CONDITIONS AND REACTIONS DEFINING DYE BACTERIOSTASIS

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The continued study of bacterial cells from the point of view which assumes that they behave as conjugated proteins (Stearn and Stearn, 1924a and b) has led to an intensive study of the action of both basic and acid dyes in their bacteriostatic and staining reactions.

The use of buffers in bacteriological media is becoming more and more common, since it is generally conceded that bacteria have very decided pH limits of growth unless slow acclimatization beyond these limits has taken place. Little of the existing data on bacteriostatic agents has taken into account that the effective dilution of these agents varies greatly with the pH of the media, and since the pH fluctuates as growth proceeds it is absolutely necessary to have sufficient buffer present for its control.

Browning, Gulbransen and Kennaway (1920) found that the sterilizing effect of diamino-acridine methyl chloride is multiplied one hundred times by a change of pH from 4 to 11. There are, however, few known organisms which have natural pH limits of growth as wide as these authors suggest. In studying the pH effect on bacteriostatic action it is essential to remain well within the limits of growth, preferably within the optimum range of H-ion concentration.

I. J. Kligler (1918) in his study of bacteriostasis by dyes attempted to control the pH of the media by the addition of 0.5 per cent K_2HPO_4. This gives an initial pH of 7.1 and protects the media against formation of acids during growth, but has no buffering power against bases. Even then this is by no means

the mostly highly effective H-ion concentration for bacteriostatic action by basic dyes. According to Dernby (1921) there are organisms such as the staphylococcus, whose optimum H-ion concentration lies between the pH limits of 7.2 and 7.6. For such an organism our data here presented will show that basic dyes would be far more inhibitive at a pH of 7.6 than at 7.1—the former being incidentally the pH of human blood.

Traube (1912) found that Na_2CO_3 accentuates the toxicity of certain stains, notably crystal violet. Prowazek (1910) noted likewise that the addition of Na_2CO_3 increased the activity of methylene blue. Dernby and Davide (1923) state that eucupin and the quinine alkaloids are more effective against the staphylococcus and diphtheria bacilli in solutions which are more alkaline than the blood.

In the cases of acidic substances, Graham-Smith (1919) found quinine more effective in acid than in neutral or alkaline solutions. Davis and White (1918) found that acid chlor-mercury fluorescein was more active in acid solution.

Data showing to what extent it is important to determine the H-ion concentration at which a specific bacteriostat is most inhibitive for a specific organism are given below, which suggest answers to other relative and cogent questions. The pH range through which the effect of any dye was studied was not arbitrarily chosen but was the range through which the organism in question has been shown to grow. Except where otherwise stated these limits were obtained from Dernby (1921). In all cases the limits of the range were tested by growing the particular strains studied in 0.2 per cent lactose broth adjusted to the upper and lower limiting values of the particular range through which they were subsequently to be studied. The media were buffered by phosphate mixtures. A minimum total salt concentration of about one-fifteenth molar was required to hold the pH constant. This latter was checked both before and after incubation by the indicator method. Doubling the concentration of buffer material did not affect the results. In the cases where gentian violet was used a 0.2 per cent lactose broth was generally employed. It was found, however, that identical results were obtained when a 1 per cent glucose broth was used in its stead.

A. BASIC DYES

As Simon and Wood (1914) have suggested, we may assume that in the structure of the bacterial organism we find receptors for either acidic or basic substances. This idea might be represented by a type formula $R\frac{NH_2}{COOH}$, the acid group acting as receptor for a basic substance and vice versa; or it might be more simply represented by the formula HPrOH.

The present authors (1923) found that at a high pH range, from about 7.6 to 8.5, bacteria as they developed in gentian violet media would settle to the bottom as a deep purple precipitate, decolorizing the solution, whereas at a lower pH range, from 4.3 to 5, the bacteria which settled were only slightly stained and the solution remained a deep purple.

The following series of equilibria may be thought of as establishing themselves in a system of bacteria and dye. We will represent any basic dye by the formula DOH.

