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The bacteriostatic action of gentian violet, crystal violet, basic fuchsin, and acid fuchsin on certain Gram positive bacteria

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THE BACTERIOSTATIC ACTION OF GENTIAN VIOLET, CRYSTAL VIOLET, BASIC FUCHSIN, AND ACID FUCHSIN ON CERTAIN GRAM POSITIVE BACTERIA

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TABLE OF CONTENTS

INTRODUCTION

The study of the bacteriostatic action of dyes is of comparatively recent origin. The early use of dyes was restricted to certain staining techniques and various investigations were conducted with compounds of the Triphenyl methane series. Later rosanilin and its derivatives were studied. These investigations sought to discover reliable methods for the observation and study of bacterial structure. The Gram stain and its many modifications, the many spore and flagella stains, attempted to demonstrate morphological characteristics. Now and then a few staining techniques were used in the differentiation of various groups of bacteria. Of all these differential methods the Gram stain is best known and has been most widely used in routine and research procedures.

The attempt to classify bacteria by means of their staining reactions to dyes, then, was clearly morphological in character. No physiological studies were conducted to show the differences in growth reactions to various dyes. Early work in this connection demonstrated that the aniline dyes, especially gentian violet, inhibited the growth of most Gram positive bacteria. This discovery became the basis for many differential media.

The Endo (22) medium, in which basic fuchsin is employed, has long been used to permit the growth of organisms of the Colon-Aerogenes Group, while occasionally other organisms in unrelated groups, including Gram positive bacteria,

are observed to grow on the medium. Such difficulties have been encountered from time to time with each medium in which dyes have been used in pure culture methods. These unusual situations have convinced the writer that a study of the tolerance of Gram positive bacteria to various dyes should be undertaken to establish the fact of tolerance and non-tolerance.

HISTORICAL

In 1903 Endo (22) first used the medium which now bears his name. He incorporated basic fuchsin in the substrate for the purpose of isolating the bacillus of typhoid fever, Eberthella typhi. Numerous papers have since been published on the use of the Endo medium for the isolation of Eberthella typhi, related organisms of the Colon-Aerogenes group, and of the dysentery group of bacilli. The medium is now included in the Standard Methods for Water Analysis to detect the presence of Escherichia coli in water samples.

The use of basic fuchsin, itself, as a bacteriostat is negligible. References in the bacteriological literature to such isolated instances of use are difficult to find. This dye has usually been combined with other substances such as sodium sulphite for its bacteriostatic effect. This is the case with the Endo medium.

Although it is usual for the Endo medium to be selective for Gram negative bacteria, this is not always so. Experience in the laboratory frequently indicates the growth of Gram positive forms on the Endo substrate. Also the medium does not always prove selective for members of the Colon-Aerogenes group of bacteria. This difficulty is frequently encountered in the bacteriological analysis of water for potability.

Consequently various explanations have arisen as to the nature of the reaction on the Endo medium. De Bord, (21) for example, postulates an acid-aldehyde production where

Escherichia coli is found to grow on the Endo medium. Kligler and Defandorfer (32) have shown that the hydrogen ion concentration of the medium must be sufficiently alkaline (pH 8.4 to pH 8.8) to permit the medium to be selective for Eberthella dysenteriae. Kahn (31) believes the reaction of Escherichia coli on the Endo medium to be the formation of an aldehyde complex. Harding and Cetenburg (29) also postulate the formation of aldehydes in the typical reddening phenomenon which is characteristic of Escherichia coli. Robinson and Rettger (37) view this phenomenon to be due to decomposition products of lactose such as lactic acid, as well as the presence of another acid, namely, acetic acid.

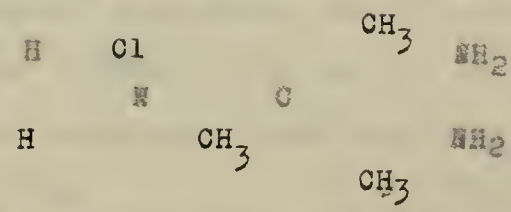
Conn (18) as chairman of the Commission on Biological Stains has had an opportunity with his associates to engage in considerable research on many dyes. Through his efforts the various biological stains and dyes have approached a more complete uniformity and standardization. It is interesting here to note the development of this standardization and uniformity out of confusion. In 1923 (15) Conn found that different samples of basic fuchsin varied as to their solubilities in alcohol. This fact was true even for products of the same manufacturer. As a result he (16) recommended the use of saturated solutions from which to begin dilutions rather than the use of dry stain. Later he (17) revised this earlier suggestion concerning the use of saturated solutions and recognized the criticism that certain inert mineral ingredients

in the dye substance may affect the saturation point of dyes to the extent of precipitating them out of solution. He understood the implications of this situation and suggested that the companies which manufacture dyes make known the actual dye and moisture content of each particular product.

Conn (17, 18) discussed basic fuchsin from the chemical point of view also. There has been a good deal of confusion in respect to this dye. Commercially it has not been chemically pure. Rather it has consisted of three different compounds. The simplest of these is pararosanilin. It is the same substance as those which have been known as basic rubin, parafuchsin, and paramagenta. Symbolically it is:

This compound becomes rosanilin with the introduction of a methyl group into its molecule. Rosanilin symbolically, then, is:

With the introduction of three methyl groups into this molecule of pararosanilin, it becomes new fuchsin which has also been known as isorubin or fuchsin N. B. Symbolically new fuchsin is:

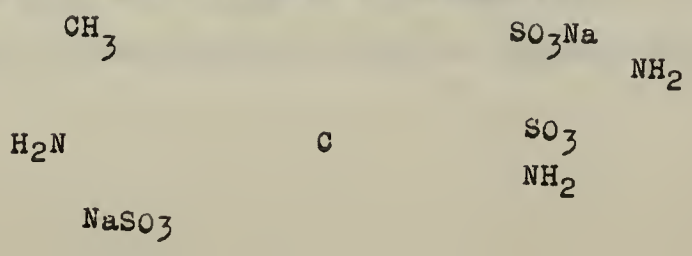


Rosanilin is not found free from pararosanilin unless it is specially prepared. The basic fuchsin mixture of rosanilin and pararosanilin has proved to be unsatisfactory for use in

the Endo medium and the Commission on Standardization of Biological Stains (17, 18) experimented with rosanilin alone and found this compound to be superior to any of the mixtures for use in the Endo medium. New fuchsin was satisfactory also.

