

EFFECT OF CULTURE MEDIA ON THE RESISTANCE AND
GROWTH OF MICROCOCCUS PYOGENES VAR. AUREUS

By

ANITA HARRIET LEAVITT

A THESIS

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

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

1952

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to Dr. W. L. Mallmann for his encouragement and advice throughout this study.

TABLE OF CONTENTS

	Page
I. INTRODUCTION	1
II. REVIEW OF LITERATURE	6
III. MATERIALS AND EQUIPMENT	16
A. Culture Media	16
B. Disinfectants	19
C. Bacterial Culture	21
IV. EXPERIMENTAL PROCEDURES	22
A. Maintenance of Culture	22
B. "Phenol Coefficient" Tests	22
1. Using broth cultures	22
2. Using washed cultures	23
C. "Semi-micro" Tests	24
D. Growth Curve Studies	25
E. Neutralization Tests	26
V. RESULTS	27
A. Effect of Culture Media on the Resistance of <u>M. pyogenes</u> var. <u>aureus</u>	27

B.	Effect of Subculture Media on the Cultivation of Disinfectant-treated Cells of <u>M. pyogenes</u> var. <u>aureus</u>	41
C.	Effect of Washed Cultures on the Resistance of <u>M. pyogenes</u> var. <u>aureus</u> as Compared to Broth Cultures	50
D.	Effect of Age of FDA Subculture Medium on the Cultivation of Disinfectant-treated Cells of <u>M. pyogenes</u> var. <u>aureus</u>	57
E.	Effect of Culture Media on Bacterial Population and on the Resistance of <u>M. pyogenes</u> var. <u>aureus</u> to Phenol	60
F.	Neutralization Tests	68
VI.	SUMMARY	71
VII.	LITERATURE CITED	74

I. INTRODUCTION

There are numerous compounds on the market purported to be disinfectants, many of which have questionable value. In order to protect the buyer, it is necessary that regulatory agencies have methods of measuring the disinfecting value of each and obtain data that will represent their relative values.

The FDA "phenol coefficient" procedure (31) has been the standard used by regulatory agencies for many years. This test was originally designed to measure the relative values of cresylic type compounds. Undoubtedly this test is reliable when applied to the evaluation of coal tar compounds; however, this procedure fails woefully when a comparative evaluation is attempted of a mercurial, a quaternary ammonium compound, sodium hypochlorite or a chloro-phenol. Tilley (35) stated that a phenol coefficient is of value only as a means of preventing the use of worthless preparations and as an aid to the manufacturer in maintaining the uniformity of his product.

The present FDA procedure may give a relative rating of the killing dilution of a disinfectant with a standardized test

culture. In this way, compounds can be classified into groups of low and high killing dilutions.

Workers in the field of disinfectant testing are well aware of the discrepancies in results that are obtained in testing the efficiency of various compounds in the laboratory. Since there is no single reliable method, it is very difficult to evaluate disinfectants on a comparative basis.

In addition to the limited application that may be made of an FDA phenol coefficient the procedure has been criticized for the fact that the usual test organisms frequently vary in their resistance to phenol as well as to the compound under test.

Standards have been established by the United States Food and Drug Administration (31) for the resistance of the test organism to be used in running phenol coefficient tests. However, difficulties are often encountered in trying to maintain a smooth culture of the required resistance. Composition of the culture media has an important influence on this. New lots of peptone and beef extract can cause the appearance of rough strains with substandard resistance (21). The hydrogen ion

concentration of the culture medium also has an important influence (26).

Semisynthetic media have been proposed by Klarmann and Wright (14) and by Wolf (38) to maintain a standard resistance of Micrococcus pyogenes var. aureus. Reddish, Wood and Burlingame (29) in their studies on these two purposed media concluded that the resistance of M. pyogenes var. aureus to phenol and Liquor Antiseptis was not maintained as satisfactorily in the semisynthetic media as in FDA broth.

Data were presented by Mallmann, Leavitt and Joslyn (19) to show that the resistance of M. pyogenes var. aureus to quaternary ammonium compounds was increased by growing the test cultures in Difco Disinfectant Testing medium as compared to that resulting from cultivation in standard FDA broth.

The writer has had difficulty in keeping a smooth resistant culture of M. pyogenes var. aureus when growing it in standard FDA broth. A two percent trypticase broth was used by Ostrolenk (20) for carrying his test cultures, and in the presentation of this paper at the 1949 meeting of the Society of American Bacteriologists, mention was made of the fact that a smooth culture could be maintained in this medium.

In order to see if a smooth resistant culture of M. pyogenes var. aureus could be maintained, Trypticase Soy (TS) broth (Baltimore Biological Laboratories) was selected as one of the culture media to be used in this study. Disinfectant Test (DT) medium and standard FDA broth were selected also to show the comparative effect of different culture media upon the resistance of M. pyogenes var. aureus to representative type disinfectants. The first two media could be obtained in a dehydrated form, thus ensuring that a uniform lot of each could be used throughout the course of the experiments. One lot of peptone was used in the preparation of the FDA culture medium.

In order to show the influence of the subculture media upon the killing dilutions, FDA broth, DT medium and a specific neutralizing medium were included in the study.

The standard FDA procedure and a modification of Klarmann and Wright's "semi-micro whole sample" procedure (15) were used. The "whole sample" method has been criticized by Rahn as being too sensitive as compared with the phenol coefficient technic (24). It was desired in this study to compare results obtained by the two methods.

The purpose of this study is to show the effect of different culture media upon the resistance of M. pyogenes var. aureus to representative type disinfectants and the effect of the subculture media on the cultivation of the disinfectant-treated cells.

II. REVIEW OF LITERATURE

Soon after the germ theory of disease had been established, efforts were made toward evaluating the action of disinfectants on bacteria. These early methods were crude, and no attempt was made at determinations on a quantitative basis. Kronig and Paul (18) were the first to realize that the relative value of disinfectants depends very largely upon the conditions under which they are tested.

In 1903, Rideal and Walker (30) proposed a method of testing disinfectants whereby the germicidal action of the disinfectant was compared with that of phenol.

A "phenol coefficient" test, known as the Hygienic Laboratory method, was proposed by Anderson and McClintic in 1911 (1).

Workers in the United States Department of Agriculture Laboratories, after working for ten years with the methods of the Rideal-Walker and the Hygienic Laboratory tests, proposed a test which incorporated the best features of both (27). This procedure, known as the FDA method (31) is commonly used today in testing the efficiency of disinfectants in the laboratory.

In this test, a phenol coefficient for a disinfectant is determined by comparing its killing power with that of phenol against a specific organism.

Wright (39) published some work to show that most of the discordant results obtained in testing disinfectants were due to slight variations in the test culture media, while the same differences in subculture media were not important. He thought that this was connected with the amount of growth of the test organism. Garrod (9) also called attention to the importance of the number of organisms used in the test.

Reddish (26) suggested standards for the resistance of Micrococcus pyogenes var. aureus to phenol. He stated that the nutritive properties and the hydrogen ion concentration of the medium are important factors in preserving the resistance of the organisms.

A great deal of work has been done on the effect of various peptones on the resistance of organisms used in "phenol coefficient" tests. Reddish and Burlingame (28) stated that the resistance of M. pyogenes var. aureus was maintained consistently when broth made from Armour's peptone was used, whereas, when other peptones were used, resistance of the organism was reduced

to a significant degree. Brewer (4) thought that variations in "phenol coefficient" results were due to the presence of phospholipids in some lots of peptone. Quisno, Foter and Gibby (21) noted that new lots of peptone or beef extract sometimes caused the appearance of rough strains with substandard phenol resistance. It was noted by Stuart (34) that when BBL trypticase was used in FDA broth instead of Armour peptone, the resistance of the organism varied.

Klarmann and Wright (14) and Wolf (38) proposed semi-synthetic media for growing M. pyogenes var. aureus in order to obtain a standard-resistant culture. Reddish, Wood and Burlingame (29) published data to show that cultures grown in FDA broth are more satisfactory than those grown in either of the two semisynthetic media.

Other factors besides media composition have been studied in an attempt to maintain a smooth culture of uniform resistance for disinfectant testing. Grubb and Edwards (11) found that if M. pyogenes var. aureus was incubated at 40 C instead of at 37 C the standard resistance returned after one to two transfers at this temperature, and that it was maintained as long

as daily transfers were incubated at 40 C. Smyth (33), in his evaluation of some of the factors influencing the phenol resistance of M. pyogenes var. aureus, noted that a two-hour difference in the age of the culture or a one-degree difference in the incubator temperature caused an important difference in the resistance of the culture. It is interesting to note the effect of the age of the culture upon the resistance. A four-hour difference in the age of the test culture is allowed by the FDA procedure.

