STUDIES ON THE METHOD OF EVALUATING DISINFECTANTS AS DEVISED BY WELCH AND HUNTER

Thesis for the Degree of M. S.

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Alma Mildred Stoll

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THESIS

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by

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OUTLINE OF CONTENTS

I. Historical Review of Methods Used in Evaluating Germicides

Very soon after the germ theory of disease had been definitely substantiated, some efforts were made toward evaluating the action of germicides on bacteria. These early methods were crude, and they made no attempt at determinations on a quantitative basis. As the science of bacteriology developed, a variety of methods for evaluating germicides were introduced, all of which endeavored to evaluate the results on a definite mathematical basis. These methods may be classified into two groups, the in vitro and in vivo methods.

A. In Vitro Methods

The first in vitro method introduced was the phenol coefficient method of Rideal-Walker in 1903. (1) Modifications of this method were developed by different workers, of which the one by Shippen, (2) was the most worthy of note. He combined the advantageous features of the Rideal-Walker and the Hygienic Laboratory methods. Later this method became known as the Food and Drug Administration method, (3) which is now official in the United States. The phenol coefficient compares the killing power of a germicide with that of phenol against a specific organism.

Many workers have used manometric methods for evaluating germicides, which measures the inhibition of the metabolic activity of a bacterial suspension by a germicide. Branham (4) modified some of these earlier methods, and placed them on a quantitative basis, using the inhibition of carbon dioxide production by yeasts as the criterion for germicidal action. Ely (5) studied the inhibition of the oxygen consumption by

various drugs upon Escherichia coli. Bronfenbrenner et al (6) not only applied the inhibition of oxygen consumption to bacteria, but also to the inhibition of proliferation of mouse liver cells, thus applying the manometric method as a measurement of toxicity as well. Further work has done by Greig and Hoogerheide (7) showing that the oxygen consumption of actively growing organisms is proportional to the bacterial content and that germicides in bacteriostatic concentrations inhibit cell multiplication, but not rate of metabolism.

As modifications of in vitro methods were gradually introduced, it was soon realized that the methods did not duplicate the actual conditions under which the test was used. It was felt that germicides which were used internally or on mucous membranes, could be tested more accurately by noting their effect on the growth of living embryonic tissue, as well as on their ability to kill bacteria. Lambert, German, Lambert and Meyer, Fuchsbaum and Bloom, (8) all used methods utilizing the inhibiting action of disinfectants on the ability of tissues to proliferate, in conjunction with the killing action on bacteria. Salle (9) continued the work along this line, developing it into a procedure for determining the toxicity index--the ratio of the highest dilution of a germicide required to prevent the growth of embryonic chicken heart tissue during 48 hours, to the highest dilution required to kill the test organism after an exposure of 10 minutes. Theoretically, the smaller the toxicity index, the more nearly perfect the germicide. Later, Salle standardized this procedure, (10) performing it in the presence of organic matter.

In a further attempt to simulate practical conditions, the ability to inhibit phagocytic activity by germicides, was introduced by Nye, (11) who correlated this with the phenol coefficient performed in the presence of organic matter, and with the intradermal injection incapable of producing necrosis at the site of inoculation. Welch and Hunter, (12)

and Welch and Brewer, (13) used the ratio of the highest dilution of germicide inhibiting phagocytosis, to the highest dilution germicidal to the test organism, for determining the toxicity index.

B. In Vivo Methods

Although the in vivo methods for testing germicides more readily simulate the actual conditions of use, not as much work has been done along this line as with the in vitro methods. This is probably due to the fact that a large number of animals are required for the work, thus entailing expense, and also to the fact that the conditions of an experiment are more difficult to control. The first method developed for the determination of toxicity of germicides by animal inoculation was introduced by Hale (14) in 1913, in an attempt to require manufacturers to use suitable poison labels on their products, and to create some standard for designating the toxicity of their products. The toxicity coefficient is expressed as the ratio of the least fatal dose of the germicide, to the least fatal dose of 5% phenol, considering the latter as 100% effective.

Realizing that in vitro tests with germicides give no indication what they will accomplish in vivo, Birkhaug (15) studied the toxicity and histologic changes produced by metaphen, following intravenous injection in rabbits. Combining both the in vitro and in vivo methods, Simmons (16) contaminated the skin of mice with anthrax spores, applied the germicidal solution, excised the skin, and then inverted and inserted it in the wound. Cultures were made from the local lesions and heart blood of the animals that died.

Rungester and Kempf (17) utilized the criterion of the prevention of infection by skin disinfectants, rather than the treatment after infection, as a basis for evaluating skin disinfectants. The tails of

mice artificially infected and subsequently treated with various dilutions of disinfectants were inserted in the peritoneal cavity. Mortality was interpreted as prevention of infection, and protection as evidence of disinfection.

With the original purpose of determining whether the fertile egg could be used as a means of testing the inactivating effect of germicides on viruses, Dunham (18) injected 7 day old chick embryos with germicides, and noted the day of death. The minimum lethal dose in grams per kilogram of body weight which killed more than half of the embryos, compared very favorably with the minimum lethal dose for man. To test the actual therapeutic value of germicides in vivo, which would measure the toxicity for both the tissue and for the test organism simultaneously, Green and Birkeland (19) infected the chorio-allantoic membrane of chick embryos, which were later subjected to various dilutions of germicides. The extent of bacterial growth in the surviving embryos determined by the agar plate method, was considered as indicative of the degree of infection, and the lack of bacterial growth in the surviving embryos as indicative of the effectiveness of the germicide in treating infected tissue.

II. Object of Study

Although the procedure for evaluating germicides by means of the phenol coefficient (3) is still standard, the variety of results obtained on the same compound by different workers, the inability of this method to test germicides chemically unrelated to the phenolic compounds, and to test the germicides under actual conditions of use, show the inadequacy of this method. Dissatisfaction is evidenced by the many attempts to introduce other procedures for the testing of germicidal compounds.

As most of the in vivo methods require a large number of animals and often special equipment, a study of one of these methods was not undertaken. The method presented by Welch and Hunter (12) is definitely an in vitro method, yet at the same time it involves the use of a living tissue, blood, which plays a vital part in in vivo experiments with wound disinfection. With the use of blood, not only is organic matter furnished, in the presence of which all germicides should be tested, but also leucocytes, which are one of the important defense mechanisms of the body against infection.

Because this method could be reproduced in the average laboratory with regard to techniques and equipment, it was studied in an attempt to determine its feasibility as a routine procedure. It was also studied to ascertain the difficulties that would be encountered in its performance, the modifications, if any, that could be suggested, and also to determine if results comparable to that of other workers could be obtained.

III. Experimental

A. Constituents of the Test

Blood. Five ml. of blood was withdrawn aseptically from the vein of apparently healthy individuals into a test tube containing .8 ml. of sterile 20% sodium citrate in .85% salt solution. After inverting the tube several times to insure proper mixing, each 5 ml. of blood was diluted to 20 ml. with sterile .85% salt solution. Guinea pig blood was withdrawn aseptically from the heart without anaesthesia, and collected in the same manner as the human blood. All blood was used within 3-5 hours after collection.

Organism. F. D. A. strain No. 209 of Staphylococcus aureus (a strain of standard resistance) was used throughout the experiment. Before use, it was transferred daily for at least 3 days, at 24 hour intervals.

Culture medium. Due to the present unavailability of some of the constituents of the standard broth (3), the dehydrated Bacto Disinfectant Test Medium of the Difco Laboratories was used. This medium consists of the following ingredients:

Proteose peptone, Difco	10 grams
Bacto-beef extract	3 "
Bacto-lactose	5 "
Sodium chloride	2 "
Ascorbic acid	.25 "
Distilled water	1,000 ml.

Final pH is 7, after autoclaving from 15-20 minutes at 15 pounds pressure. The medium was tubed in 10 ml. amounts.

Antigen Staphylococcus aureus. (F. D. A. strain No. 209) was grown in Kolle flasks on standard nutrient agar of pH 7.2 for 48 hours at 37° C. After smears were made from the growth in each flask to insure the purity of the growth, about 30 ml. of sterile .85% salt solution was added to each flask, and the growth washed off by a gentle rotary motion. The washings were pooled, measured, and treated with an equal volume of a fresh sterile sensitizing chemical compound, which had been dissolved in .85% salt solution. This treated suspension was incubated in an Erlenmeyer flask at 37° C. for from 2-3 hours. The flask was shaken at 15 minute intervals to insure thorough contact of the chemical with the organisms.

