OBSERVATIONS ON THE OXIDATION-REDUCTION PROPERTIES OF STERILE BACTERIOLOGICAL MEDIA.

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INTRODUCTION.

Avery and Neill (1) have shown that the pneumococcus cell contains active oxidation-reduction systems. It is unnecessary to present again their conception of the nature of these systems, or the different oxidation-reduction processes exhibited by them. However, it is important to point out that the bacterial cell must not be considered as a mere catalyst in these reactions. On the contrary, when the cells-substratum system is isolated, there must exist a quantitative relationship between reduction (or oxidation) of the substratum, and oxidation (or reduction) of the cells. If we keep for instance a mixture of pneumococcus cells and methylene blue under anaerobic conditions, a reduction of the dye will be observed, the cells constituting the reducing agent, and some at least of the cell constituents becoming oxidized in the process. It is to be expected that the fundamental properties of the cells are affected by such oxidation.

As will appear from the following pages, plain broth itself contains an active oxidation-reduction system. The condition of this system probably influences the condition of the cell.

In the course of studies on the initiation of growth and on the death rate of certain facultative anaerobes, and the toxic action exerted on them by certain dyes and metal salts, it was recognized that these phenomena are often controlled by the conditions of oxidation-reduction prevailing in the medium. Some knowledge of the oxidation-reduction properties of sterile bacteriological media is necessary for an understanding of these biological happenings.

HISTORICAL.

That sterile bacteriological media are not inert from the point of view of oxidation-reduction processes is an old observation. The oxidation of sterile broth by atmospheric oxygen, especially under the influence of light, has attracted attention since the beginnings of bacteriology (2). As early as 1887, Spina (3) noted the reduction of methylene blue by sterile nutrient gelatin. In 1896, Smith (4) reported that, in the closed arm of fermentation tubes, methylene blue, indigo sulfonate and litmus could be reduced by sterile media. Methylene blue was most rapidly reduced, indigo sulfonate less quickly and litmus only when sugar had been added to the medium. The addition of glucose to the broth and the application of heat always accelerated the reduction. Aqueous solutions of glucose and peptone were found to be inert. The reduction of methylene blue by sterile media was also studied by Gates and Olitsky (5); they used it as a test for the removal of oxygen from the medium, following the addition of sterile tissue.

While there are found in the literature many studies of the speed of reduction of methylene blue by bacteriological systems, an analysis of the reduction phenomena in terms of "intensity" and "capacity" does not seem to have been made. The importance of these factors was not properly emphasized until the work of W. M. Clark. It may be useful to present briefly points he has outlined that bear directly upon the present work.

Oxidation is not necessarily a reaction characterized by the addition of oxygen to a molecule; the term applies also to loss of hydrogen; more essentially, oxidation consists in a loss of electrons, and reduction in a gain of electrons. This implies that oxidation of one substance necessarily involves the reduction of an equivalent amount of another, the former giving electrons to the latter. The tendency of a system to reduce another may be measured by its tendency to give up electrons to it. This tendency expresses itself in electrometric potentials and is used as a means to measure the factor intensity in processes of oxidation-reduction. Of two systems, the one which has the greater tendency to take up electrons has an electropotential positive to that of the other. Instead of measuring the potential of two reversible oxidation-reduction systems one against the other, it is more convenient to measure them separately against a standard (the standard hydrogen electrode). We therefore speak loosely of the potential of a system (as measured against the hydrogen electrode) as a numerical index of its oxidation-reduction intensity. In the case of a definite reversible system (such as methylene blue-methylene white), the potential of this system is a function of the ratio, reductant to oxidant $\left(\frac{\text{methylene white}}{\text{methylene blue}}\right)$, of the pH of the system, of the temperature, etc. Such factors should be defined in describing the system.

