THE CHEMICAL MECHANISM OF BACTERIAL BEHAVIOR

I. BEHAVIOR TOWARD DYES—FACTORS CONTROLLING THE GRAM REACTION[1]

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I. INTRODUCTION

In a recent number of this journal (Stearn and Stearn, 1923) the authors presented some experimental evidence which indicated that the behavior of bacteria toward dyes could be explained on the assumption that it was largely determined by the properties of the bacterial protein, and that this latter is an ampholyte combining with acid dyes on the acid side of its isoelectric point and with basic dyes on the alkaline side. It was suggested at that time that an interpretation of the much discussed Gram reaction might be derived from similar considerations. In the present series of papers the authors are not interested in suggesting any modification of technique in carrying out the staining. Their object is to present data tending to correlate the Gram reaction with the general behavior of bacteria toward dyes, bacteriostasis due to dyes, the significance of the many modifications of technique suggested from time to time, and the fact that in spite of all these improvements over original methods there are yet certain cases of non-specificity toward Gram reaction especially with older cultures.

The clinical importance of this reaction has been well established. This importance can, we believe, be enhanced by technicians in whom, coupled with carefulness in execution, there is an intelligent view of the important factors influencing the reaction and their mutual relationships.

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II

A survey of some of the anomalies discovered now and then in the use of the Gram stain for diagnostic work may be of a great deal of importance especially where these cannot be explained as due to poor stains, old mordant or faulty technique in preparation of slides, and for which explanation must be sought among other uncontrolled factors.

In certain chronic cases Gram-negative organisms such as the gonococcus may hold the primary stain to such an extent that they appear Gram-positive despite the fact that culturally they act as true Gram-negative gonococci (Webster, 1920). Some of the Gram positive organisms when taken up by leucocytes seem to lose their staining characteristics and become Gram-negative. "The bacillus of Friedlander, the diphtheria bacillus, and the *Diplococcus intracellularis* are somewhat variable in their behavior toward Gram's stain, and may or may not decolorize" (Wood, 1909).

According to Czalewski (1896) anomalies arise due to the frequent use of old cultures. Grimme (1902) states that diphtheria bacilli are Gram-positive only in young cultures. In old cultures they may be weakly positive or not stained at all. Mills (1892) found that the Gram character varied somewhat with the composition of the nutrient material on which the organism is grown. Bacilli were found to be Gram-positive when taken from pus but negative when taken from culture media. Schmidt (1892) noticed that *B. coli-communis* and *B. aerogenes* from intestines rich in fat were Gram-positive, while those from intestines low in fat content were Gram-negative. The former remained Gram-positive when cultured.

Wilde (1896) isolated an organism from mice which was definitely Gram-positive but became negative when cultivated. Aronson (1897) found that in young cultures certain Gram-positive organisms acted Gram negative and he attributed this to incomplete fat formation. Burke (1922) has suggested that loss of positiveness in old cultures may be due in part to the formation of acids.

Earlier literature contains many such examples. Later work shows fewer, which may be due to the fact that the source, the habitat, the time of incubation, the nutrient medium of the organism are factors all of which are now well controlled in most laboratories to a greater extent than formerly, and the more uniform methods employed may cover factors previously uncontrolled.

III. EXPERIMENTAL ATTEMPTS AT REVERSING GRAM CHARACTER

Nikitin (1908) states that by rubbing strong acids or alkalies into Gram negative organisms they become positive. Grimme refuted this. Cedercreutz (1908) found that some Gram-negative organisms became positive when treated with fat, starch or egg white. Digestive ferments changed positive organisms to negative. Prolonged treatment with dye, iodine and heat (Neide, 1904) caused Gram-negative organisms to act as positive ones. Eisenberg showed that degenerated cultures which had been Gram-positive in the organ but reacted Gram-negatively could have their original Gram character restored by this method. Heating (Kantorowicz, 1909) or spontaneous degeneration, or the influence of changes in the animal organs (Eisenberg, 1912), cause certain organisms to lose their Gram-positiveness.