At the end of that time, the mixture was centrifuged under aseptic conditions, the supernatant fluid decanted, and the bacterial sediment was then suspended in sterile .85% salt solution, filtered through sterile cotton to remove any clumps, and diluted to give a final concentration of 1 by the Gates nephelometer.

Antiseptics: Four germicides were used in the experiment.

Name	Dilution of Stock Solution	pH	Manufacturer
Sodium orthophenylphenate	1:100 aqueous solution	11.15	Dow Chemical Co.
Phemerol	1:1,000 aqueous solution	7.5	Parke, Davis Co.
Tincture of mercresin	1:1,000 acetone-alcohol solution	6.25	Upjohn Co.
Tincture of merthiolate	1:1,000 acetone-alcohol solution	9.6	Eli Lilly & Co.

Sodium orthophenylphenate and phemerol were made up in distilled water from the dry powder. The tinctures of mercresin and of merthiolate were used in the concentrations in which they are sold in the open market. The pH of all the disinfectants was determined by the Beckman pH meter. The chemical composition of sodium orthophenylphenate is self-evident. Phemerol is one of the quarternary ammonium compounds, tincture of mercresin is composed of a mixture of .1% orthohydroxyl phenyl mercuric chloride and .1% secondary amyl tricresols in an acetone-alcohol vehicle of 10% acetone and 50% alcohol, and tincture of merthiolate is a 1:1,000 dilution of sodium ethyl mercuri-thiosalicylate in an acetone-alcohol vehicle of 10% acetone and 50% alcohol. All of the dilutions of the germicides were made in sterile .85% salt solution.

Staining Solution. A 1% solution of methylene blue in absolute alcohol was used for staining the blood smears. A buffer solution with a pH of 7.2 was made by mixing 50 ml. of m/5 KH_2PO_4 and 35 ml. of m/5 NaOH, and diluting to 200 ml. with distilled water.

B. Procedure

1. Method for Determination of Germicidal Action

After preliminary dilutions of the germicides had been made in sterile

.85% salt solution to determine their germicidal range, dilutions were made up in sterile .85% salt solution in suchvariables as to insure an accurate end point. Two-tenths of a ml. of each dilution was pipetted into sterile Kahn test tubes, and brought up to a temperature of 37°C in a water bath. After the sterile blood had been collected and diluted, each ml. was mixed with .5 ml. of a 22-26 hour broth culture of Staphylococcus aureus (strain No. 209) after it had been filtered through a sterile cotton filter to remove any clumps. The culture-blood mixture was brought up to 37°C. in the water bath, and .3 ml. added aseptically to each tube of diluted germicide. This made the final concentration of 10% for the whole blood. The diluted germicide and the infected blood were pipetted directly to the bottom of the tubes to avoid contamination of the walls, and to avoid loss by drying. Failure to observe these precautions would introduce an error in the results.

At the same time, positive and negative controls were set up, using infected and non-infected blood with sterile .85% salt solution instead of the diluted germicide. After a 30 minute incubation period at this temperature, with shaking at 10 minute intervals to insure uniform contact of the germicide with the blood, the tubes were removed from the bath.

The tubes were again shaken to insure a uniform suspension and a 4 mm. loopful was removed from each tube, and transferred to tubes containing 10 ml. of the Difco antiseptic broth. As phemerol and sodium orthophenylphenate proved to be non-bacteriostatic in action, only this initial transfer was made. However, with mercresin and merthiolate, a second broth tube was inoculated with four 4 mm. loopfuls from the first broth tube (after mixing 3 times with a pipette) to counteract bacterio-

static effects. The tubes were incubated at 37°C, and observations of growth made after 24 and 48 hours' incubation. The results of the germicidal action using human blood are presented in tables 1, 2, 3 and 4, while that with guinea pig blood is presented in tables 5, 6, 7 and 8. A study of the results in table 9 indicates that the germicidal values for human and guinea pig blood are entirely comparable.

Table 1

Germicidal Action of Sodium Orthophenylphenate

for Staphylococcus Aureus Using Human Blood

Series No.	Growth After 48 Hours' Incubation										
	Final Dilutions of Antiseptic										
	250	375	500	625	750	875	1000	1125	1250	1375	1500
1	-	-	-	-	+	+	+	+	+	+	+
2	-	-	-	-	+	+	+	+	+	+	+
3	-	-	-	+	+	+	+	+	+	+	+
4	-	-	-	-	-	+	+	+	+	+	+
5	-	-	-	-	-	+	+	+	+	+	+
6	-	-	+	+	+	+	+	+	+	+	+
7	-	-	-	-	-	+	+	+	+	+	+
8	-	-	-	-	-	-	+	+	+	+	+
9	-	-	-	-	-	+	+	+	+	+	+
10	-	-	-	-	+	+	+	+	+	+	+

Average final germicidal dilution is 1:662.

Table 2

Germicidal Action of Phemerol for Staphylococcus Aureus

Using Human Blood

Series No.	Growth After 48 Hours' Incubation																	
	Final Dilutions of Antiseptic																	
	3750	4000	4250	4500	4750	5000	5250	5500	5750	6000	6250	6500	6750	7000	7250	7500	7750	8000
1	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
2	-	-	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+
3	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+
4	-	-	-	-	-	+	-	-	+	+	+	+	-	+	+	+	+	+
5	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	-	-	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+
7	-	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+
8	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
9	-	-	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+
10	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+

Average final germicidal dilution is 1:5325.

Table 7

Germicidal Action of Tincture of Mercresin for Staphylococcus Aureus

Using Guinea Pig Blood

Series No.	Transplants	Growth After 48 Hours' Incubation																	
		Final Dilutions of Antiseptic																	
		3750	4000	4250	4500	4750	5000	5250	5500	5750	6000	6250	6500	6750	7000	7250	7500	7750	8000
1	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	Secondary	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Primary	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
	Secondary	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
3	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
	Secondary	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
4	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Secondary	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
5	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	Secondary	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
6	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	Secondary	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+
7	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Secondary	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+
8	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Secondary	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
9	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Secondary	-	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+
10	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Secondary	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+

Average final germicidal dilution is 1: 5350.

Table 4

Germicidal Action of Tincture of Merthiolate for Staphylococcus Aureus Using Human Blood

Series No.	Transplants	Growth After 48 Hours' Incubation																		
		Final Dilutions of Antiseptic																		
		2500	2750	3000	3250	3500	3750	4000	4250	4500	4750	5000	5250	5500	5750	6000	6250	6500	6750	7000	7250
1	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Primary	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	-	-	-
	Secondary	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Primary	-	-	-	-	-	+	+	+	-	+	-	+	+	+	-	+	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Primary	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
	Secondary	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Primary	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Primary	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Primary	-	-	-	-	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	Primary	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Average final germicidal dilution is 1: 2600.

Table 5

Germicidal Action of Sodium Orthophenylphenol for *Staphylococcus Aureus* Using Guinea Pig Blood

Series No.	Growth After 48 Hours' Incubation										
	Final Dilutions of Antiseptic										
	250	375	500	625	750	875	1000	1125	1250	1375	1500
1	-	-	-	-	-	-	-	+	+	+	+
2	-	-	-	-	-	-	+	+	+	+	+
3	-	-	-	+	+	+	+	+	+	+	+
4	-	-	-	-	-	-	+	+	+	+	+
5	-	-	-	-	-	+	-	+	+	+	+
6	-	-	-	-	-	-	+	+	+	+	+
7	-	-	-	-	+	+	+	+	+	+	+
8	-	-	-	-	+	-	+	+	+	+	+
9	-	-	-	-	-	-	+	+	+	+	+
10	-	-	-	-	-	-	-	+	+	+	+

Average final germicidal dilution is 1: 800.

