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Studies on the Evaluation of the Concentration Exponent of Benzalkonium Chloride

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CONCENTRATION EXPONENT — De VANZO

STUDIES ON THE EVALUATION OF THE CONCENTRATION EXPONENT OF BENZALKONIUM CHLORIDE

By

John P. DaVanzo

A Thesis

In Partial Fulfillment of the

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INTRODUCTION

In recent years many have come to believe in the exponential function of the concentration of a disinfectant to be a reliable index in evaluating the germicidal effect of that compound.

According to Topley and Wilson (1946), in her early work Chick noted that the relationship between the concentration of the disinfectant and the rate at which bacteria are killed was exponential. This exponent varies with each type of disinfectant and expresses the effect that dilution has upon the germicidal efficiency of the disinfectant. A consideration of all the common disinfectants of her time showed that the products related to phenol exhibited the greatest coefficient of dilution, while chlorine and the medicinal dyes exhibited the least. Although a one per cent solution of phenol is relatively strong as a disinfectant and is capable of destroying cultures of Salmonella typhosa within a few minutes, solutions of 0.5 per cent are but feeble germicides. In contrast, halving the concentration of chlorine only approximately doubles the time required by this disinfectant to kill microorganisms.

In 1908 Watson found by calculus that the relation between the concentration of a disinfectant and the time required to kill a bacterial population could be

expressed as,

$$C^n.t = k$$

where C represents the concentration; n, the coefficient of dilution of the disinfectant; t, the time necessary for disinfection; and k, a constant. For purposes of this paper this equation will not be derived but rather will be accepted since it is essentially the well known equation for a monomolecular chemical reaction.

In order to determine the degree to which dilution lessens the rate of disinfection, it is necessary to establish the velocity with which different concentrations of the germicide kill bacteria.

Calculation is facilitated by expressing the relation between C and t in the logarithmic form,

$$n \log C - \log t = \log k.$$

Disinfection time can be determined for a series of disinfectant concentrations and the results expressed by means of equations as shown below:

$$n \log C_1 = \log k - \log t_1$$

$$n \log C_2 = \log k - \log t_2 \text{ etc.}$$

Successive pairs of equations may then be combined and by subtraction yield the derived equations:

$$n \log C_1 = \log k - \log t_1$$

$$- n \log C_2 = - \log k - \log t_2$$

$$n \log C_1 - n \log C_2 = \log t_2 - \log t_1 \text{ ; or,}$$

$$n (\log C_1 - \log C_2) = \log t_2 - \log t_1 \text{ ; thus,}$$

$$n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

for successive pairs of equations.

Concentration exponents have been determined only occasionally since they were formally introduced by Watson in 1908. Only two extensive investigations have been made since then. The first of these was made on the action of acids by Paul, Birstein, and Reuss in 1910 (Rahn, 1945). The later was made by Tilley (1939) on the phenols.

Tilley, in 1939 (See Figure I), has shown with work on phenol against Staphylococcus aureus that an average value for n can be determined for a series of disinfectant concentrations. His results are shown in Table I. On the other hand if the same values of n are plotted according to the procedure set forth by Watson, the result is a straight line. When the successive values for log C and log t are plotted against each other the results should be in an approximately straight line (Fig. 1). The value of n may then be calculated by the equation,

$$n = \frac{y_2 - y_1}{x_1 - x_2}$$

after selection of two suitable points (x_1, y_1) and (x_2, y_2) on the straight line drawn under guidance of the plotted points representing the individual tests. This gives possible

credence to the determination of an average value for n.

It can easily be seen from data such as these that the concentration exponent could prove to be of some value in the evaluation of disinfectants. No application of this principle has been found in the literature however for quaternary compounds. Many articles have appeared on the time it takes for a germicide to kill bacteria and on new methods used for evaluating germicides, but the effort stops at this point.