It is apparent that there are two means of measuring the intensity of oxidation-reduction of a system. One is a measurement of the electrode potential, the other a determination of the percentage reduction of certain dyes which are themselves reversible systems of oxidation-reduction. The reduction intensity is usually

expressed numerically in terms of potentials against the hydrogen electrode. Clark has also introduced the symbol rH which stands for the negative logarithm of the hypothetical hydrogen pressure in equilibrium with the system in question. Both terms may be used to define the position of the indicators on the scale. The E_0' of an indicator is the "potential" of a mixture of equimolecular amounts of oxidant and reductant of that indicator, at a definite pH; the rH also corresponds to a ratio $\left(\frac{\text{reductant}}{\text{oxidant}}\right) = 1$, but is independent of the pH.

The higher its E_0' (or its rH), the more rapidly an indicator is reduced; on the other hand, the reduced forms of the dyes having the smaller E_0' (or rH) are the more readily oxidized. Any system is an oxidizing agent in relation to another with a more negative "potential," and a reducing agent for the more positive systems.

It must be understood that the potential (E_0' or rH) is only a measure of *intensity*, but not of total *capacity* to oxidize or reduce. We have here the same relation as between the intensity factor (pH) and capacity factor (total acidity) in the description of the acid-base systems. Two systems of the same oxidation-reduction potential (intensity factor) may differ greatly in the amount of substance they are capable of oxidizing or reducing (capacity factor).

The experimental observations which follow are not presented as a quantitative study of the oxidation-reduction properties of plain broth, but only as an introduction to the problems of growth and dye toxicity which form the subject of subsequent papers.

EXPERIMENTAL.

Methods.

Of the two methods of determination of the oxidation-reduction potential of a system, the electrometric one is probably preferable as it gives results without the necessity of introducing a foreign factor (dye indicator) into the medium. The indicator being itself a system of oxidation-reduction acts to "poise"[1] the medium at its own special potential. We may even conceive of a medium so poorly "poised" that it will take automatically the potential of the indicator. Furthermore, the colorimetric reading of the percentage reduction of the dye, by which the potential is determined, is only approximative.

However, the colorimetric method has been adopted in preference to the electrometric one for the following reasons: (*a*) media containing the indicators in oxidized and reduced forms have been used in certain biological studies which will

[1] A solution is said to be "poised" when it tends to resist change in oxidation-reduction potential on addition of an oxidizing or reducing system. This effect is comparable to the buffer action found in the acid-base system.

be reported later; (*b*) the indicator method allows some measure of the amount of reducing substance present in the medium.

In Table I is given a list of the indicators used in these studies. Their potentials have been measured by Clark and his coworkers (7) except in the case of Janus green, neutral red and phenosafranine. The potentials of these three have been measured by Rapkine and Würmser (8) and the values are only approximative. The Janus green molecule corresponds to two systems of oxidation-reduction; the green form changes to pink at a potential of $E_0' = -0.02$ volt (pH = 7.4), rH = 13; the pink form changes to the colorless at $E_0' = 0.26$ volt (rH = 5). Only the latter system pink ⇄ colorless is reversible; for the pink form does not turn green even if oxygen is bubbled through it. Table I also contains two dyes (litmus and malachite green) for which there are no measurements available in the literature, although they constitute reversible systems of oxidation-reduction. Considering the difficulty with which they are reduced by bacterial cultures, it may be suspected that they are negative to methylene blue and to some, at least, of the indigoes.

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	13.0
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	5.0
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 Hygienic Laboratory, Washington D. C. The other indicators were dyes used in the laboratory for staining and cytological work.

The concentrations of the dyes have been measured in terms of molar concentrations. The solutions were prepared in distilled water shortly before use. They were all autoclaved, except the four indophenols which are heat-labile. The solutions of indophenols were prepared under aseptic conditions and proved to be sterile.

Most of the dyes were rapidly decomposed in plain broth, especially under aerobic condition. This factor introduced an element of error, due to a change in the relative concentrations of the different dyes and the effect of decomposition products. Most unstable were the indophenols, especially phenol indophenol and 2 methyl indophenol; this unstability, added to the weak tinctorial power of these two dyes, was responsible for discontinuance of their use. The color of the

TABLE II.

Time Required for the Reduction of Indicators at Concentrations of 0.0002 M by "Boiled" Plain Broth (pH 7.8) Kept under Vaseline Seal.