Table 8

Germicidal Action of Tincture of Merthiolate for *Staphylococcus Aureus* Using Guinea Pig Blood

Series No.	Transplants	Growth After 48 Hours' Incubation													
		Final Dilutions of Antiseptic													
		2500	2750	3000	3250	3500	3750	4000	4250	4500	4750	5000	5250	5500	5750
1	Primary	-	-	-	+	+	+	+	+	+	+	+	+	+	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	Primary	-	-	-	-	-	-	-	-	-	+	+	+	+	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Primary	-	-	-	-	-	-	-	-	-	-	+	+	+	+
	Secondary	-	+	+	+	+	+	+	+	+	+	+	+	+	+
4	Primary	-	-	-	-	-	-	-	-	+	+	+	+	+	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Primary	-	-	-	-	-	-	-	-	-	+	+	-	+	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	Primary	-	-	-	-	-	-	-	-	-	+	-	+	+	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	Primary	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	Primary	-	-	-	-	-	-	-	-	-	-	-	+	-	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	Primary	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	Secondary	-	-	-	-	+	+	+	+	+	+	+	+	+	+
10	Primary	-	-	-	-	-	-	-	-	-	-	-	+	+	+
	Secondary	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Average final germicidal dilution: < 1: 2500.

Table 9

Comparison of Germicidal Values

with Human and Guinea Pig Blood

Germicide	Human Blood	Guinea Pig Blood
Sodium orthophenylphenate	1:662	1:300
Phemerol	1:5325	1:5675
Tincture of merthiolate	1:2600	< 1:2500
Tincture of mercresin	1:4550	1:5300

Examination of the tables on germicidal action discloses that neither growth nor killing action follows in consecutive order according to the dilutions, but is interspersed occasionally by irregularities. This may be explained in the following manner (10).

It is known that the volume of a 4 mm. loop is .005 ml. Let us assume that the particular germicide we are investigating is that of phemerol in a 1/2,200 dilution. In the germicidal test as actually performed, this gives a final dilution of 1/5,500. If one loopful or .005 ml. of this 1/5,500 dilution of phemerol were inoculated into 10 ml. of broth, the final dilution of phemerol in the broth would be $\frac{10}{.005}$ x 5,500 or a dilution of 1/11,000,000. If a minority of organisms resisted the 1/5,500 dilution of phemerol, their likelihood of survival in the broth would be very great, as the final concentration of phemerol would be so dilute as to be non-germicidal.

Let us consider the fate of the bacteria in this particular dilution of phemerol. Suppose the number of bacteria present in 1/10 ml. of broth culture is 5×10^5. This diluted in the germicidal test would give a final dilution of 1×10^5 organisms. If 99% of these organisms were killed by the

germicide,
a resistant minority of 10^3 organisms would still be left. One loopful would contain .005 x 10^3 or 5 bacteria per ml. As this number would be added to 10 ml. of broth, the final dilution of organisms in the broth would be .5 bacteria per ml.

According to the laws of chance, the possibility of transferring this number of organisms each time a loopful was transferred, as well as the possibility of such a reduced number growing in the broth, is quite remote. Due to this factor in the critical killing dilutions of a germicide, the appearance of irregularities in the growth of consecutive dilutions can be readily explained. In those cases where a secondary transfer is made to overcome bacteriostatic action, the likelihood of transferring a few viable organisms is still further reduced.

To insure the constancy of action of the staphylococcus strain that was used, its resistance to phenol was determined according to the standard procedure (3) each time the test was performed. The results of these tests are shown in Table 10. Even though this strain was supposed to show constant resistance, nevertheless there was considerable variation. At times the growth in the broth was quite floccular, showing a reversion to the rough form. This deviation in resistance would naturally influence the germicidal end point, which is one of the drawbacks to any method employing the phenol coefficient or a modification of it. However, when the results were computed, this was taken into consideration, as those that deviated too markedly from the normal were eliminated.

Table 10

Resistance of *Staphylococcus Aureus*

(F. D. A. Strain No. 209) to 5% Phenol

Dilutions of Phenol	Time Exposure to 5% Phenol		
	5 mins.	10 mins.	15 mins.
90	-	-	-
100	+	-	-
110	+	+	+
90	+	-	-
100	+	-	-
110	+	+	+
90	-	-	-
100	-	-	-
110	+	-	-
90	-	-	-
100	-	-	-
110	+	-	-
90	-	-	-
100	+	-	-
110	+	+	-
90	-	-	-
100	-	-	-
110	+	-	-
90	+	-	-
100	+	+	-
110	+	+	+
90	-	-	-
100	+	-	-
110	+	-	-
90	-	-	-
100	+	-	-
110	+	-	-
90	+	-	-
100	+	-	-
110	+	-	-
90	+	-	-
100	+	+	+
110	+	+	+
90	+	-	-
100	+	-	-
110	+	-	-

2. Method for Determination of Toxic Action

After preliminary dilutions of the germicides had been made in sterile physiological salt solution to determine their toxic range, dilutions were made up in sterile physiological salt solution in such variables as to insure an accurate end point. One-tenth ml. of each dilution was pipetted directly into the bottom of a sterile Kahn test tube, followed by the addition of .2 ml. of standardized antigen. This mixture was brought up to 37°C in a water bath, after which .2 ml. of diluted blood (previously warmed to 37°C) was added. This made the final concentration of whole blood at 10%. At the same time, a control tube was set up, using sterile .85% salt solution instead of the dilution of germicide. After an incubation period of 30 minutes at this temperature, with shaking at 5 minute intervals to insure uniform contact of the germicide and antigen with the blood, the tubes were removed from the water bath.

By means of a Wright capillary pipette, a drop of the suspension was removed from the bottom of each test tube, and smeared on clean grease-free glass slides, according to the usual technique for making blood smears. Drying of the slides was facilitated by means of an electric fan. The smears were stained for 1 minute with 15 drops of the methylene blue solution, followed by the addition of twice the amount of buffer solution for 4 minutes. To aid in mixing the solution on the slides, air was blown on them several times. The slides were then washed in tap water, and allowed to dry by draining.

In each of the critical dilutions, the number of staphylococci phagocytized by the polymorphonuclear leucocytes were counted in each of 25 cells. Only those cells were counted in which it could be definitely determined that the organisms were phagocytized and not just lying on

top of the cells. Those cells showing evidence of disintegration were eliminated from the count, unless all of the cells in that particular dilution showed evidence of disintegration due to the toxicity of the germicide. The degree of phagocytosis is interpreted as follows:

No organisms	engulfed	Absence of phagocytosis
0-20	" "	Slight phagocytosis
20-40	" "	Moderate "
20-40	" "	Marked "

Before adopting the above technique, some experiments were set up in an attempt to obtain optimum and clear cut phagocytic action. This consisted in determining the chemical most suitable for the preparation of the antigen, the length of the incubation period, and the concentration of red blood cells. In these comparative tests, human blood was used exclusively throughout.

a. Experiments on Standardization

(1) Preparation of Antigen

As Staphylococcus aureus is one of the most resistant vegetative organisms, it is necessary to artificially opsonize it in order to induce phagocytosis. One percent solutions of chromium potassium sulphate, ferric ammonium sulphate, and tannic acid were made up in .85% salt solution, and sterilized by passing through a fritted glass filter. Three different lots of antigen were made, in which the Staphylococcus aureus suspension was treated with each of the above compounds. The tests were run according to the procedure for determining toxic action, but with the use of .85% salt solution instead of the dilutions of the germicides. These results are shown in Table 11.

Not only did the leucocytes phagocytize a greater number of staphylococci with the use of the chromium potassium sulphate treated antigen, but the organisms remained separate and distinct, and did not form in

clumps, as with the tannic acid treated antigen. The poor results obtained with the ferric ammonium sulphate treated antigen, was probably due to the fact that during the 2-3 hour incubation period required for the preparation of the antigen, some oxidation took place, with the formation of ferric hydroxide. This formed such a gelatinous coating around the organisms, that it was impossible to free them of this yellow coating by subsequent washings with .85% salt solution.

Table 11

Effect on Opsonization of 10% Human Blood

(final concentration) by Three Different Compounds

Number of Organisms Phagocytized per Cell		
Chromium Potassium Sulphate	Tannic Acid	Ferric Ammonium Sulphate
7/40	0	0
7/40	0	0
7/40	7/40	2
7/40	0	0
7/40	7/40	12
7/40	7/40	6
7/40	7/40	7/40
10	7/40	7/40
7/40	7/40	0
7/40	7/40	5
7/40	0	7
7/40	7/40	0
7/40	7/40	0
0	7/40	4
7/40	7/40	0
7/40	7/40	0
7/40	10	0
7/40	30	0
7	0	0
7/40	30	8
7/40	7/40	0
7/40	7/40	0
7/40	8	5
7/40	7/40	3
7/40	7/40	0

(2) Length of Incubation Period

As all reactions necessitate a certain time period for the various constituents to be in contact with each other in order to bring the reactions to completion, it was thought advisable to perform the test for toxic action using incubation periods of 30, 45 and 60 minutes, with the three antigens. The test was performed according to the previously described technique, with the use of physiological salt solution instead of the dilutions of germicides, and with the different incubation periods as the only variant. These results are shown in Table 12.

