

71-23,820

MALONE, Winfred Francis, 1935-
EVALUATION OF QUATERNARY AMMONIUM COMPOUNDS
AS GENERAL DISINFECTANTS FOR THE HOSPITAL AND
INSTITUTIONAL ENVIRONMENT.

The University of Michigan, Ph.D., 1970
Microbiology

University Microfilms, A XEROX Company, Ann Arbor, Michigan

EVALUATION OF QUATERNARY AMMONIUM COMPOUNDS AS
GENERAL DISINFECTANTS FOR THE HOSPITAL
AND INSTITUTIONAL ENVIRONMENT

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
1970

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ABSTRACT

EVALUATION OF QUATERNARY AMMONIUM COMPOUNDS AS GENERAL DISINFECTANTS FOR THE HOSPITAL AND INSTITUTIONAL ENVIRONMENT

by

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This study is concerned with disinfection by quaternary ammonium compounds (QAC) in medical care facilities. Two microtiter disinfectant tests, one for bactericidal and the other for bacteriostatic QAC testing were developed.

Conventional microtiter components were modified in such a way that microquantities of organisms and chemicals are transferred in an analytical manner. Special microdiluters and a method of calibration with radioactive chromium 51 were developed. The use of a standard microdiluter for streaking organisms on agar plates is described.

The microtiter bactericidal test was utilized to compare various compounds, to evaluate the sensitivity of various organisms, and to evaluate effects of various environmental factors such as water hardness, temperature, and organic load on QAC activity.

The chemical neutralization of QAC activity, an important aspect of the bactericidal test, was studied and a possible mode of action suggested. Heparin, a mucopolysaccharide, was evaluated as a QAC neutralizer.

A graphical procedure is described whereby the results are represented by plotting the percentage of survivors as the integrated normal probability against logarithm of concentration. The dosage response curve can be predicted from 6 experimental measurements from a single microtiter test. The coefficient of variation for a series of tests conducted at the same session was found to be approximately 5 percent.

The microtiter bacteriostatic test made it possible to evaluate QAC bacteriostatic activity. A suspension of microorganisms was added to various concentrations of compound and the lowest concentration that inhibited growth determined. The ability to replicate determinations endows this test with advantages which can be utilized in formulation applications. A comparison with standard tube test indicated a close correlation, provided the microdiluters were calibrated for the diluent involved.

The information obtained by the microtiter techniques was related to biochemical data. Bacteria were successfully labeled with chromium 51 and a cytolytic effect of the QAC was found to be dose dependent. The effect of low levels of QAC on the incorporation of radioisotope labeled metabolic precursors was also studied. The data suggested the existence of two different thresholds of QAC action, namely, inhibition of metabolic pathways at lower concentrations and the leakage of cellular material or a lytic effect at higher concentrations. The effects related to the ability of the quaternary

ammonium compounds to act as bacteriostatic and bactericidal agents.

A delineation of the extent of microbial reduction by QAC, at several concentrations with several compounds on hospital surfaces, was accomplished. The incorporation of 0.5% polysorbate 80 and 0.07% lecithin into solid agar for QAC neutralization in surface sampling was not found to exert a significant effect on microbial recoveries.

A concentration of 1000 ppm of QAC or greater was required for effective reductions on hospital surfaces and the data indicated that the dynamics of disinfection on surfaces were considerably different from that of liquid suspensions.

The current status of hospital disinfection practice in the United States was reviewed with particular reference to disinfectant test procedures presently in use. The latest requirements for product registration by federal agencies are summarized. The establishment of a disinfection policy for individual hospitals was developed and enumerated.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. T. F. Wetzler, Chairman, for his assistance in this study. The interest and helpful criticism of Dr. W. Brown, Professor M. S. Hilbert, Dr. A. P. Jacobson and Dr. K. Cochran are sincerely acknowledged.

The guidance and encouragement of Dr. L. G. Herman, Chief, Laboratory Section, Environmental Service Branch, National Institutes of Health, is gratefully appreciated.

Acknowledgement is extended to Professor M. S. Hilbert, as Chairman of the Department of Environmental Health, for providing a traineeship.

The author is further indebted to Mr. Vinson Oviatt, Chief, Environmental Services Branch and to Dr. W. DeWitt, Director, Division of Research Services, National Institutes of Health, who provided an administrative residency and generous assistance which made possible the completion of this study.

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EVALUATION OF QUATERNARY AMMONIUM COMPOUNDS
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AND INSTITUTIONAL ENVIRONMENTS

INTRODUCTION

Safe and effective general environmental disinfectants are essential for hospital and institutional use in order to minimize the potential infection hazard from enteric, respiratory and other pathogens present on exposed surfaces. In addition, they are required for the destruction of organisms present on instruments and supplies which cannot be rendered sterile by autoclaving due to their heat sensitive characteristics. Efficient surgical antiseptics are also required to assist in the degerming of the hands and arms of the surgeon as well as the skin of the patient at least at the operative site.

The presently used disinfection tests were designed for phenolic type disinfectants. However, when used for QAC evaluation they lack reproducibility and are difficult to perform.

Supplementary tests that are relevant to hospital applications, easier to perform and more reproducible are required.

Polysorbate 80 and lecithin are presently recommended for chemical neutralization of quaternary ammonium compounds. The mode of action of the compounds has not been described.

This dissertation is aimed at developing new techniques and procedures for evaluating the effectiveness of disinfection by quaternary ammonium compounds in medical care facilities.

An assessment of the efficiency of QAC against typical microbial flora and environmental conditions of the hospital will be undertaken. It may be possible from microbial reduction studies on surfaces to suggest a practical use dilution for QAC in hospital environmental applications.

Information on the mode of action of QAC may also have relevance to their suitability for various applications. Data on the dynamics of disinfection may be related to their biochemical and biophysical effects on metabolic systems and microbial cells. Radioisotope labeling of metabolic precursors and isotope release studies will be undertaken and these aspects explored.

In the light of recent changes in regulatory requirements for hospitals, as promulgated by accrediting groups and federal legislation, present hospital disinfection practices and disinfectant requirements will be reviewed and the elements of a hospital disinfection policy enumerated.

The general objectives of this dissertation will be the development of procedures and a policy for disinfection that will result in a more dependable index of disinfection practice and in an improvement of environmental quality in medical care facilities.

HISTORICAL REVIEW

I. GENERAL CHARACTERISTICS

A. PROPERTIES

The quaternary ammonium compounds (QAC) were introduced as disinfectants for general and surface disinfection in the hospital by Domagk (1935). Within the same year approximately 25 articles appeared in foreign journals on the successful use of QAC in hospital applications (Lawrence and Block, 1968).

Quaternary ammonium compounds are a group of cationic surface active agents, many exhibiting antimicrobial properties. They have the general formula which is shown in Figure 1.

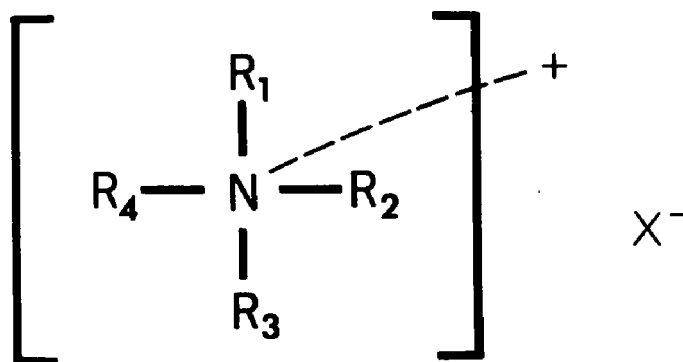


Figure 1.- General formula for the quaternary ammonium compounds. N represents nitrogen with a valence of 5. R₁, R₂, R₃, R₄ may represent hydrogen, aryl, alkyl, or heterocyclic groups and x represents the negative ion.

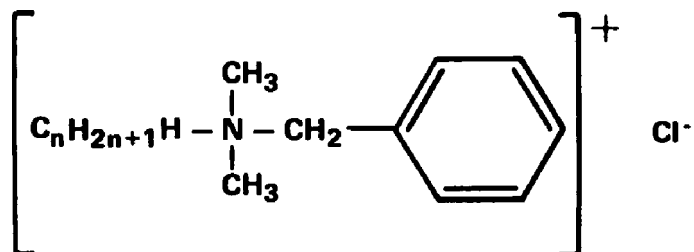
Two QAC commonly used in hospitals are listed in the XVIII edition of the United States Pharmacopeia (1970) namely, benzalkonium chloride and cetylpyridinium chloride. The chemical structures of these compounds are shown in Figure 2.

B. SUSCEPTIBILITY OF BACTERIA AND VIRUSES

Johns (1948) found that of the gram positive organisms, both staphylococci and streptococci are quite susceptible to the quaternary ammonium compounds. Lawrence (1950) reported that most of the gram negative organisms such as salmonellae are less susceptible. Chaplin (1952) reported that the pseudomonads are still less susceptible and even adaptable to one QAC formulation of 10 percent active ingredients. Merkal and Thurston (1968) found that QAC were non-inhibitory to most strains of mycobacteria in concentrations of 0.1 percent benzalkonium chloride for one day.

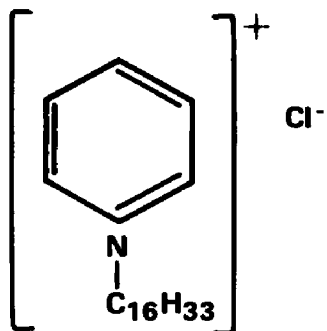
Klein and DeForest (1963), noting the interaction of QAC with lipids, suggested that hydrophilic viruses which are adsorbed to lipids proved sensitive to quaternary ammonium compounds. The results show all viruses tested, except those belonging to the picornavirus group, are inactivated by QAC. Thus, influenza, measles, rabies, psittacosis, herpes simplex, variola, vaccinia, yellow fever, Eastern and Venezuelan equine encephalitides, Japanese B encephalitis, adenovirus type 2, canine distemper, infectious meningopneumonitis, and lympho-

1. Long chain dimethylbenzyl ammonium chloride:



Benzalkonium Chloride
Aklyldimethylbenzyl ammonium chloride

2. Compounds containing heterocyclic nitrogen:



CETYLPIRIDINIUM CHLORIDE

Figure 2. — Examples of quaternary ammonium compounds in hospital applications.

cytic choriomeningitis were shown to be sensitive to QAC. On the other hand, poliovirus, Coxsackie B₁, ECHO 6, ECHO 9, foot and mouth virus and encephalomyocarditis virus which are not adsorbed to lipids were found resistant to the QAC.

C. TOXICITY

Bergmark and Rydberg (1968) extensively reported on a study of QAC action on cultured cells. Fragments of epithelial and connective tissue were exposed to various concentrations of benzalkonium chloride and cetylpyridinium chloride for 10 minutes, as these are the predominant types of tissues engaged in wound healing and therefore should be representative of tissues exposed to an open wound. Compared to controls, the growth rate of cultured cells exposed to QAC concentrations from 0.01% to 0.1% was significantly retarded. After exposure to 0.1% the inhibition of growth was almost complete, indicating massive and irreversible cell injury.

McNamara and Steinbach (1967) found no relationship between the bacteriostatic activity of QAC and human tissue substantivity. However, there appeared to be a relationship between the charged nitrogen atom and the effects of steric hinderance. As the charged nitrogen atom was progressively blocked by surrounding chemical groups, there was a reduction in substantivity.

Only a few reports of hypersensitivity to QAC have

been published. Saunder (1953) reported on a case of contact dermatitis resulting from use of QAC. Wahlberg (1962) reported two cases, for both of which the skin was affected.

Another aspect of the toxicity problem may be the effect of QAC on fish and aquatic life brought about by the ultimate environmental disposal of QAC. The Federal Water Quality Improvement Act of 1970 establishes a new standard of acceptability, and proposes significant changes in the long-accepted procedures for conducting water toxicity studies. Criteria for toxicity and biodegradability are now considerations for substances being discharged into streams, lakes and water sheds.

D. HOSPITAL APPLICATIONS

The quaternary ammonium compounds are used widely in medical care facilities. They are incorporated into general disinfectants and sanitizers for use in hospital and institutional environments. QAC detergent formulations with nonionic surfactants are appropriate for many hospital decontaminating applications. They can be used in cleaning and disinfection of environmental surfaces in corridors and patient rooms or in such areas as intensive care units or operating rooms. At high surfactant concentrations, they are used as floor finish removers, animal care facility cleaners, and for the removal of green slime from concrete floors and walls. For tile and hard surface heavy duty cleaning, QAC may be formulated in

abrasive pastes and powder cleaners. For paste cleaners the formulation of QAC and silica is sometimes employed. By absorbing a QAC on an anhydrous inorganic material such as tetrasodium pyrophosphate and blending with silica they can be used as an abrasive cleaner and disinfectant (Bell and Stoner, 1969). QAC and alkylphenylethoxylate are also employed for cleaning tile and porcelain surfaces. The formulation possesses good rinsability and will remove insoluble soap deposits. QAC are also incorporated with naphtha-based cleaners for dewaxing, degreasing and preparation of hardwood floors and surfaces for refinishing. They are being added to sweeping compounds by incorporation into sand and sawdust mixtures, to germicidal polishes in a nonionic wax emulsion, and to carpet shampoos.

QAC are commonly incorporated in hospital laundry final rinsing materials for lubricating, mildew retarding or as bacteriostats. They find application as inhibitors of slime formation and reduction of bacterial growth in physical therapy tanks or pools, and for the decontamination of inhalation therapy and anaesthesiology equipment.

They are employed as antiseptics in compounds for the treatment of superficial cuts and wounds. They enjoy widespread application as surgical scrubbing compounds for pre-operative preparation of the wound site (Price, 1950). They are even found in antiseptic throat lozenges, mouth washes, aerosol sprays, toothpastes, dentifrices and cough syrups.

Several adverse reports on the use of QAC in special and cold disinfection of medical instruments have been reported. Burdon and Whitby (1967) found several QAC germicides to be contaminated with pseudomonas. They suggested that some infections may be caused by disinfectants contaminated at the time of issue by pharmacy or later during use on the wards.

Plotkin and Austrian (1958) reported that 15 hospital patients developed pseudomonas infections which were traced to a container of QAC in which cotton pledgets were stored. Lee and Fialkow (1961) also reported on QAC solutions as a possible source of hospital acquired gram negative infections. Malizia et al. (1960) expanded on the number of cases and reviewed the literature in this context.

Myers and Lefebvre (1961) saturated cotton with benzalkonium chloride and contaminated it with Pseudomonas aeruginosa. They found viable organisms 24 hours later. Undoubtedly, the cotton absorbed the benzalkonium chloride, rendering the solution ineffective. Kundsinn and Walter (1968) reported on the removal of QAC from solution by cotton, viscose rayon, rubber and skin.

II. DISINFECTANT TESTS

Few hospital personnel are aware of the existence of the Official Methods of Analysis of the Association of Analytical Chemists (AOAC) (1970), in which are detailed some of the

official and standard tests presently available for evaluating disinfectant effectiveness. Probably because of copyright, these tests have never received wide publicity in hospital literature, although they are reproduced in pertinent reference books, Reddish (1957) and Lawrence & Block (1968).

The two AOAC official tests of relevance presently utilized are the phenol coefficient test and the use dilution confirmation test.

A. PHENOL COEFFICIENT TEST

The phenol coefficient procedure was originally described by Rideal and Walker (1903). It has been employed with various modifications for the past 67 years, the major being: Lancet Commission (1909), Anderson and McClintic (1912), American Public Health Association (1918), Reuhle and Brewer (1931), British Standards Institute (1934), and the Association of Official Agricultural Chemists (1950).

The phenol coefficient (PC) is a number obtained by dividing numerical values of greatest dilution of disinfectant capable of killing the test organisms in 10 minutes but not in 5 minutes, by greatest dilution of phenol giving the same results. An example of typical test results is presented in Table I.

For all germicides whose chemistries differ from the phenolics or cresylics, the "phenol coefficient" data may be badly distorted (Stuart et al., 1955), and in any case are

irrelevant (Klarman and Wright, 1946).

The values obtained by using the standard test organisms at 20°C are multiplied by 20 to give the maximum parts of water in which one part of the evaluative product is to be incorporated. It is implicit in the term "phenol coefficient" that the evaluative germicide can only be a relevant phenolic or cresylic compound.

B. USE DILUTION TEST

The use dilution test is most often used to support claims of germicidal effectiveness except for fungi, spores and tuberculosis organisms.

The test is based on the stipulation that 10 carriers after inoculation, partial drying and submission to the action of the disinfectant at the recommended concentration should show no survivors.

It is assumed that there must exist a fixed extinction concentration of disinfectant for a given bacterial suspension. All samples quenched before the characteristic time has elapsed, or when exposed to a concentration lower than optimal, will show growth and all with a greater concentration would be devoid of active bacteria. Such a simple view overlooks the variation which occurs between individual bacterial cells in their tolerance to disinfectants, and which must lead to marked sampling variation in the outcome of this type of test.

Jordan and Jacobs (1944) showed that bacteria will demonstrate differences in natural variation and individual tolerances to disinfectants. The number of survivors in a sample will not suddenly become zero, but will diminish more or less gradually as the dose increases. The number itself will not be subject to sampling variation, so that although there will be a mean number of survivors characteristic of a range of similar samples exposed to a similar dose, the number surviving will vary from sample to sample. In the use dilution test, the survival of even one individual will lead to a positive result and therefore the useful observation of response must cover ranges of samples in which the mean number of survivors is small and as a result the statistical reproducibility poor.

C. TEST DIFFICULTIES

Numerous attempts have been made in the past 25 years to overcome the recognized deficiencies in test procedures for determining the bactericidal effectiveness of quaternary ammonium disinfectants (Klarman and Wright, 1946; Mueller et al., 1947; Butterfield et al., 1950; Cousins, 1951; Kelsey and Mauer, 1966; Curry, 1968 and Kundsinn, 1960). There has been a growing awareness that the evaluation of disinfectant is more complicated than any single expression of activity in terms of a standard that could be found under one set of test conditions (Cook, 1967).

Apart from the dangers of error from attempting to interpret results which are not directly based on use conditions, false assumptions have often been made on the relative activity of QAC under different conditions of time, concentration, organic matter, temperature, and water hardness (Law, 1967). An even greater problem, however, arises from relating the performance of quaternary ammonium compounds to phenol which has considerably different disinfection dynamics (McCulloch, 1947).

The lack of reproducibility of both phenol coefficient and the use dilution tests has been known for some time. Rahn (1945) estimated the potential coefficient of variation as high as 360 percent for phenol coefficient test. Kelsey and Sykes (1969) reported that the coefficient of variation was as much as 30 percent, even when every attempt was made to standardize the cultural conditions and the details of the testing technique.

Beloian (1969) reported that precision based solely on a single reading of the use dilution test was too small a sample for statistical significance. He determined that a 20 percent chance existed that the highest effective dilution was inadequate. Six replications of the test were required for 95 percent confidence in most instances.

The evaluation of disinfectant data by the present methods is a challenge. Precise bacteriological test details are presented, however, there are no statistical sections.

The user of the standard AOAC procedures therefore has no guidance on the expected variability of the test or other important statistical considerations.

Disinfectants to be used in hospitals and related medical care facilities, which are shipped in interstate commerce, must be registered by U. S. Department of Agriculture (USDA) Pesticide Regulation Division which employs the results obtained in the AOAC use dilution method as the primary index to efficacy (Stuart, 1968). The applicable law is the Federal Insecticide, Fungicide, and Rodenticide Act of 1964.

Several changes in USDA requirements have occurred during the past year (Pryor, 1970). Instead of only one test, data from several tests are now required. By replicate testing, it is hoped that some statistical confidence could be placed in the endpoint determinations.

In the past, only single runs at the theoretical or claimed use dilution were required on Staphylococcus aureus FDA Strain 209, Salmonella choleraesuis ATCC #10708 and optional tests on Pseudomonas aeruginosa PRD #10. However, the number of tests now required is a minimum of 42. A breakdown of the tests, as now required, is presented in Table II.

D. APPLIED TESTS

Recognizing the need for applied tests for certain aspects of disinfectant testing, Weber and Black (1948) developed a dilution tube procedure for determining the sanitizing

efficiency of products recommended for use on food utensils.

This method was later revised by Chambers (1956) and made applicable to halogen products. This procedure is often used in determining the effects of hard water on commercial detergent sanitizers. Because of the many manipulations involved, the test has never gained the wide acceptance needed for routine applications.

Cantor and Shelanski (1951) developed a "capacity test" for disinfectants which takes into consideration the fact that organic matter reduces the effectiveness of a germicidal product. A "capacity test" was developed by Kelsey and Mauer (1966) for hospital disinfectants. Many manufactures in England have adopted the latter procedure as a supplement to the Rideal-Walker and Chick-Martin tests used in the British Commonwealth (Kelsey and Sykes, 1969).

E. MICROTITER TESTS

Sever (1962) introduced a modified microtiter system for use in serology in which precisely calibrated spiral wire loops and droppers were used to make accurate dilutions rapidly. This technique has proven valuable for a variety of complement fixation, hemagglutination, hemagglutination inhibition and metabolic inhibition tests.

This dissertation describes the application of a microtiter system to bactericidal and bacteriostatic disinfection testing.

These procedures, as developed, will be utilized to assess the quaternary ammonium compounds against typical microbial flora under varying conditions for their suitability as general disinfectants for the hospital and institutional environment.

III. MODE OF ACTION STUDIES

Disinfectants represent one of the oldest group of antimicrobial agents. For a long time they have been accepted as "general protoplasmic poisons", which means that in some way they interfere with the basic mechanisms of life without pinpointing where the effect starts.

Hotchkiss (1946) and Salton (1951) reported that bacterial cellular constituents are released by the active QAC and suggested that this is the primary mode of action.

Pethica (1958) suggested that cetyltrimethylammonium bromide (CTAB) promoted changes in the permeability of the bacterial cell membrane and that this was possibly the primary mode of action of QAC.

Gilby and Few (1960) investigated the lysis of protoplasts of Micrococcus lysodeikticus and concluded that the cationic detergents act on the phosphatidic acid complex of the protoplast membrane. Recently, Fujita and Koga (1966) reported that CTAB has a high affinity for yeast cells and absorbs rapidly to them. These reports suggest a view that quaternary compounds act generally upon the bacterial surface.

Riemersma (1966) postulated that the effect of the cationic surfactants of the quaternary ammonium type may be associated with the presence of anionic groups on the surface of the microbial cells which are capable of binding these substances.

Baker et al. (1941) postulated that QAC had a 2-fold action on bacterial cells: first disorganization of the cell membrane, and second, denaturation of proteins essential to metabolism and growth. Hamilton (1968) believed that the resistance of organisms to disinfectants was due to prevention of penetration through the cell wall.

Salton (1951) found that quaternary ammonium compounds caused the release of purines, pyrimidines, pentoses and inorganic phosphates from the cells of gram positive and gram negative organisms, and that the net charge on bacteria was reduced or reversed when excess compound was added.

An evaluation of the molecular basis of disinfection by the QAC will be undertaken in this dissertation. Certain important biochemical systems will be examined to determine the extent to which they are affected by the disinfectant. An effort will then be made to determine the order in which the reactions are affected. Correlation of the biochemical data with the bacteriostatic and bactericidal microtiter data may make it possible to deduce a primary site of action based on the sensitivity of the separate components.

IV. THE HOSPITAL ENVIRONMENT

A. THE CHANGING SCENE

The recognition and control of pathogenic microorganisms in hospitals is a problem of major concern to every patient and hospital staff member. One must assume that the presence of organisms in the environment poses a threat of cross-infection to patients, especially since the microbial population in hospitals is a dynamic universe constantly changing in response to type of patient and environmental factors.

The presently changing pattern of hospital admissions, the geriatric patient with decreased resistance, the leukemic patient on immuno-suppressive therapy, the cleaning and decontamination of complex equipment, hemodialysis units and heart lung machines are just a few of the newer problems (Dunlop, 1969).

Despite the magnitude of these problems, chemical disinfection as a means of microbial control is still a much misunderstood and neglected subject. Attention to prevention of hospital infection has shifted to physical methods of protecting patients including the recent introduction of isolators and laminar flow rooms.

B. HOSPITAL INFECTIONS

Perkins (1970) stated that there are over 900,000 hospital infections per year in the United States, that over 5

percent of all hospital patients develop an infection, and that the additional annual costs amounts to well over 100 million dollars.

There is evidence that patterns of hospital-acquired infections have been undergoing important changes recently and Ridley et al. (1970) have reported a 10-fold increase in certain highly resistant S. aureus strains. McHenry et al. (1969) reported on the increasing number of these so-called methicillin resistant S. aureus strains in hospitals. These S. aureus strains are of particular importance due to the fact that they are resistant to the semi-synthetic penicillins which have been used largely in the management of S. aureus in hospitals in the past decade. In addition to possessing high intrinsic resistance to many currently available antibiotics, they appear to be opportunistic in that debilitated and elderly patients are most prone to such infections (Willis et al., 1966). Barrett et al. (1968) in reporting one outbreak of such infections, determined that of 18 infections studied, 9 of the 18 (50%) were definitely hospital-acquired and 6 of the 18 (33%) were probably hospital acquired.

Benner and Kayser (1968) evaluated 130 cases and stated that at least 50 percent of the infections had not been acquired until after the patient had been hospitalized.

A general lack of information on the characteristics of these antibiotic resistant S. aureus organisms is evident. Because of the potential threat that they pose to hospitalized patients, a program to isolate, characterize and determine the

sensitivity of the methicillin-resistant S. aureus organisms to quaternary ammonium compounds was considered necessary. Additionally, a possible reduction in antimicrobial resistance by exposure of the organism to QAC was studied and is reported herein.

C. CHEMICAL DECONTAMINATION OF ENVIRONMENTAL SURFACES

Products utilized in hospital environmental sanitation today must clearly accomplish two functions, namely, adequate cleaning and disinfection. These products are often known as detergent-sanitizers or germicides. By definition a sanitizer is expected to reduce microbial contamination to a significant degree. Presently there are no reduction standards established for any compound recommended for use on hospital environmental surfaces.

In the monograph, Control of Infections in Hospitals (1968), published by the American Hospital Association, little if any, guidance is provided with respect to particular compounds for hospital applications, or concentrations, or contact times required for their effective utilization.

It is a generally accepted fact in the hospital field that the manufacturer's recommended use dilution, as per label on the container, is the concentration of product required for effective and practical disinfection of hospital environmental surfaces. The actual correlation of the use dilution test data to hospital environmental surface microbial levels has

never been undertaken, although it may be argued that the use dilution test does simulate actual conditions in that a product is challenged by organisms dried in the presence of organic matter (contributed by the bacteriological media on a non-porous surface).

Numerous in-use tests for qualitative and quantitative measurement of microbial contamination on hospital surfaces have been developed (Kundsin and Walter, 1961; Adams and Fahlman, 1961; Ostrander and Griffith, 1964). The techniques employed were swabbing, agar plate impression or in situ measurement of contamination made by overlapping a surface with media for microbial growth. Angelotti et al. (1964) found that cotton swabs were not satisfactory as a quantitative procedure because of poor release of microorganisms enmeshed in the cotton. Angelotti and Foter (1958) reported on a quantitative direct agar technique for measuring microbial densities on non-porous surfaces. These workers concluded that the method was capable of detecting bacteria on surfaces with a high degree of precision. Modification of this direct surface sample technique, by utilizing special sampling media, is described in this dissertation. Selected organisms are recovered successfully from the hospital environment. Quantitative determination of the efficiency of microbial reduction by quaternary ammonium compounds is studied and comparisons are made to microtiter test procedures.

CHAPTER I

DISINFECTANT TESTING

A. INTRODUCTION

Two tests are proposed which apply microtitration techniques to bactericidal and to bacteriostatic testing of quaternary ammonium disinfectants.

A suspension of viable microorganisms is added to various concentrations of the disinfectant and the number of organisms surviving after a given period of time is determined quantitatively in the bactericidal test.

The calibration of the test components, and the reproducibility of this test will be determined. The test may have application in determining the effects of such factors as temperature, water hardness, and organic system demand on QAC activity.

A study of chemical neutralization of QAC action will be conducted. Since the work of Quisno et al. (1946), neutralization of the quaternary ammonium compounds in disinfectant testing has been accomplished by the addition of 0.07% lecithin and 0.50% polysorbate 80 (polyoxyethylene sorbitan monooleate) to a solution or to solid agar for surface sampling. Very little has been published with regard to the mode of action of these disinfectant neutralizer compounds. Recently, however, Brown and Richards (1964) and Brown and Winsley (1969) have shown that in some instances polysorbate 80, enhanced

the antibacterial activity of the QAC. At levels of 0.02% polysorbate, there was increased lysis of pseudomonal organisms. When the concentration of polysorbate was increased to 0.20%, these effects were not demonstrated, indicating that the QAC had probably been inactivated by the higher concentration of polysorbate 80. It may be that the mode of action of the presently used chemical neutralizers can be described and that possible new neutralizers can be recommended.

Quaternary ammonium compounds are known to exert a highly bacteriostatic or inhibitory effect on certain strains of microorganisms (Lawrence, 1951).

Numerous techniques have been used to determine the bacteriostatic activity of various QAC. These include the standard tube dilution test, the agar streak test, zone inhibition test, the roll culture test, and the gradient plate dilution technique (Pingermann, 1968).