(1) $DOH \leftharpoondown D^+ + OH^-$

(2) $HPrOH \rightharpoonup H^+ + PrOH^-$

(3) $D^+ + PrOH^- \rightleftharpoons DPrOH$

(4) $DPrOH + H_2O \leftharpoondown HPrOH + D^+ + OH^-$

Maximum bacteriostatic action occurs with maximum formation of the un-ionized dye-protein compound, DPrOH. A study of the above equilibria would lead, among others, to three predictions. Data bearing on each of these are presented below.

I. The long arrows in the above set of equations show the direction in which the equilibrium would shift for an increase in alkalinity. This direction is obvious for equations 1 and 2, and it is clear that the effect of hydroxyl ion concentration on number 3 is only indirect. Equation 4, the hydrolysis of the dye-protein compound, takes account of the fact that the dye will be much stronger as a base than the bacterial cell protein is as an acid, and thus for comparison of effect the dye is represented as ionized compared to the protein. Increase in alkalinity would aid, directly or indirectly, the formation of DPrOH, and thus

augment the bacteriostatic effect of a basic dye, in the equilibria represented by equations 2 and 4. It would work against the formation of DPrOH in equation 1. Since, however, the dye will be much more strongly ionized than the protein the effect of an increase in alkalinity on number 1 will be much less than on number 2,[1] and we should expect an increase in alkalinity to increase the effective dilution of the basic dye.

TABLE 1

BACILLUS DYSENTERIAE (SHIGA) LIMITING pH RANGE 6.2 TO 7.6					BACILLUS TYPHOSUS LIMITING pH RANGE 5.4 TO 9.1				
Gentian violet dilution	pH				Gentian violet dilution	pH			
	5.58	6.23	7.16	7.73		6.23	6.81	7.16	7.73
45,000	−	−	−	−	150,000	−	−	−	−
50,000	+	−	−	−	200,000	+	+	−	−
60,000	+	−	−	−	300,000	+	+	+	−
100,000	+	+	−	−	500,000	+	+	+	+
200,000	+	+	+	+	No dye	+	+	+	+
BACILLUS COLI LIMITING pH RANGE 4.4 TO 7.8					BACILLUS AEROGENES OPTIMUM pH 6.5*				
Gentian violet dilution	pH				Gentian violet dilution	pH			
	5.28	6.23	7.16	7.73		5.2	6.23	7.1	7.7
10,000	−	−	−	−	5,000	−	−	−	−
20,000	+	−	−	−	10,000	+	−	−	−
30,000	+	+	−	−	30,000	+	+	−	−
70,000	+	+	−	−	50,000	+	+	+	−
100,000	+	+	+	−	100,000	+	+	+	+
200,000	+	+	+	+					

* Smith (1922).

Table 1 gives the results of experiments with gentian violet on some Gram-negative organisms. Twenty-four hour agar

[1] An example will make this clear. If we take two weakly ionized substances, one weaker than the other, say with ionization constants 10^{-6} and 10^{-9}, and calculate the effect of a change of pH from 4.0 to 8.0, we find, assuming for simplicity that they are both bases, that the ion concentration of the stronger is decreased by 10 ten times while that of the latter is decreased by a million times. I.e., the same pH change affects the weaker one 10,000 times as much as the stronger in the above case, which is fairly typical.

cultures were washed off by the addition of sterile nutrient broth. To insure the same amount of inoculation two drops of bacterial suspension were transferred by means of a sterile pipette to each tube of 0.2 per cent lactose broth containing definite amounts of dye and buffered with a phosphate mixture. The results are those obtained after seventy-two hours incubation.