TABLE I

BACTERICIDAL EFFICIENCY OF PHENOL AGAINST STAPH. AUREUS
(Tilley, 1939)

DILUTION	C PARTS /1000	TIME MINS.	LOG C	LOG T	LOG C_1 - C_2 etc	LOG t_2 - t_1 etc	n	LOG A
1:55	18.1	5.0	1.25768	0.69897	.....	.....	....	7.87
1:60	16.6	7.5	1.22011	0.87506	0.03757	0.17609	4.69	7.83
1:65	15.4	15.0	1.18752	1.17609	0.03259	0.30103	9.24	7.94
1:70	14.3	20.0	1.15534	1.30103	0.03218	0.12494	3.88	7.88
1:75	13.33	30.0	1.12385	1.47712	0.03149	0.17609	5.59	7.88
1:80	12.5	40.0	1.09691	1.60206	0.02694	0.12494	4.64	7.85
1:85	11.7	55.0	1.06819	1.74036	0.02872	0.13830	4.81	7.83
1:90	11.1	80.0	1.04532	1.90309	0.02287	0.16273	7.11	7.86
					Average Values. . . .		5.7	7.87

Experiment conducted at 20° C.

TABLE I

BACTERIOSTATIC EFFICIENCY OF PHENOL AGAINST STAPH. AUREUS
(TILLEY, 1939)

DILUTION	PARTS/1000	TIME MINS.	LOG G	LOG T	C_2 use	$\log C_1 - \log t_1$ use	n	log A
1:65	15.1	5.0	1.25768	0.69897	.....	.....	....	7.07
1:60	16.6	7.5	1.22011	0.87506	0.02757	0.17609	4.69	7.89
1:65	15.4	10.0	1.18752	1.17609	0.02589	0.30103	9.24	7.94
1:70	14.3	20.0	1.15534	1.30103	0.02518	0.12494	3.88	7.88
1:75	13.33	30.0	1.12506	1.47712	0.02419	0.17609	5.59	7.88
1:80	12.5	40.0	1.09691	1.60206	0.02595	0.12494	4.64	7.85
1:85	11.7	55.0	1.06819	1.74036	0.02012	0.13830	4.41	7.83
1:90	11.1	80.0	1.04532	1.90309	0.02287	0.16273	7.44	7.86