It is important to know the bacteriostatic as well as the bactericidal endpoint of a disinfectant. A microtiter procedure is described which enables the determination of both of these endpoints.

The minimum inhibitory concentration (MIC) for several compounds against several microorganisms will be determined utilizing the developed procedure. A comparison of the technique with the traditional tube technique will be conducted.

While the microtiter tests were devised mainly with

hospital applications in mind, it is hoped they will be applicable to disinfectants other than QAC, and to fields other than the institutional environment.

B. MATERIALS AND METHODS

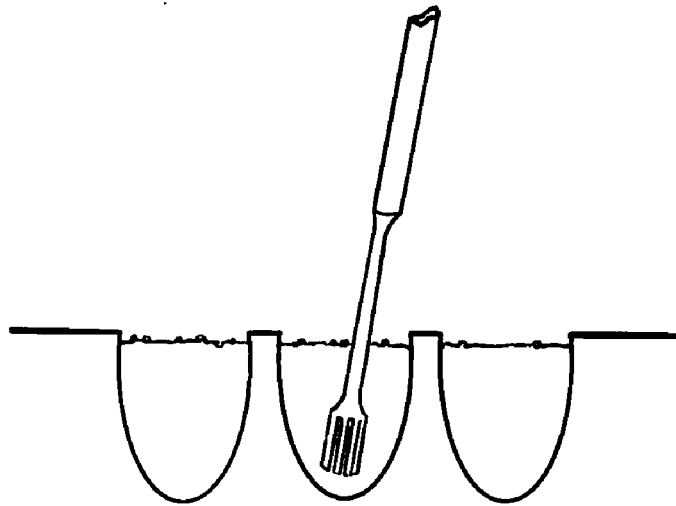
1. Design and Development of Microdiluters

Specific modifications of microtiter system* equipment were necessary in order to employ the system for disinfectant testing. In Figure 3 are presented the standard microtiter plate and microdiluter. Given a 0.25 ml volume of fluid, the 0.225 ml of broth required for 10 fold dilutions, and the 0.025 ml standard microdiluter, the microtiter plate was not deep enough to accommodate the 0.25 ml fluid volume in the presence of the standard microdiluter. Some of the solution would spill over the edges of the well. In addition, because the microdiluter must rest deep in the well, there was a residual carry-over of organisms on the shank of the diluter due to poor run-off of the suspension, when the device was moved out of the well.

These potential problems were overcome in two ways. A Micro Test II** tissue culture plate was substituted for the V-plate. This is a 96-well, flat bottom plate made of transparent polystyrene, and has the following dimensions:

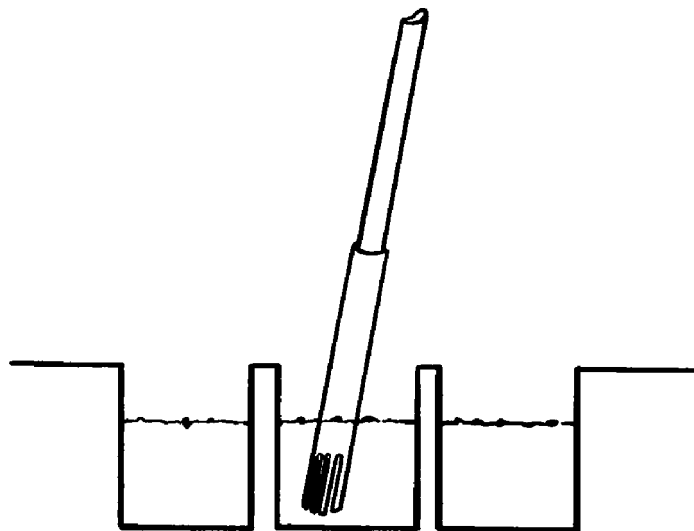
* Cooke Engineering Company, Alexandria, Virginia

** Falcon Plastics, Division of BioQuest, Cockeysville, Maryland



STANDARD MICRODILUTER + WELL

Figure 3. Regular microdiluter and the standard microtiter plate with wells.



SPECIAL MICRODILUTER + WELL

Figure 4. - Special microdiluter and plate with the wide diameter and deeper wells which were developed for use in the microtiter bactericidal disinfectant test.

Well Diameter	6 mm
Well Depth	10 mm
Well Volume	0.36 ml

There are further advantages in that the plates are sterile and individually wrapped. They are made of transparent styrene which is non-toxic to cell culture. They have a slightly raised edge around each well which provides protection against well-to-well contamination. The holding volume of the wells is greater than in the "V" well plate and thus the dilution fluid does not rise as high.

Further, specially designed microdiluters were manufactured. These do not have the beveled portion and as a result the force of gravity is utilized fully in draining the excess suspension from the outside of the microdiluters as the device is moved slowly out of the wells. An example of the specially developed microdiluter and the wells is given in Figure 4.

2. Reagents and Media

a. Culture for Test Purposes

Organisms typically used in disinfection testing by USDA were employed. They were as follows: Escherichia coli, ATCC 11229; Pseudomonas aeruginosa, PRD #10; Bacillus subtilis, FDA strain; and Staphylococcus aureus, FDA 209 ATCC 6538.

Cultures used for test purposes were grown on trypticase soy agar (TSA). Cultures were maintained in

continuous active phase by subculturing onto fresh solid medium at 24-hour intervals. For replicate day-to-day testing the culture was grown for the same time period. When required for a test, the growth was washed off with sterile distilled water, shaken with sterile glass beads, filtered through cloth to remove clumps and diluted with TSB to give a suspension of 0.4 O.D. at 550 mμ utilizing the Spectronic 20*.

b. Letheen

AOAC** Letheen broth for disinfection tests*** was prepared by suspending 25.7 g of the commercial dehydrated medium in a liter of distilled water and pH adjusted to 7.0. The formula in grams per liter of solution, is peptone, 10.0g****; beef extract, 5.0g; lecithin, 0.7g; polysorbate 80, 5.0g; and sodium chloride, 5.0g. The mixture was heated with frequent agitation until the broth boiled. The solution was cooled and dispensed as 10 ml amounts. The media tubes were then sterilized by autoclaving at 121°C for 15 minutes.

c. Glassware

Owing to the strong absorptive properties of the quaternary ammonium compounds, special care in the use

* Bausch and Lomb, Rochester, New York
 ** Official Methods of Analysis, AOAC Tenth Edition, 1970.
 *** BBL, Division of BioQuest, Cockeysville, Maryland
 **** USP Peptic digest of animal tissue

and cleaning of glassware was necessary. Disposable items were used for many purposes and where glass was employed, only low toxicity boronsilicate glassware was utilized. The glasswashing procedures employed hot chromic acid, and storage in cold hydrochloric acid overnight. Rinsing was with 0.5% sodium hydroxide, and then, in succession, deionized water, 1 N hydrochloric acid, and distilled water. Finally, the glassware was rinsed four times with glass-distilled water and sterilized at 121°C for 15 minutes.

d. Water

All water utilized in test procedures was triple glass distilled. The resistivity was measured and found to be 4 megohms. Water was dispensed into 250 ml Erlenmeyer flasks, plugged with cotton, and autoclaved at 121°C for 20 minutes.

e. Synthetic Hard Water

Since there is considerable variation among natural hard waters, the precise measurement of the deleterious effect of water hardness can be accomplished only by the establishment of acceptable chemical standards of reference for hard water. The procedure utilized in these experiments was published by Curl (1956) in the Federal Register and will provide test waters at any desired degree of hardness up to 2000 ppm.

Prepare solution A by dissolving 31.74 g MgCl_2 and 73.99 g CaCl_2 in boiled distilled water and diluting to 1 liter. Prepare solution B by dissolving 56.03 g NaHCO_3 in bottled distilled water and diluting to 1 liter. Solution A is heat sterilized, while solution B is sterilized by filtration. Place required amount of solution A in sterile 1 liter flask and add at least 600 ml sterile distilled water. Add 4 ml solution B and dilute to 1 liter with sterile distilled water. Each ml of solution A will produce a water equivalent to approximately 100 ppm hardness, calculated as $\text{Ca}(\text{CO}_3)_2$ by formula:

$$\begin{aligned} \text{Total Hardness as ppm } \text{Ca}(\text{CO}_3)_2 &= \\ 2.495 \times \text{ppm } \text{Ca}^{++} + 4.115 \times \text{ppm } \text{Mg}^{++} \end{aligned}$$

For all test waters up to 2000 ppm hardness, the pH should be 7.6 - 8.0.

f. Agar Streak Plate

TSA agar plates were poured and dried at room temperature for 72 hours prior to use to eliminate surface moisture from the plates. The bottom of each petri dish was marked with two bisecting lines, thus providing 4 areas per dish. Standard petri dishes of 100 mm diameter provided ample area to accommodate 4 sections. If the plates are sufficiently dry before use, an 0.025 ml volume will easily spread to an area of 25 mm and will be absorbed by the agar in 10-15 minutes.

3. Test Equipment

a. Pipetting Devices

An automatic nonelectric pipette, BBL 60422*, was used to dispense both the disinfectant and the suspension of organisms into the wells. The pipette is constructed of a chemically resistant plastic, Delrin, which never comes into contact with disposable tips. The plunger is set to deliver 0.1 ml and the tip is fitted for each procedure.

A Svedmyr** syringe and glass pipettes were calibrated by radiochromium and were used to deliver the 0.025 diluent to the plates. The syringe is adjusted with the screw on the push bar to deliver 0.225 ml.

b. Water Bath

The disinfection tests at 24°C were conducted in a water bath. The temperature of the water was held at $24.00 \pm 0.25^{\circ}\text{C}$ as determined by a National Bureau of Standards calibrated thermometer.

The tube containing the test organisms was immersed in the bath for 20 minutes prior to the test.

A stage was present in the bath which allowed the microtiter plate to float. The water was sufficiently

* Baltimore Biological Laboratory, Division of BioQuest, Cockeysville, Maryland.

** KIFA Industries, Salna, Sweden.

deep that the individual wells containing the disinfectant mixture were immersed yet the water was unable to come over onto the top of the microtiter plate.

c. Cold Chamber

A thermoelectric Stir-cool model SK-14 cold chamber* was utilized for disinfectant testing at temperatures below 24°C. The instrument had a 5" diameter anodized aluminum top plate with an adjustable cooling range from 25°C to -20°C using tap water through the heat exchanger at a rate of 1 liter per minute. The heat exchanger was maintained at tap water temperature by a hose connection of 1/4" tubing at the rear of the instrument. The microplate was placed on an insulated paraffin tray and equilibrium with plate was maintained by mounting with a thin layer of conductive grease.

Twenty minutes of time was allowed prior to the test for cooling the test solution to the required temperature. During this time, the plastic cover of the chamber was kept on to insure that the test solution was maintained at the desired temperature.

d. Timing Devices

Timing was begun after all the test wells had received the test culture. It took 15 seconds to add the

* Thermoelectrics Unlimited, Inc., Wilmington, Delaware

organisms to all six wells. The withdrawal of 0.025 ml samples from the six wells for neutralization and dilution was accomplished simultaneously after the commonly utilized 5 minute exposure interval.

4. Calibration of Pipettes and Syringes

A radioisotope technique was employed to evaluate and calibrate the microdiluters and syringes.

- a. 100 μ Ci of radioactive chromium 51 in standard letheen broth was taken into the devices in the standard manner of use.
- b. This quantity was subsequently released into test tubes containing distilled water and 20 minute counts of radioactivity were determined with a gamma scintillation counter*.

5. Bactericidal Tests

Two tests were conducted in one microtiter plate at one time. A typical flow sheet is as follows:

- a. Six dilutions or concentrations of the disinfectant were made individually in distilled water.
- b. A 0.1 ml quantity of each disinfectant dilution was added to a well of a plate in a temperature controlled water bath.
- c. A 0.1 ml of the test organisms was added to each well. The concentration of organisms may vary

* Autowell II, Picker Nuclear, White Plains, New York

between 10^5 to 10^8 . This step will reduce the concentration of germicide by one-half, or double the dilution.

- d. After a predetermined time interval, usually 5 minutes, 0.025 ml samples of the suspension were removed from each of the 6 wells, and decimal dilutions (10^{-1} to 10^{-8}) were made in a new plate, whose wells contain 0.225 ml of AOAC Letheen broth (5g/l Tween 80 and 0.7g/l lecithin). The contents of each well were mixed by rotation of the microdiluters for about 5 seconds.
- e. A 0.025 ml sample of the suspension in each well was removed by the 0.025 ml microdiluter starting at the highest dilution and transferred to one quarter of a standard size petri dish containing 20 ml TSA. The microdiluter was used to spread the dilution over the surface of the plate. It was held at a 75° angle and the surface was lightly touched.
- f. The plates were incubated for 24 hours at 37°C and then counted with the aid of a Quebec colony counter.

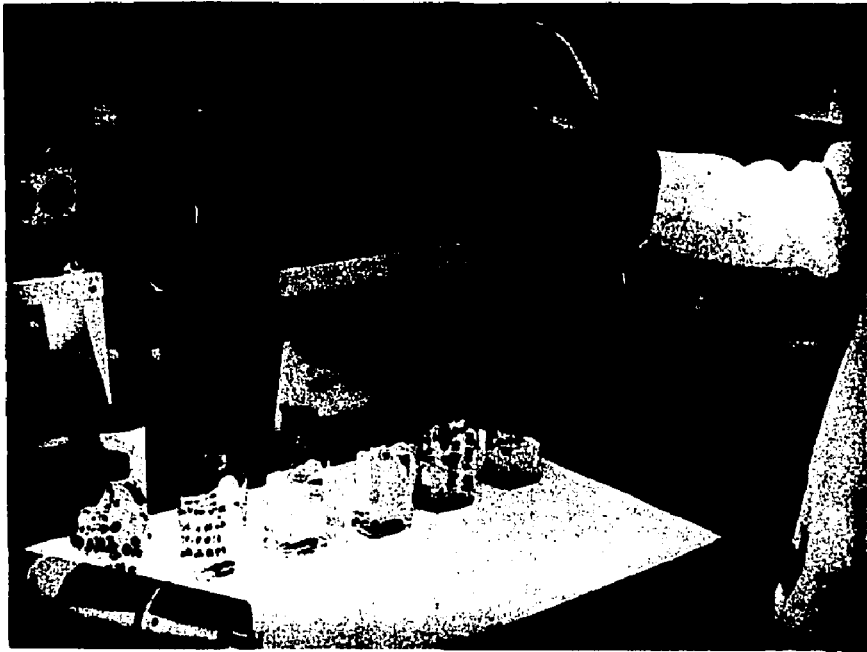
The essential details of this test are graphically presented in Plates I through VI.

6. Neutralization Studies

Sodium Heparin* at concentrations of 0, 200, 500, 1000,

* Powder from Hynson, Wescott and Dunning, Inc., Baltimore, Maryland

PLATE I

**STEPS IN THE MICROTITER BACTERICIDAL DIS -
INFECTION TEST**

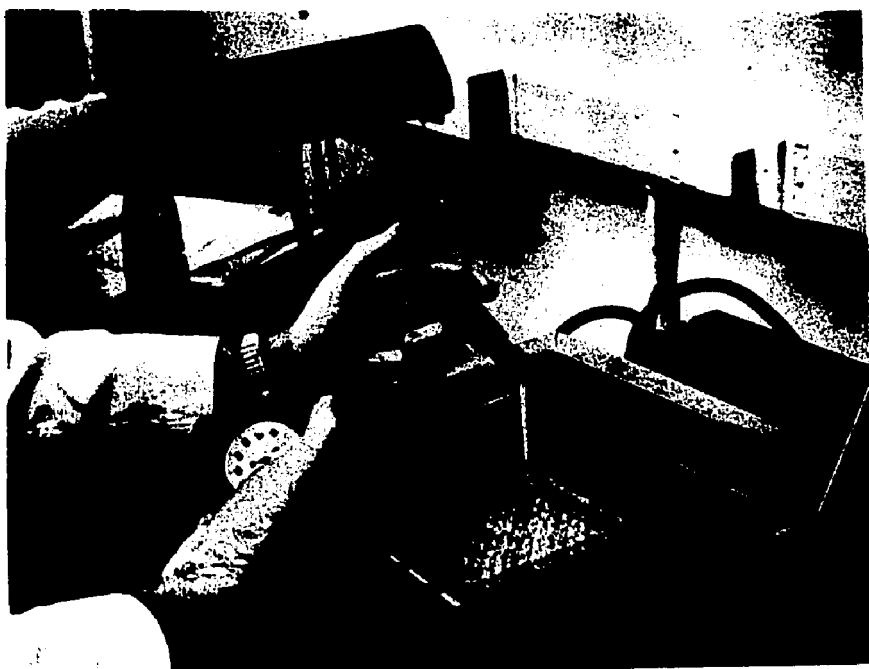
Six two-fold dilutions of the stock disinfectant are made in distilled water prior to the test.

PLATE II



Six 0.1 ml quantities of the disinfectant at various dilutions added to a well of the microtiter plate by non - electric pipette in the temperature controlled water bath or the Stir-cool cold plate.

PLATE III



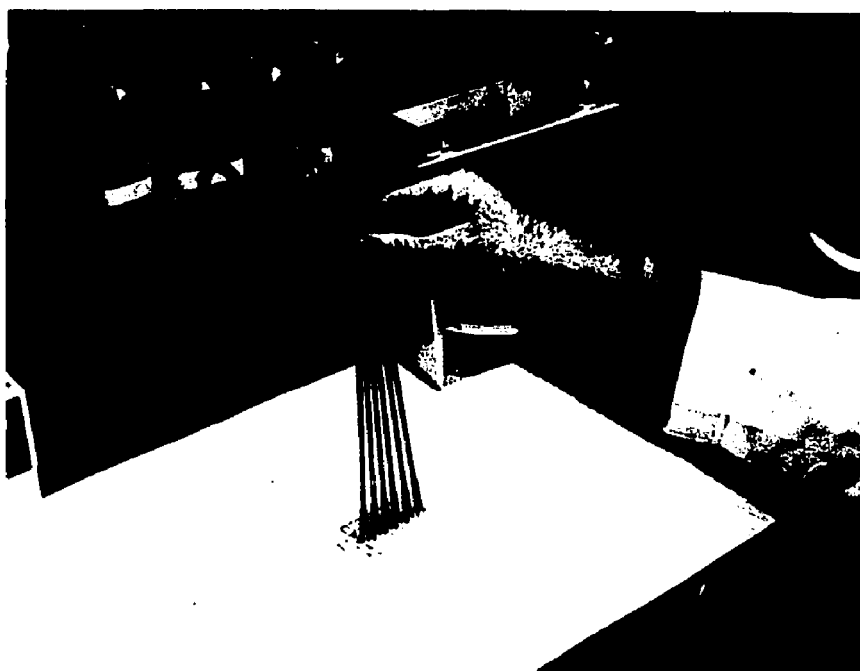
Six 0.1 ml quantities of the test organism are added to each of test wells for the exposure period.

PLATE IV



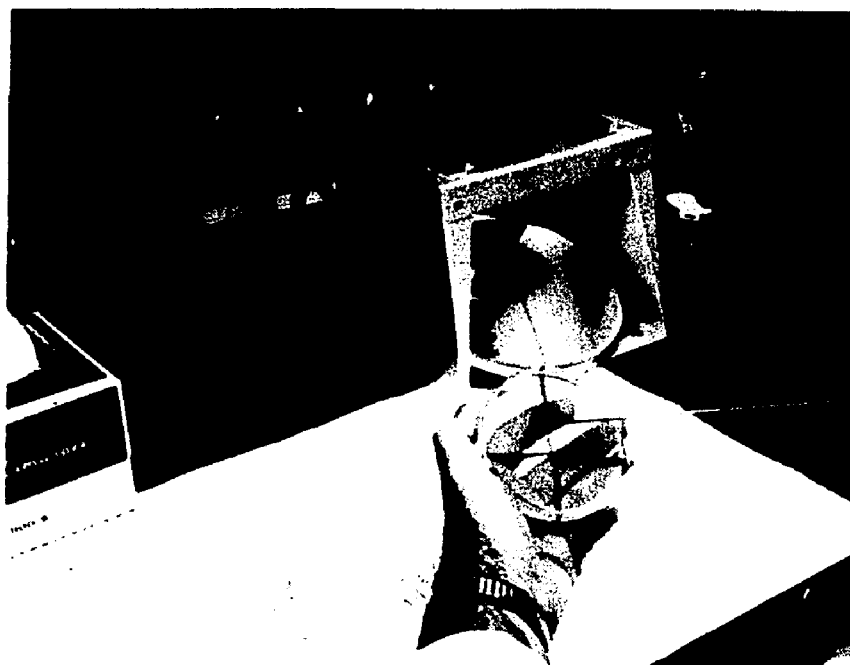
Upon completion of the exposure period, a 0.025 ml aliquot of six exposures at the various dilutions are removed by the specially developed microdiluter.

PLATE V



Neutralization of the test suspensions in 0.225 ml neutralization broth and ten - fold dilutions are accomplished by the specially developed microdiluters.

PLATE VI



Streak plating of a 0.025 ml aliquot at each of ten-fold dilutions is accomplished on one quarter of a agar plate by use of the standard 0.025 ml microdiluter. Plates are incubated and the colonies counted.

2000, and 3000 µg/ml was prepared in TSB. The concentration of QAC that resulted in approximately 50 percent survivors of Pseudomonas aeruginosa, was 62 µg/ml. This concentration was utilized in all the neutralization studies of QAC with Pseudomonas aeruginosa test organisms.

- a. A 24-hour culture of Pseudomonas aeruginosa was subjected to 62 µg/ml of BTC-824* (n-alkyldimethylbenzylammonium chloride) for 5 minutes and then inactivated in TSB containing one of five concentrations of heparin (0, 200, 500, 1000, 2000, and 3000 µg/ml), together with a control.
- b. The disinfectant test procedure was that of the previous bactericidal protocol. A 0.225 ml quantity of inactivating agent and TSB was used as the diluent in each of the microtiter dilution wells.
- c. The organisms were rapidly diluted in the inactivation media by the above procedure after the contact period and a 0.025 ml aliquot of the material was plated on TSA and incubated for 36-48 hours.

7. Disinfectant Studies

A 24-hour culture of test organisms was subjected to QAC for 5 minutes at 6 different concentrations and then inactivated in TSB and sodium heparin at 1000 µg/ml or in AOAC Lethen as specified.

* Onyx Chemical Company, 190 Warren Street, Jersey City, New Jersey

The disinfectant procedure was that previously described for microtiter bactericidal testing. A 0.225 ml quantity of inactivating agent and TSB was used as the diluent in each of the microtiter dilution wells.

The organisms were rapidly diluted in the inactivation media by the above procedure after the contact period and a 0.025 ml aliquot of the material was plated on TSA incubated for 36-48 hours and counted.

8. Bacteriostatic Tests

U bottom, hard styrene, disposable microtitration plates* were cleaned by soaking in 70% ethyl alcohol, rinsed twice in distilled water, shaken and allowed to dry. The protocol for bacteriostatic testing was as follows:

- a. 0.025 ml of diluent was added to each well in the first row by a disposable plastic micropipette.
- b. 0.025 ml of disinfectant (at four times use dilution) was added by micropipette to the well in each column and the well was mixed by rotation of a microdiluter for 5 seconds.
- c. The microdiluter was transferred to the well in column 2 and mixed.
- d. Serial 2-fold dilutions of the disinfectant were made by the same technique throughout the entire column.

* Cooke Engineering Co., Alexandria, Virginia

- e. 0.025 ml quantity of bacterial suspension was then added by micropipette to each well. The organisms were a culture grown on solid agar and transferred to TSB. The concentration was made to correspond to 0.4 OD on the Spectronic 20* organisms/ml at 550 mμ.
- f. The process was repeated in triplicate in three rows for each organism to be tested. A fourth row served as a control and no disinfectant was added to this row.
- g. The plate was covered with sealing tape and shaken gently on an automatic shaker** for 5 minutes.
- h. Plates were incubated for 25 hours at 37°C.
- i. Plates were then placed in the microtiter test reading mirror, the tape was removed. Positive well will show turbidity while negatives showing no growth or bacteriostatis will remain clear.
- j. The last clear well, or the lowest concentration in parts per million (ppm) or (μg/ml) of the test compound that inhibited growth, was reported as the minimum inhibiting concentration (MIC).

* Bausch and Lomb Company, Rochester, New York

** Thomas shaking apparatus, A. H. Thomas Co., Philadelphia, Pennsylvania

C. RESULTS

1. Standard Test Results and Registration Requirements

In Table I are presented data from a typical phenol coefficient test. In this particular example, 5 dilutions of the tested disinfectant were evaluated at 3 time periods, namely, 5, 10, and 15 minutes. Two dilutions of the phenol standard were also evaluated at the same time. The highest dilution of evaluative disinfectant in which growth occurred at 5 but not at 10 minutes of exposure is the accepted endpoint.

For the evaluative disinfectant, this endpoint was 1:350 and for the phenol standard the dilution was 1:90. The phenol coefficient is obtained by dividing former by the latter or $350/90$ which gives 3.9 as the phenol coefficient for the product involved.

In Table II a list of use dilution tests required for registration by U.S. Department of Agriculture as a hospital disinfectant is presented.

2. Calibration of Pipettes and Syringes

In Table III and IV are presented the data for the calibration of the microdiluters and syringes. The calibration was undertaken with chromium 51. A 100 μCi quantity of chromium 51 was added to 10 ml of letheen broth to give a final concentration of 10 $\mu\text{Ci/ml}$. The solution was taken up in each of the microdiluters and the Svedmyr syringe and released to a test tube containing distilled water. The tubes

TABLE I

TYPICAL PHENOL COEFFICIENT DISINFECTANT TEST RESULTS

S. typhosa At 20°C

Activity of Evaluative Disinfectant			
Dilution	5 Minute	10 Minute	15 Minute
1 – 300	0	0	0
1 – 325	+	0	0
1 – 350	+	0	0
1 – 375	+	+	0
1 – 400	+	+	+
Activity of Phenol Standard			
1 – 90	+	+ or –	0
1 – 100	+	+	+ or –

$$\text{P.C.} = \frac{350}{90} \text{ or } 3.9$$

TABLE II

**USE-DILUTION TESTS REQUIRED FOR PRODUCT REGISTRATION
AS A HOSPITAL DISINFECTANT**

Organism	No. of Tests	No. of Batches	Total Tests
<i>Staphylococcus aureus</i>	6	3	18
<i>Salmonella choleraesuis</i>	3	3	9
<i>Pseudomonas aeruginosa</i>	3	3	9
Total			36
Shelf Life Test 60 Days Later			
<i>Staphylococcus aureus</i>	3 on 2 samples	1	6
Total			42
Additional Tests – Optional on Part of Manufacturer			
Pathogenic fungi	2	1	2
Tuberculosis	1	1	1
Virus	—	—	—

TABLE III

**CHROMIUM 51 CALIBRATION OF THE STANDARD AND
SPECIAL MICRODILUTERS FOR LETHEEN BROTH**

Standard Microdiluter		
Replicate	CPM – ⁵¹Cr* (a)	Calculated Volume (ml) (a) / (b)[†] = (c)
1	1226	0.02345
2	1159	0.02217
3	1204	0.02304
4	1166	0.02230
5	1263	0.02416
6	1111	0.02125
7	1163	0.02224
8	1268	0.02425
9	1218	0.02330
10	1209	0.02312
11	1207	0.02310
12	1207	0.02309
Special Microdiluter		
1	1312	0.02510
2	1329	0.02542
3	1302	0.02490
4	1370	0.02621
5	1299	0.02485
6	1382	0.02643
7	1326	0.02536

* Counts per minute of chromium 51.

† (b) = 5227/0.1 ml, counts per minute of chromium 51 in standard.

TABLE IV
CHROMIUM 51 CALIBRATION OF 0.225 ml
SVEDMYR SYRINGE FOR LETHEEN BROTH

Replicate	CPM* — ⁵¹Cr (a)	Calculated Volume (ml) (a) / (b)[†] = (c)
1	12458	0.23833
2	12286	0.23504
3	12452	0.23822
4	12632	0.24166
5	12809	0.23505
6	12519	0.23950
7	12399	0.23721
8	12267	0.23468
9	12256	0.23447
10	12269	0.23472
11	12410	0.23742
12	12336	0.23600
13	12531	0.23973
14	12266	0.23466
15	12313	0.23556
16	12201	0.23342

* Counts per minute of chromium 51.

[†] (b) = 5226/0.1 ml, counts per minute of chromium 51 in standard.

were individually counted for 20 minutes in the well type gamma scintillation counter. The volume of fluid delivered for each instrument was calculated according to the formula:

$$c = b/a$$

c = volume (ml) of the solution delivered by the tested instruments

b = counts per minute/ml of a standard

a = counts per minute of chromium 51 from tested instruments

The mean volume (ml) of solution delivered by the microdiluters tested is presented in Table III. For the standard microdiluters the mean ($\bar{x}$) volume of the 12 tests gave a volume of 0.02298 ml. The standard deviation (s) was found to be ± 0.00083 . The coefficient of variation (cv) was calculated according to the formula:

$$cv = s/\bar{x}$$

s = standard deviation for the tests

$\bar{x}$ = mean of the tests

It was found to be 3.65%. In Table III is also presented the calculated volume (ml) for each of the 7 determinations for the specially developed microdiluters. The mean ($\bar{x}$) of the special microdiluters was 0.02254 ml and the standard deviation was ± 0.0062 . The coefficient of variation was 2.46%.

