THE RELATION OF THE BACTERIOSTATIC ACTION OF CERTAIN DYES TO OXIDATION-REDUCTION PROCESSES.

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In a preceding paper (1) it has been suggested that the growth of certain facultative anaerobes is inhibited by the existence in the medium of substances with a high oxidation potential. Accurate studies on the critical potentials for different species of bacteria will require the use of media "poised" at definite values. Even then, the results will be significant only if it is demonstrated that there exists a condition of equilibrium between the different parts of the system under consideration.

Unfortunately, we have at our disposal only very few oxidation-reduction systems, the characteristics of which are known. Among these may be mentioned the color indicators of Clark. It seems that these indicators together with a few other systems, such as hemoglobin-methemoglobin, fumaric-succinic acid, hermidin, echinochrome and some bacterial pigments, might be used to "poise" the media at their own characteristic potentials. However, the use of this technique is complicated by the toxicity of some of the dyes for certain bacterial species. It appeared possible that, in some cases, this toxicity might be partly accounted for by the high oxidation potentials of the oxidized dyes. Were this hypothesis justified, it might give us a method of determining the critical potential of the bacterial species studied, by determining which of the dyes are toxic in the oxidized form, and not toxic in the reduced form.

The bactericidal and bacteriostatic actions of certain dyes on different groups of microorganisms has been known for a long time, but the mechanism of this action is still obscure; it is likely that the mechanism varies with each type of dyes and each group of organisms. An

extensive review of the subject has been recently presented by Sartorius (2), but without any special discussion of the fundamental principles involved.

Among the few facts which have been established in this respect may be mentioned the more or less complete parallelism between the bacteriostatic of triphenylmethanes and the Gram reaction (3). On the other hand, azine and azonium dyes, such as phenosafranine and Janus green are known to precipitate proteolytic enzymes (4) and to penetrate the living cell (5). A striking example of the differential toxicity of a dye for two closely related bacterial species has been described by Sherman and Albus (6). They showed that, while the growth of hemolytic streptococci of human and bovine origin is inhibited by the presence in the medium of even low concentrations of methylene blue, the cheese strains thrive in presence of much higher concentrations of the same dye. Later, Avery (7) found that the sample of methylene blue used contained Zn as impurity, and that Zn salts alone, in corresponding concentrations, would also inhibit the human and bovine strains. However, purified methylene blue retained the same property. Some time later, Brown (8) reported "that streptococci which were inhibited by methylene blue in the presence of oxygen grew well in media containing methylene blue which was reduced."

Burnet (9) studied the growth of certain organisms on acid fuchsin agar plates and found that the growth of "isolated" cells of *Staphylococcus aureus*, diphtheroid bacillus and Friedländer's bacillus was inhibited on such media. (Isolated cells were obtained by spreading a dilute suspension of the culture over the plate.) However, growth developed in the vicinity of colonies of *Staphylococcus aureus* obtained by local heavy inoculation. Burnet concluded from his experiments "that the fuchsin interferes directly with the production or utilization of peroxide by the organism. The presence of products of growth whose primary function is to destroy peroxide, protects isolated organisms against otherwise inhibiting concentrations of the dye. . . . . Fuchsin must act by rendering the organism sensitive to traces of peroxide produced in its own metabolism."

Although not dealing directly with the toxicity of dyes for bacterial cultures, the following three studies may be mentioned here.

W. M. Clark and associates (10) have indicated that the halogens lose the greater part of their toxicity when added under conditions which prevent the attainment of oxidation potentials positive to the indophenols. Voegtlin and collaborators (11), working with normal and cancer tissues, found that the "toxic action of dyes depends to some extent on their oxidizing power for reduced glutathione, and also on other features of their molecular structure" and demonstrated by toxicity tests "the existence of a biological antagonism between reduced glutathione and methylene blue." In the course of their studies on the intracellular oxidation-reduction potentials of *Amœba dubia*, Cohen, Chambers and

Reznikoff (12) observed that "the reductants of toxic oxidants were usually non-toxic" and that "the diazines were decidedly toxic."

The ultimate object of the following experiments was to find out whether indicators of oxidation-reduction can be used to determine the critical oxidation potential of the medium above which the growth of Pneumococcus and Streptococcus cannot take place. The problem which has been most directly considered here is an analysis of the mechanism by which such indicators exert a bacteriostatic action on these species.

