AN INVESTIGATION OF THE EFFICIENCY OF THE QUATERNARY AMMONIUM GERMICIDES IN THE PRESENCE OF HARD WATER

By

FRANK ROBERT PEABODY

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

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Department of Bacteriology and Public Health

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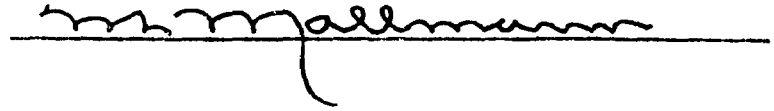
Approved 

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The survivor-curve test of Weber and Black was used for the first phase of the studies. The interfering action of the college tap water with the germicidal action of a quaternary ammonium compound (QAC) was shown to be a retarding of the action rather than a direct neutralization of the germicide. When the water was softened by different methods, no difference in the interfering action was noted except when using Versene. The addition of this complexing agent resulted in a marked reduction in the interfering action of the hard water. Subsequent testing with Versene in distilled water proved that the action is more than the softening action on the water. A definite increase of germicidal action resulted from the addition of Versene to the QAC in distilled water whether the Versene was added to the organisms or to the germicide.

When repeated dosages of organisms were added to the same disinfectant solution, a reserve of germicidal action was demonstrated even in the presence of tap water. To illustrate

this more readily, the exhaustion test was designed in which successive increments of organisms were added to the same disinfectant solution and samples taken ten minutes after the addition of each increment. In this way, the number of increments that could be added before the germicidal action was "exhausted" could be determined.

By utilizing the strong complexing action of the Versene, the germicidal action of the QAC which had been inactivated by the hard water could be released again to exert a germicidal action comparable to that of QAC in distilled water. This release of germicidal action was also demonstrated without the use of Versene by allowing an extended period for the germicide to be released from its combination with the interfering factor in the hard water.

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INTRODUCTION

The rapidly increasing use during the past decade of the group of germicides known as the quaternary ammonium compounds has presented many problems from the standpoint of practical applications as well as the laboratory evaluation of their germicidal efficiency. Although their first use was in the field of medicine, as antiseptics for skin and wound treatment, many of the more recent applications have been as germicidal agents in the area of public health; more specifically, as sanitizers for dairy equipment both on the farm and in the dairy plants, for treatment of food-processing equipment, for use as a chemical rinse treatment in the sanitization of multiple-use containers in the restaurant, and many other applications. It was in this area of disinfection that many of the most controversial problems were encountered.

Although these difficulties are many and complex, one which is pertinent to this study is the method of evaluating the germicidal action of this group of compounds. It soon became evident that evaluation of these compounds by the FDA phenol coefficient method gave misleading results when compared to that of a

phenolic type of compound for which the test was designed, and also presented many technical problems in duplicating results, avoiding bacteriostasis, and other factors. This led to the development of many "modifications" and new tests for assessing some germicidal value to these compounds. Wyss (25) has classified these many techniques into three groups according to the fundamental theory involved. At one extreme are experiments designed to measure the effect of varying individual factors and predicting the performance of the compound under any condition of application. At the other extreme is found the test in which the compound is taken into the field and its effectiveness tested under the conditions where it is to be used. Intermediate to these is the phenol coefficient method and its various modifications. The Weber and Black Method (23) and the Cantor-Shelanski Capacity Test (3) which are used in this study are based to a certain degree on practical considerations of the ultimate use of the compounds to be tested.

Another problem to receive a great deal of attention was the determination of substances which would inactivate these compounds, or at least interfere with their germicidal activity. A particular phase of this difficulty was encountered when the quaternary

ammonium compounds were applied as sanitizing agents in the restaurant, dairy, and other places where concentrated solutions or powders were made into a use dilution at the time of application. In these cases when the Weber and Black Method was used for evaluation, many of the waters used for the preparation of the sanitizing solutions would interfere with the germicidal action of the quaternary ammonium compounds. This thesis is concerned primarily with this problem of the interference of certain hard waters with the germicidal efficiency of the quaternary ammonium compounds. As a means of studying certain phases of this problem, an "exhaustion test" is used which is a modification of the capacity test previously mentioned and a field test method of Mallmann, Kivela, and Turney (11).

LITERATURE REVIEW

Due to the several fields of application of the quaternary ammonium compounds and the many workers interested, the literature on these compounds is very extensive. There are several excellent reviews which cover this material such as those by Rahn and Van Eseltine (17), Wyss (25), and Lawrence (9). In this review, an attempt has been made to review only that literature which pertains to the interfering action of hard waters upon the germicidal activity of the quaternary ammonium compounds.

The first mention of the possible effect of the hardness of the diluting water was made by Hanne (7) in 1937 in a review of compounds suitable for hospital use. He noted that when using alkyldimethylbenzyl ammonium chloride in a concentration of 1:10,000, a time interval of 16 minutes was necessary to kill a culture of Escherichia coli when tap water was used as the diluent, while the same effect was obtained in 8 minutes when using distilled water. A concentration of 1:5,000 was necessary to achieve kill in the 8-minute period when using tap water. It would appear that this information was not available to workers in this country

at that time, as it was not until 1947 that further mention of this situation was made in the literature.

The Sanitary Engineering Division of the U. S. Public Health Service, Washington, D. C., presented a statement at the request of the State Sanitary Engineers at their meeting in May, 1947, which included a section on interfering substances, organic and inorganic, in the waters in which the bactericidal agents were used (21). The data which they presented are given in Table 1. They indicate that there is a very marked reduction in the germicidal action of these compounds when they are used in the presence of the two tap waters as judged by the tests used for evaluation. In addition, it is shown that the beneficial effect of detergents added to the quaternary ammonium compounds when tested in distilled water may give the opposite result in the presence of tap water. Attention was also called to the fact that the manufacturer's recommended dilution was apparently based upon tests performed with distilled water, and the margin of safety calculated with the pure water was now diminished or lost entirely. It was concluded that "pending the development of residual tests which provide an accurate measure of bactericidal efficiency, anyone contemplating the use of such compounds for disinfection would be well advised to make

Table 1. Interfering action of tap waters on germicidal properties of quaternary ammonium compounds. (Report of the U. S. Public Health Service, Sanitary Engineering Division, prepared for Conference of State Sanitary Engineers meeting, Washington, D. C., May 7-8, 1947.)

Compound Designated	Ppm Required to Produce 100% Kill of *E. coli* in 1 min. When Compound is Dissolved in			Ppm Recommended for Use by Manufacturer
	Pure Water	Cincinnati Tap	Norwood Tap	
A	20	100	250	234
B	10	70	220	190
C	5	500	3200+	78

bacteriological tests of the product under consideration in the water to be used and under the conditions in which it will be used." This had the effect of practically eliminating the quaternary ammonium compounds from many fields such as the dairy industry, where it would be very impractical to attempt to test the compound in the well water of each individual producer. The result was that many health departments eliminated them entirely on the basis of this report.

At about this same time there were several other investigators working on the problem of interfering substances. In 1948 Ridenour and Armbruster (18) reported their studies on the effects of various waters using distilled water, a softened water (lime-soda treatment) with a soap hardness of 80 ppm and a moderately hard, untreated water with a soap hardness of 275 ppm. They found that to obtain a 99.9 per cent kill, the following concentrations of quaternary ammonium compound were necessary in each of the waters: 34, 75, 95 ppm. They ran a survivor-curve type of test, and a study of the shape of the curves indicates that there is some sort of neutralization by the water substances. This was especially true in the moderately hard water, in which the reduction in bacteria was very small up to a concentration of 50 ppm

of the germicide. Above that point, the rate of increase of per cent reduction was quite rapid with an increase in the concentration. They concluded that above a certain minimum, the rate of kill was about the same, although the amounts required were, of course, greater in the case of the hard water. They continued this investigation to include an examination of certain factors which might be responsible for this interference. This included the cations calcium, magnesium, sodium, and potassium, and the anions sulfate, nitrate, chloride, normal phosphate, and carbonate. They found that the calcium and magnesium gave a definite adverse effect on the germicidal action. The sodium and potassium had little effect. With the anions, the effect was about the same for all of them up to a salt concentration of 70 ppm, with the exception of phosphate and carbonate, which showed an increase in germicidal activity. Since these two were tested at extremes of the pH range, 9.0 and 1.0 respectively, this was believed to be the cause of this reaction.