Although there was a slight increase in the number of organisms phagocytized for the longer incubation periods, it was felt that the slightly increased numbers did not sufficiently affect the results to warrant the additional time involved in performing the test. Action of the three compounds in affecting phagocytic action, remained relatively the same with regard to each other, regardless of the time period.

(3) Concentration of Red Blood Cells

As it was found by Welch and Brewer (13) that a final concentration of 40% red blood cells proved to be too much organic matter to test the germicidal activity of most germicides, they employed a final concentration of 10% red blood cells for the germicidal tests, and a final concentration of 40% red blood cells for the toxicity tests (12). They stated that it was necessary to use different concentrations of red blood cells in the two phases of the test, in order to obtain definite end points for a greater number of germicides used. Since toxicity indices are based on the ratio of toxic action to germicidal action, it seemed logical that the same final concentration of red blood cells should be used for both tests.

In view of this fact, toxicity tests were set up in duplicate with various dilutions of the four germicides, using human red blood cells in

Table 12

Effect on Opsonization of 10% Human Blood (final Concentration)

by Three Different Compounds at Three Different Incubation Periods

Chromium Potassium Sulphate			Tannic Acid			Ferric Ammonium Sulphate		
30 mins.	45 mins.	60 mins.	30 mins.	45 mins.	60 mins.	30 mins.	45 mins.	60 mins.
740	740	740	0	0	740	0	1	0
740	15	8	0	740	740	0	0	2
740	740	740	740	740	740	0	0	2
740	740	740	0	740	740	0	740	0
740	5	740	740	740	20	12	0	1
740	26	740	740	0	740	6	2	0
740	740	740	740	740	740	740	0	0
10	740	740	740	740	30	740	740	0
740	740	740	740	10	19	0	0	740
740	740	740	740	0	740	5	740	0
740	740	740	0	740	740	7	0	2
740	740	740	740	0	740	0	0	0
740	740	740	740	0	0	0	18	0
0	740	740	740	740	20	4	0	0
740	740	740	740	740	740	0	3	0
740	740	740	740	740	30	0	0	740
740	740	740	10	740	0	0	0	5
740	740	740	30	35	7	0	0	0
7	740	13	0	30	740	0	1	0
740	740	740	30	740	740	8	0	8
740	740	740	740	740	740	0	0	740
740	740	740	740	740	0	0	0	0
740	740	740	8	740	740	5	0	0
740	740	740	740	740	740	3	0	20
740	740	740	740	740	740	0	3	0

final concentrations of 10% and 40%. Otherwise, the tests were performed according to the previously described method for determining toxic action. These results are shown in tables 13, 14, 15 and 16.

Examination of these tables shows that the toxic end point as well as the point at which the germicide is no longer toxic, is much sharper with the use of the final concentration of 10% human red blood cells, than with the 40% red blood cells. This may be explained by the fact that the quantity of germicide and the number of organisms is constant, but with a greater number of polymorphonuclear leucocytes in the 40% blood cell suspension, there will be a fewer number of bacteria phagocytized per cell. With the 40% red blood cell suspension, more organic matter is present, and due to the greater viscosity, the germicide is not brought into such direct contact with the cells, either due to the mechanical effect of improper mixing, or due to the decreased amount of electrolyte in the suspension. In the more concentrated dilutions of the germicides, the polymorphonuclear leucocytes are affected by the toxic action of the germicide due to its extreme concentration, but in the more critical dilutions, the other effects are more obvious.

Table 13

Effect of Final Concentrations of 10% and of 40% Human Blood Cells on Toxic Action of Sodium Orthophenylphenate

10% Red Blood Cells									40% Red Blood Cells				
Final Dilution of Germicide													
500	1000	1500	2000	2500	3000	3500	4000	Control	500	1000	1500	2000	Control
Impossible to count due to disintegration of leucocytes	Impossible to count due to disintegration of leucocytes	0	20	0	740	740	740	740	Impossible to count due to disintegration of leucocytes	35	0	740	740
		0	38	740	30	740	740	740		0	740	740	740
		0	0	30	23	740	740	740			5	740	740
		0	740	32	30	740	740	740		20	38	22	740
		0	740	20	740	740	740	740		25	28	740	40
		0	0	0	740	740	740	740		23	740	740	740
		1	10	740	740	740	740	740		40	0	740	740
		0	30	0	740	740	740	740		40	740	740	740
		0	0	4	8	740	740	740		25	740	740	740
		28	15	0	740	740	740	740		740	35	740	740
		0	3	40	740	740	740	740		0	40	740	20
		0	5	0	740	28	740	740		0	740	740	740
		17	0	0	740	740	740	740		38	12	16	740
		0	740	35	740	740	740	740		0	740	740	740
		0	20	0	740	30	740	740		1	0	740	740
		0	740	1	740	740	740	740		0	740	740	740
		15	740	0	740	740	740	740		22	740	740	740
		25	0	40	740	740	740	740		8	0	740	35
		0	0	0	35	740	740	740		5	40	740	740
		0	0	740	35	23	740	740		0	0	740	740
		0	4	1	30	740	740	740		740	1	740	740
		0	0	0	740	740	740	740		740	740	740	740
		26	0	35	740	35	740	740		0	40	740	740
		0	740	13	0	740	740	740		0	740	740	740
		0	32	38	740	740	740	740		0	740	740	740

Table 14

Effect of Final Concentrations of 10% and of 40% Human Blood Cells on Toxic Action of Tincture of Mercresin

10% Red Blood Cells					40% Red Blood Cells					
Final Dilution of Germicide										
5000	10000	15000	20000	25000	5000	10000	15000	20000	25000	30000
0	0	35	7/40	7/40	1	4	0	7/40	16	7/40
0	0	0	7/40	7/40	0	3	0	20	23	7/40
0	0	0	7/40	7/40	0	0	40	0	6	7/40
0	0	0	3	7/40	0	0	13	0	37	7/40
0	0	0	7/40	7	20	5	3	7/40	21	7/40
0	0	0	7/40	7/40	8	3	1	30	22	7/40
0	0	0	7/40	7/40	0	4	6	0	7/40	7/40
0	0	31	7/40	32	3	0	3	0	0	5
0	0	20	0	7/40	8	0	3	17	6	7/40
0	4	1	28	7/40	15	0	4	14	36	7/40
0	0	4	7/40	7/40	10	3	7/40	0	7/40	3
0	0	28	7/40	7/40	13	0	3	5	0	7/40
0	0	7	7/40	7/40	0	0	7/40	40	12	40
0	0	22	7/40	7/40	11	0	0	16	7/40	7/40
0	0	3	30	7/40	0	6	1	11	0	0
0	0	0	7/40	7/40	7	0	0	26	1	7/40
0	0	0	17	7/40	3	0	0	0	35	7/40
0	0	0	7/40	7/40	0	8	4	7/40	40	6
0	0	7/40	7/40	0	1	0	1	9	40	7/40
0	0	0	0	7/40	10	0	1	7/40	7/40	7/40
0	0	7/40	0	7/40	0	0	5	1	20	0
0	0	0	0	7/40	1	2	5	1	7/40	7/40
0	0	0	7/40	7/40	6	1	0	1	13	7/40
0	0	0	7/40	7/40	9	4	0	7/40	7/40	7/40
0	0	25	7/40	7/40	2	1	0	9	35	7/40

Table 15

Effect of Final Concentration of 10% and 40% Human Blood Cells on Toxic Action of Tincture of Merthiolate