Some attention has been paid to the variation in the amount of material removed by the 4-mm loop owing to the different surface tension values of various types of disinfectants (5). However, Rahn (24) believed that the fluctuation in the number of cells was so small that it could not influence the results, as long as standard methods were followed.

With so many factors influencing the resistance of a test organism, it is remarkable that consistent results are ever obtained. The author has consistently had trouble in maintaining a smooth resistant culture when the organisms were cultured in FDA broth. The Difco Laboratories developed a medium (Disinfectant Testing medium) which they claim gives a culture

with constant resistance. The Baltimore Biological Laboratory prepared a medium (Trypticase Soy broth) which they claim will maintain a smooth culture for a considerable period of time.

The author decided to compare the above-mentioned three media to determine their relative values in maintaining a culture of constant resistance.

It is known that some disinfectants have such great bacteriostatic activity that slight traces of disinfectant carried over may prevent growth of the test organisms without killing them. Rahn (24) stated that this danger seemed rather remote when the FDA "phenol coefficient" technic was followed. In this procedure, one 4 ml loopful (0.01 ml) is transferred from the medication tube to 10 ml of broth, resulting in a 1-1000 dilution of the disinfectant. No disinfectant is known which produces bacteriostasis in 1/1000 of the lowest bactericidal concentration.

Rahn (25) stated that sterilization of bacteria is brought about by a chemical reaction between the disinfectant and some vital part of the cell. This reaction may take place in several steps, the first of which may be reversible. After a definite

period of contact with the disinfectant, all cells contain a certain amount of the disinfectant, but a fatal reaction may not have been produced. Transfer into fresh broth may not cause the disinfectant to diffuse quickly out of the cell, and the cell may die in the new medium although it was not dead at the time of transfer. In such a case, the use of an antidote or neutralizing medium would save the life of the organism which would be considered dead by the standard technic. For this reason a neutralizing medium, as well as Special FDA broth and DT medium, was used for subculturing the test organism.

James (13) listed the specifications for an ideal neutralizer: (1) it destroys all the germicidal effects to be exerted by disinfectant remaining in solution, (2) it removes from unkilld bacterial cells all the disinfectant and restraining influences, (3) it introduces no germicidal or bacteriostatic effect and (4) it resuspends unkilld bacteria in fluid substrate.

Gegenbauer (10) found that staphylococci that had been treated with mercuric chloride could be revived by repeated washing, provided that the contact with the disinfectant had

been less than one hour. After that time, washing could not save the cells, as the mercury ions had reacted with some of the cell constituents. However, hydrogen sulfide reversed this chemical reaction by forming insoluble mercuric sulfide within the cell. The reversion, which restored the power of reproduction, was still possible after sixteen, but not thirty-three, hours, if the disinfectant contained one percent mercuric chloride. Fildes (7) also used hydrogen sulfide for the chemical inactivation of the mercury ion. There are many sulfur-containing compounds which can neutralize the bacteriostatic activity of mercurials. Sodium thioglycollate was used by Salle, Catlin and Westley (32) and by Heinemann (12). Thioglycollate broth was selected as the neutralizing medium for Metaphen in this study because of its availability in a powdered form.

With phenol it had not been thought necessary to use a neutralizing agent in the subculture medium. Flett et al. (8) conducted studies in which they used ferric chloride as a chemical inactivator for phenol. They found that the killing dilution of phenol was lowered when ferric chloride was added to the subculture medium. Tilley (36) was unable to duplicate

these results. He found no difference with ferric chloride added to the FDA broth as compared with FDA broth alone. In this study, it was decided to check the use of ferric chloride as an inactivator for the phenolic compounds, Dowicide A and Dowicide C.

Many substances have been suggested for the inactivation of quaternary ammonium compounds. Domagk (6) was the first to call attention to the fact that anionic detergents will inactivate the surface-active cationic compounds. Baker, Harrison and Miller (3) showed that certain synthetic anionic detergents also inactivated the germicidal properties of cationic compounds. Klein and Kardon (16) showed that "reversal" of quaternary ammonium activity could not be produced once the organisms had been exposed to germicidal concentrations of the compounds. They found that the addition of an anionic detergent to the treated bacterial suspensions interrupted the action of the cationic disinfectants, thereby permitting surviving organism to grow.

Weber and Black (37) tested lecithin, naphuride sodium, Duponal W. A., Tergitol W. A. 7, Triton x200, Triton x300, sodium oleate, sodium thiosulfate, among other compounds. They

found that lecithin and naphuride sodium were the only ones of the inhibitors tested that showed no bacteriostatic effect against a mixed culture.

Favorable results on the use of lecithin alone or in combination with other substances for the inactivation of cationic disinfectants have been reported also by Quisno, Gibby and Foter (23), Armbruster and Ridenour (2) and others. Quisno, Gibby and Foter (23) formulated Letheen broth in which lecithin acts as the chief neutralizer while Tween 80 acts as a solubilizing and dispersing agent for the lecithin. The concentration of lecithin and Tween 80 used did not inhibit the growth of any organism that would grow in FDA broth. Therefore "Letheen" broth was picked as the inactivating medium to be used for testing quaternary ammonium compounds in this study.

There has been a great deal of discussion about the sampling technic of the "phenol coefficient" procedure. Some workers (15) have felt that in this method of "random sampling" a representative sample of the disinfectant-culture mixture is not removed for subculturing. Methods have been proposed in which the whole mixture is cultured (15, 22). Some workers (24) feel that this "whole sample" method is too sensitive a

test as compared to the standard FDA procedure. To determine the relative value of this technic as compared to the 'phenol coefficient' procedure, a slight modification in the 'semi-micro' technic of Klarmann and Wright was used along with the FDA procedure.

III. MATERIALS AND EQUIPMENT

A. Culture Media

The following media were used for daily transfers of M. pyogenes var. aureus. Subsequent tests were run upon the organisms grown in these three media.

1. FDA broth

Armour peptonum siccum, Lot No. 100976	10.0 gm
Bacto beef extract	3.0 gm
Sodium chloride	5.0 gm
Distilled water (pH was adjusted to 6.8 to 7.0)	1000 ml

2. Difco Disinfectant Test medium (DT medium), Lot No. 380974

Proteose peptone	10.0 gm
Bacto beef extract	3.0 gm
Sodium chloride	2.0 gm
Lactose	5.0 gm
Ascorbic acid	0.025 gm
Distilled water (final pH was 7.0±)	1000 ml

3. Trypticase Soy broth (TS broth), Lot No.
2751 (Baltimore Biological Laboratories)

Trypticase	17.0 gm
Phytone	3.0 gm
Sodium chloride	5.0 gm
Dipotassium phosphate	2.5 gm
Dextrose	2.5 gm
Distilled water (final pH was 7.2-7.4)	1000 ml

The following media were used as subculture media for
"phenol coefficient" and "semi-micro" tests.

1. Special FDA broth

Difco peptone	10.0 gm
Bacto beef extract	3.0 gm
Sodium chloride	5.0 gm
Distilled water (pH adjusted to 6.8 to 7.0)	1000 ml

2. Difco Disinfectant Test medium

Formula the same as given in A

3. Lethen broth

Part A	
Tween 80 (Atlas Powder Co.)	5.0 gm

18

Azolectin (American Lecithin Co.) 0.7 gm

Hot distilled water 400 ml

Part B

Beef extract 5.0 gm

Sodium chloride 5.0 gm

Armour peptonum siccum, Lot No.
100976 10.0 gm

Distilled water 600 ml

Mix parts A and B and boil for ten
minutes.

(pH adjusted to 6.8 to 7.0)

4. Difco Brewer's thioglycollate medium

Beef, infusion from 500 gm

Sodium chloride 5.0 gm

Dipotassium phosphate 2.0 gm

Proteose peptone 1.0 gm

Dextrose 5.0 gm

Sodium thioglycollate 0.5 gm

Bacto-agar 0.5 gm

Bacto-methylene blue 0.002 gm

Distilled water 1000 ml

5. Special FDA broth plus 0.03% ferric chloride

The following media were used in Section E under Results, for the studies on growth curves and resistance of M. pyogenes var. aureus to phenol.