2–6 Chloroindophenol	2 hours	2 hours or less	Indigo monosulfonate	Not reduced after a month
Phenol indophenol	2 "		Janus green (pink⇄colorless)	
2-Methyl indophenol	2 "		Neutral red	
1-Naphthol-2-sulfonate indophenol	7 "		Phenosafranine	
Indigo tetrasulfonate	20 "		Litmus	
Methylene blue	52 "		Malachite green	
Janus green (green→pink)	72 "			
Indigo trisulfonate	110 "			
Indigo disulfonate*	288 "			

* The broth-indigo disulfonate mixture presented a very slight greenish tinge which did not disappear in the course of a month.

indophenols and indigoes rapidly faded in aerobic tubes of broth (in less than 12 hours). As the color could not be restored by the addition of potassium ferricyanide, it is apparent that this change in color was not due to reduction by the broth; it seems on the contrary that oxidation by air results in a breaking down of the molecule since the dye-broth mixtures kept under vaseline seal largely recovered their original color following addition of potassium ferricyanide. The sample of indigo monosulfonate partly precipitated out in plain broth, and the results obtained with such preparations may be open to question.

To prevent the access of air, the tubes were sealed with a 2 cm. layer of sterile vaseline.

Most of the experiments were carried out with a batch of meat infusion broth (pH 7.8) prepared 2 to 4 weeks before use. Fairchild's peptone (lot 280630) was used.

Reduction of Dyes by "Boiled" Broth.

As reported in the historical review, sterile plain broth is capable of reducing methylene blue when protected from the action of oxygen. The object of the first experiment was to establish whether other indicators of oxidation-reduction would be reduced, and if so, in what order.

Experiment 1.—Sterile plain broth (3 weeks old) was tubed in amounts of 5 cc. The tubes were boiled for half an hour to deaerate the broth, rapidly cooled down and enough of the dyes added to give a final concentration of 0.0002 M. The tubes were then sealed with vaseline and incubated at room temperature (25°C.). The time required for reduction in each instance is given in Table II.

A first conclusion to be drawn is that, under the conditions of the experiment, the reduction time of equimolecular concentrations of the different indicators decreased with the increase of the electrode potentials, thus confirming Voegtlin's conclusion that "the indicators if tested by biological methods arrange themselves in the same order as that obtained by means of the purely physical electrode measurements" (6). Especially remarkable is the fact that Janus green comes immediately after methylene blue, although its reduction (green → pink) is not a reversible process; this seems to offer some support to the theory of "apparent oxidation-reduction potentials" of irreversible systems, suggested by Conant (9).

Concerning the order of reduction of the different dyes, the only anomaly is that of indigo tetrasulfonate which was reduced in less time than methylene blue. This fact has been confirmed by duplicate experiments; its probable explanation is that a partial breaking down of indigo tetrasulfonate takes place in presence of the broth, a fact already discussed.

The reduction of indigo monosulfonate must have been only very slight; the partial precipitation of the dye prevented accurate reading. Neutral red, phenosafranine, malachite green and litmus were not reduced at all, and Janus green was not reduced from the pink to the colorless form. These facts indicate that the potentials of litmus and malachite green will be found on the negative side of indigo disulfonate.

Indigo disulfonate was only slowly reduced and it is even possible that its reduction was never complete as the broth-dye mixture

maintained a faint greenish tinge. It seems, therefore, that the plain "boiled" broth kept under vaseline seal slowly reaches a potential corresponding to a very advanced reduction of indigo disulfonate (at pH 7.8).

The fact that indigo disulfonate perhaps was not completely reduced may be an indication that, although the broth is capable of reaching more reducing potentials, it does not contain enough reducing sub-

TABLE III.

Time Required for the Reduction of Different Concentrations of Indigo Disulfonate by "Boiled" Plain Broth under Vaseline Seal.