The adjustable Svedmyr syringe was also calibrated with chromium 51. The results for 16 replicate volumetric deter-

minations is presented in Table IV. The calculated mean is 0.23723 and the standard deviation is equal to ± 0.00622 . The coefficient of variation is 1.31%. The device was recalibrated to deliver the 0.2250 ml of letheen broth as required for accurate utilization in the microtiter bactericidal testing procedure.

3. Bactericidal Studies

In Table V are presented the data for evaluation of the sensitivity of Staphylococcus aureus and Pseudomonas aeruginosa to a quaternary ammonium compound using the microtiter bactericidal test. The percent survivors was calculated from the following formula:

$$\text{Survivors/ml} \times 10^2 / \text{Initial inoculum/ml}$$

In Figure 5 the data are presented in the graphical manner whereby the results are represented by plotting the percentages of survival as integrated normal probability against the logarithm of the disinfectant concentration.

This test was conducted with Staphylococcus aureus and Pseudomonas aeruginosa PRD #10, 5 minute exposure at 24°C. The S. aureus population was almost 80% killed by 20 µg/ml of disinfectant in 5 minutes. This had increased to 99% killed at 60 µg/ml. The figure demonstrates that the disinfectant solutions made from QAC are fairly effective against gram positive bacteria.

In the case of Pseudomonas aeruginosa there was a much greater resistance to the effect of the QAC. Although 75% of

TABLE V
EVALUATION OF EFFECTIVENESS OF QAC AGAINST SEVERAL
ORGANISMS TYPICAL OF THE HOSPITAL ENVIRONMENT

5 Minute Exposure To QAC* At 24°C

<i>S. aureus</i>		
QAC $\mu\text{g/ml}$	Survivors/ml	Percent Survivors
0	1.40×10^6	100.0
20	3.22×10^5	22.0
40	5.18×10^4	3.7
80	4.75×10^3	0.3
<i>Ps. aeruginosa</i>		
0	2.00×10^6	100.0
20	5.40×10^5	27.1
40	3.46×10^5	17.3
80	1.96×10^5	9.8
160	8.20×10^4	4.1
320	3.60×10^4	1.8

* BTC 824 Onyx Chemical Company, 190 Warren Street, Jersey City,
 New Jersey.

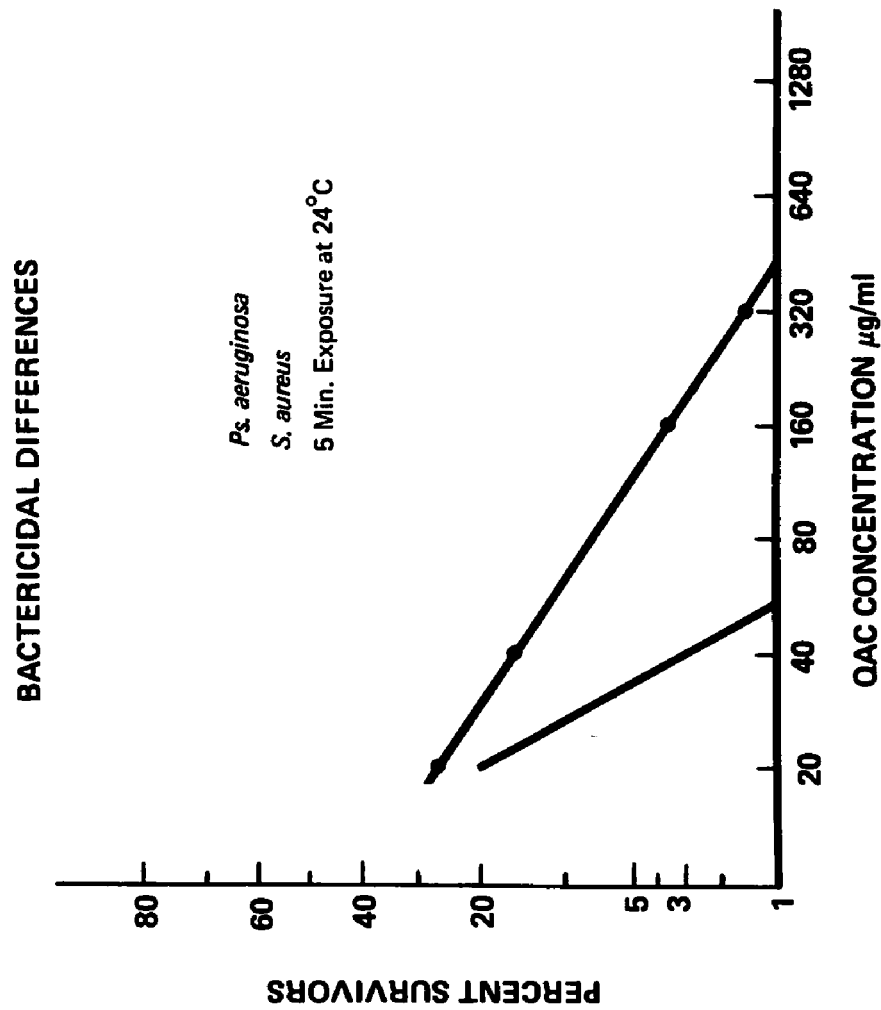


Figure 5. - The bactericidal disinfection of several organisms by a quaternary ammonium compound. The test was conducted with Pseudomonas aeruginosa PRD #10 and Staphylococcus aureus, 5 min. exposure at 24°C. Presented are the percent survivors as the integrated normal probability and the logarithm of the concentration.

the population was killed at 20 µg/ml, as much as 480 µg/ml was required for a 99% reduction. There was an increased disinfectant resistance by this pathogen when compared to S. aureus.

In Table VI are presented the data when a phenolic type hospital disinfectant was evaluated by the microtiter bactericidal procedure at various dilutions of the product against S. aureus at 24°C for 5 minutes. A plot of the data is shown in Figure 6. The rapid dilution of the suspension after initial exposure reduced the disinfectant concentration and eliminated any residual bacteriostatic effect. Many phenyl-phenolic compounds are not particularly effective as bacteriostats thus reducing any possible inhibitory effects of low concentrations of the agent in the first several dilutions. To insure the rapid removal of the phenol, polysorbate 80 was incorporated into the neutralizing medium, since Lawrence (1951) claimed this compound was an effective neutralizer of phenolic disinfectants.

This particular phenolic disinfectant, Ves-Phene*, is normally utilized at a 1:128 dilution. The results indicate that reductions greater than 99% are obtainable with this compound at these levels. In addition, the microtiter test was shown to be applicable to the testing of phenolic compounds as bactericides as well as it is to the quaternary

* Vestal Laboratories, Inc., St. Louis, Missouri

TABLE VI
USE OF THE MICROTITER BACTERICIDAL PROCEDURE FOR EVALUATING
THE EFFECTIVENESS OF A PHENOLIC* DISINFECTANT

S. aureus 5 Minute Exposure At 24°C

Dilution	Survivors/ml	Percent Survivors
—	3.60×10^6	100.0
1:6400	3.52×10^6	97.8
1:3200	2.94×10^6	81.7
1:1600	1.27×10^6	35.3
1:800	2.92×10^5	8.1
1:600	6.85×10^4	1.9
1:500	4.32×10^4	1.2
1:400	2.23×10^4	0.6

* Ves-Phene® — 9.4%, o-phenylphenol, 4 chloro 2 phenylphenol, 6 chloro 2 phenylphenol, p-tertiary amyphenol. Vestal Laboratories, W.R. Grace & Co., St. Louis, Missouri.

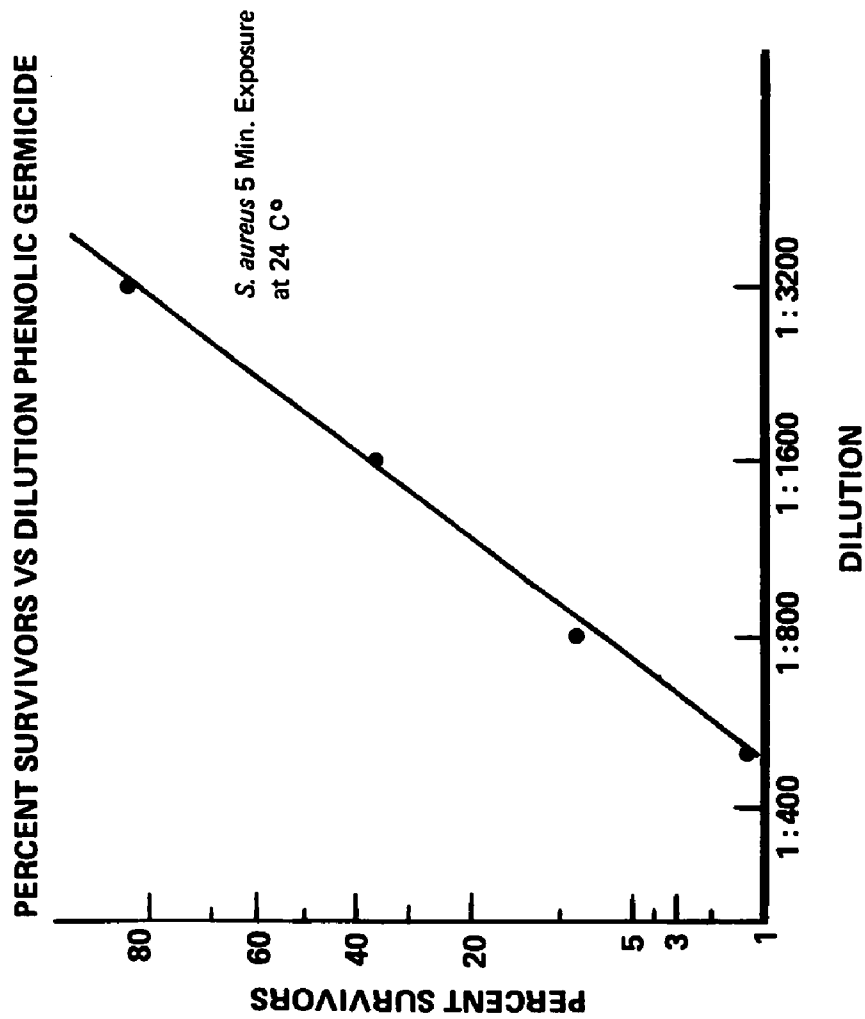


Figure 6. - The bactericidal disinfection curve for a typical phenolic disinfectant. The test was conducted with *S. aureus* with a 5 min. exposure at 24°C. Presented are the percent survivors as the integrated normal probability and the logarithm of the dilution.

ammonium compounds.

4. Comparison of Products

In Figure 7 are presented the disinfection dynamics data when two quaternary ammonium compounds were evaluated concurrently and the percentage survivors plotted against the logarithm of the concentration. The Effective Dose₅₀ (ED₅₀) for Product A was 22 µg/ml and the ED₅₀ for Product B was 60 µg/ml. The test was conducted with Pseudomonas aeruginosa and the exposure interval was 5 minutes. Product A was a N-alkyldimethylbenzylammonium chloride and N-alkyldimethylethylbenzylammonium chloride mixture containing 4.0% active ingredients. Product B was N-alkyltotylmethyltrimethylammonium chloride and listed as 2.8% active material. Product A was a mixture of QAC with a highly alkaline pH which may have accounted for its greater activity against the organisms in question. The protocol enabled the concurrent evaluation of the two compounds at six different concentrations. Product A was superior in all cases to Product B. There was an overall greater reduction in the percent survivors at all concentrations of this particular product. The procedure thus permits the evaluation of two compounds under the same test conditions, and permits a valid judgment as to product effectiveness at various concentrations.

In Table VIII are presented the results for the evaluation of two different types of hospital disinfectants which were evaluated simultaneously against the same organism. As

TABLE VII
COMPARISON OF BACTERICIDAL ACTIVITY OF TWO QUATERNARY
AMMONIUM COMPOUNDS

Pseudomonas aeruginosa 5 Minute Exposure at 24°C

Compound A*		
QAC Conc. ppm	Survivors/ml	Percent Survivors
0	2.00 x 10⁶	100.0
15	1.14 x 10⁶	57.0
30	8.56 x 10⁵	42.8
60	5.92 x 10⁵	29.6
120	3.86 x 10⁵	19.3
Compound B†		
0	2.00 x 10⁶	100.0
15	1.47 x 10⁶	73.5
30	1.34 x 10⁶	67.0
60	1.14 x 10⁶	57.2
120	7.45 x 10⁵	37.2

* Bact-clean® 4.0% n-alkyl dimethyl benzylammonium chloride and n-alkyl dimethethyl benzylammonium chloride, Dameron Enterprises, Inc., Louisville, Kentucky.

† Sanikleen® 2.8% n-alkyl totyl methyl trimethyl ammonium chloride, West Chemical Products, Inc., New York, New York.

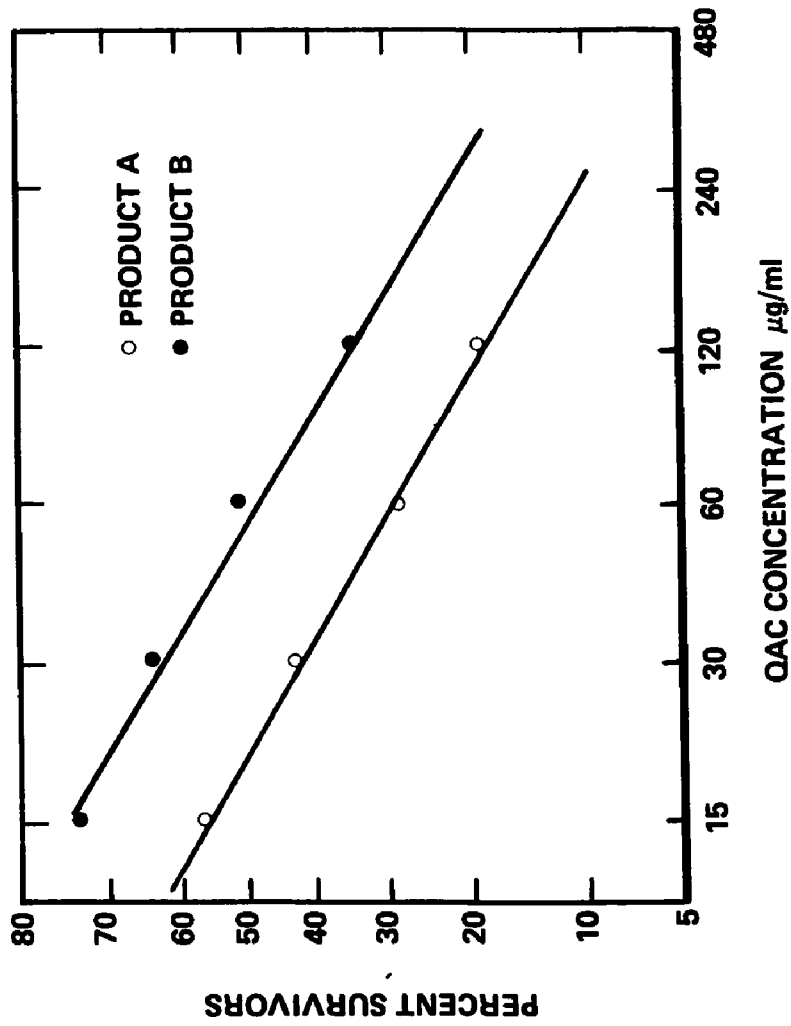


Figure 7. – Comparison of the bactericidal effectiveness of two quaternary ammonium compounds. The test was conducted with *Pseudomonas aeruginosa* PRD #10. 5 minute exposure at 24° C. Presented are the percent survivors as the integrated normal probability and the logarithm of the concentration.

TABLE VIII

DISINFECTION DYNAMICS OF A QAC AND A PHENOLIC DISINFECTANT

Pseudomonas aeruginosa 5 Minute Exposure At 24°C

QAC*		
ppm	Survivors/ml	Percent Survivors
0	2.10×10^6	100.0
15	1.50×10^6	71.4
30	1.24×10^6	59.0
60	9.10×10^5	43.3
120	5.15×10^5	24.5
240	2.73×10^5	13.0
480	1.30×10^5	6.2
Phenolic†		
0	2.10×10^6	100.0
45	1.96×10^6	93.3
60	1.76×10^6	83.8
110	2.22×10^5	10.6

* BTC 824 Onyx Chemical Company.

† Ves-Phene® — 9.4%, o-phenylphenol, 4 chloro 2 phenylphenol, 6 chloro 2 phenylphenol, p-tertiary amylphenol. Vestal Laboratories, W.R. Grace & Co., St. Louis, Missouri.

shown in Figure 8, at 60 $\mu\text{g/ml}$ of the phenolic type compound there were 78% survivors and at 90 $\mu\text{g/ml}$ there were 20% survivors. A small increase in the concentration resulted in a large change or decrease in the number of survivors.

In Figure 9 the concentration of a QAC was changed from 60 $\mu\text{g/ml}$ to 90 $\mu\text{g/ml}$. At 60 $\mu\text{g/ml}$, there were 48% survivors, but at 90 $\mu\text{g/ml}$ there were 42% survivors. A small change in concentration resulted in only a small decrease in the number of survivors. Thus, the activity of the phenolic disinfectant did not change linearly, but changed as some power of the concentration.

The results indicate that the slope of the disinfection survival curve gives a statement of the change in the activity of a compound for a given change in concentration. A knowledge of the slope of the curve of a disinfectant enables one to determine if a disinfectant can be easily diluted (low numerical slope) or whether dilution results in drastic changes in bactericidal efficiency (high numerical slope). The slope provides an index to the effect dilution has upon the germicidal efficiency of a disinfectant.

This study points out the relevance of this type of information for hospital applications. If the hospital staff were to indiscriminately lower the concentration of a product for economic reasons, or because of a preparation error, unexpected hazards to staff and patient could result especially with regard to certain disinfectants.

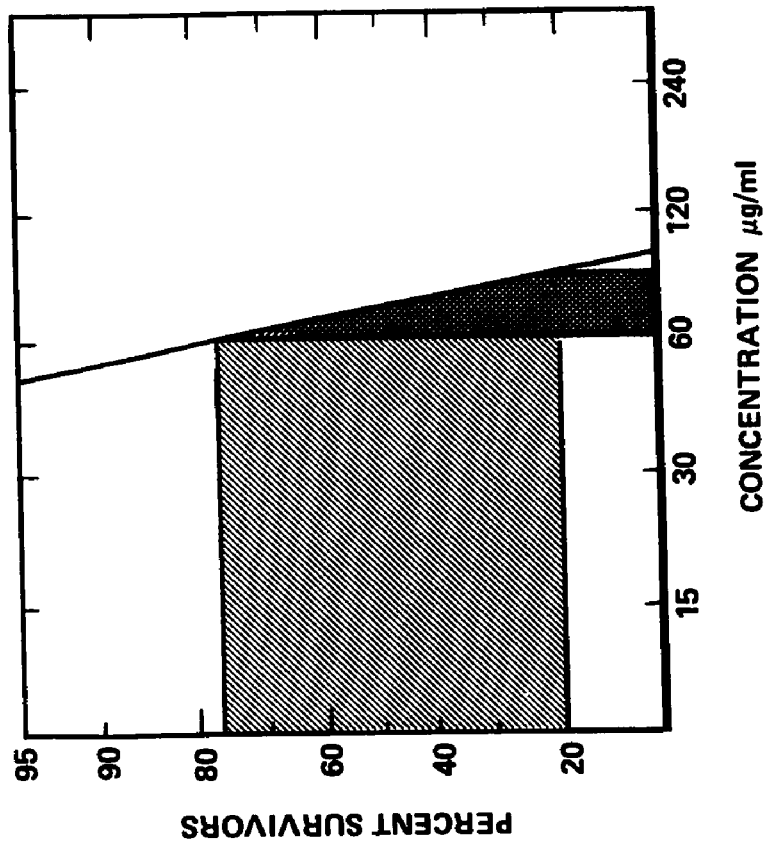


Figure 8. - The disinfection dynamics of a phenolic disinfectant. Pseudomonas aeruginosa PRD #10, 5 min. exposure at 24°C. Presented are the percent survivors as the integrated probability and the logarithm of the concentration.

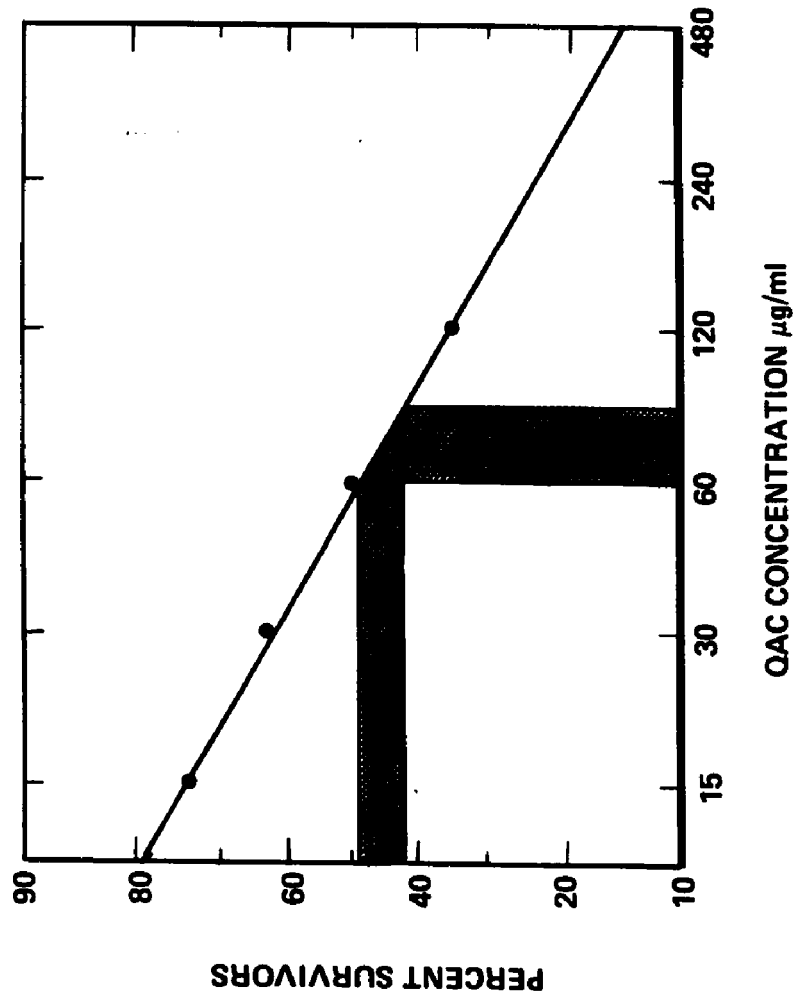


Figure 9. - The disinfection dynamics of a quaternary ammonium compound against *Pseudomonas aeruginosa* PRD #10, 5 min. exposure at 24° C. Presented are the percent survivors as the integrated normal probability and the concentration as its logarithm.

5. Bacteriophage Testing

In Table IX are presented data on the viricidal effectiveness of a phenolic and a quaternary hospital disinfectant against the T2 bacteriophage of E. coli. The two tests were conducted simultaneously, 1.66×10^5 phage/ml were exposed to the two disinfectants for 5 minutes at 24°C. After the exposure period a 0.025 ml sample from each treatment well and a control well was neutralized in letheen broth and diluted in 10 fold dilutions from 10^{-1} to 10^{-6} in individual microtiter wells. Instead of streaking a 0.025 ml quantity of suspension on a standard bacteriological plate as is done in the microtiter bactericidal tests, 0.025 ml of the phage - QAC - letheen mixture was added to 1 ml of warm 0.8% agar containing 0.025 ml of 1.0×10^8 /ml of E. coli. After mixing, this suspension was poured over the surface of a dry TSA plate, incubated overnight, and the number of plaque forming units (PFU) were counted. As shown in Figure 10, at the 1:100 dilution of products, often used for hospital environmental disinfection, 63.5% of bacteriophage was apparently killed by the phenolic while at the same dilution of QAC, 99.2% was killed. The T2 bacteriophage of E. coli was more susceptible to the QAC than to the phenolic disinfectant. The total concentrations of active ingredients at 1:100 dilutions were 550 ppm of QAC and 2050 ppm of phenolic, which demonstrates the greater viricidal efficiency by the QAC for this particular bacteriophage. In the case of the quaternary ammonium compound, a 95-99% reduction occurred in the regions of typical

TABLE IX

**TESTING THE T2 BACTERIOPHAGE OF *E. COLI* SUSCEPTIBILITY TO
TWO HOSPITAL DISINFECTANTS BY MICROTITER PROCEDURES**

5 Minute Exposure At 24°C

QAC*		
Dilution	PFU/ml†	Percent
Control	1.66×10^5	100.0
1:100	1.34×10^3	0.8
1:200	2.06×10^3	1.2
1:300	4.99×10^3	3.0
1:400	8.26×10^3	5.0
1:500	1.39×10^4	8.4
Phenolic‡		
Control	1.66×10^5	100.0
1:100	6.05×10^5	36.4
1:200	7.20×10^4	43.4
1:300	8.80×10^4	53.0
1:400	9.90×10^4	59.6
1:500	1.10×10^5	66.2
1:600	1.28×10^5	77.1

* Germerase® — 5.5% alkyl dimethylbenzylammonium chloride,
Horizon Industries, Cleveland, Ohio.

† Plaque forming units/ml

‡ 1 Stroke Ves-Phene® — 20.5% o-phenylphenol, o-benzyl-p-chlorophenol,
and p-tertiary amylphenol, Vestal Laboratories, W.R. Grace & Co.,
St. Louis, Missouri.

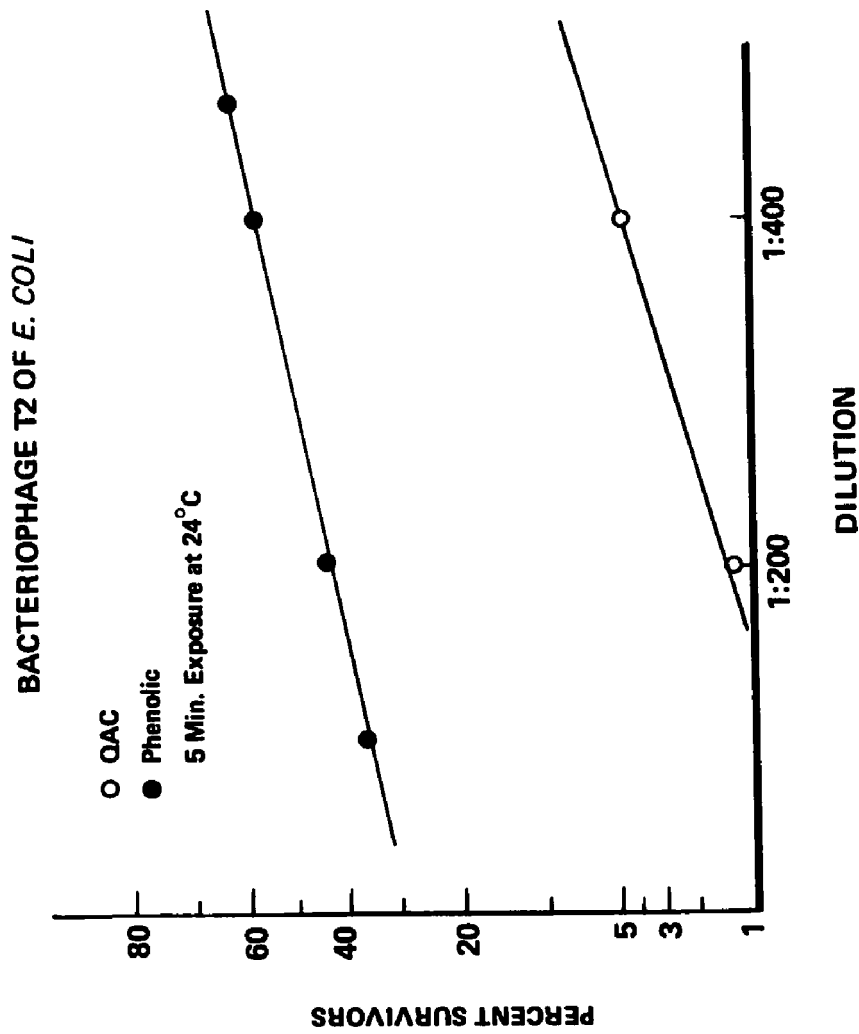


Figure 10. - Comparison of the virucidal effectiveness of a phenolic disinfectant and a quaternary ammonium compound. The test was conducted with the T2 bacteriophage of *E. coli*, a 5 min. exposure at 24°C. Presented are the percent survivors as the integrated normal probability and the logarithm of the concentration.

hospital disinfectant concentrations. For the phenolic disinfectant, 40-70% reduction occurred in five minutes under similar conditions.

6. Neutralization of Disinfectant

In Table X are presented the data on the effectiveness of sodium heparin on the neutralization of a quaternary ammonium compound as demonstrated by the survival numbers when neutralization and dilution of the QAC were accomplished in various concentrations of heparin and TSB. The test was conducted with a 24 hour culture of Pseudomonas aeruginosa PRD #10, at 1.0×10^6 /ml, in TSB. The exposure was for 5 minutes at 24°C.