In a comparative study of the properties of the quaternary ammonium compounds with the hypochlorites, reported in 1948, Shere (20) pointed out a marked reduction of the germicidal properties of the former group when used in the presence of hard

water. He tested a series of quaternary ammonium germicides in two waters of 0 ppm hardness and 400 ppm hardness, and found that the increase in concentration necessary to give kill in 10 minutes varied from two to four hundred times in the case of the hard water. When a water-conditioning agent (trisodium phosphate) was added in the amount of 200 ppm, the necessary amount of quaternary ammonium compound increased in the case of the hard water. Shere also noted that in the first part of the test there was no indication other than bacteriological data to show that any change had taken place in the presence of the hard water. Chemical tests showed the concentration of quaternary ammonium compound to be the same in both series.

In 1948, Mueller and Seeley (15) reported that they had undertaken a study to obtain more information on the controversial subject of the constituents in water as they affect germicidal activity. They reported no close correlation between the water hardness (as determined by soap) and the germicidal action. The differences in pH found in the natural waters tested were not significant. With as much as 1,000 ppm of calcium or magnesium present, they were able to show that 200 ppm of quaternary ammonium compound was able to give approximately 100 per cent

kill in a period of 8 minutes. However, as much as 10 ppm of ferric iron completely inactivated the quaternary ammonium compound. They concluded that the results to that date would indicate that 200 ppm of the germicide has sufficient reserve germicidal potential for most jobs.

The following year, Mueller (14) reported that this effect of the ferric iron can be removed by the use of a balanced detergent sanitizer, although he does not state what ingredients would be included in this formulation. The report of a detailed study was made later by Mueller and Seeley (16) on the effect of metallic ions on the germicidal activity of the quaternary ammonium germicides. The list of ions which they tested against Roccal included potassium, sodium, calcium, magnesium, zinc, nickel, manganese, barium, cupric copper, ferrous and ferric iron, and aluminum in the form of the chloride salts. Potassium, sodium, lithium, and ferric sulfates were included, and also sodium nitrate. The test organism they employed was E. coli in a concentration of about 10×10^6 organisms per ml. After varying intervals (usually 1 and 5 minutes) the material was neutralized with sodium naphuride. In studying the direct effect of pH, they found that there was no evidence of reduction in bacterial numbers between

the pH levels of 2.0 and 10.0. The germicidal action of the Roccal was decreased in a lowered pH.

Of the monovalent salts tested, none showed interference with 200 ppm of the quaternary ammonium compound. A 2.0 M solution of sodium chloride gave a marked reduction, but this was far in excess of any practical situation. Of the divalent salts, a 300 to 450 ppm concentration of calcium and magnesium was necessary to show any reduction in the germicidal action of a 200 ppm solution of the Roccal, and then only in the 30-second, one- and two-minute intervals. It was necessary to go to a salt concentration of 800 ppm to give a reduction of activity in the 5-minute test. In the case of the trivalent ions, only a small quantity was necessary to inactivate the germicide. They determined that this effect increased in a ratio of 1:100:10,000 for the mono-, di-, and trivalent salts. This means that a 2.0 M sodium chloride, 0.02 M manganese chloride and 0.0002 M aluminum chloride would all give about the same interference pattern. In summation, they state that all of the cations tested interfered with the germicidal action and the factor of valence had the most effect in determining the degree of this interference.

About this time other workers, Weber and Black (24) and Foster (5), noted the effect of the hard water ions in interfering with action of the quaternary ammonium compounds.

A continuation of the studies cited in the statement of the U. S. Public Health Service was reported by Butterfield, Wattie, and Chambers (2), in which they showed the results of tests on six different tap waters. They found that on these waters the concentration in ppm of germicide that was necessary to give 100 per cent kill in a period of 1 minute varied from 60 to 320 ppm. Using distilled water buffered to 8.0, 7.0, and 6.6, the amount required was less than 50 in all cases; while a sample of Cincinnati tap water, to which a trace of sodium borate had been added, required 500 ppm of the germicide. This study was extended to include combinations of chemical constituents in distilled water, but none was found that could reproduce consistently the results in natural waters. It was also noted that any modification of the natural water (boiling, aeration, autoclaving, or filtration) altered the degree to which the quaternary ammonium germicide was affected by the water. No accompanying change in pH or turbidity nor precipitation occurred to give visual evidence of alternation. Thus, they stated that the change in bactericidal efficiency must

be due to factors other than the chemical constituents ordinarily determined.

The remainder of this study was carried out on three waters--distilled water buffered with Clark and Lubs buffer to adjust the pH from 7.0 to 9.5; Cincinnati tap water; and Norwood, Ohio, tap water. These waters were reported by Lawrence (9) to be approximately 155 ppm and 445 ppm hardness. The studies of Butterfield et al. were carried out in a volume of 500 ml using a concentration of E. coli strain 198 of about 1,000 to 2,000 organisms per ml. Their criterion of satisfactory disinfection was 100 per cent kill in 1 minute. This they based upon the Ordinance and Code (22) which provides for a 2-minute contact time. They observed that perhaps this criterion was too lenient and the end point should be set at 15 or 30 seconds to give a sufficient margin of safety. Bacteriological examinations were made at the end of 1, 2, and 5 minutes in most of the tests by pipetting the desired amount into a Petri dish and pouring the agar at the end of the exposure period.

In a series of tests on fifteen different quaternary ammonium compounds, it was found that the average concentrations in ppm necessary to give the 100 per cent kill in 1 minute on the distilled,

Cincinnati, and Norwood Waters were 52:211:324, or a ratio of about 1:4:6. This was typical of most of the compounds not only in the 1-minute test, but for the 2- and 5-minute results as well. There was one exception in the case of an alkyldimethylbenzyl ammonium chloride which required a lower concentration with the Norwood water than with the Cincinnati water to give 100 per cent reduction in the 5-minute period. In using a quaternary ammonium compound plus a detergent, a very marked effect was noted in that the Norwood water required a concentration of over 15,000 ppm to give 100 per cent reduction in any of the time intervals. The germicide alone had given only slightly lower than average results, but the combination gave an exaggerated effect.

These workers also investigated the problem as to whether this interference was an instantaneous or a progressive one. In a series of sixty-six tests, they could find no case in which the interference seemed to increase after the first 1 or 2 minutes--the shortest intervals that were tested after the dilutions of the germicide were prepared from the tap waters. They concluded that these compounds are stable in the diluting water after the first reduction in germicidal efficiency has taken place.

Lawrence (9) has reported findings to confirm the adverse effect of hard waters upon the germicidal action of several commercial quaternary ammonium compounds using gram-negative bacteria (E. coli, Salmonella typhosa) as the test organisms. Using the phenol coefficient technique, he found that the coefficients were reduced from about 250 to 55 in one case and 22 in another. Because of the use of these compounds in situations where gram-positive organisms predominate, a series of tests was run using strain 209 of Micrococcus pyogenes var. aureus in the presence of hard water containing 350 ppm of hardness. These data unexpectedly reveal that, with one exception, the germicidal activity was not impaired when using the gram-positive test organism in the same waters that had shown marked reduction in the action against the gram-negative group. Subsequent studies with synthetically prepared hard waters confirmed this observation.

Further studies by this investigator show that the addition of small amounts (0.05 per cent was found to be optimum) of trisodium phosphate will enhance the germicidal activity of the alkyldimethylbenzyl ammonium chloride he was using so that it compares with the action in distilled water.

Johns (8) also observed the effect of hard waters on this type of compound. Using both gram-negative and gram-positive organisms, he noted that in the case of the latter, there was no appreciable reduction in the germicidal efficiency from that observed in distilled water. When using the E. coli as a test organism, his results were comparable to those previously cited.

One of the more recent reports on the problem of hard waters is that of Botwright (1). This is primarily a field study comparing the use of an alkaline cleaner and hypochlorite system against the use of a detergent-sanitizer used on a group of dairy farms. However, the observations on the effect of the hard waters encountered is pertinent to this discussion. The hardness of the waters varied from 14 to 180 ppm at one station, and from 24 to 350 ppm (determined by the soap hardness test) at the other. In both cases, the hardness of the water on farms using the cleaner-sanitizer #5 exceeded that of the farms using the chlorine system. He stated that the hardness did not appear to affect adversely the quality of the milk as measured by the pasteurized milk counts. As a matter of interest, the producers having the hard water tended to produce a better milk.