Concentration of the dye	0.002 M	0.001 M	0.0005 M	0.0002 M	0.0001 M
Time at which the final stage of reduction was reached	16 days 50 per cent reduction	16 days	14 days	12 days	8 days
		All tubes reach the same very faint greenish tinge			

TABLE IV.

Reducing Capacity of "Boiled" Plain Broth for Dyes of Different E_0'.

Concentration of the dye	0.002 M	0.001 M	0.0005 M	0.0002 M
	per cent reduction			
2-6 Chloroindophenol	60	Complete reduction		
1-Naphthol-2-sulfonate indophenol	80	"	"	
Methylene blue	50	"	"	
Indigo tetrasulfonate	70	"	"	
Indigo disulfonate	70	Very faint greenish tinge		

stances to reduce a 0.0002 M concentration of the dye. Experiment 1 was therefore repeated, using different concentrations of indigo disulfonate (Table III).

It appears that the broth used in these experiments reaches a final potential corresponding to almost complete reduction of indigo disulfonate. However, with another lot of broth, prepared only 24 hours before the experiment, complete reduction of the dye was obtained in 3 days.

It is possible to conceive of a medium containing several systems of oxidation-reduction which have not reached a condition of equilibrium,

and which it is permissible to call "independent systems." If the broth used in these experiments contains several such "independent systems," it may be expected that they will not all have the same reduction potential; under such circumstances, the broth would reduce a larger amount of dyes with higher E_0', than of dyes with a lower E_0'.

To test this possibility, the reducing *capacity* of the broth for dyes of different E_0' was measured, concentrations of dyes varying from 0.002 M to 0.0002 M being used.

Experiment 2.—The tubes of broth-indicator mixtures (5 cc. per test-tube) were sealed with vaseline and incubated at room temperature. The final readings were made after 3 weeks (Table IV).

With the possible exception of indigo disulfonate (the case of which has already been discussed), the broth reduces completely all the dyes in concentrations of 0.001 M but only partially the 0.002 M concentrations. The differences between the different dyes may be accounted for by the partial breaking down of some of them in the presence of broth (see under Methods).

On the whole, the experiment seems to indicate either (*a*) that the broth contains only one active system of reducing substances (at least in appreciable amounts), or (*b*) that, if there are several "independent" systems, their rH is about the same.

The boiling of media has usually been resorted to as a means of driving out the oxygen in solution, and it has been considered that we were dealing here with a purely mechanical action. There is considerable biological and chemical evidence which suggests that this is not the case. The biological evidence will be presented in a subsequent paper.

The facts observed when the oxidized indicators are added to "boiled" broth will now be considered in some detail.

Experiment 3.—Test-tubes containing 5 cc. of plain broth (prepared 1 week before the experiment) were boiled for 1 hour (or autoclaved), and rapidly cooled down; enough dye was then added to give a final concentration of 0.00025 M.

The 2-6 chloroindophenol turned colorless immediately after addition to the "boiled" broth, but the original color could be restored by the addition of potassium ferricyanide; this shows that the dye had been reduced and not decomposed.

Another tube similarly treated, but without addition of potassium ferricyanide, was aerated by shaking, but the dye remained colorless. The tube was kept opened (with only a cotton plug to insure sterility) and it took more than 6 hours before the dye turned blue again.

The results were the same when phenol indophenol was used. With 1-naphthol-2-sulfonate indophenol, the reduction was only partial and the reoxidation more rapid. With methylene blue, the phenomenon was hardly noticeable, although there was for a few minutes, a partial reduction of the dye. Nothing was observed with the other indicators.

These results are hard to explain on the assumption that the only effect of boiling is a mechanical removal of oxygen. If it were so, the dye should be oxidized immediately after readmittance of oxygen (as provided by shaking). Three possibilities which may be suggested are, that boiling results in (1) the breaking down of some oxidizing substance in the broth, (2) the liberation of some reducing substance, (3) the reduction of some reversible system of oxidation-reduction; the third hypothesis would of course imply the oxidation of some other constituent of the broth.

