CHEMICAL CRITERIA OF ANAEROBIOSIS WITH SPECIAL REFERENCE TO METHYLENE BLUE

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The literature of anaerobic technology contains frequent references to various criteria of anaerobiosis aside from growth of organisms. To be sure, the successful cultivation of a known anaerobic micro-organism under given conditions, in contrast with the failure of growth of the same organism on the surface of solid media of similar composition in free contact with air, constitutes a satisfactory biological criterion of anaerobiosis for the particular organism used in the test and under the special conditions thereof. But there are circumstances in which it is desirable to correlate other means of determining oxygen tension reduction. An obviously useful physical means is the vacuum manometer, but most helpful of all are chemical criteria, which are usually based upon coloration changes.

One of the earliest indicators used, and a notable exception to the rule of indicators with coloration changes, was phosphorus, whose failure to ignite was employed by Gratama, a student of Gunning’s (1877).

A mixture of alkali with pyrogallic acid, as used in reducing oxygen tension for the cultivation of anaerobic organisms, is also frequently mentioned as affording a criterion of successful anaerobiosis since in the absence of oxygen the solution remains nearly or quite colorless. But it is scarcely possible for this

1 This essay is based upon an investigation completed during the writer’s tenure of the Logan Fellowship at the University of Chicago and is one of a series awarded the Howard Taylor Ricketts Memorial Prize for 1920.
reagent to serve both as a means of removing oxygen and as a
criterion of removal at the same time, so that the latter purpose
can be achieved only when combined with other means of oxygen
tension reduction.

Fermi and Bassu (1904), using alkaline pyrogallol as a criterion,
encountered extreme difficulty in demonstrating complete anaerobi-
sis. For example, they found that boiling media under
paraffine oil for over one hour does not prevent the darkening
of an alkali-pyrogallol mixture placed therein and a similar
statement was made respecting the passage of hydrogen and
carbon dioxide through media. It appears from my own exper-
iments that the colored compounds formed by the action of
oxygen upon alkali-pyrogallol mixtures are quite stable and the
reactions irreversible, for neither very weakly alkaline-solutions
which show only a trace of color with pyrogallic acid nor strongly
alkaline deep colored solutions can be decolorized by prolonged
boiling. Therefore the difficulty of making the mixture without
obtaining some coloration and the further impossibility of remov-
ing it previous to actual test distinctly limit the practicability
of alkaline pyrogallol as a criterion of anaerobiosis, notwith-
standing its great value as a means of oxygen tension reduction.

More extensive use has been made of substances which in the
absence of free oxygen are reduced to leucobases. Some of these
can be used, not only in media during the active growth of
organisms, but separately as well, for estimating the suitability
of special apparatus. Among such indicators may be mentioned
potassium ferro-ferro cyanid, litmus, indigo (sodium indigo
sulphonate) and methylene blue.

Potassium ferro-ferro cyanid \(\{(\text{K}_2\text{Fe (Fe Cy}_6)\}\) is of slight
historical, but no practical, importance. It was used by Gunning
(1877) (1878) (1879) and is said to become colorless \(\text{[Fe}_2\text{Fe Cy}_6\)
when air is eliminated.

The earliest authentic reference to the bacteriological use of
litmus appears to be that of Würtz (1892) who introduced litmus
lactose agar as a differential medium for \textit{Bact. coli} and \textit{Bact.
typhosum}. It was impossible to confirm Novy's (1893) allusion
[copied by Hunziker (1902)] to Buchner (1885) and Cohen (?)
as first to use litmus to indicate acid and reduction changes respectively, the last reference apparently being altogether erroneous. The decolorization of indigo and methylene blue in culture media were studied by Spina (1887) whose interest in these dyes hinged rather upon their reduction by bacterial growth though he recognized the phenomenon as occurring most vigorously in the depths and noted the return of color on exposure to the air. Kitasato and Weyl (1890) confirmed this observation so far as regards sodium indigo sulphate. The decolorization of all three dyes by sterile culture media under anaerobic conditions, as well as by living aerobic and anaerobic cultures, was especially investigated by Smith (1893) (1896) who noted the necessity of some organic substance such as glucose or peptone and an alkaline reaction in the case of sterile media decolorized by heat.

I found neutral litmus solutions unaffected in color by heating for twenty minutes in a boiling water bath, and the same is true of litmus with 1 per cent glucose. Litmus solutions with 1 per cent glucose and HCl stronger than N/8 were precipitated by heating and the precipitate was not redissolved on cooling; weaker acid solutions were unaffected except for reddening. Strong alkali N/2 to N/32 caramelized the sugar and decolorized the dye permanently; weaker solutions decolorized on boiling for a few minutes and regained their original blue color only on exposure to air.

The recoloration of such decolorized solution of litmus, indigo and methylene blue by exposure to air indicates reversible reactions and constitutes the key to the use of such dyes as criteria of anaerobiosis.

McLeod (1913) cleverly utilized the blue laboratory pencil mark as a criterion of anaerobiosis upon the basis of its decolorization in the absence of air. Some pencils fail to respond, however, according to my experience.

METHYLENE BLUE AS A CRITERION OF ANAEROBIOSIS

The most valuable and most extensively advocated chemical criterion of anaerobiosis is methylene blue. Introduced as an ingredient of culture media by Spina (1887), studied as an indi-
cator of anaerobiosis by Smith (1893) and others, it has been used to a certain extent by almost every serious investigator of anaerobiosis since. Smith (1893) (1896) noted its decolorization in the closed arm of the fermentation tube. Sanfelice (1893) and Liefmann (1908) defended the use of glass slips on the basis of the decolorization of methylene blue in the underlying agar. Trenkmann (1898) and Rivas (1902) used it in their culture tests with Na₂S as a reducing agent. Kabihel (1899) used it in his bell jar device for plates and thereby showed the necessity of removing the covers for efficacious absorption of oxygen by alkaline pyrogallol; he also showed its value as an indicator of the anaerobiosis of deep culture media, liquid and solid. It was used by Petri (1900) in connection with oxygen tension reduction by hydrogen and alkaline pyrogallol, by Sellards (1904) with phosphorus, by Fremlin (1903) (1904), Stüler (1904), Berner (1904), Lentz (1910) with various plating devices, by Wrzosek (1907) (1909), Liefmann (1907), Hata (1908), Guillemot and Szczawinska (1908), Zinsser, Hopkins and Gilbert (1915) with plant and animal tissues, by Laidlaw (1915), and McIntosh and Fildes (1916) in the use of spongy platinum and palladium black as hydrogen-oxygen catalysts, by Wilson (1917) in the use of coal gas, by Douglas, Fleming, and Colebrook (1917) in connection with many porous substances and by a great many others.