EXPERIMENTAL PROCEDURES

There were two types of tests used in these studies. The first was a survivor-curve type of test following the procedures given by Weber and Black (23). This test was selected because much of the information on the interference of hard waters was obtained by the use of this technique. It was designed to evaluate the practical performance of germicides used in the food industry. The second test used was an "exhaustion" type of test, which is a modification of the "capacity" test presented by Cantor and Shelanski (3) and the field test for the evaluation of restaurant sanitizers used by Mallmann, Kivela, and Turney (11).

In the Weber and Black method, Escherichia coli, Cincinnati strain #198, is used as the test organism. The concentration of the inoculum is 100×10^6 organisms per ml in the reaction mixture. This number was selected on the basis of laboratory tests using an artificially contaminated dish water and a swab-rinse count of glasses which had been dipped in it and allowed to drain for 15 minutes. By making plate counts of both, it was found that the ratio of bacteria per ml of dish water to bacteria per glass was 100/1 in both heavily and lightly contaminated water.

They considered that high counts on the swab-rinse plates would be from several hundred thousands to one or two millions, and so the 100×10^6 organisms per ml would represent a heavy load of contamination in dishwashing. The organisms were taken from an agar slant by washing and suspending in the water to be tested and then diluted such that the final concentration will have the desired number of organisms. A slight variation from their technique was introduced here by shaking the culture in the standard dilution bottle for 2 minutes in place of the twenty-five times as recommended. This was done only in the one dilution prior to adding the suspension to the medication pot. At all other times the standard technique for making dilutions was followed.

As in any type of a speed reaction test, it is necessary to have an effective neutralizer to stop the action of the germicide at the exact end of the time interval being tested. There have been many suggested for this purpose, but the Weber and Black method indicates that asolectin in Tween 80 is satisfactory. Since a study of neutralizers was beyond the range of this study, this method of neutralization was adopted for all of the tests performed.

The exposure times they selected were 15, 30, 60, 120, and 300 seconds in order to give data comparable to the situations encountered in sanitizing dishes. The total volume of material in the test is 10 ml, and all of the solutions are held in a water bath at 25 C.

The testing procedure is as follows: a 5 ml amount of the bacterial suspension containing double the amount of organisms desired is placed in a medication pot, being careful that none of the organisms are splashed on the sides of the tube. In a second medication pot in the water bath is placed about 10 ml of the germicide being tested, adjusted to twice the desired concentration in the water under consideration. Five ml of the germicide solution is then rapidly pipetted into the suspension of organisms and the tube quickly swirled to aid in mixing. One ml of the suspension is then removed, and at 15 seconds from the time of mixing, it is quickly discharged into the dilution blank containing 9 ml of the asolectin solution. This is repeated at the end of 30, 60, 120, and 300 seconds from the initial time of mixing. Appropriate dilutions are then made from each of the blanks to insure accurate counting of the surviving organisms. A count is also made of a sample taken from the unused portion of the suspension which had

not been in contact with the germicidal agent and this is used as the initial count, or number of organisms at "0 seconds." All the plates were prepared with tryptone glucose extract agar (Difco) with no added milk, but containing 100 mg of lecithin (asolectin in Tween 80) per 100 ml of medium.

Additional controls are described by Weber and Black, but work by Mallmann, Alegnani, and Cope (13) using this technique indicated that these gave little additional information.

Plate counts were made at the end of 24 hours' incubation at 37 C. The results may be reported as actual densities of organisms surviving or as per cent reduction after each interval of exposure. The "endpoint" is 100 per cent kill of the test organisms.

The "exhaustion" test employed in these studies is similar to the "capacity" test of Cantor and Shelanski. In describing their technique (3), they defined the term "capacity" as "an attribute of germicides which determines the maximum number of incrementally added microorganisms, plus rigidly defined extraneous matter in solution or suspension, sterilizable by a given quantity of the germicide." Their test was designed to simulate more closely the actual conditions under which a germicide is used.

As two extremes of this they cite the use of antiseptics in wound treatment and the use of sanitizers in dishwashing. In both cases the contamination, both bacteria and extraneous matter, is being added incrementally. With this in mind, they outlined a procedure which would attempt to evaluate a situation analagous to this in the laboratory. Inasmuch as they are not interested in eliminating the presence of organic matter from the test, the culture recommended is the standard S. typhosa grown in FDA phenol coefficient broth. This furnishes a standard inoculum of organisms plus controlled extraneous matter. In those cases in which it was desired to test the germicide under adverse conditions, the amount of soil or organic matter was increased by mixing an equal quantity of sterilized whole milk with the broth culture of the test organism. This resulted in a broth suspension containing 2×10^8 bacteria per ml and approximately 6 per cent whole milk solids.

The test was performed by adding increments of 2.5 ml of the broth suspension (a total of 5×10^8 organisms) to 500 ml of the germicide and sampling at the end of 15 and 30 seconds. Ten minutes after the addition of each increment another was added and the 15- and 30-second samplings repeated. This was continued for a total of ten increments. For purposes of their

discussion, they also added the total of the ten increments (50 x 10^8 organisms) at one time to compare the ability of the germicide under these two types of situations. Plates were made of the samples in order to determine the per cent reduction in each case.

Although the "exhaustion" test parallels this technique to some extent, the fundamental information sought is slightly different. In this case, it was desired to determine how many organisms could be added to a germicidal solution before all of the germicidal activity had been depleted, or "exhausted." Since the speed with which the compound could react is not as important as the presence of some germicidal action, the time interval used in this case was 10 minutes. The organisms were prepared in a suspension such that 1 ml contains the number of cells suitable for addition at one time--usually as close to 200 x 10^8 as possible. When added to 200 ml of the germicidal solution, this gives an inoculum of 100 x 10^6, which is comparable to the concentration used in the Weber and Black tests.

The volume of 200 ml of the germicide was selected so that the removal of 1 ml of the solution for sampling and the addition of 1 ml of the bacterial suspension for each stage of the test would

not appreciably alter the concentration of the germicide in the reaction flask. In a series of fifteen increments, the reduction in concentration of the germicide would be approximately 7 per cent. The actual concentrations resulting in the flask at each successive stage are given in Table 2. Dilutions of the germicide were made in the various types of water under test. The culture suspension was made in distilled water so the amounts added would not change the hardness of the water to an appreciable degree. In addition, the decrease in concentration of germicide and hardness of the water might be considered as compensating errors. There was no extraneous matter added in this case because it was desired to follow the interaction of the germicide and tap water in the presence of the bacterial cells alone.

The test is performed by adding 1 ml of the bacterial suspension to the reaction flask, mixing well by swirling, and allowing it to remain in the 25 C water bath for 10 minutes. At the end of this interval, 1 ml is removed and placed in an asolectin-Tween 80 dilution blank containing 9 ml of the neutralizer. Although time which elapsed between the individual stages of the test was not considered important, the following increment was usually added 1 minute after sampling, or at intervals of 11 minutes

Table 2. Actual concentrations of germicide present in each successive stage of the exhaustion test when the initial concentration was 200 ppm.

Increment	Concentration in ppm
1	200.
2	199.
3	198.005
4	197.060
5	196.075
6	195.095
7	194.115
8	194.145
9	192.180
10	191.219
11	190.263
12	189.312
13	188.366
14	187.425
15	186.489

throughout the testing period. Suitable dilutions of the samples were made and plated on tryptone glucose extract agar to which the asolectin in Tween 80 had been added in the concentration 100 mg per 100 ml of medium. The concentration of the bacterial suspension was determined by taking a sample about midway in the testing period and plating as for the other samples.

This type of test is similar to the one used by Mallmann, Kivela, and Turney in assessing the value of sanitizers in beverage establishments (11). Although not specifically described as an "exhaustion" test, their method was to sample every tenth glass as it went through the washing and sanitizing process to determine the number of glasses that could be sanitized in a given concentration and volume of germicide before the germicidal activity began to diminish. In this way, as high as eight hundred glasses could be examined consecutively to determine whether or not the sanitizer would become exhausted.

