THE RELATION OF OXIDATION-REDUCTION POTENTIAL TO THE GROWTH OF AN AEROBIC MICROÖRGANISM

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INTRODUCTION

The possible relationship between the oxidation-reduction potentials of culture media and the ability of microorganisms to initiate growth has recently interested bacteriologists. The work of Aubel and Aubertin (1927), Dubos (1929a), Fildes (1929), Knight and Fildes (1930), Plotz and Geloso (1930), and others, has established the fact that the growth of certain anaerobes is greatly influenced by the oxidation-reduction potential of the medium. In the case of aerobic bacteria, however, conflicting results have been reported. Allyn and Baldwin (1930, 1932) demonstrated that the potential of the medium was of considerable importance in determining whether or not small inocula of Rhizobium, an aerobic organism, could initiate growth. Knaysi and Dutky (1934), on the other hand, finding that Bacillus megatherium would not grow in the absence of dissolved oxygen, even when the potential of the medium was varied over a considerable range, concluded that “the limiting factor in the growth of Bacillus megatherium in vacuum is the oxygen content and not the oxidation-reduction potential of the culture medium.” Their experiments offer no information, however, as to whether or not the potential of the medium affects the growth of this organism under aerobic conditions.

The purpose of this paper is to report: (a) experimental evi-

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dence indicating that the growth of an aerobe (Bacillus megatherium) in the presence of adequate oxygen is influenced by changes in the oxidation-reduction conditions of the medium, and (b) roughly quantitative data as to the range of potential in which such an aerobic organism most readily initiates growth.

EXPERIMENTAL

The oxidation-reduction conditions in the culture medium were varied in two ways: first, by the addition of "poising agents" in the form of reversible oxidation-reduction systems, such as the indicator dyes, which alter the capacity of the medium at definite potential zones; second, by the addition of K$_2$SO$_3$, which reduces the medium and so changes its potential.

Freshly prepared broth adjusted to a pH of 7.2 ($\pm$0.1) was used in all experiments unless otherwise stated. Its composition was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>3 grams</td>
</tr>
<tr>
<td>Peptone</td>
<td>5 grams</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2 grams</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 cc</td>
</tr>
</tbody>
</table>

The indicators used were manufactured by the La Motte Chemical Products Company, with the exception of phenosafranine, neutral red, and methylene blue, which were purchased from the National Aniline and Chemical Company, Inc. The indicators were prepared in sterile water under aseptic conditions immediately before use. The aerobe studied was Bacillus megatherium; all cultures were incubated at 37°C.

I. The effect of oxidation-reduction indicators on growth

Dubos (1929b) studied the effect of a group of oxidation-reduction indicators upon the growth of several facultative anaerobes, and found that the dyes with $E'_0$ values above including that of methylene blue, when in the oxidized form, were toxic to pneumococci and hemolytic streptococci of human and bovine origin. On the other hand, when reduced, the dyes were no longer toxic.²

² Cohen, Chambers, et al. (1929) noted the relative non-toxicity of these dyes in the reduced form when injected into marine ova.
The dyes having $E'_o$ values below that of methylene blue showed no bacteriostatic action, with the exception of phenosafranine, neutral red, and Janus green, which were shown to have bactericidal properties other than those which could be attributed to their effect upon oxidation-reduction conditions, and were therefore not included in the final data. It should be emphasized that since the indicators are added in small concentrations in the oxidized form, they do not change the potential of the medium appreciably. The effect is rather to add "capacity" to the medium at a definite zone of potential (dependent upon the particular dye), and thus to stabilize the potential at this level when the medium is being reduced by the organism. His results led Dubos to offer the hypothesis that "the 'inhibiting' dyes 'poise' the medium at a potential outside the range in which the inhibited organism can grow."

A technique similar to that described by Dubos was used in testing the effect of oxidation-reduction indicators upon the growth of *Bacillus megatherium*. The indicators used are listed in table 1 in the order of the electromotive series. The action of

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>$E'_o$ (VOLT) AT pH 7.2 AND 30°C</th>
<th>GROWTH IN 24 HOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-Chlorophenol indophenol</td>
<td>+0.218</td>
<td>-</td>
</tr>
<tr>
<td>Phenol indophenol</td>
<td>+0.212</td>
<td></td>
</tr>
<tr>
<td>o-Cresol indophenol</td>
<td>+0.180</td>
<td></td>
</tr>
<tr>
<td>1-Naphthol-2-sulfonate indophenol</td>
<td>+0.111</td>
<td></td>
</tr>
<tr>
<td>Thionine</td>
<td>+0.056</td>
<td></td>
</tr>
<tr>
<td>Methylene blue</td>
<td>+0.004</td>
<td></td>
</tr>
<tr>
<td>Indigo tetratosulfonate</td>
<td>-0.055</td>
<td>+</td>
</tr>
<tr>
<td>Indigo trisulfonate</td>
<td>-0.091</td>
<td>+</td>
</tr>
<tr>
<td>Indigo disulfonate</td>
<td>-0.134</td>
<td>+</td>
</tr>
<tr>
<td>Indigo monosulfonate</td>
<td>-0.165</td>
<td>+</td>
</tr>
</tbody>
</table>