TABLE I.

List of Dyes Used.*

Name of oxidant	E_0'(pH = 7.4) (in volts)	rH
2-Chloroindophenol (o-chlorophenol indophenol)	+0.233	21.8
Indophenol (phenol indophenol)	+0.228	21.6
2-Methyl indophenol (o-cresol indophenol)	+0.195	20.5
1-Naphthol-2-sulfonate indophenol	+0.123	18.1
Methylene blue	+0.011	14.4
Janus green (green → pink) (approx.)	−0.02	13
K_4 indigo tetrasulfonate	−0.046	12.5
K_3 indigo trisulfonate	−0.081	11.3
K_2 indigo disulfonate	−0.125	9.9
K indigo monosulfonate	−0.182	7.5
Janus green (pink ⇄ colorless) (approx.)	−0.26	5
Neutral red (approx.)	−0.31	3.7
Phenosafranine (approx.)	−0.525	−3.5
Litmus	?	?
Malachite green	?	?

* The indophenols, the methylene blue, and 3 of the indigoes were obtained from La Motte Chemical Company. Indigo trisulfonate was obtained through the courtesy of Dr. B. Cohen of the Hygiene Laboratory, Washington, D. C. The other indicators were dyes used in the laboratory for staining and cytological work.

EXPERIMENTAL.

Methods.

12 hour plain broth cultures of the following organisms were used in the test

Pneumococcus: smooth virulent: Type I (1/200/4)
Type II (D/39/43)
Type III (A/66/69)

Rough avirulent derived from Type I (1/193/R)
Type II (D/39/R)
Type III (M/3/R)
Streptococcus hæmolyticus: human strains: L, S/43, S/23/Glossy, S/23/Matt
bovine strains: C/64, C/67
cheese strains: P, M

Since, in all cases, the results have been the same for all the strains of the same type of organism, only the results dealing with representatives of each type will be given.

The experiments were carried out in meat infusion broth, prepared 1 to 2 weeks before use.

The dyes used may be found in Table I.

The behavior of these dyes in plain broth has been studied in a preceding paper. It may be well to review briefly some of these results.

All the dyes studied function as reversible systems of oxidation-reduction, with the exception of the green → pink reduction of Janus green which is irreversible. The rH of all but two of them are known. When added to plain broth in concentrations not exceeding the reduction capacity of the medium, all indicators positive to indigo disulfonate, *i.e.* with an rH higher than 10, are reduced, if the system is kept under vaseline seal. The time of reduction for equimolecular concentrations of the dyes increases progressively as the rH becomes more negative. Although no electrometric measurements are available for malachite green and litmus, the fact that they were not reduced by plain broth suggests that their rH is smaller than that of indigo disulfonate.

Many of the indicators, when added to broth, are rapidly decomposed under aerobic conditions, but remain stable under vaseline seal; the indophenols are especially unstable (particularly methyl indophenol, and phenol indophenol).

The dye solutions were autoclaved, except the solutions of indophenols which were prepared (with aseptic technique) and proved to be sterile.

Aerobic Growth of Pneumococcus and Hemolytic Streptococcus in the Presence of Oxidized Dyes.

Experiment 1.—To a series of test-tubes each containing 5 cc. of broth, sufficient amounts of the different dyes were added to give final concentrations of 0.001 M, 0.003 M, 0.0001 M. The tubes were inoculated with 0.1 to 0.01 cc. of

TABLE II.

Growth of Pneumococcus and Hemolytic Streptococci in the Presence of rH Indicators.

Indicator		Pneumococcus R strain derived from Type II (D/39/R)		*Streptococcus hæmolyticus.* Human strain. L		*Streptococcus hæmolyticus.* Cheese strain P	
		Inoculum cc.		Inoculum cc.		Inoculum cc.	
	M	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}
2-Chloroindophenol	0.001	−	−	−	−	+*	+†
	.0003	−	−	−	−	+	+
	.0001	+	−	−	−	+	+
Phenol indophenol	.001	−	−	−	−	+†	+†
	.0003	−	−	−	−	+	+
	.0001	+	−	−	−	+	+
2-Methyl indophenol	.001	Erratic results due to breaking down of the dye in the broth				+	+
	.0003					+	+
	.0001					+	+
2-Sulfonate-1-naphthol indophenol	.001	−	−	−	−	+	+
	.0003	−	−	+	−	+	+
	.0001	+	−	+	+	+	+
Methylene blue	.001	−	−	−	−	+	+
	.0003	−	−	−	−	+	+
	.0001	+	−	−	−	+	+
Janus green	.001	−	−	−	−	+	+
	.0003	−	−	−	−	−	−
	.0001	−	−	−	−	−	−
Indigo tetrasulfonate	.001	+	+	+	+	+	+
	.0003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Indigo trisulfonate	.001	+	+	+	+	+	+
	.0003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+

In this table + indicates that growth developed in 24 hours; − indicates no growth.