Conn and Darrow (19) have shown that results with the Endo medium have been unsatisfactory and conflicting because of differences in saturation of various lots of basic fuchsin. Margolena and Hansen (36) agree with this finding. Reed and Genung, (24) who studied the effect of certain triphenyl methane dyes on Staphylococcus aureus and Escherichia communior, used sixteen different dyes and learned that the bacteriostatic effect on these organisms was constant for each dye. Escherichia communior was able to adapt itself to increasing concentrations of the dyes, whereas this was not true for Staphylococcus aureus. This work has not yet been published in detail.

The study of acid fuchsin has not been extensive. Theoretically twelve different acid fuchsins are possible, since this dye is a sulfonated derivative of basic fuchsin of which there are four possible forms according to the degree of methyl substitution. Normally samples of acid fuchsin are mixtures of several compounds. Usually, however, acid fuchsin or synonymously Fuchsin S, SN, SS, ST, S3, acid magenta, or acid rubin, is represented symbolically as:



Churchman, (9, 10) working with acid fuchsin as a bacteriostat, demonstrated that this dye has a reverse selective bacteriostatic power. By this he means that Gram negative bacteria are inhibited, whereas gentian violet inhibits Gram positive organisms. His work was not quantitative in respect to dilutions of acid fuchsin. It is interesting to observe that he cites no literature. Churchman mentioned another phenomenon peculiar to acid fuchsin. He found in cases of extrinsic bacteriostasis, in which any dye is added to the medium, that the Gram positive spore bearing bacteria are inhibited; and in instances of intrinsic bacteriostasis, where the dye is added directly to the bacteria before they are inoculated on unstained media, that the Gram negative forms of bacteria are inhibited and the Gram positive spore bearing organisms are unaffected. Churchman in this experiment worked with comparatively few organisms and only one, Bacillus anthracis, was Gram positive.

The use of gentian violet for staining purposes is well known. Gram (26) in 1884 first used the dye in the original staining technique which bears his name. The utilization of gentian violet for bacteriostatic purposes was delayed until 1902 when Conradi and Drigalski (20) introduced it into a medium in order to inhibit the growth of Gram positive fecal strains of bacteria. It was not until 1912, however, that a systematic study of the effect of gentian violet on Gram positive ferms and Gram negative forms of bacteria was conducted.

This work was done by Churchman (7) who was the first to observe the effect of a dye on the "cessation of motility, genistasis, inhibition of sporulation, suspension of animation" among bacteria. He first used the term "bacteriostasis" to nonnote these effects which are characteristic of basic materials. Churchman (7, 10) concluded that approximately ninety percent of Gram positive organisms are inhibited by a concentration of one to one hundred thousand of gentian violet, while Gram negative forms of bacteria are relatively unaffected by this concentration. He found that closely related strains of bacteria reacted in the same way to various basic dyes, and therefore he assumed the presence of certain specific protoplasmic groups in the bacterial structure which became saturated with the dye. He (8) abandoned this point of view in 1921 and concluded that physical as well as chemical forces were a factor. Benians (4), Shumaker (38), Burke and Barnes (6), had begun to study possible mechanical differences between cell membranes of various organisms and their relative permeability and non-permeability. Churchman undertook similar studies and came to a similar position.

The chemical theory as opposed to the physical theory to explain the fundamental reasons for bacteriostatic action first arose in 1923. Traube (49) in 1912 had observed that by adding $NaHCO_3$ to gentian violet, the bacteriostatic effect of the dye was enhanced. Stearn and Stearn (42, 43, 44) cognizant of this fact and many other phenomena which changed the chemical aspects of bacteriostatic activity, developed a chemical theory.

In 1923 (41) they expounded their first so-called chemical theory and in 1925, 1926, 1928, and 1930 they enlarged upon the theory. Ingraham (30) accepted certain of the premises of Stearn and Stearn. Yet she felt that an intimate relationship exists between bacteriostasis and oxidation-reduction potentials.

Hall and Ellefson (27) became interested in the use of gentian violet to eliminate spurious presumptive tests for Escherichia coli in water. Hall (28) later continued similar investigations with aerobic and anaerobic bacteria.

As is the case with other dyes, there have been many conflicting results in the use of gentian violet. Here as elsewhere the reason is the lack of standardization of the dye. This factor again is stressed by Conn (18). He states that gentian violet is a mixture and not a pure compound. Crystal violet is an individual compound, hexamethyl pararosanilin. More uniform results have been obtained with the crystal violet. The work of Conn (15, 16, 17, 18), Genung (23, 24), and many others, demonstrated that a standardization of crystal violet is relatively simple in contrast to the more complex mixture of gentian violet.

EXPERIMENTAL

The bacterial cultures which have been used in this study are as follows:

Streptococcus hemolyticus (a) Mass.

Streptococcus hemolyticus (b) Mass.

Streptococcus hemolyticus R 4

Streptococcus non-hemolyticus R 10

Streptococcus X 95

Streptococcus X 99

Streptococcus X

Streptococcus saprophytic

Staphylococcus aureus

Staphylococcus aureus Kr 29

Staphylococcus aureus (Reddish)

Staphylococcus aureus (Walker)

Staphylococcus aureus (Nigard)

Staphylococcus albus

Staphylococcus albus (N.Y.U.)

Staphylococcus citreus

Staphylococcus citreus (Yale)

Staphylococcus aurantiacus

Sarcina lutea (a)

Sarcina lutea (b)

Sarcina aurantiacus (a)

Sarcina aurantiacus (b)

Micrococcus cereus

cultures---(continued)

Micrococcus tetragena (a)

Micrococcus tetragena (b)

Micrococcus flavus

Micrococcus varians

Escherichia coli

Pseudomonas fluorescens

Neisseria catarrhalis

The medium, on which the bacteria were grown was that of Ayers and Johnson (1) for the cultivation of coccus forms. The medium was prepared as follows:

A

Beef extract..... 5 grams

Peptone.....10 grams

$Na_2HPO_4 \cdot 2H_2O$..... 2 grams

Distilled water.....500 c.c.