The indicators were added to tubes of broth to give a final concentration of $1.8 \times 10^{-4}$ molar. The tubes were then inoculated with 0.1 cc. of a twenty-four-hour broth culture of *B. megatherium* diluted 1:10, and observed for growth after twenty-four hours' incubation at 37°C.
phenosafranine and neutral red was also studied, but these tests were not included in the final data for reasons similar to those mentioned by Dubos. The dyes were added to 5 cc. portions of medium to give final concentrations of from $2 \times 10^{-4}$ to $1.8 \times 10^{-1}$ molar, and the tubes were inoculated with 0.1 cc. of a twenty-four-hour broth culture at dilutions of 1:10, 1:100, and 1:1000. After incubation for twenty-four hours, the tubes were examined for growth. Representative results of duplicate experiments with the highest concentration of dye and the largest inoculum have been recorded in table 1. When smaller concentrations of the dyes were used, it was found that the larger inocula were able to overcome the bacteriostatic effect of the "inhibiting" dyes. In the case of these latter, additional tests were made, in which sufficient quantities of K$_2$SO$_3$ were added to the dye-medium mixture to reduce the dye to the colorless form. None of the dyes inhibited growth when so reduced. The results obtained with Bacillus megatherium were thus strikingly in accord with those of Dubos with the facultative anaerobes.

In the hope of obtaining further evidence as to the nature of the inhibitory action of the dyes, experiments were carried out to determine (a) the effect of the dyes on the growth curve, and (b) whether or not the dyes, in the concentrations used, actually poised the medium to any appreciable extent.

II. Time-potential relationships

Allyn and Baldwin in 1930 pointed out the importance of oxidation-reduction potential in determining the length of the lag phase of Rhizobium. Ingraham (1933) concluded that gentian violet owes its bacteriostatic action, at least in part, to its ability to poised the potential of the medium and showed that it affects growth only during the lag phase. If the inhibitory oxidation-reduction indicators act through a similar mechanism, it should be possible, by using suitable concentrations of the "inhibiting" dye, to demonstrate an increase in the length of the lag phase.

Due to the difficulty of making accurate direct counts on Bacillus megatherium because of its tendency to grow in chains and to form clumps, an indirect electrometric technique was used
in studying the length of the lag phase. The method employed was based upon the common observation (Frazier and Whit-tier, 1931) that the beginning of the logarithmic phase of growth practically coincides with a sharp drop in the potential of the medium. This fact was confirmed in the case of Bacillus megatherium in broth culture by simultaneous potentiometric measurements and microscopic counts. Although the counts were made with some difficulty, the results were sufficiently uniform to show clearly that the first swift drop in potential coincided with the beginning of the logarithmic phase of growth. Certain of the dyes which were shown earlier to inhibit growth were added to the culture medium to make final concentrations of from $2 \times 10^{-5}$ to $1.6 \times 10^{-4}$ molar. Each 5 cc. of medium then received an inoculum of 0.1 cc. of a twenty-four-hour broth culture diluted 1:10. Potential measurements were made at intervals over a seventy-hour period of incubation. The potentiometric set-up was similar to that described by Allyn and Baldwin in 1932. All potentials are referred to the hydrogen electrode. Time-potential curves for inoculated medium containing different amounts of 1-naphthol-2-sulfonate indophenol are shown in figure 1. Similar results were obtained with methylene blue.

The time-potential curves show that increasing concentrations of an "inhibiting" dye tend to lengthen the lag phase. That the dye, in the concentrations used, is effective only during the lag phase may be demonstrated by adding the dye to the medium at various intervals following inoculation. Inhibition of growth occurs only in those tubes receiving the dye before the onset of the logarithmic phase of growth.

The question may be raised as to whether the dyes have been added in sufficient concentration to have any appreciable poising effect on the medium. The time-potential curves of figure 1 show that in the two cases where sufficient dye was added to lengthen the lag phase markedly, a distinct "plateau" was encountered in the region of $+0.100$ volt. Since the $E'_0$ of the dye lies in this range of potential, it is evident that the "plateau" is caused by the poising action of the dye. The interpretation is substantiated by the fact that the length of the plateau in each
curve is roughly proportional to the dye concentration. Similar curves were obtained by Cannan, Cohen, and Clark (1928) when potentials were followed on a culture of yeast to which methylene blue had been added.