* The dyes are arranged in the order of the electromotive series (see Table I).

† The growth of the cheese strains of *Streptococcus hæmolyticus* was delayed in the presence of 2-chloroindophenol and phenol indophenol but finally developed in 36 hours.

TABLE II—*Concluded.*

		Pneumococcus R strain derived from Type II (D/39/R)		*Streptococcus hæmolyticus.* Human strain L		*Streptococcus hæmolyticus.* Cheese strain P	
		Inoculum cc.		Inoculum cc.		Inoculum cc.	
		10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}	10^{-2}
	M						
Indigo disulfonate	.001	+	+	+	+	+	+
	.0003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Indigo monosulfonate	.001	+	+	+	+	+	+
	.0003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Neutral red	.001	−	−	−	−	−	−
	.0003	−	−	−	−	+	−
	.0001	−	−	−	−	+	+
Phenosafranine	.001	−	−	−	−	−	−
	.0003	−	−	−	−	+	+
	.0001	−	−	−	−	+	+
Malachite green	.001	+	+	+	+	+	+
	.003	+	+	+	+	+	+
	.0001	+	+	+	+	+	+
Litmus	?	+	+	+	+	+	+

TABLE III.

Time Required for the Growth of Cheese Strain (P) of Hemolytic Streptococcus to Develop in the Presence of Indophenols.

Dye	Concentration	Inoculum cc.			
		10^{-3}	10^{-4}	10^{-5}	10^{-6}
	M	*hrs.*	*hrs.*	*hrs.*	*hrs.*
2-Chloroindophenol	0.001	28	48	48	48
Phenol indophenol	0.001	28	28	28	28
1-Naphthol-2-sulfonate indophenol	0.001	12	18	20	20

the various cultures and growth was recorded after 36 hours incubation at 37°C. Typical protocols are given in Table II.

The results were definite except in the case of the cheese strain of *Streptococcus hæmolyticus*, the growth of which was retarded in the presence of some of the indophenols but finally developed. The question was investigated further by using smaller inocula of this strain (Table III).

Judging from the color of the solution, a good deal of the 2-chloroindophenol was decomposed in 48 hours, so that it may be concluded that the growth of cheese strains of *Streptococcus hæmolyticus* is at least partially inhibited by 2-chloroindophenol, and also perhaps by phenol indophenol.

Summarizing the results of Tables II and III, it appears that the growth of Pneumococcus and of human strains of *Streptococcus hæmolyticus* is inhibited by all the dyes positive to indigo tetrasulfonate (*i.e.* with an rH higher than 12.5), and by neutral red and phenosafranine. The cheese strains of *Streptococcus hæmolyticus* are inhibited only and more or less completely by the most positive indophenols and by Janus green, neutral red and phenosafranine.

Toxic Action of Some of the Oxidized Dyes on Cultures of Streptococcus hæmolyticus.

It is probable that the effect of the addition of dyes to broth is manifold; it is known for instance, that bacteriostatic and bactericidal action of dyes do not always run parallel (3). Before attempting to find whether the bacteriostatic action of the oxidized forms of rH indicators is due in many cases to a "poising" of the medium at a high oxidation potential, it is necessary to eliminate from our tests those indicators which can be shown to exhibit a primary toxicity, not related to oxidation-reduction phenomena. While the object of Experiment 1 was to establish which of the dyes would prevent cell multiplication, Experiment 2 served to establish whether any of these dyes would be toxic for the cells. This was tested by adding dyes to a fully developed culture and studying their effect.

Experiment 2.—12 hour broth cultures of *Streptococcus hæmolyticus* were transferred to test-tubes in amounts of 2 cc. per tube; sufficient amounts of the

dyes were then added to give a final concentration of 0.005 M. After exposure of the cultures to the dyes for different lengths of time, subcultures were made on blood agar plate to determine the viability. The test was not performed with Pneumococcus because the cells of this organism autolyze too rapidly.