The progressive reduction of dyes by "boiled" broth can be shown more rapidly by the following technique:

Experiment 4.—Tubes of broth containing 0.0002 M of the different dyes were immersed in a bath of boiling water. The indophenols could not be used in this experiment because they are not heat-stable. Under such conditions, the dyes were reduced in the following order: indigo tetrasulfonate, methylene blue, Janus green (green → pink), indigo trisulfonate, indigo disulfonate. The reduction of indigo disulfonate was only partial and took several hours, while the first three dyes were reduced after 1 to 2 hours boiling. The dyes negative to indigo disulfonate were not affected.

Reduction of Dyes by "Unboiled" Broth.

It has been shown that a progressive drift toward high reduction potentials can be observed in broth-dye mixtures sealed with vaseline. Even though the broth was boiled immediately before the experiment, it had time to dissolve some oxygen before being sealed. As all the dyes down to indigo disulfonate were maintained in the reduced condition under the vaseline seal, it may be supposed that the oxygen in solution had combined with some element of the broth. This fact is much more clearly evidenced when "unboiled," instead of "boiled" broth is used.

Experiment 5.—The same procedure described in Experiment 1 was used, except that half of the tubes of broth were not boiled, while the other half were boiled for 1 hour immediately before the experiment. The broth was 3 weeks old. The dyes were used in final concentrations of 0.001 M, 0.0005 M, 0.0002 M. The tubes, sealed with vaseline, were incubated at room temperature. In Table V are given the number of hours required for 30 per cent, 60 per cent and 90 per cent reduction of the indicators.

TABLE V.

Time (in Hours) Required for the Reduction of Dyes by "Boiled" and "Unboiled" Broth.

Dye	Concentration	"Unboiled" broth			"Boiled" broth		
		30 per cent, red	60 per cent, red	90 per cent, red	30 per cent, red	60 per cent, red	90 per cent, red
	M						
2-6 Chloroindophenol	0.001	—	—	—	—	3	7
	0.0005	5	20	144	—	3	7
	0.0002	3	15	30	—	—	3
1-Naphthol-2-sulfonate indophenol	0.001	—	72	144	7	20	72
	0.0005	7	20	94	—	7	20
	0.0002	3	20	72	—	3	7
Methylene blue	0.0005	94	144	288	29	48	192
	0.0002	48	72	144	3	7	72
Janus green	0.0002	168	240	292	—	20	72
Indigo tetrasulfonate	0.001	94	144	168	48	72	144
	0.0005	17	48	168	7	20	48
	0.0002	—	48	72	—	—	20
Indigo trisulfonate	0.0002	48	94	240	21	48	110
Indigo disulfonate	0.001	—	192	336	21	144	336
	0.0005	72	172	336	72	144	336
	0.0002	48	94	288	—	—	288

Numerals = time in hours
— = test not made

Several conclusions may be drawn from this experiment.

Whether or not oxygen is present in large amounts in the broth at the beginning of the experiment, the final reduction intensity reached under vaseline remains the same (at least as far as could be measured by the colorimetric method). The order of reduction is much more regular in "boiled" than in "unboiled" broth. The time of reduction is greater in "unboiled" than in "boiled" broth. The difference is

considerable with the dyes having a high E_0', but is almost insignificant in the case of the indigo disulfonate. The following considerations may account for this fact. The indophenols are rapidly reduced, but reoxidized as fast as reduced by the oxygen present in the broth. On the contrary, the process of reduction of the indigo disulfonate is extremely slow. While it is going on, the oxygen itself combines with some autoxidizable agent of the broth, so that only a negligible fraction of the reduced dye is reoxidized by free oxygen.

One fact of special interest is that the experiment demonstrates the presence in plain broth of some autoxidizable substance which combines with free oxygen. The pressure of oxygen corresponding to the amount dissolved in the broth has not been computed, but this pressure is certainly too high to be in equilibrium with the reduced dyes. That free oxygen has combined in some way is therefore not doubtful. This oxygen may perhaps combine with the reducing system responsible for the dye reduction. If this system is reversible, the presence of different concentrations of oxygen in the broth may affect the final potential, and the difference would be revealed by potentiometric measurements. On the other hand, the oxygen may combine with some substance other than that responsible for the reduction of the dye, and form a highly oxidized agent of the nature of a peroxide.

