THE USE-DILUTION METHOD OF TESTING DISINFECTANTS

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Marjorie Ellen Hanes

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THESIS

This is to certify that the

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by

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Introduction

There is a need for a better method of measuring the effectiveness of chemical agents used in the destruction of bacteria. Many methods have been devised but due to the wide diversity in chemical constituents of the various agents no one method has been satisfactory.

The need for testing the efficiency of substances which prevent or retard bacterial action was felt even before it was known that disease and decay are caused by micro-organisms. The development of methods started with the early history of bacteriology.

Robert Koch [6] in 1881 was the first to test disinfectants by using a pure bacterial culture as a standard. Silk threads which had been impregnated with organisms were exposed to disinfectant action and transferred to a suitable medium to determine the presence or absence of growth. This was a primitive method and the results obtained were not accurate due to the carry-over of the disinfectant into the broth. It, however, aroused the interest of scientists who later worked to develop better methods.

Kronig and Paul [7] in 1897 were the first to recognize that time is a function of disinfection and that bacterial kill is a gradual process and not an instantaneous one. Garnets of standard size were dipped into a culture of micro-organisms and then dried completely before exposure to the disinfectant. After exposure to the disinfectant each garnet was rinsed to eliminate the remaining disinfectant and then incubated in tubes of broth.

In 1898 Hill [5] devised a method using long glass rods which were sterilized in stoppered glass tubes, dipped into a broth culture or smeared from a slant culture and then exposed to a disinfectant. The

period of exposure and the type of organisms used was purely arbitrary. The rods were placed into tubes of a suitable medium and incubated.

Phenol was introduced as a basis of comparison by Rideal-Walker[1d] in 1903. Varying dilutions of the disinfectant and phenol were used so that a mathematical expression could be obtained by a comparison of the dilutions of each required to kill bacteria in a given period. Experimental error was minimized by the application of standard temperature, organisms, media and time-periods.

Chick and Martin [2] in 1908 demonstrated that temperature, variations in number and age of organisms, and culture medium have a tremendous effect on the accuracy of results obtained when testing disinfectants. They suggested that one specific bacterial culture should be selected and used for testing all disinfectants to eliminate confusion in evaluating the effectiveness of the disinfectant. A set time period of thirty minutes for exposure of the organisms to the action of a disinfectant was used because it was recognized that the speed of disinfection varies. Sulfide was used to neutralize the bacteriostatic effect of the disinfectant in the testing of mercuric compounds. Chick and Martin also suggested a procedure for testing the disinfectant in the presence of 5 per cent organic matter to simulate the actual conditions under which the disinfectant would be utilized.

The Reddish technic [1] described in 1927 represents the best method of testing devised in the series of procedures based on the original Rideal-Walker method. Since the development of the Rideal-Walker method all later developments such as the Lancet Method, Hygienic Laboratory Method, American Public Health Association Method and the Reddish procedures were merely adaptations pertaining to test organisms, media, and other

minutia. All of these technics determine the minimum concentration of disinfectant that will kill under standard conditions. None of these present conditions of testing bear any resemblance to the conditions under which the compounds will be used for disinfection purpose. The attempt to determine a numerical expression in which all compounds are compared to phenol is fallacious and misleading. A mercurial, a quaternary ammonium chloride, and a phenol are not comparable. The important issue to be determined is whether or not the compound under test will kill effectively when applied in the process of disinfection.

Jensen and Jensen (5) in 1933 devised the cover-slip method in an effort to improve on the phenol coefficient. This procedure offers an interesting development, not in the fact that it offers another means of evaluating disinfectants numerically in comparison to phenol, but the fact that it suggests a technic more comparable to conditions found in actual practice. In this technic a loopful of a saline suspension of Staphylococcus aureus is placed on a cover-glass and dried prior to use. In disinfection the organisms encountered are dried on the surface of the object being disinfected and piled in layers from continual exposure of the object to contamination. This technic departs from the Rideal-Walker method and returns to the original technics of Kronig and Paul, and Hill. Killing dilutions of the disinfectants tested are much lower than those obtained by the Reddish phenol coefficient method.

It is evident that present methods of testing disinfectants are not without discrepancies and the necessity of improvement upon these methods is of primary importance. The phenol coefficient method is used as a criteria for evaluating the efficiency of a disinfectant. A compound, therefore, which produces bacterial kill only at low dilutions is designated as a poor disinfectant regardless of the fact that it may be very

effective at full strength or at a low dilution and could be used at that concentration with exemplory results. The manufacturers of chemical disinfectants specify the dilution at which the compound should be used under given conditions. It is this dilution, the use-dilution, which should be used in evaluating the disinfectant. If the disinfectant at the dilution specified by the manufacturer should fail to kill bacteria in a time period which would correspond to the period of exposure in actual use, the disinfectant could be termed ineffective. A lower dilution which produces the desired results could then be suggested for use.

When the phenol coefficient of a compound is used to evaluate its effectiveness a great error may be introduced if the dilution coefficient is not taken into consideration, as demonstrated by Hyma [4]. When the concentration of a disinfectant is reduced to one-half of its original concentration the disinfecting ability may be reduced anywhere from two to sixty-four times depending upon the disinfectant. If a disinfectant is tested at its use-dilution the problem of the dilution-coefficient is eliminated since this is the concentration actually used by the consumer and no further dilution takes place. The use-dilution method of testing disinfectants was devised in an effort to improve upon the phenol coefficient method.