**CHEMISTRY OF METHYLENE BLUE**

Discovered by Caro in 1876 and used empirically for many years in the arts, methylene blue, as a chemical compound, was studied most authoritatively by Bernthsen. He showed (1883) that Na₂S₂O₄ reduces it to its colorless leuco base, methylene white, which may be crystallized out of ether and whose aqueous solution becomes dark blue again in acid solution with iron chloride. Möhlau (1883) expressed the rule that methylene white is changed to methylene blue by oxidizing agents in acid solution. Bernthsen (1883) engaged in a brief polemic with Möhlau (1883) and Erlenmeyer (1883) as to the structural formulae of these compounds and finally (1884) set down methylene blue chloride as
which is reduced (Bernthsen 1885) by the action of zinc or zinc chloride with HCl or H₂SO₄ and in alkaline solution with ammonium sulfate to leuco-methylene blue

These formulae are generally accepted now, practically the only disagreement being as to the direct bond between two of the nitrogen atoms.

Landauer and Weil (1910) also obtained leuco-methylene blue by treating a solution of the blue salt in alcohol, with phenylhydrazine, warming and cooling under CO₂. It has a melting point of 185°C, and is not oxidized even by pure oxygen in an atmosphere free from acid and in strongly alkaline solutions is not acted upon by permanganate or hydrogen peroxide. The following equation represents the reaction

\[
\begin{align*}
C₆H₅-N(CH₃)₂ & \xrightarrow{NH₂NHC₆H₅} C₆H₅-N(CH₃)₂Cl \\
\text{(blue)} & \xrightarrow{C₆H₅ + N₂ + HCl} \text{(colorless)}
\end{align*}
\]

Excepting Landauer and Weil (1910) chemists have studied methylene blue largely from the standpoint of action of inorganic compounds upon it. We turn now to a consideration of its behavior in the presence of those factors which enter into bacteriological culture media, since the custom, not altogether defensible,
as I shall show, has grown up of adding a trace of this dye to the culture medium—either with or without inoculation—as a criterion of anaerobiosis.

We have already referred to the fundamental observations of Smith (1893) (1896) on the decolorization of methylene blue in alkaline solutions containing glucose or peptone under anaerobic conditions induced by heating. Kabrhol (1899) and Hammerl (1901) used such a solution along with their cultures as an indication of the successful exclusion of oxygen and the latter showed that the sugar might be replaced with sodium formate. Fremlin (1904) found an alkalinized methyl alcohol solution of methylene blue more delicate than an aqueous solution but recognized the possible inhibitory action of the volatile spirit upon bacterial growth.

As Bernthsen has shown, commercial methylene blue is likely to be a mixture with methylene azure, the latter being formed by the action of alkalis. Underhill and Closson (1905) have given methods for the purification of both, which however is not necessary in using methylene blue as a criterion of anaerobiosis since both compounds yield colorless leuco-bases under similar conditions; furthermore methylene azure is formed from methylene blue under conditions of alkalinity such as obtain in the test.

EXPERIMENTAL WORK

Decolorization—Preliminary discussion

The writer's interest in methylene blue as a criterion of anaerobiosis dates from the invention of the constricted tube and marble device (Hall, 1915). It was possible to show by its use that certain shipments of tubes contained 4 per cent with defective bore so that they could not be used. A properly made tube containing a methylene blue solution of certain composition, with a good marble seal will not permit the return of color below the marble for several days after decolorization by heating. One must not fall into the error of assuming, however, that decolorization of methylene blue necessarily indicates suitability for anaerobic growth; there are many factors, aside from the reduc-
tion of oxygen tension, in the cultivation of anaerobes. However, the failure of a *properly balanced* solution to remain decolorized indicates a defect in the method of air exclusion proposed. Decolorization of methylene blue probably occurs at a definite point during the abstraction of oxygen—a point yet to be determined. So decolorization may indicate suitability for some organisms and not for others. Methylene blue tests with McLeod's (1913) plate were satisfactory yet the bacteriological use of this plate in our hands was never satisfactory. Therefore, while we must admit that the decolorization of methylene blue solution, delicately adjusted, frequently correlates with successful anaerobic cultures, it is more important to recognize the limits and conditions of this test and to appreciate that the factors which enter into the successful decolorization of methylene blue are not necessarily common to the growth of all obligative anaerobes. It should be emphasized especially that acidification, probably through absorption of carbon dioxide from the air, may account for a return of color to decolorized methylene blue solutions and that in this case the dye cannot be bleached again without re-alkalinization.

*Essential factors in decolorization*

Preliminary experiments had to do with tests of Grübler's methylene blau für Bacillen in two culture media commonly used for the cultivation of anaerobes—magnesium carbonate glucose broth (Hall, 1915) and neutral (phenolphthalein) glucose agar. A trace of methylene blue in either of these media is easily decolorized by heating in a boiling water bath. In the open air such decolorized solutions quickly recover their original blue color but protected from air remain decolorized indefinitely. Thus in the constricted tube filled with glucose broth the color returns above but not below the marble seal; in deep glucose agar the color returns to the upper layers first and gradually descends. But it was noticed in certain cases of methylene blue glucose broth allowed to stand for several days that heating failed to decolorize the dye although it had done so originally. The outcome was a series of experiments to determine the
principal factors of decolorization and return of color in methylene blue solution.

Considering each of the ingredients of magnesium carbonate glucose broth as possible single factors in the decolorization of the dye, it was first shown that only those solutions slightly alkanilized, as by means of magnesium carbonate or sodium or potassium hydroxide, lose color on boiling. The use of magnesium carbonate referred to involves addition of an excess and the removal of the undissolved residue by filtration after boiling: only a trace of magnesium goes into solution and the reaction is made faintly alkaline (pH = about 8). With such adjustment it was found possible to dispense with the salt and any two of the other three factors (meat infusion, peptone, and glucose) without interfering with decolorization. But the clearest cut results were obtained with glucose present. Furthermore, rather prolonged boiling is required for decolorization if the glucose be added to the filtrate from a heated MgCO₃ suspension in water, i.e., is not heated in the presence of an excess of MgCO₃; a slightly alkaline solution of 2 per cent agar was also decolorized easily. Further experiments were then undertaken to determine the effect of variation in reaction upon methylene blue solutions in the presence of these various organic substances.