10% Red Blood Cells						40% Red Blood Cells								
Final Dilution of Germicide														
5000	10000	15000	20000	25000	30000	5000	10000	15000	20000	25000	30000	35000	40000	45000
0	0	10	0	7/40	7/40	0	0	0	7/40	27	35	7/40	14	7/40
0	0	10	0	7/40	35	0	0	22	7/40	0	0	37	22	7/40
0	0	6	22	7/40	11	0	0	6	0	7/40	0	7/40	3	7/40
0	0	6	21	22	7/40	0	0	9	11	0	7/40	7/40	23	7/40
0	0	7/40	11	7/40	7/40	0	0	7	29	7/40	0	7/40	20	7/40
0	1	3	12	7/40	7/40	0	0	7/40	0	7/40	7/40	7/40	21	0
0	0	8	0	0	7/40	0	0	0	30	23	7/40	3	7/40	7/40
0	3	0	15	7/40	7/40	0	0	18	3	25	7/40	17	7/40	36
0	0	4	26	7	7/40	0	1	0	21	7/40	7/40	7/40	15	7/40
0	0	21	7/40	7/40	7/40	0	0	1	5	7/40	0	7/40	7/40	7/40
0	0	15	5	1	7/40	0	0	0	30	22	0	7	19	7/40
0	4	8	30	7/40	7/40	0	0	0	7	0	0	18	25	7/40
0	0	7/40	6	7/40	0	0	1	2	16	35	0	7/40	0	8
0	0	8	7/40	7/40	7/40	0	0	3	35	7/40	7	0	7/40	7/40
0	1	7/40	0	30	7/40	0	0	0	0	0	30	2	7/40	7/40
0	0	0	30	7/40	7/40	0	0	0	0	7/40	20	7/40	7/40	7/40
0	0	0	32	7/40	7/40	0	0	2	7/40	7/40	7	7/40	7/40	7/40
0	0	3	0	7/40	7/40	0	1	2	15	0	35	7/40	7/40	7/40
0	0	9	7/40	7/40	7/40	0	0	9	10	7/40	1	0	25	7/40
0	5	0	4	15	7/40	0	0	13	14	7/40	7/40	0	0	20
0	0	0	7	7/40	7/40	0	0	0	0	3	27	7/40	7/40	7/40
0	0	2	7	7/40	7/40	0	0	0	7/40	7/40	35	4	0	7/40
0	3	6	7/40	7/40	7/40	0	0	11	13	0	7/40	7/40	23	7/40
0	0	5	7/40	7/40	7/40	0	0	14	20	7/40	7/40	12	7/40	8
0	0	0	0	7/40	7/40	0	0	2	2	0	0	7/40	0	7/40

Table 16

Effect of Final Concentration of 10% and of 40% Human Blood Cells on Toxic Action of Phemerol

10% Red Blood Cells			40% Red Blood Cells			
Final Dilution of Germicide						
5000	10000	15000	5000	10000	15000	20000
0	7+40	7+40	25	1	7+40	7+40
0	7+40	7+40	0	7+40	10	7+40
0	7+40	7+40	2	0	0	7+40
0	1	7+40	0	0	7+40	7+40
0	7+40	0	2	7+40	7+40	13
0	7+40	7+40	0	0	7+40	40
0	7+40	7+40	0	8	4	7+40
0	7+40	7+40	0	0	7+40	32
0	15	7+40	0	3	1	7+40
0	7+40	7+40	0	2	7+40	0
0	7+40	0	0	2	7+40	7+40
0	7+40	7+40	23	4	7+40	18
0	7+40	7+40	0	7+40	7+40	7+40
0	7+40	7+40	0	7+40	7+40	7+40
0	7+40	7+40	40	1	6	7+40
0	18	7+40	0	7+40	7+40	7+40
0	15	0	0	8	7+40	20
0	7+40	7+40	0	4	7+40	7+40
0	7	7+40	23	7+40	24	7+40
0	7+40	7+40	0	2	7+40	7+40
0	5	7+40	0	1	7+40	7+40
0	0	7+40	1	7+40	7+40	7+40
0	14	7+40	0	0	11	7+40
0	7+40	7+40	0	7+40	7+40	7+40
0	7+40	7+40	0	0	1	7+40

b. Results of the Toxic Action on Human Blood

Toxicity tests were performed with the four germicides, with the adopted modifications. The results shown in tables 17, 18, 19, 20 and 21 are based on the average of the results obtained on five series of tests with each germicide. The toxic end point was selected as the lowest dilution of the germicide in which all phagocytic action was inhibited. In those cases where one or two cells showed less than five organisms phagocytized, those dilutions were also considered as toxic, as that slight amount of phagocytosis was considered negligible. The non-toxic end point was selected as the highest dilution of the germicide in which over 70% of the cells showed marked phagocytosis.

The degree to which phagocytosis takes place may be interpreted in the following manner (11). In those dilutions in which phagocytosis was completely inhibited, the leucocytes were killed immediately by the germicide due to its extreme concentration, so that the normal function of the leucocyte was not possible. In those dilutions in which phagocytosis was slight, the killing action of the germicide did not take place immediately, so that phagocytic action was initiated. However, after a short time the germicide began to act, and stopped further phagocytic activity by the leucocyte. In those dilutions in which phagocytosis was marked, the germicide had been diluted to the point where it no longer interfered with the normal phagocytic activity of the cell (20). Complete inhibition of phagocytosis cannot be interpreted as complete destruction of the cell, as this state can also be due merely to loss of function.

From a study of table 21, it will be noted that there is a marked variation in phagocytic activity of the controls, from 56-92% of the cells showing marked phagocytosis. Sometimes this decreased phagocytic activity did not affect the final results materially, as in Series 2, but in some

cases it affected the results markedly. The day on which Series 4 was performed, a duplicate series was run, with blood from two different individuals as the only variable factor. The control on Series 4 showed that 92% of the cells displayed marked phagocytosis, while the control on the duplicate series only showed 62%. This decreased phagocytic activity affected the final results to such an extent that these results had to be discarded. Since leucocytes are constantly undergoing destruction in the blood under normal conditions, the presence of a few cells in the control tests showing complete lack of phagocytic activity could easily be attributed to this phenomenon.

Another observation made while performing the toxicity tests, was that in the more concentrated dilutions of the germicides, the red blood cells were lysed, and the leucocytes showed evidences of disintegration, particularly in those dilutions that proved to be toxic. However with phemerol, a surface tension depressant, the leucocytes and their phagocytic activity were unaffected, even though coagulation of the plasma, and lysis of the red blood cells took place.

Toxicity Tests with

Series 1						Series 2							Series 3		
1250	1500	1750	3250	3500	3750	1500	1750	2000	3250	3500	3750	4000	1500	1750	3500
0	2	0	7⅖0	7⅖0	7⅖0	0	18	0	7⅖0	0	7⅖0	7⅖0	0	30	7⅖0
0	0	16	7⅖0	30	1	0	0	7⅖0	7⅖0	0	7⅖0	4	0	26	7⅖0
0	0	0	7⅖0	7⅖0	7⅖0	0	0	0	0	0	7⅖0	0	0	1	7⅖0
0	0	26	0	0	7⅖0	0	0	0	0	7⅖0	0	7⅖0	0	0	0
0	0	0	7⅖0	7⅖0	7⅖0	0	0	0	7⅖0	7⅖0	7⅖0	0	0	7⅖0	30
0	0	9	⅖0	7⅖0	7⅖0	0	27	7⅖0	0	7⅖0	7⅖0	7⅖0	0	11	7⅖0
0	0	15	33	7⅖0	7⅖0	0	0	2	7⅖0	7⅖0	7⅖0	7⅖0	0	0	7⅖0
0	0	0	7⅖0	7⅖0	0	0	0	0	7⅖0	7⅖0	7⅖0	7⅖0	0	4	0
0	15	35	0	7⅖0	0	0	23	0	7⅖0	7⅖0	0	7⅖0	0	0	0
0	0	0	0	7⅖0	7⅖0	0	0	0	0	7⅖0	7⅖0	0	0	0	7⅖0
0	0	7⅖0	7⅖0	7⅖0	4	0	0	28	0	7⅖0	7⅖0	7⅖0	0	7⅖0	25
0	0	0	7⅖0	7⅖0	7⅖0	0	0	0	0	7⅖0	7⅖0	7⅖0	5	0	7⅖0
0	1	7⅖0	7⅖0	0	7⅖0	0	36	0	7⅖0	0	7⅖0	7⅖0	0	17	7⅖0
0	0	0	7⅖0	7⅖0	7⅖0	0	0	0	7⅖0	7⅖0	0	0	0	0	7⅖0
0	0	12	7⅖0	0	7⅖0	0	36	0	7⅖0	0	7⅖0	7⅖0	0	7⅖0	0
0	1	0	0	7⅖0	7⅖0	0	0	30	7⅖0	7⅖0	7⅖0	7⅖0	0	3	0
0	0	4	7⅖0	7⅖0	7⅖0	0	0	12	0	7⅖0	0	0	0	19	7⅖0
0	0	0	0	7⅖0	20	0	0	0	0	0	7⅖0	7⅖0	0	17	7⅖0
0	0	0	7⅖0	0	7⅖0	0	0	7⅖0	0	7⅖0	0	7⅖0	0	7⅖0	0
0	3	0	7⅖0	25	7⅖0	0	0	15	0	0	16	0	0	0	7⅖0
0	0	0	7⅖0	1	7⅖0	0	12	0	7⅖0	0	7⅖0	7⅖0	0	23	0
0	0	0	⅖0	27	7⅖0	0	0	0	0	7⅖0	7⅖0	22	0	7⅖0	14
0	0	7	0	7⅖0	7⅖0	0	0	7⅖0	7⅖0	7⅖0	0	7⅖0	0	5	0
0	1	27	25	7⅖0	7⅖0	0	18	0	7⅖0	7⅖0	7⅖0	7⅖0	0	0	3
0	0	0	12	7⅖0	7⅖0	0	0	0	7⅖0	0	7⅖0	7⅖0	0	6	0