Death of bacteria is held by most bacteriologists to be the failure to grow after the test bacteria have been planted into a favorable medium and incubated for a reasonable length of time. The reliability of such a concept is questionable due to several factors which must be taken into consideration; namely: bacteriostasis, extended lag phase and small inoculum. Bacteriostasis of a bacterial cell may result from the adsorption of certain chemical disinfectants upon the surface of the cell. Under these conditions no growth occurs but the cell is alive

and may remain so for a prolonged period of time. The cell may in time, be able to overcome bacteriostasis naturally or it may require the addition of a neutralizing agent to restore normal cell activity. Unfavorable conditions brought about by exposure to a disinfectant may extend the lag phase of the culture so that no growth is evident within the expected period of incubation. If only a few bacterial cells are living after disinfection the number of organisms present may be too small to initiate growth in the volume of medium to which they have been transferred. The enzymes and accessory food products of the culture become diluted to such an extent that they are no longer of sufficient concentration to support growth and reproduction of the bacterial cells. In this thesis it is recognized that no method has been devised to ascertain death of bacteria so lack of bacterial growth after a reasonable period of incubation will be accepted as sufficient evidence of sterilization.

Terminology used in describing the process of killing bacteria and the killing agents is not only confusing to the laity but also the scientist. A disinfectant is generally defined as a chemical agent that kills organisms capable of producing disease. An antiseptic is defined as a chemical agent that prevents the growth of disease-producing bacteria but does not necessarily cause kill. The research workers in disinfection and the manufacturers of disinfectants define a disinfectant as a chemical agent used to destroy pathogenic bacteria on inanimate objects. Antiseptics are defined as chemical agents used to destroy or prevent the development of pathogenic bacteria on skin and in tissues of the body. In this thesis the latter definitions will apply to the usage of the terms disinfectant and antiseptic.

Experimental Studies

The selection of suitable apparatus for the conveyance of the bacterial culture into the disinfectant and then to the culture medium was the first consideration. In the method devised by Koch the disinfectant clung to the surface of the silk threads causing a carry-over of the disinfectant into the culture medium. Kronig and Paul selected garnets because of their impregnable surface but the garnets themselves were difficult to handle. The cover-slips used by Jensen and Jensen also presented difficulties in handling. Hill used long glass cylinders which were very suitable for transfer into tubes of broth. Although the rods were of comparable size the entire surface of the rods were not inoculated with the culture and uniformity of the number of organisms present was questionable. Dr. W. L. Mallmann suggested the use of a metal or glass cylinder one inch in length and one-fourth inch in diameter which could be made with a loop at one end to facilitate handling. Cultures dried on a glass rod would be more comparable to the surface of inanimate objects than broth cultures because organisms on such a surface would be dry and piled in layers.

In a series of tests made with these rods, a tentative procedure of examination was set up based largely on past experiences with present methods. The rods were dipped into 24 hour broth cultures and placed in sterile Petri dishes to dry. After drying the rods were dropped into medication pots containing 10 cc. of disinfectant solution. After varying periods of exposure at 20°C. the rods were lifted from the tubes with a sterile wire and dipped into a tube of sterile water to remove the excess disinfectant and then dropped into sterile nutrient media. The tubes were shaken vigorously to release the organisms from the rods

and suitable dilutions were plated to measure quantitatively the extent of kill. The rods were incubated in the broth for 24 hours to determine any resulting growth and act as a check upon negative plates which showed no colony formation. It is possible that negative plates could be obtained when viable organisms existed in the broth due to the error introduced in random sampling. If the number of organisms present in the broth were relatively small a one cc. amount could be removed which contained no living organisms. Tubes showing turbid growth were indicated as innumerable and not plated. Tubes not showing growth were plated to determine the presence of viable organisms. The media selected for these studies were those now used in this laboratory for phenol coefficient determination. Staphylococcus aureus was grown in Difco disinfectant testing medium and Eberthella typhosa was grown in standard broth described in F. D. A. Bulletin 198 (11). These two media have proved very satisfactory for use in this laboratory, particularly for growing the test organisms. Staph. aureus and E. typhosa were selected as the test organisms because they have been successfully used in the phenol coefficient determination and no objection to their use could be found. The Staph. aureus cultures when grown in Difco disinfectant testing medium are always neutralized with NaOH to eliminate changing the pH of the disinfectant solution in the usual phenol coefficient procedure. However, in these studies, this was unnecessary because the cultures were always dried and no material change in pH would result when the inoculated rods were introduced into the disinfectant dilution. The test organisms selected showed the following resistance to phenol:

Staph. aureus,

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

E. typhosa,

Dilution	5 min.	10 min.	15 min.
1:80	-	-	-
1:90	+	-	-
1:100	+	+	+

Preliminary tests with these rods were so favorable that the following studies were made to ascertain the feasibility of the procedure for the evaluation of disinfectants, particularly in use-dilution.

Experiment I -- To determine the adherence of a bacterial culture to metal and glass cylinders.

The first experiments were made to determine whether or not the organisms would adher to the rods in sufficient numbers to give a dependable result when carried from the disinfectant to the rinse and then to the final broth. To measure the loss of organisms, sterile water was substituted for a disinfectant in the first tube to eliminate kill. This tube could be plated to determine the number of organisms removed mechanically by dropping into the liquid. In like manner, the rinse tube and the final broth could be checked. The results are presented in Tables 1 and 11. These experiments were repeated using sodium lauryl sulfate instead of sterile water to determine the effect of detergent action upon the mechanical loss of organisms. Escherichia coli was chosen as the test organisms because lauryl sulfate

has no inhibitory effect on it. The results obtained are shown in Tables 111 and 1V.