The following facts stand out as a result of many experiments.

Neutral aqueous solutions of Grübler's Methylene blau für Bacillen containing 0.0001 gram or more per cubic centimeter are not decolorized in a water bath boiling hard for twenty minutes. Neither the inorganic acids, HCl, H₂SO₄, HNO₃, nor the organic acids, oxalic, acetic, lactic, citric, butyric, succinic, formic, and propionic, in a concentration of N/10, have any visible effect when heated in weak solutions of methylene blue. Yet methylene blue is decolorized slowly in a solution of HCl acting on zinc in the presence of platinum. N/10 NH₄OH has no visible effect while the equivalent concentrations of BaOH, NaOH and KOH produce a violet lavender color only—indicating, no doubt, the formation of methylene azure.

Neutral glucose solutions ranging from 1 to 10 per cent and faintly or deeply colored with methylene blue are not decolorized
during thirty minutes in a boiling water bath. The same is true of 2 per cent Witte's peptone solutions and of 2 per cent agar solutions. Neither does the addition of 0.5 per cent glucose to any of the acid solutions mentioned above result in decolorization on heating; likewise 2 per cent agar and 2 per cent Witte's peptone in graded hydrochloric acid solutions up to \( \frac{N}{10} \) for agar (which fails to solidify), and up to \( \frac{N}{2} \) for peptone, refuse to decolorize on heating. The neutral sodium salts of the above acids formed by adding equivalent amounts of standardized NaOH do not alter the result; none decolorize on heating.

On the other hand an extremely small excess of alkali causes the heated glucose, agar or peptone solution of methylene blue to lose its color completely. Even such traces of alkali as may be dissolved from the glassware may cause the decolorization of methylene blue in glucose solutions on heating. Incidentally we recall that Laird (1913) found the reaction time for Fehling's solution reduced by boiling glucose, laevulose, galactose, maltose and lactose in various makes of German glassware, owing to the abstraction of calcium hydroxide from the glass. All experiments reported herein were made with glassware carefully cleaned with chromic acid cleaning fluid and rinsed in distilled water. The use of such weak concentrations of alkali, which were approximated only by dilutions of standardized \( \frac{N}{1} \) solutions, involves the possibility of other factors of error, as for example, atmospheric CO\(_2\) and non-neutral distilled water, which do not enter so fully with higher concentrations. Repeated tests of the distilled water by colorimetric tests with phenolsulphonephthalein showed the limits of pH value to be 6.8 and 7.0; thus this possible factor of error was excluded. The CO\(_2\) factor of error was reduced as far as possible by the use of freshly boiled distilled water for the preparation of solutions and checked as a disturbing factor in the interpretation of results. The great difficulty in adequately and exactly controlling the very slight alkalinity of the solutions in different experiments without the use of buffer substances may account for some nonsignificant discrepancies between the results with high dilutions of alkali in different tests. It should be made quite clear that decolorization of
alkaline methylene blue solutions in the presence of these certain organic ingredients of culture media is quite independent of the presence or absence of the acid ions mentioned.

Neutral and $\text{N}/20 \text{ HCl}$ solutions of 1 per cent levulose, glucose, lactose, maltose, sucrose, raffinose, inulin, dextrin, mannitol and, dulcitol, (all Merck's highest purity), with 0.00005 gram methylene blue were tested also for decolorization by heating in a boiling water bath for ten minutes, with negative results. Glucose,

<table>
<thead>
<tr>
<th>TABLE 1 Correlation of Fehling's test and decolorization of methylene blue by alkalinized sugar solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>------------------</td>
</tr>
<tr>
<td>Levulose</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Lactose</td>
</tr>
<tr>
<td>Maltose</td>
</tr>
<tr>
<td>Sucrose</td>
</tr>
<tr>
<td>Raffinose</td>
</tr>
<tr>
<td>Inulin</td>
</tr>
<tr>
<td>Dextrin</td>
</tr>
<tr>
<td>Mannitol</td>
</tr>
<tr>
<td>Dulcitol</td>
</tr>
</tbody>
</table>

* Quoted from Hawk-Practical Physiological Chemistry. Blakiston, Philadelphia, 1907.
Reduction indicated by +.
No reduction during ten minutes in boiling water bath indicated by –.

levulose, lactose, and maltose solutions decolorized methylene blue, however, in one or two minutes in $\text{N}/1000 \text{ NaOH}$, but raffinose, inulin and dextrin solution required $\text{N}/100 \text{ NaOH}$ while sucrose, mannitol, and dulcitol, failed to decolorize methylene blue in even $\text{N}/3 \text{ NaOH}$.

These results with ten representative carbohydrates of reputable purity suggested the following attempt to correlate methylene blue reduction with that of copper sulphate in Fehling's test as in Table 1.

There is apparently a well defined correspondence between those carbohydrates whose reducing action is shown in Fehling's
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Test and those which reduce methylene blue in \( \frac{n}{1000} \) NaOH. These carbohydrates are also most susceptible to alkaline-hydrolysis. The trisaccharid raffinose and the poly-saccharids inulin and dextrin are generally considered not to give Fehling's test; they are less easily hydrolysed by alkalis, and they require therefore a stronger concentration of alkali to reduce methylene blue. The disaccharide, sucrose, and the alcohols, dulcitol and mannitol, are especially resistant to alkalis: they respond therefore to neither Fehling's nor the methylene blue test. But preliminary treatment of sucrose with \( \frac{n}{100} \) HCl readily hydrolyses it and the overneutralization of such a mixture to \( \frac{n}{100} \) alkalinity causes it to decolorize methylene blue quickly on heating.

Quantitative relations

In many of the experiments up to this point the importance of quantitative relations was recognized.

We have just seen that a minute quantity of alkali (\( \frac{n}{1000} \) NaOH) suffices to insure decolorization of certain carbohydrates in 1 per cent solution with 0.00005 gram methylene blue per cubic centimeter. The rapidity of decolorization of glucose solution varies according to the concentration of alkali, which, if sufficiently strong, effects the destruction of color without heating: furthermore, less alkali is required to effect the loss of color under anaerobic conditions than in the presence of the air. The following is abstracted from a protocol covering an experiment with 1 per cent glucose, 1:10,000 methylene blue, of varying degrees of alkalinity as indicated, placed in constricted tubes with marble seals and read after twenty-four hours incubation at 37°C. without preliminary heating.