Toxic 1: 1250
Non-toxic 1: 3750

Toxic 1: 1500
Non-toxic 1: 3750

Toxic 1: 1500
Non-toxic 1:

Toricity Tests with

Series 1						Series 2						Series	
17500	20000	22500	37500	40000	42500	10000	12500	15000	27500	30000	32500	7500	10000
0	5	8	7½0	7½0	7½0	0	1	1	7½0	7½0	7½0	0	5
0	5	0	7½0	7½0	7½0	0	2	3	7½0	7½0	7½0	0	0
0	2	0	0	7½0	0	0	0	15	14	7½0	7½0	0	1
0	0	0	7½0	0	7½0	0	0	12	7½0	7½0	7½0	0	3
0	2	3	17	0	7½0	0	0	2	7½0	7½0	7½0	0	0
0	0	4	7½0	7½0	7½0	0	5	0	7½0	0	7½0	0	0
0	6	0	7½0	18	7½0	0	0	3	7½0	0	0	0	0
0	0	0	7½0	14	7½0	0	3	7	7½0	0	7½0	0	0
0	1	4	7½0	7½0	7½0	0	0	1	0	7½0	7½0	0	0
5	3	7	7½0	0	7½0	2	3	4	7½0	0	7½0	0	3
0	8	7	0	0	7½0	0	0	9	7½0	0	7½0	0	0
0	3	0	7½0	7½0	7½0	0	0	5	0	7½0	7½0	0	0
0	0	8	7½0	0	7½0	0	0	4	7½0	7½0	7½0	0	0
0	0	12	3	0	7½0	0	0	15	0	7½0	0	0	0
0	0	0	7½0	7½0	7½0	0	0	5	7½0	7½0	7½0	0	0
3	0	0	7½0	7½0	7½0	2	0	0	7½0	7½0	7½0	0	0
0	0	0	7½0	7½0	0	0	2	4	0	0	7½0	0	0
0	0	13	7½0	0	7½0	0	0	7	7½0	7½0	7½0	0	2
0	0	0	0	7½0	7½0	0	0	6	3	7½0	7½0	0	0
0	0	0	22	7½0	7½0	0	1	0	7½0	0	7½0	0	0
0	3	0	7½0	0	7½0	0	4	14	7½0	7½0	7½0	0	0
0	2	6	7½0	7½0	7½0	0	0	6	0	7½0	7½0	0	0
0	1	4	31	7½0	7½0	0	1	1	7½0	0	7½0	0	1
0	0	4	0	7½0	7½0	0	1	1	7½0	0	7½0	0	0
0	3	3	7½0	7½0	7½0	0	0	5	7½0	0	7½0	0	3

Toxic 1: 17500
Non-toxic 1: 42500

Toxic: 1: 10000
Non-toxic 1: 32500

Toxicity Tests with Phemerol

Series 1					Series 2					Series 3					Series 4						Series 5				
6000	7000	10000	11000	12000	6000	7000	10000	11000	12000	6000	7000	8000	11000	12000	13000	6000	7000	8000	10000	11000	12000	5000	6000	10000	11000	12000
0	0	7⅖	7⅖	⅖	0	0	7⅖	7⅖	7⅖	0	0	25	5	7⅖	7⅖	7⅖	7⅖	7⅖	7⅖	7⅖	7⅖	0	0	0	0	7⅖
0	0	0	7⅖	24	0	12	4	7⅖	15	0	0	5	7⅖	0	7⅖	0	0	10	0	7⅖	7⅖	0	0	7⅖	7⅖	7⅖
0	5	7⅖	7⅖	7⅖	0	18	7⅖	7⅖	7⅖	0	0	0	12	7⅖	7⅖	0	18	10	20	7⅖	7⅖	0	9	7⅖	7⅖	7⅖
0	13	0	7⅖	7⅖	0	16	21	7⅖	7⅖	0	0	3	10	7⅖	7⅖	0	0	0	7⅖	0	7⅖	0	16	0	7⅖	30
0	0	7⅖	7⅖	7⅖	0	6	7⅖	7⅖	7⅖	0	5	7⅖	0	7⅖	7⅖	0	0	6	7⅖	7⅖	0	0	0	26	7⅖	7⅖
0	0	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖	0	0	9	25	7⅖	7⅖	0	0	7	7⅖	7⅖	7⅖	0	0	21	7⅖	7⅖
0	22	21	10	7⅖	0	0	24	14	7⅖	0	0	7⅖	7⅖	7⅖	7⅖	0	9	0	0	7⅖	7⅖	0	0	0	12	7⅖
0	0	7⅖	7⅖	7⅖	0	4	7⅖	7⅖	18	0	5	31	0	0	7⅖	0	0	0	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖
0	10	7⅖	12	7⅖	0	7⅖	7⅖	26	7⅖	0	0	7⅖	7⅖	7⅖	7⅖	0	0	4	7⅖	0	7⅖	0	7	7⅖	7⅖	7⅖
0	0	7⅖	7⅖	0	0	36	7⅖	35	7⅖	0	0	6	3	7⅖	7⅖	0	10	0	7⅖	7⅖	7⅖	0	0	21	0	7⅖
0	4	0	21	7⅖	0	0	0	0	7⅖	0	0	8	7⅖	7⅖	7⅖	0	0	7⅖	14	7⅖	7⅖	0	37	24	7⅖	7⅖
0	2	0	0	7⅖	0	24	0	0	12	0	3	0	0	7⅖	7⅖	0	0	3	6	7⅖	7⅖	0	0	28	7⅖	7⅖
0	0	7⅖	0	7⅖	0	5	7⅖	0	7⅖	0	0	0	7⅖	7⅖	7⅖	0	7⅖	0	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖
0	3	12	7⅖	7⅖	0	0	22	7⅖	7⅖	0	0	5	7⅖	7⅖	7⅖	0	0	20	0	0	7⅖	0	2	7⅖	0	7⅖
0	0	7⅖	7⅖	7⅖	0	27	7⅖	7⅖	7⅖	0	0	0	7⅖	7⅖	7⅖	0	7⅖	6	0	7⅖	7⅖	0	0	7⅖	7⅖	1
5	0	7⅖	7⅖	0	0	0	7⅖	7⅖	20	0	0	0	0	0	7⅖	0	0	0	7⅖	7⅖	0	0	8	0	7⅖	7⅖
0	0	0	0	7⅖	0	25	8	0	7⅖	0	8	7	10	7⅖	7⅖	0	0	0	4	7⅖	7⅖	0	0	7⅖	7⅖	7⅖
0	0	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖	0	0	0	18	7⅖	7⅖	0	5	7⅖	7⅖	7⅖	7⅖	0	6	7⅖	7⅖	7⅖
0	0	3	7⅖	7⅖	0	0	34	7⅖	7⅖	0	0	18	7⅖	0	7⅖	0	0	8	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖
0	3	7⅖	7⅖	21	0	0	7⅖	7⅖	0	0	0	3	7⅖	7⅖	7⅖	0	20	0	0	8	7⅖	0	21	7	7⅖	7⅖
0	6	7⅖	7⅖	7⅖	0	17	7⅖	7⅖	7⅖	0	10	0	7⅖	7⅖	7⅖	0	7⅖	0	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖
0	0	19	7⅖	7⅖	0	1	11	7⅖	7⅖	0	0	1	7⅖	0	7⅖	0	0	10	7⅖	7⅖	17	0	9	7⅖	7⅖	7⅖
0	5	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖	0	0	7⅖	0	7⅖	7⅖	0	12	7⅖	7⅖	7⅖	7⅖	0	7⅖	14	7⅖	7⅖
0	0	0	7⅖	2	0	32	7⅖	7⅖	7⅖	0	0	25	8	7⅖	7⅖	0	0	0	0	25	7⅖	0	0	7⅖	7⅖	7⅖
0	0	0	7⅖	0	0	0	7⅖	7⅖	7⅖	0	0	7	7⅖	0	7⅖	0	0	0	7⅖	7⅖	7⅖	0	0	7⅖	7⅖	7⅖