An evaluation of the results obtained demonstrates conclusively that there is a mechanical loss of organisms from the rod into the various liquids with which it comes in contact. The number of organisms removed by detergent action is much greater than by water. However, the number of organisms remaining on the cylinders when they are placed in the broth appeared to be of sufficient magnitude to demonstrate the amount of bacterial kill effected by the disinfectant as demonstrated in later experiments.

Experiment 11--To determine whether there is any difference in the results when using metal or glass rods.

Both metal and glass rods were used in the first experiments. It soon became evident that experimentation could be simplified if one type of rod were selected for use in further studies. To measure the difference in results obtained, metal and glass rods were dipped in broth bacterial cultures, dried, and exposed to disinfectant action for periods of one, five, ten and 30 minutes. The cylinders were rinsed to remove the remaining disinfectant and then put in broth. The broth was plated and the tubes of broth containing the rods and the plates were incubated for 24 hours at 37°C. Any tubes not showing growth in 24 hours were replated to determine the presence of any viable organisms. Tables V and Vl show the results obtained.

Upon examination of results it is evident that the degree of variation in results between the two types of rods is negligible and either could be used with accurate results. However, after further investigation it was decided that the glass rods are more practical because they are easier to clean and sterilize, the organisms dry faster on the glass surface, and the glass rods are more simple and less expensive to make. Although the metal rods are made of Monel metal and the probability of cor-

TABLE I - The adherence of a bacterial broth culture of Escherichia coli to metal rods in passage through tubes containing water.

Time in Medication Tubes	Number of Organisms Removed			
	Medication tube Sterile Tap Water	Distilled Water (First Rinse)	Broth	Distilled Water (Final Rinse)
1 minute	1,300	140	9,500	420
5 minutes	5,000	900	8,500	150
10 minutes	440	390	7,000	80
30 minutes	6,000	210	2,500	0

TABLE II - The adherence of a bacterial broth culture of Escherichia coli to glass rods in passage through tubes containing water.

Time in Medication Tubes	Number of Organisms Removed			
	Medication Tube Sterile Tap Water	Distilled Water (First Rinse)	Broth	Distilled Water (Final Rinse)
1 minute	15,000	750	9,000	175
5 minutes	9,500	900	10,500	250
10 minutes	9,000	800	8,500	300
30 minutes	5,500	275	2,500	60

TABLE III - The adherence of a bacterial broth culture of Escherichia coli to metal rods in passage through tubes containing a detergent.

Time in Medication Tubes	Number of Organisms Removed			
	Medication Tube Na Lauryl sulfate 1:1000	Distilled Water (First Rinse)	Broth	Distilled Water (Final Rinse)
1 minute	12,000	4,900	57,000	250
5 minutes	23,000	1,800	7,200	140
10 minutes	14,500	1,500	5,000	0
30 minutes	20,000	500	1,000	70

TABLE IV - The adherence of a bacterial broth culture of Escherichia coli to glass rods in passage through tubes containing a detergent.

Time in Medication Tubes	Number of Organisms Removed			
	Medication Tube Na Lauryl sulfate 1:1000	Distilled Water (First Rinse)	Broth	Distilled Water (Final Rinse)
1 minute	25,000	1,500	8,000	700
5 minutes	22,000	3,000	10,500	400
10 minutes	17,500	1,500	9,500	350
30 minutes	11,500	2,500	7,000	500

TABLE V - A comparison of metal and glass rods as carriers of organisms in testing disinfectants, using Roccal 1:1000 as test disinfectant.

Time of Exposure	Number of Surviving Bacteria							
	Glass Rods				Metal Rods			
	Staph. aureus Trial		E. typhosa Trial		Staph. aureus Trial		E. typhosa Trial	
	1	2	1	2	1	2	1	2
1 Minute								
Plated immediately	275	154	0	0	571	190	0	0
Incubated 24 hours*	Innum.	Innum.	Innum.	Innum.	Innum.	Innum.	0	0
5 Minutes								
Plated immediately	31	0	0	0	0	0	0	0
Incubated 24 hours	Innum.	Innum.	Innum.	Innum.	Innum.	Innum.	0	0
10 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	140,000 Innumerable		180,000 Innumerable		160,000 Innumerable		140,000 Innumerable	

* Rods incubated in broth 24 hours and then plated. Turbid growth in tubes were not plated. Such tubes are indicated as innumerable.

TABLE VI - A comparison of metal and glass rods as carriers of organisms in testing disinfectants, using Dowicide "A", 1:200 as the test disinfectant.

Time of Exposure	Number of Surviving Bacteria							
	Glass Rods				Metal Rods			
	Staph. aureus Trial		E. typhosa Trial		Staph. aureus Trial		E. typhosa Trial	
	1	2	1	2	1	2	1	2
1 Minute								
Plated Immediately	1,000	570	15	0	1,500	1,000	0	0
Incubated 24 hrs.	Innum.	Innum.	Innum.	0	Innum.	Innum.	0	0
5 Minutes								
Plated Immediately	690	145	0	0	465	560	0	0
Incubated 24 hrs.	Innum.	Innum.	0	0	Innum.	Innum.	0	0
10 Minutes								
Plated Immediately	27	0	0	0	197	47	0	0
Incubated 24 hrs.	Innum.	0	0	0	Innum.	Innum.	0	0
30 Minutes								
Plated Immediately	0	0	0	0	0	0	0	0
Incubated 24 hrs.	0	0	0	0	0	0	0	0
Control								
Plated Immediately	180,000		180,000		200,000		200,000	
Incubated 24 hrs.	Innum.		Innum.		Innum.		Innum.	