Heat until dissolved and then adjust reaction to pH 7.8

B

Distilled water.....150 c.c.

Casein (pure)..... 5 grams

$Na_2HPO_4 \cdot 2H_2O$..... 2 grams

Heat until dissolved. Add to A and to the resulting mixture of A and B add 10 grams of gelatin.

C

Heat mixture of A and B (including gelatin) in the autoclave for ten minutes at fifteen pounds pressure. Add 0.5 grams of glucose. Filter through paper. Reaction pH 7.6

D

Add 1.5 per cent agar and 0.5 grams sodium citrate. Make up to 1000 c.c. Tube and sterilize in the autoclave for twenty minutes at fifteen pounds pressure. Final pH 7.5

The writer found it to be just as good a procedure to combine steps A and B; and steps C and D.

The dyes used in this study were:

1. Gentian Violet (improved). Dye content 75 per cent.
   The Coleman and Bell Company.
2. Crystal Violet. Dye content 85 per cent.
   The Coleman and Bell Company.
3. Basic Fuchsin (pure crystals) Dye content 93 per cent.
   The Coleman and Bell Company.
4. Acid Fuchsin. Dye content 62 per cent.
   National Aniline and Chemical Company.

The scheme of dilutions for each of the four dyes used was as follows:

1. Gentian violet (aqueous). 1 gram per 100 c.c. This solution is equivalent to one containing 0.01 grams per c.c.

2. Crystal violet. Stock solution equals 1 gram per 100 c.c. water or 0.01 grams per c.c.

3. Basic fuchsin. Stock solution equals 2.5 grams per 100 c.c. of ethyl alcohol.

4. Acid fuchsin. Stock solution A equals 1.5 grams per 100 cc. of water, or 0.015 grams in 1.0 cc. Dilute the stock solution A up to 150.0 cc., thus giving solution B which contains 1.5 grams of acid fuchsin per 150 cc. of solution, or 0.01 gram in 1.0 cc. A dilution of 1-100,000

is obtained by adding 1 cc. of solution B to one litre of medium. The concentrations of dye in this study have been increased in a geometric ratio. This accounts for some of the unusual fractional dilutions which are only theoretically possible. The writer admits the technical difficulty in practice, of obtaining such fractional dilutions; nevertheless, these dilutions were approximated with reasonable accuracy.

The procedure of adding the various dyes to the hot agar was uniform in each experiment. The medium was measured out in terms of cubic centimeters, and the amount of the dye solution in terms of cubic centimeters was added to the measured medium to make the desired proportion of dye to medium. Bacteriostasis, where it occurred, was extrinsic (9).

The stock medium already described was tubed in ten cc. amounts and slanted to make agar slants. Large tubes were used for this purpose. The stock medium was the only substrate used in the bacteriostatic studies.

Before the bacteria were inoculated on the medium containing dye, they were cultivated for 48 hours on dye-free stock agar slants. From these slants the bacteria were inoculated into sterile saline blanks to give an approximate density of 1.5 according to the nephelometer (McFarland) scale. A loopful of this saline suspension streaked on the slanted dye-containing medium. Tubes of the medium thus inoculated were incubated for various periods of time as shown in the accompanying tables.

No tube was discarded until an incubation period of ninety-six hours had elapsed. Observations were made when the growth of the organisms had definitely shown their tolerance to dye.

GENTIAN VIOLET

Table 1 sumarizes the tolerance of the bacteria to gentian violet. Inhibition of growth is symbolized by minus signs; growth is designated by plus signs. It will be seen from the table that the dilutions of 1-500,000, 1-400,000, and 1-300,000 were ineffective in inhibiting the visible growth of the organisms. A dilution of 1-100,000 was successful in inhibiting only ten of the twenty-eight strains employed in the gentian violet studies. At a dilution of 1-75,000 twenty of the strains failed to grow; dilutions of 1-50,000 and 1-25,000 each inhibited the growth of twenty of the twenty-eight strains. At a dilution of 1-10,000 twenty-three of the strains were inhibited, while a 1-5,000 dilution inhibited the growth of all of the strains employed, including Escherichia coli, the one Gram negative organism employed.

Churchman (7) made the statement that a dilution of 1-100,000 of gentian violet inhibited 90 per cent of the Gram positive bacteria he studied. The writer found that only thirty-seven per cent of the Gram positive bacteria studied were inhibited at a dilution of 1-100,000. The dilution of 1-75,000 in the writer's experiments inhibited 75 per cent of the Gram positive strains.

Table 1

Growth of organisms in the presence of varying concentrations of gentian violet

Organisms	1-6000		1-10000		1-26000		1-50000		1-75000		1-100000		1-200000		1-300000	
	48	96	48	96	48	96	48	96	48	96	48	96	48	96	48	96
Streptococcus hemolyticus (a)	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus hemolyticus (b)	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus hemolyticus N 4	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Streptococcus non-hemolyticus N 10	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X 95	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X 99	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus saprophytic	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Staphylococcus aureus Nr 20	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Staphylococcus aureus (Reddish)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Staphylococcus aureus (Walker)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Staphylococcus aureus (Migard)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Staphylococcus albus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Staphylococcus albus (N.Y.U.)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+

Table 1 (continued)

Growth of organisms in the presence of varying concentrations of gentian violet

Organisms	Dilutions of dye															
	1-5000		1-10000		1-25000		1-50000		1-75000		1-100000		1-200000		1-300000	
	48	96	48	96	48	96	48	96	48	96	48	96	48	96	48	96
Staphylococcus citreus (a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Staphylococcus citreus (b)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Staphylococcus aurantiacus	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Sarcina lutea (a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Sarcina lutea (b)	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Sarcina aurantiacus	-	-	-	-	-	-	-	-	-	-	+-	+-	+	+	+	+
Sarcina aurantiacus (Yale)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Micrococcus cereus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Micrococcus tetragena (a)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Micrococcus tetragena (b)	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
Micrococcus flavus	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
Micrococcus varians	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Escherischia coli	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+