As shown in Figure 11, there was 30% survival of the sample that was diluted only in TSB. The highest percent survival, 45%, occurred in the sample containing 1000 µg/ml in TSB. At concentrations of 2000 µg/ml and 3000 µg/ml the percentages of organisms surviving were 32 and 25% respectively. The latter percentage is below the control, indicating that at high concentrations of the heparin there may be some toxic effects to the organisms especially after exposure to the QAC. It was concluded from these results that the percent survivors recovering from the QAC exposure varied according to the concentrations of heparin in the diluent indicating that there was an effect on recovery that was related to the concentration of the heparin. A concentration of 1000 µg/ml of heparin in TSB was selected for further evaluation as a

TABLE X
EFFECTIVENESS OF SEVERAL CONCENTRATIONS OF HEPARIN AS A
QAC* NEUTRALIZER

Pseudomonas aeruginosa 5 Minute Exposure At 24° C

QAC Conc. μg/ml	Heparin Conc. μg/ml in TSB	Survivors/ml	% Survivors
0	0	1.0 x 10 ⁶	100.0
62	0	3.0 x 10 ⁵	30.0
62	200	3.1 x 10 ⁵	31.0
62	500	3.4 x 10 ⁵	34.0
62	1000	4.5 x 10 ⁵	45.0
62	2000	3.2 x 10 ⁵	32.0
62	3000	2.5 x 10 ⁵	25.0

* QAC – BTC 824 Onyx Chemical Company at 62 μg/ml.

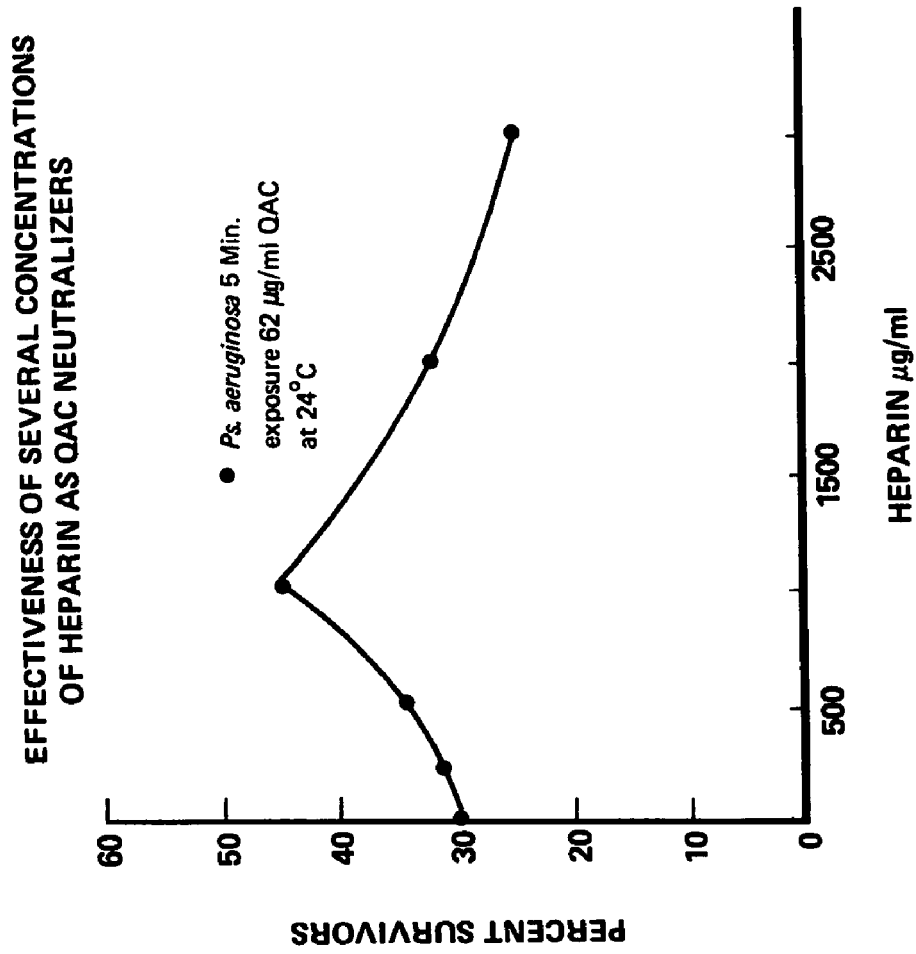


Figure 11. - Effectiveness of various concentrations of heparin as a quaternary ammonium compound neutralizer. The percent survivors of the test organism and the concentration of heparin are presented.

QAC neutralizer and in subsequent studies its effectiveness was compared with letheen for dilution and neutralization after exposure to various QAC concentrations.

In Figure 12 are presented the findings when heparin, letheen and a control without neutralizer were evaluated simultaneously as QAC neutralizers. Pseudomonas aeruginosa was exposed for 5 minutes to QAC. At high concentrations of the QAC the shape of the curve, with letheen as the neutralizer, tended to decline indicating decreasing effectiveness of the letheen system to neutralize higher levels of the QAC disinfectant. The transformed shape of the disinfection curve was linear in all cases where heparin was utilized as the inactivator.

The control containing TSA, without incorporation of neutralizing compounds, yielded lower results. This is probably due to the carry-over of QAC at especially high QAC concentrations resulting in a bacteriostatic effect. The results demonstrate higher percentages of survivors as opposed to the control when either heparin or letheen were utilized for the 10-fold dilutions.

Studies on the neutralization of QAC by heparin, letheen and the control with S. aureus as the test organism are presented in Table XII and Figure 13. S. aureus is very sensitive to QAC action and one would expect recognizable differences if either neutralizer was ineffective. However, this was not the case. If heparin had been more effective in

TABLE XI
EVALUATION OF LETHEEN AND HEPARIN FOR NEUTRALIZATION
OF QAC IN MICROTITER TESTS

Pseudomonas aeruginosa 24°C For 5 Minutes

Trypticase Soy Broth (TSB)		
Conc. QAC* μg/ml	Survivors/ml	Percent Survivors
0	2.20 x 10 ⁶	100.0
15	1.15 x 10 ⁶	52.3
31	6.23 x 10 ⁵	28.3
62	1.54 x 10 ⁵	7.0
125	8.80 x 10 ²	0
AOAC Letheen		
0	2.20 x 10 ⁶	100.0
15	1.59 x 10 ⁶	72.3
31	1.10 x 10 ⁶	50.0
62	5.96 x 10 ⁵	27.1
125	2.02 x 10 ⁵	9.2
250	1.54 x 10 ³	0.1
Heparin (1000 μg/ml) in TSB		
0	2.10 x 10 ⁶	100.0
15	1.86 x 10 ⁶	88.6
31	1.44 x 10 ⁶	68.5
62	1.00 x 10 ⁶	47.6
125	6.10 x 10 ⁵	29.1
250	1.65 x 10 ⁵	7.9

* BTC 824 Onyx Chemical Company.

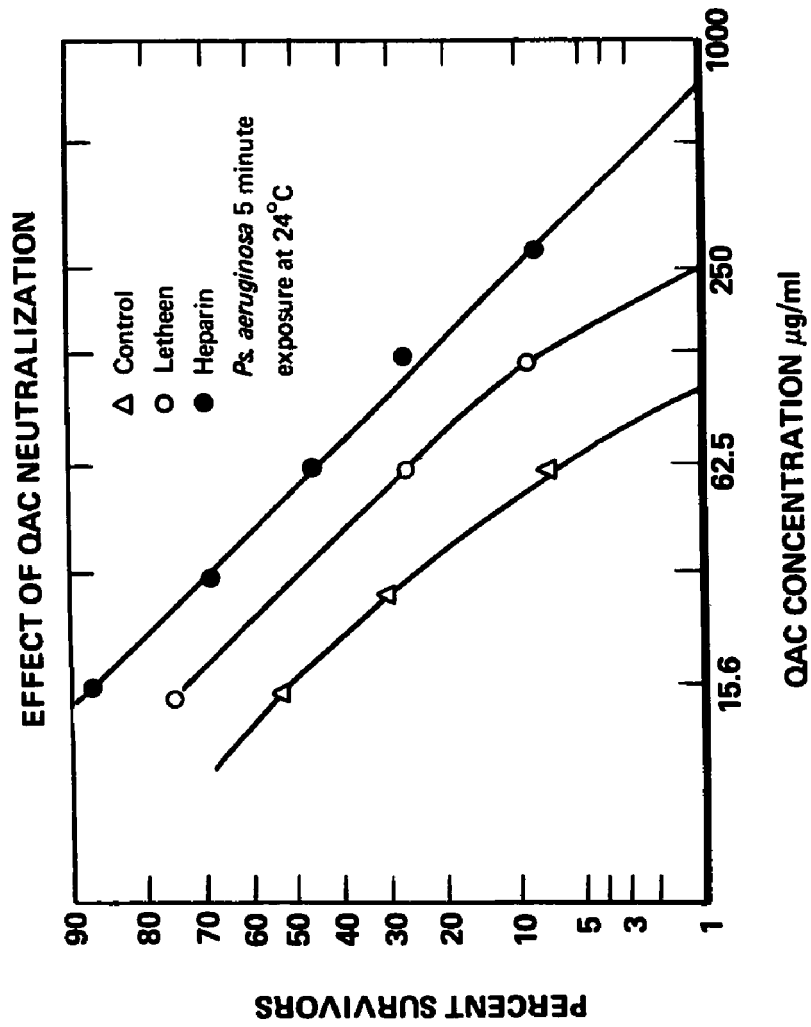


Figure 12. - Comparison of quaternary ammonium compound neutralization by heparin and letheen with *Pseudomonas aeruginosa* as test organism. Presented are the percent survivors as the integrated normal probability and the logarithm of the concentration.

TABLE XII
EVALUATION OF LETHEEN AND HEPARIN FOR NEUTRALIZATION
OF QAC IN MICROTITER TEST
S. aureus 24°C For 5 Minutes

TSB		
Conc. QAC* μg/ml	Survivors/ml	Percent Survivors
0	2.20×10^6	100.0
20	4.37×10^5	19.9
40	4.85×10^4	2.2
60	4.40×10^3	0.2
Letheen – AOAC		
0	2.00×10^6	100.0
20	5.20×10^5	29.6
40	8.00×10^4	4.0
60	3.42×10^4	1.7
80	2.00×10^2	0.1
Heparin (1000 μg/ml) in TSB		
0	2.10×10^6	100.0
20	6.97×10^5	33.2
40	1.32×10^5	6.3
60	4.20×10^4	2.0
80	1.33×10^3	0.6

* BTC 824 Onyx Chemical Company

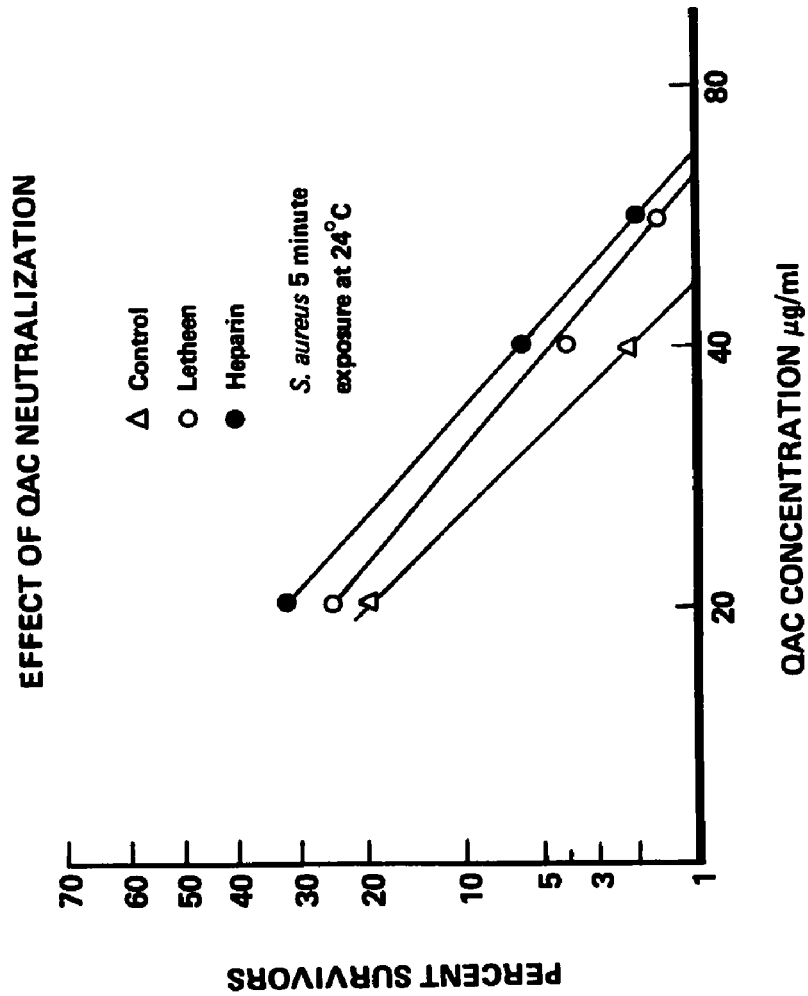


Figure 13. – Comparison of quaternary ammonium compound neutralization by heparin and lethene with *Staphylococcus aureus* as the test organism. Presented are the percent survivors as the integrated normal probability and the logarithm of the concentration.

blocking QAC action a greater difference in the slopes of the survival curves might have been observed. While the heparin nevertheless was demonstrated to be a better neutralizer than letheen, it appeared to be more effective in disinfection tests involving pseudomonas organisms.

7. Environmental Parameters

The effect of environmental factors, water hardness, temperature and organic load, on the efficiency of quaternary ammonium compounds was studied.

In Table XIII are presented the data for the effect of 300 µg/ml of water hardness on the bactericidal activity of QAC. The test organism was a 24 hour culture of E. coli at 2.80×10^6 /ml of initial inoculum.

In Figure 14 the results indicate that there was a greater percentage of survivors in the presence of the added inorganic ions. The slope of the hardness curve was slightly affected and the intercept differed considerably. The intercept of the 0 hardness curve was 110 µg/ml of QAC while the intercept of the 300 µg/ml hardness curve was nearly 320 µg/ml of QAC. There was a considerable decrease in bactericidal activity due to 300 µg/ml concentration of water hardness. The interference of divalent cations on the bactericidal efficiency of QAC was evident.

In Table XIV are presented data on the effect of temperature on QAC activity. The test organism was E. coli at 2.80×10^6 /ml. The organisms were exposed to 100 µg/ml of QAC

TABLE XIII

EVALUATION OF THE EFFECT OF WATER HARDNESS* ON THE
ACTIVITY OF QAC†*E. coli* 5 Minute Exposure at 24°C

Control – No Hardness		
QAC Conc. μg/ml	Survivors/ml	Percent Survivors
0	2.80×10^6	100.0
25	1.39×10^6	49.8
50	3.92×10^5	14.0
100	3.36×10^5	1.2
Test – 300 μg/ml Hardness		
0	2.80×10^5	100.0
50	1.49×10^6	54.5
100	5.86×10^5	21.0
200	1.67×10^5	3.8

* Preparation of hard water according to protocol.

† BTC 824 Onyx Chemical Company.

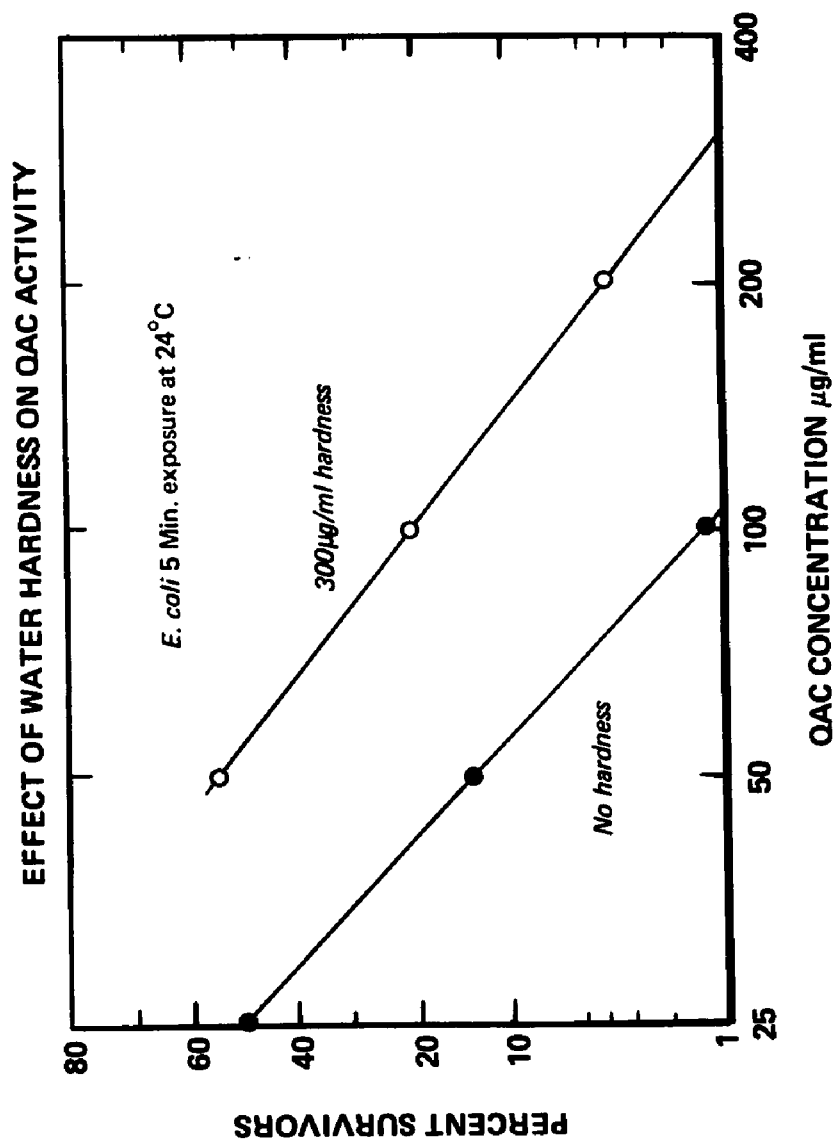


Figure 14. - The effect of water hardness on QAC activity. Presented are the percent survivors as the integrated normal probability and the logarithm of the QAC concentration.

TABLE XIV
EVALUATION OF EFFECT OF TEMPERATURE ON THE ACTIVITY OF QAC
E. coli 100 µg/ml QAC*

44° C Test		
Time	Survivors/ml	Percent Survivors
0 sec.	2.80 x 10⁶	100.0
15 sec.	2.16 x 10⁶	77.1
30 sec.	9.35 x 10⁵	33.4
45 sec.	8.12 x 10⁴	2.9
1 min.	9.52 x 10³	0.3
37° C Test		
0 sec.	2.80 x 10⁶	100.0
15 sec.	2.69 x 10⁶	95.4
30 sec.	2.41 x 10⁶	86.1
45 sec.	1.91 x 10⁶	68.2
1 min.	1.32 x 10⁶	47.1
1 min. 15 sec.	6.68 x 10⁵	23.8
1 min. 30 sec.	2.40 x 10⁵	8.6
1 min. 45 sec.	3.92 x 10⁴	1.4

* BTC 824 Onyx Chemical Company.

TABLE XIV — Continued

24° C Test		
Time	Survivors/ml	Percent Survivors
0 sec.	2.80×10^6	100.0
30 sec.	2.65×10^6	94.6
1 min.	2.24×10^6	80.1
1 min. 30 sec.	1.55×10^6	55.4
2 min.	8.37×10^5	29.9
2 min. 30 sec.	2.96×10^5	10.4
3 min.	7.84×10^4	2.8

and 0.025 ml samples withdrawn at 15 second intervals.

The percent survivors versus the temperature in degrees centigrade are presented graphically in Figure 15.

The intercept of 99% reduction at 44°C was 46 seconds, at 37°C the intercept was 1 minute 54 seconds and at 24°C the intercept was 3 minutes 16 seconds.

In this particular series of tests, samples were removed at fixed time intervals at one constant temperature. This is a change from the normal testing procedure where time was fixed and effects of various concentrations studied. Because of the capabilities of the microtiter procedure, samples can be withdrawn from the test suspension in intervals as short as 15 seconds and subsequently inactivated. Such rapid sampling and neutralization is not available when utilizing standard testing procedures. Observations can be conducted over any desired time interval and the results recorded.

In Table XV are presented the data on the effect of fetal calf serum or protein at various concentrations on the dynamics of QAC activity. The test organism was E. coli, 2.80×10^6 /ml at 24°C for a 5 minute exposure.

In the British Standards Institution QAC test, the organic matter may be added pre-mixed with organisms or added as a separate step.

The activity of the QAC was tested against several organic loads which included 0.5% and 5.0% protein.

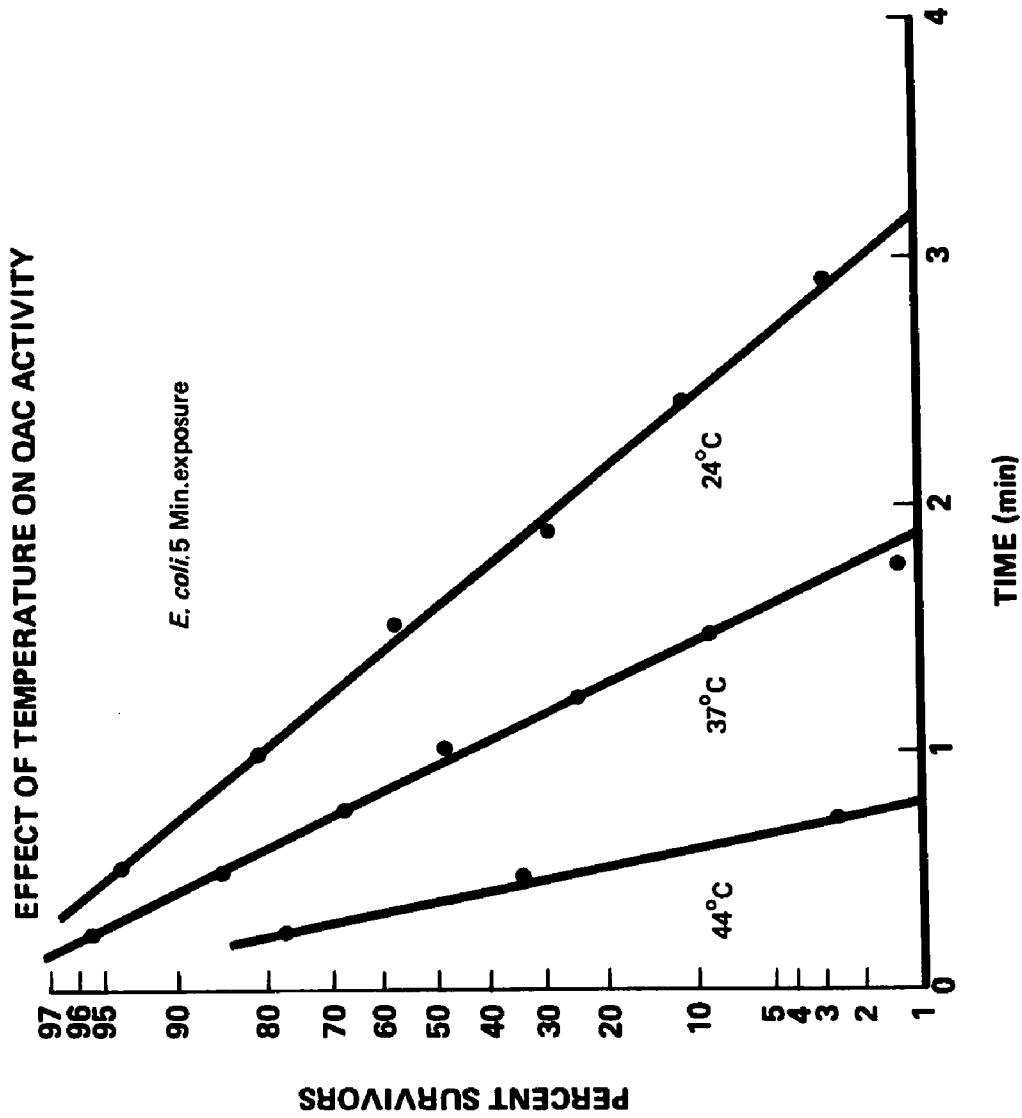


Figure 15. - The effect of temperature on quaternary ammonium compound activity. Presented are the present survivors as the integrated normal probability and the time in minutes.

TABLE XV
EVALUATION OF EFFECT OF ORGANIC LOAD*
ON THE ACTIVITY OF QAC†

E. coli 5 Minute Exposure At 24°C

Control – No Protein		
QAC Conc. μg/ml	Survivors/ml	Percent Survivors
0	2.80×10^6	100.0
25	1.49×10^6	53.2
50	3.92×10^5	14.0
100	3.26×10^4	1.2
Test – 0.5% Protein		
0	2.80×10^6	100.0
25	2.03×10^6	72.5
50	1.04×10^6	37.1
100	3.46×10^5	12.4
200	5.04×10^4	1.8
Test – 5.0% Protein		
200	4.07×10^5	14.5
400	2.71×10^5	9.7
800	1.78×10^5	6.4
1600	1.06×10^5	3.9
3200	5.90×10^4	2.1

* Newborn Calf Serum, Microbiological Associates, Bethesda, Maryland.

† BTC 824 Onyx Chemical Company.

As seen in Figure 16, the activity of the QAC under 0.5% organic load increased the intercept from 110 µg/ml to over 200 µg/ml. The 0.5% organic load would represent rather severe environmental situations.

8. Reproducibility of Bactericidal Test

In Table XVI are presented the data for 6 microtiter bactericidal tests conducted at one session. In Table XVII are presented data for 6 replications of same tests conducted on 6 consecutive days.

The purpose of these studies was to evaluate the reproducibility of the microtiter bactericidal disinfection test. The tests were conducted with Pseudomonas aeruginosa PRD #10 and the QAC concentrations were 0, 15.62, 31.25, 62.50, 125.00 and 250.00 µg/ml for 5 minute exposures at 24°C. The percent killed is presented instead of percent survivors because it facilitates statistical evaluation by the method of Cornfield and Mantel (1950). The percent killed was calculated from the following formula:

$$(\text{Initial Inoculum} - \text{Survivors} \times 10^2) / \text{Initial Inoculum}$$

The initial inoculum is estimated by the number of organisms at the zero dose.

The responses in Table XVI are relatively uniform, indicating that the techniques are good and can lead to reproducible results. The largest percentage range of viable counts is only 1.2 percentage points, both occurring at the lowest dose of 15.62 µg/ml and 31.25 µg/ml.

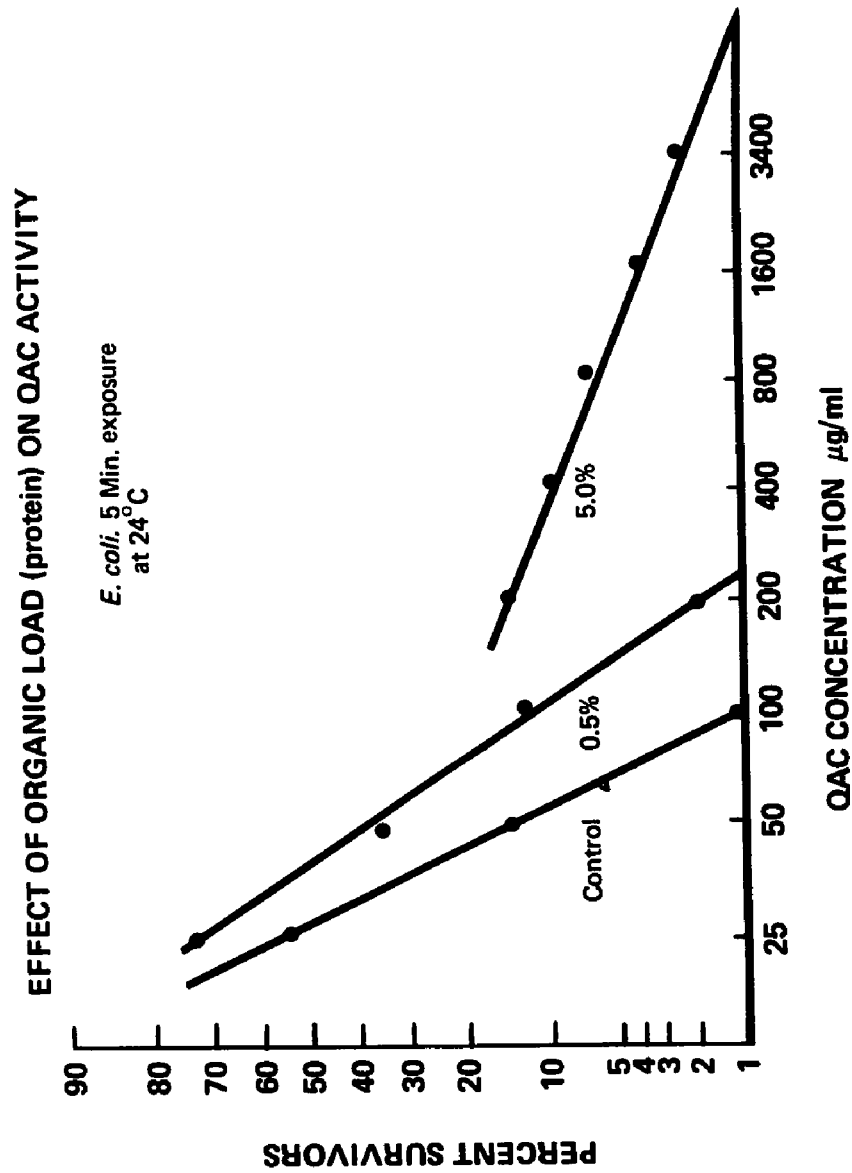


Figure 16. - The effect of organic load (protein) on quaternary ammonium compound activity. Presented are the percent survivors as the integrated normal probability and the logarithm of the quaternary ammonium concentration.