1. Difco Disinfectant Test medium, Lot No: 365853
2. Difco Disinfectant Test medium, Lot No. 380974
3. Disinfectant Test medium, made in the laboratory using Difco Peptone, Lot No. 406935
4. FDA broth made with Difco peptone, Lot No. 406935
5. FDA broth made with Difco peptone, Lot No. 405934
6. FDA broth made with Armour peptonum siccum, Lot No. 100976
7. Trypticase Soy broth, Lot No. 3639 (Baltimore Biological Laboratories)

The following Sorensen's buffer, pH 7.0, was used for the washed cultures

6.0 ml Na_2HPO_4 (11.876 gm $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$ in one l of solution)

4.0 ml KH_2PO_4 (9.078 gm KH_2PO_4 in one l of solution)

Tryptone glucose extract agar was used for all plating purposes.

B. Disinfectants

The disinfectants used in the study were:

1. Dowicide A Flake, which is composed of 97 percent sodium-o-phenylphenate as the active ingredient. It is manufactured by the Dow Chemical Company.
2. Dowicide C, which was made from Dowicide 31 by adding sodium hydroxide. In order to make the compound water soluble, Dowicide 31 was converted into the sodium salt as directed by the Dow Chemical Company, manufacturers of this product. It contains 85 percent chloro-o-phenylphenol as the active ingredient.
3. Metaphen, an anhydride of 4 nitro-3, 5-biacetoxy-mercuri-2 cresol, which is manufactured by Abbot Pharmaceutical Company.
4. BTC, which is composed of 10 percent alkyl-dimethylbenzyl ammonium chloride as the active ingredient with the alkyl radical ranging from C8 to C18. It is manufactured by Onyx Oil and Chemical Company.
5. Phenol (Baker and Adamson Quality), which was obtained from the Allied Chemical and Dye Corporation. A 5-percent solution was made and stored in a dark bottle at 4 C.

C. Bacterial Culture

The test organism was M. pyogenes var. aureus No. 209,
as approved by the Food and Drug Administration.

IV. EXPERIMENTAL PROCEDURES

A. Maintenance of Culture

The stock bacterial culture was kept on plain nutrient agar slants. Stock subcultures were made every four weeks.

The cultures used in the experiments were employed only after a minimum of four daily subtransfers in the proper broth after transfer from the stock agar slant. After four weeks of use, the test organism was once again taken from the month-old agar stock culture. Cultures were incubated at 37 C. The test organism was a 22-26 hour culture of M. pyogenes var. aureus. The experiments were conducted using pooled cultures of, at least, three tubes of the appropriate broth cultures. Just before the test was started the pH of the cultures grown in DT medium and TS broth were adjusted to 6.8-7.2, using a Beckman potentiometer.

B. "Phenol Coefficient" Tests

1. Using broth cultures. The technic used is described by Ruehle and Brewer (31). This technic is known as the FDA

method. In order to have comparable results, dilutions of the disinfectant to be tested were made up in sterile 250-ml Erlenmeyer flasks and 5-ml amounts of each dilution were then pipetted into each of three sterile seeding pots. In this manner, seeding pots were prepared for three "phenol coefficient" tests at one time. One test was run using Special FDA broth as the subculture medium; in the second test, DT medium was used for subculturing and in the third test a neutralizing medium was employed. The same pooled culture was used in all three tests. Seeding pots were held in a water bath at 20 C for the test. After the tests were completed, suitable dilutions of the test organism were plated. These plates were incubated at 37 C for 48 hours and then the colonies were counted. Readings were made on the "phenol coefficient" tests after 24, 48, 72 hours' and 6 days' incubation at 37 C. The six-day reading was taken as the final result. The killing dilution was reported as that dilution which caused "kill" in ten but not in five minutes.

2. Using washed cultures. Broth cultures of M. pyogenes var. aureus, 22-26 hours old, were centrifuged at 1800 RPM for 30 minutes to one hour depending upon the medium in which the organisms were growing. Thirty minutes were sufficient to

throw down the organisms growing in DT medium and TS broth, while it took one hour to throw down the organisms grown in FDA broth. The supernatant was then discarded, and the organisms suspended in 10 ml of buffer, pH 7.0. These cultures again were centrifuged, the supernatant discarded, and the organisms suspended in buffer for the test. Pooled samples of, at least, three tubes were used as the test organism. The "phenol coefficient" tests were conducted and results read in the same manner as with the broth cultures.

C. "Semi-micro" Tests

A slight variation in the technic of the "semi-micro" modification by Klarmann and Wright (15) was used.

One ml of each disinfectant dilution was placed in each of three large test tubes. Three dilutions of phenol and seven of the disinfectant to be tested were used. At 15-second intervals 0.1 ml of a culture of M. pyogenes var. aureus was added to each tube and the tube was swirled. At the end of 5, 10 and 15 minutes' exposure to the disinfectant, 20 ml of Special FDA broth, DT medium or the appropriate neutralizing medium was

added. Tubes were incubated at 37 C and readings made at 24, 48, 72 hours and 6 days.

The tests were conducted at 20 C. Cultures were prepared in the same way as those for the 'phenol coefficient' tests.

D. Growth Curve Studies

A suspension of M. pyogenes var. aureus was made by taking a small amount of growth from the stock nutrient agar slant and putting it in saline. The tube was shaken well to break up all the clumps. One standard 4-mm loopful of this suspension was placed in each of the seven media, the names of which are given in (C) under Materials and Equipment. Appropriate dilutions were made and plated of each new culture. These were considered to be zero hour counts. At the end of six hours' and 24 hours' incubation at 37 C, dilutions were again plated. After 24 hours' incubation, one standard 4-mm loopful of culture was transferred from each of the seven tubes into new broth tubes of the same kind of medium. Proper dilutions of these new cultures were plated at zero, six and 24 hours. After 24 hours' incubation, one standard loopful was

transferred from the second transfer cultures into a new set of the seven media. Platings again were made at zero, six and 24 hours. All plates were incubated at 37 C and counted at the end of 48 hours.

E. Neutralization Tests

M. pyogenes var. aureus, grown in DT medium, was used as the test organism. The subculture media were Special FDA broth, DT medium and the specific neutralizing medium for the disinfectant being used.

One ml of each disinfectant dilution was placed in a large test tube. Twenty ml of the subculture medium then was poured into each tube. The mixture of disinfectant dilutions and subculture media was inoculated with 0.1 ml of the culture. Examinations for growth were made after 24, 48, 72 hours' and 6 days' incubation at 37 C.

V. RESULTS

A. Effect of Culture Media on the Resistance of M. pyogenes var. aureus

FDA "phenol coefficient" tests and "semi-micro" tests were run using as the test organism, M. pyogenes var. aureus which had been grown in (A) standard FDA broth, (B) DT medium, and (C) TS broth. Two tests, differing only in the subculture medium used, were run on each compound. Special FDA broth was used as the subculture medium in one test, while DT medium was used for the other, in order to see if the subculture medium would affect the comparative results.

Four compounds, Dowicide A (sodium-o-phenyl phenate), Dowicide C (sodium chloro-o-phenyl phenate, Metaphen (4 nitro-3, 5-biacetoxy-mercuri-2 cresol) and BTC (alkyl-dimethyl-benzyl-ammonium chloride) which were chosen as representative disinfectants, were used in the comparative study.

The results obtained are shown in Tables I-VIII inclusive. Examination of the "phenol coefficient" test values for the four compounds shows that lower phenol coefficient values were obtained when the test organism was grown in DT medium, showing

a more resistant organism than when it was grown in standard FDA broth or TS broth.

Although much has been said about the importance of the amount of growth of the test culture upon its resistance (39), the number of organisms present in the test culture would not appear to be the influencing factor here. Bacterial counts were made of each culture at the time of testing and in every case a higher bacterial count was obtained when the organism was grown in TS broth than in either FDA broth or DT medium. Table IX lists some of the bacterial counts obtained. In most cases the culture grown in FDA had a lower count than the one grown in DT medium. Examination of Tables I-IV inclusive shows that the culture grown in FDA broth was more resistant than the one grown in TS broth when the compounds tested were phenolics.

When Metaphen and BTC were the compounds tested, it would appear that the culture grown in TS broth was as resistant or more so than the one grown in FDA broth. It is difficult to draw conclusions when no neutralizing media are used with these two compounds.

