gelatinase, which is capable of liquefying gelatin. Gelatin which is an indiffusible protein is hydrolyzed by this enzyme so that the cells may utilize it internally.

Since gelatin melts at temperatures about 20°C, in order to

determine whether gelatin has been liquefied or not, the cultures under observation are cooled below this temperature if their incubation temperatures were above 20°C. Where gelatin has been hydrolyzed, it will remain liquid even at low temperatures (Salle, 1961, and Bradshaw, 1963).

It was found that ADEBAC had an inhibitory effect on the gelatinase enzyme of the resistant strains of A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris, P. aeruginosa, and P. saliciperda. The sensitive strains of these test organisms liquefied gelatin, but the resistant strains did not.

It would appear that both the sensitive and the resistant strains of P. fragi lacked gelatinase as they did not liquefy gelatin. On the other hand, both the sensitive and the resistant strains of P. fluorescens MI, P. syringae, and S. marcescens liquefied gelatin.

Although Breed, Murray and Smith (1957) reported that A. aerogenes, A. metalcaligenes, E. coli, and P. saliciperda do not liquify gelatin, and that P. fragi does, quite the opposite results were obtained for these test organisms in this study.

The fermentative properties of each of the ADEBAC resistant strains of the test organisms appear to have been affected in a different way by this quaternary ammonium compound.

The ADEBAC sensitive and the ADEBAC resistant strains of

A. aerogenes 12658 produced both acid and gas from glucose, sucrose, lactose and maltose. This was also true in the case of E. coli 198. The ADEBAC apparently had no inhibitory effect on the carbohydrate splitting enzymes of these organisms.

Although Bergey's Manual of Determinative Bacteriology (1957) reports that A. metalcaligenes has no action on carbohydrates the results obtained in the present study showed otherwise. With the exception of an alkaline reaction in lactose, the sensitive strains of this organism produced acid and gas from glucose and maltose. It would appear, therefore, that ADEBAC had an inhibitory action on the fermentative enzymes of the resistant strains of this test organism.

ADEBAC apparently had no effect on the fermentative properties of the resistant strains of P. vulgaris. Both the sensitive and the resistant strains gave identical results.

The sensitive strains of P. aeruginosa produced acid and gas from glucose and maltose; and only acid from sucrose. Lactose was not fermented. The resistant strains had no effect on sucrose, lactose or maltose, apart from giving an alkaline reaction. Acid and gas were produced from glucose. These results differ from those listed by Breed, Murray and Smith (1957) for this organism. It is supposed to have no effect on glucose, sucrose, lactose or maltose. ADEBAC appears to have inhibited the glucose, sucrose, and maltose

splitting enzymes of the resistant strains.

The sensitive strains of P. fluorescens MI produced acid and gas from glucose, lactose and maltose, and only acid from sucrose. The resistant strains had no effect on these carbohydrates, which would indicate that ADEBAC inhibited the enzymes capable of splitting glucose, sucrose, lactose and maltose.

ADEBAC appears also to have inhibited the enzymes of the resistant strains of P. fragi, as these strains had no effect on glucose, sucrose, lactose or maltose, other than to give alkaline reactions in the latter three. The sensitive strains produced acid and gas from glucose, lactose, and maltose, and only acid from sucrose. This also is contrary to the results listed by Breed, Murray and Smith (1957) for this organism.

The resistant strains of P. saliciperda had no effect on glucose, sucrose, lactose or maltose, while the sensitive strains produced acid from glucose, sucrose and maltose. The ADEBAC apparently inhibited the glucose, sucrose, and maltose splitting enzymes of the resistant strains.

Since identical results were obtained in the case of P. syringae, the only exception being that the sensitive strains, in addition to acid, also produced gas in glucose, ADEBAC apparently had the same effect on the fermentative enzymes of this organism as on

those of P. saliciperda.

ADEBAC apparently had no effect on the enzymes of the resistant strains of S. marcescens, as both the sensitive and the resistant strains produced acid from glucose, sucrose, and maltose, and in the case of lactose, also gas.

Certain bacteria may have a two-fold action on litmus milk. They can reduce the litmus indicator present in the milk as a result of their respiratory activities, and they can also bring about certain changes in the milk itself. Neutral or alkaline litmus milk is purple. When the litmus dye becomes reduced by the test organisms the litmus milk becomes white. The litmus becomes pink when the reaction is acid, and a deeper purple when the reaction is alkaline.

Milk consists mainly of the proteins casein, lactalbumin and lactoglobulin, the sugar lactose, and various vitamins and minerals. During their growth processes, bacteria may bring about certain changes in the milk. When they ferment the milk sugar, lactose, a soft, acid curd is formed. This may be accompanied also by the production of gas, which then separates the curd. A hard, rennet curd is produced when the bacterial enzyme rennin clots the milk.