The difference between the results of Churchman (7) and those of the writer is easily explained. In the first place Churchman (7) used a saturated aqueous solution of gentian violet. The writer used a solution containing exactly 1 gram of gentian violet in 100 c.c. of water. The previously noted objections of Conn (15, 16, 18) to the use of saturated solutions convinced the writer that a dye solution containing a known number of grams per cubic centimeter should be used. In the second place Churchman does not mention the actual dye content of the gentian violet. Differences in actual dye content account for differences in bacteriostatic effectiveness of dye. It will be remembered that of 448 strains of bacteria studied by Churchman (7), 26 were streptococci. Reference to the writer's Table 1 indicates that streptococci are tolerant to greater concentrations of gentian violet than are the other Gram positive strains employed. Churchman's results are in agreement with the writer's observations.

The apparent difference between Churchman's (7) and the writer's results thus becomes a striking agreement when the differences of the conditions of the two experiments and the streptococcus variations are considered. The inhibition of Escherichia coli by a dilution of 1-5000 of gentian violet is shown in Table 1. This fact is also consistent with Churchman's point of view that prolonged staining of bacteria or the addition of gentian violet in sufficient concentrations will inhibit Gram negative as well

as Gram positive bacteria. Much of the confusion and subsequent misunderstanding and dispute concerning bacteriostatic experiments has arisen because the many workers in the field have failed to state exactly the conditions under which the experiment has been conducted. Differences in actual dye and mineral content of dye substances may significantly influence the results of the experiment. Conn (17, 18) and his associates have pointed out that differences in mineral content of a dye materially effect changes in the saturation point of any dye which has been studied by the Commission on Biological Stains. Saturations vary with so many other conditions that the writer has been careful to use only known definite amounts of dye of known dye content.

The chemical relationship of gentian violet to crystal violet has already been discussed in detail. Gentian violet is a mixture of many compounds, some of which may be unknown, whereas crystal violet is a chemically pure substance: hexamethyl pararosanilin. The use of crystal violet as a substitute for gentian violet as a bacteriostatic agent made it seem desirable that more should be known concerning the actual bacteriostatic powers of crystal violet. The writer, therefore, undertook to study the effect of crystal violet on the Gram positive organisms which had been used in the gentian violet study. Two Gram negative bacteria, Pseudomonas fluorescens and Neisseria catarrhalis were added. Escherichia coli had already been used in the gentian violet experiment. The

acid producing power of Escherichia coli is well known: Its tolerance to the basic dye, gentian violet, had already been demonstrated. Pseudomonas fluorescens and Neisseria catarrhalis have not the same degree of acid producing ability as Escherichia coli. Later in this thesis the relationship of tolerance to dye and acid production by the bacteria, will be discussed. For this reason Pseudomonas fluorescens and Neisseria catarrhalis were added to the number of organisms.

Since the stock solution of gentian violet contained one gram of gentian violet in one hundred cubic centimeters of water, the crystal violet solution was also a one per cent aqueous solution. The conditions of the study with gentian violet and crystal violet were as nearly identical as possible. The dye was added to a medium of the same composition as previously used in the gentian violet work and identical methods of inoculation were employed.

CRYSTAL VIOLET

The results of the crystal violet experiment are noted in Table 2. A dilution of 1-100000 inhibited the growth of twenty-two, or eighty-one per cent, of the twenty-seven Gram positive organisms employed. None of the Gram negative organisms were inhibited. A greater degree of bacteriostasis has also been observed with a 1-50000 dilution of crystal violet as compared to the same concentration of gentian violet. In twenty-four hours of incubation all of the Gram positive strains were inhibited by a dilution of 1-50000 of crystal violet. Five of these Gram positive bacteria succeeded in growing feebly after forty-eight hours of incubation. This fact indicated that crystal violet was successful in prolonging the lag period of these bacteria. The dilution of 1-25000 of crystal violet inhibited all of the Gram positive bacteria for forty-eight hours, and only one organism subsequently showed any growth. The growth of this one organism was not visible until an incubation period of seventy-two hours had elapsed. The growth was feeble and even after ninety-six hours incubation, though definite, growth was only slight. Escherichia coli grew vigorously at a dilution of 1-25000 of crystal violet. The other Gram negative organisms, Pseudomonas fluorescens and Neisseria catarrhalis, were inhibited at this dilution. A dilution of 1-25000 of gentian violet inhibited only seventy-four per cent of the Gram positive strains. A concentration of 1-5000 of gentian violet was necessary to inhibit all of the Gram

Table 2

Growth of organisms in the presence of varying concentrations of crystal violet

	1-25000				1-50000				1-100000			
	24	48	72	96	24	48	72	96	24	48	72	96
Streptococcus hemolyticus (a)	-	-	-	-	-	+-	+	+	-	-	-	-
Streptococcus hemolyticus (b)	-	-	+-	+	-	+	+	+	+	+	+	+
Streptococcus hemolyticus R 4	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus non-hemolyticus R 10	-	-	-	-	-	+-	+	+	+	+	+	+
Streptococcus X 95	-	-	-	-	-	+-	+	+	+	+	+	+
Streptococcus X 99	-	-	-	-	-	-	-	-	+	+	+	+
Streptococcus X	-	-	-	-	-	+-	+	+	+	+	+	+
Streptococcus saprophytic	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus Kr 29	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (Reddish)	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (Walker)	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (Migard)	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus albus	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus albus (N.Y.U.)	-	-	-	-	-	-	-	-	-	-	-	-

Table 2 (continued)