IV. PREVIOUS WORK ON THE RELATION OF ACIDITY TO GRAM CHARACTER

Burke (1922) states 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. The failure of Gram-positive organisms from old cultures or from the genital urinary tract to retain the violet dye may be due in part to the presence of certain acids."

Kopeloff and Beerman (1922) advise adding $NaHCO_3$ to the primary stain to neutralize acidity and to improve the intensity of the stain in Gram-positive organisms. They advocate using

an iodine solution to which has been added NaOH since "the free hydroxyl ion may aid in intensifying the stain."

According to Sheppe and Constable (1923) Gram-positive organisms do not retain the primary stain when mordanted with Lugol's iodine solution which has become acid (through light or heat). They found that when $NaHCO_3$ was added to such Lugol's solution normal Gram characters were restored.

Atkins (1920) states that addition of aniline sulphate to gentian violet solution, and of NaOH to iodine, retard decolorization of Gram-positive organisms.

V. EXPERIMENTAL

The experimental data presented fall under two heads: (*A*) The rôle of pH in determining the Gram character of an organism, and (*B*) the function of mordants.

For determining the Gram character of an organism the following modifications of the classic technique were adopted as giving most consistent results: (1) carbol gentian violet was used as primary stain; (2) Lugol's iodine solution was used as mordant; (3) acetone was used as decolorizer. Inasmuch as pure cultures were employed no counterstain was used.

A. The rôle of pH in determining the Gram character of an organism

Table 1 gives data obtained by staining three kinds each of organisms classified as Gram-positive and Gram-negative. The smears were stained two minutes at varying values of pH, mordanted two minutes with the solutions indicated, and decolorized with acetone. No counterstain was used. To insure uniform treatment smears of *B. diphtheriae*, *Bact. typhosum*, *Bact. coli* and *Bact. aerogenes* were made on the same slide. *Streptococcus pyogenes* and *Staphylococcus pyogenes-aureus* were run subsequently.

The significant fact brought out by these data is that any of the organisms, regardless of their nominal Gram character, can be rendered positive to either the basic dye gentian violet or the acid dye acid fuchsin, or negative to either one. The presence or absence of the iodine does not seem to affect the general behavior greatly, as will be brought out more clearly later.

In accordance with the idea that bacteria behave as ampholytes, just as Loeb has shown proteins to behave, all of these organisms in acid solution tend to retain acid dyes. The nominal Gram character is seen to make no difference. In alkaline solution all of these organisms tend to retain the basic gentian violet.

TABLE 1

MORDANTS	GRAM-POSITIVE ORGANISMS			GRAM-NEGATIVE ORGANISMS		
	Streptococcus	Staphylococcus	*B. diphtheriae*	*Bact. coli*	*Bact. aerogenes*	*Bact. typhosum*
Carbol gentian violet used as stain						
HCl, pH = 0.3	neg.	neg.	neg.	neg.	neg.	neg.
HCl plus Lugol's solution, pH = 0.3	pos.	pos.	neg.	neg.	neg.	neg.
HAc, pH = 2.4	pos. (pale)	pos. (pale)	neg.	neg.	neg.	neg.
HAc plus Lugol's solution, pH = 2.4	pos.	pos.	neg.	neg.	neg.	neg.
$NaHCO_3$ plus Lugol's solution, pH = 10.2	pos.	pos.	pos.	pos.	pos.	pos.
NaOH plus Lugol's solution, pH = 11.2	pos.	pos.	pos.	pos.	pos.	pos.
NaOH, pH greater than 11.	pos.	pos.	pos.	pos.	pos.	pos.
Carbol acid fuchsin used as stain						
HCl, pH = 0.3	pos. (pale)	pos. (pale)	pos.	pos.	pos.	pos.
HCl plus Lugol's solution, pH = 0.3	pos. (pale)	pos. (pale)	pos.	pos.	pos.	pos.
HAc, pH = 2.4	pos.	pos.	pos.	pos.	pos.	pos.
HAc plus Lugol's solution, pH = 2.4	pos. (very pale)	pos. (very pale)	pos.	pos.	pos.	pos.
$NaHCO_3$ plus Lugol's solution, pH = 10.2	neg.	neg.	neg.	neg.	neg.	neg.
NaOH Lugol's solution, pH = 11.2	neg.	neg.	neg.	neg.	neg.	neg.
NaOH, pH greater than 11.	neg.	neg.	neg.	neg.	neg.	neg.