The water hardness determinations were made using the Versenate method as outlined in the Technical Bulletin No. 2, of the Bersworth Chemical Company (26). The test is based upon the principle that the salts of ethylene diamine tetra acetic acid (referred to as Versene) will form very stable chelates with the

calcium and magnesium. Other less stable chelates with the calcium and magnesium are decomposed by the Versene. In addition to this there is an azo dye, Eriochromeschwartz T, which forms a colored complex compound with magnesium. Like the other less stable complexes, this also is broken down by the Versene to form the more stable Versene magnesium chelate.

The dye itself has a deep blue color at a pH of 10, but the magnesium complex formed is wine red. When Versene is added to a solution containing the red-colored chelate, a sharp change in color to the blue is noted when there is sufficient Versene to chelate all of the magnesium. Although the calcium does not form this chelate with the dye, it is included in the titration with the magnesium, since the Versene will chelate first with the calcium ions. In making the standard Versene solution for titration, a small amount of magnesium chloride is added to form the color reaction with the dye in case the water or solution being titrated contains only calcium and no magnesium.

The method is much simpler and more accurate than the soap hardness titration formerly used. For the determination of the amount of hardness in the samples used in these studies, the Versene titration method has been employed.

The determinations of the pH of the various test waters were made using a Beckman pH meter. Since most of these values were in the range 6.9 to 7.5, no specific mention is made of the pH in the results except in those cases which deviate from this range.

Each table giving results of a survivor-curve test represents a typical set of data selected from many trials to insure consistent results and is representative of many replicates. The data on the exhaustion tests correlate well with others in the series and represent few trials, in addition to those reported.

Other equipment and methods used were those accepted as standard for bacteriological work.

PRESENTATION OF DATA

According to the literature previously cited showing the interference of the activity of the quaternary ammonium compounds (QAC) in hard waters, it would appear that this group should be eliminated from use in this class of waters or that the concentration should be increased to the point where they will give results comparable to those obtained with distilled water. Practical experience in the field has shown, however, that this type of compound has been used routinely in hard water areas with very satisfactory results. Specific cases are the studies of Mallmann and associates on milking machine sanitizers (10), in restaurant utensil sanitation (12), and the studies of Botwright previously cited (1). There is also the experience of one of the local dairies that has been using a QAC in a hard water area for the past five years on its producers' farms with very satisfactory results. The first phase of this work was an attempt to investigate the reasons for these apparently conflicting situations.

As an approach to this problem, it seemed desirable to determine, if possible, whether or not the reaction between the QAC and the interfering factor in the water was a direct chemical

neutralization. It was postulated that if this were the case, the following situation would exist: When a solution of 200 ppm of QAC is made up in hard water, the germicidal activity might be comparable to that obtained with a concentration of 50 ppm of the same compound in distilled water depending upon the particular water used. If there existed a direct reaction between the factor in the water and the 150 ppm of QAC which was lost, then a concentration of 350 to 400 ppm of the QAC should give results comparable to a 200 ppm solution tested in distilled water. That this is not the case may be observed in Table 3.

These data show that using 50 ppm concentration of QAC in distilled water gives results that are comparable to the 200 ppm concentration used in the college tap water having a hardness of 385 ppm. By increasing the concentration in the distilled water by 150 ppm, there is more than enough germicidal potential to give complete kill in 60 seconds. Starting from the point of 200 ppm of QAC in the tap water, an increase in concentration of 300 ppm is not sufficient to give this degree of kill in the same time interval.

As further evidence of the relationship between the activity in distilled water as compared to tap water, the data in Table 4

Table 3. The effect of increasing the concentration of quaternary ammonium compounds in the presence of both distilled water and tap water.

Exposure Time in Seconds	Distilled Water		
	50 ppm*	100 ppm	200 ppm
	(organisms per ml remaining)		
0	165,000,000	165,000,000	165,000,000
15	55,000,000	26,300	40
30	39,000,000	2,480	270
60	4,700,000	350	0
120	36,000	10	0
300	1,150	0	0

* Concentration of quaternary ammonium compound.

Table 3 (continued)

Tap Water (385 ppm hardness)			
100 ppm	200 ppm	300 ppm	500 ppm
(organisms per ml remaining)			
34,000,000	34,000,000	34,000,000	34,000,000
64,000,000	17,500,000	8,000,000	3,400,000
12,700,000	10,000,000	700,000	620,000
10,000,000	710,000	80,000	38,000
3,500,000	98,000	38,000	0
950,000	180	0	0

Table 4. A comparison of the germicidal effect of QAC using distilled and tap water as the test waters.

Exposure Time in Seconds	Distilled Water (0 ppm)	
	100 ppm*	100 ppm
	(organisms per ml remaining)	
0	107,000,000	76,000,000
15	1,960	170,000,000
30	620	100,000,000
60	70	130,000,000
120	10	56,000,000
300	0	145,000

* Concentration of QAC.

Table 4 (continued)

Tap Water (330 ppm)		
200 ppm	300 ppm	400 ppm
(organisms per ml remaining)		
76,000,000	76,000,000	76,000,000
33,000,000	5,800,000	680,000
15,000,000	110,000	245,000
700,000	2,000	1,000
320	60	10
0	0	0

are presented. The hardness of the water had decreased at this time to 330 ppm, but the results are comparable to those at the slightly higher level.

These data all tend to confirm the reports in the literature that there is a certain degree of interference with the action of the QAC when used in certain hard waters. In order to find how high a concentration would be necessary to achieve the standard of complete kill in 60 seconds, using the Weber and Black technique, additional runs were made at increasing concentrations until this point was reached. These data are given in Table 5. They indicate that in order to use a compound of this type in the waters of this area, it would be necessary to increase the concentration of germicide to approximately 800 ppm or about four times the concentrations usually employed.

Additional study of these tables would indicate that in place of a direct "neutralization" of the QAC by the interfering substances in the hard water, there is rather a retarding of the germicidal action. Thus, in a situation where there is an extended period for the germicide to exert its effect (more than 1 or 2 minutes) it might be possible that the end result would be entirely satisfactory as far as a practical application of a QAC is concerned.

Table 5. To determine the concentration necessary to obtain 100 per cent kill in 60 seconds, using tap water as the diluent.

Intervals of Exposure in Seconds	Tap Water (300 ppm); QAC (200 ppm)	Tap Water (330 ppm); QAC (400 ppm)	Tap Water (310 ppm); QAC (800 ppm)
	(organisms per ml surviving)		
0	93,000,000	76,000,000	40,000,000
15	10,300,000	680,000	10
30	4,500,000	245,000	0
60	3,100,000	1,000	0
120	820,000	10	0
300	0	0	0

The next phase of the work was to study various factors associated with water hardness to determine the importance each might have in connection with the over-all problem. The first step in this series was to determine the interference that might be due to the presence of simple salts and organic matter. For this purpose the Weber and Black technique was run with the test solution containing 250 ppm of sodium chloride in one series and 250 ppm peptone in the other. The data are given in Table 6. The results are in accordance with the work of Mueller and Seeley (16) in regard to monovalent ions having very little inhibiting effect on the QAC.

The next point of attack was a determination of the relationship between the hardness of the water and the degree of interference with the germicidal action. To obtain a series of waters of this type, the tap water was softened by the addition of varying amounts of the complexing agent Versene, the tetra sodium salt of ethylene diamine tetra acetic acid. The resulting waters tested had a hardness of 270, 220, and 140 ppm, respectively. Although there are no outstanding differences, it can be seen in Table 7 that there is a consistent trend toward an increased germicidal efficiency in the softer water. This is in accord with previous

Table 6. The effect of the addition of sodium chloride and peptone in various concentrations of QAC.

Exposure Time in Seconds	Distilled Water (0 ppm)	NaCl (250 ppm)		
	100 ppm*	100 ppm	200 ppm	400 ppm
	(organisms per ml remaining)			
0	21,000,000	25,000,000	25,000,000	25,000,000
15	16,800	38,000	40	10
30	3,600	28,000	100	40
60	2,250	2,250	0	0
120	670	30	0	0
300	550	0	0	0

Exposure Time in Seconds	Distilled Water (0 ppm)	Peptone (250 ppm)		
	100 ppm*	100 ppm	200 ppm	400 ppm
	(organisms per ml remaining)			
0	91,000,000	98,000,000	98,000,000	98,000,000
15	11,500	24,000	360	580
30	1,700	51,000	390	10
60	3,950	5,100	0	0
120	750	2,950	0	0
300	20	20	0	0

* Concentration of QAC.