Growth of organisms in the presence of varying concentrations of crystal violet

Organisms	Dilutions of dye					
	1-20000		1-30000		1-100000	
	24	48	24	48	24	48
Staphylococcus citreus (a)	-	-	-	-	-	-
Staphylococcus citreus (b)	-	-	-	-	-	-
Staphylococcus aurantiacus	-	-	-	-	-	-
Sarcina lutea (a)	-	-	-	-	-	-
Sarcina lutea (b)	-	-	-	-	-	-
Sarcina aurantiacus	-	-	-	-	-	-
Sarcina aurantiacus (Yale)	-	-	-	-	-	-
Micrococcus cereus	-	-	-	-	-	-
Micrococcus tetragena (a)	-	-	-	-	-	-
Micrococcus tetragena (b)	-	-	-	-	-	-
Micrococcus flavus	-	-	-	-	-	-
Micrococcus varians	-	-	-	-	-	-
Escherichia coli	+	+	+	+	+	+
Pseudomonas fluorescens	-	-	+	+	+	+
Neisseria catarrhalis	-	-	+	+	+	+

positive organisms. Since a dilution of 1-25000 of crystal violet was sufficient to inhibit all of the Gram positive bacteria after forty-eight hours of incubation and a dilution of 1-5000 of gentian violet was necessary to inhibit all of the Gram positive strains after a similar period of incubation, the writer concluded that in consideration of the conditions of the experiment crystal violet is a much more effective bacteriostatic agent than gentian violet.

The inhibiting effect of basic fuchsin on the growth of Gram positive cocci was also studied. The same medium of Ayers and Johnson (1) was used. The stock solution of basic fuchsin consisted of 2.5 grams of the dry stain dissolved in 100 c.c. of alcohol. This solution of dye was definitely less than saturation. The same organisms which had been used in the gentian violet and crystal violet studies were employed in the basic fuchsin experiments.

BASIC FUCHSIN

The observations of growth and inhibition of the bacteria in the presence of varying concentrations of basic fuchsin are recorded in Table 3. It is interesting to note that much lower concentrations of basic fuchsin were required than had been necessary with gentian violet and crystal violet to inhibit the growth of a majority of the organisms. A basic fuchsin dilution of 1-500000 inhibited the growth of twenty of the twenty-seven Gram positive cocci employed, or seventy-four per cent of them, in ninety-six hours. This same figure held true for a dilution of 1-400000. At a dilution of 1-300000 twenty-one organisms were inhibited after forty-eight hours incubation. One of the organisms, Streptococcus hemolyticus R 4, succeeded in growing rather well after ninety-six hours of incubation had elapsed.

In the presence of basic fuchsin in a 1-100000 dilution, twenty-four of the organisms were inhibited after forty-eight hours of incubation. After ninety-six hours had elapsed, one of these organisms, Streptococcus hemolyticus (a), succeeded in growing. Thus, the number of organisms inhibited at a dilution of 1-100000 after ninety-six hours incubation was twenty-three, or approximately eighty-five per cent of the Gram positive cocci employed. This figure compares with thirty-seven per cent for gentian violet and seventy-four per cent for crystal violet. A concentration of 1-75000 of basic fuchsin inhibited the growth of twenty-four of the Gram positive cocci after forty-eight hours of incubation. After

TABLE 3

Growth of organisms in the presence of varying concentrations of Basic Fuchsin

Organisms	Dilutions of dye															
	1-25T		1-50T		1-75T		1-100T		200T		300T		400T		500T	
	48	96	48	96	48	96	48	96	48	96	48	96	48	96	48	96
Streptococcus hemolyticus (a)	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+	+
Streptococcus hemolyticus (b)	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Streptococcus hemolyticus R 4	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
Streptococcus non-hemolyticus R 10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Streptococcus X 95	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X 99	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
Streptococcus X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus saprophytic	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus Kr 29	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (Reddish)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (Walker)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aureus (Wigard)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus albus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus albus (N.Y.U.)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 3 (continued)

Growth of organisms in the presence of varying concentrations of Basic Fuchsin

Organisms	Dilutions of dye															
	1-25T		1-50T		1-75T		1-100T		1-200T		1-300T		1-400T		1-500T	
	48	96	48	96	48	96	48	96	48	96	48	96	48	96	48	96
Staphylococcus citreus (a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus citreus (b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Staphylococcus aurantiacus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sarcina lutea (a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sarcina lutea (b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sarcina aurantiacus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sarcina aurantiacus (Yale)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus cereus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus tetragena (a)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus tetragena (b)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus flavus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus varians	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Escherischia coli	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Neisseria catarrhalis	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

ninety-six hours of incubation had elapsed, Streptococcus hemolyticus (a) succeeded in growing. At a dilution of 1-50000 twenty-four of the organisms were inhibited and with a concentration of 1-25000 bacteriostasis was complete for all of the Gram positive cocci. Two of the Gram negative organisms, Pseudomonas fluorescens and Neisseria catarrhalis failed to grow in forty-eight hours, but grew in ninety-six hours.

ACID FUCHSIN

Churchman's (9, 10) work in 1923 had suggested that acid fuchsin did not have any appreciable effect on Gram positive bacteria. Because three basic dyes had already been studied in this investigation, it seemed advisable to learn the comparative differences between the inhibiting capacities of these basic dyes and an acid dye. Acid fuchsin was selected for this purpose.

The same organisms and medium which had been used in the other bacteriostatic studies were used in studying the bacteriostatic power of acid fuchsin. The stock solution of acid fuchsin was a one per cent aqueous solution. The first dilution of the dye was 1-100000. The concentrations of dye were increased in a geometric ratio until a dilution of 1-781.25 was reached. The bacteriostatic power of the dye in each dilution was observed.