<table>
<thead>
<tr>
<th>TUBE NUMBER</th>
<th>NaOH</th>
<th>ABOVE MARBLE</th>
<th>BELOW MARBLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( \frac{n}{60} )</td>
<td>Nearly colorless</td>
<td>Slightly yellow</td>
</tr>
<tr>
<td>2</td>
<td>( \frac{n}{120} )</td>
<td>Blue</td>
<td>Colorless</td>
</tr>
<tr>
<td>3</td>
<td>( \frac{n}{240} )</td>
<td>Blue</td>
<td>Nearly colorless</td>
</tr>
<tr>
<td>4</td>
<td>( \frac{n}{480} )</td>
<td>Blue</td>
<td>Blue</td>
</tr>
</tbody>
</table>
On boiling five minutes all were decolorized above and below except tube 4. This experiment thus illustrates not only the point just mentioned but also our frequent observation that very weakly alkaline solutions are likely to fail to decolorize if allowed to stand exposed to the air long before use, probably owing to neutralization by CO₂. This is a point to which we shall return.

As to variations in dye content with \( \frac{N}{1000} \) NaOH and 1 per cent glucose, 1:1000 and 1:10,000 methylene blue failed to decolorize in this particular experiment while solutions containing 1:100,000 did so. The weaker the concentration of dye, the less alkali is required.

With \( \frac{N}{1000} \) NaOH and 1:10,000 methylene blue variations in glucose from 0.15 to 20 per cent appeared to make little or no difference in decolorization, yet further dilution and variations in alkalinity and dye content showed distinct effects, to which reference will now be made for it is apparent that the three reagents necessary in a test for the decolorization of methylene blue by heating bear a definite quantitative relation, one to another. Briefly, the amount of alkali required bears an inverse relation to that of glucose but the necessary amounts of these two reagents bear a direct relation to that of methylene blue. The more alkali the less glucose is required and vice versa, but the more methylene blue the more glucose or alkali is required. Those relations are best displayed in the following experiment:

For the purpose of this and several similar experiments a copper water bath with a support providing for 10 rows of 10 perforations each to hold test tubes was used. The tubes were of uniform size as to length and bore; they were carefully cleaned and placed in the support in rows corresponding to the record marks of table 2, one tube for each mark. To each were first added 7 cc. neutral distilled water and 1 cc. of an aqueous methylene blue solution 10 times the strength required in that particular section of the experiment. Solutions 10 times the strength of glucose required in each of the vertical rows and of sodium hydroxide in each of the horizontal rows were prepared and of these 1 cc. each was added to each tube in the test. In such
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an experiment it is always important to add the alkali last to avoid any considerable action of a concentration greater than that indicated by the recorded data. The total volume of liquid in each tube was 10 cc.

The support with the tubes was then placed in the bath filled with boiling water and the boiling continued for ten minutes, when the support with the tubes was removed and the color or lack of color in the solutions recorded. The reading was repeated five and fifteen minutes after removal from the bath.

To facilitate the manipulation and observation of so many tubes when an important time element is involved it was necessary to divide the experiment in point of time into three sections corresponding to the different quantities of methylene blue used; conditions were duplicated as far as possible in each section with the exception of the quantity of dye, even to the use of dilutions from identical solutions of the three reagents. Also while the data submitted were secured during the space of one afternoon, the tests were repeated several times on other occasions with essentially similar results.

The lines drawn in table 2 indicate the division at each reading between those tubes showing definite color and those not showing color. Next the line on the colored side there were always tubes partially decolorized. As the tests were exposed to room temperature and the air the division line had to be moved in the direction of stronger alkali and stronger glucose, in short, those solutions containing least sugar and least alkali were last to decolorize and first to regain their color.

Table 2 shows also that larger quantities of glucose and alkali are required for the decolorization of a larger quantity of methylene blue and, further, that a decrease in alkali is compensated for by an increase in glucose. Roughly, within certain limits a five-fold increase in glucose permits halving the alkali and vice versa. It is not difficult to understand the direct correspondence between the amount of dye decolorized and the amounts of glucose and alkali required upon the theory that a definite quantity of some substance or substances produced by the action of alkali on glucose and other susceptible carbohydrates is necessary
as a matter of chemical equivalence but an attempt to apply the Guldberg-Waage mass law was not successful.

The present status of our knowledge of the changes which monosaccharids undergo in the presence of alkalis, so well sum-

TABLE 2
Decolorization and recoloration of varying concentrations of methylene blue in relation to varying concentrations of glucose and alkali

<table>
<thead>
<tr>
<th>NaOH</th>
<th>Methylene Blue 1:1000</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>N/100</td>
<td>-</td>
<td>After 5 minutes</td>
</tr>
<tr>
<td>N/200</td>
<td>-</td>
<td>At once</td>
</tr>
<tr>
<td>N/400</td>
<td>-</td>
<td>Per cent glucose</td>
</tr>
<tr>
<td>N/800</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N/1600</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>N/3200</td>
<td>0.004 0.02 0.1 0.5 1.0 2.0</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methylene Blue 1:10,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
</tr>
<tr>
<td>N/100</td>
</tr>
<tr>
<td>N/200</td>
</tr>
<tr>
<td>N/400</td>
</tr>
<tr>
<td>N/800</td>
</tr>
<tr>
<td>N/1600</td>
</tr>
<tr>
<td>N/3200</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Methylene Blue 1:100,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH</td>
</tr>
<tr>
<td>N/100</td>
</tr>
<tr>
<td>N/200</td>
</tr>
<tr>
<td>N/400</td>
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<tr>
<td>N/800</td>
</tr>
<tr>
<td>N/1600</td>
</tr>
<tr>
<td>N/3200</td>
</tr>
</tbody>
</table>

- indicates no reduction—a blue solution.
+ indicates reduction—a colorless solution.

marized by Woodyatt (1915, 1918), indicates a tremendous variety of reactions according to the sugars concerned, the concentration of hydroxyl ions, degree and time of heating, presence
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and absence of air, etc. In general, the basis laid by Lobry de Bruyn (1895), Lobry de Bruyn and Van Ekenstein (1895, 1896, 1897) Nef (1907), Mathews (1909), Henderson (1911), Glattfeld (1913), and others, indicates two groups of products resulting from alkali treatment, first, isomers as a result of the action of weak concentrations, low temperature, etc., and second, split products as a result of stronger concentration and higher temperature. Weak alkalis are transformative, strong alkalis destructive. The literature indicates clearly that glucose ionizes in the presence of alkali as a weak acid, which can be readily shown by colorimetric determination of the change in H⁺ ion concentration of alkaline buffer solutions to which glucose is added. Alkali upsets the stability of the molecule causing the formation not only of all the possible isomers, but of metallic glucosates, and sugars of one, two, three, four, and five carbon atoms as well as oxy-acids.