Table 7. Effects of varying amounts of Versene added to tap water using a concentration of 100 ppm QAC.

Exposure Time in Seconds	Distilled Water	Versene Softened Tap Water		
		140 ppm	220 ppm	270 ppm
	(organisms per ml surviving)			
0	95,000,000	93,000,000	86,000,000	90,000,000
15	1,980	21,500,000	64,000,000	56,000,000
30	840	1,400,000	25,000,000	52,000,000
60	30	160,000	1,700,000	18,500,000
120	90	310,000	36,000	1,200,000
300	0	0	10	1,320

work, but other data in this study did not seem to show as much effect with the same amount of change in the amount of hardness. To determine the effect of other methods of softening, a series was tested using distilled water, tap water (315 ppm), a 1:1 ratio of tap and distilled (165 ppm), a zeolite-softened water (180 ppm), and a Versene-softened water (160 ppm). The data in Table 8 indicate that there is no appreciable difference in the first three "softened" waters, but in the case of the Versene-treated sample, there is a definite increase in the germicidal action. When this was repeated on this group of waters using 400 ppm of the QAC, any differences (including the tap water of 345 ppm) were not great enough to be significant. However, the previous series did raise the question as to whether there was some factor present in the case of the Versene which was enhancing the germicidal activity of the QAC independently of the effect upon the hardness of the water.

To investigate this possibility, two series of tests were set up using only distilled water and adding the Versene to one group. The concentrations of germicide used were 50, 100, and 200 ppm. The data in Table 9 illustrate very clearly the increase in the germicidal action of this QAC in the presence of the Versene. It

Table 8. The effect of softening methods on the interference with germicidal activity of a 200 ppm concentration of Quaternary Ammonium Compound.

Exposure Time in Seconds	Distilled* (6 ppm)	Tap* (315 ppm)
	(organisms per ml surviving)	
0	500,000,000	530,000,000
15	40	> 100,000
30	20	> 100,000
60	0	> 100,000
120	0	> 100,000
300	0	290

* pH value of solutions:

Distilled	7.5
Tap	7.2
Zeolite softened	7.2
Tap + distilled	7.2
Tap + Versene	6.4

Table 8 (continued)

Zeolite Softened* (180 ppm)	Tap + Distilled* (165 ppm)	Tap + Versene* (160 ppm)
	(organisms per ml surviving)	
470,000,000	650,000,000	480,000,000
> 100,000	> 100,000	40,000
> 100,000	> 100,000	50,000
> 100,000	> 100,000	1,900
5,700	530	500
0	0	< 100

Table 9. Effect of Versene on Germicidal Activity of a Quaternary Ammonium Compound in distilled water.

Exposure Time in Seconds	Distilled Water - pH 6.9		
	50 ppm*	100 ppm	200 ppm
	(organisms per ml remaining)		
0	165,000,000	165,000,000	165,000,000
15	55,000,000	26,300	40
30	39,000,000	2,480	270
60	4,700,000	350	0
120	36,000	10	0
300	1,150	0	0

* Concentration of QAC.

Table 9 (continued)

Distilled Water and Versene, pH 6.9 - 7.0		
50 ppm	100 ppm	200 ppm
(organisms per ml remaining)		
95,000,000	95,000,000	95,000,000
190,000	9,000	30
13,000	30	0
4,000	0	0
0	0	0
0	0	0

may also be noted that at a concentration of 200 ppm in distilled water, there is sufficient germicidal power that the added effect of the Versene is not noticeable, the same thing that was noted in Table 8.

Since only the end result was studied in each of these cases, there was no indication as to whether the Versene was acting upon the QAC or whether it was altering the bacterial cell in such a way as to make it more susceptible. This was studied in both distilled and tap water by adding the Versene to the germicide in one series and to the suspension of organisms in the other for each of these test waters. These data, together with the controls for each water, are given in Table 10. Although there is some indication from this one trial that the combination of the Versene and culture gave a greater reduction, the differences are not great enough to draw any definite conclusions. Since this was somewhat of a digression from the main problem of the thesis, no additional trials were made.

This increased activity of the QAC when combined with Versene is in accordance with the data cited in the manufacturer's Technical Bulletin No. 2 (26).

Table 10. Effect of varying the initial contact point of Versene.

Exposure Time in Seconds	Distilled Water (5 ppm); QAC (50 ppm)		
	Control	Versene + QAC	Versene + Culture
	(organisms per ml remaining)		
0	210,000,000	210,000,000	180,000,000
15	10,000,000	13,600	400
30	6,000,000	40	20
60	1,900,000	10	20
120	148,000	0	0
300	280	0	0

Table 10 (continued)

Tap Water (300 ppm); QAC (200 ppm)		
Control	Versene + QAC	Versene + Culture
	(organisms per ml remaining)	
170,000,000	170,000,000	240,000,000
11,600,000	1,850,000	220,000
900,000	11,000	6,400
19,000	360	1,850
320	20	10
0	0	0

Briefly, the work that has been presented has shown that there is an interference with the germicidal action of the QAC in the presence of hard water, although this does not seem to vary proportionally to the degree of hardness of the water. The method by which the water is softened has little effect except in the case of the Versene. This effect has been shown to be independent of the softening process. However, from these data there is no indication of the reaction which might occur in this interference, nor the physical state of the QAC after it has been added to the hard water. There is a possibility that the QAC which has been "tied up" so that it cannot give the same reaction that takes place in distilled water, is still present in the solution and may have the same germicidal potential. An attempt to assess the amount of this unused potential was made by running a series of three consecutive survivor-curve tests using the same germicidal solution. This could also be considered as a test to determine how many organisms could be added to the germicidal solution before the action became depleted--or exhausted. Inasmuch as the total volume in the medication pot is only 10 ml, and the amount removed for sampling in each series is 5 ml, the concentration of the germicide in each successive series would be

50 per cent of that in the preceding one, due to the 5 ml of culture suspension added each time. This necessitated a separate control for each stage of the test. These data are presented in Table 11.

Because of the difficulties involved in this technique, the volume of germicidal solution was increased to 200 ml and the volume of suspension reduced to 1 ml. The concentrations of both germicide and bacterial suspension were adjusted to give the same ratio as that sought in the standard Weber and Black technique. Several trials were made with this technical arrangement and the results presented in Table 12. These data show that the germicidal activity of the solutions is almost the same in each stage of the test. As judged by the endpoint of 100 per cent kill, there is very little difference. However, if the entire curve is examined, it will be seen that the 15- and 30-second survivors increase with each stage. This would indicate that the germicidal capacity of the QAC is decreasing with each increasing increment of bacteria, but not enough in three stages to show up at the 5-minute period. This raised the question of how long can this dosage of organisms be repeated before the germicide becomes exhausted. For the purpose of studying this question, the "exhaustion" test was evolved.

Table 11. The effect of adding three successive increments of bacteria to a solution of QAC.

Decreasing Concentration of QAC Dilution	Exposure Time in Seconds	Surviving Organisms After Added Increments	Control for Each QAC Concentration (org./ml)
	(1st increm.)	91,000,000	(200 ppm) 91,000,000
200 ppm	15	120	30
	30	40	0
	60	10	10
	120	0	10
	300	0	0
	(2nd increm.)	91,000,000	(100 ppm) 91,000,000
100 ppm	15	Innumerable	23,600
	30	Innumerable	520
	60	260	270
	120	10	0
	300	0	0
	(3rd increm.)	91,000,000	(50 ppm) 91,000,000
50 ppm	15	Innumerable	Innumerable
	30	Innumerable	Innumerable
	60	Innumerable	Innumerable
	120	Innumerable	2,900
	300	Innumerable	300

Table 12. Germicidal efficiency after repeated dosages of organisms.