Is the Reduction of Dyes "Catalyzed" by Broth?

Although the reduction of methylene blue by plain broth has been used as a test for the "absence" of oxygen in anaerobic jars, the mechanism of this reduction has been little understood. The object of the following experiment was to test whether broth can act as a catalyst in the reduction of methylene blue in the presence of hydrogen.

Experiment 6.—Sterile broth containing 0.0002 M of the different dyes was transferred in 10 cc. amounts to test-tubes which were closed by a rubber stopper with glass connections to allow the bubbling of hydrogen gas (Fig. 1), all points of contact between glass and rubber being sealed with vaseline. The hydrogen was not completely oxygen-free as it had been washed only through potassium permanganate.

When hydrogen was bubbled through the broth-dye mixture (at a pressure of 2 lbs.), all the dyes with a rH positive to that of indigo trisulfonate were completely

reduced. Indigo trisulfonate and disulfonate were not reduced even after 10 hours treatment. The bubbling was discontinued at that time.

When palladinized asbestos was added to the broth-dye mixture, and hydrogen bubbled under the same conditions, all the dyes were rapidly reduced.

This experiment shows that the broth is not capable of catalyzing the reduction of dyes by hydrogen.

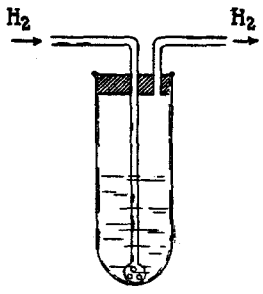

FIG. 1

It may be of interest to mention that there is in broth a substance which, under certain conditions, can increase the velocity of reduction of methylene blue by cysteine.

Experiment 7.—5 cc. portions of a $\frac{1}{100,000}$ (by weight) solution of methylene blue in phosphate buffer (pH 7.5) were transferred to test-tubes, and enough cysteine hydrochloride (neutralized with NaOH) added to bring its final concentration to 0.05, 0.03, 0.02, and 0.01 per cent respectively. Some of the tubes received also 0.1 cc. of broth (recently prepared), some 0.1 cc. yeast extract and others were kept as controls. The tubes were sealed with vaseline and incubated at 37°C. The times of reduction of the methylene blue in the different tubes are given in Table VI.

TABLE VI.

Time (in Minutes) Required for the Reduction of Methylene Blue (Concentration 1/100,000) by Cysteine.

	Concentration of cysteine				
	0.05 per cent	0.03 per cent	0.02 per cent	0.01 per cent	0
Phosphate buffer (pH 7.5)....	60	120	150	Reduction only partial	No reduction
Phosphate buffer (pH 7.5) + 2 per cent broth.....	12	30	40	"	"
Phosphate buffer (pH 7.5) + 2 per cent yeast extract.....	12	25	40	"	"

This experiment brings out the interesting fact that the reduction of methylene blue by cysteine is activated by small amounts of broth and yeast extract. To study now the mechanism of this activation would lead us too far astray.

Is the Reducing Power of the Broth Due to a Reversible System of Oxidation-Reduction?

It is a matter of common observation by those who use broth-methylene blue mixtures as indicators of removal of oxygen from anaerobic jars, that the same tube of methylene blue broth may be used a number of times, the methylene blue being alternately oxidized when brought in contact with the air and reduced again in the anaerobic jar. If there exists an equilibrium between the methylene blue and the reducing substances of the broth, this fact is an indication that there exists in the broth a reversible system of oxidation-reduction. But it is also possible that each reduction of the methylene blue corresponds to the irreversible oxidation of an equivalent amount of reducing substances in the broth and that the process would stop after a certain number of reductions.

