THE EVALUATION OF THE GERMICIDAL EFFICIENCY OF QUATERNARY AMMONIUM COMPOUNDS

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The Evaluation of the Germicidal Efficiency of Quaternary Ammonium Compounds

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Introduction

Present methods of evaluating disinfectants leave much to be desired. The phenol coefficient procedure of the Food and Drug Administration yields questionable results when used as a basis of comparison among chemical agents of different structures and behavoirs. A phenol coefficient as a comparative evaluation of a quaternary ammonium compound, sodium hypochlorite, chlorophenate, or a mercurial is valueless. The use of the phenol coefficient is particularly inapplicable for testing quaternary ammonium compounds, not only because of their lack of relationship to phenol but because of the difficulty of reproducible results. A phenol coefficient is applicable only when compared to phenolic derivatives (17).

Several factors contributing to difficulties experienced in testing surface active agents by the phenol coefficient method have been recognized. Procedures to compensate for these difficulties have been advocated, but do not eliminate the basis for error; that of comparing compounds of different types, and with different modes of action with phenol.

In order to eliminate bacteriostasis resulting in the carry over of the disinfectant to the subculture broth, variations based on dilution and neutralization have been introduced. Shippen (20) introduced the procedure of carrying over a loopful of the first subculture into a second tube of broth. Although it was not originally designed for this purpose, it has found a use in the testing of quaternary ammonium compounds. Shippen's modification, while diluting out the disinfectant has the disadvantage of also diluting out the organisms so

that the second broth tube may not be inoculated even though the first tube has a few viable organisms present.

Klarmann and Wright (9) in their "semi-micro" modification of the phenol coefficient use 200 ml. of broth to dilute their medication mixtures. They stated that even this high dilution did not eliminate bacteriostasis.

The use of lecithin as a neutralizing agent in the testing of these cationic detergents was suggested by Quisno, Gibby and Foter (16). Pressman and Rhoades (15) neutralized their medication-culture mixtures with sodium stearate before making subcultures.

Tobie and Orr (22) found that erratic results reported in testing surface active germicides were due partially to the formation of drops on the transfer loops which were not equal in size. The resulting reduction in the number of bacteria taken for inoculation is especially important after the numbers of bacteria have been greatly reduced. To avoid making the germicides seem better than they actually are, the authors suggested transferring .02 ml. by pipette instead of making transfers by loop.

Several writers report that variations in results obtained when testing quaternary ammonium compounds are due to clumping (3,8) and to the adherence to the walls of the tubes by the bacterial cells (8). This prevents a uniform exposure period and representative sampling of the medication mixture.

The hypothesis has been advanced (3,8) that on exposure to cationic germicides a large number of bacteria tend to adhere to each other forming clumps. Only the outside layers are in direct contact with

the cations so that complete kill is sometimes impossible in the time of exposure. However, as reported by Kivela, (7) no clumping of Salmonella typhosa or Micrococcus pyogenes takes place in any dilutions from 1:1,000 to 1:100,000 using Roccal, Hyamine, and BTC as the cationics.

That the original F. D. A. method is not suitable for measuring the germicidal action of this class of compounds is claimed by Klarmann and Wright (8). They noted that suspended bacterial cells enter into some combination with the quaternary ammonium compounds which causes them to be attached to carriers, such as glass, making them practically unavailable for transfer by the F. D. A. loop. Klimek and Umbreit (10) failed to confirm the results of Klarmann and Wright, finding no evidence that a significant number of viable organisms remained adsorbed on glass in a tube to make them unavailable for transfer.

Several methods for the assaying of the effectiveness of surface active agents have been recommended by workers in the field. The use-dilution method (12), in which practical working dilutions are tested under conditions approaching those encountered in the field, is a relatively simple means of evaluation. The use-dilution was selected as a test in Part II of this thesis where it is discussed in greater detail

Johns (5) has developed a glass slide technique in which partially dried glass slides are immersed in the disinfectant to be tested, rinsed with tap water to minimize bacteriostatic action and the slide plated in a nutrient medium. By means of controls, a 99.9 per cent kill is taken as the end-point.

Cade (2) uses a combination of the standard phenol coefficient test combined with a plate count as a means of bringing about the determination of a more definite end point. The standard F. D. A. loop does not always pick up viable organisms in the medication mixture, so that a cotton swab is used to remove organisms adhering to the sides of the tube, the swab is then swished in a tube of melted agar and plated. Per cent kill is calculated by this method.