The results of Experiment 2 are given in Table IV.

These results indicate that, under the conditions of the experiment,

TABLE IV.

Toxic Action of rH Indicators on Streptococcus hæmolyticus.*

Dyes in 0.005 M concentration	*Streptococcus hæmolyticus*						
	Human strain					Cheese strain	
	Time of exposure (hrs.)					Time of exposure (hrs.)	
	2	4	7	10	28	10	28
2-Chloroindophenol	+	+	+	+	+	+	+
Phenol indophenol	+	+	+	+	+	+	+
1-Naphthol-2-sulfonate indophenol	+	+	+	+	+	+	+
Methylene blue	+	+	+	+	+	+	+
Janus green	−	−	−	−	−	±	−
Indigo tetrasulfonate	+	+	+	+	+	+	+
Indigo trisulfonate	+	+	+	+	+	+	+
Indigo disulfonate	+	+	+	+	+	+	+
Indigo monosulfonate	+	+	+	+	+	+	+
Neutral red	+	±	−	−	−	+	−
Phenosafranine	+	+	+	+	+	+	+
Litmus	+	+	+	+	+	+	+
Malachite green	+	+	+	+	+	+	+

* The dyes are arranged in the order of the electromotive series (see Table I).

− indicates no growth on blood agar, ± indicates poor growth on blood agar, + indicates good growth on blood agar.

Janus green, neutral red and phenosafranine exerted a definite toxic action on the cells of *Streptococcus hæmolyticus*. On the contrary, the cells remained alive much longer in the indophenols, methylene blue, the indigoes, malachite green and litmus. Further work was therefore limited to this latter group of dyes.

Growth of Pneumococcus and Hemolytic Streptococcus in the Presence of Reduced Indophenols.

If the bacteriostatic action of the oxidized indophenols and methylene blue is due to the fact that they "poise" the medium at a high oxidation potential, the reduced forms of these dyes should be inactive.

Experiment 3.—The bacteriostatic action of the reduced dyes was tested in the apparatus represented in Fig. 1. This apparatus is a slight modification of the one described in a previous article (1) Fig. 2, the tube through which the hydrogen enters the system being shorter in order to avoid bubbling gas through the culture.

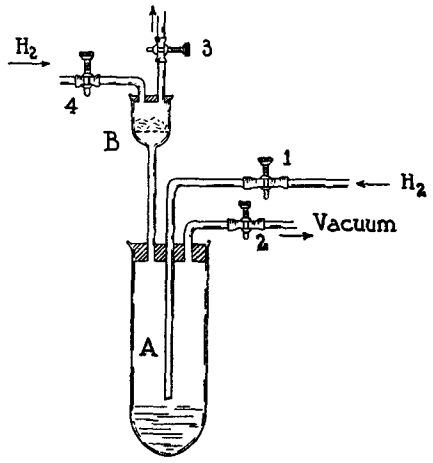

FIG. 1.

The experiment was carried out as follows:

10 cc. portions of recently boiled broth were transferred to each of 36 large tubes; the reduced dyes were added to 12 of them which were filled with the reduction apparatus (Fig. 1); 12 other tubes were treated with the oxidized dyes and the last 6 did not receive any treatment. The dyes (2-chloroindophenol and 1-naphthol-2-sulfonate indophenol) were used in concentrations of 0.0005 M. All the tubes had been inoculated before treatment. Growth was recorded after 24 hours incubation at 37°C. (Table V).

This experiment confirms the complete inhibition of growth of Pneumococcus and human strains of *Streptococcus hæmolyticus* by the oxidized indophenols and the partial inhibition of the cheese strains by the oxidized 2-chloroindophenol. On the contrary, all the cultures grew well in the presence of the reduced dyes.

The Effect of the Addition of Oxidized Dyes to Plain Broth on the Growth of Pneumococcus and Streptococcus hæmolyticus Incubated under Anaerobic Conditions.

It has been shown (13) that the indophenols and methylene blue are reduced by plain broth when the system is kept under vaseline seal. In consideration of the results of Experiments 3, it appears likely there-

fore that the bacteriostatic action of the dyes would be greatly decreased under vaseline seal.