Both Metaphen and BTC have high bacteriostatic properties, causing inhibition of the test organism in very high dilutions. In high dilutions, "skips" in the data, by which is meant the obtaining of a negative result where a positive result is indicated, and "wild pluses," which are positive results in the data where negative results are indicated, often occur. The bacteriostatic property of Metaphen and BTC, coupled with "skips" and "wild pluses" account for the fact that results obtained with these two compounds are not as easily interpreted as with Dowicide A and Dowicide C. However, the culture grown in DT medium showed a greater resistance to all the compounds tested, phenolics and nonphenolics, than the cultures grown in standard FDA broth or TS broth when the FDA "phenol coefficient" technic was used.

The subculture medium did not affect the comparative results obtained, although a lower phenol coefficient value was obtained when DT medium was used as the subculture medium than when Special FDA broth was used.

Results obtained by the "semi-micro" technic were not as consistent as the ones obtained by the FDA procedure. The killing dilutions for Dowicide A and Dowicide C checked fairly

well with the ones obtained by the FDA procedure. However, organisms grown in any one of the three culture media tested cannot be said to be consistently more resistant.

Much higher killing dilutions were obtained for Metaphen and BTC by the "semi-micro" technic than with the standard FDA procedure. This would be expected as both Metaphen and BTC have high bacteriostatic properties, and without the use of a neutralizing substance, inhibiting action would persist after the exposure period of the test was completed. The fact that the whole sample is cultured in the "semi-micro" technic allows a greater ratio of the disinfectant to the diluent so that bacteriostatic activity exists to a greater extent than in the "phenol coefficient" technic in which one 4-mm loopful of disinfectant-culture mixture is removed for subculturing. Klimek and Umbreit (17) found that "skips" and "wild pluses" occurred with the "semi-micro" technic as well as with the FDA procedure.

With Metaphen and BTC as well as with Dowicide A and Dowicide C, the killing dilutions were lower when DT medium was used as the subculturing medium than when Special FDA

broth was used. More about the effect of the subculture media will be presented in the next section.

To summarize the material presented in this section, it can be stated that with the FDA procedure, a more resistant culture of M. pyogenes var. aureus was obtained when the organisms were grown in Disinfectant Test medium than when they were grown in either FDA broth or Trypticase Soy broth. No definite conclusions can be drawn as to the resistance of the test culture when the "semi-micro" technic is used.

TABLE I

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF

M. PYOGENES VAR. AUREUS

Test compound--Dowicide A

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS broth

Subculture medium--DT medium

Phenol Coefficient Test		Semi-micro Test	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A 1-700	10	>1-200<1-300	5
B >1-300<1-400	5	1-400	10
C 1-900	15	1-400	7

TABLE II

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF

M. PYOGENES VAR. AUREUS

Test compound--Dowicide A

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS broth

Subculture medium--Special FDA broth

Phenol Coefficient Test			Semi-micro Test	
	Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A	>1-1000<1-1200	12	>1-800<1-900	10
B	1-400	5	>1-400<1-500	6
C	>1-1200<1-1400	16	>1-600<1-700	8

TABLE III

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF
M. PYOGENES VAR. AUREUS

Test compound--Dowicide C

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS broth

Subculture medium--DT medium

Phenol Coefficient Test			Semi-micro Test	
	Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A	1-4000	57	>1-2000<1-4000	42
B	1-2000	33	1-2000	36
C	1-4000	61	>1-2000<1-4000	75

TABLE IV

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF

M. PYOGENES VAR. AUREUS

Test compound--Dowicide C

Test organism--M. pyogenes var. aureus grown in:

- A. FDA broth
- B. DT medium
- C. TS broth

Subculture medium--Special FDA broth

Phenol Coefficient Test			Semi-micro Test	
	Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A	>1-4000<1-6000	71	>1-2000<1-4000	50
B	>1-2000<1-4000	46	>1-2000<1-4000	60
C	1-6000	85	>1-4000<1-6000	71

TABLE V

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF

M. PYOGENES VAR. AUREUS

Test compound--Metaphen

Test organism--M. pyogenes var. aureus grown in:

- A. FDA broth
- B. DT medium
- C. TS broth

Subculture medium--DT medium

Phenol Coefficient Test		Semi-micro Test	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-30,000<1-40,000	466	<1-50,000	<769
B >1-20,000<1-30,000	384	>1-50,000<1-60,000	1375
C >1-30,000<1-40,000	466	>1-60,000<1-70,000	1625

TABLE VI

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF

M. PYOGENES VAR. AUREUS

Test compound--Metaphen

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS broth

Subculture medium--Special FDA broth

Phenol Coefficient Test		Semi-micro Test	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-50,000<1-60,000	687	>1-100,000	>1300
B >1-30,000<1-40,000	466	>1-100,000	>1300
C 1-50,000	768	>1-100,000	>1300

TABLE VII

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF
M. PYOGENES VAR. AUREUS

Test compound--BTC

Test organism--M. pyogenes var. aureus grown in:

- A. FDA broth
- B. DT medium
- C. TS broth

Subculture medium--DT medium

Phenol Coefficient Test			Semi-micro Test		
Killing	Dilution	Phenol Coefficient	Killing	Dilution	Phenol Coefficient
A	>1-15,000<1-20,000	250	>1-20,000<1-30,000		384
B	1-8,000	100	>1-20,000<1-30,000		415
C	1-15,000	214	>1-20,000<1-30,000		625

TABLE VIII

EFFECT OF CULTURE MEDIA ON THE RESISTANCE OF

M. PYOGENES VAR. AUREUS

Test compound--BTC

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS broth

Subculture medium--Special FDA broth

Phenol Coefficient Test		Semi-micro Test	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-20,000<1-25,000	321	1-50,000	833
B 1-10,000	125	>1-40,000<1-50,000	750
C 1-20,000	266	>1-30,000<1-40,000	636

TABLE IX

BACTERIAL COUNTS, EXPRESSED IN MILLIONS, ON TEST CULTURES OF M. PYOGENES VAR. AUREUS, CULTURED IN (A) STANDARD FDA BROTH, (B) DIFCO DISINFECTANT TEST MEDIUM AND (C) TRYPTICASE SOY BROTH

A	B	C
130	140	775
140	480	1070
140	300	760
82	380	650
98	570	980
200	400	810
480	210	900
56	530	740

B. Effect of Subculture Media on the Cultivation
of Disinfectant-treated Cells of
M. pyogenes var. aureus

In the study of subculture media used for growing the organisms after they had been subjected to the action of the various disinfectants, only the data are presented from the experiments in which DT medium was used for growing the organism preparatory for the disinfectant tests. The DT medium results were selected primarily because the organisms grown in this medium were the most resistant.

The subculture media used were DT medium, Special FDA broth and a neutralizing medium that was specific for each type of compound used in the study. Standard FDA broth plus 0.03 percent ferric chloride (8) was used as the neutralizing medium for Dowicide A and Dowicide C, both of which are phenolic derivatives. Thioglycollate broth (32) was used for Metaphen, and Lethen broth (23) was used for BTC.

Examination of Tables X and XI shows that when DT medium was used as the subculturing medium, a lower phenol coefficient was obtained with the FDA procedure than when Special FDA broth or the FDA broth plus ferric chloride was

used. No difference was observed in the killing dilution of either Dowicide A or Dowicide C with Special FDA broth and FDA broth plus ferric chloride. This substantiates the work of Tilley (36) who found no difference with FDA plus ferric chloride and FDA broth. This would indicate that the ferric chloride had no effect as a chemical inactivator for the phenolic compounds as previously suggested by Flett et al. (8).

Metaphen, which is highly bacteriostatic, was effectively inactivated by the thioglycollate broth. The killing dilution was less than 1-500 which is the dilution at which the mercurial was obtained from the manufacturer.

The antibacterial action of mercury is thought to be due to its combination with the -SH groups in the bacterial cell, thus depriving the cell of -SH groups which are essential for its metabolism (7). The antibacterial action of mercury has been specifically neutralized by -SH compounds. Thus, it would be expected that a much lower killing dilution would be obtained when thioglycollate broth was used than when Special FDA broth or DT medium was used. Observation of Table XII shows that a lower killing dilution was obtained when DT medium was used

for subculturing than when Special FDA broth was used, which is consistent with data previously presented.

The data on BTC are presented in Table XIII. Lethen broth was demonstrated to be a more effective inactivator for this compound than was DT medium or Special FDA broth. A phenol coefficient value of 85 was obtained with the Lethen broth, while one of 100 with DT medium and one of 125 with Special FDA broth resulted.