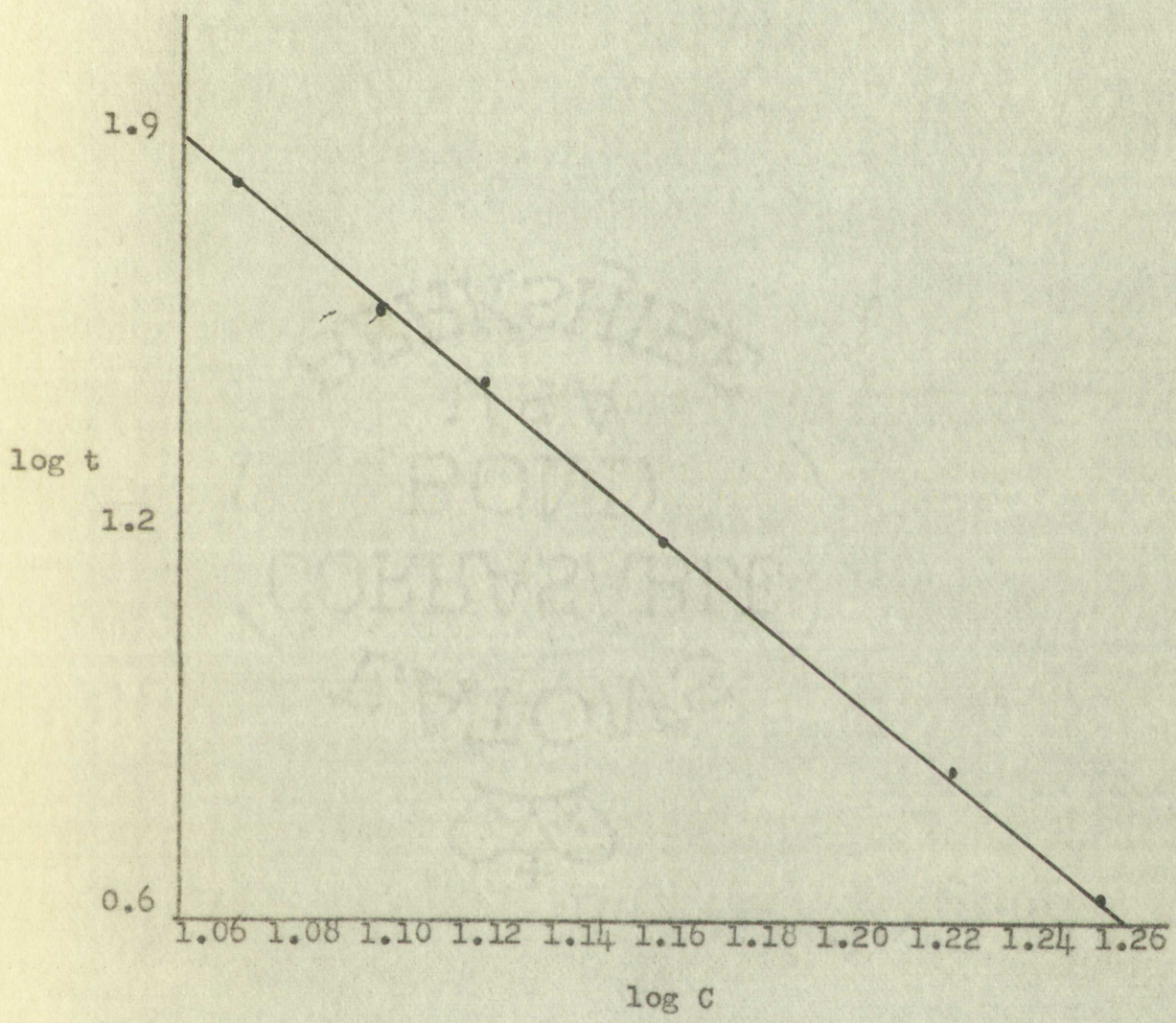
Average values. . . . 5.7 7.87

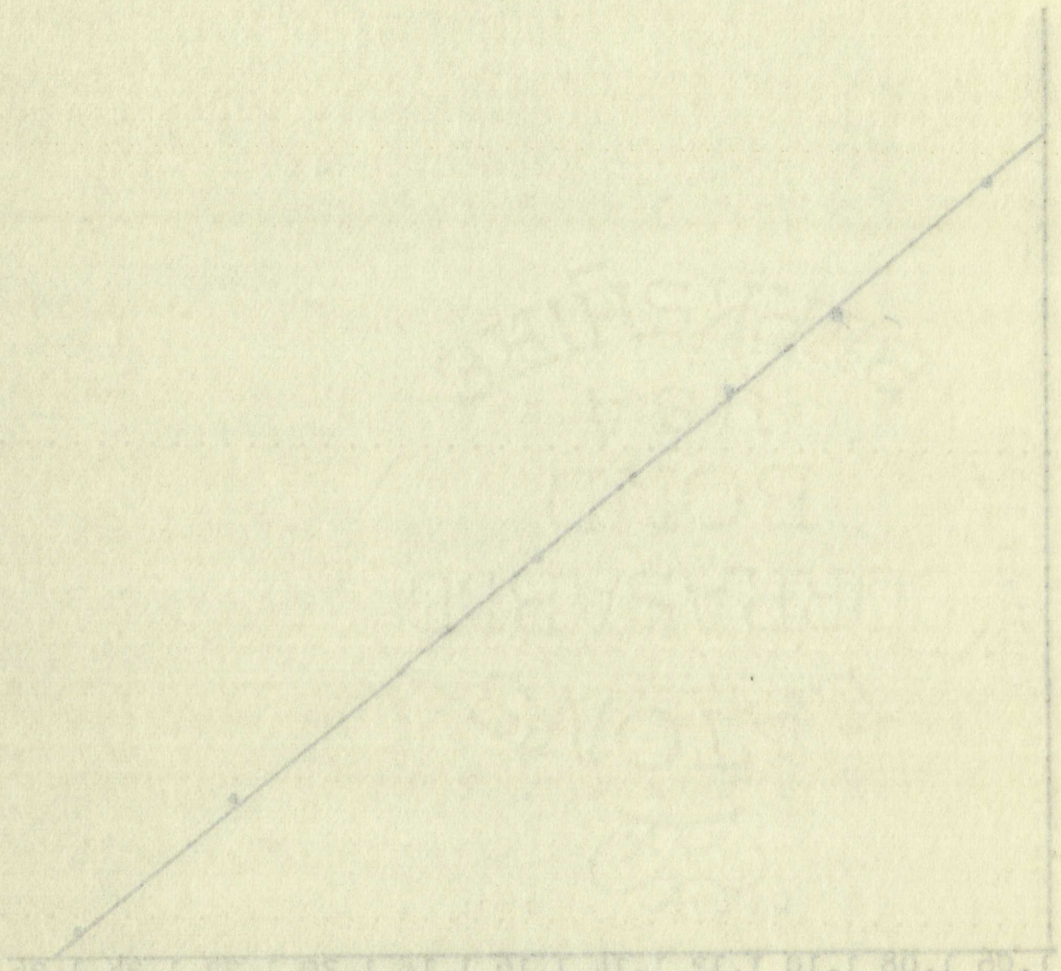
Experiment conducted at 20° C.