It is also known that methylene blue is reduced in plain broth when the mixture is kept in anaerobic jars. As was to be expected, the indophenols are also reduced under such conditions.

Experiment 4.—The toxicity of methylene blue and indophenols was tested under vaseline seal and in anaerobic jars. In the course of the experiments, it was found preferable to use smaller concentrations of methylene blue than of indophenols for the two following reasons; (*a*) methylene blue is reduced only very slowly in high concentration, (*b*) methylene white being but little soluble precipitates out.

TABLE V.

Influence of the Addition of Oxidized and Reduced Indophenols on the Growth of Pneumococcus and Hemolytic Streptococci.

Dyes in 0.0005 M concentration	Pneumococcus		*Streptococcus hæmolyticus*			
	Inoculum cc.		Human strains		Cheese strains	
			Inoculum cc.		Inoculum cc.	
	10^{-1}	10^{-2}	10^{-1}	10^{-2}	10^{-1}*	10^{-2}*
2-Chloroindophenol, oxidized	−	−	−	−	+	−
2-Chloroindophenol, reduced	+	+	+	+	+	+
1-Naphthol-2-sulfonate indophenol, oxidized	−	−	−	−	+	−
1-Naphthol-2-sulfonate indophenol, reduced	+	+	+	+	+	+
Controls	+	+	+	+	+	+

* As already pointed out in Experiment 1, the bacteriostatic action of oxidized indophenols on cheese strains of *Streptococcus hæmolyticus* can be recognized only when very small inocula are used.

On the other hand, both the reduced and oxidized forms of the indophenols are very soluble and the oxidized forms are reduced rapidly even in concentrations as high as 0.0005 M; furthermore, these dyes decompose rapidly under aerobic conditions in the presence of broth, and it is necessary to use high concentrations to obtain sharp results.

In this experiment, the indophenols and indigoes were used in 0.0005 M concentrations and methylene blue in 0.00006 M concentrations.

The broths used were 6, 3 and 9 days old, respectively. The cultures were Pneumococcus (D/39/R), *Streptococcus hæmolyticus*, human strain L and cheese strain P. The size of inocula used and the results are given in Tables VI, VII and VIII. The tubes marked "vaseline" were sealed with a 2 cm. layer of vaseline; the anaerobic jars were incubated for 48 hours before being opened.

The results of Tables VI, VII and VIII constitute a new demonstration of the bacteriostatic action of the oxidized forms of methylene blue and the indophenols on Pneumococcus and human strains of

TABLE VI.

Growth of Pneumococcus (D/39/R) in the Presence of Dyes, under Aerobic and Anaerobic Conditions.

Dye	Aerobic				Anaerobic											
					Vaseline seal						Jar					
	Inoculum cc.				Inoculum cc.						Inoculum cc.					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
2-Chloroindophenol 0.0005 M	−	−	−	−	+	+	+	+	−	−	+	+	+	−	−	−
1-Naphthol-2-sulfonate indophenol 0.0005 M.	−	−	−	−	+	+	+	−	−	−	+	+	−	−	−	−
Methylene blue 0.00006 M	−	−	−	−	+	+	−	−	−	−	+	+	+	+	−	−
Indigo tetrasulfonate 0.0005 M	+	+	−	−	+	+	+	+	+	−	+	+	+	−	−	−
Plain broth	+	+	−	−	+	+	+	+	−	−	+	+	+	−	−	−

+ indicates that growth developed, − indicates no growth developed.

TABLE VII.

Growth of Human Strains (L) of Streptococcus hæmolyticus in the Presence of Dyes, under Aerobic and Anaerobic Conditions.

Dye	Aerobic				Anaerobic											
					Vaseline seal						Jar					
	Inoculum cc.				Inoculum cc.						Inoculum cc.					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
2-Chloroindophenol 0.0005 M	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−
1-Naphthol-2-sulfonate indophenol 0.0005 M.	−	−	−	−	+	+	−	−	−	−	+	−	−	−	−	−
Methylene blue 0.00006 M	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−
Indigo tetrasulfonate 0.0005 M	+	+	+	−	+	+	+	+	+	+	+	+	+	+	−	−
Plain broth	+	+	+	−	+	+	+	+	+	−	+	+	+	+	−	−

+ indicates that growth developed, − indicates no growth developed.