Lecithin in the Lethen broth acts as the chief neutralizer. Negatively charged fatty acid ions have been found capable of neutralizing the germicidal properties of positively charged cationic germicides (23).

A lower killing dilution was obtained when the test organisms were subcultured in DT medium than in Special FDA broth as also occurred with the other disinfectants tested.

The results obtained with the "semi-micro" technic tend to substantiate those obtained with FDA procedure in that FDA broth plus ferric chloride is no more effective in inactivating Dowicide A and Dowicide C than was DT medium or Special FDA broth.

Although a lower killing dilution was obtained for Dowicide A when DT medium was used for subculturing than when Special FDA broth was used, the culture used was more resistant to phenol, which caused a higher "phenol coefficient" value to result.

Lower killing dilutions and "phenol coefficient" values resulted when DT medium was used in the "semi-micro" tests with Dowicide C, Metaphen and BTC as the test compounds.

Results obtained with the neutralizing media, thioglycollate broth and Lethen broth, correlated with those obtained by the phenol coefficient technic. Lower killing dilutions and phenol coefficient values resulted for Metaphen and BTC when the neutralizing media were used.

Summarizing the material in this section, it can be stated that FDA broth plus ferric chloride was no more effective than FDA broth and in most cases less effective than DT medium as a subculture medium in inactivating Dowicide A and Dowicide C. Thioglycollate broth was effective in both methods used in neutralizing the Metaphen. A lower killing dilution for BTC was obtained when Lethen broth was used for subculturing than when FDA broth or DT medium was used.

DT medium was shown to be more effective as a sub-culture medium than was Special FDA broth in "phenol coefficient" tests on all four compounds.

TABLE X
EFFECT OF SUBCULTURE MEDIA ON THE CULTIVATION
OF DISINFECTANT-TREATED CELLS OF
M. PYOGENES VAR. AUREUS

Test compound--Dowicide A

Test organism--M. pyogenes var. aureus grown in Disinfectant

Test Medium (DTM)

Subculture media

A. Special FDA broth

B. DT medium

C. FDA broth plus 0.03% FeCl_3

Phenol Coefficient Test			Semi-micro Test	
	Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A	1-400	5.7	>1-400<1-500	6.4
B	>1-300<1-400	5.0	1-400	10.0
C	1-400	5.7	>1-400<1-500	9.0

TABLE XI

EFFECT OF SUBCULTURE MEDIA ON THE CULTIVATION
OF DISINFECTANT-TREATED CELLS OF
M. PYOGENES VAR. AUREUS

Test compound--Dowicide C

Test organism--M. pyogenes var. aureus grown in Disinfectant

Test Medium

Subculture media

A. Special FDA broth

B. DT medium

C. FDA broth plus 0.03% FeCl_3

Phenol Coefficient Test			Semi-micro Test	
Killing Dilution	Phenol Coefficient		Killing Dilution	Phenol Coefficient
A >1-2000<1-4000	46		>1-2000<1-4000	60
B 1-2000	33		1-2000	40
C >1-2000<1-4000	42		>1-2000<1-4000	50

TABLE XII
EFFECT OF SUBCULTURE MEDIA ON THE CULTIVATION
OF DISINFECTANT-TREATED CELLS OF
M. PYOGENES VAR. AUREUS

Test compound--Metaphen

Test organism--M. pyogenes var. aureus grown in Disinfectant

Test Medium

Subculture media

A. Special FDA broth

B. DT medium

C. Thioglycollate broth

Phenol Coefficient Test		Semi-micro Test	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-30,000<1-40,000	466	>1-100,000	>1428
B >1-20,000<1-30,000	384	>1-50,000<1-60,000	1375
C <1-500	<7.0	<1-500	<9.0

TABLE XIII

EFFECT OF SUBCULTURE MEDIA ON THE CULTIVATION
OF DISINFECTANT-TREATED CELLS OF
M. PYOGENES VAR. AUREUS

Test compound--BTC

Test organism--M. pyogenes var. aureus grown in Disinfectant

Test Medium

Subculture media

A. Special FDA broth

B. DT medium

C. Lethen broth

Phenol Coefficient Test		Semi-micro Test	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A 1-10,000	125	>1-40,000<1-50,000	750
B 1-8,000	100	>1-20,000<1-30,000	454
C >1-4000<1-8000	85	>1-5000<1-10,000	187

C. Effect of Washed Cultures on the Resistance
of M. pyogenes var. aureus as
Compared to Broth Cultures

As early as 1897, it was recognized that organic matter played an important role in interfering with the germicidal activity of disinfectants (18). In most cases of disinfection, proteins are present, and frequently other organic and inorganic substances. Therefore, it is important to know to what extent foreign materials interfere with sterilization.

Different types of disinfectants will be affected differently by the same foreign substance. This is one of the reasons why there are so many kinds of disinfectants on the market today.

Most methods of testing the efficiency of disinfectants involve the use of a broth culture of the test organism. In the FDA "phenol coefficient" procedure 0.5 ml of the bacterial culture is placed in 5.0 ml of disinfectant solution. A considerable amount of organic matter is introduced, approximately 1500 ppm of peptone and meat extract. This is sufficient to interfere with the action of disinfectants that otherwise would be capable of killing bacteria in very low concentrations (25).

In order to eliminate the effect of foreign material that would be introduced by the broth along with the cells of the test organism, tests were conducted using washed cells of M. pyogenes var. aureus that were resuspended in Sorensen's phosphate buffer, pH 7.0, for the test.

Tests were conducted using the FDA procedure and the "semi-micro" technic. Only the data of the tests in which DT medium was used for subculturing are presented. This medium was shown in the preceding section to be more effective as a subculture medium than was Special FDA broth.

Data for comparing the results obtained using washed cultures and broth cultures are recorded in Tables XIV-XVII inclusive. Examination of the data on the "phenol coefficient" method shows that a higher killing dilution was obtained when the washed cells were used as the test organism than when the broth cultures were used. This was true for all of the compounds tested.

Results obtained by the "semi-micro" technic do not show consistent differences between tests run using the washed cells and those using the regular broth cultures. This correlates with the results obtained in preceding sections.

Washed cells of M. pyogenes var. aureus that had been grown in DT medium were shown to be more resistant in most cases than the washed cells that had been grown in FDA broth and TS broth.

TABLE XIV
EFFECT OF WASHED CULTURES ON THE RESISTANCE
OF M. PYOGENES VAR. AUREUS AS COM-
PARED TO BROTH CULTURES

Test compound--Dowicide A

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS medium

Subculture medium--DT medium

PHENOL COEFFICIENT TEST

Broth Culture		Washed Culture	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A 1-7000	10	1-1000	14
B >1-300<1-400	5	1-900	13
C 1-900	15	>1-1400<1-1600	21

SEMI-MICRO TEST

Broth Culture		Washed Culture	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-200<1-300	5	>1-300<1-600	10
B 1-400	10	>1-500<1-600	8
C 1-400	7	1-600	11

TABLE XV
EFFECT OF WASHED CULTURES ON THE RESISTANCE
OF M. PYOGENES VAR. AUREUS AS COM-
PARED TO BROTH CULTURES

Test compound--Dowicide C

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS medium

Subculture medium--DT medium

PHENOL COEFFICIENT TEST

Broth Culture			Washed Culture	
Killing	Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A	1-4000	57	>1-8000<1-10,000	100
B	1-2000	33	1-6000	85
C	1-4000	61	1-8000	100

SEMI-MICRO TEST

Broth Culture			Washed Culture	
Killing	Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A	>1-2000<1-4000	42	1-4000	53
B	1-2000	36	>1-1000<1-2000	25
C	>1-2000<1-4000	75	>1-2000<1-4000	60

TABLE XIV
EFFECT OF WASHED CULTURES ON THE RESISTANCE
OF M. PYOGENES VAR. AUREUS AS COM-
PARED TO BROTH CULTURES

Test compound--Metaphen

Test organism--M. pyogenes var. aureus grown in:

A. FDA broth

B. DT medium

C. TS broth

Subculture medium--DT medium

PHENOL COEFFICIENT TEST

Broth Culture		Washed Culture	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-30,000<1-40,000	466	1-50,000	625
B >1-20,000<1-30,000	384	1-30,000	461
C >1-30,000<1-40,000	466	1-40,000	533