Table 2, giving results for strongly Gram-positive organisms, while in accord with the well known fact that this group of organisms is in general more sensitive to basic dyes than the Gram-negative shows clearly that the behavior toward these dyes under varying conditions is perfectly analogous to that of the Gram-negative organisms. The culture of staphylococcus used was isolated from an infected knee and was found to grow

TABLE 2

STAPHYLOCOCCUS AUREUS LIMITING pH RANGE 5.6 TO 8.1					STREPTOCOCCUS HEMOLYTICUS LIMITING pH RANGE 5.5 TO 8.0					
Gentian violet dilution	pH				Gentian violet dilution	pH				
	6.4	7.0	7.2	7.6		6.4	7.1	7.3	7.7	8.0
2,000,000	−	−	−	−	4,000,000	+	−	−	−	−
3,000,000	+	−	−	−	5,000,000	+	+	+	−	−
4,000,000	+	+	+	−	6,000,000	+	+	+	+	−
6,000,000	+	+	+	+	8,000,000	+	+	+	+	+
					10,000,000	+	+	+	+	+

in a concentration of gentian violet 1:2,000,000 at a pH of 6.4 even though that was beyond its optimum limits of growth. The growth limits are given as 5.6 to 8.1 but the optimum lies between 7.2 and 7.6.

Here also the data represent the results of seventy-two hours incubation.

Table 3 gives results obtained with two other basic dyes brilliant green, which is more strongly basic than gentian violet, and para-rosaniline, which is less strongly basic than gentian violet. The organism studied was *Bacillus coli*. Data for both forty-eight and seventy-two hours incubation are included. The peculiar behavior of the brilliant green should be especially noted. Its behavior was normal up to a pH of about 7, beyond

which its color faded out. Up to the point of this change it exerts an extremely powerful bacteriostatic action, but beyond this point this power is largely lost. The point is rather sharp since at 6.8 to 6.9 perfectly normal action is encountered while at 7.16 peculiar results are obtained.

Another striking phenomenon, which, unfortunately, it is impossible to show in our tables, is the *comparative quantity* of growth. In general it may be said that in such cases as repre-

TABLE 3

BRILLIANT GREEN					PARA-ROSANILINE				
Dye dilution	pH				Dye dilution	pH			
	4.95	5.28	6.46	7.16		5.28	6.23	7.16	7.73
50,000	−	−	−	−	5,000		(See note)		
	−	−	−	−					
75,000	−	−	−	−	7,500	+	+	−	−
	+	−	−	+		+	+	−	−
150,000	+	+	−	−	10,000	+	+	−	−
	+	+	−	+		+	+	−	−
300,000	+	+	+	−	15,000	+	+	+	+
	+	+	+	+		+	+	+	+
600,000	+	+	+	+					
	+	+	+	+					

Note: At this concentration there was growth in all tubes as evidenced by slight precipitation. This was very slight at the lower values of pH gradually increasing to the higher.

sented in table 1 for those concentrations of dye where there is growth in all tubes at all H-ion concentrations studied, the amount of growth falls off as the alkalinity increases, as one might expect from the remainder of the table. These "gradations" in quantity of growth with the pH are almost as striking as the "gradations" in the limiting dilution of dye with the pH, and furnish an equally striking substantiation of the prediction as to the pH effect.

II. Reversibility. The equilibria represented in equations 1 to

4 above should be reversible. As already pointed out (Stearn and Stearn, 1924b) the bacteria are probably not killed by these dyes except perhaps at fairly high concentrations, but are merely fixed and rendered temporarily powerless to multiply. The following is the evidence that this prediction is true.

Tubes containing dye in which no growth appeared after seventy-two hours incubation were treated with a solution containing the same concentration of dye but so acidified that the final pH would be lower than that at which the original incubation took place, and would be one at which decided growth had been shown to take place. Invariably, after twenty-four hours incubation, growth took place, shown not only by clouding of the media but also by streaking on agar and microscopically

TABLE 4

pH OF ORIGINAL BROTH (NO GROWTH OCCURRED)	APPROXIMATE pH OF ADJUSTED BROTH (GROWTH AFTER TWENTY-FOUR HOURS)	DYE DILUTION	ORGANISM
7.3	6.0	50,000*	*B. coli*
8.0	6.0	100,000*	*B. coli*
7.0	6.0	10,000*	*B. aerogenes*
7.7	6.0	7,500†	*B. coli*

* Gentian violet.
† Para-rosaniline.

examining the growth. This guaranteed that the cloudiness of the broth was due to the original organisms themselves which had again manifested their reproducing power, and not to the presence of a contaminating form. Experiments were made with both gentian violet and para-rosaniline. Table 4 includes a few of the many experiments performed along this line. In the case of the adjusted broth the control of the pH is not quite as close as in the other cases.