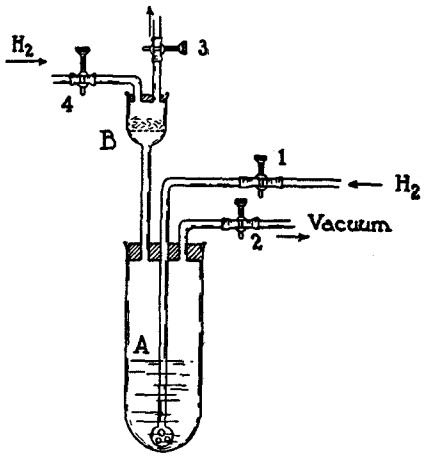

FIG. 2

The following experiment bears on this point.

Plain broth, kept under vaseline seal, reduces all the dyes with an E_0' greater than the potential of indigo monosulfonate, but does not affect the more negative dyes. If the oxidation-reduction system of the broth is reversible, it should be able to oxidize the reduced forms of these dyes. This was shown to be true by the use of the apparatus presented in Fig. 2.

Experiment 8.—*A* is a test-tube containing the broth to be studied, *B* a Jena glass filter containing palladinized asbestos and the dye to be reduced. The glass rubber connections were sealed with vaseline; the hydrogen was bubbled at a pressure of 2 lbs., this hydrogen had been washed only through potassium permanganate.

The procedure was as follows: The whole apparatus was washed through with hydrogen, then, the stop-cocks 2 and 4 being closed, a current of hydrogen was

passed for 1 hour through the broth which had been boiled immediately before; this stream of hydrogen served to deaerate the broth and reduce the dye in *B*.

After the dye had been completely reduced, the stop-cocks 2 and 3 were closed, and 2 and 4 opened. The reduced dye was thus forced down into the broth through the glass filter, enough dye being used to give a final concentration of 0.0002 M.

The method could not be used for methylene blue owing to the very low solubility of the leuco base and also to the absorption by the glass filter.

The following indicators were used: 2–6 chloroindophenol, indigo tetrasulfonate, neutral red, phenosafranine. The results may be summarized as follows: Reduced neutral red and phenosafranine were oxidized as soon as they came in contact with the deaerated broth. The other dyes remained reduced.

DISCUSSION.

As mentioned in the introduction, the object of this survey was not a systematic study of the oxidation-reduction system of plain broth, but only a *description* of the behavior of broth in the presence of dyes, under the conditions used in other biological studies. However, it may be of interest to analyze the results as a means of developing a working hypothesis for a systematic and accurate investigation of the problem.

The term "reducing power" of the broth has been resolved into its three components: the intensity factor (reduction potential), the capacity factor (amount of dye reduced) and the time factor.

When the system plain broth is protected from oxygen, it reaches an ultimate reduction potential corresponding to reduced indigo disulfonate; dyes with a more negative E_0' are not reduced. Equimolecular quantities of the dyes are reduced, indicating that there is either a single system active in the broth, or that if several "independent" systems are present, they all have the same reduction potential. The indicators are reduced progressively in the order of the electromotive series, with the exception of indigo tetrasulfonate which is reduced faster than would be expected; this exception is probably accounted for by a partial breaking down of the dye in the broth.

As regards the nature of the reducing substance present in the broth, it has usually been considered to be a reducing sugar (10); the following considerations throw some doubt on this assumption: The lot of broth used in this work could reduce an amount of dye

corresponding to a concentration of about 0.0017 M. Other determinations show that this reducing capacity is unusually low and that other lots of broth can reduce at least 4 times as much. If this reduction were due to a sugar, the broth should contain an equivalent amount of this reducing sugar, *i.e.*, 0.025 to 0.1 per cent in terms of glucose. In view of the great improvement of growth (with such an organism as Pneumococcus) obtained by the addition of 0.03 per cent glucose to plain broth, it seems unlikely that such broth already contains 0.025 to 0.1 per cent glucose, although the possibility remains that broth contains a sugar very different from glucose in its biological properties.

It has been seen that a number of dyes are reduced by plain broth when the mixture is kept under a vaseline seal. As already mentioned, it is hardly possible that the dyes exist in the reduced form in presence of the oxygen dissolved in the broth, and, consequently, it is concluded that this oxygen combines with some autoxidizable substance of the broth. If this reaction goes on under vaseline seal, it must also go on under aerobic conditions.