Exposure Time in Seconds	Distilled Water; QAC (100 ppm)	Trial 1
	(organisms per ml)	
1st Increment		
0	83,000,000	110,000,000
15	14,300	171,000
30	1,110	14,600
60	0	2,000
120	0	110
300	0	-
2nd Increment	83,000,000	110,000,000
15	16,100	Innumerable
30	120	3,600,000
60	0	25,000
120	0	140
300	0	0
3rd Increment	83,000,000	
15	6,200	
30	170	
60	0	
120	0	
300	0	

Table 12 (continued)

Tap Water (315 ppm); QAC (200 ppm)		
Trial 2	Trial 3	Trial 4
(organisms per ml)		
84,000,000	790,000,000	800,000,000
185,000	45,000	360,000
21,000	9,200	430
2,400	60	40
580	0	0
0	0	0
84,000,000	790,000,000	800,000,000
14,000,000	6,000,000	7,800,000
210,000	20,000	570
3,500	600	60
-	4,000	240
-	0	10
84,000,000	790,000,000	800,000,000
42,000,000	26,000,000	33,000,000
2,250,000	2,460,000	2,100,000
113,000	170,000	41,000
1,800	10	180
2,750	0	0

Since the speed of kill was not the important factor here, but rather the ability of the germicide to kill in a reasonable length of time, the survivor-curve type of test was not used. In place of the series of time intervals, tests were made only at an interval of 10 minutes after the addition of each increment of the culture. The specific techniques of this "exhaustion" test have been described in a previous section. The first series was run on distilled water, tap water, and two intermediate mixtures of these two with the resulting levels of 5, 115, 220, and 315 ppm hardness. Each sample was plated in duplicate, and the average of the two is reported in Table 13. In all four cases, the germicide was able to give the same degree of germicidal activity in the seventh increment as in the first. In other words, the exhaustion point of 100 ppm of the QAC used is beyond the limit of this series in which a total of more than 500×10^8 organisms was added. In order to obtain any differential information, it would be necessary to go through more increments than was done in this case.

A series of these exhaustion tests was performed on different waters using 75, 100, 150, and 200 ppm of the QAC to determine the endpoint of these various concentrations of the

Table 13. Results of the exhaustion test applied to four different waters of varying hardness, using 200 ppm QAC.

Increment	300 ml Distilled (5 ppm)	200 ml Distilled + 100 ml Tap (115 ppm)	100 ml Distilled + 200 ml Tap (220 ppm)	300 ml Tap (315 ppm)
	(organisms per ml remaining 10 minutes after each successive dosing)*			
1st	25	10	0	30
2nd	150	10	10	320
3rd	5,000	0	5	20
4th	0	10	50	10
5th	10	0	2,300	10
6th	5	0	35	20
7th	0	10	-	460

* Average of two plates.

germicide. These data are presented in Tables 14 through 17. A study of these tables reveals that, as the concentration of the germicide increases, there is a corresponding increase in the number of increments of culture which can be killed in a 10-minute period. This is true of both the distilled and the tap water. It also shows that even in the case of the highest concentrations tested in the series, the germicidal efficiency of the QAC in tap water is rapidly exhausted under these conditions.

However, these tests still gave no indication as to the condition of the QAC which obviously had been removed from action. Since previous tests had shown the activity to be increased by the addition of Versene, and since this is a strong complexing agent which might have some effect in releasing any QAC which might be tied up but still unaltered chemically, another series was run. In this case the plan was to start an exhaustion test and continue it for several increments. Then some of the Versene would be added in a quantity sufficient to soften the water to 0 ppm hardness. As controls, three other series accompanied this one. These consisted of one series using distilled water, a second with tap water containing the same amount of Versene that was to be added to the third series, which was started in tap water alone,

Table 14. The exhaustion test applied to two waters using a concentration of QAC of 75 ppm.*

Increment Number	Distilled Water	Tap Water (310 ppm)
	(organisms per ml remaining after each successive dosing)	
1	20	Innumerable
2	0	Innumerable
3	0	Innumerable
4	Innumerable	Innumerable
5	16,000	Innumerable
6	240	Innumerable
7	26,500	Innumerable
8	40,000	Innumerable
9	Innumerable	Innumerable
10	Innumerable	Innumerable
11	Innumerable	Innumerable
12	Innumerable	Innumerable

* Concentration of organisms per ml added at the beginning of each increment - 51,000,000.

Table 15. The exhaustion test applied to two waters using a concentration of QAC of 100 ppm.*

Increment Number	Distilled Water	Tap Water (310 ppm)
	(organisms per ml remaining after each successive dosing)	
1	0	Innumerable
2	500	Innumerable
3	40	Innumerable
4	0	Innumerable
5	30	Innumerable
6	20	Innumerable
7	14,000	Innumerable
8	5,800	Innumerable
9	0	Innumerable
10	30	Innumerable
11	500	Innumerable
12	35,000	Innumerable

* Concentration of organisms per ml added at the beginning of each increment - 48,000,000.

Table 16. The exhaustion test applied to two waters using a concentration of QAC of 150 ppm.*

Increment Number	Distilled Water	Tap Water (310 ppm)
	(organisms per ml remaining after each successive dosing)	
1	0	60,000
2	0	9,800
3	10	300,000
4	10	Innumerable
5	0	Innumerable
6	0	Innumerable
7	0	Innumerable
8	0	Innumerable
9	10	Innumerable
10	10	Innumerable
11	0	Innumerable
12	30	Innumerable
13	0	Innumerable
14	0	Innumerable
15	0	Innumerable

* Concentration of organisms per ml added at the beginning of each increment - 76,000,000.

Table 17. The exhaustion test applied to two waters using a concentration of QAC of 200 ppm.*

Increment Number	Distilled Water	Tap Water (310 ppm)
	(organisms per ml remaining after each successive dosing)	
1	0	370
2	0	300
3	0	660
4	0	8,400
5	10	60,000
6	10	Innumerable
7	4,100	Innumerable
8	20	Innumerable
9	0	Innumerable
10	0	Innumerable
11	0	Innumerable
12	0	Innumerable
13	0	Innumerable
14	0	Innumerable
15	0	Innumerable

* Concentration of organisms per ml added at the beginning of each increment - 58,000,000.

and a fourth series using only tap water. The results are given in Table 18.

In the three "control" runs, the results are as would be expected from the previous data. The tap water was only carried out to seven increments, as this had been shown to be sufficient to reach an endpoint. The interesting thing about these data is the reaction following the addition of the Versene to the reaction flask prior to the fifth inoculation. Not only were almost all of the organisms added at that time destroyed, but also those which must have been accumulating from the first four increments. Apparently, the QAC which is inactivated by some factor in the hard water is still capable of exerting germicidal action if it can be released.

It is interesting to note also that in the case of the flask in which the hard water sample had been discontinued after seven increments, the count was reduced to 60 organisms per ml after this mixture had stood for approximately 90 minutes. This would suggest the possibility that over an extended period such as this that the germicidal activity of the QAC is also released.

Table 18. The exhaustion test as applied to several waters using 100 ppm concentration of QAC.[1]

Increment Number	Distilled Water (0 ppm)	Tap + Versene[2] (0 ppm)	Tap Water (310 ppm)	Tap Water (310 ppm)
		(organisms per ml remaining after each successive dosing)		
1	0	0	Innumerable	Innumerable
2	0	0	Innumerable	Innumerable
3	20	0	Innumerable	Innumerable
4	0	0	Innumerable	Innumerable
			(Versene added[2])	
5	0	0	170	Innumerable
6	0	10	0	Innumerable
7	50	0	0	Innumerable[3]
8	310	0	0	
9	0	0	0	
10	10	10	20	
11	0	0	60	
12	10	0	20	
13	10	0	70	
14	2,100	0	20	
15	25,000	0	0	

[1] Concentration of organisms per ml added at the beginning of each increment - 18,000,000.

[2] 500 mg Versene added in each case.

[3] 90 minute sampling - 60 organisms per milliliter.

DISCUSSION

A study of the literature on the subject of interfering substances in hard water, especially in those studies where the Weber and Black technique was employed, indicates that the quaternary ammonium compounds are not suitable for use in a hard water. Using this method of testing, it has been shown that the water in the Lansing area falls in this category. During the period of testing, the hardness of the Michigan State College water supply has varied from 300 to 385 ppm. According to the Weber and Black test, the quaternary ammonium compounds could not be used in this water unless the concentration were increased to 800 ppm (Table 5). It has been previously stated that this is not in accordance with the actual experience in the field with this group of compounds.