III. Basic strength of dye. A third prediction one might make is that the greater the basic strength of the dye the more effective a bacteriostat it will be at any one pH. This is brought out in equation 1, for the stronger the dye the greater the concentration of dye ion D^+. While no accurate data on the ionization constants of the dyes used are available, the effect on the basic or

acidic strength of the substitution of various groups in various positions in the molecule is more or less definitely known, and thus comparative basic strengths can be determined. (For a somewhat more full discussion of this question applied to the triphenylmethane dyes, see Stearn and Stearn, 1924b.) Table 5 compares the limiting dilutions of the basic dyes used on *Bacillus coli* at the same pH. The order is that of decreasing basic strength. It should be pointed out that from consideration of their formulas there is probably little difference between the basic strength of gentian violet and methyl violet. They should be nearly the same. The former has in it one more methyl group and might be a shade the stronger.

TABLE 5

DYE	LIMITING DILUTION		
	pH 5.2	pH 6.2	pH 7.7
Brilliant green	200,000	250,000	Power destroyed
Gentian violet	15,000	25,000	150,000
Methyl violet	10,000	15,000	70,000
Para-rosaniline	5,000	5,000	10,000

B. ACID DYES

A set of equilibria, analogous to those given for basic dyes, could be given for acid dyes. From such a set the three analogous predictions might be made. Data bearing on these points are presented below. These data are not as extensive as in the case of basic dyes but the general behavior is indicated almost as conclusively. Perhaps the most important feature regarding these data, one which has been mentioned but which should be emphasized, is that in this work the authors have limited their range of study to conditions of solutions in which the organisms are known to thrive in the absence of dye, so that the data are less equivocal than much of the material at present available in this field.

1. Increase in alkalinity with acid dyes should have the

opposite effect to that with basic dyes. Table 6 gives results for eosin, acid violet 5B, and acid fuchsin. Using a buffered 0.2 per cent lactose broth, a loopful of *Bacillus coli* suspension was inoculated and incubated for seventy-two hours. In the more alkaline solutions the dyes are seen to be less effective.

TABLE 6

DILUTION	pH						
	5.28	6.23	7.16	7.73	4.95	6.23	7.15
				Acid fuchsin			
25	−			+			
50	−	−	+	+			
75	−	−	+	+			
100	−	+	+	+			
				Acid violet			
25					−		+
50					−	−	+
100					−	+	+
200					+	+	+
				Eosin			
25	−			+			
50	−	+	+	+			
75	−	+	+	+			
100	+	+	+	+			

TABLE 7

pH OF ORIGINAL BROTH (NO GROWTH OCCURRED)	pH OF ADJUSTED BROTH (GROWTH IN TWENTY-FOUR HOURS)	DYE DILUTION	ORGANISM
6.0	7.0	100*	*B. coli*
6.0	7.0	200*	*B. coli*

* Acid fuchsin.

II. Reversibility. As in the case of basic dyes, it was possible to take a tube in which no growth had occurred after seventy-two hours incubation, and, by adding alkaline dye solution, keeping the concentration of dye constant, to obtain vigorous growth in twenty-four hours. Table 7 is analogous to table 6.

III. Acidic strength of dye. Table 6 is arranged in the probable order of decreasing acidic strength of the dyes. A glance at these data will show the increase in bacteriostatic power with the acidic strength, keeping the pH constant.

C. EFFECT OF THE PREVIOUS ENVIRONMENT OF THE ORGANISM ON ITS SENSITIVITY TO DYES

The importance of this problem, from the point of view both of classification or identification and of resistance to bacteriostats and bacteriocides, is apparent. Below are presented the results of a few preliminary experiments in this direction. They will not be discussed at this time.

I. A twenty-four-hour growth of *Staphylococcus aureus* was stained for one minute with a sterilized solution of gentian violet by thoroughly mixing while still suspended on the agar slant. The stained organisms were then inoculated into nutrient broth, one tube at a pH of 6.2 and another at a pH of 7.7. After twenty-four hours incubation vigorous growth of the organism had developed in the broth at a pH of 6.2, but there was no growth whatsoever in the other tube.