The above results were repeatedly confirmed. All the organisms gave a normal reaction when the usual method of Gram staining was employed.

The only direct experimental data recorded bearing on the above results are presented in a paper by Eisenberg (1912). He found that Gram-negative organisms had a greater tendency

to retain acid dyes, such as cyanosin, than did the Gram-positive ones. Though there was some overlapping, he suggested a possible new classification of cyanosin-positive and cyanosin-negative organisms.

These acid dyes he mordanted with "salts of an acid nature," as for example, alum, picric acid, phenol, salts of the rare heavy metals and even H_2SO_4 at a concentration as high as ten per cent. This latter mordant, besides rendering the stain more firmly fixed,

TABLE 2

Showing the effect of a mixture of carbol gentian violet and carbol acid fuchsin at varying values on pH on both Gram-positive and Gram-negative organism

Time of staining was three minutes. The decolorizer was acetone

pH OF DYE MIXTURE	APPEARANCE OF SMEAR					
	Gram-positive organisms			Gram-negative organisms		
	Streptococcus	Staphylococcus	*B. diphtheriae*	*Bact. typhosum*	*Bact. coli*	*Bact. aerogenes*
3.21	deep pink	deep pink	deep pink	deep pink	deep pink	deep pink
3.47	pale pink	pale pink	pale pink	pale pink	pale pink	pale pink
6.35	pale purplish	pale purplish	very pale purplish	decolorized	decolorized	decolorized
7.3	pale purple	pale purple	pale purple	decolorized	decolorized	decolorized
8.0			pale purple	pale lavender	pale lavender	pale lavender
9.0	pale purplish blue	pale purplish blue	pale purple	pale purple	pale purple	pale purple

The original dye mixture had a pH of 2.6 and in all cases stained all the organisms bright red.

tended to intensify the color, due possibly to sulphonation. These mordants, he states, do not play the same rôle as those ordinarily employed in the Gram reaction.

So far as we have been able to determine, no one has appreciated the significance of these results, and even from his own discussion it is obvious that he himself had little idea of their possible meaning.

In table 1 it will be seen that there is a large jump between the least acid and the least alkaline solutions, i.e., from a pH of 2.4

to one of 10.2. At a pH less than 2.4 all organisms retained the acid dye, and at a pH above 10.2 all retained the basic dye. It would seem that there should be a point, the isoelectric point, at which no dye is retained. Table 2 presents data for this intermediate range.

Slides were stained three minutes with a mixture of basic gentian violet and acid fuchsin buffered at varying values of pH, measured electrolytically. No mordant (other than buffering the dye) was used. The slides were then decolorized with acetone and examined. Confirmation of the facts brought out in table 1 is apparent. Even when both dyes are present the acid dye is retained in acid solution and the basic dye in alkaline solution. In no case was the color as intense as seen in the slides from which table 1 was constructed, due to the fact that each dye was diluted by half on mixing and again by half on adding an equal volume of buffer solution, so that the final effective dye concentration was only one fourth that used in preparing the slides for table 1.