Klarmann and Wright (8,9) have developed a semi-micro adaptation of the phenol coefficient test and a filter paper transfer technique. In the semi-micro method 0.5 ml. of medication mixture and 0.05 ml. of the test culture are allowed to react before adding the entire mixture to 20 ml. of nutrient broth. In this way the entire solution is subcultured resulting in a more representative sample and yet avoiding the necessity of using large volumes of broth. The filter paper transfer technique introduces pieces of filter paper into the culture before adding 5 ml. of the various dilutions to the disinfectant. At the end of five, ten and fifteen minute intervals, respectively, one piece of filter paper is transferred to 20 ml. of nutrient broth. These are retransferred into a second tube, also containing 20 ml. of broth, to eliminate bacteriostasis.

In vivo methods of evaluating germicides have been devised to avoid the inconsistencies encountered in the in vitro tests. Nungester and Kempf (14) have designed an "infection-prevention" test using mice as test animals. In this test the tails of anesthetized mice are immersed in a culture highly pathogenic to mice. The contaminated tail is exposed to the disinfectant for two minutes then the tip of the tail

(1 cm.) is cut off and implanted aseptically in the peritoneal cavity. The efficiency of the disinfectant is determined by survival or death of the animals.

Green and Birkeland (4) used the developing chick embryo as a means of determining the effective dilutions of quaternary ammonium compounds used as wound disinfectants. In this procedure the chorio-allantoic membrane is inoculated with a 23-25 hour culture of Staph. aureus 209, the membrane is then treated with disinfectant for five consecutive days. On the sixth day, the membrane is stroked with a moist cotton swab and streaked on nutrient agar for an estimation of remaining viable organisms.

Although not doing away with variations in the resistance of the organism, the modification of the phenol coefficient procedure devised by Kenner et al (6) did away with wild plusses and always produced a definite end point. In this test, using cetyl pyridinium chloride as the disinfectant, and Salmonella typhimurium as the test organism, subcultures were inoculated into mice instead of into F. D. A. broth. The recovery of organisms from the heart blood of the infected mice was taken as the end point.

As yet, none of the methods proposed for the evaluation of the bactericidal efficiency of the quaternary ammonium compounds has been universally accepted despite the obvious shortcomings of the Food and Drug Administration method. Some further modifications must be made in existing tests, or a new test devised, which will compensate for the peculiarities of this type compound. It is important to recognize that such factors as temperature, variations in number and

resistance of organisms, and the composition of the culture medium play an important part in the results obtained in testing disinfectants. The many uses of disinfectants, with their accompanying different conditions, makes obvious the fact that no one test can fulfill all the requirements of these conditions. There is, however, an urgent need for a reliable basis of comparison for even such limited conditions as can be duplicated practically for a large number of laboratory tests.

Recognized sources of difficulty in present methods of comparison which have caused confusion as to the relative merits of quaternary ammonium compounds have been summarized by Klimek and Umbreit (10). The difficulties are: variation in composition and components of the test medium; genetic mutation or transitory dissociation of the test culture; variations in the number of organisms which may be transferred to subcultures when a standard 4 mm. loop is employed; and lastly, the habitual comparison of chemically unrelated substances with phenol.

These are factors which cause wide variations in the results obtained by the phenol coefficient procedure, and which exist to plague the worker in other methods devised for testing this type compound. Variations which occur in the resistance of the test organisms to the quaternary ammonium type compound while the resistance to phenol remains unchanged (1,13), have made the assignment of phenol coefficient values meaningless when applied to the cationic class of disinfectants. A coefficient arrived at today in one laboratory may not be duplicated in another laboratory tomorrow, and may even differ

in the same laboratory when tested by the same worker. At present there is no method of maintaining the disinfectant testing strains of Salmonella typhosa or Micrococcus pyogenes var. aureus at a standard resistance to the quaternary ammonium compounds. This then brings up the question of what the one right coefficient is since so many different values may be obtained by skilled technicians working under the same conditions.

The search for a basis of comparison which would nullify the effects of these factors, and produce a method that would yield reproducible results regardless of the resistance of the test organisms led to the work done in this thesis.

Experimental

The selection of a basis of comparison that will yield similar results regardless of the resistance of the organism is of prime importance in the development of a new method. Not only is the killing dilution of the quaternary ammonium compounds not comparable to the killing dilution of phenol in calculating the use-dilution, but the resistance of the test organisms; namely, Sal. typhosa and M. pyogenes var. aureus,may vary widely to the quaternary ammonium compounds and still not vary to phenol. Mallman (11) showed that two strains of Sal. typhosa obtained from the same culture gave identical resistances to phenol, but gave resistances of 1:15,000 and 1:45,000 to Phemoral, a quaternary ammonium compound. Mallman, Leavitt, and Joslyn (13) demonstrated that M. pyogenes var. aureus when grown in F. D. A. broth and Difco disinfectant testing broth yielded killing dilutions of 1:4,000 and 1:20,000 although the resistance to phenol was the same for both cultures.