-rosion occurring was negligible the use of glass rods would eliminate any possible oligodynamic action. In the following experimental studies the glass rods are used exclusively.

Experiment three- To determine the most effective way of drying the broth bacterial culture on the surface of the glass rods.

In the first experiments the glass rods which had been dipped in a broth bacterial culture were allowed to dry in a sterile Petri dish. The excess broth culture on the rod drained to the surface of the plate and made drying in the alloted time period difficult. To overcome this effect, a piece of sterile filter paper cut to fit the bottom of the Petri dish was used. The inoculated rods were placed on this adsorbent surface. Rods which had been dried on the glass surface and those which had been dried on filter paper were exposed to the action of a disinfectant in the usual manner and the results obtained are presented in Table Vll. It is obvious since the number of organisms retained on the glass rods after drying on filter paper is greater than those on the rods dried on glass, that the organisms were well dried and resisted the washing effect of the disinfectant to a greater extent.

Experiment four- To determine the length of time required to dry the broth bacterial culture on the surface of the glass rod.

In an effort to determine the actual time period necessary to dry the broth culture on the glass surface of the cylinder the inoculated rods were dried for periods of 30 minutes, one hour, two hours, three hours and 15 hours before exposure to the disinfectant. The results obtained from the rods which had been dried for 30 minutes and the rods which had been dried for 15 hours are shown in Table Vlll. Upon examination of the results it was found that there is very little difference in the number of organisms retained on the rods after the short and long drying periods. It is evident, therefore, that the 30 minute drying period is sufficient.

Table V11 The comparative effect of drying the inoculated glass rods on a glass surface and on filter paper.*

	Cylinders dried on filter paper		Cylinders dried on glass	
Time of Exposure	Staph. aureus	E. typhosa	Staph. aureus	E. typhosa
1 Minute	14,000*	10,500	9,000	6,0000
5 Minutes	7,000	5,000	6,000	4,000
10 Minutes	3,000	1,500	2,000	2,000
30 Minutes	2,000	600	900	600
Control	230,000	210,000	170,000	120,000

* Test disinfectant- Phemerol 1:10,000
* Average of ten tests

Table VIII The effect of drying time upon the number of organisms retained on the glass rods.*

Time of Exposure	Glass rods dried 1/2 hour				Glass rods dried 15 hours			
	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	12,000	9,000	1,000	752	11,000	3,000	2,000	613
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
5 Minutes								
Plated immediately	7,500	5,500	621	214	5,500	2,500	900	221
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
10 Minutes								
Plated immediately	3,500	3,000	47	0	2,000	1,500	821	14
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
30 Minutes								
Plated immediately	973	1,000	0	0	1,000	487	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	Innum	Innum
Control								
Plated immediately	170,000		190,000		150,000		180,000	
Incubated 24 hours	Innum		Innum		Innum		Innum	

* Test disinfectant-- Phemerol 1:10,000

Experiment five- To determine the effect of organic matter upon the activity of disinfection.

It is well recognized that the presence of organic matter interferes with disinfectant action. Organic matter may 1. react with the disinfectant to form inactive compounds, 2; adsorb some of the disinfectant from solution, 3. neutralize a portion of the disinfectant, or, 4. form a mechanical barrier around the organisms which prevents penetration of the disinfectant. (8). To determine the effect of organic matter upon disinfection, ten per cent horse serum was added to the disinfectant solution. The bacterial count obtained after exposure of the inoculated glass rods to the disinfectant containing organic matter was slightly increased as shown in Tables 1X and X. However, it appeared that no appreciable change occurred when organic matter was present at that concentration. In previous attempts to determine the effect of organic matter upon disinfection serum was added to the bacterial culture and the glass rods inoculated with this mixture. When these rods came in contact with the disinfectant the organic matter sluffed off carrying the bacterial cells with it into the disinfectant solution. The possibility that the organic matter of the broth in which the test organisms were being grown might act as a protective agent against the disinfectant was questionable. However, to insure accurate results in later studies, broth cultures of the two test organisms were centrifuged and washed several times with sterile physiological saline solution to remove the organic matter. When the washed organisms were dried upon the glass rods and exposed to disinfectant action the results obtained varied little from the results obtained using a broth culture. This is shown in Tables X1 and X11.

It may be that the amount of organic matter adhering to the glass rods with the bacterial cells is very small and would therefore be unable to exert an observable degree of protective action. The cells themselves may act as a protective agent. The bacterial cells when dried on the rods are not in a single layer upon the glass surface but form a film several layers thick. The outer layer of cells would form a shield which could protect the inner layers if the disinfectant were not powerful enough to penetrate the barrier and attack all the bacteria.

Experiment six- To determinethe necessity of using neutralizing agents to counteract bacteriostasis.