The fact that decolorization occurs in the presence of minute quantities of alkali might seem to speak strongly for some isomer as responsible for decolorization. But since isomers as well as the original sugar are destroyed by higher concentrations of alkali and these decolorize more readily than low concentrations we cannot entertain this idea seriously. Also the decolorization of methylene blue in similar concentrations of glucose and levulose depends upon identical concentrations of alkali; thus 0.1 per cent solutions of these sugars were decolorized in N/800 NaOH but not in N/1600 NaOH during ten minutes boiling.

Similarly the temptation to explain the possible reduction of alkalinity in the test almost to the vanishing point, by increasing the glucose content, as a result of the adulteration of glucose with effective isomers or split products is checked by the observation that even 20 per cent solutions of glucose without alkali fail to decolorize methylene blue on prolonged boiling.

On the other hand we are unable to exclude split products as the effective agency when alkali is present. Methylene blue solutions caramelized by boiling a few minutes in N/10 or stronger NaOH, and, when neutralized or even slightly acidified, and allowed to regain their color (yellow + blue = green), can be
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decolorized (yellow) in this condition by further boiling; furthermore, prolonged boiling of glucose, levulose, and lactose in strongly acid solutions also results ultimately in more complete decolorization of methylene blue.

Some of the organic acids were noted above as furnishing conditions necessary for the decolorization of heated methylene blue solutions even in the presence of glucose. Alone in N/10 concentration, neutralized with equivalent amounts of N/10 NaOH, and alkalinized to N/10 NaOH, they also fail. Neither formaldehyde, a building stone of glucose, nor ethyl alcohol, one of the most frequent fermentation products of glucolysis, in 5 per cent solution, acidified with HCl to N/10, neutral, or alkalinized to N/10 NaOH, causes the decolorization of methylene blue solutions containing 1 part per 100,000 on boiling. Other products of alkali glucolysis must be tested if we are to fasten the responsibility for the decolorization of methylene blue upon a definite single substance. Our present speculations lead us to suspect that decolorization of methylene blue depends upon those conditions which liberate nascent hydrogen and, that the formation of metallic glucosates by alkalis is somewhat analogous in this respect to the action of HCl on zinc. Or, it may be that the hydrogen required for the reduction of methylene blue to its leuco-base is derived from the dissociation of water and corresponds to the equivalent oxygen uniting with the residue of the sugar molecule, according to Nef's theory.

Two per cent Witte's peptone solutions and 2 per cent agar solutions with 1:100,000 methylene blue are decolorized by heating with alkali. But with peptone, at least 1 part N/1 NaOH in 128 had to be present, owing possibly to the considerable buffer action of peptone. With agar solutions (pH = 7) decolorization occurred with 1 part N/1 NaOH per 200 agar but not with 1 part per 250, although agar is supposed to have little or no buffer action according to Clark and Lubs (1917). Addition of 0.5 per cent glucose did not permit decolorization in less alkali than in controls without glucose, in fact the presence of agar inhibits decolorization in concentrations of alkaline glucose solution which will readily decolorize without the agar.
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In the decolorization of methylene blue temperature is a factor; heat plays a double rôle, driving out oxygen by lowering the solubility point and accelerating the chemical reaction between alkali and organic matter.

Sunlight effects the decolorization of methylene blue but this factor is mentioned here only as a disturbing influence which has been avoided in the experimental work. Lasareff (1912) and Gebhard (1912) have shown that the bleaching effect of light is most intense in the absence of oxygen; the color returns in the dark in the presence of oxygen providing exposure was to wave lengths less than 620 μ but otherwise does not.

Table 3 displays the results of an experiment showing that the return of color to decolorized methylene blue agar in bright sunlight is considerably less rapid than in diffuse light or in the dark.

**Table 3**

*Depth of colored band at top of 2 per cent agar with N/100 NaOH at different time intervals after decolorization, in varying light intensities*

<table>
<thead>
<tr>
<th></th>
<th>15</th>
<th>30</th>
<th>45</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
<td>mm.</td>
</tr>
<tr>
<td>Sunlight</td>
<td>0.5</td>
<td>0.6</td>
<td>1.0</td>
<td>1.5</td>
<td>2.6</td>
<td>3.4</td>
<td>4.1</td>
<td>4.9</td>
</tr>
<tr>
<td>Diffuse-light</td>
<td>0.5</td>
<td>1.1</td>
<td>2.0</td>
<td>2.5</td>
<td>3.8</td>
<td>5.0</td>
<td>5.5</td>
<td>6.0</td>
</tr>
<tr>
<td>Dark</td>
<td>0.8</td>
<td>1.5</td>
<td>2.1</td>
<td>3.0</td>
<td>4.1</td>
<td>5.2</td>
<td>5.6</td>
<td>6.2</td>
</tr>
</tbody>
</table>

As to the decolorization of methylene blue by living cells this discussion does not particularly concern itself further than to note with Jordan that "anaerobes will grow in media where . . . . reduced methylene blue shows no trace of reoxidation." They will grow also in undecolorized methylene blue but observations of many tests have shown no instance where such growth was unaccompanied by decolorization. While, as Ricketts (1904) has mentioned, we cannot regard the reduction of methylene blue as a definite test for living cells, as Ehrlich and others have suggested, since methylene blue becomes leucomethylene blue when its affinities for hydrogen have been satisfied, whether through reduction by living or non living matter,
yet in the known absence of non living reducing agents, the
decolorization of methylene blue in culture media may be taken
as a fair indication of anaerobic growth where the conditions of
anaerobiosis are such as not in themselves to decolorize the dye.
The failure of certain streptococci to decolorize methylene blue
in milk as sherman and Albus (1918) found, appears to be a
matter of inhibition; it is interesting to note Brown's (1920)
observeration that some of these forms will develop in the depths
of agar containing decolorized methylene blue but not in the
colored band near the surface; contrary-wise it is possible for
many organisms to grow aerobically upon media colored with
methylene blue without decolorization. The possible rôle of
adsorption of methylene blue by bacterial bodies in its relation
to true reducing processes still remains to be investigated.