At a pH of 3.47 retention of acid fuchsin is very slight, while, with the exception of the Staphylococcus and Streptococcus, the same is true for gentian violet at a pH of 8.0 or below. Between these limits, or more probably from about 3.8 to 7.5 for the Gram-negative organisms and within narrower limits for the others, there is a distinct range through which little if any dye is retained. This may be thought of for the present as an "isoelectric range." This range has much significance in the theory presented in the following paper and will be discussed there.

There is always a little equivocation in using dye mixtures since one is never exactly sure that the effect of one dye on the other may not have some influence on results. In the above experiments the range through which the organisms seemed completely decolorized might correspond to conditions in which neither dye combines with the protoplasm, or it might represent conditions in which both would tend to combine with it if they did not mutually combine with each other.

To study the behavior of bacteria a little more closely a series of buffer solutions was carefully made up and adjusted to varying values of pH. Instead of using a dye mixture the effect of each

dye was studied independently. The organisms chosen were *Staphylococcus pyogenes-aureus*, a typical strongly Gram-positive organism, *Bact. coli*, a typical Gram-negative one, and *B. diphtheriae*, which is generally listed as Gram-positive but which is known to vary, and which in table 2 is seen to behave in a manner which looks like a "middle course" between the behavior of the Staphylococcus and Streptococcus and of the other organisms.

Smears of all three organisms were made on the same slide and stained. By the clinical technique employed both the Staphylococcus and the *B. diphtheriae* were Gram-positive though the latter reacted rather weakly so. The *Bact. coli* was negative. Slides with the three organisms were then stained three minutes with solutions of carbol gentian violet in one series and with carbol acid fuchsin in another. They were then mordanted for three minutes with the buffer solutions at pH values varying by one unit. The results are indicated in figure 1. The broken lines indicate action toward acid fuchsin, the unbroken ones toward gentian violet. As abscissae are plotted the pH values of the buffering solutions while the ordinates represent intensity of color after decolorizing with acetone on an arbitrary comparative scale.

These curves, it seems to the authors, give a clue to the prime difference in behavior between Gram-positive and Gram-negative organisms. The Staphylococcus is seen to retain the basic dye from a pH of 3 up, while *Bact. coli* only begins to retain it to any extent at a pH of 8.0, and unmordanted *B. diphtheriae* shows but little greater tendency to do so.

Several points of interest are brought out by these curves. It has already been mentioned that Sheppe and Constable (1923) found that addition of hydriodic acid to a pH of about 3 caused typical Gram-positive organisms to react as amphophiles. A glance at the curve for Staphylococcus shows that it retains basic dye to just about this pH, but below this point it tends to become decolorized. The authors (Stearn and Stearn 1923) showed that cultures of *Bact. coli* in media at a pH above 8.0 tended to remove gentian violet from such media, while below this value of pH some of the dye remained in solution.