FIGURE I

BACTERICIDAL EFFICIENCY OF PHENOL AGAINST STAPH. AUREUS

n = 5.75




1.9
1.2
0.6

MATERIALS AND METHODS

The quaternary ammonium compound benzalkonium chloride was selected for this work because of its ready availability and because it already enjoys widespread use as a chemical sanitizing and disinfecting agent. The test organism of choice was Escherichia coli strain 198, obtained from the U. S. Public Health Service and originally used by Weber and Black (1948) for quaternary testing. In order to reduce variation in the organism to a minimum all cultures used throughout the experiment were maintained under oil in the refrigerator.

A constant temperature water bath of fourteen gallon capacity was employed in this work. An immersion type Calrod heater was placed at one end of the water bath with an electric stirrer at the opposite end to provide for even distribution of heat. A microset, differential range type thermoregulator with an adjustable column of mercury and an adjustable tungsten contact was inserted into the system with a supersensitive mercury relay. The tungsten contact was filed to a sharp point to insure more precise temperature control. A bureau of standards calibrated thermometer calibrated to one-tenth of one degree was suspended in the middle of the bath. All the runs were carried out at 37° C. with an accuracy of plus or minus 0.1° C.

The test was patterned after that of Weber and Black (1948) although the test organism was not prepared

as advocated by these authors. They proposed growing the test organism on an agar slant for twenty-four hours after which 5 ml of sterile distilled water were added to the slant. The organisms were then loosened and diluted to 100 ml. This was supposed to give an initial count of 300,000,000 organisms per cc. After preliminary investigation, this method proved awkward and gave inconsistent counts. To obtain a greater degree of consistency, the test organism was inoculated directly into 10 ml of broth containing 0.5 per cent casamino acids and 0.25 per cent dextrose (adjusted to pH 7.4 after autoclaving) and allowed to grow at 37° C. for twelve hours. At the end of that time the organisms were washed twice by centrifugation in 10 ml of sterile distilled water and the volume adjusted to 100 ml. Five ml of this suspension were transferred to a medication tube which was then placed in the water bath and warmed to 37.0° C. Appropriate dilutions from the original 100 ml aliquot were made at this time and plated with tryptose glucose beef extract agar plating medium in order to see how many organisms were initially present. The organisms in this initial count usually averaged 300,000,000. Only those tests where the initial count ranged from 250,000,000 to 350,000,000 were considered when calculating the concentration exponents.

The germicide was prepared by pipetting the appropriate aliquot of benzalkonium chloride concentrate directly into a 99 ml distilled water blank, shaking thoroughly, and allowing

it to warm to 37.0° C. in the water bath. The portions of the benzalkonium chloride used to calculate the concentration exponents were 0.03 ml, 0.05 ml, and 0.07 ml. This represented dilutions of 15 ppm, 25 ppm, and 35 ppm respectively. The benzalkonium chloride solution used throughout the test procedures was taken from the same batch in order to insure consistency of results.