Knowing that such marked discrepancies may occur, it is apparent that unless the approximate killing dilution of quaternary ammonium compound was known it would be impossible for a bacteriologist to know whether his results were dependable. The mere fact that his phenol resistance was standard is of no value in determining that the resistance to the quaternary ammonium preparation is correct.

In as much as the quaternary ammonium compounds are basically similar it would seem logical to assume that any shift in the resistance of the test organism to one would be comparable to another, even though their germicidal activities might be quite different. For this

reason it was thought that the selection of a pure product such as Hyamine 1622, a di-isobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride might be used as a reference compound in the same manner that phenol has been used in the F. D. A. phenol coefficient. If, however, the organism need not have a standard resistance to the Hyamine 1622, then the test would be even more simple than the F. D. A. phenol coefficient because the standard killing dilution could shift with the resistance of the test organism.

The selection of a basis of comparison that will give similar results, regardless of the resistance of the organism, is of prime importance in the development of a new method. To determine whether resistance of test organisms to all quaternary ammonium compounds varied with a constant ratio, several different types of compounds were tested against organisms of varied resistance. The compounds tested were Hyamine 1622, a di-isobutyl-phenoxy-ethoxy-ethyl-dimethyl-benzyl-ammonium chloride; Bional E. C., an alkyl-dimethyl-ethyl-ammonium bromide; Tetrosan, an alkyl-dimethyl-dichloro-benzyl-ammonium chloride; BTC, an alkyl-dimethyl-benzyl-ammonium chloride; and LPC, lauryl pyridinium ammonium chloride. Hyamine 1622 was chosen as a basis of comparison since it is a pure compound, readily soluble, and stable in a stock solution of 1:100. This list includes compounds which have their highest effective dilutions falling over a wide range; thus including compounds of varied efficiencies for the test.

Existing tests were chosen for the determination of the influence of varied resistance on the ability of the compounds to maintain a constant ratio of effectiveness. The phenol coefficient

test was chosen for the first set of experiments since it is a familiar test to all and has the official sanction of the Food and Drug Administration. In part II the use-dilution method was selected as offering an available method for ascertaining the critical dilutions with a greater degree of accuracy. This method has been adopted by the Food and Drug Administration (21).

Experiment I

The Determination of a Quaternary Ammonium Compound Coefficient by means of the F. D. A. Method.

The test organisms used were 22-26 hour broth cultures of a standard disinfectant testing strain of Salmonella typhosa, (Hopkins) and Micrococcus pyogenes var. aureus (209) incubated at 37° C. To obtain organisms of a greatly varied resistance to quaternary ammonium compounds M. pyogenes var. aureus was grown in Difco disinfectant testing broth (13), and in F. D. A. broth. The presence of lactose in Difco broth enables M. pyogenes var. aureus to withstand substantially higher concentrations of quaternary ammonium disinfectants. First tests with Sal. typhosa were carried out with an organism transferred for five consecutive days in F. D. A. broth from an agar slant stock culture of the organism. Daily transfers for several weeks significantly altered the resistance of the organism, and it was this less resistant form that was used in a second series of tests. A resistant and a susceptible form of each organism was used in this experiment, although both forms originally came from the same source.

Tests were carried out at 20° C. following the directions set forth in the F. D. A. Circular 198 (19), except that Hyamine 1622 was used to check the organisms for standard resistance. In all cases the test organisms showed the following resistance to phenol at 20° C.

M. pyogenes var. aureus

Dilution	5	10	15
1:60	+	-	-
1:70	+	-	-
1:80	+	+	+

Sal. typhosa

Dilution	5	10	15
1:90	-	-	-
1:100	+	-	-
1:110	+	+	+

Five ml. of appropriate dilutions was transferred to medication pots (1 in. by 3 in.) and placed in a 20° C. water bath until the temperature of the bath was reached. Five-tenths ml. of the test culture, previously shaken for fifteen minutes, was added to each of the dilutions at a suitable time interval. At five, ten, and fifteen minute intervals from the time of seeding the medication pots, transfers were made to tubes of broth. The broth was of the same composition as that used to grow the organisms.

From the results of the tests quaternary ammonium compound coefficients were calculated for the various types of compounds. The The quaternary ammonium compound coefficients are calculated by dividing the highest dilution of disinfectant capable of killing in ten minutes but not in five minutes, by the greatest dilution of Hyamine 1622 showing the same results. The quaternary ammonium compound coefficients are expressed to the nearest whole number to avoid fictitious accuracy. Hereafter, these will be referred to as QAC coefficients.