The test organisms may also utilize the proteins casein or lactalbumin as sources of energy. In such a case, these proteins are peptonized, that is hydrolyzed, by the bacterial enzymes. The

pH of the milk then becomes alkaline, since large amounts of ammonia are liberated when the milk proteins are digested (Bradshaw, 1963).

The results obtained in the present study showed that there were no marked differences between the ADEBAC sensitive and ADEBAC resistant strains of A. aerogenes 12658, P. aeruginosa, P. fluorescens MI, P. fragi, P. syringae and S. marcescens, as far as their activity in litmus milk was concerned. Apparently the quaternary ammonium compound had no inhibitory effect on the enzymes of the resistant strains of these organisms. However, it appears to have had some effect on the resistant strains of A. metalcaligenes, E. coli 198, P. vulgaris and P. saliciperda.

Many bacteria possess the enzyme nitratase which enables them to reduce nitrate salts to their nitrite counterparts. This is due to the fact that oxygen is split from the nitrate molecule and combined with hydrogen from the substrate to form water and a nitrite. Some bacteria are also capable of converting nitrates to molecular nitrogen.

The occurrence of nitrites is indicative of nitrate reduction, while the presence of gas indicates that the nitrite has been reduced to ammonia and later to nitrogen.

Since some bacteria reduce nitrates beyond the nitrite stage

rather quickly, the test for nitrate reduction should be read at frequent intervals. Otherwise, it might be assumed that the test is negative (Salle, 1961, and Bradshaw, 1063).

From the experimental results obtained in this study, it was found that neither the ADEBAC sensitive nor the ADEBAC resistant strains of P. fragi and S. marcescens possess the enzyme nitratase as they all gave a negative nitrate reduction test.

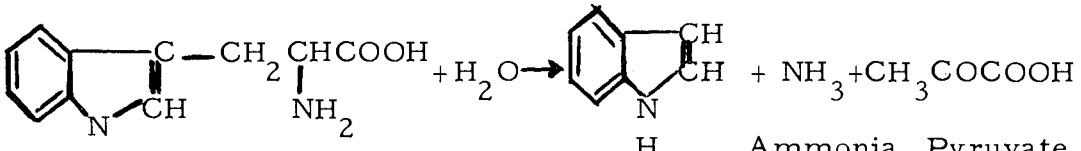
The sensitive and the resistant strains of A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris and P. fluorescens MI apparently do possess this enzyme as they all reduced nitrates. In the case of the sensitive and the resistant strains of P. fluorescens MI, nitrate reduction appears to have gone beyond the nitrite stage.

The sensitive strains of P. aeruginosa, P. saliciperda and P. syringae reduced nitrates, while the resistant strains did not. Apparently, ADEBAC inhibited the nitratase enzyme of these resistant strains. On the other hand, it is quite possible, especially in the case of P. aeruginosa, that the resistant strains reduced the nitrate beyond the nitrite stage faster than the sensitive strains do. Breed, Murray and Smith (1957) reported that P. aeruginosa does reduce nitrates to nitrites and then to nitrogen.

The results obtained in this study do not all compare with those noted by Breed, Marray and Smith (1957) for P. syringae and

S. marcescens. They noted that P. syringae does not reduce nitrates, while S. marcescens does. This is the opposite of the results obtained for these organisms in this study.

Many bacteria, during the process of putrefaction, are capable of degrading proteins which contain the amino acid tryptophan, to produce indole, as shown in the following reaction catalyzed by tryptophanase.



Tryptophan Indole Ammonia Pyruvate

The indole that is produced remains in the medium, and since the only amino acid, which occurs naturally that possesses the indole ring is tryptophan, the test for indole then also becomes a test for the presence of tryptophan. This test is quite useful in the identification and classificiation of various bacteria.

The medium used in the performance of the test is of great importance, as tryptophan is not present in all proteins. Therefore, a peptone medium high in tryptophan is usually used (Gershenfeld, 1945, Gunsalus and Stanier, 1961, Salle, 1961, and Bradshaw, 1963).

From the experimental results obtained in the present study, it was found that neither A. aerogenes 12658 nor S. marcescens possess the enzyme tryptophanase, since neither the ADEBAC

sensitive nor the ADEBAC resistant strains of these organisms produced indole. The sensitive and the resistant strains of E. coli 198 and P. vulgaris, on the other hand, do possess this enzyme since they both produced indole.

The sensitive strains of P. aeruginosa, P. fluorescens MI, P. fragi, P. saliciperda and P. syringae also produced indole. However, the resistant strains of these organisms did not. It would, therefore, appear that the ADEBAC has an inhibitory effect on the enzyme tryptophanase of the resistant strains of these test organisms.

The results obtained for indole production in this study differ somewhat from those listed by Breed, Murray and Smith (1957) for P. fluorescens, P. fragi, and P. saliciperda; these organisms are reported as being indole negative.

Certain bacteria have an enzyme which permits them to split off the sulfur atoms from such sulfur containing amino acids as cystine and methionine that are present in proteins. The sulfur atoms become reduced to hydrogen sulfide upon the addition of hydrogen from the substrate.