Toxic 1: 6000
Non-toxic 1: 11000

Toxic 1: 6000
Non-toxic 1: 11000

Toxic 1: 6000
Non-toxic 1: 12000

Toxic 1: 6000
Non-toxic 1: 11000

Toxic 1: 5000
Non-toxic 1: 11000

Toxicity Tests with Tincture of Mercresin

Series 1							Series 2						Series 3							Series 4						Series 5		
12500	15000	30000	32500	35000	37500	40000	10000	12500	15000	30000	32500	35000	37500	12500	15000	27500	30000	32500	35000	10000	12500	15000	27500	30000	32500	7500	10000	20000
0	32	740	740	740	740	740	0	0	0	740	740	740	740	0	0	740	20	740	740	0	0	0	0	740	740	0	0	740
0	0	740	14	740	0	740	0	0	0	0	740	0	740	0	4	740	740	740	0	0	0	0	31	740	740	0	20	0
0	7	740	740	740	0	740	0	0	12	740	740	0	2	0	2	740	740	740	740	0	6	16	740	740	740	0	0	740
0	0	16	740	740	740	740	0	0	0	740	740	740	740	0	28	0	740	0	740	0	0	4	740	6	740	0	0	22
0	0	740	740	740	740	740	0	2	20	0	740	740	740	0	0	740	740	740	0	0	0	0	32	740	740	0	0	740
0	0	740	740	740	740	740	0	0	0	740	16	740	740	0	35	0	740	740	740	0	0	15	25	740	0	0	2	0
0	14	740	740	3	740	740	0	0	12	740	740	0	740	0	0	0	740	4	0	740	0	10	26	740	7	740	0	0	740
5	19	33	31	740	10	740	0	0	0	740	740	740	740	740	0	0	740	740	740	740	0	0	5	0	740	740	0	2	740
0	10	740	740	34	740	740	0	0	19	740	740	740	740	740	4	0	740	740	740	740	0	0	24	740	740	740	0	0	740
0	25	740	740	740	36	740	0	0	18	0	9	0	740	0	25	0	740	740	0	740	0	4	2	740	740	740	0	0	740
0	0	740	6	740	740	740	0	3	740	740	740	0	740	0	0	740	3	2	740	0	0	11	0	22	13	0	2	740
0	0	5	740	23	740	740	0	0	1	740	740	740	740	0	0	0	740	740	740	0	0	15	740	740	740	0	0	16
0	0	8	740	23	28	740	0	5	28	10	32	740	0	0	0	740	740	740	1	0	0	0	740	740	740	0	8	740
0	15	740	17	0	740	740	0	0	10	740	740	13	740	0	13	740	740	8	740	0	10	32	0	740	740	0	2	26
0	0	0	740	2	740	740	0	1	0	740	14	740	740	0	740	0	740	740	740	0	0	0	740	17	740	0	0	15
0	0	0	740	740	740	740	0	0	24	35	740	740	740	0	0	0	0	740	740	0	0	0	740	740	740	0	0	740
0	20	740	740	740	0	740	0	0	0	740	740	740	740	0	21	740	740	0	0	0	2	22	0	740	740	0	2	740
0	0	740	10	740	740	740	0	740	0	0	0	740	740	0	8	0	740	740	740	0	0	4	740	740	740	0	0	10
0	0	740	740	740	740	740	0	0	4	740	740	13	0	0	0	740	0	740	3	0	0	15	0	740	740	0	0	740
0	4	20	0	740	740	740	0	0	740	0	5	740	740	0	740	6	740	0	740	0	3	4	740	740	22	0	2	740
0	16	0	14	740	7	740	0	0	0	740	740	740	740	0	15	0	740	740	740	0	0	9	19	740	740	0	0	740
0	12	0	740	740	740	740	0	0	6	0	1	740	740	0	0	740	0	740	0	0	0	0	740	740	740	0	0	740
0	1	0	740	740	740	740	0	4	0	740	740	740	740	0	0	740	740	740	740	0	0	0	5	740	740	0	2	24
0	740	0	740	740	740	740	0	0	740	6	740	740	740	0	16	740	740	740	740	0	0	11	740	740	740	0	0	740
0	12	38	740	30	740	740	0	0	0	740	35	740	740	0	12	740	0	740	740	0	0	12	740	740	740	0	0	740

Toxic 1: 12500
Non-toxic 1: 32500

Toxic 1: 10000
Non-toxic 1: 35000

Toxic 1: 12500
Non-toxic 1: 30,000

Toxic 1: 10000
Non-toxic 1: 30000

Toxic 1: 7500
Non-toxic 1: 22500

Table 21

Controls of Toxicity Tests

Number of Organisms Phagocytized per Cell				
Series 1	Series 2	Series 3	Series 4	Series 5
740	740	740	740	740
740	740	740	740	740
740	20	740	740	740
740	7	0	740	740
740	740	740	740	25
740	0	740	740	740
740	740	740	740	740
740	0	740	0	740
740	740	740	740	740
2	740	0	740	740
740	740	740	740	3
740	0	740	740	740
740	740	740	740	740
740	0	740	740	740
740	740	2	740	740
740	740	740	740	0
740	27	740	0	740
740	0	740	740	740
0	740	740	740	740
740	740	0	740	740
740	28	740	740	740
740	740	740	740	0
740	0	740	740	740
740	0	740	740	740
740	740	740	740	740

3. Determination of Toxicity Indices

Using the same method for calculating toxicity indices as that proposed by Salle, (10) the toxicity indices were calculated on the basis of the average results obtained in the germicidal and toxicity tests. These results are shown in table 22, and those obtained by Welch and Hunter (12) and Welch and Brewer (13) in table 23.

A study of these tables shows that the results obtained were very comparable and the amount of deviation was no greater than that which would be expected in the hands of different workers. Although they did not make any studies on phemerol, the results which they obtained on zephiran are comparable, since both are quarternary ammonium compounds. The slight differences obtained in the results with sodium orthophenylphenate may be due to the differences in hydrogen ion concentration of the two solutions. They do not give the hydrogen ion concentration of the solution they used, while the one I used had a pH of 11.15. Some of the higher orthophenols are insoluble in water, but are soluble in an excess of alkali. (21) However, an excess of alkali reduces the germicidal power.

These tables emphasize the fact that an efficient germicide is a compound which is non-toxic in the dilution in which it is germicidal, and also the importance of using germicides only in the particular dilution in which they are germicidal. If germicides are used in the dilution in which there is absolutely no toxicity toward tissue, they are no longer germicidal.

Table 22

Toxicity Indices in this Study

Germicide	A Toxic Dilution	B Germicidal Dilution	Non-toxic Dilution	A/B Toxicity Index
Sodium Orthophenylphenate	1: 1150	1: 662	1: 3750	1.7
Phemerol	1: 4600	1: 5325	1: 11200	.86
Tincture of merthiolate	1: 11500	1: 2600	1: 30500	4.42
Tincture of mercresin	1: 10500	1: 4550	1: 30000	2.3

Toxicity Indices of Welch, Brewer and Hunter

Sodium Orthophenylphenate	1: 900	1: 900	Not determined	1.
Phemerol	1: 3000	1: 6250	" "	.48
Tincture of merthiolate	1: 17000	1: 2980	" "	5.7
Tincture of mercresin	1: 7500	1: 3000	" "	2.5

4. Experiments with Guinea Pig Blood

As guinea pigs are easy to handle and readily available laboratory animals, studies were attempted using their blood, with a view toward determining their toxicity indices. Previous to setting up the experiments with the germicides, control tests were set up, using .85% salt solution. The tests were performed in every way as in the procedure outlined for human blood, but in the smears of blood samples from three different guinea pigs, only from one to two cells showed phagocytosis, and those had only phagocytized from two to five organisms.