TABLE XVI
 REPRODUCIBILITY OF MICROTITER BACTERICIDAL TEST
Pseudomonas aeruginosa PRD #10 5 Minute Exposure To QAC* At 24°C

Replicate Tests Same Session						
Dose μg/ml	#1		Replicate #2		#3	
	Survivors†	Percent Kill	Survivors†	Percent Kill	Survivors†	Percent Kill
0	4933	-†	5012	-	5111	-
15.62	3067	37.8	3102	38.1	3118	39.0
31.25	1583	67.9	1620	67.7	1614	68.4
62.50	791	84.0	801	84.0	810	84.2
125.00	320	93.5	340	93.2	334	93.5
250.00	94	98.1	100	98.0	98	98.2

Dose μg/ml	#4		Replicate #5		#6	
	Survivors†	Percent Kill	Survivors†	Percent Kill	Survivors†	Percent Kill
0	5004	-	4981	-	5090	-
15.62	3080	38.4	3098	37.8	3104	39.0
331.25	1590	68.2	1579	68.3	1631	68.0
62.50	798	84.1	791	84.1	796	84.4
125.00	339	93.2	322	93.5	310	93.9
250.00	91	98.2	92	98.2	93	98.2

* BTC 824 Onyx Chemical Company in distilled water.

† Survivors/.05 ml. † Control.

In Table XVII are presented the data for replicates on different days. As anticipated, the results are not as homogeneous as those from the replications from a single session. There is a difference of 10 percentage points between the highest and lowest response. Both of the maximum ranges occurred at the lowest dose (15.62 $\mu\text{g/ml}$) of QAC.

Table XVII demonstrates that the average total inoculum of the five experiments resulted in a standard deviation of 396.6, which gives a coefficient of variation of 7.1 percent. However, the Median Effective Doses (ED_{50}) fell in the interval between the two lowest doses (15.62 $\mu\text{g/ml}$ and 31.25 $\mu\text{g/ml}$) of QAC just as they did in the same session replicates.

The greater part of this day to day variability can be removed by several methods. For example, by replicating the zero dose inoculum a number of times and using the average of these as the base for calculating the percentage kill, or by assaying a substance through the use of a so-called standard, or both of these. Thus, the inherent day to day variability of the bactericidal test can be made relatively small and the results highly reproducible.

In Table XVIII are presented the computations for the statistical information for replicate number 1, same session, which are presented in Table XIX. The values for Δ and w' are taken from the tabulations of the paper of Cornfield and Mantel (1950). The doses were coded to facilitate the computations on a desk calculator. However, when a digital computer is used, it is neither necessary to code the doses

TABLE XVII

REPRODUCIBILITY OF MICROTITER BACTERICIDIAL TEST
Pseudomonas aeruginosa PRD #10 5 Minute Exposure To QAC* At 24°C

Replicate Tests on Different Days						
Dose μg/ml	#1		Replicate #2		#3	
	Survivors†	Percent Kill	Survivors†	Percent Kill	Survivors†	Percent Kill
0	4981	—†	6001	—	5500	—
15.62	3098	37.8	3546	40.9	3494	36.5
31.25	1579	68.3	1882	68.6	1737	68.4
62.50	791	84.1	984	83.6	691	87.4
125.00	332	93.3	450	92.5	370	93.3
250.00	92	98.2	201	96.7	120	97.8

Replicate Tests on Different Days						
Dose μg/ml	#4		Replicate #5		#5	
	Survivors†	Percent Kill	Survivors†	Percent Kill	Survivors†	Percent Kill
0	5491	—	5862	—	—	—
15.62	3241	41.0	3140	46.4	46.4	46.4
31.25	1631	70.3	1661	71.7	71.7	71.7
62.50	781	85.8	721	87.7	87.7	87.7
125.00	462	91.6	397	93.2	93.2	93.2
250.00	31	99.4	91	98.4	98.4	98.4

* BTC 824 Onyx Chemical Company in distilled water. † Survivors/.05 ml. ‡ Control.

TABLE XVIII

COMPUTATIONAL SCHEME* FOR OBTAINING THE DOSE-RESPONSE LINE†

Dose	Coded Log Dose x (1)	f (2)	fx (1) x (2) (3)	fx^2 (1) x (3) (4)	1st Cycle			2nd Cycle		
					Probit (5)	Δ (6)	w' (7)	Probit (8)	Δ (9)	w' (10)
15.625	0	3067	—	—	4.7	— .617	.566	4.78	— .603	.586
		1866	—	—		.998	.697		.943	.682
31.25	1	1583	1583	1583	5.2	— .929	.678	5.36	— 1.040	.708
		3350	3350	3350		.675	.591		.584	.551
62.50	2	791	1582	3164	5.7	— 1.291	.762	5.93	— 1.469	.793
		4142	8284	16568		.412	.458		.314	.391
125.00	3	320	960	2880	6.2	— 1.688	.823	6.51	— 1.947	.851
		4613	13839	41517		.219	.311		.137	.225
250.00	4	94	376	1504	6.7	— 2.110	.866	7.09	— 2.453	.891
		4839	19356	77424		.098	.177		.046	.098

2 x 6	A = $\Sigma f\Delta$ = 2191.86	= 175.86	L = $b + \delta b = b = 0.577$	= 0.60
3 x 6	B = $\Sigma fx\Delta$ = 4675.08	= 582.17	M = $\frac{5-J}{L} = M = 0.381$	= 0.400
2 x 7	C = $\Sigma fw'$ = 11225.34	= 10151.49	N = $C/H = .00006948$	
3 x 7	D = Σfxw = 16898.32	= 13622.82	O = $\sigma_b = \sqrt{N} = 0.0083$	
4 x 7	E = $\Sigma fx^2w'$ = 43340.78	= 32673.58	P = $1/C = .00009851$	
	F = AE-BD = 15995924	= 2184821	Q = $X = D/C = 1.342$	
	G = BC-AD = 15440611	= 3514184	R = $(M-Q)^2 = 0.887364$	
	H = CE-D^2 = 200961772	= 146104296	S = $P + NR = 0.00016016$	
	I = F/H = $\sigma a = 0.080$	= 0.015	T = $\sqrt{S} = 0.0126556$	
	J = $a + I = a = 4.78$	= 4.76	U = $\sigma_m = T/L = 0.021$	
	K = G/H = $\sigma b = 0.077$	= 0.024		

* According to procedure of Cornfield and Mantel (1950).

† Calculations are for Replicate #1, same session, Table XVI.

(except to convert them to logarithms) nor to have a table for Δ and w' ; these can be calculated directly through function subroutines.

Tables XIX summarizes the values of the Median Effective Doses (ED_{50}) (M), the slopes of the dose-response lines (L), and their Standard Errors (U), and (O), for all the replicate tests of Tables XVI and XVII. The coded ED_{50} 's can be reconverted to absolute dosages, for these sets of data by the formula:

$$ED_{50}(\text{Dosage}) = 15.62 (2^x)$$

where x is the coded ED_{50} . Applying this formula to the ED_{50} of Table XIX the absolute Median Effective Dose (ED_{50}) for Replicates 1-6 respectively, were found to be 20.62, 20.32, 19.77, 20.24, 20.38 and 19.91 $\mu\text{g/ml}$ of QAC. Similarly for the data of the lower portion of Table XIX the absolute Median Effective Doses are: 20.38, 18.40, 20.43, 18.85 and 16.23 $\mu\text{g/ml}$ of QAC for Replicates 1-5.

It is evident from Table XIX that the coded ED_{50} endpoints are quite reproducible when the determinations are made at the same session. The largest difference between the coded ED_{50} was 0.061 which amounts to a relative activity of 112 percent of Replicate 3 relative to Replicate 1.

Table XIX shows that the slopes for the same session data were quite homogeneous and are approximately 0.59 probits per two fold dilution. The coefficient of variation (cv) for each of the Replicates was determined from Table XIX as follows:

TABLE XIX
MEDIAN EFFECTIVE DOSES (CODED) SLOPES AND STANDARD ERROR

Same Session Replicate Test				
Replicate Number	Coded E.D. 50 (M)	Standard Error (U)	Slope (L)	Standard Error (O)
1	0.400	0.021	0.60	0.008
2	0.379	0.022	0.58	0.008
3	0.339	0.022	0.59	0.008
4	0.373	0.022	0.59	0.008
5	0.383	0.022	0.60	0.008
6	0.350	0.021	0.60	0.008
Different Days Replicate Test				
1	0.383	0.022	0.60	0.008
2	0.264	0.023	0.53	0.007
3	0.387	0.020	0.62	0.008
4	0.271	0.022	0.59	0.008
5	0.055	0.024	0.55	0.008

$$cv = \text{Standard Error} \times 10^2 / \text{Coded ED}_{50} =$$

$$0.021/0.400 = 5.25\%$$

for Replicate 1.

The cv of the other 5 Replicates, 2 through 6, were 5.80, 6.48, 5.89, 5.71 and 5.74% respectively, for the same session data. The closeness of these percentages together with the fact that the values are low, indicates the excellent reproducibility of the same session tests.

The lower portion of Table XIX brings out the effects of day to day error or, possibly, day to day bias. Both the slopes and the ED_{50} are affected. Judging through the ED_{50} , Replicate 5 is the most active. Replicates 2 and 4 would be judged as of equal activity, less active than Replicate 5 but more active than Replicates 1 and 3. Judging through the slopes, Replicates 2 and 5 are the flattest while the remaining three can be considered as of approximately equal gradients or, possibly, Replicate 3 has a slightly different slope from the others.

9. Determination of Minimum Inhibitory Concentrations

In Table XX are presented data on the bacteriostatic endpoint or Minimum Inhibition Concentration (MIC) (ppm) of four QAC disinfectant compounds determined according to the microtiter bacteriostatic procedure. Two fold dilutions of the product in TSB were accomplished with the standard microtiter in a microtiter plate. A 1.0×10^8 /ml suspension of the test organisms in TSB was added to each well, the plates

TABLE XX

MICROTITER BACTERIOSTATIC ENDPOINTS OR MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF SEVERAL QAC DISINFECTANTS

QAC	Test Organisms			
	<i>S. aureus</i>	<i>E. coli</i>	<i>Ps. aeruginosa</i>	<i>B. subtilis</i>
	MIC in ppm			
A*	0.25	15	64	1.00
B	0.94	62	200	0.94
C	0.12	15	63	0.25
D	0.20	16	31	0.28

*A Hyamine 1622 – 50% active ingredients – Diisobutyl phenoxy ethoxy ethyl dimethyl benzyl ammonium chloride; Rohm and Haas, Philadelphia, Pennsylvania.

B BTC 824 – 50% active ingredients – n-alkyl dimethyl benzyl ammonium chloride; Onyx Chemical Company.

C Hyamine 3500 – 50% active ingredients – alkyl dimethyl benzyl ammonium chloride; Rohm and Haas.

D Hyamine 2389 – 50% active ingredients – 80% methyldodecylbenzyl trimethyl ammonium chloride; and 20% trimethyl ammonium chloride; Rohm and Haas.

covered with plastic, small holes punched in each top for ventilation and the plates placed in a 37°C incubator for 24 hours. The plates were then placed on the reading mirror and the minimum inhibitor concentration (MIC) or the last clean well (no growth) determined. The tests indicate that organisms have a different MIC for each particular QAC compound but that generally, the sensitivities are in distinct ranges.

In Table XXI are presented the MIC (ppm) for four compounds often utilized in hospital antiseptic formulations. The compounds were cetyl trimethyl ammonium bromide (CTAB) and myristyl trimethyl ammonium bromide (MYTAB), trichlorocarbanilide (TCC) and Temasept I which is a mixture of tribromosalicylanide (TBS) and dibromosalicylanide (DBS).

The results indicated that without additional formulation the four compounds were quite effective inhibitors of the gram positive organisms but much less so against the gram negatives. In Tables XXII and XXIII a study of the effectiveness of combinations of the QAC formulations with the two other antimicrobials is presented.

In Table XXII the additive effect of the initial addition of 2 percent TCC on CTAB and MYTAB was studied. The MIC for the gram negative organisms: E. coli, Ps. aeruginosa and S. typhosa were particularly affected.

In Table XXIII are presented the MIC (ppm) for CTAB and MYTAB in the presence of the initial addition of 2 percent

TABLE XXI

MICROTITER BACTERIOSTATIC ENDPOINTS OR MINIMUM INHIBITORY CONCENTRATIONS (MIC) OF SEVERAL HOSPITAL ANTISEPTICS

Antiseptic	Test Organisms				
	<i>S.</i> <i>aureus</i>	<i>E.</i> <i>coli</i>	<i>Ps.</i> <i>aeruginosa</i>	<i>S.</i> <i>typhosa</i>	<i>B.</i> <i>subtilis</i>
	MIC in ppm				
Cetyl trimethyl ammonium bromide*	5	660	1770	270	5
Myristyl trimethyl ammonium bromide†	5	180	1380	190	5
Trichlorocarbinilide‡	4	540	1250	612	3
Tribromosalicylanide§ + Dibromosalicylanide	3	318	780	91	4

* Bromat – CTAB – cetyl trimethyl ammonium bromide; Fine Organics, Inc., 205 Main Street, Lodi, New Jersey.

† Mytab – MYTAB – myristyl trimethyl ammonium bromide; Fine Organics, Inc.

‡ – TCC – 3, 4, 4'-trichlorocarbanilide; Monsanto Chemical Company, St. Louis, Missouri.

§ Temasept I – TBS – 3, 4', 5-tribromosalicylanide and DBS – 4', 5-dibromosalicylanide; Fine Organics, Inc.

TABLE XXII

THE EFFECT OF 2% TRICHLOROCARBANILIDE* (TCC) ON THE
MINIMUM INHIBITORY CONCENTRATION (MIC) ON TWO QAC

QAC	Test Organisms			
	<i>S. aureus</i>	<i>E. coli</i>	<i>Ps. aeruginosa</i>	<i>S. typhosa</i>
	MIC in ppm			
Cetyl trimethyl ammonium bromide	0.1	8	40	7
Myristyl trimethyl ammonium bromide	0.1	8	36	3

* TCC – 3, 4, 4'-trichlorocarbaniide; Monsanto Chemical Company,
St. Louis, Missouri.

TABLE XXIII

THE EFFECT OF 2% TEMASEPT I* ON THE MINIMUM INHIBITORY
CONCENTRATION (MIC) ON TWO QAC

QAC	Test Organisms			
	<i>S. aureus</i>	<i>E. coli</i>	<i>Ps. aeruginosa</i>	<i>S. typhosa</i>
	MIC in ppm			
Cetyl trimethyl ammonium bromide	0.1	6	38	8
Myristyl trimethyl ammonium bromide	0.1	4	29	4

* Temasept I — TBS — 3, 4', 5-tribromosalicylanide and DBS — 4', 5-dibromosalicylanide; Fine Organics, Inc., 205 Main Street, Lodi, New Jersey.

Temasept I to the initial QAC formulation. The MIC for the gram negative organisms were even lower than with TCC. The findings indicate that with the microtiter bacteriostatic procedure it is possible to rapidly evaluate replicate samples or combinations of compounds. This may be significant for hospital applications where contamination of antiseptics, creams, lotions, etc. with gram negative organisms is a problem.

10. Reproducibility of Bacteriostatic Test

A comparison of the microtiter and standard tube bacteriostatic tests endpoints is presented in Table XXIV. A total of 106 individual microtiter tests and 31 tube dilution tests were conducted at 8 different dilutions of QAC. The individual percentages were calculated for each dilution, these in turn were added together to give the total percentage of tubes showing growth at each dilution. The cumulative percentages for both methods at seven of the concentrations of the QAC are presented in Figure 17. The curves are close together and demonstrate a close correlation between the two methods, provided, of course, that calibration of the microdiluters is first accomplished.

TABLE XXIV
CORRELATION OF MICROTITER AND TUBE DILUTION BACTERIOSTATIC TEST ENDPOINTS
E. coli at 1×10^8 /ml in TSB

Test	No. of Tests	Two Fold Dilutions of QAC* in Parts per Million (ppm)						
		480	240	120	60	30	15	7.5
Microtiter								
Endpoints †	106	0	0	0	16	59	31	0
Percent		0	0	0	15.1	55.7	29.2	0
Cumulative Percent		0	0	0	15.1	70.8	100.0	100.0
Tube Dilution								
Endpoints	31	0	0	0	9	15	5	2
Percent		0	0	0	29.0	48.8	16.1	6.5
Cumulative Percent		0	0	0	29.0	77.4	100.0	100.0

* BTC Onyx Chemical Company.

† Number of minimum inhibitory concentration (MIC) endpoints in ppm of QAC at each dilution.

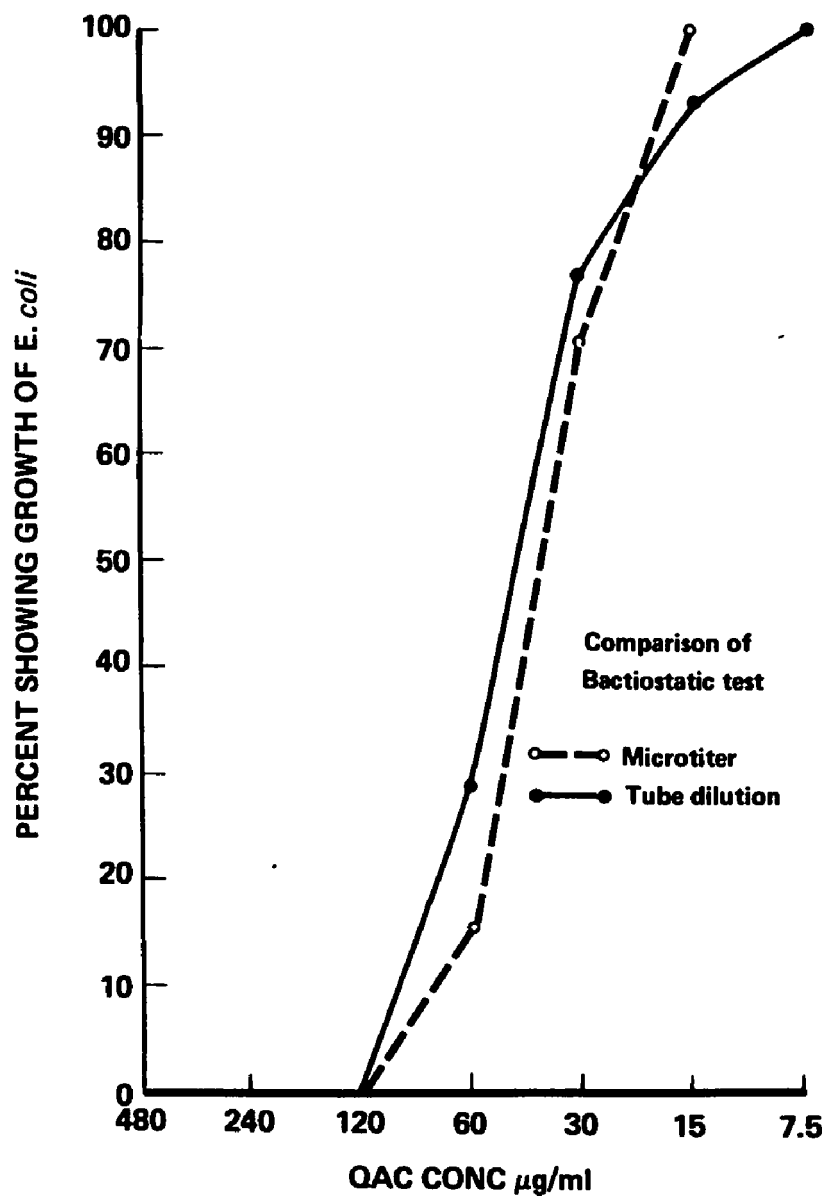


Figure 17. - Comparison of the microtiter and standard tube test minimum inhibitory endpoints (MIC).

D. DISCUSSION

It is especially important for hospital personnel to be able to estimate from laboratory tests the potential and practical value of a disinfectant. However, the ability to obtain reliable data and to present the information in a meaningful manner by presently available test procedures is so difficult that proper developments in the field of hospital disinfection have been seriously and adversely affected.

1. Bactericidal Tests

Two contributions to the solution of this problem are presented. One is the development of a microtiter test procedure which permits the evaluation of six tests simultaneously and the other is the application of a graphical procedure for presentation of disinfection data.

The microtiter bactericidal method incorporates the suspension type features of the British Standard for Laboratory Evaluation of Quaternary Ammonium Compounds (1960). In addition, it overcomes a number of difficulties associated with older procedures in that rapid dilution and improved chemical neutralization have resulted.

Kinetic data have been employed for the design of sterilization equipment for hospitals (Perkins, 1969) for canning processes in the food industry (Ball and Olsen, 1957) and in spacecraft sterilization (Bruch, 1966). The application of such principles to the determination of practical disinfection processes have been applied in this study.

It was found that microbial survival curves for QAC when plotted on conventional types of logarithmic paper were sigmoidal in shape. Conversion of the curves to a straight line could be accomplished by use of the transformation of Berkson (1953). This was accomplished with special graph paper*, whereby the results are represented by plotting the percentages of survival as integrated normal probability against the concentration. When the arithmetic concentration is used as the abscissa, curves of various shapes are obtained; but the curves are transformed to symmetrical sigmoids, when the logarithm of the concentration is used. This is in agreement with suggestions of Dodd (1970) who stated that handling the percent survivors in transform is absolutely essential in disinfection testing. Thus, transformation of the data by means of the Berkson function simplified the information and extended its scope.

The microtiter bactericidal procedure was employed to compare the efficiency of several QAC, to study the sensitivity of organisms to QAC, to evaluate QAC dilution and neutralization by various compounds and to evaluate the effects of environmental factors on QAC activity.

Because of the low numerical value for the slope of the QAC curve (Table XVIII) neutralization by dilution is impractical and chemical neutralization is required.

* Special graph paper No. 32.451, Codex Book Company, Inc. Norwood, Massachusetts. Paper designed by J. Berkson, Mayo Clinic, Rochester, Minnesota.

The findings of Figures 12 and 13 indicated that polysorbate 80 and lecithin were effective QAC neutralizers. Kakemi et al., (1965) suggested that the polysorbate 80 consists of large spherical micelles of many monomers. They suggested that the molecules may be oriented with the hydrocarbon chains inward forming semi-liquid cores with the hydrophylic polyoxyethylene chains facing toward the water. A possible model of polysorbate 80 micelles in water is presented in Figure 18. It is suggested that the alkyl group of the QAC may become embedded in the central region of the micelle with the polar portion of the QAC extending into the palisade region.

A postulated model for polysorbate 80 and lecithin acting together for QAC neutralization is presented in Figure 19. The apolar group of lecithin may be embedded in the central region with the polar anionic portion available for electrical interaction with the QAC. Neutralization or inactivation of a QAC by such a system would occur by two modes - solubilization and/or electrostasis. The inactivation of the QAC in the polysorbate 80 - lecithin system was effective at lower concentrations of the QAC. However, it experienced difficulties at higher QAC concentrations.

The mode of neutralization of the quaternary ammonium compounds by heparin may be due to a high charge in density or to dissociable acidic groups on the polyelectrolyte.

Scott (1962) reported that heparin was a nonhomogeneous mixture of giant molecules, with an average molecular weight

**SUGGESTED MODEL FOR INACTIVATION OF QUATERNARY
COMPOUND BY POLYOXYETHYLENE SORBITAN MONOOLEATE
(POLYSORBATE 80)**

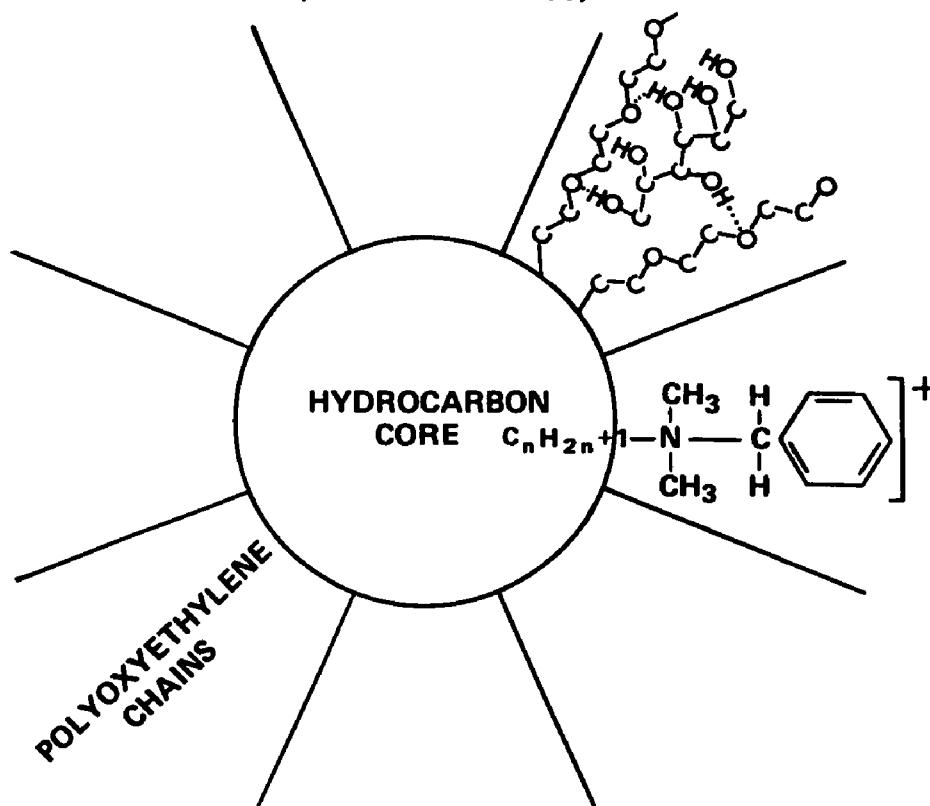


Figure 18. - A postulated polysorbate 80 configuration which may account for for QAC neutralization. Spherical micelle of polysorbate 80 with nonpolar alkyl group of QAC embedded in the central region and the polar portion of molecule extending into palisade region.

**SUGGESTED MODEL FOR INACTIVATION OF QUATERNARY AMMONIUM
COMPOUND BY POLYOXYETHYLENE SORBITAN MONOOLEATE
(POLYSORBATE 80) AND LECITHIN**

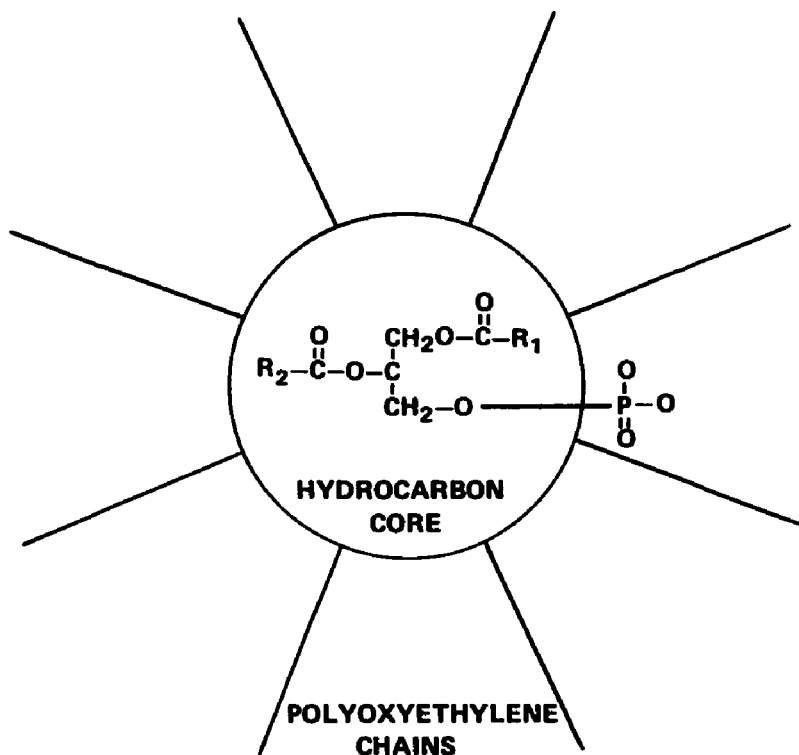


Figure 19. — A postulated polysorbate 80 - lecithin configuration which may account for QAC neutralization. Lecithin is solubilized in spherical micelle of polysorbate 80 with the nonpolar group of lecithin embedded in the central region and the polar anionic portion of molecule available for electrical interaction with the cationic QAC.

of 16,000 to 20,000 and that it contained approximately 80-100 negatively charged sulfate and sulfonic groups per molecule. Owing to the spatial relationship of the various parts of the large heparin molecule, probably 50 percent or less of the sulfate and sulfonic groups are in a favorable position to be bound to the QAC molecules.

A complex may result from the attraction of the strong negative charges of the sulfonic and sulfate groups of the heparin to the strong positive charge of the pentavalent nitrogen of the QAC. This can be demonstrated grossly by mixing solutions of heparin and QAC, and noting the heavy white precipitate that forms. Also the insoluble complex can be demonstrated by the addition of toluidine blue and ferric oxide suspended in water. In both cases there is a distinct color change which is easily demonstrated. Heparin is freely soluble in water and micelles are not produced.