SEMI-MICRO TEST

Broth Culture		Washed Culture	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A <1-50,000	<769	>1-50,000<1-60,000	1100
B >1-50,000<1-60,000	1375	140T	2000
C >1-60,000<1-70,000	1625	>1-50,000<1-60,000	1375

TABLE XVII
EFFECT OF WASHED CULTURES ON THE RESISTANCE
OF M. PYOGENES VAR. AUREUS AS COM-
PARED TO BROTH CULTURES

Test compound--BTC

Test organism--M. pyogenes var. aureus grown in:

- A. FDA broth
- B. DT medium
- C. TS broth

Subculture medium--DT medium

PHENOL COEFFICIENT TEST

Broth Culture		Washed Culture	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-15,000<1-20,000	250	1-20,000	266
B 1-8000	100	1-10,000	133
C 1-15,000	214	1-20,000	286

SEMI-MICRO TEST

Broth Culture		Washed Culture	
Killing Dilution	Phenol Coefficient	Killing Dilution	Phenol Coefficient
A >1-20,000<1-30,000	384	>1-20,000<1-30,000	384
B >1-20,000<1-30,000	415	>1-20,000<1-30,000	312
C >1-20,000<1-30,000	625	>1-20,000<1-30,000	625

D. Effect of Age of FDA Subculture Medium on the
Cultivation of Disinfectant-treated Cells of
M. pyogenes var. aureus

During the course of this study, it was noted that sometimes wide discrepancies occurred in the phenol coefficient killing dilution when Special FDA broth was used as the subculturing medium. No such discrepancies were noticed when DT medium was used. This phenomenon was first observed when duplicate tubes were run on Dowicide A using two lots of FDA as the subculture medium. As far as could be determined the ages of the subculturing media were the distinguishing factors. The same lots of peptone and beef extract had been used in the preparation of the media. The pH of the two lots was the same.

Examination of Table XVIII shows that the killing dilution of Dowicide A ranged from 1-400 to more than 1-2400 when the organism was grown in DT medium. Three lots of FDA broth were used. A killing dilution of 1-400 was obtained when a lot of FDA broth that was 18 days old was used while a killing dilution 1-800 was obtained on the same test when a different lot of subculture medium, four days old, was used. A

dilution of more than 1-2400 was obtained when a lot of FDA broth, one day old, was used.

"Phenol coefficient" tests also were performed, using the test organism grown in FDA broth. The Special FDA subculturing medium was one day old at the time of the first test. This lot of medium was refrigerated to retard evaporation; further tests were run after 20 and 30 days, using this same lot of medium. Examination of Table XVIII shows that the killing dilution was 1-2000 when the subculturing medium was one day old, while it was $>1-1000 < 1-1200$ and 1-1000 respectively on the 20- and 30-day-old medium.

It is interesting to note that Quisno, Foter and Gibby (21) observed that the use of a freshly prepared medium was of value in maintaining the standard resistance and smooth phase of the test organism. They theorized that the partial oxygen tension might be a factor. The writer failed to find any reference as to the effect of the age of the subculture medium on the values obtained for phenol coefficients. No reasons for the variations observed in this study can be presented at this time. There is need for further study.

TABLE XVIII
EFFECT OF AGE OF FDA SUBCULTURE MEDIUM ON
THE CULTIVATION OF DISINFECTANT-TREATED
CELLS OF M. PYOGENES VAR. AUREUS

Test compound--Dowicide A

TEST ORGANISM GROWN IN DT MEDIUM

Date Medium Prepared	Date of Test	Killing Dilution
2-5-51	2-23-51	1-400
2-19-51	2-23-51	1-800
7-30-51	7-31-51	>1-2400

TEST ORGANISM GROWN IN FDA BROTH

Date Medium Prepared	Date of Test	Killing Dilution
7-30-51	7-31-51	1-2000
	8-20-51	>1-1000 < 1-1200
	8-30-51	1-1000

E. Effect of Culture Media on the Bacterial Population and on the Resistance of M. pyogenes var. aureus to Phenol

In the standard FDA "phenol coefficient" procedure it states that the test culture must be taken from a nutrient agar slant and transferred into broth at least three successive times before it is ready to be used. In order to determine the effect of the daily transfers on the bacterial population, growth curves were run on the first, second and third day transfers into broth from the stock nutrient agar slant. "Phenol coefficient" tests were run on the second and third day transfers, to determine if there was a change in the resistance to phenol of the test organism after the second day transfer from the stock culture.

Seven different culture media were included in the study. They are listed in Table XIX. Two lots of DT medium, made from the dehydrated form, plus one lot made in the laboratory were used; three lots of FDA broth, two made with different lots of Difco peptone and one made with Armour peptone, and one lot of TS broth were included in the study. In this way the effect of different lots of peptones on the bacterial population and on the resistance of M. pyogenes var. aureus to phenol could be compared.

The bacterial counts expressed in logarithms are plotted on Graph 1. Examination of the graph shows that TS broth (G) has the highest bacterial count of the seven media, and that there is not much change in the count from the second to the third day transfer. Standard FDA broth (F) shows the lowest 24-hour bacterial count on all three transfers. There is not much difference in the bacterial population of the three lots of DT media (A, B, C). They show a lower count than the two lots of FDA broth made with Difco peptone (D, E), but have a higher count than FDA broth made with Armour peptone. Lower counts were obtained on the third day than on the second day transfers for all of the seven media used.

Results of the "phenol coefficient" tests, made on the second and third day broth transfers are shown in Table XX. Examination of the Table shows that the resistance of the test organism to phenol varied. It showed no linear relationship to the bacterial count although FDA broth (F) had the lowest bacterial population and also, on the third day transfer, the organisms grown in this medium showed the lowest resistance to phenol. A culture grown in a lot of FDA broth, made with Difco peptone (E) showed the same resistance to phenol, but

had the third highest bacterial count. The organisms grown in TS broth, with the highest bacterial count, showed the same resistance to phenol as the ones grown in DT medium (B) which had a much lower bacterial population. This would indicate that there is no linear connection between the number of viable cells present in the populations studied and the resistance of M. pyogenes var. aureus to phenol.

It is interesting to note that only one medium (D) produced a culture of the required resistance to phenol. The FDA circular on methods of testing antiseptics and disinfectants (31) states that any strain of M. pyogenes var. aureus used in the FDA procedure must survive a 1-60 dilution of phenol for five minutes and a 1-70 dilution for 15 minutes.

It is very difficult to maintain a culture of the required resistance. Most workers in this field have encountered this difficulty at some time. In order to determine if too high resistance is demanded of the test organism, a study was made of the killing dilutions of phenol that were obtained when standard FDA broth, DT medium and TS broth were used for growing M. pyogenes var. aureus.

Special FDA broth and DT medium were used for subculturing. Table XXI shows the mean and standard deviation of the values obtained. Examination of the table shows that the most resistant culture was the one grown in TS broth and subcultured in DT medium. Even this was not as resistant as is required by the specifications set up by the FDA procedure. The culture grown in FDA broth was the least resistant of the three cultures tested.

In every case a higher killing dilution was obtained when Special FDA broth was used for subculturing than when DT medium was used. This is shown not only in the values obtained for each individual test culture medium, but also for the figure obtained when the killing dilution values for the three culture media were pooled.

TABLE XIX

MEDIA USED IN STUDY ON BACTERIAL POPULATION AND
RESISTANCE OF M. PYOGENES VAR. AUREUS TO PHENOL

A	Difco Disinfectant Test medium, lot No. 365853
B	Difco Disinfectant Test medium, lot No. 380974
C	Difco Disinfectant Test medium, made up in the laboratory
D	FDA broth, made with Difco peptone, lot No. 406935
E	FDA broth, made with Difco peptone, lot No. 405934
F	FDA broth, made with Armour peptone, lot No. 100976
G	BBL Trypticase Soy broth, lot No. 3639

LOG. COUNTS OF
MICROCOCOCCUS PYOGENES VAR. *AUREUS* AT
 0, 6 AND 24 HRS. ON 1ST, 2ND AND 3RD DAY
 BROTH TRANSFERS FROM STOCK AGAR SLANTS.