Since these samples of guinea pig blood showed so little phagocytic activity, it was thought that these guinea pigs might be abnormal, so immediately upon withdrawal of the blood from one of the pigs, a blood smear was made, and stained with Wright's stain. On the basis of 200 leucocytes counted, the following differential was obtained.

Polymorphonuclears	20%
Lymphocytes	76%
Eosinophiles	2%
Monomuclears	2%

Many of the red blood cells showed marked anisocytosis and poikilocytosis, an occasional cell showed achromia and polychromatophilia, and an occasional normoblast was found. Many of the lymphocytes also showed an abnormality in the form of an oval inclusion cell, which was sometimes smooth in appearance, and sometimes so disintegrated that only the cell wall remained. Usually only one body was found in a cell, but occasionally several small ones were found. These bodies took the acid stain.

As judged by the standards for normal values of human blood, this smear showed evidences of a pathological condition, both with regard to the leucocytes and the erythorocytes. This belief was further substantiated by the fact that all three guinea pigs died about one month later.

(As judged by the standards for normal values of human blood, this smear showed evidences of apathological condition, both with regard to the leucocytes and the erythrocytes. This belief was further substantiated by the fact that all three guinea pigs died about one month later.)

Following that time interval, another guinea pig was bled, and efforts were again made toward performing the toxicity test. At this time 100 leucocytes were counted, of which 19% showed phagocytosis. The action was very weak, however, as 18% showed slight phagocytosis, while only one cell phagocytized 3 organism. At the same time, another control was set up in which the incubation period was extended to 60 minutes. This time only 23% of the cells showed phagocytosis, most of which showed only slight phagocytosis, and only 4% showed marked phagocytosis. As the normal phagocytic activity of the cells was so low, it was not deemed advisable to continue the toxicity tests using guinea pig blood. These results are not in agreement with those of Welch (22) who states that the average of the control tests from ten normal guinea pigs showed that 56% of the cells exhibited marked phagocytosis (7-40 organisms per cell).

A blood smear was also made from this guinea pig, and on the basis of 200 cells counted; the following differential was obtained:

Polymorphenuclears	42.5%
Lymphocytes	56.5%
Large mononuclears	1 %

An occasional red blood cell showed anisocytosis, achromia, and polychromatophilia, and an occasional normoblast was seen. Many of these lymphocytes also showed the presence of the inclusion cells.

Klieneberger (23) gives the following average normal values and the range of normal values for guinea pig blood:

Normal Values		Range of Values	
Hb 79%; R.B.C. 5,270,000; W.B.C. 15,000.		Hb 83-107%; R.B.C. 4,430,000-6,150,000; W.B.C. 6,900-13,400.	
Differential		Differential	
Polymorphonuclears	38.5 %	Polymorphonuclears	9-48%
Lymphocytes	46.5 %	Lymphocytes	33-88%
Eosinophiles	13 %	Eosinophiles	1-10%
Mononuclears	.5 %	Mononuclears	0-1.5%
Basophiles	.85%	Basophiles	0-1%
Transitionals	.65%	Transitionals	.5-3%

The blood findings of the normal guinea pig show (23) that the red blood cells exhibit considerable anisocytosis, frequent evidences of polychromatophilia, an occasional stippled cell, and very rarely an erythroblast. The hemoglobin and red cell values are approximately the same as in human blood, but the leucocytes are slightly higher, with the lymphocytes in the majority. In the blood smear, lymphocytic cells are quite regularly found with eccentric nuclei and oval inclusion cells. In the same animal, cells of this type are found with a small nucleus and a large inclusion body, and cells with a large nucleus and a small inclusion body. It is not certain whether these cells belong to the lymphoid or myelocyte system, and whether this change in the size of the inclusion cells is a cycle in the development of the cell or not.

Downey (24) states that a peculiar body, oval in shape, and suggestive of a phagocytized erythrocyte is found in the lymphocytes and monocytes of guinea pig blood. Usually one is present in a cell, and sometimes several may occur. He ascribes the name of Kurloff bodies to these inclusion cells, and states that they have also been described in the lymphocytes of wild Cavidae.

As the above references show, the normal guinea pig blood deviates considerably, particularly in the differential count, and the inclusion bodies in the lymphocytic cells, and the evidences of anemia are normally present. It cannot be assumed then, that the guinea pigs I bled showed any pathological condition. However, these findings do not help to explain the weak phagocytic action of guinea pig cells. Klieneberger (23) states that a thorough anatomical study, ad a study of the diet and living conditions would have to be made, in order to explain the frequent anemia found in the guinea pig, which is otherwise difficult to explain. If these are consi lered as possible contributory factors to anemia in the guinea pig, might not they also in some way affect the phagocytic activity?

Although Spink (25) et al in their experiments could not show that ascorbic acid influenced antibacterial activity, yet in a review of experiments performed by other workers, results were shown that the activity of the complement was directly related to ptimal concentrations of ascorbic acid. The experimental work of Maccari (26) and Ardy (27) offers further proof that the opsonic power of guinea pigs is increased by the injection of vitamin C, and that in scorbutic animals the opsonic power is almost zero. Welch (22) showed that a thermolabile substance in the complement of the guinea pig was an essential factor in producing phagocytosis in the method for determining the toxicity of germicides. It is generally known that guinea pigs require vitamin C in their diet, for without it, they soon show evidences of nutritional diseases. On the basis of the evidence furnished, the conjecture could be made that the failure of guinea pig cells to show normal phagocytosis ~~is needed to substantiate this statement.~~ was due To lack of vitamin C. However, further proof is needed To substantiate This statement.

Toxicity indices could not be calculated on guinea pig blood, as I was unable to perform tests showing the toxic action of germicides on guinea pig blood.

IV. Summary

The method of evaluating germicides according to their toxicity indices, that is, the highest possible germicidal efficiency with the least possible toxicity for human cells, is a method that can be reproduced in most laboratories with fairly consistent results. This is substantiated by the similarity in results obtained in this study and that obtained by Welch, Brewer, and Hunter on the same compounds.

It reproduces to some extent the actual conditions encountered in wounds by testing germicides in the presence of organic matter, blood, which not only is a tissue normally found in the body, but is also an important defensive mechanism against bacterial invasion by means of the phagocytic activity of the leucocytes. Other actual conditions simulated are the temperature (body temperature) at which the test is performed, and the use of physiological salt solution as a diluent for all of the suspensions.

Actual conditions as they naturally occur are not simulated, for the germicides are not tested according to their internal action, on mucous surfaces, or on the other tissues of the body. However, by testing germicides according to their toxic action on one of the body tissues, they are classified on an entirely different basis than when classified solely by their phenol coefficients. As the toxicity index is entirely independent of the phenol coefficient, the former method of rating places a narrower limitation on the use of germicides.

By the retention of a modified phenol coefficient method for testing the germicidal action, all the deficiencies of the phenol coefficient method are encountered. Among the most important of these deficiencies may be mentioned the limitation of testing with only a few organisms occurring in natural infections, variability in resistance and numbers

of organisms, proportion of culture to disinfectant, inaccuracy of loop, influence of composition of medium on growth, testing in the absence of organic matter, and the inapplicability of use on compounds unrelated to phenol.

With regard to the actual performance of the test, the end points for determining toxic action are very sharp, and fairly consistent, but the process of making the smears, staining, and counting the ingested bacteria is not only time-consuming, but very tedious. However, the use of slides furnishes a permanent record which can be referred to and checked at any time. The necessity of having freshly drawn human blood available, and the fact that it is not constant in its phagocytic ability, are other obstacles in this test. Although guinea pig blood is available at any time, its weak phagocytic activity, and the differences in its constituents as compared to that of human blood, does not make its use feasible for determining toxic action, or for use in simulating the activities and reactions of human blood. The possibility of vitamin C deficiency as an explanation of weak phagocytic action, offers the opportunity for further investigation along this line.

In conclusion, I believe that this method serves only as a tentative in vitro method for classifying germicides on the basis of their germicidal and toxic effects, preparatory to testing them by in vivo methods in experimental and natural infections.

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