Recoloration of methylene blue

Whereas we are able only to speculate as to the basic expla-
nation of these various phenomena a knowledge of them enables
us to guage correctly the concentration of ingredients in the use
of methylene blue as a criterion of anaerobiosis. Such use
depends, as already noted, upon the recoloration of decolorized
methylene blue in the presence of air, and the failure of recolor-
ation when air is excluded. But recoloration does not occur in
glucose solutions in alkali stronger than N/32 in which marked
caramelization has occurred, nor in peptone more strongly
alkaline than N/16, nor in agar sufficiently alkalinized to prevent
solidification; neutralization of such glucose solutions permits
recoloration, however (Yellow + blue = green).

As a general rule, the delicacy of methylene blue as a criterion
of anaerobiosis varies directly as the kind and amount of reducing
agent employed, and the temperature used to effect decoloriza-
tion, and inversely as the alkalinity of the solution. As shown
in table 2 those decolorized solutions last to lose their color were
first to regain it. In general a moderate concentration of glucose,
e.g., 0.5 to 2 per cent with a low concentration of alkali (N/500
to N/1000 NaOH) gives the best results for tests involving
liquids; 2 per cent neutral agar, plus 1 part N:1 NaOH per 100 is satisfactory for tests involving solid media.

There is a possible fallacy in the use of too weakly alkaline solutions, namely, that on standing they cannot be decolorized by boiling. By exposing all the seven possible combinations of one, two, or all, of the three factors, glucose, alkali, and dye, for forty-eight hours, and then adding those lacking in each of six of these, it can be shown readily that only those originally containing alkali deteriorate; that is, deterioration consists in loss of alkalinity. The test solution must be freshly alkinized, though the glucose methylene blue or agar methylene blue may be kept as stock solutions. Loss of ability to decolorize might conceivably be attributable to acid in the glassware though I have never encountered this factor knowingly. The change of reaction is most reasonably attributed to absorbtion of atmospheric carbon dioxide. An easy proof of change in reaction of faintly alkaline solutions on exposure to air is afforded if one heats $\frac{N}{1000}$ NaOH colored with phenolphthalein in a constricted tube with marble seal in a bath of boiling water; this dye is not affected by such heating. But on cooling for several hours the color above the marble fades while that below remains. Or, drawing air through such a colored solution causes it to fade, through change of reaction, but if the air be washed by bubbling through several tubes of strong lye to remove CO$_2$, with the efficacy of such removal tested by passage through clear lime water, the phenolphthalein test solution remains alkaline. A repetition of this last experiment with a decolorized methylene blue solution gives the same result, i.e., recoloration, with air containing CO$_2$ and air freed therefrom, except that the solution recolorized with the latter continues susceptible to repeated decolorization longer than with the former. This proves that CO$_2$ is not the only factor in recolorization of methylene blue as it is in the change of reaction in the phenolphthalein experiment. If the air be carefully washed in several successive mixtures of alkaline pyrogallol so as to remove both carbon dioxide and oxygen its passage through a decolorized methylene blue solution does not cause the return of color. In short, there
are two possible factors in the recoloration of methylene blue by exposure to air, oxygen and carbon dioxide—two processes, oxidation and acidification.

Since the reaction rests unquestionably upon a quantitative basis, even though we know nothing of the absolute values in oxygen and carbon dioxide concerned, the volumes of test solution in relation to surface exposure, where time marks the progress of recoloration, is of great importance in comparative tests. With equal surface exposure large volumes regain their color more slowly than small volumes. In all cases care has been taken to use the same size tubes and identical volumes in a given experiment unless otherwise stated. Differences in volume between experiments account readily for certain apparent discrepancies in actual observations of time required for recoloration.

Application to methods of culture

With these data at hand tests have been made of a great many methods of cultivation, in which connections I gladly acknowledge the aid of my student, Miss Margaret Eakin. Here, as in the culture of anaerobic microorganisms, we have to distinguish between the factors of oxygen tension reduction and of reduced oxygen tension maintenance.

We have referred already to the literature on biological reduction of methylene blue; to this we may add that our experiments show the general possession by living cells of the property of reduction. This property is common to many aerobes and anaerobes, so in symbiotic mixtures, methylene blue is reduced as a matter of course. With a broth culture of hay bacillus in an external rubber stoppered tube and a smaller internal tube containing slanted 2 per cent agar with 1:100,000 methylene blue and \( \text{N}/100 \text{NaOH} \) analogous to the method of Salomonson (1889), only partial reduction was obtained in twenty-four hours at 37°C and similar tubes of nutrient agar inoculated with \( B. \ botulinus \), \( B. \ tetani \), \( B. \ welchii \) and other obligate anaerobes failed to yield satisfactory surface growth. Failure of complete decolorization here is correlated with refusal of strict anaerobes
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to multiply. This method is well known to be adapted to the culture of microphilic aerobes, however.

Plant and animal tissues also reduce methylene blue in the depths of liquid media. In one instance a piece of sterile guinea pig kidney under mineral oil kept methylene blue decolorized in its immediate neighborhood at 37°C. for 196 hours whereas the control without tissue but with an equivalent depth of oil was completely recolored in thirty minutes.

Many investigators, as already noted, have referred to the decolorization of methylene blue by animal and plant tissues as well as by various inert substances in culture media as indicating anaerobic conditions therein. Of these, Zinsser, Hopkins and Gilbert (1915) recognized most clearly that we have to deal here with another process in addition to reduction, namely adsorption. They were unable, by extraction of animal organs, to secure any reducing substance whatever apart from the tissues and concluded that adsorption is mainly responsible for the loss of color in media containing methylene blue in the presence of such agents. This conclusion was strengthened by their observation that heated tissues are nearly, if not quite, equal to unheated tissues for this purpose. Similar observations were previously made by Wrzosek (1907), Liefmann (1907), Guillemot and Szczawinska (1908), and Hata (1908) but it is doubtful if any of these workers appreciated the important rôle of adsorption.

It is possible, as I shall show presently, to extract reducing substances from both plant and animal tissues, and in this important respect their action upon methylene blue differs from that of inert particulate substances such as sand.

When a small piece of potato was placed in an aqueous solution of methylene blue (1:100,000) at room temperature, the solution adjacent to the tissue lost its color within two to three hours and within a few more hours the test tube showed a lightly colored bluish liquid in which the potato fragment was slightly tinged with blue, most prominently at its uppermost end. No recoloration occurred in such a partially decolorized solution on exposure in a Petri dish nor could it be decolorized by boiling except on alkalization. In contrast, the potato fragment became mark-
edly bluer on exposure, and if cut into displayed a decreasing intensity of dye in the interior, the color deepening rapidly in contact with the air. Such an experiment may be interpreted as indicating adsorption plus reduction, the latter occurring mainly, if not exclusively, within the plant tissue.