This oxidation by molecular oxygen seems to be a slow process. It is possible, but not certain, that oxygen combines with the reduced system responsible for the reduction of the dye. If this system gives rise to a potential determined by the ratio $\frac{\text{reductant}}{\text{oxidant}}$, the final potential should be affected by the amount of oxygen which has combined with the system, in other words, by the age and aeration of the broth; one would then expect the potential of the completely reduced broth to be more negative than indicated by the results reported here. In fact, some experiments recently carried out with broth freshly prepared gave a *complete* and rapid reduction of indigo disulfonate.

On the other hand, the problem is complicated by another fact. When broth or nutrient agar are exposed to the action of air, at least in the presence of light, an oxidation takes place which seems to result in the formation of a substance having the characteristics of a peroxide. This factor should of course be taken into account. It is even possible that, as a result of oxidation, the same substance may give rise to the oxidized form of a reversible oxidation-reduction system and, at the same time, to a peroxide-like compound. Thurlow (11) has shown

for instance that, in the course of the autoxidation of glutathione by air, a peroxide is formed.

Finally, it may be proper to point out a fact of possible significance for anaerobic technique. It has been assumed that reduction of methylene blue in a medium or in an anaerobic jar corresponds to *complete* removal of oxygen (5). As a matter of fact, conditions may be obtained under which the broth is reduced enough to maintain methylene blue in the reduced condition, but not enough to reduce other dyes. In fact, Pasteur, in his work on anaerobiosis, used the reduction of indigo carmine as an index of anaerobic conditions. It is interesting to note that his standard was therefore much more exacting than ours, and it might be useful to come back to it in the cultivation of certain strict anaerobes.

SUMMARY.

Sterile plain broth contains an active oxidation-reduction system, the characteristics of which have been analyzed.

1. Intensity factor: Under vaseline seal, the lot of broth used in these experiments reaches a reduction potential corresponding to reduced indigo disulfonate (rH = 10). All the indicators with a more positive E_0' are reduced, the others are not affected.

It seems probable that fresh broth, which has not undergone oxidation by molecular oxygen, would give a higher reduction potential.

2. Capacity factor: The maximum amounts of different indicators that can be reduced correspond to equimolecular concentrations. This seems to indicate either (*a*) that the broth does not contain several "independent" reducing systems (at least in appreciable amounts), or (*b*) that these hypothetical "independent" systems all have about the same reduction potential.

3. Time factor: The different indicators of oxidation-reduction potentials are reduced in the order of the electromotive series.

4. Nature of the system: The system seems to be reversible (this not excluding the possibility of irreversible autoxidations) and does not appear to be of the nature of a sugar.

The relation of these phenomena to the cultivation of different species of bacteria will be reported later.

Addendum.—While this article was in course of publication, there appeared a paper by Coulter (12). By the electrometric method, he showed that if broth (pH 7.6) is deaerated by a current of oxygen-free nitrogen, this broth passes progressively from an initial potential between +0.250 and +0.150 volt, to a potential between −0.050 and −0.060 volt. "This appears to be a limiting value for the majority of lots of bouillon examined, and may be regarded as a characteristic potential for the system." If the flow of nitrogen is discontinued before the broth has reached the ultimate reduction potential, and the vessel sealed against the entrance of oxygen, "the potential continues to fall slowly and after 5 days or longer attains the limiting value above noted of − 0.060 to −0.050 volt."

Our own experiments confirm this drift toward a region of greater reducing intensity. However, the final potentials obtained by Coulter were appreciably positive to the ones reported in our experiments (the difference being in the neighbourhood of 0.100 volt). It is difficult to interpret the significance of these differences as long as comparative measurements by the electrometric and colorimetric methods have not been made. It is sufficient to recall here that, while electrometric measurements give to glutathione a reduction potential corresponding to a voltage of −0.063 volt, this substance can reduce indigo carmine, the E_0' of which is only −0.125 volt. For an explanation of this anomaly, see Kendall and Loewen (13).

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