The results for the acid fuchsin study are recorded in Table 4. Acid fuchsin in a dilution of 1-100000 had little effect on the organisms studied. Only four strains were inhibited after an incubation period of twenty-four hours. They were: Staphylococcus citreus (a), Sarcina aurantiacus (Yale), Micrococcus cereus, and Micrococcus varians. After forty-eight hours had elapsed Micrococcus cereus succeeded in growing. The other three organisms were inhibited even after seventy-two hours of incubation had elapsed. Three organisms failed to grow at a concentration of 1-50000. They were Staphylococcus citreus (a), Sarcina aurantiacus (Yale), and Micrococcus varians. Dilutions of

Growth of organisms in the presence of varying concentrations of acid fuchsin

Organisms	Dilutions of dye																							
	1-781.25			1-1562.5			1-3125			1-6250			1-12500			1-25000			1-50000			1-100000		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
Streptococcus hemolyticus (a)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus hemolyticus (b)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus hemolyticus R 4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus non-hemolyticus R 10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X 95	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X 99	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus X	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococcus saprophytic	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus Kr 29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus (Reddish)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus (Walker)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aureus (Wigard)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus albus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus albus (N.Y.U.)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus citreus (a)	-	+	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-

Table 4 (continued)

Growth of organisms in the presence of varying concentrations of acid fuchsin

Organisms	Dilutions of dye																							
	1-781.25			1-1562.5			1-3125			1-6250			1-12500			1-25000			1-50000			1-10000		
	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72	24	48	72
Staphylococcus citreus (b)	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Staphylococcus aurantiacus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sarcina lutea (a)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sarcina lutea (b)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sarcina aurantiacus	-	-	-	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+
Sarcina aurantiacus (Yale)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Micrococcus cereus	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	
Micrococcus tetragena (a)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Micrococcus tetragena (b)	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Micrococcus flavus	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+
Micrococcus varians	-	+	+	-	+	+	-	+	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	-
Escherischia coli	-	+-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fluorescens	-	+-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Neisseria catarrhalis	-	+-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

1-25000, 1-12500, 1-6250, and 1-1562.5 inhibited only one organism, Sarcina aurantiacus (Yale). After an incubation period of twenty-four hours had elapsed Sarcina aurantiacus was inhibited by all the dilutions mentioned in the previous sentence. It grew at forty-eight hours, however. Staphylococcus citreus (a) was inhibited by these same dilutions after an incubation period of forty-eight had passed. Staphylococcus citreus (b) was inhibited by dilutions of 1-6250, 1-3125, 1-1562.5, and 1-781.25 after twenty-four hours of incubation. The organism grew at all these mentioned dilutions after forty-eight hours had elapsed.

Sarcina aurantiacus was inhibited even after seventy-two hours of incubation by a dilution of 1-781.25. Micrococcus cereus was inhibited by dilutions of 1-3125, 1-1562.5, 1-781.25 after twenty-four hours of incubation had elapsed, but it grew after forty-eight hours in all these dilutions. Micrococcus tetragena (b) was inhibited after twenty-four hours of incubation had elapsed, but grew in forty-eight hours at dilutions of 1-1562.5 and 1-781.25. Micrococcus flavus failed to grow after twenty-four hours at dilutions of 1-12500, 1-6250, 1-3125, 1-1562.5, and 1-781.25 but grew well after forty-eight hours of incubation had elapsed.

Acid fuchsin, on the whole, had little bacteriostatic effect on the Gram positive bacteria. In some of the cases cited above the dye succeeded merely in prolonging the lag period of some of the bacteria which grew well after

forty-eight or seventy-two hours. These observations confirmed Churchman's (9) work with acid fuchsin. He noticed that acid dyes have little or no bacteriostatic effect on Gram positive bacteria. The writer's study bore this out in every detail.

ACID PRODUCTION

In all of these bacteriostatic experiments Escherichia coli has been studied with the other organisms. Pseudomonas fluorescens and Neisseria catarrhalis were employed in the crystal violet, basic fuchsin, and acid fuchsin investigations. The reactions of these organisms were compared to those of Escherichia coli. Escherichia coli is capable of producing considerable quantities of acid. This organism is Gram negative and was shown to be tolerant to all the dyes employed, except 1-5000 gentian violet. In consideration of the known tolerance of the acid producing Gram negative organisms to dyes the writer proceeded to investigate the production of acid possible by the cocci employed , and any relationship between their dye tolerance and acid production in suitable media. The media selected for this acid study were litmus milk and brom cresol purple milk.

The reactions of the organisms in litmus milk and brom cresol purple milk are recorded in Table 5. Acidity production is designated by the plus sign and lack of acidity by the minus sign. Records were made for acidities after twenty-four and forty-eight hour periods of incubation. No changes were noted between the twenty-four and forty-eight hour readings. The organisms which produced a definite acidity were as follows:

CHART 5

Acidity Production in Litmus and Bron Cresol Purple Milk

	Litmus milk		Bron cresol purple milk	
Organisms	24	48	24	48
Streptococcus hemolyticus (a)	+	+	+	+
Streptococcus hemolyticus (b)	+	+	+	+
Streptococcus hemolyticus N 4	+	+	+	+
Streptococcus non-hemolyticus N 10	+	+	+	+
Streptococcus X 95	+	+	+	+
Streptococcus X 99	+	+	+	+
Streptococcus X	+	+	+	+
Streptococcus saprophytic	+	+	+	+
Staphylococcus aureus	-	-	-	-
Staphylococcus aureus Er 20	-	-	-	-
Staphylococcus aureus (Reddish)	-	-	-	-
Staphylococcus aureus (Walker)	-	-	-	-
Staphylococcus aureus (Migard)	-	-	-	-
Staphylococcus albus	-	-	-	-
Staphylococcus albus (N.Y.U.)	-	-	-	-
Staphylococcus citreus (a)	-	-	-	-
Staphylococcus citreus (b)	-	-	-	-
Staphylococcus aurantiacus	-	-	-	-

\+ acidity

\- lack of acidity

Table 5 (continued)

Organisms	Litmus milk		Bromcresol purple milk	
	24	48	24	48
Sarcina lutea (a)	-	-	-	-
Sarcina lutea (b)	-	-	-	-
Sarcina aurantiaca	-	-	-	-
Sarcina aurantiaca (Yolo)	-	-	-	-
Micrococcus cereus	-	-	-	-
Micrococcus tetragena (a)	-	-	-	-
Micrococcus tetragena (b)	-	-	-	-
Micrococcus flavus	-	-	-	-
Micrococcus varians	-	-	-	-
Escherichia coli	+	+	+	+
Pseudomonas fluorescens	+	+	+	+
Neisseria catarrhalis	+	+	+	+

\+ acidity

\- lack of acidity

Streptococcus hemolyticus (a)

Streptococcus hemolyticus (b)

Streptococcus hemolyticus R 4

Streptococcus non-hemolyticus R 10

Streptococcus X 95

Streptococcus X 99

Streptococcus X

Streptococcus saprophytic

Staphylococcus aurantiacus

Escherichia coli

Pseudomonas fluorescens

Neisseria catarrhalis

In brom cresol purple milk the above mentioned microorganisms behaved in the same way as in litmus milk. Of the Gram positive bacteria the streptococcus forms produced acid to a greater extent than any of the other Gram positive cocci. This is in agreement with the observations of Langwill (34).