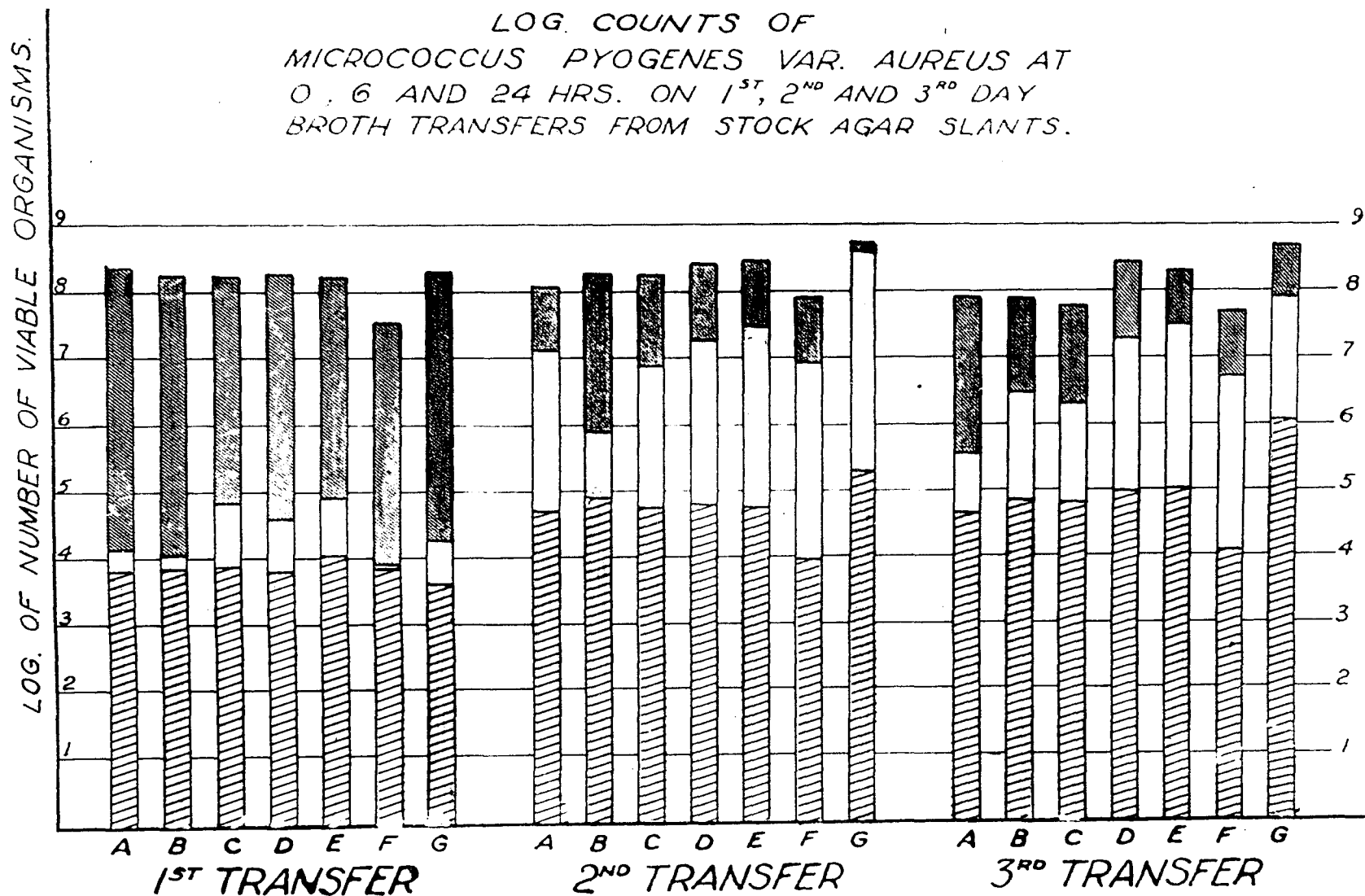


TABLE XX

KILLING DILUTIONS OF PHENOL ON SECOND AND THIRD
 DAY BROTH TRANSFERS FROM NUTRIENT AGAR
 SLANT OF M. PYOGENES VAR. AUREUS

Culture media--listed in Table XIX

Subculture medium--DT medium

	Second Transfer	Third Transfer
A	1-70	1-70
B	>1-70<1-80	>1-60<1-70
C	1-70	1-70
D	>1-60<1-70	1-60
E	>1-60<1-70	1-80
F	>1-70<1-80	1-80
G	1-60	>1-60<1-70

TABLE XXI

KILLING DILUTIONS* OF PHENOL

Test organism--M. pyogenes var. aureus grown in:

- A. FDA broth
- B. DT medium
- C. TS medium
- D. FDA broth, DT medium and TS broth

	Subcultured in Special FDA Broth	Subcultured in DT Medium
A	1-82.2 $\pm$ 8.55	1-74.0 $\pm$ 3.14
B	1-76.5 $\pm$ 5.0	1-71.5 $\pm$ 5.25
C	1-78.0 $\pm$ 7.25	1-67.5 $\pm$ 7.75
D	1-77.1 $\pm$ 7.25	1-70.45 $\pm$ 5.5

* Values obtained by calculation of the mean of killing dilutions of phenol by "phenol coefficient" tests.

F. Neutralization Tests

Tests were made to see if the neutralizing media used stopped the germicidal or bacteriostatic action of the disinfectant being tested. A culture of M. pyogenes var. aureus which was grown in DT medium was used as the test organism.

Since the largest amount of disinfectant-dilution that was used in any of the tests was one ml, this amount was used in the neutralization tests. Klarmann and Wright (15) recommended that 20 ml of medium be used in their "semi-micro" test to stop the action of the disinfectant. In order to determine if this amount was sufficient, the tests were conducted by adding 20 ml of medium to each of the disinfectant dilutions. One-tenth ml of the culture was then added immediately to each of the tubes containing the disinfectant-medium mixture. Results were recorded at the end of 24, 48, 72 hours' and six days' incubation at 37 C. Data are reported on the six-day reading.

Special FDA broth and DT medium were used as well as the specific neutralizing medium for each disinfectant. In this way, it would be demonstrated whether the neutralizing medium used had a greater effect than the other two media on stopping the germicidal or bacteriostatic activity of the disinfectant.

Letheen broth as formulated by Quisno, Gibby and Foter (23) was used as the neutralizing medium for BTC and sodium thioglycollate broth was used for Metaphen. FDA broth plus 0.03 percent ferric chloride as suggested by Flett et al. (8) was used for Dowicide A and Dowicide C.

It can be seen by examining Table XXII that both FDA broth and DT medium are as effective as the FDA broth plus ferric chloride in neutralizing Dowicide A and Dowicide C, thus allowing the test organism to grow.

Metaphen is neutralized in the 1-500 dilution with the 20 ml of sodium thioglycollate broth, while the FDA broth and DT medium are not able to stop the bacteriostatic action of Metaphen in a dilution of 1-2000.

With BTC, Letheen broth is much more effective in neutralizing the disinfectant than the other two media used. It was effective at a 1-1000 dilution, while DT medium was effective at a dilution of 1-40,000 of BTC, and Special FDA broth only allowed growth of the organism at a 1-80,000 dilution of BTC.

TABLE XXII

RESULTS OBTAINED BY NEUTRALIZATION TEST

Dowicide A			
Dilution	FDA Broth	Subcultured in: DT Medium	FDA Broth + FeCl_3
1-200	-	+	-
1-300	+	+	+
1-400	+	+	+
Dowicide C			
Dilution	FDA Broth	Subcultured in: DT Medium	FDA Broth + FeCl_3
1-1000	-	-	-
1-2000	+	+	+
1-4000	+	+	+
Metaphen			
Dilution	FDA Broth	Subcultured in: DT Medium	Thioglycollate Broth
1-500	-	-	+
1-1000	-	-	+
1-2000	-	-	+
BTC			
Dilution	FDA Broth	Subcultured in: DT medium	Lethen Broth
1-1000	-	-	+
1-20,000	-	-	+
1-40,000	-	+	+
1-80,000	+	+	+

VI. SUMMARY

A study was made of the effect of the culture media on the resistance of M. pyogenes var. aureus to disinfectants and of the effect of the subculture media on the cultivation of bacterial cells after they had been treated with disinfectants.

"Phenol coefficient" and "semi-micro" tests were conducted on four representative-type disinfectants.

When the "phenol coefficient" technic was used, DT medium was shown to produce a culture that was more resistant to all the disinfectants tested whereas the culture in TS broth showed a greater resistance to phenol. However, the killing dilution with the TS broth culture showed a greater standard deviation from the mean than did the killing dilution with the FDA broth or the DT medium cultures. This would indicate that values obtained with the TS broth were not as consistent as those obtained with the other two media.