Many classes of disinfectants have marked bacteriostaic power. This is so marked in the mercurials that even a loopful from the medication pots to the broth medium in the F. D. A. procedure is sufficient to inhibit growth. With such compounds it has been customary to use the Shippen modification to dilute the compound beyond its bacteriostatic titre or to add some neutralizing agent to the medium to eliminate bacteriostatic activity. It was necessary to test the use-dilution technic to determine whether or not bacteriostatic activity occurred in the test. It is conceivable that sufficient disinfectant might be carried over to the inoculating medium on the rod even though a water rinse were used between the medication pot and the inoculating medium. Two methods of neutralizing the bacteriostatic effect were tried: the use of agar and broth culture media containing the neutralizing agent in the case of mercurials and the addition of the neutralizing compoind to the rinse material when testing cationic disinfectants. Linden's Fluid Thioglycollate Medium and a solid medium made by adding 15 grams of agar /liter to Linden's formula were used as the culture

Table IX The Effect of the addition of 10% horse serum to phenol upon its disinfectant action.

	Phenol 1:100 + 10% horse serum				Phenol 1:100			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	9000	7000	182	2000	9000	10,000	30	75
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
5 Minutes								
Plated immediately	7500	4500	0	0	7500	6500	0	0
Incubated 24 hours	Innum	Innum	Innum	0	Innum	Innum	Innum	0
10 Minutes								
Plated immediately	6000	4000	0	0	4000	3500	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	0	0
30 Minutes								
Plated immediately	3000	2500	0	0	2500	635	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	0	0
Control								
Plated immediately	130,000		140,000		170,000		200,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

Table X The effect of the addition of 10% horse serum to Phemerol upon its disinfectant action.

	Phemerol 1:5000 + 10% serum				Phemerol 1:5000			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		F. typhosa	
1 Minute								
Plated immediately	2500	200	740	800	1000	2500	419	83
Incubated 24 hours	Innum	Innum	Innmu	Innum	Innum	Innum	Innum	Innum
5 Minutes								
Plated immediately	1500	900	175	321	825	1000	0	0
Incubated 24 hours	Innum	Innum	0	Innum	Innum	Innum	0	0
10 Minutes								
Plated immediately	347	450	0	0	316	496	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	0	0
30 Minutes								
Plated immediately	79	0	0	0	21	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	190,000		160,000		140,000		150,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

Table XI The comparative effect of a disinfectant upon bacterial cells free from organic matter and a broth bacterial culture.*

	Washed cells				Broth culture			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	210	301	0	0	500	400	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	Innum	Innum
5 Minutes								
Plated immediately	0	0	0	0	121	0	0	0
Incubated 24 hours	0	0	0	0	Innum	Immun	0	0
10 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	160,000		170,000		170,000		150,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

* Test disinfectant- NaOCl 200ppm.

Table XII The comparative effect of a disinfectant upon bacterial cells free from organic matter and a broth bacterial culture.*

	Washed cells				Broth culture			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	283	190	53	17	2500	914	3	0
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	0	0
5 Minutes								
Plated immediately	140	76	0	0	1000	249	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	0	0
10 Minutes								
Plated immediately	0	0	0	0	223	163	0	0
Incubated 24 hours	0	0	0	0	Innum	Innum	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	190,000		190,000		180,000		190,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

* Test disinfectant- Roccal 1:1000.

media when testing mercuric compounds. The active sulhydryl group of the sodium thioglycollate incorporated in these media removes the bacteriostatic power of the metallic compound, thus permitting the growth of the organisms which had been suspended by bacteriostasis.

Weatherwood To counteract the bacteriostatic effect of cationic compounds 0.1% soap solution was added to the rinse material. In evaluating the results shown in Tables XIII and XIV it was found that complete bacteriostasis did not occur because some bacterial growth appeared when no neutralizing agent was used. The bacterial counts were lower under those circumstances and occurred only after a short period of exposure to the disinfectant. It is apparent that some bacteriostasis occurred and was sufficient to inhibit the small number of viable organisms present after being exposed to the disinfectant for five minutes and interferes with bacterial growth enough to present a lower count after one minute exposure. Therefore, the use of a neutralizing agent in either the rinse material or the culture media is desirable in testing compound having bacteriostatic power to eliminate a misinterpretation of the disinfecting ability of the compound.

Experiment seven- <u>To determine the amount of variation in the number of organisms present on the inoculated glass rods.</u>

The accuracy of results obtained when using glass rods as a means for transporting the bacterial culture depends to a great extent upon the maintenance of a standard number of organisms on the surface of the rods. To determine the variations between the bacterial counts obtained from the inoculated glass rods, the control counts of the tests made were noted and tabulated in Table XV. Controls are run by dropping the culture-covered rods directly into tubes of broth and plating in suitable amounts. It is to be expected that there are variations in the bacterial count due to the random sampling but these errors merely account for the variations in results obtained from seemingly identical tests and do not influence greatly the accuracy of

Table XIII The effect of a neutralizing agent on bacterial kill using sodium thioglycollate to neutralize Merphenyl Nitrate 1:1500.*

	Neutralizing agent				No neutralizing agent			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	493	714	0	0	32	175	0	0
Incubated 24 hours	Innum	Innum	0	0	0	Innum	0	0
5 Minutes								
Plated immediately	21	400	0	0	0	0	0	0
Incubated 24 hours	Innum	Innum	0	0	0	0	0	0
10 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	190,000		170,000		150,000		170,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

*Sodium thioglycollate incorporated in agar and broth.