The results of laboratory tests by the Weber and Black technique which indicate that the quaternary ammonium compounds would be incompatible in the college water supply are in opposition to the results of field tests showing that dairy farms in the adjacent area are successfully using quaternary ammonium compounds. However, these two findings are not incompatible, because the degree of

kill attained and the initial inoculum must be considered. The death curves obtained by applying a germicide to a bacterial culture are always characterized by a so-called resistant minority of organisms that extends the time of total kill markedly beyond the normal curve. In the case of death curves obtained with quaternary ammonium compound, the resistant minority appears to survive for a longer period than with most germicidal agents, although this group is relatively small in number--sometimes less than 10 organisms out of an original inoculum of 100,000,000 or more. To avoid this seemingly long period for total kill, many workers have suggested using an endpoint of 99, 99.9, or even 99.9999 per cent reduction as the endpoint. This would bring the laboratory results more in line with the field experience in this case. A closer examination of the data presented shows that in the case of the tests made using 200 ppm of the germicide, there was almost always at least a 50 per cent reduction at the end of 15 seconds. By the end of the 1-minute interval, the kill had reached a point of approximately 99 per cent. Looking now at the practical side of the situation, there are two factors to be kept in mind. The first is that in many instances, even where only a contact time of 1 or 2 minutes is allowed, a reduction of 99 per cent is a very

acceptable result. The second is the general situation in which a sanitizer would be used. Thorough cleaning should always precede the use of a sanitizing solution. This is necessary in order to remove the organic matter, debris, grease, or whatever type of soil might be present on the surface to be sanitized. If this is not done, there will be large amounts of material present that will either react with the germicide to chemically inactivate it, or act as a barrier between the germicide and the bacterial cell so that it cannot get to the bacteria even if there is some germicidal potential remaining. As a result of this thorough cleaning which is necessary to remove the soil that is present, most of the bacteria will be removed by mechanical action. This means that when the sanitizer is applied under the proper conditions, there should be a very low concentration of bacteria to be destroyed. Since the numbers of bacteria with which we are now dealing have thus been reduced to a very low number, a per cent reduction of 90 or perhaps even less would give a very satisfactory end result. For example, if the rate of kill from an actual survivor curve is applied to a theoretical inoculum of 1,700 organisms per ml, the following might occur:

Time in Seconds	Actual "Death Curve"	Theoretical "Curve" if Initial Inoculation were 1,700 org./ml
0	170,000,000	1,700
15	11,600,000	116
30	900,000	9
60	19,000	-
120	320	-
300	0	-

Thus, the 100 per cent endpoint would have been achieved in 60 seconds using the small initial inoculum, and an insignificant number was attained in 30 seconds. Another way of looking at this situation is that the amount of germicide that is usually employed is far in excess of that which is actually needed, and would therefore have a reserve of germicidal action which is not used. It is apparent from the data presented that in the case of distilled water, the use-dilution of 200 ppm quaternary ammonium compound is far in excess of that needed even when the initial dosage is about 100×10^6. In the presence of the hard water, this reserve is reduced, but it is apparent from the survivor-curve tests in which three successive increments were added that

the germicide has almost the same activity after each load of bacteria has been destroyed. If the same per cent reduction were applied in a situation in which the initial inoculum was much less--as would be the case with proper cleaning methods--then the end result would be such that there would be no public health hazard.

Another observation made from these tables is that the interfering action of the hard water has more effect upon the rate of kill than on the total kill. This would indicate a slightly different type of reserve which is not immediately available but may be released more slowly to exert its germicidal action over a longer period of time. This being the case, there is the possibility that in many practical situations the actual contact time would be much greater than the time allowed for the sanitizer to be in contact with the objects being treated. That is, in the field there is no attempt made to stop the germicidal action at the end of the contact time although there are situations where this would occur. That the germicide still has a potential capacity to kill large numbers of cells is demonstrated by the results given in Table 18 in the case of the tap water sample. When the test was discontinued at the end of the seventh increment, the organisms surviving were

such that a plate prepared from a dilution of 10^{-2} was completely overgrown. However, after 90 minutes, the germicide was able to reduce the number of cells from the total of 126×10^{6} that had been added to 60 organisms per ml (as represented by six colonies on the 10^{-1} dilution plate).

In actual practice, there are many instances where a relatively slow action from a sanitizer is a very acceptable situation. The sanitization of beverage glasses may be cited as an example. After the proper cleaning has been accomplished, the glasses carry a very small number of bacteria when they reach the germicidal rinse. Assuming the volume of the rinse tank to be about 5 gallons, the majority of the bacteria remaining on the first glasses to be introduced into a fresh solution would be removed from the glass by dilution. These organisms that had been washed off would then be attacked by the germicide. That is, much of the germicidal action would be taking place in the solution as well as on the surface of the glasses. As long as the bacterial count of the rinse water approached zero, the dilution effect would continue to remove organisms from the surface of the glasses and the end product--the bacterial count on the glass--would be satisfactory. The function of the germicide in this case would be to keep the

bacterial count of the rinse solution very low. To accomplish this, only a sufficient speed of kill would be necessary to destroy all of the organisms removed from one glass, or group of glasses, before the next was placed in the rinse water. As long as the rinse water does not become overloaded due to insufficient washing or some other factor, a relatively slow-acting germicide would be just as effective as one with a very rapid kill. If this situation of overloading did exist, the fast-acting compound would probably not be effective either due to the inactivating action of the soil present.

In each of these cases discussed, there is a portion of the compound which has been made germicidally inactive. The work of Salton in Australia (19) has shown that the bacterial cell adsorbed more of the germicide than was necessary to kill it. His test organisms were Pseudomas flourescens 542, and Micrococcus pyogenes var. aureus. Using four pH levels from 5.2 to 8.2 and germicide concentrations of 100, 200, 400, and 800 ppm of quaternary ammonium compound, he was able to show that the amount of quaternary ammonium salt adsorbed was the same for all four pH levels, but the germicidal action was dependent upon the pH of the solution. This indicates that the

process of adsorption on the surface of the cell is somewhat independent of the germicidal action taking place.

Based upon this work and the fact that the adsorption is dependent upon the isoelectric point of the cell which can be altered by a change in pH, an attempt was made to release this material in the following manner: Using a modified Weber and Black method, two neutralizing solutions were run in parallel, and inoculations were made simultaneously into each. One of these solutions was kept at the usual pH of 7.2, and the other was adjusted to 3.8. The data presented in Table 19 are evidence that there was little difference between the two pH levels in two separate trials. Although these data are inconclusive insofar as this particular problem is concerned, they are presented here to show that the neutralizer is apparently doing a good job. If there were some bacteriostasis taking place because of the germicide that was adsorbed on the cell surface, then the inocula in the acid blank should have shown increased growth because of the release of the quaternary ammonium molecules from the surface and their reaction with asolectin.

Table 19. A comparison of acid and neutral inhibitor solutions.

Exposure Time in Seconds	Distilled Water (0 ppm); QAC (100 ppm)		Tap Water (300 ppm); QAC (200 ppm)	
	pH 7.2	pH 3.8	pH 7.2	pH 3.8
	(organisms per ml surviving)			
0	98,000,000	120,000,000	66,000,000	66,000,000
30	120	550	1,250,000	830,000
60	20	0	120,000	98,000
120	0	0	2,500	1,700
300	0	0	0	0
	Second Trial			
0	240,000,000	240,000,000	250,000,000	250,000,000
30	21,000	19,000	140,000	120,000
60	300	230	11,000	7,200
120	30	0	100	70
300	0	0	0	10

Dennis (4) showed what happens when an in vivo test is used concurrently with an in vitro method. He employed two highly mouse-virulent organisms, Streptococcus pyogenes, strain C-203, and Pasteurella boviseptica as the test organisms. Using an exposure period of 2 minutes, he was able to show that the germicide in the presence of Norwood water gave a greater protection than when diluted in distilled water--another case in which the interfering action of the hard water did not seem to affect the end result.