Acid (N/100 HCl) and alkaline (N/100 NaOH) solutions gave similar results. Heating such a series immediately in the boiling water bath resulted in decolorization of the alkaline solution only. Exposed to the air in the tube the color returned to this solution on cooling in an intensity practically equal to that of the neutral and acid solutions. Adsorption proceeded in all three and did not seem to be notably accelerated by the heating. Previous boiling of the potato fragment seemed to have no influence on the result.

Extraction by boiling a 1 gram fragment of potato in 1.0 cc. of N/10 HCl, neutral water, or N/10 NaOH for ten minutes and decanting the supernatant fluid yielded a solution containing a reducing substance for methylene blue which could be demonstrated by its decolorization on boiling in N/20 alkaline solution. The color readily returned to such solutions on exposure to the air in a Petri dish. The method of extraction suggested that the substance extracted was probably starch which assumption was substantiated by the iodine test. Starch reduces methylene blue on boiling in alkaline solutions.

Experiments with animal tissues, such as rabbit and guinea pig liver, in aqueous solutions of methylene blue gave results apparently identical with those recorded for plant, i.e., potato tissues. The solutions, acid, neutral and alkaline, became decolorized in the immediate neighborhood of the tissues within a few hours and almost completely, throughout, in twenty-four hours. The nearly colorless solutions separated from their tissues did not regain their color on exposure to the air nor could they be completely decolorized by heating except in the case of the alkaline solution. Alkalization of the neutral and acid solutions, however, facilitated their rapid decolorization by heat. The tissues became slightly tinged with blue during contact with the dye solution and quickly colored on exposure to the air,
both on the surface and in the exposed depths. These phenomena coincide exactly with those observed for the plant tissue and point to the same two processes, adsorption and reduction.

But when it came to extraction of the reducing substance from the animal tissues it was found that the solutions from freshly boiled liver, whether acid (N/10 HCl), neutral, or alkaline (N/10 NaOH), failed to decolorize methylene blue added to them, even when strongly alkanized and heated further. Immediate decolorization upon the addition of a trace of glucose proved the suitability of the general conditions of the test for the proof of a reducing agent. But kept in the ice chest overnight either with or without previous boiling, and in acid, neutral or alkaline solutions and then further boiled immediately previous to separation of the clear supernantant fluids, guinea-pig liver yielded a reducing substance to the fluid capable of decolorizing methylene blue under the influence of heat in alkaline solutions. Guinea pig kidney also gave a similar result in neutral distilled water; acid and alkaline extractions of kidney were not tried.

The results with these animal tissues differ from those with potato, both in respect to the relative ease of extraction of the reducing agent in the latter case, and probably in regard to its chemical nature. There is little reason to doubt that the reducing substance extracted from potato is starch; the chemical nature of that from the animal tissues is only conjectural. We may say definitely that it belongs to the non-heat-coagulable extractives, that it is not materially affected by relatively strong acids and alkalises, and that it escapes from the tissue into the solution during sixteen hours maceration in the ice chest or at room temperature, with or without previous boiling. Furthermore, and this may be the point overlooked by Zinsser, Hopkins and Gilbert (1915), a necessary condition for decolorization of methylene blue by heat in the presence of either the reducing substance from potato or that from rabbit and guinea pig liver and kidney is an alkaline reaction. Solutions so decolorized regain their color on exposure to air.

Thus in considering the action of such plant and animal tissues in anaerobic culture media from the standpoint of their effect
on methylene blue we have to recognize that both adsorption and reduction are concerned.

To complete a representative study of porous substances used in the cultivation of anaerobic organisms I have selected white sea sand. There has been a strong suggestion in such recent work as that of Douglas, Fleming and Colebrook (1917) that the principal value in plant and animal tissues added to culture media for the cultivation of obligative anaerobes lies in their provision of interstices which by their minute size serve to prevent diffusion of oxygen as well as to afford secluded foci for the initiation of growth, and this view has much to commend it. They have shown, indeed, and others as well as ourselves have confirmed, the value of various inert insoluble substances added to media in place of animal and plant tissue.

When I attempted the treatment of simple methylene blue solutions with sand, results startlingly like those with tissues were secured except that there was no reduction in the depths of the sand. In brief, adsorption is the sole process concerned here, and it occurs aerobically as well as anaerobically. In a Smith fermentation tube filled with an aqueous methylene blue solution and shaken up with sand which settled into the neck, marked decolorization occurred in both the open and the closed arms.

Of course it was impossible to "extract" a reducing agent from sand. But so far as the solution itself was concerned it behaved exactly like that treated with tissue; with this exception, that some reducing agent such as glucose, as well as an alkaline reaction had to be provided in order to secure complete decolorization by heating.

Whereas sand of itself has no true reducing action, there is no doubt of its efficacy as a means of maintaining reduced oxygen pressure, as we may judge from the persistence for many days of decolorization in the closed arm of a fermentation tube provided with a slightly alkaline glucose solution of methylene blue and a sand seal in the neck of the tube. The sand seal with suitable culture media in the fermentation tube is also, quite satisfactory from the cultural standpoint.
In summary, the difference between plant and animal tissues and inert substances such as sand are the differences between more or less soluble organic substances and insoluble inorganic substances. No doubt, plant and animal tissues may serve the same mechanical purposes as sand; in addition they may supply nutrients to the medium, buffer substances and possibly even "vitamins." With plant and animal tissues in media, otherwise lacking in reducing substances, these may be of supplementary importance in the cultivation of obligate anaerobes. Finally, unless we are willing to concede some importance to adsorption as a factor in anaerobiosis, sand and other inert porous substances may serve only as a means of maintenance of reduced oxygen tension, i.e., as seals, whereas tissues may serve not only this purpose but may actually aid in the reduction of oxygen tension in addition to the nutritive functions they fulfill. We may emphasize the importance of heat in this connection since the existence of a true self active reducing agent as distinguished from the phenomenon of adsorption seems as yet unproved.