Table XIV The effect of a neutralizing agent on bacterial kill using 0.1% soap solution to neutralize Phemerol 1:2500.*

	Neutralizing agent				No neutralizing agent			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	1000	1500	0	0	197	0	0	0
Incubated 24 hours	Innum	Innum	0	0	0	0	0	0
5 Minutes								
Plated immediately	465	192	0	0	0	0	0	0
Incubated 24 hours	Innum	Innum	0	0	0	0	0	0
10 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	190,000		190,000		170,000		180,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

*Soap solution added to rinse material.

Table XV Number of viable organisms obtained from the surface of inoculated glass rods.

Staph. aureus		E. typhosa	
110,000	180,000	120,000	170,000
120,000	180,000	130,000	170,000
120,000	180,000	140,000	170,000
130,000	180,000	140,000	180,000
150,000	180,000	140,000	180,000
150,000	180,000	150,000	180,000
160,000	180,000	150,000	180,000
160,000	190,000	150,000	180,000
160,000	190,000	150,000	180,000
160,000	190,000	160,000	180,000
160,000	190,000	160,000	180,000
170,000	190,000	160,000	180,000
170,000	190,000	160,000	190,000
170,000	190,000	160,000	190,000
170,000	190,000	160,000	190,000
170,000	190,000	170,000	190,000
170,000	190,000	170,000	190,000
170,000	190,000	170,000	190,000
170,000	190,000	170,000	190,000
170,000	200,000	170,000	200,000
180,000	200,000	170,000	200,000
180,000	200,000	170,000	200,000
Average = 177,000	Mean = 170,000	Average = 170,000	Mean = 170,000

of the data from the majority of the tests.

Experiment eight- The effect of phenol resistance variation of the test organisms on the results obtained in testing disinfectants.

The test organisms Staph. aureus and E. typhosa are checked frequently to determine any variations from the pattern of resistance set for them in the phenol coefficient. In this manner a standard culture may be maintained for use. To determine the effect a slight variation of the phenol resistance of a test organism might have on the results obtained in testing disinfectants, slightly irregular cultures were used in parallel tests with standard cultures. In Table XVl a standard Staph. aureus culture and one which is more resistant (phenol kills in five minutes but not in ten at a dilution of 1:60) are used in testing tincture of metaphen. The results obtained are comparable. A less resistant E. typhosa culture (1:100 dilution of phenol kills in five minutes but not in ten) and a standard culture present little variation in results when testing merthiolate 1:1000 as shown in Table XVll. It is evident that these findings present a great advantage. If a bacterial culture which does not strictly adher to the phenol resistance set for it can be used for testing disinfectants with results comparable to those obtained when using cultures which comply to the standard phenol coefficient, the necessity of eliminating slightly irregular cultures from use is no longer present. It is recognized, however, that a great degree of variation from the phenol resistance is indicative of dissociation of the organism and such cultures must be excluded from use.

Table XVI A comparison of results obtained when using a test organism which varies from the standard phenol coefficient.*

Time of Exposure	Standard Staph. aureus		Irregular Staph. aureus	
1 Minute				
Plated immediately	613	123	49	320
Incubated 24 hours	Innum	Innum	Innum	Innum
5 Minutes				
Plated immediately	47	0	31	73
Incubated 24 hours	Innum	Innum	Innum	Innum
10 Minutes				
Plated immediately	0	0	0	0
Incubated 24 hours	0	0	0	0
30 Minutes				
Plated immediately	0	0	0	0
Incubated 24 hours	0	0	0	0
Control				
Plated immediately	190,000		160,000	
Incubated 24 hours	Innumerable		Innumerable	

* Test disinfectant- Tincture of Metaphen 1:200

Table XVll A comparison of results obtained when using a test organism which varies from the standard phenol resistance.*

Time of Exposure	Standard E. typhosa		Irregular E. typhosa	
1 Minute				
Plated immediately	162	114	137	150
Incubated 24 hours	Innum	Innum	Innum	Innum
5 Minutes				
Plated immediately	49	96	82	79
Incubated 24 hours	Innum	Innum	Innum	Innum
10 Minutes				
Plated immediately	15	23	33	56
Incubated 24 hours	Innum	Innum	Innum	Innum
30 Minutes				
Plated immediately	7	0	20	4
Incubated 24 hours	Innum	Innum	Innum	Innum
Control				
Plated immediately	170,000		170,000	
Incubated 24 hours	Innumerable		Innumerable	

* Test disinfectant- Merthiolate 1:1000

The preceding studies were made to determine a simple efficient method of testing disinfectants. From an evaluation of these studies the following procedure is suggested:

APPARATUS

A water bath for the maintenance of constant temperature is used. A lid should be provided with holes of a suitable diameter to hold the medication pots upright in the bath. The medication pots are glass tubes one inch in diameter and three inches in length. Solid glass cylinders one-fourth inch in diameter and one inch in length are provided with a loop at one end. An ordinary wire transfer needle bent at the end is used for manipulating the glass rods. Sterile physiological saline blanks for rinsing the rods and sterile distilled water for making dilutions should be provided in 90cc and 99 cc. volumes..Petri dishes of ordinary size are used. Those used for drying the glass rods should be provided with sterile filter paper on the floor of the dish. All pipettes should be graduated and sterilized. Subculture tubes may be of any convenient size. It is preferable to have test tubes of 20 cc. volumes so that the cylinders will be completely immersed in the broth.