It is generally well known that calcium and magnesium interfere with the germicidal properties of quaternary ammonium compounds. In the experiments in which the effect of calcium on the system was to be determined, a solution of $CaCl_2$ was added which was calculated to give forty parts per million of the calcium ion per ml of germicide. A similar technique was employed using $MgCl_2 \cdot 6H_2O$ as a source of magnesium ions.

After the temperature of the suspension in the medication tube and the germicide reached 37° C., 5 ml of benzalkonium chloride solution were removed with a 5 ml pipette and released into the medication tube which was then swirled several times. After the appropriate time intervals, 1.0 ml was removed from the mixture and quickly expelled into a 9 ml blank containing an inactivator for the quaternary ammonium compound. A solution containing 2.2 grams lecithin, 5.6 ml Tween 80, and 1.25 ml $\frac{1}{4}$ M phosphate buffer per liter of distilled water, adjusted to pH 7.2 after sterilization, was used to inactivate the

benzalkonium chloride. The time intervals in which the organisms were exposed varied from fifteen seconds to 600 seconds depending upon the concentration of germicide employed.

After inactivation of the germicide, the number of surviving organisms was determined by means of plate counts. Each dilution tube was thoroughly shaken and 1 ml and 0.1 ml aliquots were transferred to petri dishes and plated on tryptone glucose beef extract agar to which asolecithin and Tween 80 were added. The plates were incubated for twenty-four hours after which time the colonies were counted. Sterility was conceived to have been reached if no colonies appeared in either the 1.0 ml or 0.1 ml plates.

EXPERIMENTAL RESULTS

Repeated tests in which the results were duplicated revealed that the 15 ppm dilution of germicide gave complete sterility in 300 seconds. In like manner, the 25 ppm and 35 ppm dilutions gave complete sterility in 90 and 30 seconds respectively as shown in Table II. The values for time in the 25 ppm and 15 ppm dilutions were substituted in the formula

$$n_1 = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

where t_1 = 90 sec, t_2 = 300 sec, C_1 = 25 ppm, and C_2 = 15 ppm. The value of n_1 came out to be 2.35. In like manner the values for time in the 35 ppm and 25 ppm dilutions were substituted in the formula

$$n_2 = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

where t_1 = 30 sec, t_2 = 90 sec, C_1 = 35 ppm, and C_2 = 25 ppm. The value for n_2 came out to be 3.26.

Finally, the values for time in the 35 ppm and 15 ppm dilutions were substituted in the formula

$$n_3 = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

where t_1 = 30 sec, t_2 = 300 sec, C_1 = 35 ppm, and C_2 = 15 ppm. The value of n_3 was 2.72. The three exponents, 2.35, 3.26, and 2.72 were then averaged giving a value of 2.77 for the concentration exponent.

TABLE II

TIME FOR COMPLETE KILLING OF *ESCHERICHIA COLI* WITH THREE DILUTIONS OF 10% BENZALKONIUM CHLORIDE

Ml Benzalkonium Chloride	Dilution	Time for Complete Sterility
0.03 ml	15 ppm	300 seconds
0.05 ml	25 ppm	90 seconds
0.07 ml	35 ppm	30 seconds

After the concentration exponent for the benzalkonium chloride was arrived at, the entire series was redone with 40 parts per million per ml of the calcium ion put into the germicide. The effects that the calcium ion had on the efficiency of the germicide can readily be seen in Table III. The 15 ppm dilution now took 525 seconds to completely kill the suspended culture. Likewise the 25 ppm dilution and the 35 ppm dilution required 220 seconds and 120 seconds respectively for complete sterility. When these time values were substituted into the formula as previously described, the values for n_1, n_2, and n_3 were 1.70, 1.71, and 1.77 respectively. The concentration exponent was therefore averaged to be 1.73.