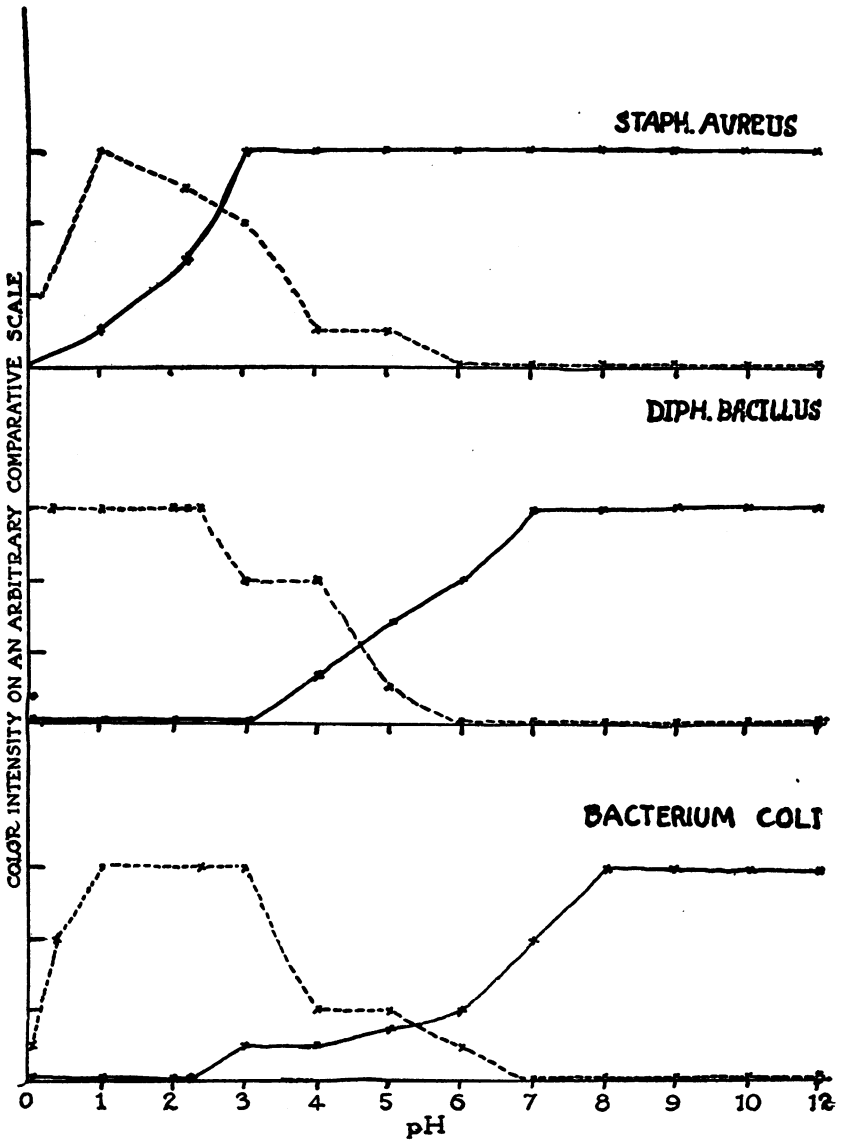

FIG. 1. SHOWING THE RETENTION OF ACID (BROKEN LINE) AND BASIC (UNBROKEN LINE) DYE AT VARYING VALUES OF pH

Discrepancies in measured isoelectric points may be explained at least in part by the behavior shown by these bacteria. The isoelectric point of a pure protein has a fairly definite value, but careful workers have not been able to agree on corresponding points for the same living cells to within as much as two or three pH units. The point found will generally depend on the method employed. In working with *B. diphtheriae*, for instance, these curves indicate that the dye method would point to a value of about 4.5. for the isoelectric point, while cataphoresis experiments would probably indicate a point near a pH of 3.0 The latter is probably a true isoelectric point; the former, however, is probably not. The significance of these points will be touched on in a following paper.

The data presented here have convinced the authors of the following points:

1. Gram-positive organisms can be rendered negative by increasing acidity.
2. The opposite effect can be produced by increasing alkalinity.
3. Acid dye positive organisms can be rendered negative by increasing alkalinity.
4. The opposite effect can be produced by increasing acidity.
5. There is a certain range—the isoelectric range—through which there is little tendency for any stain to be retained. This range is characteristic of each organism.
6. This range has, in general, wider pH limits in the case of Gram-negative organisms than in the case of Gram-positive organisms.

B. The function of mordants

A very significant fact regarding all the mordants which have been recommended for clinical Gram staining is that they are all mild oxidizing agents. Even though the formation of a dye-mordant precipitate has been stressed as the function of the mordant, it will be seen that the following list, which constitutes all of those ever seriously proposed, contains nothing but mild oxidizing agents. Besides bromine and iodine there have been proposed picric acid, trinitrocresol, trinitrobenzoic acid, trinitro-

resorcin, dinitrophenol, dinitrobenzene, trinitrotoluol, trinitroxylol, trinitraniline, orthometa, and para. Even when the iodine is dissolved in tenth normal NaOH as suggested by Atkins (1920), the resulting hypoiodite is still a mild oxidizing agent. In most cases the action of such agents must not be too strong, or the color may be bleached by the destruction of the dye.