Streptococcus hæmolyticus; as expected, the cheese strain of *Streptococcus hæmolyticus* was inhibited only by high concentrations of 2-chloroindophenol and when the inoculum was small. Under anaerobic

conditions (vaseline seal or anaerobic jar), methylene blue and the indophenols lost a great part of their bacteriostatic action. In the tubes kept under vaseline seal, it was possible to observe that, in the

TABLE VIII.

Growth of Cheese Strain (P) of Streptococcus hæmolyticus in the Presence of Dyes, under Aerobic and Anaerobic Conditions.

Dye	Aerobic					Anaerobic									
						Vaseline seal					Jar				
	Inoculum cc.					Inoculum cc.					Inoculum cc.				
	10^{-1}	10^{-2}	10^{-3}	10^{-5}	10^{-7}	10^{-1}	10^{-3}	10^{-5}	10^{-6}	10^{-7}	10^{-1}	10^{-2}	10^{-3}	10^{-5}	10^{-7}
2-Chloroindophenol 0.001 M	−	−	−	−	−	+	+	+	+	+	+	+	+	+	−
1-Naphthol-2-sulfonate indophenol 0.001 M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Plain broth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+ indicates that growth developed, − indicates no growth developed.

TABLE IX.

Influence of 0.00002 M Methylene Blue on the Growth of Type II Pneumococcus (D/39/3) in "Boiled" and "Unboiled" Broth under Aerobic and Anaerobic Conditions.

Inoculum	Aerobic				Anaerobic Jar			
	"Unboiled" broth		"Boiled" broth		"Unboiled" broth		"Boiled" broth	
	No methylene blue	Methylene blue	No methylene blue	Methylene blue	No methylene blue	Methylene blue	No methylene blue	Methylene blue
cc.								
10^{-1}	+	+	+	+	+	+	+	+
10^{-2}	+	−	+	+	+	+	+	+
10^{-3}	−	−	+	−	−	+	+	+
10^{-4}	−	−	+	−	−	+	+	+
10^{-5}	−	−	+	−	−	+	+	+
10^{-6}	−	−	+	−	−	−	+	+

+ indicates that growth developed, − indicates no growth developed.

presence of these dyes, growth did not develop as long as the dye was not reduced. On the contrary, growth developed in the presence of the oxidized form of indigo tetrasulfonate.

The unexpected observation has been made that the initiation of growth of Pneumococcus is facilitated in the anaerobic jar when traces of methylene blue are added to the broth. The most characteristic example of such an effect is given in Table IX.

Experiment 5.—One half of the broth used in this experiment had been boiled immediately before inoculation. The "boiled" and "unboiled" samples were divided again into two series; one of them was inoculated as such, the other one only after addition of 0.00002 M methylene blue. The inoculated tubes were incubated either under aerobic conditions, or in an anaerobic jar. The observations were made after 36 hours incubation (Table IX).

Similar results have been obtained with 6 different strains of Pneumococcus. They confirm that methylene blue is not toxic, when reduced in the broth. In the anaerobic jar, growth developed only with 10^{-1} and 10^{-2} cc. inoculum in the plain "unboiled" broth, while it took place with as little as 10^{-5} cc. inoculum in the presence of 0.00002 M methylene blue. However, even such a minute concentration of the dye is bactericidal in the oxidized form, as appears from the results obtained under aerobic conditions. (This is especially striking in the case of "boiled" broth.) The growth of very small inocula in the presence of traces of methylene blue in the anaerobic jar must be due therefore to an indirect effect. It can be explained by assuming that methylene blue, being such an actively reversible system, does accelerate the reduction of the broth itself.

Comparative Growth of Human and Cheese Strains of Streptococcus hæmolyticus in Plain Broth.

It has been established that the growth of human strains of *Streptococcus hæmolyticus* is completely checked by the presence in the medium of the oxidized forms of methylene blue and the indophenols, while the growth of the cheese strains of *Streptococcus hæmolyticus* is inhibited only by the 2-chloroindophenol, the most positive of the indicators tested. On the other hand, it has been suggested elsewhere (1) that the growth of small inocula of human strains of *Streptococcus hæmolyticus* may be inhibited by the presence of oxidized substances in plain broth. It was interesting to compare the growth of small inocula of human and cheese strains of *Streptococcus hæmolyticus* in this medium.