The magnesium ion was found to reduce the effectiveness of the germicide to a slightly greater degree than did the calcium. The 15 ppm dilution took 560 seconds for complete sterility, whereas the 25 ppm and 35 ppm dilutions took 300 seconds and 180 seconds respectively. The values for n_1, n_2, and n_3 in this case were 1.22, 1.52, and 1.34, yielding an average concentration exponent of 1.36 for the germicide plus magnesium. The values of K (Table IV) were arrived at by substitution in the formula

$$C^n t = K$$

where C = 15 ppm, n = 2.77, and t = 300 seconds.

TABLE III

THE EFFECTS OF CALCIUM AND MAGNESIUM ON THE TIME FOR COMPLETE STERILITY OF ESCHERICHIA COLI BY BENZALKONIUM CHLORIDE

Ml Benzalkonium Chloride		Dilution	Time for Complete Sterility
0.03 ml		15 ppm	300 seconds
0.03 ml	40 ppm Calcium	15 ppm	525 seconds
0.03 ml	40 ppm Magnesium	15 ppm	560 seconds
0.05 ml		25 ppm	90 seconds
0.05 ml	40 ppm Calcium	25 ppm	220 seconds
0.05 ml	40 ppm Magnesium	25 ppm	300 seconds
0.07 ml		35 ppm	30 seconds
0.07 ml	40 ppm Calcium	35 ppm	120 seconds
0.07 ml	40 ppm Magnesium	35 ppm	180 seconds

TABLE IV

BACTERICIDAL EFFICIENCY OF BENZALKONIUM CHLORIDE AGAINST

ESCHERICHIA COLI

C (ppm)	Time Secs	Log C	Log T	Log $C_1 - C_2$ etc	Log $t_2 - t_1$ etc	n	Aver n	K	Aver K
35 B.C.	30	1.5441	1.4771	0.3680	1.0000	2.72		659,000	
25 B.C.	90	1.3979	1.9542	0.1462	0.4771	3.26		576,000	
15 B.C.	300	1.1761	2.4771	0.2218	0.5229	2.35	2.77	543,000	592,000
35 B.C.-40 Ca	120	1.5441	2.0792	0.3680	0.6410	1.77		562,900	
25 B.C.-40 Ca	220	1.3979	2.3424	0.1462	0.2632	1.71		576,500	
15 B.C.-40 Ca	525	1.1761	2.7202	0.2218	0.3778	1.70	1.73	568,800	569,400
35 B.C.-40 Mg	180	1.5441	2.2553	0.3680	0.4929	1.34		226,600	
25 B.C.-40 Mg	300	1.3979	2.4771	0.1462	0.2218	1.52		238,900	
15 B.C.-40 Mg	560	1.1761	2.7482	0.2218	0.2711	1.22	1.36	222,600	229,366

DISCUSSION AND INTERPRETATIONS

Application of the concentration exponent requires that all factors other than the time and concentration be strictly nonvariable. To maintain this consistency is difficult. For this reason, valid objections could be raised to the use of the exponent in speculation as to the action of disinfectants. Experienced investigators have had difficulty in securing consistent values of n. Difficulties inherent in the method limit the rigorous control of variable factors other than time and concentration of the disinfectant.

Weber and Black, using a 200 ppm concentration of quaternary ammonium germicide, obtained complete sterility in 300 seconds when using distilled water to suspend their organisms and dilute the germicide. When using tap water, the organisms were too numerous to count at 300 seconds. This emphasizes the effect of interfering ions on the germicidal properties of quaternary ammonium compounds.

We obtained complete sterility in 300 seconds using 15 ppm benzalkonium chloride and washing the organisms with a high-grade distilled water (Table III). The same type of distilled water was used to dilute the germicide. With 35 ppm benzalkonium chloride, and again using distilled water throughout, complete sterility was arrived at after only 30 seconds. The average value for n was found to be 2.77 (Table IV). Tilley arrived at a concentration exponent of 5.7 (Table I) using phenol as the germicide and Staphylococcus aureus as the test organism. The reason for his high value for n can be explained

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by his choice of test organism. Staphylococcus aureus is more resistant to chemical disinfectants than is Escherichia coli.