Examination of the data in Tables I and II does not show any basis for the establishment of a QAC coefficient. Variations in the resistance of the organism to the quaternary ammonium compounds used in this series of tests gave as widely divergent results in the calculation of a QAC coefficient as for a phenol coefficient. In the case of Bional E. C. a constant ratio was maintained when compared to Hyamine 1622 regardless of the resistance of the organism. With BTC, Tetrosan, and LPC the coefficients obtained varied by as much as 33 to 50 per cent. Phenol coefficients calculated from the killing dilutions of the disinfectants against organisms grown in F. D. A. broth varied to a slightly greater extent than did the QAC coefficients, ranging from 33 to 56 per cent.

A quaternary ammonium compound coefficient determined by the F. D. A. procedure is as accurate a method for evaluating this type compound as a phenol coefficient. It varies over almost as wide a range, but there is a more solid basis in comparing like compounds with each other.

Differences in the QAC coefficients obtained may be due to factors inherent in testing by the F. D. A. phenol coefficient procedure. The effect of surface tension on the size of the drops on the transfer loops, clumping, adherence to the side of the vessel, and bacteriostasis still exist to make the use of the phenol coefficient impractical. Wide ranges of dilutions are necessary to avoid wild plusses which make it impossible to obtain a definite end point. All these factors exist to make it impossible to ascertain the exact killing dilutions for these compounds, thus making this method

inappropriate for determining either a phenol or a QAC coefficient of any value.

Data obtained from Phenol Coefficient Tests at 20° C. using S. typhosa var. carrier strain in Difco and F D A broth as the test organism

Compound	Killing Dilutions		R. A. C. Coefficient		Phenol Coefficient	
	Difco	F D A	Difco	F D A	Difco	F D A
Pyradine 1632	1:1T *	1:2OT	1	1	1.4	2.6
[illegible]	1:1T	1:35T-1:45T	1	2	14	50.0-64.3
Bionol A. C.	1:1T-1:25T	1:2OT	1	1	14-17	2[illegible]
Tebrosan	1:3T	1:7OT-1:75T	3	4	42	100.0-107.1
LPC	1:5T	1:75T-1:85T	5	4	71	107.1-121.0

* T = 1,000

Data obtained from two series of Phenol Coefficient tests at 20° C. using Bd. typhosa as test organism:

Compound	Killing Dilutions		R. A. C. Coefficients		Phenol Coefficients	
	1st test	2nd test	1st test	2nd test	1st test	2nd test
Uyvanine 1522	1:12.5T *	1:17.5T	1	1	125	175
[illegible]	1:15T-1:20T	1:30T	1-2	2	150-200	300
Phenol U. S. C.	1:7.5T-1:10T	1:15T	1	1	75-100	150
Petrowan	1:200-1:250T	1:400-1:450T	2-3	2	200-250	400-450
LPC	1:300T-1:400T	1:300-1:750T	2-3	3	300-400	300-750

* T = 1,000

Experiment II

The determination of a Hyamine Coefficient by means of the Use-Dilution Method

A second set of tests were run using the use-dilution (12) method as a check on the results obtained by the phenol coefficient method.

The test organisms used were the same as those in the tests using the F. D. A. phenol coefficient method.

In the use-dilution method 22-26 hour broth cultures were shaken for fifteen minutes to break up bacterial clumps. Sterile glass rods $\frac{1}{4}$ inch in diameter and 1 inch in length having a flattened "nail" head for handling were dipped into the broth culture and then laid on sterile filter paper in petri dishes for drying. Care was taken to avoid rolling while drying. A drying period of thirty minutes was allowed at room temperature.

Desired dilutions of the disinfectant were made and 10 ml. amounts placed in each of the medication pots (1 inch by 3 inches). A 10 ml. amount of sterile physiological saline was placed in another set of medication pots. The pots were then placed in a 20° C. water bath and brought to the temperature of the bath.

Since the end-point in these tests was to be the highest dilution to kill in ten minutes but not in five, a departure was made from the method set up by Mallman and Hanes (12). Only two glass rods were immersed in the disinfectant to be tested, eliminating the 1 min. and 30 min. exposure periods. At the end of the five and ten minute exposure periods a rod was removed and immersed in the physio-

logical saline for one minute. It was then placed in a tube containing 10 ml. of nutrient broth. The tube was shaken vigorously to remove all organisms from the rod and 1 ml. amounts plated.