CULTURE

The test organisms are 22 to 26 hour cultures of E. typhosa and Staph. aureus, of strains designated in the Food and Drug Administration Circular 198 (12), incubated at 37°C. and grown in nutrient broth. Difco disinfectant test medium is used for culturing Staph. aureus and E.typhosa is grown in the medium described in the F. D. A. Circular 198 (12). The broth cultures are transferred daily and fresh transfers are made monthly from the stock agar slant culture. The resistance of the organisms to phenol is determined at weekly intervals to ascertain the maintenance of cultures which comply with the standards set for them in the F. D. A. Circular 198(12).

PROCEDURE

24 hour broth cultures of Staph. aureus and E. typhosa are shaken for fifteen minutes to eliminate bacterial clumps and insure even dispersal of the organisms throughout the medium. The sterile glass rods are submerged in the culture and then placed on sterile filter paper in a covered Petri dish and allowed to dry for thiry minutes.

Desired dilutions of the disinfectant are made and ten cc. amounts placed in each of the glass seeding tubes. The tubes are placed in a 20°C. water bath and allowed to come to the temperature of the bath. Since the tests are run in duplicate, two tubes of the disinfectant are needed for each test organism.

Controls are run by placing one of the glass rods which has been inoculated directly into a tube of broth.

Four of the bacteria-covered glass cylinders are placed in each of the tubes of disinfectant and removed at intervals of one, five, ten and thirty minutes. The cylinders after removal from the disinfectant are immersed in sterile physiological saline solution and then placed in 10 cc. of nutrient broth. The tubes are shaken vigorously to remove the organisms from the glass rods and the broth is plated in one cc. amounts. The controltubes are diluted 1:10 and 1:100 before plating to insure an accurate count. The broth tubes and the agar plates are incubated at 37°C. for 24 hours. Any tubes not showing growth in 24 hours are plated. In this manner it is possible to discern growth at time intervals which show no colony formation on the plates made immediately after exposure.

PRESENT TESTS ON CHEMICAL DISINFECTANTS
Varying Dilutions

The practicality of the method developed in this thesis can only be determined by actual use in testing disinfectants. To determine the most effective concentration of a disinfectant varying dilutions of the disinfectant were tested using the procedure just described. No attempt is made to determine the phenol coefficient of the disinfectant; the experiments are done simply to determine the dilution of the disinfectant which produces the greatest bacterial kill in the shortest period of time.

One of the most commonly used coal-tar disinfectants, lysol, has a phenol coefficient of five and is designated for use at a dilution of 1:320. From the results shown in Tables XVIII, XVIV and XX it can be demonstrated that even at a dilution of 1:300 lysol fails to prevent the growth of Staph. aureus after 30 minutes exposure. E.typhosa, a less resistant organism, is killed after ten minutes exposure at this dilution. At a dilution of 1:50 lysol produces immediate bacterial kill and could be used as a disinfectant effectively at this concentration provided that no harmful results to the skin or surfaces being disinfected occurred.

The use of phenol as a disinfectant has been generally discontinued because of its low killing power and toxicity. It has, however, been maintained as a standard for the comparison of disinfectants. The results obtained from testing varying dilutions of phenol are shown in Tables XXI and XXII. Phenol is most commonly used for disinfection at a dilution of 1:20. From the results obtained employing the use-dilution technic it is evident that a 1:50 dilution of phenol produces bacterial kill in less than one minute and could be used as effectively as a 1:20 dilution.

Phemerol, a representative cationic disinfectant, produces immediate bacterial kill at its use-dilution, 1:1000. To determine whether higher dilutions might prove as effective, varying dilutions were tested by the use-dilution technic. After evaluating the results shown in Tables XXIII and XXIV one could conclude that at a dilutions of 1:2500 phemerol could be used for disinfection if the exposure time were extended to ten minutes.

Table XXIII The use-dilution technic applied to various dilutions of Phemerol.

	1:1000				1:2500			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	97	0	0	0	950	1000	0	0
Incubated 24 hours	0	0	0	0	Innum	Innum	0	0
5 Minutes								
Plated immediately	0	0	0	0	371	293	0	0
Incubated 24 hours	0	0	0	0	Innum	Innum	0	0
10 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	120,000		180,000		180,000		180,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

Table XXIV The use-dilution technic applied to various dilutions of Phemerol.

	1:5000				1:7500			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	2500	2000	740	800	11,000	9500	1500	1000
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
5 Minutes								
Plated immediately	1500	900	175	321	6500	6000	700	630
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
10 Minutes								
Plated immediately	347	450	0	0	2500	3500	41	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	Innum	Innum
30 Minutes								
Plated immediately	79	0	0	0	2000	1500	0	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	0	0
Control								
Plated immediately	190,000		160,000		200,000		180,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

Table XX The use-dilution technic applied to various dilutions of Lysol.

	1:300				1:400			
Time of Exposure	Staph. aureus		E. typhosa		S taph. aureus		E. typhosa	
1 Minute								
Plated immediately	3500	5000	315	299	7500	9000	3000	4500
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
5 Minutes								
Plated immediately	940	1500	45	51	4500	6500	1500	2000
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
10 Minutes								
Plated immediately	320	217	0	0	920	1500	820	760
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
30 Minutes								
Plated immediately	14	32	0	0	490	750	0	0
Incubated 24 hours	Innum	Innum	Innum	Innum	Innum	Innum	Innum	Innum
Control								
Plated immediately	180,000		150,000		190,000		160,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

The Use-dilution

It is interesting to know the most effective dilution of a disinfectant but it is the determination of the efficiency of the use-dilution which is most important to the consumer. Commercial disinfectants are used by the laity and professional people for all types of disinfection. It is imperative, therefore, that the disinfectant at the dilution suggested by the manufacturer for use comply with the claims made for it. The use-dilution technic is a method devised to test the disinfectant power of a disinfectant at its use-dilution.