Experiment 6.—Varying amounts of 12 hour cultures of human (L) and cheese (P) strains of *Streptococcus hæmolyticus* were seeded into test-tubes containing 5 cc. of plain broth (1 week old). Growth was recorded after 24 hours (Table X).

The results of Table X indicate that, while a fairly large inoculum is necessary to insure the growth of human strains of *Streptococcus hæmolyticus* in plain broth, the cheese strains develop in the same medium following inoculation of a very few cells only.

DISCUSSION.

Janus green, neutral red and phenosafranine form a special class among the indicators of oxidation-reduction potential used in this work.

TABLE X.

Growth of Human (L) and Cheese (P) Strains of Streptococcus hæmolyticus in Plain Broth.

Streptococcus hæmolyticus	Inoculum cc.						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Cheese strain P	+	+	+	+	+	+	+
Human strain L	+	+	−	−	−	−	−

+ indicates that growth developed, − indicates no growth developed.

They rapidly kill cultures of hemolytic streptococci to which they are added in sufficiently high concentrations. These cultures remain alive for a longer time when put in contact with the same concentrations of the other indicators. This toxic action of Janus green, neutral red and phenosafranine is not related to phenomena of oxidation-reduction. The discussion will therefore be limited to the other indicators.

The results concerning the bacteriostatic action of the indophenols, methylene blue, the indigoes, malachite green and litmus on Pneumococcus and *Streptococcus hæmolyticus* are summarized in Table XI.

In Table XI, the dyes are arranged in the order of their oxidation-reduction potentials (electromotive series). It is apparent that Pneumococcus and human strains of *Streptococcus hæmolyticus* are inhibited only by the indophenols and methylene blue; *i.e.* by the dyes having an rH higher than 12.5. The cheese strains of *Streptococcus*

hæmolyticus are inhibited by 2-chloroindophenol and perhaps phenol indophenol, but not by the others, indicating that their "bacteriostatic potential" is above rH 21.

The bacteriostatic action of the indophenols and methylene blue is completely or partly corrected when (*a*) they are added to the medium in the reduced form, (*b*) the mixture broth-dye is incubated under anaerobic conditions (the concentration of dyes being low enough to allow their reduction in a reasonable time).

TABLE XI.

Growth of Pneumococcus and Hemolytic Streptococci in the Presence of Different rH Indicators Used in the Oxidized Form under Aerobic Conditions.

Dye*	Pneumococcus	*Streptococcus hæmolyticus*	
		Human strain	Cheese strain
2-Chloroindophenol	−	−	+−
Phenolindophenol	−	−	+− (?)
1-Naphthol-2-sulfonate indophenol	−	−	+
Methylene blue	−	−	+
Indigo tetrasulfonate	+	+	+
Indigo trisulfonate	+	+	+
Indigo disulfonate	+	+	+
Indigo monosulfonate	+	+	+
Malachite green	+	+	+
Litmus	+	+	+

* The dyes are arranged in the order of the electromotive series (see Table I).
− indicates complete inhibition of growth, +− indicates partial inhibition of growth, + indicates normal growth.

It has already been suggested that, before an organism can multiply, the medium in which it is present must reach a critical reduction potential. It is attractive to suppose that, for Pneumococcus and human strains of hemolytic streptococcus, the critical potential is between indigo tetrasulfonate (rH = 12.5) and methylene blue (rH = 14.4) while it is somewhere below the chloroindophenol for the cheese strains of *Streptococcus hæmoyticus*. The corollary of such a view is that all the dyes or other substances with an oxidation potential higher than that of indigo tetrasulfonate would be bacteriostatic for Pneumococcus and human strains of hemolytic streptococcus, while

only the ones positive to phenol indophenol would have such as action on cheese strains of hemolytic streptococcus (provided that the dye is not primarily toxic and that conditions of equilibrium exist between the dye and the culture).

In terms of this hypothesis, the large inocula required for obtaining a growth of Pneumococcus and of human strains of *Streptococcus hæmolyticus* in plain broth, serve the purpose of reducing the broth to a potential (rH = 13) corresponding to reduced methylene blue. The cheese strains of *Streptococcus hæmolyticus* on the contrary grow with such a small inoculum because the potential of aerated broth is within the range in which these organisms can multiply. In fact, the oxidation potential of the broth is certainly not much higher than that of oxidized 2-chloroindophenol, since the dye remains reduced for a long time in broth that has been recently boiled, and aerated afterwards. In fact, 2-chloroindophenol is the only dye tested which exerts a definite bacteriostatic action, and even then, this action is only partial, indicating that we are just on the border of the critical potential.