Controls were run by dropping rods covered with the dried organisms into the saline tubes for one minute and then into tubes containing 10 ml. of nutrient broth. Suitable dilutions of the broth were plated.

Since the data obtained from the plate counts is not pertinent to this paper the results are not included. Due to errors in random sampling some negative plates were obtained where the tubes showed growth at the end of forty-eight hours. All plates and tubes containing the rods were incubated at 37° C. for forty-eight hours.

The organisms were checked for standard resistance by the phenol coefficient method.

The use-dilution was designed originally to evaluate disinfectants under conditions approximating those encountered in actual use, and was meant to divide disinfectants into two categories: satisfactory and unsatisfactory. However, in these tests a comparison was set up between BTC, Bional, Tetrosan, and LPC; again using Hyamine 1622 as a basis of comparison. No standards have been set up for the resistance of M. pyogenes var. aureus and Sal. typhosa to phenol when tested by the use-dilution method. In this group of experiments only a QAC coefficient was calculated.

Examination of the QAC coefficients obtained (Tables III and IV) by the use-dilution method show a close correlation regardless of the resistance of the organism. Only with LPC did the relative ratios

of the disinfectant powers of the compounds fail to remain constant. The coefficients obtained with Lauryl Pyridinium Chloride against M. pyogenes var. aureus differed by twenty-five per cent, against Sal. typhosa only twenty per cent. With the other compounds tested an exact ratio was maintained when tested against these organisms. Although the resistance to Hyamine 1622 differed from 1:5,000 to 1:10,000 in the case of each organism, the resistance of the organisms to BTC, Bional E. C., and Tetrosan changed to the same degree so that a constant ratio was maintained.

The data indicate that a reliable evaluation of quaternary ammonium compounds was possible by a QAC coefficient obtained by the use-dilution method, using Hyamine 1622 as a basis of comparison. By this method the need for standardizing the test organism has been eliminated. Since the resistance of the organism to the reference compound varies to the same degree to the compound being tested, a coefficient representing their relative merits may be obtained that will remain constant even when tested against organisms of widely varied resistance.

Data obtained from Use-dilution Tests at 20° C. using L. [illegible] var. [illegible] grown in Difco and F D A broth as the test organism

Compound	Killing Dilutions		Ø. A. C. Coefficient	
	Difco	F D A	Difco	F D A
Hyamine 1622	1:5T *	1:10T	1	1
BTC	1:5T	1:10T	1	1
Dimal E. C.	1:5T	1:10T	1	1
Tetrosan	1:10T	1:20T	2	2
LPC	1:15T-1:20T	1:25T-1:30T	3-4	3

* T = 1.0 c.c.

Data obtained from two series of Use-dilution Tests at 20° C. using 24 hr. broths of varied resistance as the test organism

Compound	Killing Dilutions		R. A. C. Coefficient	
	1st test	2nd test	1st test	2nd test
Hyamine 1622	1:5T *	1:10T	1	1
PCP	1:10T	1:15T-1:20T	2	2
Phenol U. C.	1:5T	1:10T	1	1
Tetrosan	1:15T	1:25T-1:30T	3	3
LPC	1:25T	1:40T	5	4

* T = 1,000

Summary

1. The Food and Drug Administration phenol coefficient procedure, and any modifications of it yet proposed, are not suitable for evaluating the germicidal efficiency of quaternary ammonium compounds.

2. A quaternary ammonium compound coefficient obtained by the use-dilution method offers a reliable means of obtaining a quantitative and qualitative evaluation of the germicidal efficiency of quaternary ammonium compounds.

Discussion

The development of a suitable method for the evaluation of quaternary ammonium compounds is of considerable interest. That the present F. D. A. technique is found lacking as a basis of comparison is obvious from a study of the literature. In this thesis Hyamine 1622 was used as a basis for comparison of other compounds of the same type and of the same mode of action. In the first group of experiments the use of Hyamine 1622 was the only departure from the standard phenol coefficient procedure. The results of the first experiment show no basis for the establishment of a QAC coefficient in preference to the F. D. A. procedure, except as a comparison of similar compounds.

In the group of experiments using the use-dilution as a test method many of the sources of error which produced variable results have been eliminated or reduced. Variations in the resistance of the organism have become of lesser importance since the organism changes its resistance to the standard in approximately the same ratio as it does to the disinfectant to be tested. By rinsing the rods in physiological saline the need for excessive dilution or for the addition of chemical neutralizing substances is eliminated. A representative sample is assured by removing all viable organisms with the glass rod.

Since the conditions of the test more closely resemble those encountered in practice it is felt that a QAC coefficient obtained by the use-dilution method offers a more practical method for the evaluation of cationic germicides.

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