In order to facilitate the detection of hydrogen sulfide gas, the test medium usually contains an iron or lead salt. As the hydrogen sulfide is evolved, it reacts with one of these salts to produce an

insoluble iron or lead compound that is dark in color in the test medium along the line of inoculation (Salle, 1961, and Bradshaw, 1963).

The experimental results obtained in the present study show that hydrogen sulfide was produced by the ADEBAC sensitive and the ADEBAC resistant strains of A. aerogenes 12658, E. coli 198, and P. vulgaris. Both the sensitive and the resistant strains of P. aeruginosa, P. fragi, and P. saliciperda did not produce hydrogen sulfide. This would indicate that the former test organisms possess the enzyme that gives rise to hydrogen sulfide (and that it was not inhibited by ADEBAC in the resistant strains) while the latter test organisms do not.

In the case of A. metalcaligenes, the sensitive strains did not produce hydrogen sulfide, while the resistant strains did.

ADEBAC, apparently, had an inhibitory effect on the enzymes of the resistant strains of P. fluroescens MI, P. syringae, and S. marcescens since they did not produce hydrogen sulfide, while the sensitive strains of these organisms did.

Although Breed, Murray and Smith (1957) noted that P. syringae does not produce hydrogen sulfide, it was found to do so in the present study.

SUMMARY AND CONCLUSIONS

Seventeen different bacteria: A. aerogenes 12658, A. metalcaligenes, B. linens, C. lividum, E. coli 198, M. caseolyticus, M. flavum, M. lacticum, P. vulgaris, P. aeruginosa, P. fluorescens MI, P. fragi, P. saliciperda, P. syringae, S. marcescens, S. aureus 209, and S. lactis E were grown on agar slants containing various concentrations of alkyl dimethyl ethyl benzyl ammonium chloride (ADEBAC), and alkyl dimethyl benzyl ammonium chloride (ADBAC). The degree of resistance developed to ADEBAC by each test organism was not markedly different from the degree of resistance developed to ADBAC by those same organisms.

The quaternary ammonium sensitive and resistant strains of the test organisms were stained with Sudan black B. The resistant strains appeared to possess more fat bodies per cell than the sensitive strains.

The gram-negative test organisms showed higher development of resistance to ADEBAC and ADBAC than the gram-positive test organisms. This appears to bear out the theory that the gram-negative bacteria are more resistant to the deleterious effects of quaternary ammonium compounds than the gram-positive bacteria, due to their higher lipid content. The lipid surface coat of the gram-negative bacteria acts as a protective barrier.

The ADEBAC sensitive and the ADEBAC resistant strains of the following gram-negative bacteria were used in carrying out various comparative tests: A. aerogenes 12658, A. metalcaligenes, E. coli 198, P. vulgaris, P. aeruginosa, P. fluorescens MI, P. fragi, P. saliciperda, P. syringae and S. marcescens.

The quaternary ammonium compound did not appear to affect, in any way, the motility or the growth patterns on TGY agar slants or in nutrient broth, of the resistant strains. The results obtained were identical for both the sensitive and the resistant strains.

Changes in the growth temperature, above the optimum, may affect both the reaction of a biochemical test, as well as pigment production. The latter was observed in the present study. Sensitive strains of S. marcescens that grew at 37 ^{o}C, which is above their optimum temperature, did not produce the characteristic pink pigment. There is a possibility that the lack of pigment production may be ascribed to a decrease in the cell's enzymatic composition as a result of a change in the growth temperature.

The results of this study also tend to bear out the theory that quaternary ammonium compounds may have an inhibitory effect on bacterial enzymes. ADEBAC appears to have had such an effect on the enzymes: gelatinase, carbohydrases, nitratase, tryptophanase, and sulfur-splitting enzymes of some of the resistant strains

of the test organisms.

It may be concluded from the results obtained in the present study that gram-negative bacteria are more resistant to quaternary ammonium compounds, and that the gram-positive bacteria are more susceptible. Also, it was shown that the quaternary ammonium compounds produce an inhibitory effect on certain bacterial enzymes. This inhibitory effect may be the result of one or more things. Should the quaternary ammonium compounds tie up metallic activators essential to these enzymes, their activity will become impaired. On the other hand, the enzymes themselves may be extracellular or surface oriented (e.g. permease) and thereby become affected when the quaternary ammonium compounds are firmly adsorbed to the bacterial surface. It is also quite possible that some enzymes, being dependent on labile sulfhydryl or other groups may be inhibited in their activities as a result of the effect of the quaternary ammonium compounds on these groups making up the active center of the enzyme.

It is clear that more work needs to be done on the individual enzymes to determine the exact nature of the interaction that takes place between them and the quaternary ammonium compounds. It also would be of interest to determine the nature of the lipid present in the resistant cells, and also the nature of its metabolism.

The effect that the quaternary ammonium compounds have on the permeases should also be studied.

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