All these factors presented indicate that the survivor-curve test as outlined by Black and Weber would not be a satisfactory test to use in the evaluation of a quaternary ammonium compound in hard water, especially when the contact period is more than 1 or 2 minutes.

Although the correlation between this laboratory method and the actual application seems questionable, the data presented do confirm the fact that there is some interference with the germicidal action of this group of compounds by the presence of hard water. It has also been shown that by the use of Versene, the germicidal efficiency of these compounds can be increased in hard water so that the speed of kill is comparable to that of the same concentration

of quaternary ammonium compound in distilled water. For several formulations of this type of combination, reference may be made to the bulletin of the manufacturer (26).

Additional studies on this compound (Table 9) have shown that there is also an increase in the germicidal activity of the quaternary ammonium compounds diluted in distilled water when the Versene is added. This indicates that the action of this compound is more than a simple removal of the hard water ions. No satisfactory explanation of this is known at the present time. It is reported (6, 26) that there is a zone at which the concentration of quaternary ammonium compound and Versene react with each other to form a cloudiness or precipitate accompanied by a sharp drop in germicidal activity. This can be recovered by increasing the concentration of the Versene beyond the zone.

Considering again the reaction of the Versene in the hard water solutions, there are several things which might be proposed as a result of this action. A simple equation for the chelating reaction of this compound can be given in this way:

Versene + metal ions $\longrightarrow$ Metal-Versene complex + sodium ions

This is given more in detail in Figure I. Two points from the

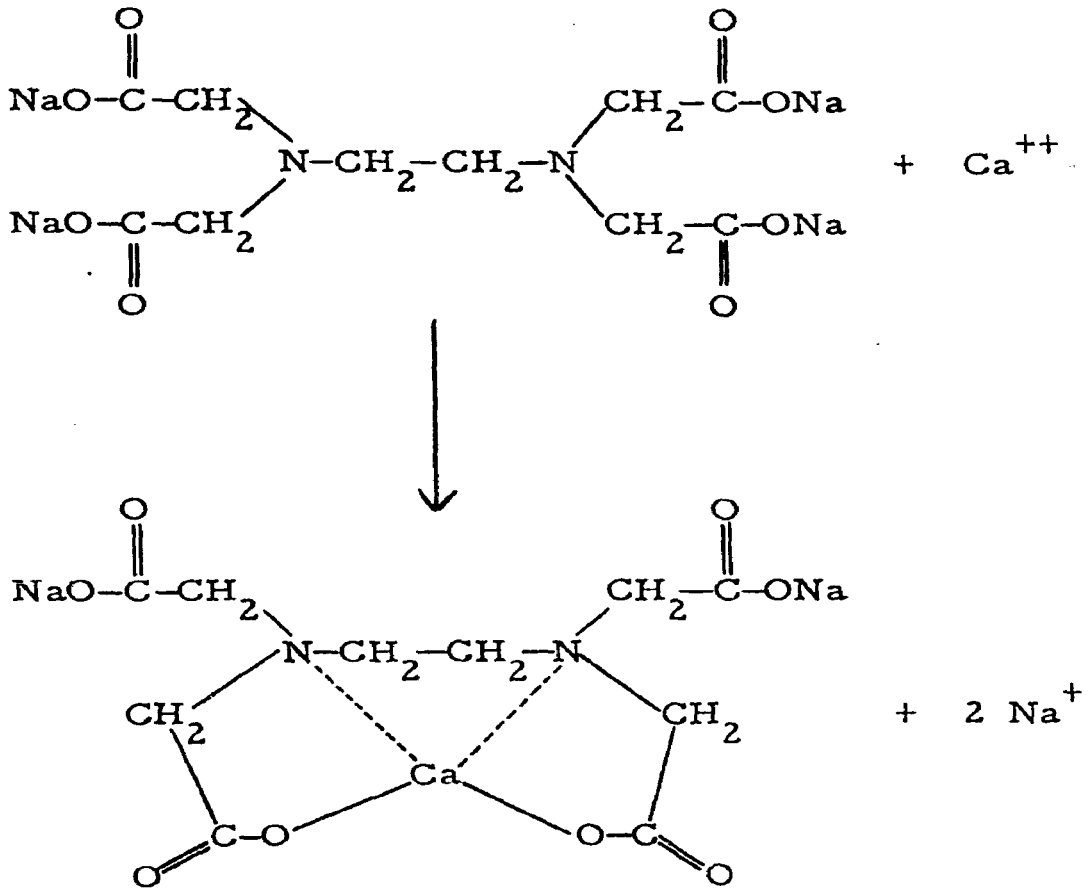

Figure I. The chelating reaction of Versene.

Technical Bulletin No. 2 are of interest here. The Versenes are nonspecific in their complexing action in that they will inactivate practically any metallic ion. Also, this complex is such that the Versene will break down simpler complexes and metal salts even though they may be insoluble.

Although it has been shown that there is more to the action than that of softening, it is likely that the main activity of the Versene in hard water is a complexing out of the factor that is the interfering substance. Thus, in the case of the exhaustion test in which the Versene was added after four increments, it seems likely that the action is due to this property of destroying complexes of a more simple nature to form a Versene complex. Although the combination of the interfering factor and the quaternary ammonium compound can only be speculated upon, it is evident that there is little, if any, alteration of the germicidal action of the quaternary ammonium compound taking place. This has been shown by the addition of the Versene resulting in the immediate release of a large amount of the germicidal agent.

Studying the information given in Table 10, it is not possible to determine whether the enhanced action of the Versene is due to some action on the bacterial cell or an alteration of the

germicide itself to make it more germicidal. The only differences indicate that there is slightly better germicidal action when the Versene is added to the bacterial culture first. This would be indicative of an action which altered the bacterial cell in some way to make it more vulnerable. However, it would be inadvisable to draw any definite conclusions from these limited data, especially since some of the work by Geotchius (6) would indicate that the action is better when the Versene is added to the germicide first. From a practical standpoint, the main thing is the increased germicidal activity which results; however, a continuation of these studies might be of interest from the standpoint of determining the mode of action of this phenomenon.

The use of the exhaustion test for the evaluation of quaternary ammonium compounds in the presence of hard water gives more information which correlates with the practical situation than does the survivor-curve type of test of Weber and Black. It is possible to show that there is a reserve of germicidal potential present even in the case of hard water that is not evident from the results of the Weber and Black method. This is demonstrated by the fact that the subsequent increments of culture will be killed until an endpoint is reached with the quaternary ammonium

compound in the "normal" state in the hard water. The test was easily adapted to the technique with the addition of Versene which has been discussed previously.

The time interval was selected with the idea that 10 minutes would give sufficient time for the action of the germicide to be completed. In this way an evaluation could be made of the "total" action taking place. That this was a false assumption can be seen by the results with the tap water as shown in Table 18. When the solution was allowed to stand for about 90 minutes, there was a marked increase in the germicidal action over the 10-minute period. These time intervals could be adjusted to give information for any time period desired from the 15- and 30-second intervals used by Cantor and Shelanski up to any desired interval. The repeated increments could be used in the same way with any interval.

By the interpretation of the results of the exhaustion test, it is possible to obtain an evaluation of a compound in the presence of hard water which is much closer to the actual conditions than the survivor-curve type of test. Although extensive tests would be necessary, it might be possible to establish a certain criterion as an endpoint by a comparison with the results of actual field

studies. Thus, a laboratory evaluation of the quaternary ammonium compounds in the presence of hard water could be made.

CONCLUSIONS

1. The interference of the hard water of the college water supply was shown to affect the rate of kill of the quaternary ammonium compounds to a greater extent than the total kill.

2. Data are presented to show that the germicide had been inactivated, but not completely destroyed.

3. The addition of Versene after several stages of the exhaustion test released the quaternary ammonium compound that had been inactivated so that it exerted a germicidal action comparable to that observed in distilled water.

4. Using a concentration of 200 ppm of quaternary ammonium compound, the addition of 870×10^6 organisms per ml in fifteen increments did not exhaust the germicidal capacity of this compound in the presence of distilled water. When diluted in tap water, the endpoint of germicidal action was noted after the addition of 340×10^6 organisms per ml.

5. The exhaustion test is presented as a method of obtaining a more realistic evaluation of the germicidal properties of the quaternary ammonium compounds in the presence of hard water.

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