With 40 ppm calcium ion, the average value for n turned out to be 1.73 whereas a solution containing 40 ppm magnesium ion gave an average value of 1.36 for n. An examination of these figures shows that these interfering ions have their greatest retarding effect upon the lower concentrations of germicide employed and further that this interfering action is approximately threefold with each halving of germicidal concentration.

Having established the value of K (Table IV) the concentration exponent can be put to practical use. Any unknown time for sterilization can be found by simple substitution in the formula

$$C^n t = K$$

Thus, if we wanted to know what the effect of doubling the concentration of benzalkonium chloride would be on the time for sterilization, we would merely select the values for n and K from Table IV, substitute in the above formula, and solve for t:

$$30^{2.77} t = 593{,}000.$$

The time for sterilization, t, is thus found to be 70 seconds using 30 ppm benzalkonium chloride in distilled water. It will be noted from Table III that when using 15 ppm benzalkonium chloride, the time for sterilization was 300 seconds. In like manner when we increased the concentration from 30

to 35 ppm, the time for sterilization was found to be 30 seconds. There is thus an inverse exponential relationship between concentration of germicide and time for sterilization.

The purity of the lecithin used to inactivate the quaternary ammonium compound is believed to be a factor that influences the time required for sterility in this test. For this work a relatively impure grade of lecithin obtained from the Amend Drug and Chemical Co., Inc., New York (Batch No. C-30090) was used for inactivation of the germicide. It was found, however, that when a more pure form of lecithin was used, the time for sterility was considerably less than when the impure form was used. The concentration exponent was not worked out for the germicide when the latter inactivator was used.

It would appear that some variation is caused by the presence of more resistant members in the original test culture inoculum. These would be variably inhibited in their growth properties after contact with the germicide depending upon the degree of their refractoriness toward it.

To quote Tilley (1939): ".... it is impossible to control with mathematical precision such factors as the resistance and numbers of the bacteria in the test cultures and the medication mixtures, and examination of the results presented in the tables and figures shows many irregularities due to unavoidable experimental errors."

The hydrogen ion concentration must also be care-

fully controlled in experiments of this type. It is known that hydrogen ion will affect the action of quaternaries. The most obvious effect is increase in interference as the pH is lowered.

According to Mueller and Seeley (1951), the effects of metallic ions on the germicidal qualities of various quaternary ammonium compounds have been studied by the U. S. Health Service. There is general agreement that magnesium interferes with the bactericidal qualities of these compounds to a slightly greater degree than does calcium. The investigation of Mueller and Seeley (1951) confirms these results and further shows that iron inhibits quaternaries more than does calcium and magnesium. The ferric ion is apparently more deleterious than is the ferrous ion. The effect of iron on the germicidal effect of benzalkonium chloride was not undertaken in this investigation but the effects of calcium and magnesium are in agreement with previous investigators.

The actual time for sterility of Escherichia coli culture with benzalkonium chloride is somewhat shorter than that reported by Weber and Black. The exact conditions for the experiment, as mentioned previously, are difficult to duplicate and it may be that a variable which is not immediately evident has been introduced in this work. However, the number of times that these results have been replicated establishes the validity of the concentration exponents, at

least under the conditions in which this work has been performed. More important, it re-emphasizes the feasibility of evaluating disinfectants by use of the concentration exponents. There is room for future work of this kind to develop a more satisfactory and consistent method of carrying out the experimental procedures involved in testing quaternary ammonium compounds.

SUMMARY

A known number of Escherichia coli were exposed to various concentrations of the quaternary ammonium compound, benzalkonium chloride, for various periods of time. From the formula,

$$n = \frac{\log t_2 - \log t_1}{\log C_1 - \log C_2}$$

the concentration exponent, n, was found to be 2.77 for this particular germicide.

The effects of calcium and magnesium on the germicide were also determined and it was found that a 40 parts per million solution of the calcium ion changed the concentration exponent of benzalkonium chloride to 1.73 and a 40 parts per million solution of the magnesium ion altered it to 1.36.

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