The fact that the dyes are not toxic in the reduced form is of course in agreement with the hypothesis.

Let us now consider a few of the objections that may be made to this point of view. When the dyes are added in the reduced form, they remain reduced in the medium only if the broth is at an oxidation potential below, or equal to, that of the reduced dye. On the other hand, if oxidized indophenols or methylene blue are added to reduced broth, and the system kept under aerobic conditions to prevent reduction of the dye, it is likely that the broth will oxidize rapidly. Consequently, when we are dealing with oxidized or reduced dye, we are dealing at the same time with oxidized or reduced broth. As we know that the condition of the broth is of the greatest importance for initiation of growth (1), it is hard to know whether the dye is no longer toxic because present in the reduced form or only because the reduced broth is a more favorable medium. To this objection one can answer by pointing out that according to the hypothesis, both oxidized broth and the oxidized indicators under consideration are bacteriostatic, *not as broth or dye*, but by virtue of their *oxidizing intensity*. In both cases, fundamentally, the mechanism of action is the same, and a solution of the problem will be possible only when we have at our disposal media which do not function themselves as active systems of oxidation reduction.

Another weak point is that toxicity stops exactly with the indigoes. Is it not possible that toxicity is related, not to the oxidation-reduction characteristics of the dyes, but to their structure? A convincing proof would be to find an organism inhibited only by some of the indigoes, and not by the ones with a more negative rH. An interesting evidence of this sort is supplied by the fact that cheese strains of *Streptococcus hæmolyticus* are inhibited only by the most positive of the indophenols tested. In that respect, it would be important to test the action of other indophenols.

As they stand, our experiments do not supply any fact to contradict the hypothesis that methylene blue and oxidized indophenols are bacteriostatic because they "poise" the medium at an oxidation potential outside the range in which Pneumococcus and human and bovine strains of hemolytic streptococcus can grow. The problem of the mechanism whereby such a medium, poised at a high oxidation potential, becomes bacteriostatic for certain species, can be considered from at least two points of view. On the one hand, we may suppose that the presence of oxidized dyes in the medium results in such a change in the condition of some of the constitutents of the broth, that they are no longer available for growth. On the other hand, the action of the dye may be not on the broth, but on the bacterial cells themselves. We know for instance, that the pneumococcus cell contains a dual system of oxidation-reduction consisting of a thermolabile cellular constituent, and thermostable autoxidizable substances which can be removed by washing (14). By means of this system, the Pneumococcus cell is able to carry on a series of oxidations and reductions, the thermostable components becoming reversibly reduced or oxidized in the process, while, under aerobic conditions, the cellular component is inactivated by oxidation. It may be considered that the oxidized dyes bring the autoxidizable substances, or the thermolabile cellular constituent, to an oxidation potential where they are no longer adjusted to the metabolic activities of the cell.

Whatever its explanation may be, the fact remains that the indicators studied are much less toxic under anaerobic than under aerobic conditions. The significance of this observation for the use of dyes in therapeutics is evident since their action *"in vitro,"* under aerobic conditions, is little indication of what it will be in the presence of living tissues which are known to be active reducing agents. It is to be

expected that some dyes will be very effective when used on superficial wounds, but not when injected.

Undoubtedly, the mode of action of dyes is manifold, but the observations presented above may help in the analysis of this action, by pointing to one of the factors to be controlled.

SUMMARY.

Oxidized indophenols and methylene blue are bacteriostatic for Pneumococcus and hemolytic streptococci of human and bovine origin, while the indigoes, malachite green and litmus are not toxic.

2-Chloroindophenol, the most positive of the indicators of oxidation-reduction potentials used, is also the only one to have a bacteriostatic action on cheese strains of *Streptococcus hæmolyticus*.

Methylene blue and the indophenols are no longer bacteriostatic when present in a reduced form in a medium capable of maintaining them in such a condition.

A comparison of these results with the growth in plain broth of the organisms studied suggests that the "inhibiting" dyes "poise" the medium at an oxidation potential outside the range in which the inhibited organisms can grow.

The validity of this hypothesis is discussed.

The significance of these observations for the use of dyes in therapeutics is considered.

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