Study of Table 1 shows that of the Gram positive cocci tolerant to gentian violet in the stronger concentrations the majority were streptococci. This was also true for crystal violet and basic fuchsin. The only exceptions were Streptococcus non-hemolyticus R 10 which was inhibited by basic fuchsin in a dilution of 1-500000, and Streptococcus hemolyticus (a) and Streptococcus hemolyticus R 4 which were inhibited by crystal violet in a dilution of 1-100000.

In general, it is true that those microorganisms which were capable of producing acid in detectable quantities were also tolerant to the dyes in the stronger concentrations.

DISCUSSION

Four dyes were employed in this investigation. They were: gentian violet, crystal violet, basic fuchsin, and acid fuchsin. The bacteriostatic effect of these dyes was observed on the Gram positive bacteria previously mentioned. Each of the basic dyes was shown to have a bacteriostatic influence on all of the organisms. There was no significant bacteriostatic action by acid fuchsin. Crystal violet was a more powerful bacteriostatic agent than gentian violet. The results with crystal violet were more definite and clear cut. The advantages of using crystal violet instead of gentian violet are evident, due to the fact that crystal violet is a chemically pure compound of known structure. Its composition is more uniform and more constant results may be expected with this dye than with gentian violet.

The staphylococci, sarcinae, and micrococci were relatively non-tolerant to the dyes employed in this study. The streptococci were the most tolerant of all the Gram positive organisms. The exceptions among the streptococci were Streptococcus hemolyticus (a), Streptococcus non-hemolyticus R 10, and Streptococcus hemolyticus R 4. These cultures were less tolerant to gentian violet, crystal violet, and basic fuchsin than the other streptococci employed. In general, however, streptococci are less sensitive to the bacteriostatic effect of dyes than any other types of Gram positive cocci. These observations are in accord with Churchman's (7) findings.

Comparative differences in bacteriostatic effect were observed among the dyes employed. Gentian violet proved to be the least effective, crystal violet more effective, and basic fuchsin most effective. The inert materials present in gentian violet are considerable and the consequent bacteriostatic effect of this dye would be mitigated. The actual dye content of the gentian violet employed was only seventy-five per cent. The actual dye content of crystal violet was eighty-five per cent. This factor accounts for the observed difference in bacteriostatic effect of the two dyes. The actual dye content of the basic fuchsin employed was ninety-three per cent, and pure crystals of basic fuchsin were used. This is in keeping with the progressively more potent bacteriostasis observed with gentian violet, crystal violet, and basic fuchsin.

There are two theories as to the mechanism of bacteriostasis of dyes. One is the physical theory, the other the chemical theory. Benians (4), Burke and Barnes (6), and Churchman (8) have expounded the physical theory. This theory states that bacteriostasis occurs when the cell membrane of the bacteria exerts a selective action in adsorbing a dye which may be used for bacteriostatic purposes. Where the cell membrane adsorbs the dye material bacteriostasis occurs; where the cell membrane does not adsorb the dye, the microorganism grows and is not inhibited by dye. The Gram negative bacteria generally do not adsorb the dye on the cell membrane; the Gram positive

bacteria generally adsorb the dye and bacteriostasis occurs. Briefly, this is the gist of the physical theory.

Stearn and Stearn (42, 43) pointed to the fact that staining reactions of bacteria show that they behave as ampholytes retaining basic dyes in alkaline solution and acid dyes in acid solution. The Gram stain is an illustration; Gram positive bacteria tend to retain basic dye and Gram negative bacteria do not retain basic dye. Burke (5) found that "the addition of $NaHCO_3$ results in a greater concentration of methyl violet being present in the Gram positive organism after decolorization, and lactic acid causes the opposite effect." Kopeloff and Beerman (33) suggested adding $NaHCO_3$ to the primary (Gram) stain to neutralize acidity and to intensify the stain in Gram positive organisms. They advocated using an iodine solution to which NaOH had been added since "the free hydroxyl ion may aid in intensifying the stain." Atkins (2) found that the addition of aniline sulphate to gentian violet solution, and of NaOH to iodine solution, retarded decolorization of Gram positive organisms.

In consideration of these chemical changes which may be effected in the Gram character, Stearn and Stearn (41, 44, 46, 47, 48) have undertaken an exhaustive study in which they arrive at a chemical theory to explain the fundamental mechanism of bacteriostasis. These authors have attacked the problem of bacteriostasis from the physico-chemical point of view. The inhibition of bacteria by basic substances such as gentian violet, crystal violet,

and basic fuchsin, takes place to a greater extent in alkaline media than in acid media. The writer found his results to be in agreement with the chemical theory of bacteriostasis. Acid fuchsin had relatively no bacteriostatic effect in the alkaline media which was used in the writer's experiments. Beckwith (3) (1921) reported similar results. The dilutions of basic dyes which were necessary for bacteriostatic action were more concentrated in the writer's experiments than in experiments reported by Churchman (7), and Kligler (32). The medium used by the writer contained ten grams of gelatin per liter, a strong concentration considering the other ingredients of the medium employed. Graham-Smith (25), showed that the presence of gelatin, whose isoelectric point is at a pH of 4.7 and whose properties thus would be acidic in neutral solution, tends to weaken the bacteriostatic effect of basic dyes.

A more detailed explanation of the chemical theory is necessary so that the implications of the writer's and Ingraham's (30) work may be better understood. The writer can think of no better way to express the succinct ideas of the chemical theory than to quote directly from Stearn and Stearn (44).