II. For two weeks a culture of *Bacillus coli* grew in nutrient broth whose pH was kept at 6.2. Another culture was grown at a pH of 7.7. They were twice transferred to new media and kept at 37°. At the end of this period a loopful of the culture media was inoculated into methyl violet—0.2 per cent lactose broth. The results are included in table 8. Readings were made after twenty-four, forty-eight, and seventy-two hours. While the final readings were the same in the case of both cultures, the twenty-four-hour and even the forty-eight-hour readings were not. The culture which had been kept in the more alkaline medium at a pH of 7.7 is rendered temporarily more sensitive to the action of the methyl violet, and it is only after some time that it recovers its normal ability to multiply in the presence of the dye.

The results under "a" were from the culture kept for two weeks in a broth at a pH of 6.2, those under "b" were from the culture at 7.7 described above. The results under "c" came

from inoculations from a suspension prepared from an agar slant kept at a pH of about 7.0 for forty-eight hours.

TABLE 8

DILUTION OF METHYL VIOLET	pH											
	5.2			6.2			7.1			7.7		
	a	b	c	a	b	c	a	b	c	a	b	c
10,000	+	−	−	−	−	−	−	−	−	−	−	−
	+	+	−	−	−	−	−	−	−	−	−	−
	+	+	+	−	−	−	−	−	−	−	−	−
20,000	+	+	−	+	−	−	−	−	−	−	−	−
	+	+	+	+	−	−	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
30,000	+	+	+	+	+	+	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
	+	+	+	+	+	+	−	−	−	−	−	−
70,000	+	+	+	+	+	+	+	+	−	+	−	−
	+	+	+	+	+	+	+	+	−	+	+	−
	+	+	+	+	+	+	+	+	+	+	+	−
100,000	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
200,000	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+
	+	+	+	+	+	+	+	+	+	+	+	+

D. SUMMARY AND DISCUSSION

1. In defining the bacteriostatic strength of a dye it is necessary to state the pH at which it is to act.

2. A method of proving the reversibility of a dye bacteriostatic reaction has been shown. This indicates an added reason why it is essential to give repeated injections of gentian violet in its use intravenously.

3. For a series of organisms not too widely different in character, the same concentration of the same dye might easily be

given as the limiting inhibitive dilution merely by altering the reaction of the medium. This is brought out in table 9 using data obtained with gentian violet.

4. In several cases, with Gram positive organisms, unexpected results were obtained. For instance in certain series in which *Streptococcus hemolyticus*, isolated from the spinal fluid in a case of meningitis, was being studied, it was found that at a pH of 5.9 and in a concentration of gentian violet 1:2,000,000 growth occurred. Upon examination the culture proved to contain long chains of streptococci about half of which were Gram positive and the remainder distinctly and strongly Gram negative. Even after subculturing on agar chains of the cocci would exhibit this characteristic; i.e., a deeply stained blue coccus would be united to a deeply stained red one, etc. The growth

TABLE 9

ORGANISM	pH	
	Limiting dilution, 50,000	Limiting dilution, 100,000
Bacillus aerogenes	7.73	8 plus
Bacillus coli	7.16	7.73
Bacillus typhosus	6.81	7.16
Bacillus dysenteriae	Less than 5.0	Less than 5.0

in this particular tube seemed quite instructive and peculiar since no such thing had taken place at even higher dilutions of gentian violet and higher values of pH. Similar results were obtained with *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*. Growth of any of these distinctly Gram positive organisms in scattered tubes of large series we found meant the presence of strains of so-called mutants which might develop other unlooked for characteristics. The study of these mutating strains is now in progress, and with it a study of the effective cause which stimulated this pronounced and peculiar action.

In conclusion it is a pleasure to acknowledge the suggestions of Dr. B. F. Sturdivant, Director of the Laboratories of the Pasadena Hospital, whose continued interest has been a source of inspiration.

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