There was a possibility that the quaternary ammonium heparin complex may itself be bactericidal. There was also the possibility that excess and unaltered QAC might be entrapped temporarily in the precipitate or attached loosely to weakly negative carboxy radicals. However, rapid dilution and good mixing have avoided this possibility.

Heparin may also have had a bacteriostatic effect against certain bacteria. However, this was observed only when the exposure was for an extended period of time (over 24 hours).

Toxic effects to the organisms were not demonstrated during the short exposure time of the organisms to the agent

at the 1000 µg/ml heparin level. The total counts for the organisms in the presence of heparin were not significantly lower than the controls without the agent, or in the presence of lecithin-polysorbate 80.

Liquid solutions of sodium heparin often contain 1000 µg/ml of a phenolic as a preservative. This could have drastic adverse effects on any disinfection test and the acquisition of the material as a dry powder, without microbial inhibitors, is a necessity for bactericidal tests.

The effectiveness of the compound for other types of QAC is not known but structural features might influence the ability to form complexes. The data have proven the compound effective for the alkyldimethylbenzylammonium type disinfectant which is the most widely used QAC in hospital disinfection practice. The molecular weight, or degree of branching of either the QAC or heparin, could prove important for other molecular configurations. Those personnel contemplating use of heparin as a disinfectant neutralizer should assure themselves that the product is of a high degree of purity.

The evaluation of the effectiveness of QAC against viruses has proven difficult. Koski (1968) reported that the cell systems employed to culture viruses after QAC exposure are more sensitive to the compounds than the viruses themselves, and residual amounts of the QAC lead to difficulties in the enumeration of survivors.

The inactivation of viruses that contaminate environ-

mental surfaces of the hospital, presents problems which have been considered only recently. Although the T2 bacteriophage is not relevant to the hospital, the experiment demonstrated that at the concentration presently used, some germicides were rather ineffective for T2 bacteriophage inactivation. Such findings have also been shown in other environmental applications by Lothrop and Sproul (1969). The testing of the action of QAC against bacteriophages by the microtiter procedure has overcome a number of the above problems. It is possible that certain bacteriophages might serve as representative indicators and they might be employed as screening agents to determine relative effectiveness of particular formulations intended for use in hospital applications.

Preliminary cleaning of a hospital floor may not completely eliminate soil or other residue which might be present from waxes, strippers or sealers.

Tolerances of QAC to hard water, protein, temperature, and pH take on special significance in that an appropriate use dilution must provide a margin of safety by insuring disinfection under the practical range of environmental conditions.

As reported by Armbruster and Ridenour (1949) and quantitated by the microtiter procedure, water hardness had a decided effect on QAC activity. The reduction in the activity of quaternary ammonium compounds was due to the presence of Ca^{++} and Mg^{++} ions in solution. These ions may react with anionic sites present on the surface of the bacterial cell

thus reducing the possibility of quaternary compounds interacting with the cell surface.

The microtiter procedure makes it possible for personnel evaluating a QAC product for a particular hospital application to take this factor into consideration in determining a use dilution.

The impact of organic load on QAC activity was reported by Ridenour and Armbruster (1947). However, it was Malizia (1960) who pointed out the danger in hospital applications. The microtiter procedure makes it possible to rapidly determine the effect of a particular QAC at several levels of organic load. In this particular study the effect of 0.5% and 5.0% was studied. The 5.0% level is much higher than would be found in most hospital environmental applications but might be encountered in containers of excreta, laboratory wastes, blood, protein, or animal products. As demonstrated by Figure 18, under conditions of 5.0 percent organic load the concentration of the QAC required for acceptable microbial reductions would be unacceptable for environmental surfaces because the concentrations required would present a toxicity hazard to the user. However, for sanitization of containers, these levels might be acceptable.

Disinfection testing at the 5.0 percent level proves difficult. The dynamics of disinfection are considerably different and extrapolation of data to other situations is not advisable.

The use of 0.5 percent organic load for studying effects on QAC activity was most satisfactory and is in agreement with the report of Mitchell (1963) who recommended this level for grading the activity of QAC compounds in applied situations. An important point evident from these studies is that it is necessary for a surface to be relatively free of organic load and gross contamination if maximum disinfection is desired.

There are two important criticisms for presenting the microtiter bactericidal data as outlined. They are the loss of information due to the conversion of actual count of viable organisms to a percentage response and the assumption that the zero dose is an accurate count of the initial inoculum on which to base the percentage kill.

The main reason for using the percentage response rather than fitting a linear relationship between the dose and actual count of the organisms is that there is neither a theoretical or actual reason not to expect a significant departure from linearity. To use a second or higher degree curve could only give rise to other, perhaps severer criticisms than the above. The log-dose transformation can also guarantee a rectification of the response ogive particularly when the dose regimen is confined to the linear part of the ogive. In addition, the calculation of the parameters of the dose-response relationship in the manner presented endows the microtiter bactericidal test with a theoretical advantage in that a useful interpretation of the transformation can be made. This

is sufficient to lessen the first criticism.

The assumption that the number of organisms in the initial inoculum are the same for all doses applies equally whether this transformation is used or a dose-response relationship is fitted directly to the counts of the viable organisms. Thus, the second main criticism applies equally to other methods of data presentation and to other test procedures.

The results from Tables XVI and XIX give sufficient evidence to indicate that with the appropriate techniques it is possible to obtain replicable accuracy especially at the same session. It is possible to reduce day to day variation by either replicating the zero dose inoculum a number of times and using the mean count as the base for computing percentages or through the use of a standard. The latter method can be used both to obtain an estimate of activity of a preparation relative to a standard or to judge the relative activity of two preparations through a standard. This latter method implies that random processes are operating on the test and standard alike but the potency remains constant. Thus, for example, the ED_{50} of replicate on day 5 (Table XVII and Table XIX) is estimated as significantly lower than the other replicates, but had there been a test performed on a standard at the same time, it is possible that the ED_{50} would have been low, too, and the relative potency would not differ significantly from that estimated on other days.

The criticisms are inherent to other tests and as yet cannot be overcome. In spite of these criticisms this method is more valid in terms of reproducibility and elimination of bias than any method currently used.

2. Bacteriostatic Tests

The microtiter bacteriostatic procedure makes it possible to evaluate QAC activity in which a suspension of viable microorganisms is added to various concentrations of disinfectant, and the lowest concentration that inhibited growth is reported as the minimum inhibitory concentration (MIC).

The influence of vehicles, surfactants and other agents on QAC activity was demonstrated and should be utilized to the fullest in obtaining a product with the greatest bacteriostatic effectiveness. For example, in Table XXII it is demonstrated that the cetyltrimethylammonium bromide (CTAB) required to inhibit Pseudomonas aeruginosa dropped from 1770 ppm to 40 ppm in the presence of added TCC. For E. coli, the inhibition dropped from 660 ppm to 8 ppm, and for S. aureus from 5 ppm to 0.1 ppm. The advantages of formulation studies are considerable and point out opportunities for development of specific products for specific hospital situations.

The statistical evaluation of the microtiter bacteriostatic test with the conventional tube dilution test indicated that there is close correlation between the two methods provided, of course, that calibration of the microdiluters is first accomplished.

The chromium 51 calibration method is recommended as a method for calibration of the microdiluters.

The choice of organisms depends on the intended compound application and is limited only by growth requirements and availability.

Two-fold dilutions with 0.025 ml dilution loops were found to be the more accurate, as the 0.05 ml dilution loops gave erratic results due to the increased surface activity of many of the tested compounds.

A large number of compounds can be quickly evaluated by microtiter bacteriostatic procedures. Not only is information derived as to the minimum inhibitory concentration, but knowledge with regard to the biochemical mode of action can be obtained. These assets of the bacteriostatic test will be presented in greater detail in a later chapter.

The microtiter methods have proved highly versatile and adaptable to bactericidal and bacteriostatic disinfectant testing. They are rapid and economical tests which improve the productivity of a testing program. The microdilution loops can be easily sterilized with alcohol and flame as opposed to cleaning and sterilization of many glass pipettes with the standard tube system. One disposable microtiter plate is used instead of many dilution bottles and test tubes. Numerous tests can be accomplished in a short period and the test occupies only a small area of laboratory space. Because of the manipulations involved with tube testing, only one set

of experimental factors usually can be evaluated at any one test run. The microtiter procedure allows for several sets of experimental parameters being evaluated simultaneously or tested in replicate. The information obtained provides a basis for a more dependable index of product effectiveness.

The microtiter tests could have wide application in formulation research and it is hoped will contribute to higher standards of hospital disinfection practice.

CHAPTER II

APPLICATIONS IN BASIC BIOLOGY - MODE OF ACTION

A. INTRODUCTION

With the large variety of toxic chemicals being released into the environment, it is particularly advisable to understand the mode of action of various disinfectants. It is with these thoughts in mind, that an assessment of the mode of action of the quaternary ammonium compounds is undertaken.

The mode of action of QAC may fall into one or more of the following categories: (1) alteration or destruction of lipoprotein membranes, (2) inhibition of nucleic acid synthesis, including both DNA and RNA, (3) inhibition of protein synthesis, and (4) inhibition of energy metabolism or energy coupling.

Chromium 51 has been utilized successfully for labeling erythrocytes, platelets, leukocytes, and lymphocytes (Kleine and Hiemkl, 1965; Scarfe and Brahee, 1967; and Boccaccio, 1969). Its application for labeling bacteria and studying QAC effects on bacterial cells will be evaluated.

The in vitro interaction of QAC with nucleic acids was demonstrated by Scott (1962), who showed that quaternary ammonium compounds form complexes with polyanions. Zugible and Fin (1966) demonstrated the formation of a red-colored ion-association-complex when quaternary ammonium-polyanion

(DNA, RNA) complexes were treated with ferric thiocyanate. Young (1968) utilized the QAC interaction for extraction of ribosomal nucleic acids.

An investigation was undertaken to determine the possible effects of quaternary ammonium compounds on various metabolic functions of bacteria. The study involved the effects of QAC on the incorporation of metabolic precursors into microbial systems. From the results obtained, it was hoped that an elaboration of the possible effects of QAC on DNA, RNA and protein synthesis could be formulated.

B. MATERIALS AND METHODS

1. Chromium 51 Studies

The isotope, in the form of $\text{Na}_2^{51}\text{CrO}_4^*$, was made up in sterile saline solution. The radiochemical purity of the isotope was greater than 99 percent. Chromium 51 has a half-life of 27.8 days and is a gamma emitter of 0.325 meV.

- a. Bacterial cultures were grown for 24 hours on agar plates previously discussed. They were transferred to TSB and a suspension of $1.0 \times 10^{6-8}$ /ml prepared in 10.0 ml.
- b. Suspension was centrifuged at 3400 rpm for 15 minutes. The supernatant was drawn off and the pellet suspended in 1.0 ml TSB in a standard test tube.

* Amersham Searle, The Radiochemical Centre, Amersham, Buckinghamshire, England.

- c. A 0.1 ml aliquot (100 μ Ci/ml) of chromium 51 in saline was added to the suspension by a special pipette.
- d. The suspension was incubated at 37°C for 15 minutes.
- e. Suspension was centrifuged for 10 minutes at 3400 rpm, washed twice in 2.0 ml of TSB and finally resuspended in saline.
- f. 1.8 ml aliquots of the disinfectant were prepared to have final concentrations of 0, 25, 50, 100, 200, 400, 800, 1600, and 3200 μ g/ml of QAC for the pseudomonas evaluation, and 0, 5, 10, 20, 40, 80, 160 and 320 μ g/ml for the S. aureus study.
- g. A 0.2 ml aliquot of chromium 51 organisms in TSB suspension was added.
- h. After 5 minutes of exposure of the organisms to the disinfectant at 24°C, 8.0 ml of AOAC letheen broth prepared as previously described was added as a QAC neutralizer.
- i. The suspension was rapidly passed through a 0.22 μ millipore filter and washed with saline.
- j. Filter papers were removed from the millipore vacuum apparatus* and placed in standard test tube for gamma counting.
- k. All measurements for radioactivity were made with a well type crystal scintillator detector**.

* Millipore Filter Co., New Bedford, Massachusetts

** Autowell II Picker Nuclear Co., White Plains, New York

1. The microtiter bactericidal test was in accordance with procedures given in Chapter I.

2. Radioactive Tracer Incorporation Studies

The liquid scintillation counting fluid was prepared by adding 42 ml of Liquiflow* to a liter of toluene. Each liter of solution contained 100.00 grams of PPO (2, 5-diphenyl-oxazole) and 1.25 grams of POPOP (p-bis[2-(5-phenyloxazolyl)]-benzene). Fifteen ml of this counting fluid were added to each vial. The POPOP accepts the energy emitted by PPO and re-emits it at a wave length more closely matched to the peak sensitivity of the photomultiplier tube.

- a. To determine the effect of QAC on synthesis of DNA, RNA and protein in microorganisms, a 12 hour culture of 1.0×10^8 /ml of Escherichia coli was suspended in 10.0 ml of fresh TSB medium.
- b. Amounts of sterile aqueous solutions of thymidine-methyl- ^3H , uracil- ^3H , or phenylalanine- ^1C were added to individual tests so as to result in a final concentration of 2 $\mu\text{Ci/ml}$.
- c. 20 $\mu\text{g/ml}$ of QAC** was used in all these tests. The material and the cells were incubated for various periods of time.

* New England Nuclear Corp., Albany Street, Boston, Massachusetts

** BTC 824, Onyx Chemical Corp., 190 Warren Street, Jersey City, New Jersey

- d. Cells were incubated in a water bath at 37°C.
Every 10 minutes the tubes were shaken for 30 seconds to resuspend the culture and allow for proper mixing.
- e. A 0.1 ml aliquot of the suspension was drawn off by use of a calibrated non-electric pipette at selected intervals and placed on the millipore filter (HA 0.45 μ)*. Filters were wet with 0.01% of the appropriate non-radioactive precursors.
- f. The filter was washed with 50.0 ml of sterile saline solution followed by 10.0 ml 50% trichloroacetic acid reagent (TCA) and ethyl alcohol.
- g. Filter papers were removed from the millipore vacuum apparatus and placed in 15.0 ml of the above liquid scintillation solution in the special vials for radioactive counting.
- h. Each sample was counted for 20 minutes on a Beckman CS-250 liquid scintillation spectrometer**.
- i. The microtiter bacteriostatic test was in accordance with procedures given in Chapter I.

C. RESULTS

1. Chromium 51 Labeling of Bacteria

Table XXV shows the incorporation of chromium 51 into

* Millipore Filter Corp., New Bedford, Massachusetts
** Beckman Instrument Company, Los Angeles, California

TABLE XXV

UPTAKE OF CHROMIUM 51 BY BACTERIA *PSEUDOMONAS AERUGINOSA*
PRD 10 AT 24°C

Time (min)	CPM ⁵¹ Cr/ml*
0	0
5	1515
10	3007
15	3134
30	3992
45	4552
50	5027
60	5333
70	5149

* Data expressed as counts per minute of chromium 51/ml.

the bacteria. In this study a 24 hour culture of Pseudomonas aeruginosa PRD #10 (1.0×10^8 /ml) was tagged according to the previously developed procedure. Aliquots were removed at 5, 10, 15, 30, 60 and 75 minute intervals. Samples of 0.1 ml were placed on 0.22 μ millipore filter and washed with 200 ml sterile saline to remove free chromium 51 from solution. Organisms remaining on the filter were counted for radioactivity. A 60.8% uptake of chromium 51 occurred within 15 minutes of the time of exposure to the radioisotope.

2. Chromium 51 Release

In Table XXVI a study of the percent chromium 51 remaining with Pseudomonas aeruginosa after exposure to various concentrations of QAC is shown along with the survival of the same organisms as determined by the bactericidal microtiter procedure. The two tests were conducted simultaneously. After five minutes of exposure the suspension was neutralized with broth. The resulting mixture was passed through a 0.22 μ millipore filter and washed. The filter paper was removed and counted in a well type scintillation counter for 20 minutes and the results were reported in counts per minute of chromium 51/ml. The percent chromium 51 remaining on the filter paper was determined by dividing the counts per minute per ml at the tested concentration by the counts per minute per ml for the control. The microtiter survival data were obtained in accordance with the bactericidal test presented in Chapter I.

TABLE XXVI

**EVALUATION OF CHROMIUM 51 REMAINING WITH
PSEUDOMONAS AERUGINOSA AND MICROBIAL
 SURVIVAL AFTER QAC* EXPOSURE†**

Chromium 51 Test		
QAC μg/ml	CPM ⁵¹ Cr/ml† Remaining with Cells	Percent ⁵¹ Cr Remaining with Cells
0	3313	100.0
25	3230	97.5
50	3120	94.2
100	2945	88.3
200	2630	79.8
400	795	24.0
800	656	19.8
1600	645	19.8
3200	629	18.9
Microtiter Survival Test		
QAC μg/ml	Survivors/ml	Percent Survivors
0	1.80 x 10 ⁶	100.0
25	1.52 x 10 ⁶	84.4
50	1.06 x 10 ⁶	58.9
100	5.75 x 10 ⁵	31.9
200	1.67 x 10 ⁵	9.3
400	2.34 x 10 ⁵	1.3

* BTC 824 Onyx Chemical Company.

† 5 Minutes at 24°C.

† Data expressed as counts per minute of chromium 51/ml.

In Table XXVII are presented the results of a study similar to the previous one when S. aureus was utilized as the test organism. A 1.3×10^6 /ml suspension of S. aureus organisms was utilized in both the chromium 51 and microtiter test procedures.

3. Radioactive Tracer Incorporation Studies

In Tables XXVIII, XXIX and XXX are reported the effects of QAC on the incorporation of the radioactive precursors, thymidine-methyl- ^3H , uracil- ^3H and phenylalanine- ^{14}C into DNA, RNA and protein of E. coli. A subinhibitory concentration of QAC, n-alkyldimethylbenzylammonium chloride, 20 $\mu\text{g/ml}$, was selected as the concentration for all three studies. This concentration was selected after determination of the minimum inhibitory concentration (MIC) of the strain of E. coli by the microtiter bacteriostatic test as outlined in Chapter I. The MIC for the E. coli was found to be 31 $\mu\text{g/ml}$ QAC, therefore, a concentration below this figure or 20 $\mu\text{g/ml}$ was selected.

Each of the above radioactive precursors was added to the 10.0 ml TSB and E. coli suspension to give a final concentration of 2 $\mu\text{Ci/ml}$. The QAC was added and 0.1 ml aliquots of test suspension were removed at the selected intervals and placed on a 0.45 μ filter paper in the filter apparatus and washed with 50.0 ml of sterile saline followed by 10.0 ml of 50% trichloroacetic acid reagent (TCA) and ethyl alcohol. The TCA was utilized to precipitate the protein, nucleic acid and lipids. The radioactive unincorporated precursors and

TABLE XXVII

**EVALUATION OF CHROMIUM 51 REMAINING WITH *S. AUREUS* AND
MICROBIAL SURVIVAL AFTER QAC* EXPOSURE†**

Chromium 51 Test		
QAC μg/ml	CPM ⁵¹ Cr/ml‡ Remaining with Cells	Percent ⁵¹ Cr Remaining with Cells
0	4630	100.0
5	3890	84.0
10	3020	65.3
20	1793	38.7
40	709	15.3
80	459	9.9
160	420	9.1
320	495	10.7
Microtiter Survival Test		
QAC μg/ml	Survivors/ml	Percent Survivors
0	1.30 x 10 ⁶	100.0
5	7.58 x 10 ⁵	58.3
10	4.94 x 10 ⁵	32.0
20	1.74 x 10 ⁵	13.4
40	2.21 x 10 ⁴	1.7

* BTC 824 Onyx Chemical Company.

† 5 Minutes at 24°C.

‡ Data expressed as counts per minute of chromium 51/ml.

small molecules were washed through the filter. The filters were removed and placed in the liquid scintillation vials, the scintillation fluid added and the vials counted in accordance with the listed procedure.

In Table XXVIII the data for the effect of QAC on the incorporation of thymidine-methyl- ^3H into deoxyribonucleic acid (DNA) of E. coli are presented. The effect was quite rapid with 89.7% inhibition occurring within 15 minutes. The percentage changed very little rising only to 94.3% in the following 60 minute period.

In Table XXIX the data on the effect of QAC on uracil- ^3H incorporation into ribonucleic acid (RNA) of E. coli was evaluated. The effect was initially rapid, 42.7% inhibition in 7.5 minutes, however, this rose only slightly to 51.3% after 90 minutes. The effect on uracil- ^3H incorporation into RNA was much less than on thymidine-methyl- ^3H incorporation.

In Table XXX the effect of 20 $\mu\text{g/ml}$ QAC on phenylalanine- ^{14}C incorporation into protein is reported. Initially the rate of incorporation was slowed to 51.3% of normal after 7.5 minutes of exposure. This increased during the next 30 minutes and reached a plateau in approximately 45 minutes.

D. DISCUSSION

1. Chromium 51 Uptake and Release Studies

In Figure 20 the uptake of chromium 51 by Pseudomonas aeruginosa over a given period of time is demonstrated. The

TABLE XXVIII

THE EFFECT OF QAC* ON THE INCORPORATION OF THYMIDINE-
METHYL-3H[†] INTO DNA OF *E. coli* AT 37°C

Time (min)	Control DPM/ml [†] Mean $\pm$ S.E.	Exposed DPM/ml [†] Mean $\pm$ S.E.	Percent Inhibition
15	2736 $\pm$ 301	284 $\pm$ 39	89.7
30	9505 $\pm$ 800	359 $\pm$ 62	96.2
45	11063 $\pm$ 1008	638 $\pm$ 98	94.2
60	12842 $\pm$ 1131	643 $\pm$ 81	95.0
75	12850 $\pm$ 1312	664 $\pm$ 70	94.8

* BTC 824 at 20 μ g/ml, Onyx Chemical Company

[†] Thymidine-Methyl-³H was precursor for DNA.

[†] Data expressed as disintegrations per minute DPM/ml. Each number represents the mean and the standard error of the mean of 4 determinations.

TABLE XXIX

THE EFFECT OF QAC* ON THE INCORPORATION OF URACIL - ^3H †
 INTO RNA OF *E. COLI* AT 37°C

Time (min)	Control DPM/ml† Mean $\pm$ S.E.	Exposed DPM/ml† Mean $\pm$ S.E.	Percent Inhibition
7.5	2,150 $\pm$ 250	1231 $\pm$ 154	42.8
15.0	3,200 $\pm$ 340	1600 $\pm$ 204	49.8
30.0	4,125 $\pm$ 590	2115 $\pm$ 231	48.7
45.0	4,200 $\pm$ 440	2006 $\pm$ 321	52.2
60.0	4,227 $\pm$ 540	2081 $\pm$ 281	50.8
75.0	4,231 $\pm$ 561	2030 $\pm$ 234	52.0
90.0	4,291 $\pm$ 521	2091 $\pm$ 267	51.3

* BTC 824 Onyx Chemical Company

† Uracil - ^3H was the precursor for RNA.

‡ Data expressed as disintegrations per minute/ml. Each number represents the mean and the standard error of the mean of 4 determinations.

TABLE XXX

THE EFFECT OF QAC* ON THE INCORPORATION OF PHENYLALANINE —
¹⁴C† INTO PROTEIN OF *E. COLI* AT 37°C

Time (min)	Control DPM/ml† Mean ± S.E.	Exposed DPM/ml† Mean ± S.E.	Percent Inhibition
7.5	82 ± 20	40 ± 10	51.2
15	260 ± 42	97 ± 13	62.7
30	1180 ± 180	306 ± 40	74.1
45	2730 ± 320	434 ± 44	84.1
60	4715 ± 790	540 ± 81	88.6
75	6210 ± 594	589 ± 179	90.5
90	7821 ± 800	702 ± 126	91.0

* BTC 824 Onyx Chemical Company.

† Phenylalanine — ¹⁴C was the precursor for protein.

‡ Data expressed as disintegrations per minute/ml. Each number represents the mean and standard error of the mean of 4 determinations.

TABLE XXXI
DEOXYRIBONUCLEIC ACID BASE RATIOS AND MICROTITER
BACTERIOSTATIC ENDPOINTS (MIC) ($\mu\text{g/ml}$)
FOR SEVERAL MICROORGANISMS

Organisms	G.C. Ratio*	MIC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i>	31	0.5
<i>Proteus vulgaris</i>	37	1.7
<i>Escherichia coli</i>	50	31.0
<i>Salmonella typhosa</i>	50	122.0
<i>Pseudomonas aeruginosa</i>	64	200.0

* GC Ratio were furnished by Herbert Manning, Department of Microbiology,
University of Maryland, College Park, Maryland.

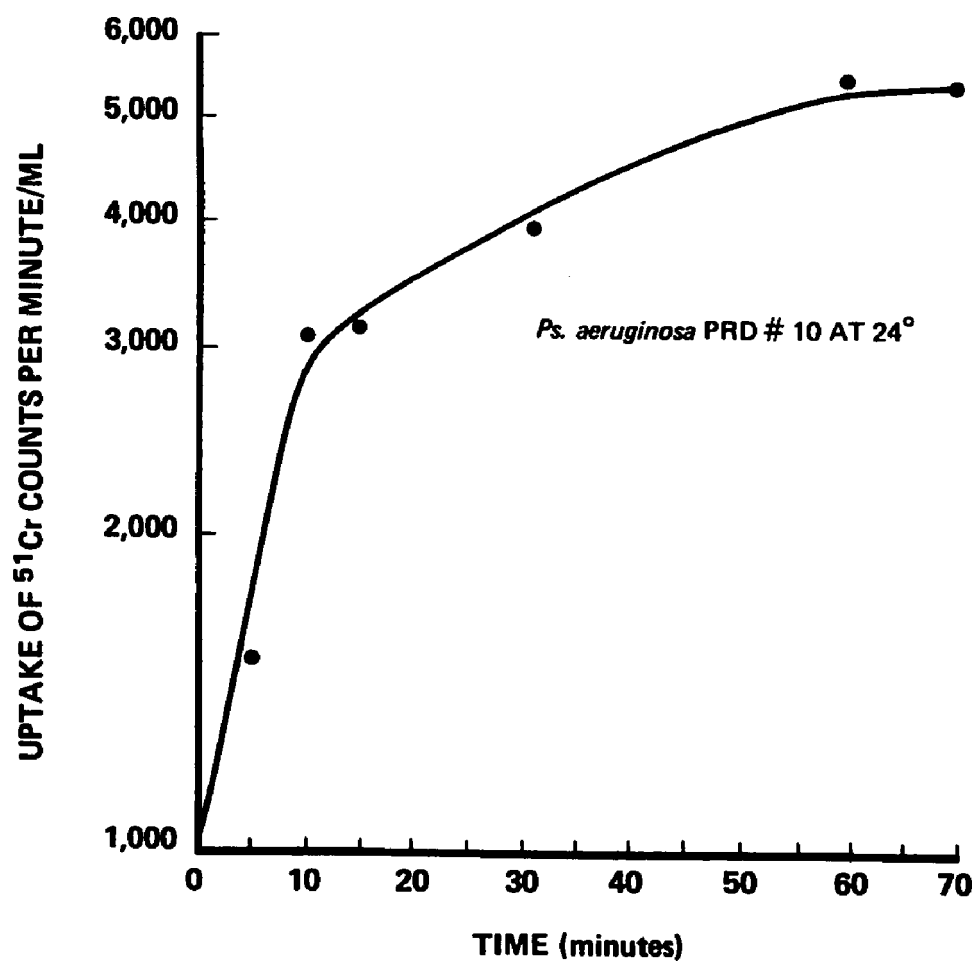


Figure 20. - The incorporation of chromium 51 into *Pseudomonas aeruginosa*. Presented are the logarithm of counts per minute/ml of chromium 51 incorporated and the time in minutes.

figure indicates an exponential rate of incorporation in first 10 minutes followed by a plateau after 15 minutes. The data indicate that organisms can be tagged with chromium 51. The data suggests that a possible cellular binding site had either become saturated with radiochromate or had reached an equilibrium after this period. Scarfe and Brahee (1967) in studies with other cellular systems indicated that hexavalent chromium is oxidized to the trivalent form after incorporation into cellular systems. The trivalent form is less soluble than the hexavalent form and is not readily re-utilizable.

This is a decided advantage for chromium 51 over phosphorus 32 or other isotopes which might re-enter cells and even metabolic pathways after release. As shown in Figure 21, the effective concentrations at which events took place are related to biophysical effects which can be observed when the microtiter bactericidal test data are correlated with the chromium 51 test data.

Figure 21 indicates that the release of chromium 51 from S. aureus is correlated with the microtiter survival data. The data indicate that cells were lysed at all concentrations of the compound. S. aureus is highly sensitive to QAC action and its MIC is very low (less than 0.5 µg/ml QAC - see Chapter I). The QAC concentration resulting in maximum release can be determined from the point at which the curve begins to level off. Beyond this point there is no additional chromium 51 released.