In the comparison of the subculture media, it was found that FDA broth plus ferric chloride was no more effective than Special FDA broth alone in the cultivation of the disinfectant-treated cells of M. pyogenes var. aureus. Thioglycollate broth

and Lethen broth were shown to be more effective for Metaphen and BTC, respectively, than Special FDA broth or DT medium. DT medium was shown to be more effective as a subculture medium than was Special FDA broth in "phenol coefficient" tests on all four compounds.

Results obtained using the "semi-micro" technic were not as consistent throughout the whole study as those obtained by the FDA procedure. This indicates that this method is not as reliable as the "phenol coefficient" technic and that it would not serve as a satisfactory substitute for the FDA procedure.

Washed cells of M. pyogenes var. aureus were shown to be less resistant than those in broth cultures normally used for the "phenol coefficient" tests. This is due to the fact that the foreign material introduced by the broth interfered with the action of the disinfectant, allowing the organism to appear more resistant.

The age of the FDA broth subculture medium appeared to have an important effect upon the cultivation of the disinfectant-treated cells of M. pyogenes var. aureus. Media that were freshly prepared were not as effective in allowing the organisms to grow as media that had been prepared several days prior to use. The

oxygen tension of the medium may have had some effect on the results obtained.

VII. LITERATURE CITED

1. Anderson, J. F., and McClintic, T. B. 1911. A method for the bacteriological standardization of disinfectants. J. Inf. Diseases, 8, 1.
2. Armbruster, E. H., and Ridenour, G. M. 1947. A new medium for study of quaternary bactericides. Soap and Sanit. Chem., 23, 119.
3. Baker, Z., Harrison, R. W., and Miller, B. F. 1941. The bactericidal action of synthetic detergents. J. Exptl. Med., 74, 611.
4. Brewer, C. 1943. Variations in phenol coefficient determinations of certain disinfectants. Am. J. Pub. Health, 33, 261.
5. _____. 1944. Report on disinfectants. J. A. O. A. C., 27, 554.
6. Domagk, G. 1935. Eine neue Klasse von Desinfektion-smitteln. Deut. Med. Wochschr., 61, 829.
7. Fildes, P. 1940. Mechanism of antibacterial action of mercury. Brit. J. Exptl. Path., 21, 67.
8. Flett, L., and Haring, R.; Geuteras, A., and Shapiro, R. 1945. Revival of organisms presumably killed by phenol. J. Bact., 50, 591.
9. Garrod, L. 1935. Effect of bacterial numbers on minimum bacteriostatic concentrations. J. Inf. Diseases, 57, 247.
10. Gegenbauer, V. 1921. Studien uber die Desinfektionwirkung des Sublimates. Arch. fur Hyg., 90, 23.

11. Grubb, T. C., and Edwards, M. 1946. A method of restoring and maintaining the phenol resistance of certain strains of S. aureus. J. Bact., 51, 205.
12. Heinemann, B. 1943. Comparison of methods used for evaluating the bactericidal property of mercuric compounds. J. Am. Pharm. Assoc., Sci. Ed., 32, 298.
13. James, L. H. 1947. Quaternaries vs. phenolics. Soap and Sanit. Chem., Nov., Dec., 125.
14. Klarmann, E. G., and Wright, E. S. 1945. Synthetic and semi-synthetic media for disinfectant testing. Soap and Sanit. Chem., Jan., 113.
15. _____. 1946. An inquiry into the germicidal performance of quaternary ammonium disinfectants. Soap and Sanit. Chem., Jan., 125.
16. Klein, M., and Kardon, Z. G. 1947. The "reversal," neutralization, and selectivity of germicidal cationic detergents. J. Bact., 54, 245.
17. Klimek, J. W., and Umbreit, L. E. 1948. On the germicidal behavior of a quaternary ammonium compound. Soap and Sanit. Chem., Jan., 137.
18. Kronig, B., and Paul, T. L. 1897. Die chemischen Grundlagen der Lehre von der Giftwirkung und Desinfektion. Zeitschr. fur Hyg., 25, 1.
19. Mallmann, W. L., Leavitt, A. H., and Joslyn, D. A. 1948. Effect of medium on resistance of S. aureus to quaternary ammonium compounds. Proc. Soc. Exptl. Biol. and Med., 68, 401.
20. Ostrolenk, M. 1950. Comparison of in vitro germicide test methods. J. Am. Pharm. Assoc., Sci. Ed., 39, No. 2.

21. Quisno, R., Foter, M. J., and Gibby, I. W. 1946. A technique for maintaining standard resistance for S. aureus. J. Bact., 52, 499.
22. Quisno, R., Foter, M. J., and Rubenkoenig, H. 1947. Quaternary ammonium germicides. Soap and Sanit. Chem., June, 145.
23. Quisno, R., Gibby, I. W., and Foter, M. J. 1946. Neutralizing medium for evaluating the germicidal potency of the quaternary ammonium salts. A. J. Pharm., 118, 320.
24. Rahn, O. 1947. Advisability of some proposed changes for the evaluation of disinfectants. J. Am. Pharm. Assoc., Sci. Ed., 36, 134.
25. _____. 1945. Injury and death of bacteria by chemical agents. Biodynamica Publishers.
26. Reddish, G. 1925. Resistance to phenol of S. aureus. Am. J. Pub. Health, 15, 534.
27. _____. 1927. Examination of disinfectants. Am. J. Pub. Health, 17, 320.
28. Reddish, G., and Burlingame, E. M. 1938. Effect of peptone on resistance of S. aureus. J. Am. Pharm. Assoc., 27, 331.
29. Reddish, G., Wood, L., and Burlingame, V. 1946. Resistance of S. aureus cultured in semi-synthetic media. Am. J. Pharm., 118, 4.
30. Rideal, S., and Walker, J. T. A. 1903. The standardization of disinfectants. J. Royal Sanit. Inst., 24, 424.
31. Ruehle, C. L. A., and Brewer, C. M. 1931. U. S. Food and Drug Administration methods for testing antiseptics and disinfectants. USDA Circ. 198.

32. Salle, A. J., Catlin, B. W., and Wesley, B. 1947. Profile evaluation of germicides. J. Am. Pharm. Assoc., Sci. Ed., 36, 129.
33. Smyth, H. F., Jr. 1934. Evaluation of some factors influencing the phenol resistance of S. aureus. J. Bact., 28, 333.
34. Stuart, L. S. 1947. N. A. I. D. M. - USDA report on quaternary ammonium testing. Soap and Sanit. Chem., Sept., 135.
35. Tilley, F. W. 1921. Phenol coefficients. Am. J. Pub. Health, 11, 513.
36. _____. 1948. Influence of subculture media on results obtained in disinfectant testing. J. Bact., 56, 479.
37. Weber, G., and Black, L. 1948. Inhibitors for neutralizing the germicidal action of quaternary ammonium compounds. Soap and Sanit. Chem., Sept., 137.
38. Wolf, P. A. 1945. A medium containing an acid casein hydrolysate for use in testing disinfectants. J. Bact., 49, 463.
39. Wright, J. H. 1917. Importance of uniform culture media in bacteriological examination of disinfectants. J. Bact., 2, 346.

EFFECT OF CULTURE MEDIA ON THE RESISTANCE AND
GROWTH OF MICROCOCCUS PYOGENES VAR. AUREUS

By

Anita Harriet Leavitt

AN ABSTRACT

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

DOCTOR OF PHILOSOPHY

Department of Bacteriology and Public Health

Year 1952

Approved

M. Mallman

ANITA HARRIET LEAVITT

ABSTRACT

Micrococcus pyogenes var. aureus was grown in standard FDA broth, Difco Disinfectant Test (DT) medium and Trypticase Soy (TS) broth. FDA "phenol coefficient" tests and "semi-micro" tests were run using the organisms grown in the above-mentioned media. A phenolic, a chloro-phenol, a mercurial and a quaternary ammonium compound were selected as test compounds. DT medium, FDA broth and a specific neutralizing medium were used for subculturing when the tests were run.

Results indicated that the organisms grown in DT medium were more resistant to the four compounds tested than were the organisms that were grown in FDA broth or TS broth. A smoother culture was maintained in DT medium and TS broth than was in FDA broth, but variations in the resistance to phenol occurred with the organisms grown in each of the three media.

Results obtained with the "semi-micro" tests were not as consistent as those obtained by the "phenol coefficient" method.