Data presented below indicate that not only the above named substances, but practically any other mild oxidizing agents are

TABLE 3

Action of oxidizing agents as mordants

ORGANISM	$K_2Cr_2O_7$	$KMnO_4$	BLEACHING POWDER	NEUTRALIZED H_2O_2	BROMINE WATER	COMMERCIAL H_2O_2	LUGOL'S IODINE
Gram-positive organisms							
Streptococcus	strong pos.	pos.	pos.	pos.	pos.	weak pos.	pos.
Staphylococcus	strong pos.	pos.	pos.	pos.	pos.	weak pos.	pos.
B. diphtheriae	strong pos.	pos.	pos.	pos.	pos.	weak pos.	pos.
Gram-negative organisms							
Bact. coli	strong pos.	pos.	pos.	weak pos.	weak pos.	weak pos.	neg.
Bact. aerogenes	strong pos.	pos.	pos.	very pale	weak pos.	weak pos.	neg.
Bact. typhosum	strong pos.	pos.	pos.	very pale	weak pos.	weak pos.	neg.

very satisfactory as mordants in Gram staining. These data (table 3) were obtained by staining slides with carbol gentian-violet for two minutes, mordanting two minutes with the various oxidizing agents and decolorizing with acetone. No counterstain was used. Approximately N/50 solutions of the mordants were used as this is about the concentration of Lugol's iodine solution. The solutions are arranged in the table in the probable order of their chemical oxidizing potential, the stronger agents coming

first and the milder ones last. In no case was anything added to change the reaction of the aqueous solution except that of the neutralized hydrogen peroxide, which was neutralized with $NaHCO_3$. The other solutions would give for the most part a nearly neutral or slightly acid reaction, especially bromine water and the commercial hydrogen peroxide.

Potassium dichromate is by far the most effective mordant we have used. Its use is seen to render even the Gram-negative organisms positive, at least so far as retaining stain is concerned. This table further shows that the "general" behavior of both types of organisms is essentially the same. The milder oxidizing agents act selectively on the organisms which tend of themselves to be Gram amphophile, but a more vigorous action affects even such typical Gram-negative organisms as those above listed.

If the mordant action is an oxidation, its effect should be reversed by reducing action. That this is the case is indicated by the following experiment. It was found (table 1) that at a pH of 0.3 a certain culture of *Streptococcus pyogenes* was decolorized. Mordanting with Lugol's solution at this pH caused the Streptococcus to retain the stain. Three smears of this culture were stained and mordanted with Lugol's iodine adjusted to a pH of 0.3 with HCl. Slide 1 decolorized with acetone, retained the stain. Slide 2 was treated with a pure acid solution at a pH of 0.3, while no. 3 was treated for the same length of time (ten minutes) with a saturated solution of stannous chloride at this same pH. Slide 2 retained the stain while No. 3 readily gave up its deep color upon the addition of the first few drops of acetone and was completely decolorized. The stannous chloride, a typical and commonly used reducing agent had counteracted the mordanting effect of the iodine. That its action was not due to the dissolving of any dye-iodine precipitate is evident from the fact that stannous chloride itself precipitates gentian violet.

Thus the subsequent reduction had lessened the affinity of the protein for the basic dye, which affinity the previous oxidation with iodine had strengthened. Reducing agents, when allowed to act on protein which had not been previously oxidized, seemed to have little effect one way or the other on the affinity of the protein for dye.