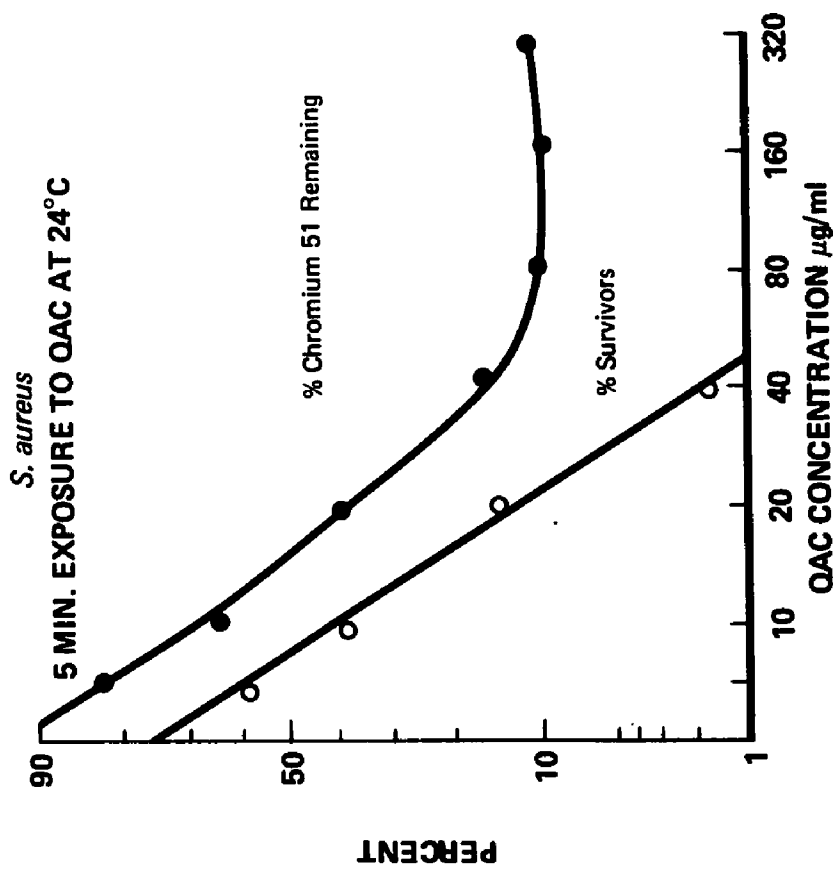


Figure 21. - Correlation of microtiter bactericidal survival with chromium 51 remaining with *S. aureus* organisms after exposure to quaternary ammonium compound.

Figure 22 indicates that the release of chromium 51 from Ps. aeruginosa is also correlated with microtiter survival data. Comparing Figures 21 and 22, it can be seen that lesser amounts of chromium 51 were released in this evaluation than was the case with the S. aureus test. There was also a leveling off in the release of chromium 51 at high QAC concentrations.

The resistance of pseudomonas organisms to QAC action may be due to the inability of the QAC to readily lyse the pseudomonas cell. This may be related to the structure of the cell wall or membrane.

Ivanov, et al. (1964) reported that the pseudomonas organisms have a greater quantity of phospholipids than S. aureus. The resistance of the pseudomonas organisms to the QAC action may be related to the amount of phospholipids in the cell membrane. The phospholipids may solubilize the QAC in a manner similar to that previously discussed for QAC neutralization in solution. Beyond a certain saturation point events occur which result in lysis of the cells. Higher concentrations of QAC would be required for a cytolytic response in bacteria with greater quantities of phospholipids. These findings are also in agreement with the work of Davis (1968) who reported bacterial lysis by QAC as observed by electron microscopy.

Another hypothesis for the resistance of pseudomonas organisms observed, however, at bacteriostatic concentrations of QAC will be reported later in this chapter.

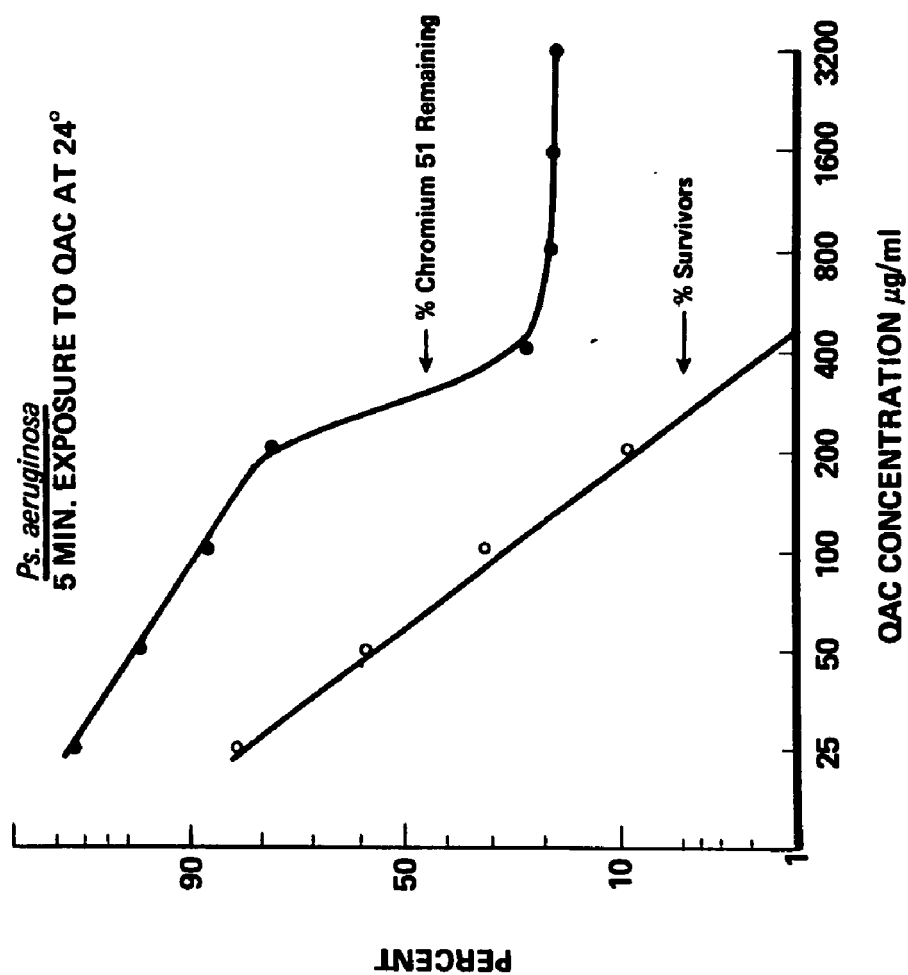


Figure 22. - Correlation of microtiter bactericidal survival with chromium 51 remaining with *Ps. aeruginosa* after exposure to quaternary ammonium compound.

Isotope labeling and the measurement of cytolysis through the release of the chromium 51 provides a simple, objective method for the study of the effect of particular QAC on bacteria. The tests do not have to be counted immediately but can be left for a more suitable occasion. Parallel tests can be run at the same time, the microtiter tests being completed first so as to prevent errors from extended delays in streaking plates.

2. Metabolic Inhibition Studies

In Figures 23, 24 and 25 are presented the curves for the time course of incorporation of the radioactive precursors thymidine-methyl- ^3H into DNA, uracil- ^3H into RNA and phenylalanine- ^{14}C into protein.

In Figure 23 the first part of the curve demonstrates increased inhibition with time, but thereafter a second mechanism may have come into play which accounted for the very slight decrease. Hugo (1970) has reported such an effect with other drugs and explains it as being due to progressive coagulation of the cytoplasmic membrane rendering it slightly impermeable to the incorporation of the metabolic precursor with time. This slight decrease in inhibition was only noted in the thymidine-methyl- ^3H incorporation study.

Figure 26 summarizes the inhibition rates of DNA, RNA and protein. The incorporation of thymidine-methyl- ^3H into DNA was most inhibited by the QAC. The extent of inhibition of phenylalanine- ^{14}C incorporation into protein was slow in

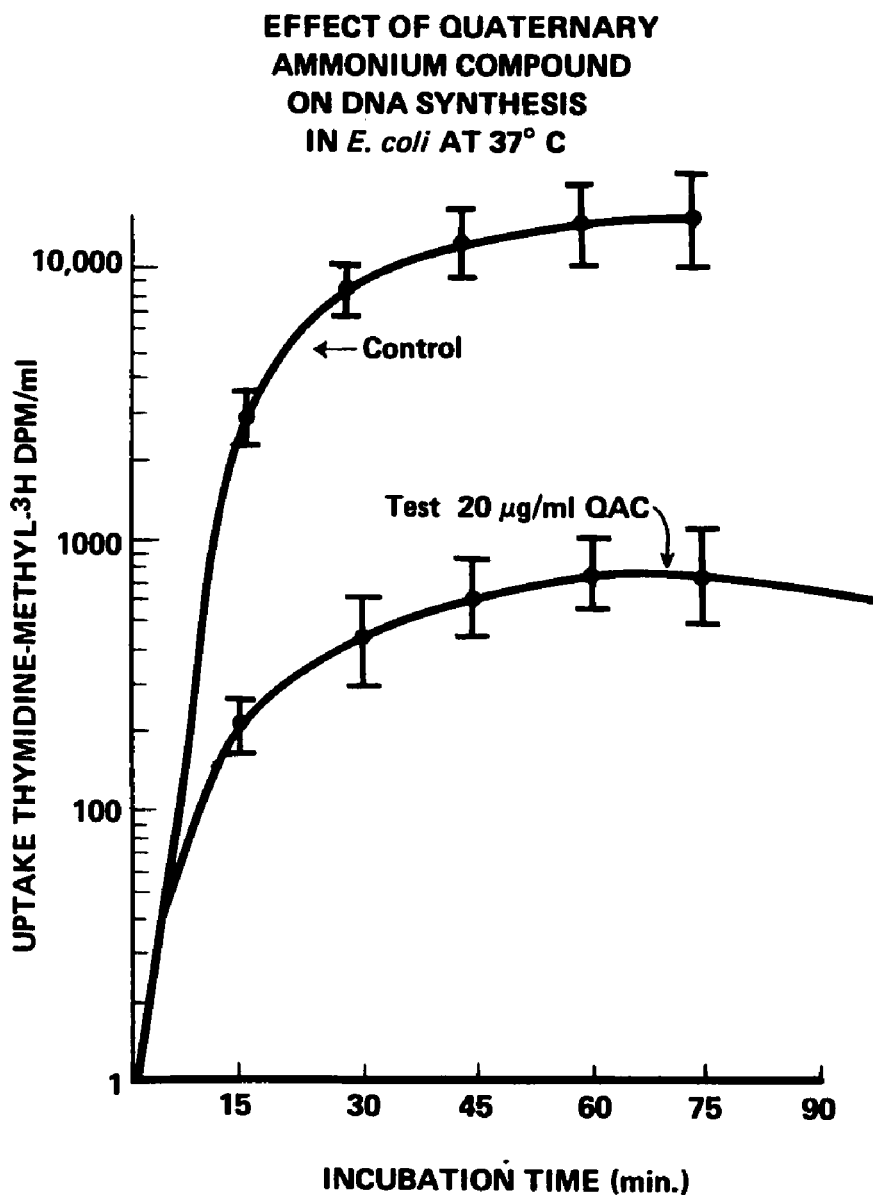


Figure 23. - The effect of a sub-inhibitory concentration of quaternary ammonium compound on incorporation of thymidine-methyl-³H into DNA. Each point represents the mean of 4 determinations. The standard error (S.E.) of the means is indicated by the vertical bars.

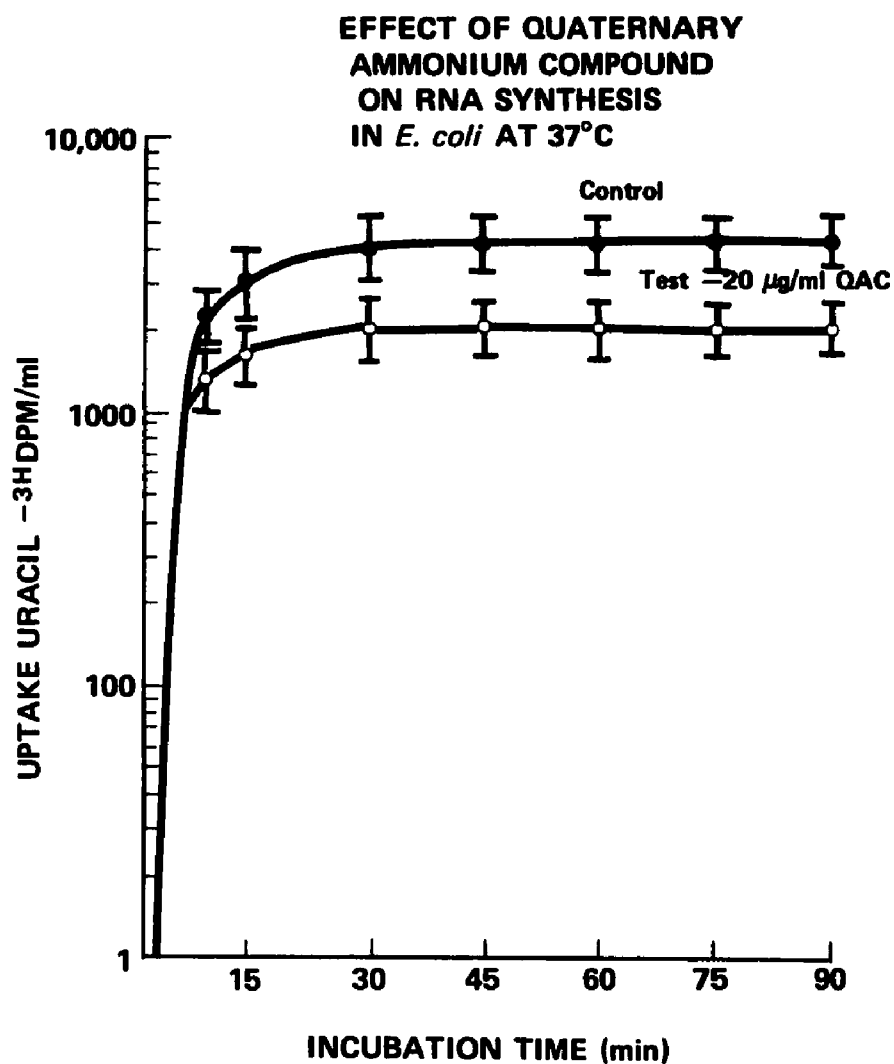


Figure 24. - The effect of a sub-inhibitory concentration of quaternary ammonium compound on incorporation of uracil-³H into RNA in *E. coli*. Each point represents the mean of 4 determinations. The standard error (S.E.) of the means is indicated by vertical bars.

**EFFECT OF QUATERNARY
AMMONIUM COMPOUND
ON PROTEIN SYNTHESIS
IN *E.coli* AT 37°C**

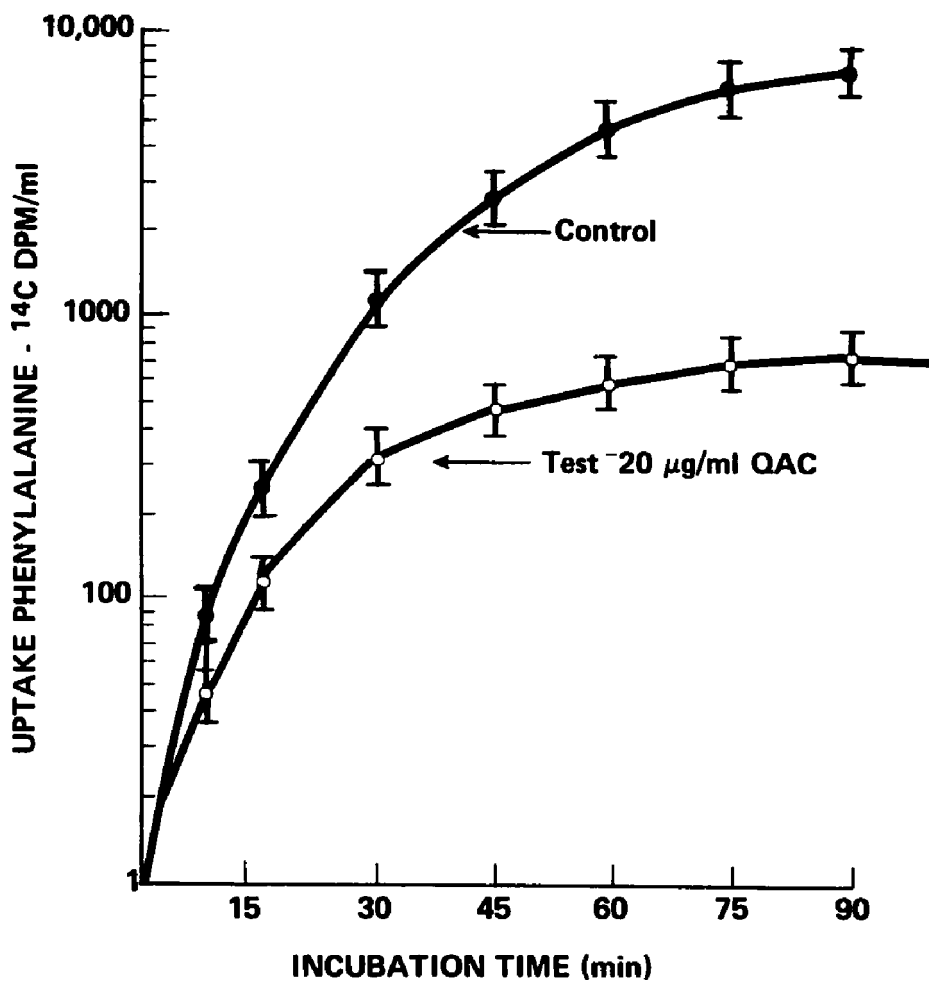


Figure 25. - The effect of a sub-inhibitory concentration of quaternary ammonium compound on incorporation of phenylalanine- ^{14}C into protein of *E. coli*. Each point represents the mean of 4 determinations. The standard error of the means (S.E.) is indicated by the vertical bars.

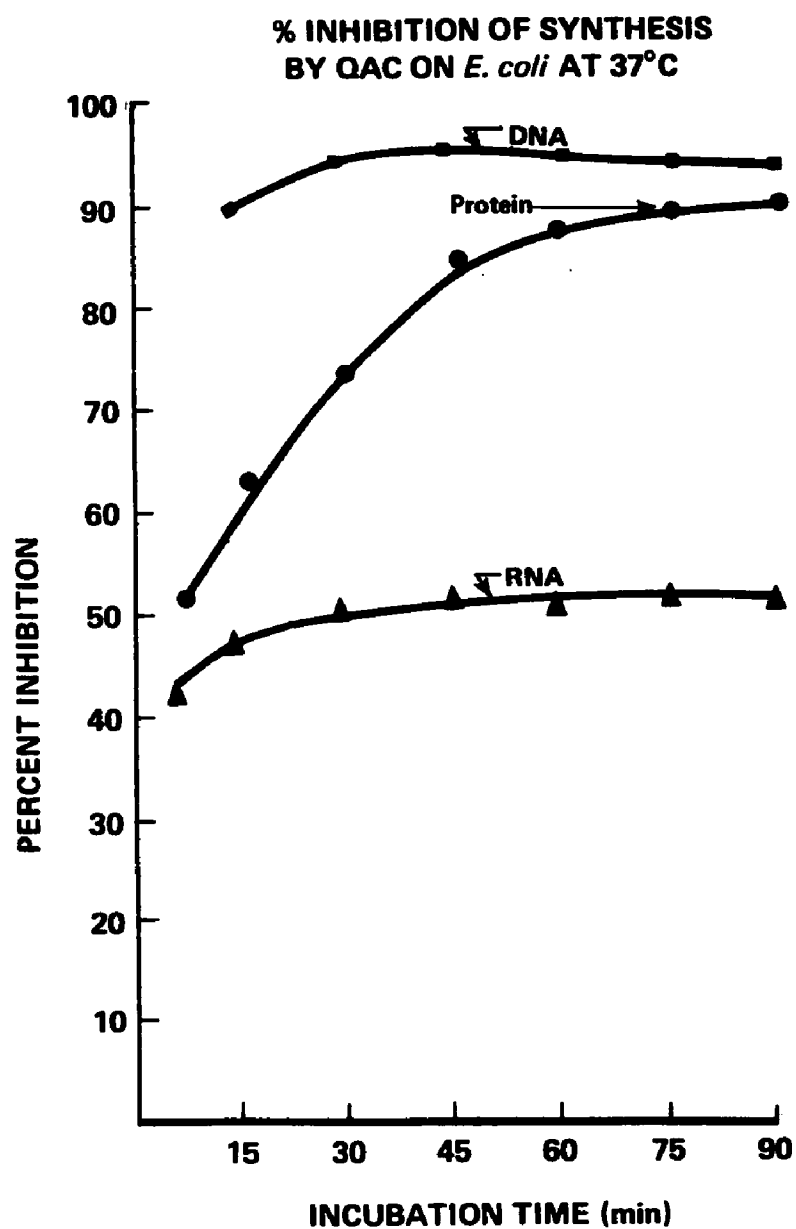


Figure 26. - Summary of the percent inhibition of DNA, RNA, and protein synthesis by a sub-inhibitory concentration of quaternary ammonium compound on *E. coli*.

the beginning but increased with the passage of incubation time. Least affected was the incorporation of uracil-³H into ribonucleic acid.

From these data it might be speculated that the primary action of QAC may be on the synthesis of DNA since when equal concentrations of QAC were used a much greater inhibition of DNA than RNA was observed. The action on protein synthesis may not be a direct effect on the QAC. The amount of protein inhibition was greater than that of RNA but occurred at a slower rate during the incubation period.

These observations suggest that the inhibition of protein synthesis and RNA synthesis occurred subsequent to DNA synthesis. Therefore, they may not be a primary site of action but a secondary one being led by the inhibition of DNA synthesis.

3. Correlation of Microtiter Test Data With Metabolic Studies

The microtiter bacteriostatic test data also give evidence for a metabolic effect, possibly involving DNA at low QAC concentrations, as demonstrated from the following information.

In Figure 27, a finding that the bacteriostatic endpoint (MIC) of QAC may be related to the base composition of the DNA is demonstrated. If the logarithm of the MIC obtained for several species of bacteria is plotted against the Guanine-Cytosine (GC) ratio, a linear relationship is obtained.

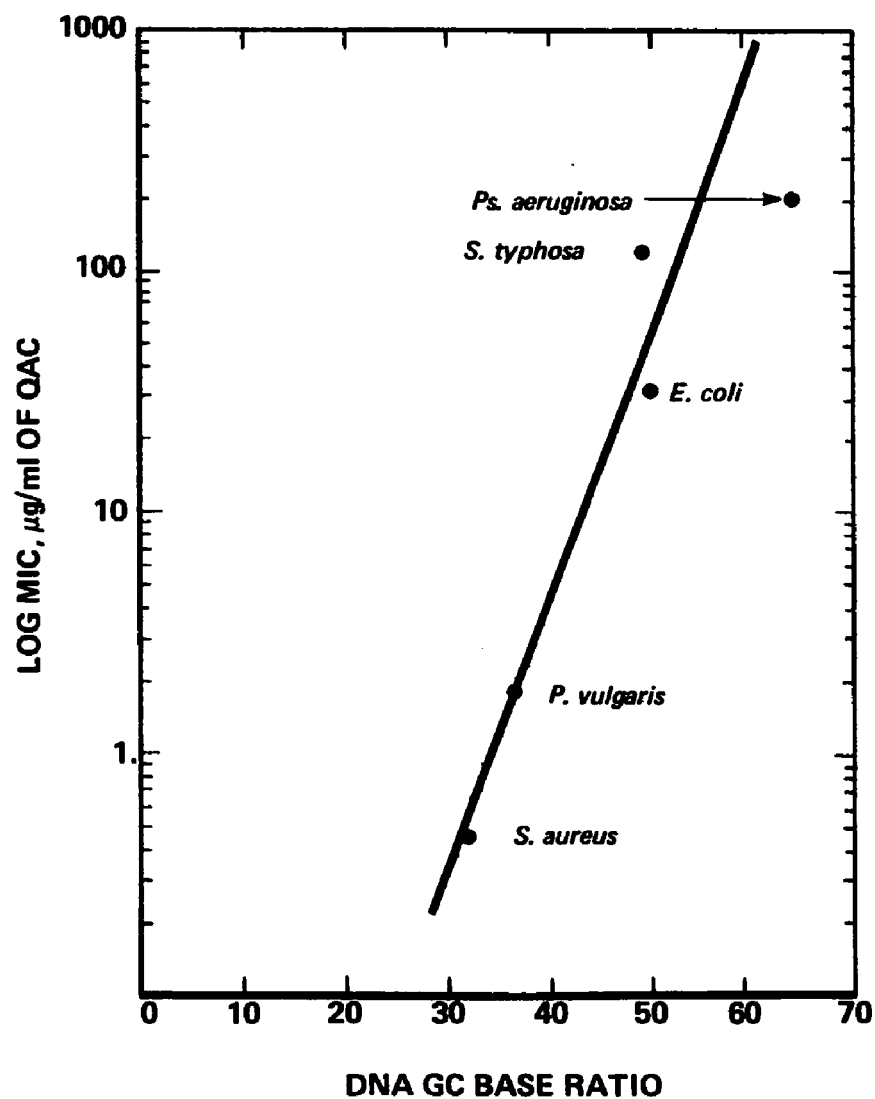


Figure 27. Relationship between the minimum inhibitory concentration of quaternary ammonium compound ($\mu\text{g/ml}$) and the DNA base composition for several microorganisms.

Based on this information the following generalization can be made. The sensitivity of S. aureus to QAC (MIC 0.2-0.5 µg/ml, Table XX, Chapter I) may be due to stronger binding to adenine-thymine (AT) rich DNA. *Pseudomonas* has high resistance to QAC as demonstrated by the microtiter test (MIC of 200-500 µg/ml, Table XX, Chapter I), moreover, it has a low AT ratio and a high GC ratio. It may be that binding to GC rich DNA may be much less than binding to AT rich nuclear material. There is also a possibility that the inhibition of DNA might influence changes in permeability.

Evidence for changes in permeability characteristics of the organisms at various QAC concentrations was demonstrated by the chromium 51 release study.

Nucleic acids are needed for the synthesis of protein components of the cell membranes. Protein synthesis was shown to be indirectly affected. Any relationship, however, between the nucleic acid synthesis and permeability properties of the cell are only speculative but might be a factor especially when one considers that microorganisms with a high AT ratio were also extremely sensitive to QAC bactericidal action.

Correlation of microtiter bacteriostatic and bactericidal endpoints with biochemical data makes it possible to suggest the following hypothesis on the mode of action of quaternary ammonium compounds. The generalization is based on the biochemical data from the chromium 51 release studies, the meta-

bolic inhibition studies, and the information from the microtiter test procedures.

Individual cells take up the QAC at the surface of the cell membrane, possibly by solubilization into the phospholipid components of the cell. Some of the molecules penetrate the cell resulting in a slow-down of metabolic pathways as evidenced by the inhibition of DNA, protein and RNA synthesis. The microtiter bacteriostatic test findings along with the neutralization data indicate that at low QAC concentrations the integrity of the cell is not altered, that surface absorption is reversible and that cell death is minimal. Leakage of cellular constituents is low, probably due to blockage of natural exchange processes.

In the presence of higher QAC concentrations more molecules are solubilized in the surface of the cell, a greater number of molecules penetrate through the inner cell membrane and leakage of cellular material and cellular death result. The data suggest the existence of two different thresholds of QAC action, namely inhibition of metabolic pathways and leakage of cellular material or a lytic effect.

It was not possible to demonstrate a single mode of action by the quaternary ammonium compounds on microorganisms. However, it was possible to determine susceptibility and the order in which various metabolic systems and cell components were effected and to relate these affects to the ability of quaternary compounds to act as bacteriostatic and bactericidal agents.

CHAPTER III

FIELD APPLICATIONS IN THE HOSPITAL ENVIRONMENT

A. INTRODUCTION

1. Qualitative Studies

Altemeier (1967) called attention to the need for applied studies with regard to the microbial contamination of the hospital environment. He pointed out that the number of hospital-acquired infections has continued to increase and he attributed the lack of tangible epidemiological evidence to a failure in design of investigations. Lack of proper sampling and inadequate culture techniques, in addition to poor epidemiological markers for recovery of specific target organisms contributed to the difficulties.

The modern hospital is faced with an increasing number of highly resistant organisms resulting from the selective pressures of continuously applied chemotherapeutic measures. The emergence of drug resistant S. aureus strains has been described by Benner and Kayser (1968) as the major hospital treatment problem of the decade.

Whereas there is a large amount of general information on the biological characteristics of these organisms, there is a particular lack of specific information. Utilizing the capabilities of the previously developed microtiter bacteriostatic and bactericidal test procedures (See Chapter I), it is possible to determine the specific concentrations of QAC

required for effective kill of selected organisms and to evaluate the possibility of a reduction in antimicrobial resistance of these organisms after sublethal exposure to quaternary ammonium compounds.

2. Quantitative Studies

Any assessment of the microbial contamination of hospital surfaces must also involve accurate quantitative studies of the bacterial levels on these surfaces. When germicides are used as part of a general cleaning program, it is assumed that the objective would be the reduction of the microbial levels, hopefully resulting in better control of hospital-acquired infections. To date these objectives have been rarely demonstrated. Finegold et al. (1962) found that floors washed with disinfectants alone were microbially no cleaner than when detergent-sanitizers were used. Vesley and Michaelsen (1964) found that adding disinfectants to floor-cleaning solutions neither improved the bacterial counts immediately after cleaning, nor did it affect the rate at which the floor counts subsequently increased. Ayliffe et al. (1967) did find a slightly greater reduction in floor counts after using disinfectants than after soap and water alone, but they, too, found that microbial levels continued to increase due to normal fallout and shoe sole contamination after disinfection.