"Simon and Wood (40) have come the nearest to what we believe to be the chemistry of bacteriostasis. For the action of basic dyes they say, 'The most plausible inference would by to assume the existence of corresponding acid groups in the structure of the organism with which basic groups would tend to unite.' "

"The amino acid composition of proteins and allied

amphoteric substances furnishes just such acid groups. If we think of the protein molecule as having the property of an amino-acid and write a type formula, as Loeb (35) does

$$R\begin{matrix}NH_2\\ \\ COOH\end{matrix}$$

we have, in the language of Simon and Wood (39,40), receptors for either acidic or basic substances.

"On the alkaline side of the isoelectric point the reaction with the basic dye would be represented thus:

$$R\begin{matrix}NH_2\\ COOH\end{matrix} \text{ plus } \underset{(\text{basic dye})}{DOH} \text{ yields } H_2O \text{ plus } R\begin{matrix}NH_2\\ COOD\end{matrix}$$

"The dye molecule is only very difficultly destroyed so that the organism finds itself unable to liberate that portion of the dye which it might utilize in its own metabolism, and by this liberation free again this "receptor" or point of attack for basic nitrogenous nutrient material. This would bring about starvation of the organism, not necessarily to the extent of actually killing it at once, but in the sense of inhibiting growth and multiplication.

"On the acid side of the isoelectric point it might be expected that acid dyes should be just as effective as are basic dyes on the alkaline side. For a simple protein they might be, but the authors have pointed out that staining characteristics of bacteria indicate an isoelectric range between the isoelectric points of bacterial protein and bacterial lipoid, through which the protein would act as a base and the lipoid as an acid.

"Basic dyes find their task very simple, for a solution on the alkaline side of the isoelectric point of bacterial protein is also on the same side of the isoelectric point of the much more strongly acidic lipoid, and thus there is no interference with action. Throughout the isoelectric range, however, the lipoid is working against an acid dye and even though the reaction is favorable for dye-protein combination it is also favorable for lipoid-protein combination into a conjugate protein, and we have both lipoid and acid competing for the protein. These two reactions which tend to take place simultaneously may be represented:

$$R\begin{matrix}NH_2\\COOH\end{matrix} \text{ plus } (\underset{\text{acid dye}}{HD}) \text{ yields } R\begin{matrix}NH_3D\\COOH\end{matrix}$$

(Analagous to the reaction:

$$NH_3 \text{ plus } HCl \text{ yields } NH_4Cl)$$

$$R\begin{matrix}NH_2\\COOH\end{matrix} \text{ plus } \underset{\text{(lipoid)}}{HLi} \text{ yields } R\begin{matrix}NH_3Li\\COOH\\\text{(conjugate protein)}\end{matrix}$$

and a very much greater quantity of acid dye would have to be used for the same inhibitive effect than of basic dye under the former set of conditions.

"For acid dyes to have an effect commensurate with the well known effectiveness of basic dyes when used properly, it would be necessary to lower the pH to a value below the isoelectric point not only of the protein but also of the lipoid. In general this will throw the reaction over to an acidity so great that inhibition of

growth will take place without the addition of dye at all, so that the practical application of high dilutions of acid dyes seems limited."

The writer's experiments with acidity production were conducted before the publication of Ingraham's (30) work on oxidation-reduction potentials. The writer, to repeat, found that those organisms which were capable of producing a definite acidity in brom cresol purple milk and in litmus milk were also relatively tolerant to dyes. A statement that a microorganism is capable of producing quantities of acid is equivalent to saying that the organism has reduced the oxidation-reduction potential or poised the potential at a lower level. This becomes evident when the method for deriving pH is calculated from the observed potential as expressed in terms of volts. Ingraham's (30) results showed that in those cases where a bacterial form is capable of poising the oxidation-reduction potential at a reduced level, the organism is also dye-tolerant. She shows, further, that dye is toxic in many cases only during the lag period when the bacterial cells are adjusting the oxidation-reduction potential and pH to a favorable level. The writer agrees with this statement.

The writer's results seen as a whole are consistent with other work in the field of bacteriostasis, and in consequence the physical theory of bacteriostasis is given little credence. On the contrary, the chemical

theory of Stearn and Stearn (41, 42, 43, 44, 45, 46, 47, 48) is given support. The work of Ingraham (30) which does not conflict with that of Stearn and Stearn, but rather extends their work to a greater horizon, is also supported and confirmed by the writer's results as far as they go. The implications for future work in bacteriostasis the writer considers to be in the field of physical chemistry where the large and fundamental answers will probably be found.

Summary

1. The bacteriostatic effects of gentian violet, crystal violet, basic fuchsin, and acid fuchsin in various dilutions were studied on a group of Gram positive cocci.

2. Of the dyes employed basic fuchsin exerted the most pronounced bacteriostatic effect on all of the Gram positive organisms; crystal violet and gentian violet, with respect to their bacteriostatic potency, followed in the descending order named. Acid fuchsin was almost without bacteriostatic effect on the bacteria studied. This was due, no doubt, to the alkaline reaction of the medium used. The acid character of the dye, according to other investigators, requires a definitely acid substrate for effective bacteriostatic action.

3. The bacteriostatic potency of a dye depends on its chemical purity and dye content. The basic fuchsin employed contained 93 per cent of dye; crystal violet, 85 per cent of dye; and gentian violet, 75 per cent of dye. In addition gentian violet is not a pure compound.

4. Streptococci were more tolerant than the other Gram positive cocci to the dyes employed.

5. The acid-producing powers of the various bacteria were studied in litmus milk and in brom cresol purple milk. Those organisms, like the streptococci, which are capable of producing appreciable quantities of acid were relatively more tolerant to the dyes than the organisms which produced less acid or none at all.

6. The results indicated in the preceding paragraph agree with those of Ingraham (30) concerning the reduced oxidation-reduction potentials effected by dye-tolerant

organisms, and also tend to support the chemical theory of bacteriostasis as developed by Stearn and Stearn (41 to 43 inclusive.).

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We, the undersigned, members of Mr. Rogosa's thesis Committee, approve this thesis.

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