![Time-potential curves for B. megatherium cultures in plain broth, and in broth containing various concentrations of 1-naphthol-2-sulfonate indophenol](image)

**Fig. 1. Time-potential curves for B. megatherium cultures in plain broth, and in broth containing various concentrations of 1-naphthol-2-sulfonate indophenol**

**III. Zone of optimum potential for growth initiation**

The above experiments indicate that, in general, only those dyes which poise the potential of the medium above a certain definite level inhibit the growth of Bacillus megatherium. The relation of the initial potential of the medium to the ability of the organism to initiate growth was then investigated.
An inorganic reducing agent, K$_2$SO$_3$, was added in varying quantities to obtain media of different potentials. It was found potentiometrically that the potentials of sterile medium thus obtained covered a range of from +0.450 volt to -0.050. Slight jarring of tubes containing a highly reduced medium (the surface of which was not protected from the air) was observed to cause a marked rise in the potential, due undoubtedly to the entrance of oxygen. In all subsequent work, suitable precautions were taken to avoid unnecessary jarring of the tubes. Potentiometric measurements showed that a considerable period of time was required for the sulfite to reduce the medium to a stable potential. In some cases stable potentials were not attained for nearly twenty-four hours. Once a state of equilibrium was reached, a relatively constant potential was maintained for well over twenty-four hours, provided the tubes were carefully handled.

Experiments were carried out in which a series of broth tubes was prepared, to which sulfite had been added to make final concentrations of from 0.003 to 0.03 per cent. The tubes were allowed to stand for forty-eight hours at 37°C, so that stable potentials might be reached. They were then inoculated and the time of first appearance of turbidity was recorded. The differences observed with heavy inocula were too slight to be conclusive, and the results obtained with highly diluted inocula lacked uniformity, probably because of clumping. However, inoculation with a twelve-hour broth culture diluted 1:1000 to 1:10,000 gave reproducible results. Representative readings are shown in figure 2, and indicate that broth containing from 0.015 to 0.021 per cent K$_2$SO$_3$ is considerably more suitable for growth initiation than is the untreated broth. The potential of the medium exhibiting the most favorable conditions was determined both potentiometrically and colorimetrically, and was found to be roughly between 0.00 and -0.05 volt. This observation becomes significant when it is recalled that of all the dyes studied, only those which tend to poise the medium at potentials above 0.00 volt inhibited growth.

* In preliminary experiments no growth appeared within twenty-four hours in tubes containing more than 0.025% sulfite.
Confirmation of this work using other reducing agents is of course to be desired, and should be sought in subsequent investigations.

![Graph showing the effect of potassium sulfite in broth on growth initiation by B. megarherium.](image)

**FIG. 2. THE EFFECT OF POTASSIUM SULFITE IN BROTH ON GROWTH INITIATION BY B. MEGATHERIUM**

K₂SO₃ was added to a series of broth tubes in the amounts indicated, and the tubes were allowed to stand without agitation for forty-eight hours. They were then inoculated with a diluted broth culture of *B. megarherium* and observed for first appearance of turbidity.

**DISCUSSION**

An interesting working hypothesis has been suggested to explain the manner in which oxidation-reduction conditions affect bacterial suspensions. Evidence has been amassed by various investigators that bacteria can multiply only in media having a potential below a fairly definite "critical" value. The well-known
fact that bacteria can lower the potential of common media suggests that an organism ordinarily lowers the potential of the medium in its immediate vicinity to the "critical" value before starting to multiply. The addition of a reagent which oxidizes the medium, or an oxidation-reduction system which can poise it above the "critical" potential zone, would be expected to make it more difficult for the organism to reduce the medium locally, and therefore cause a certain degree of bacteriostasis, which will depend upon the concentration of the oxidizing or poising agent, and the size and degree of dispersion of the inoculum (Dubos, 1929a, b; Allyn and Baldwin, 1930). Conversely, if the medium has been reduced to a potential below the "critical" value at the time of inoculation, the organism may begin to multiply almost immediately, and the lag phase may be appreciably shortened (Allyn and Baldwin, 1932). The ability of the organism to initiate growth will thus depend upon the relation of the oxidation-reduction conditions of the medium to the "critical" potential.

Whether or not this hypothesis is ultimately proved to be correct, the evidence afforded on the one hand by the indicator experiments and on the other by the sulfite results here reported seems to support this line of reasoning. In conclusion, the data presented indicate that the growth of Bacillus megatherium in the presence of oxygen sufficient for growth is definitely influenced by the oxidation-reduction conditions of the culture medium.

SUMMARY

1. Reversible oxidation-reduction indicators positive to, and including, methylene blue inhibit the growth of Bacillus megatherium when in the oxidized form. Indicators negative to methylene blue in the electromotive series fail to inhibit growth. None of the compounds which inhibit growth in the oxidized form are bacterio-static when reduced.

2. Potentiometric measurements indicate (a) that the bacterio-static compounds studied affect growth only during the lag phase, and (b) that in these experiments sufficient indicator was added to the medium to have an appreciable poising effect. Such observations support the hypothesis suggested by Dubos that
the bacterio-static action of these compounds is a function of their
ability to poise the potential of the medium.

3. Beef-extract broth to which suitable amounts of an inorganic
reducing agent has been added, is more favorable for growth
initiation by Bacillus megatherium than untreated broth. The
potential range of the medium in which growth is most readily
initiated is in the neighborhood of 0.00 to −0.05 volt.

REFERENCES

Service.
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