A quantitative evaluation involving the application of two QAC detergent germicides to occupied patient rooms was conducted in order to study the effect of the various con-

centrations of the compounds and to determine the possibility of neutralizing the agent to permit recovery of any remaining viable organisms.

These studies, together with the bacteriostatic and bactericidal microtiter studies, have provided a basis for determining a practical use dilution of the QAC for hospital environmental applications.

B. MATERIALS AND METHODS

1. Qualitative Studies

a. Isolation

(1) Surveys of environmental surfaces were conducted over a two-month period with Rodac plates containing egg yolk agar to isolate staphylococci.

(2) Egg yolk agar plates were prepared in accordance with the procedure of Herman and Morelli (1961) using a modified Chapman-Stone #110 medium.

(3) Patients' rooms, corridors, nursing stations, treatment rooms, sinks, ventilation systems, cart wheels, shoes and clothing of hospital personnel were sampled. Sixteen Rodac plate samples were collected from each nursing unit.

b. Identification

(1) Organisms picked from Rodac egg yolk plates were streaked on TSA and Blood agar and incubated for

24 hours. The slide coagulase test was performed on all suspicious S. aureus colonies according to the procedure of Ness (1957).

(2) Antibiotic sensitivity was determined by the disk sensitivity test according to the procedure of Petersdorf and Sherris (1965).*

(3) Bacteriophage typing was according to the procedure of Blair and Williams (1961).

c. Sensitivity Tests

(1) The Minimum Inhibitory Concentration (MIC) for isolates of resistant S. aureus isolated from the hospital environment was determined according to previous microtiter bacteriostatic test method protocol (See Chapter I).

(2) A bactericidal evaluation of the isolates of resistant S. aureus was determined according to microtiter bactericidal test method protocol (See Chapter I). Inactivation of residual QAC and dilution were accomplished in letheen broth.

2. Quantitative Studies

a. Microbial Reductions

Two QAC products, Micro-quat**, an n-alkyldimethyl-

* Sensi-Disk - sensitivity test disk manufactured by BBL Division of BioQuest, Cockeysville, Maryland
** Economics Laboratory, Inc., Osborn Building, St. Paul, Minnesota

benzylammonium chloride, 9.4% active ingredients and A33*, an n-alkyldimethylbenzylammonium chloride, 14.0% active ingredients, were evaluated as hospital surface disinfectants in occupied patient rooms. The 1X concentration of the first product is 500 ppm and the 2X concentration is 1000 ppm of active ingredients. For the second product, the 1X concentration is 600 ppm and the 2X concentration is 1200 ppm. The floors of 38 occupied patient rooms were surveyed. The germicide was applied by the one-bucket mopping system in accordance with NIH Cleaning Procedure Manual (1966). A freshly filled bucket was used for each test and a 16 ounce sterile mop was used. Contact (Rodac) agar plates filled with 16.5-17.5 ml TSA and TSA with 0.07% lecithin and 0.50% polysorbate were used for all surface sampling. Thirty Rodac samples with each medium were collected in each room by a random numbers technique prior to cleaning, and 30 samples were collected with each medium 10 minutes after the cleaning procedure. Plates were incubated for 24 hours at 37°C and then promptly counted.

* Airkem, a Division of Airwich Industries, Inc., Commerce Road, Carlstadt, New Jersey

C. RESULTS

1. Qualitative Studies

a. Isolation and Identification

The Rodac surface sampling technique was utilized with egg yolk for recovery of methicillin resistant S. aureus strains from hospital environmental surfaces.

In Table XXXII are listed 10 isolates of resistant S. aureus obtained during surveys of hospital nursing units. The particular growth characteristics of these organisms provided a handy marker for their isolation and enumeration. They grew well on egg yolk media and produced a rich deep yellow pigment. Ten isolates were evaluated for phage type, five were non-typable and four were type 83 and one was type 83/85.

The sensitivities to antibiotics were determined by disk sensitivity tests. The antibiogram for all 10 strains were the same in that they were sensitive to chloramphenicol, bacitracin, cephalothin, lincomycin, kanamycin, and neomycin. They were resistant to penicillin, tetracycline, erythromycin, streptomycin, and methicillin. The control, S. aureus FDA strain 209, was sensitive to all the antibiotics.

b. Microtiter Tests

The bacteriostatic and bactericidal endpoints for the methicillin resistant S. aureus isolates obtained

TABLE XXXII
ANTIBIOTIC SENSITIVITIES AND PHAGE TYPES OF *S. AUREUS* ISOLATES FROM
HOSPITAL ENVIRONMENT

Source	No.	Antibiogram*											Phage	
		Pen	TC	Chl	Ba	Er	St	Cf	Linc	K	Neo	Me	Type	RTD†
Corridor Floor	13	R	R	S	S	R	R	S	S	S	S	R	NR	
Nursing Station	37	R	R	S	S	R	R	S	S	S	S	R	83++	
Patient	38	R	R	S	S	R	R	S	S	S	S	R	83++	
Nursing Shoes	60	R	R	S	S	R	R	S	S	S	S	R	83++	
Weather-master	A1	R	R	S	S	R	R	S	S	S	S	R	NR	
Cart Wheels	8B	R	R	S	S	R	R	S	S	S	S	R	NR	
Patient	492	R	R	S	S	R	R	S	S	S	S	R	83++/85+	
Sink	493	R	R	S	S	R	R	S	S	S	S	R	NR	
Patient	540	R	R	S	S	R	R	S	S	S	S	R	NR	
Patient Floor	840	R	R	S	S	R	R	S	S	S	S	R	83++	
Control		S	S	S	S	S	S	S	S	S	S	S	Not typed	

* Antibiogram

Pen	Penicillin	Cf	Cephalothin
TC	Tetracycline	Linc	Lincomycin
Chl	Chloramphenicol	K	Kanamycin
Ba	Bacitracin	Neo	Neomycin
Er	Erythromycin	Me	Methicillin
St	Streptomycin		

† Phage Type RTD — Routine Test Dilution. Phage Typing — courtesy of Bacteriology Section, Laboratory Branch, Center for Disease Control, Atlanta, Georgia.

from the patients and hospital environmental surfaces were then examined for sensitivity to quaternary ammonium compounds.

In Table XXXIII are presented data on the determination of the sensitivity of these S. aureus organisms to quaternary ammonium compounds. Both the bacteriostatic concentration (MIC) ($\mu\text{g/ml}$) and the bactericidal endpoint ($\mu\text{g/ml}$) required for 99% reduction were determined in 5 minute exposure to 24°C to BTC 824 - an n-alkyldimethylbenzylammonium chloride. A concentration of 0.2-0.4 $\mu\text{g/ml}$ of QAC was required for a bacteriostatic or inhibitory effect and a concentration of 58-70 $\mu\text{g/ml}$ of quaternary ammonium compound during a 5 minute exposure at 24°C resulted in a 99% kill of $1 \times 10^6/\text{ml}$.

2. Quantitative Studies

a. Environmental Surface Sampling

Utilizing the Rodac sampling method with a specialized media, quantitative microbial sampling of 38 hospital rooms was conducted.

In Table XXXIV are summarized the results from sampling the floor surfaces of 10 patient rooms. Thirty Rodac surface samples were collected before the application of the product and 30 Rodac surface samples were collected 10 minutes after the application. The concentration of QAC was 500 ppm in all cases. In the last five rooms the average percent reduction was 55.9

TABLE XXXIII

**MICROTITER BACTERIOSTATIC AND BACTERICIDAL ENDPOINTS
OF RESISTANT STRAINS OF *S. AUREUS* FROM THE HOSPITAL
ENVIRONMENT**

Number	Bacteriostatic Endpoint MIC ($\mu\text{g/ml}$)	Bactericidal Endpoint 1.0 Percent Survivors ($\mu\text{g/ml}$)
13	0.2	65
37	0.4	70
38	0.2	62
60	0.2	69
A - 1	0.2	61
8 - B	0.4	59
492	0.4	58
493	0.2	62
540	0.2	68
840	0.4	62
Control	0.2	60

TABLE XXXIV

**QUANTITATIVE MICROBIAL SAMPLING OF ENVIRONMENTAL SURFACES
BEFORE AND AFTER WET MOPPING IN PATIENT ROOMS**

QAC 1,* Rodac plate method 500 ppm

No Inactivators			
Test	Before	After	Percent Reduction
1	201.2	81.5	59.5
2	160.3	69.1	56.9
3	158.2	64.6	59.2
4	135.8	62.5	54.0
5	129.1	59.0	54.3
			56.8
Inactivators			
6	170.4	80.2	52.9
7	140.3	67.5	51.9
8	120.6	50.9	57.8
9	190.2	87.5	54.0
10	182.0	67.8	62.8
			55.9

*** QAC 1 – Micro-Quat ®, 9.4% n-alkyl dimethyl benzyl ammonium chloride,
Economics Laboratory, Inc., Osborn Building, St. Paul, Minnesota.**

indicating a slightly lower recovery of organisms in the presence of the neutralizers.

In Table XXXV are summarized the results from quantitative sampling of floor surfaces of 10 patient rooms. In this case the product was QAC 2 at 600 ppm active QAC. In the five rooms sampled with the neutralizers in the sampling media the average percent reduction was 67.3. In the second five rooms the average percent reduction was 68.0 indicating no significant difference in the percent recoveries in the presence of the incorporated neutralizers. There was an increased percent reduction of microbial decontamination in both cases over the reductions found in Table XXXIV.

In Table XXXVI are shown data obtained from quantitative sampling of floor surfaces in eight patient rooms. In this case, QAC 1 at 1000 ppm of active ingredients was utilized. The average percent reduction was 75.5 for the first four rooms utilizing TSA as sampling media and 78.5 in the last four rooms. The sampling media in the last four rooms contained the inactivators. There was no significant reduction of microbial contamination when quantified with the sampling media containing the neutralizers.

In Table XXXVII are summarized the results when 1200 ppm of QAC 2 were utilized for environmental sur-

TABLE XXXV
QUANTITATIVE MICROBIAL SAMPLING OF ENVIRONMENTAL SURFACES
BEFORE AND AFTER WET MOPPING IN PATIENT ROOMS

QAC 2, *Rodac plate method 600 ppm

No Inactivators			
Test	Before	After	Percent Reduction
11	118.0	43.7	63.0
12	164.0	64.0	61.0
13	132.0	42.0	68.2
14	183.0	47.5	74.1
15	172.0	50.8	70.5
			<u>67.3</u>
Inactivators			
16	230.0	72.4	68.5
17	152.0	56.5	62.8
18	124.0	29.8	76.0
19	181.0	59.2	67.3
20	134.0	46.5	65.4
			<u>68.0</u>

* QAC 2 – A-33®, 14.0% n-alkyl dimethyl benzyl ammonium chloride, Airkem,
 Commerce Road, Carlstadt, New Jersey.

TABLE XXXVI
QUANTITATIVE MICROBIAL SAMPLING OF ENVIRONMENTAL SURFACES
BEFORE AND AFTER WET MOPPING IN PATIENT ROOMS

QAC 1, Rodac contact plate 1000 ppm QAC

No Inactivators			
Test	Before	After	Percent Reduction
21	187.2	29.5	79.0
22	159.6	38.2	76.1
23	175.5	42.6	75.8
24	164.7	47.4	71.2
			<u>75.5</u>
Inactivators			
25	169.1	34.4	79.7
26	132.4	29.3	77.9
27	145.1	36.6	74.8
28	167.8	30.9	81.6
			<u>78.5</u>

TABLE XXXVII
QUANTITATIVE MICROBIAL SAMPLING OF ENVIRONMENTAL SURFACES
BEFORE AND AFTER WET MOPPING IN PATIENT ROOMS

QAC 2, Rodac contact plate 1200 ppm

No Inactivators			
Test	Before	After	Percent Reduction
29	120.2	20.4	83.0
30	187.1	30.8	83.5
31	176.7	17.5	90.1
32	134.2	22.8	83.0
33	146.2	19.7	86.5
			<u>85.2</u>
Inactivators			
34	121.3	19.6	83.9
35	110.4	16.6	84.9
36	108.0	16.4	84.8
37	129.4	23.2	82.1
38	116.2	22.4	80.7
			<u>83.3</u>

face decontamination. The average percent reduction was 85.2% in the first five rooms using TSA for the sampling and 83.3% in the last five rooms, in which the neutralizers were incorporated into the sampling media. The dosage of QAC used here (1200 ppm) was the highest concentration used in the study and it gave the greatest percentage of microbial reduction.

D. DISCUSSION

1. Qualitative Surface Sampling

The Rodac surface sampling technique was utilized for qualitative and quantitative sampling of flat non-porous hospital surfaces. The plates are commercially available and designed so that when filled with 16.5-17.5 ml selected agar, a raised convex surface is provided which facilitates contact with any smooth dry surface. Because of the simplicity and compactness of the plates, they can be utilized for all hospital areas with little inconvenience to patients or staff.

In Table XXXIII are presented the data on sensitivity to QAC of isolates of methicillin resistant S. aureus. The study indicates that all isolates were very sensitive to quaternary ammonium action. The microtiter bacteriostatic endpoint was below 0.5 µg/ml and the bactericidal endpoint between 58-70 µg/ml QAC for all the isolates tested.

The isolation of methicillin resistant organisms from

environmental surfaces demonstrate that contamination can be readily distributed laterally into corridors and other areas of a medical care facility. In addition, it emphasizes the implication of environmental surfaces as an element in direct dynamic contact with the other hospital areas.

The microtiter test data also demonstrate that proper use of QAC disinfectants could result in control and destruction of the staphylococcus organisms, thus reducing the chances for these organisms becoming a hazardous component of the hospital flora.

Bouanchaud et al. (1969) reported that the survivors following exposure of S. aureus to low levels of ethidium bromide, a quaternary ammonium compound, resulted in a reduction or elimination of antibiotic resistance. A check of the susceptibility of methicillin-resistant S. aureus organisms to methicillin, after exposure to n-alkyldimethylbenzylammonium chloride, was therefore initiated. Six isolates of S. aureus isolated from the hospital environment were involved in this study. The MIC for methicillin was determined before QAC treatment by the microtiter bacteriostatic procedure. All 6 isolates were grown overnight at 37°C in TSA containing 0.18 µg/ml subinhibitory concentrations of QAC. They were transferred daily for 7 consecutive days. After 7 transfers the MIC for methicillin was re-determined. The MIC for methicillin was found to be within a 25-50 µg/ml range, indicating little change in their resistance to this semisynthetic penicillin. A curing effect or reduction of

methicillin resistance in these isolates was not demonstrated.

The genetic basis of methicillin resistance is unknown, and the relationship of the new strains which are untypable, Type 83, or in one case, Type 85, to older hospital resistant types (80, 81) is presently not understood.

If drug resistance of S. aureus is acquired from other bacteria, it must develop only where there is a mixed flora. The role of the environment is not known in this regard. However, with regard to a patient situation, areas such as superficial infections might be considered possible sites for resistance transfer.

In the case of these resistant strains which may be presented in lesions of infected persons or present in healthy carriers, such as physicians, nurses or housekeeping personnel, they would be competent to donate drug resistance factors by spontaneous transduction to sensitive organisms. The epidemiologic consequences of resistance transfer are of considerable interest to those concerned with the hospital environment. Since these resistant strains might appear more readily to colonize normal individuals than sensitive strains, they would be in a more favorable position to be transmitted to susceptible persons.

These isolates did not appear to be particularly virulent. However, they must be considered as a source for more virulent strains acquiring resistance. Novick (1969) reported that S. aureus plasmids may be transmitted by transducing

prophages, therefore, it is possible that this resistance could be transmitted to highly virulent strains already part of the hospital environment. These are certainly issues which must be considered if effective environmental control measures are to be developed.

It is important that every precaution be taken to insure that infectious transfer does not become an important factor in the occurrence or dissemination of strains of S. aureus with multiple resistance. Early recognition of resistant strains can lead to their eradication by appropriate chemotherapy of patients and by adequate disinfection procedures.

The level of resistant organisms present in the hospital environment must always be kept as low as possible. Implementation of a vigorous disinfection policy by various hospital departments must be used to control dissemination of such organisms.

2. Quantitative Surface Sampling

Control of microbial contamination of hospital environmental surfaces entails not only those measures required to isolate and identify microorganisms but it also requires quantitative studies to determine the effective destruction or inhibition of the organisms by adequate disinfection practice. The delineation of the extent of microbial reduction by quaternary ammonium compounds at several concentrations and with several products was accomplished. In addition,

the effectiveness of neutralization by polysorbate 80 and lecithin in solid agar for surface sampling was evaluated.

The results indicate that QAC products utilized at a concentration of 500-600 ppm active ingredients gave a percentile reduction of 55-68%. With concentrations within the 1000-1200 ppm range the percentile microbial reduction was in the 75-85% order.

The results indicate that for adequate hospital environment surface decontamination, QAC concentrations of greater than 1000 ppm are needed to give maximum bacterial reductions.

Unfortunately, at all of the concentrations of QAC studied, there did not appear to be any significant difference between the contact plates employing the neutralization system and the plates without the neutralization system. These conclusions can be explained in terms of the previously discussed microtiter test findings and the neutralization model. Kakemi et al. (1965) showed the critical micelle concentration of polysorbate 80 in aqueous solution to be below 0.1%. However, in TSA agar the critical micelle concentration (CMC) is probably never reached. Inactivation of QAC by the polysorbate 80 and lecithin system involves solubilization of the QAC by micelles (See Chapter I). Because of the presence of the solid or agar phase the micelles probably do not occur to any great extent and as a result no difference in the percent survivors for either system is evident. The incorporation of 0.5% polysorbate 80 and 0.07% lecithin in TSA solid media for surface sampling is therefore not

justified and cannot be recommended.

As a result of these conclusions, caution must be exercised in invoking Rodac surface sampling as factual statements on the actual percent microbial reductions obtained for QAC. The results obtained undoubtedly include both bactericidal and bacteriostatic effects, the latter contributing to what might appear to be increased efficiency, when actually this might not be the case.

The major problem with the Rodac surface sampling technique involves primarily the use of a suitable neutralization media to remove the bacteriostatic effect which will result from residual amounts of the germicide present on environmental surfaces. The QAC exerts a bacteriostatic effect on highly sensitive organisms such as S. aureus and vegetative B. subtilis which was demonstrated by the microtiter bacteriostatic test.

Thus, there are limitations on the use of Rodac surface technique for quantitative studies involving QAC on environmental surfaces. Comparison of the effectiveness of phenolic disinfectants, which exerts a low bacteriostatic effect, to effects by quaternary ammonium compounds by the above surface sampling procedures may not be valid.

The percentile reductions of microbial contamination on the environmental surface can be used to quantify the efficiencies of QAC at various concentrations. However, the absolute counts should also be considered in any study of the extent of environmental surface contamination.

Routine microbial sampling or surveillance of the hospital environment is not necessary. But qualitative sampling surveys oriented toward epidemiological investigations with a defined purpose have definite advantages. In addition, quantitative studies, such as evaluating a disinfectant concentration or cleaning technique, may have merit if consideration is given to the compounds being evaluated, on the organisms exposed and surviving the sampling media.

3. Establishing a Disinfection Policy

Most hospitals have some sort of disinfection policy, but the choice of disinfectants for similar purposes in different hospitals differs considerably. Some policies are much more extensive than others and strict observance of the policies is rare, either as regards the type of disinfectant or routine in-use concentration.

Federal Medicare requirements and the new Standards for Hospital Accreditation (Joint Commission of Hospital Accreditation) stipulate written policies for most hospital functions. These include guidelines for decontamination performed by such departments as central service supply and for the cleaning of equipment and measurement of disinfectant chemicals in house-keeping services. Based on this study the major points considered necessary for an adequate disinfection policy are as follows:

- a. List the important environmental areas and surfaces in which chemical disinfectants are currently being

used.

- b. Select a disinfectant likely to be effective for specific use and clearly designate the product.
- c. Study and ascertain active ingredients, formula, phenol coefficient, use dilution, and toxicity; review tests on various bacteria, particularly Staphylococcus aureus and Pseudomonas aeruginosa. Determine the most effective pH. Consider limitations such as hard water, organic load and other environmental parameters particular to the hospital in question.
- d. Draw up a written policy considering the above factors. Designate the time intervals for application. Specify the manner in which the use dilution will be made. Specify the number of rooms or footage that will be completed prior to solution discard. Arrange for distribution of the disinfectant to points of use. Evaluate frequently to insure that the product is being used at the correct use dilution. Insure that if the product is received in bulk lots that the concentration supplied as a working solution is correct.
- e. Instruct personnel in the above policy. Constantly make in-house checks to insure that the system has not broken down through lack of good communications.
- f. Evaluate the effectiveness of the program by the

microtiter test procedures and the in-use surface sampling technique.

In this chapter, the QAC products evaluated at various concentrations have produced a germicidal effect immediately after application. Although this effect may be fleeting in character, due to the concentrations used, the surface treated, and the microbial flora present, there nonetheless exists support for the proposition that regular concurrent disinfection of premises with suitable concentrations of QAC represents an important measure that can be directed against vegetative microbial populations in the hospital environment.

SUMMARY

This dissertation is concerned with disinfection by quaternary ammonium compounds in medical care facilities. Two microtiter disinfection tests, one for QAC bactericidal testing and the other for bacteriostatic QAC testing were developed.

The microtiter bactericidal procedure is intended and designed simply for assessing disinfectant activity of a quaternary ammonium compound under selected conditions by a prescribed suspension technique. Environmental factors such as water characteristics, temperature and organic loads were taken into consideration. Within a given set of variables, the procedure is particularly applicable for grading and comparing various quaternary ammonium compounds, in terms of relative disinfectant activities.

The microtiter bactericidal procedure enables evaluation of the disinfectant activity of the product, or group of products, in which a suspension of viable microorganisms is added to various concentrations of the disinfectant and the number of organisms surviving after a given period of time is determined quantitatively.

The microtiter components were modified in such a way that micro-quantities of organisms and chemicals are transferred in an accurate and analytical manner. Special micro-diluters were developed.

A method of calibration of the microdiluters with radioactive chromium 51 was developed. The application of the standard microdiluters for streaking of suspensions of organisms on plates was employed.

A graphical procedure is described whereby the results of disinfection testing by the microtiter bacteriocidal procedure are conveniently represented by plotting the percentages of survivors as the integrated normal probability against the logarithm of the disinfectant concentrations. The dosage response curve is predicted from 6 experimental measurements which are available from a single microtiter disinfection test. Utilizing the transformation, the distribution was generally linear throughout the experimental range 1.0-99.0% survivors.

The application of slope determinations for sensitivity and compound comparisons is recommended.

Environmental factors such as the effect of water hardness, temperature and organic load on QAC activity were evaluated. A reduction of QAC activity in presence of Ca^{++} and Mg^{++} was demonstrated by the microtiter bacteriocidal procedure.

The effect of organic load (fetal calf serum) on bacteriocidal activity was apparent. The data indicate that organic material had a significant effect on the activity of the QAC. The importance of insuring that environmental surfaces be free of heavy organic load and gross contamination prior to application of QAC was demonstrated.

The microtiter test was utilized for bacteriophage susceptibility testing. The experiments demonstrated that hospital germicides at presently used concentrations are rather ineffective for inactivation of the T2 bacteriophage of E. coli.

The capability to neutralize the effects of residual quantities of the QAC after completion of the exposure period was studied and heparin was found to be an effective neutralizer of QAC action. The anionic portion of the heparin molecule was believed to react with the cationic nitrogen of the quaternary compound. The use of polysorbate 80 and lecithin for QAC neutralization, long traditional in QAC disinfectant studies, was re-evaluated. The reason for the effectiveness of these compounds was postulated to be involved with solubilization of the QAC into the micelles of the polysorbate 80, along with a possible ionic interaction by the cationic nitrogen of the QAC with the phosphate group on the lecithin.

Replicate testing of microtiter bactericidal test at the same session and different sessions was conducted in order to evaluate the reproducibility of the test. The differences noted especially with regard to the data from different days were believed to be due to estimation of the initial inoculum. The reproducibility of the test can be improved by replication of the control. The coefficient of variation of the same session replicates was approximately 5.0% indicating the excellent reproducibility of the test.

The microtiter bacteriostatic test made it possible to evaluate the QAC activity in which a suspension of viable microorganisms was added to various concentrations of the compound and the lowest concentration that inhibited growth was reported as the minimum inhibitory concentration (MIC).

Several QAC disinfectants and antiseptics were evaluated and the minimum inhibitory concentration for four organisms determined. The results indicated different MIC for each of the compounds evaluated, but the sensitivities remained in distinct ranges. The gram positive organisms were inhibited to a much greater extent than the gram negatives. The effect of additional antimicrobials on the MIC of the two QAC compounds was also studied.

The simplicity of the test, together with the ability for replicate determinations, endows the microtiter bacteriostatic test with distinct advantages which can be utilized in formulation research.

The microtiter bacteriostatic test was compared with the tube dilution test. The results indicated close correlation of the methods provided the microdiluters were calibrated for the diluent involved.

A chromium 51 procedure was developed for the tagging of bacteria. The technique allows for the study of effects of QAC on bacterial cells. It was found that an increase in leakage of chromium 51 from cells occurred with increased dosage of QAC. The leakage was correlated with microtiter

bactericidal survival data. It was found that at low concentrations of QAC there was less disruption of cells and that less leakage occurred in cells having a greater resistance to QAC action. The evidence tended to indicate that the mechanism of QAC action at higher QAC levels was due to physical damage to the cell membrane resulting in lysis.

The effects of sub-inhibitory concentrations of QAC as determined by the microtiter bacteriostatic test on the incorporation of the metabolic precursors, thymidine-methyl- ^3H , uracil- ^3H and phenylalanine- ^{14}C on E. coli were studied.

The time course of incorporation for each of the above metabolic precursors showed that there were differences in the rates of inhibition among the three cases. The incorporation of thymidine-methyl- ^3H into DNA was most inhibited by the QAC, whereas the extent of inhibition of uracil and phenylalanine incorporation into RNA and protein respectively increased with the passage of incubation time.

When equal concentrations of QAC were used, a much greater inhibition of DNA than RNA was observed. The inhibitory action of QAC on protein synthesis may not be a direct effect of the QAC. The amount of inhibition of RNA was greater than that of protein but the latter occurred at a slower rate during the incubation period. The observations suggest that the inhibition of RNA and protein synthesis occurred subsequent to DNA synthesis. Therefore, they were not a primary site of action but a secondary one being modified by the inhibition of DNA synthesis.

The microtiter bacteriostatic minimum inhibitory concentrations (MIC) of QAC for several strains of microorganisms appear to correlate with the DNA base composition. In situations where there were lesser amounts of adenine and thymine there were greater concentrations of QAC required for inhibition. In situations where there were greater amounts of adenine and thymine, such as with S. aureus, very low concentrations of QAC resulted in rapid inhibition of the organisms.

A hypothesis was derived that individual cells take up the QAC at the surface of the cell membrane, possibly by solubilization into the phospholipid components of the cell. Some of the molecules penetrate the cell resulting in a slow-down of metabolic pathways as evidence by the inhibition of DNA, protein and RNA synthesis. The microtiter bacteriostatic test findings, along with the neutralization data, indicate that at low QAC concentrations the integrity of the cell is not altered, that surface absorption is reversible and that cell death is minimal. Leakage of cellular constituents is low, probably due to blockage of natural exchange process.

In the presence of higher QAC concentrations more molecules are solublized in the surface of the cell, a greater number of molecules penetrate through the inner cell membrane and leakage of cellular material and cellular death results. The data suggest the existence of two different thresholds of QAC action, namely, inhibition of metabolic pathways and leakage of cellular material or a lytic effect.

The Rodac surface sampling technique was utilized for

microbial sampling in qualitative and quantitative procedures. Utilizing the Rodac plate for qualitative studies, in conjunction with the microtiter bactericidal and bacteriostatic procedures, selected isolates of resistant S. aureus from the hospital environment were evaluated.

Ten isolates were obtained from 7 environmental sites by surface sampling and 3 from patients. The environmental isolates were obtained from the corridor floor, nursing station, ventilation system, cart wheels, patient room sink and patient room floor. They were found to have a bacteriostatic endpoint at less than 0.5 µg/ml and 58-70 µg/ml of QAC as the bactericidal 99.0% endpoint. The sensitivity was not significantly different from control strains. The resistance of isolates to the antibiotic, methicillin, was not affected by the QAC.

The Rodac sampling procedure was also utilized in quantitative microbial sampling of hospital environment surfaces. The application of quaternary ammonium compounds to environmental surfaces revealed that 500-600 ppm of active ingredients resulted in microbial reduction of 55-68%. Concentrations greater than 1000 ppm are needed to give most effective microbial reductions on environmental surfaces.

No effect was found when polysorbate 80 and lecithin were employed for neutralization of QAC in solid agar media. The reason for a lack of significant differences might be explained by the fact that micelles are not formed in solid agar, therefore, the effectiveness of these neutralizers in

non-aqueous systems is limited.

The fact that 1000 ppm of QAC, or greater, was required for effective microbial reductions on environmental surfaces tends to indicate that the dynamics of disinfection on environmental surfaces are considerably different than those in liquid suspensions.

The current status of hospital disinfection practice was reviewed. The latest requirements for product registration were summarized. The establishment of a disinfection policy for hospitals was enumerated and discussed. The implementation of the policy and test procedures may result in a more dependable index of disinfection practice and in an improvement of the environmental quality in medical care facilities.

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