Barde-Parker solution is a chemical disinfectant used at full strength for cold sterilization of surgical instruments. From the results obtained from frequent testing of this solution it was found that bacterial kill is effected in less than one minute, as shown in Table XXV. The phenol coefficient of this compound is relatively low indicating that it is a poor disinfectant yet from the results obtained by the use-dilution technic it could be concluded that Barde-Parker solution at its use dilution is an effective disinfectant.

Complex compounds of mercury have been given much attention in past years as excellent disinfectants. Their efficiency has been overrated, for although they may produce bacterial kill at low dilutions they are not bactericidal at the high dilutions indicated in earlier investigations of these compounds. Three mercurials were tested at their use-dilution using the use-dilution technic. Tincture of metaphen, an organic mercuric compound, is designated for use at a dilution of 1:200. This concentration fails to kill Staph. aureus after an exposure time of five minutes as shown in Table XXV. Merphenyl nitrate,

a double mercuric salt, at its use-dilution of 1:1500 inhibits E. typhosa immediately but Staph. aureus is able to grow after an exposure of five minutes to this disinfectant. The results are shown in Table XXVl. Both E. typhosa and Staph. aureus are able to resist the bactericidal effect of merthiolate at its use-dilution of 1:1000 after 30 minutes exposure to the disinfectant as shown in Table XXVll. A disinfectant which is not able to produce bacterial kill after such an extended period of exposure would be completely ineffective for all practical disinfection purposes.

Tincture of iodine, employed at a use-dilution of 5%, is capable of killing bacteria in less than one minute. This corresponds to the results obtained by Nungester when using Tincture of iodine to disinfect the skin of a mouse (9). Table XXVl shows the results obtained when testing tincture of iodine by the use-dilution technic.

The use-dilution technic was applied in testing hexylresorcinol (S.T 37) at its use-dilution 1:1000. As the results shown in Table XXVll indicate, no germicidal effect was evident even after thirty minutes exposure of the organisms to the disinfectant.

Dowicide A (orthophenyl phenol) use-dilution 1:100, and Dowicide C, use-dilution 1:1000, were tested to determine germicidal efficiency. Both compounds at their use-dilution were able to effect kill of E. typhosa in less than one minute and Staph. aureus in ten minutes. It is evident from an evaluation of the results shown in Table XXVlll that these compounds would be satisfactory disinfectants if an exposure time of ten minutes were used. A lower dilution which would produce bacterial kill of Staph. aureus upon immediate contact would, however, be preferable.

It is evident from the results obtained by the use-dilution technic that disinfectants may be classified as either satisfactory or unsatisfactory for disinfection purposes. The mercurial compounds and hexylresorcinol (S.T. 37) were found to be unsatisfactory disinfectants. Tincture of iodine, phemerol, Barde-Parker solution and the dowicides are satisfactory for disinfection at their use-dilution. Phenol and lysol are effective as disinfectants only if used at low dilutions. However, lysol, at its use-dilution, would be classed as an unsatisfactory disinfectant.

Table XXV The use-dilution technic applied to Barde-Parker solution and Tincture of Metaphen

	Barde-Parker (full strength)				Tincture of Metaphen			
Time of Exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	0	0	0	0	72	435	0	0
Incubated 24 hours	0	0	0	0	Innum	Innum	0	0
5 Minutes								
Plated immediately	0	0	0	0	5	0	0	0
Incubated 24 hours	0	0	0	0	Innum	Innum	0	0
10 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	200,000		190,000		170,000		180,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

Table XXVlll The use-dilution technic applied to Dowicide A and Dowicide C.

	Dowicide A 1:100				Dowicide C 1:1000			
Time of exposure	Staph. aureus		E. typhosa		Staph. aureus		E. typhosa	
1 Minute								
Plated immediately	193	47	0	0	1000	491	32	0
Incubated 24 hours	Innum	Innum	0	0	Innum	Innum	0	0
5 Minutes								
Plated immediately	21	0	0	0	418	29	0	0
Incubated 24 hours	Innum	0	0	0	Innum	Innum	0	0
10 Minutes								
Plated immediately	0	0	0	0	21	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
30 Minutes								
Plated immediately	0	0	0	0	0	0	0	0
Incubated 24 hours	0	0	0	0	0	0	0	0
Control								
Plated immediately	190,000		190,000		170,000		190,000	
Incubated 24 hours	Innumerable		Innumerable		Innumerable		Innumerable	

SUMMARY

1. A method was developed as a means of determining the killing powers of a disinfectant at its use-dilution.

2. This use-dilution technic was also found to be effective in evaluating various dilutions of the disinfectant and determining the most effective concentration.

3. The use-dilution technic is found to be more simple than the phenol coefficient method and is more practical because it evaluates the disinfectant under conditions comparable to actual use.

4. The use-dilution technic makes it possible to divide disinfectants into two categories: satisfactory and unsatisfactory.

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