The chemical effect of oxidation is in general to render the substance oxidized more acidic in character. Thus any affinity for a basic dye is increased. Figure 2 shows the effect of

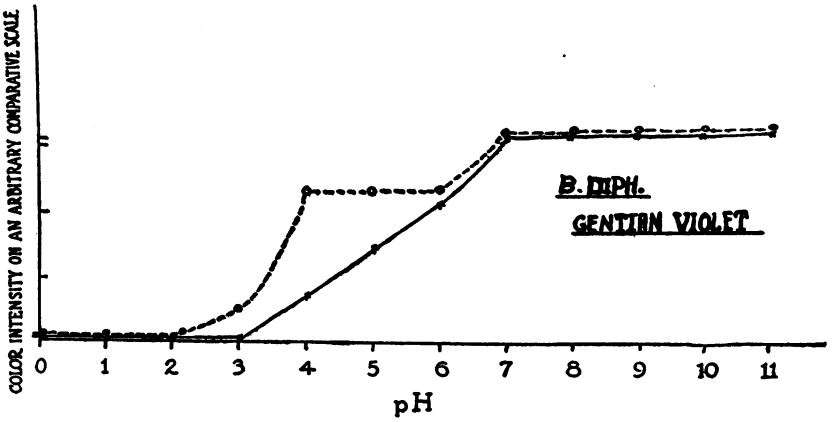

FIG. 2. SHOWING THE EFFECT OF MORDANTING ON THE RETENTION OF BASIC DYE BY AN ORGANISM OF VARIABLE GRAM CHARACTER AT DIFFERENT pH VALUES

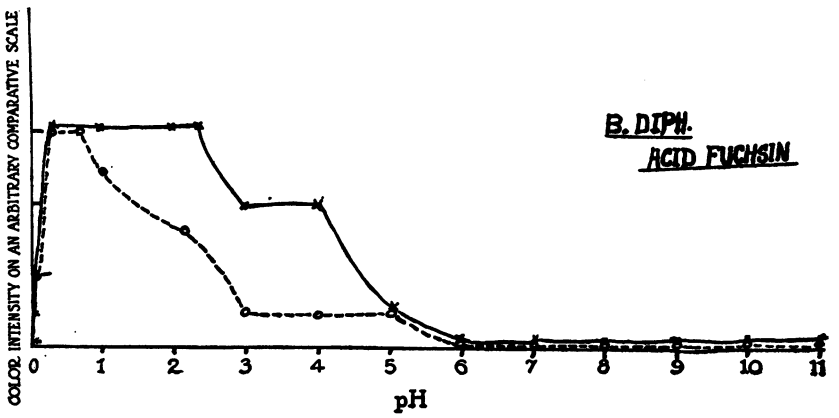

FIG. 3. SHOWING THE EFFECT OF MORDANTING ON THE RETENTION OF ACID DYE BY AN ORGANISM OF VARIABLE GRAM CHARACTER AT DIFFERENT pH VALUES

mordanting *B. diphtheriae*. The coordinates are the same as those in figure 1. The dotted line represents the mordanted, and the unbroken line the unmordanted, smears. The effect is to shift the isoelectric range toward the acid side of the curve.

Naturally the effect of such mordanting action on the affinity of protein for acid dye should be opposite, i.e., at any particular pH this affinity should be decreased. This is shown in figure 3 which also represents the behavior of *B. diphtheriae*. Superposition of figures 2 and 3 will bring out the shift in the isoelectric range mentioned above.

SUMMARY

The theory evolving from our experimental data and developed in the following paper explains the following points:

1. The decolorizing effect of acids on organisms stained with basic dyes.
2. The stabilizing effect of mild alkalies on the affinity of protein for basic dye.
3. The variability of the Gram character of certain bacteria.
4. The function of the pH.
5. Gradations of positiveness in both groups of organisms.
6. The known facts of bacteriostasis.
7. The extension of the artificial variability of bacteria as to Gram character.
8. The somewhat greater affinity of all organisms, regardless of Gram character, for basic dye than for acid dye through the ordinary pH range.
9. The function of mordants in Gram staining.

The theory is also in accord with the newer ideas as to the mechanism of permeability, as well as with the known chemistry of proteins and conjugate proteins.

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