As to physical reduction, i.e., ebullition, the data already presented bear testimony to the efficacy of boiling. In these tests, as in the actual culture of anaerobes, boiling is often an essential preliminary procedure in the test. The use of both liquid and solid deep media so decolorized shows the first return of color at the top and proves the importance in the case of liquid solutions, of such factors as narrowness of bore in the tube volume of solution, the effect of diffusion currents, etc. With solid media these are not so important.

In 1, 2 and 3 per cent agar, with \( \text{N/100 NaOH} \) and \( 1:100,000 \) methylene blue, decolorized by boiling in standard culture tubes of 1.5 cm. bore, the depth of the blue band at the top of the agar at various intervals appeared as in table 4.

Apparently variation of agar content, within the limits of 1 to 3 per cent makes only a little difference in the rate or depth of recoloration. We may point out that the depth of blue color at the top of the agar column corresponds roughly to that in a deep glucose agar stab or shake culture which is free from growth though there are doubtless variations according to species, and
perhaps nutrient conditions, as Burke (1919) has mentioned. On standing longer the blue band thickens, and it is suggested that the distance from the surface at which anaerobic growth commences is determined partly by the rapidity of multiplication permitted by the nutritional conditions of the culture.

Deep tubes of agar, in which recoloration of decolorized methylene blue is occurring, present the phenomenon of rhythmic banding, i.e., Liesegang's rings. This subject has been studied recently by Holmes (1918) in other cases, but no one, so far as I am aware, has investigated the phenomenon in the case of methylene blue, for which no really satisfactory explanation is available.

TABLE 4

Depth of returning blue band in decolorized methylene blue agar of varying densities

<table>
<thead>
<tr>
<th>AGAR per cent</th>
<th>MINUTES</th>
<th>5 mm.</th>
<th>10 mm.</th>
<th>20 mm.</th>
<th>30 mm.</th>
<th>40 mm.</th>
<th>50 mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1</td>
<td></td>
<td>0.4 mm.</td>
<td>0.5 mm.</td>
<td>0.6 mm.</td>
<td>1.8 mm.</td>
<td>2.6 mm.</td>
<td>3.3 mm.</td>
</tr>
<tr>
<td>2 2</td>
<td></td>
<td>0.4 mm.</td>
<td>0.5 mm.</td>
<td>0.7 mm.</td>
<td>1.8 mm.</td>
<td>2.5 mm.</td>
<td>2.8 mm.</td>
</tr>
<tr>
<td>3 3</td>
<td></td>
<td>0.6 mm.</td>
<td>0.8 mm.</td>
<td>0.8 mm.</td>
<td>2.5 mm.</td>
<td>3.5 mm.</td>
<td>4.5 mm.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>AGAR per cent</th>
<th>HOURS</th>
<th>1 mm.</th>
<th>2 mm.</th>
<th>4 mm.</th>
<th>10 mm.</th>
<th>21 mm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 1</td>
<td></td>
<td>3.6 mm.</td>
<td>5.5 mm.</td>
<td>7.5 mm.</td>
<td>11.2 mm.</td>
<td>16.5 mm.</td>
</tr>
<tr>
<td>2 2</td>
<td></td>
<td>3.2 mm.</td>
<td>5.3 mm.</td>
<td>7.2 mm.</td>
<td>11.0 mm.</td>
<td>16.0 mm.</td>
</tr>
<tr>
<td>3 3</td>
<td></td>
<td>5.0 mm.</td>
<td>7.5 mm.</td>
<td>10.0 mm.</td>
<td>14.0 mm.</td>
<td>19.8 mm.</td>
</tr>
</tbody>
</table>

Other methods of deep culture involving solid or semi-solid media present phenomena analogous to those observed with deep agar tubes. Thus gelatin and deep brain media with methylene blue remain decolorized in the depths for several days after heating but the immediate coloration in the uppermost layer gradually extends downward as oxygen and carbon dioxide are absorbed. Corresponding to the usual failure of cultural tests with unprotected liquid media, alkaline glucose solutions of methylene blue regain their color on exposure to
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air soon after boiling. But large flasks of solution or very slender deep tubes of such decolorized solutions remain decolorized for some hours—sufficiently long, indeed, for anaerobic growth to be initiated in suitable media heavily inoculated under similar circumstances. In the Smith fermentation tube efficacy for anaerobic culture, or maintenance of decolorization of methylene blue, depends largely upon the bore at the bend and satisfactory results for either cannot be secured without the use of a special seal, such as sand, tissues, etc. In some unprotected tubes the color returned to the solution in the closed arm in fifteen minutes, in others in sixty minutes and in still others after several hours; with sand seals the dye color returned only in the open arm and then gradually faded even here through adsorption.

The method of deep colony culture—between the nested halves of a Petri dish when tested with methylene blue showed a progressive recoloration of the decolorized dye from the periphery inward, except when protected by a paraffine or vaseline seal.

There is no phase of the methylene blue problem to which we have given more careful attention than its use in connection with insoluble liquid seals, i.e., hydrocarbon oil, wax and grease. The widespread use of these substances as a means of excluding oxygen together with the theoretical and practical objections to their use, especially in the case of oil, have justified a searching examination of this matter. The results with methylene blue only serve to support the conclusion that liquid hydrocarbons are to a degree superfluous and inefficacious means of maintaining anaerobiosis; on the other hand the waxes and semisolid grease seals are more satisfactory for certain purposes, from the standpoint of air exclusion, though inferior to mechanical seals in the matter of convenience and cleanliness.

In the first place an alkaline glucose solution of methylene blue does not decolorize at 37°C. under 2.5 cm. depth of mineral oil, although it may be mentioned here that it does so readily in a few hours under the marble seal in a constricted tube, or under a cover slip in a plain tube, or under a thin layer of paraffine.

I have shown repeatedly with different samples of mineral
oil that the color returns to alkaline glucose methylene blue solution decolorized under varying depths of oil by heating in a boiling water bath almost as soon as without the oil. The following instance illustrates this point. Two per cent glucose (Pfanstiehl) with N/500 NaOH and 1:100,000 methylene blue (Grübler) in aqueous solution was placed in equal depth (3 cm.) in similar culture tubes of \( \frac{1}{2} \) inch diameter and covered to the depths noted with "Fulmor" oil, a white neutral mineral oil prepared by the Fuller Morrison Company of Chicago; a constricted tube with the same dye solution and marble seal was included for a control. It should be noted that the diameter of this tube was about twice, and the surface exposure of liquid therefore 4 times, that of the other, thus offering even greater opportunities for rapid recoloration, which was observed above the seal. All were decolorized throughout by heating two minutes in the boiling water bath and readings made as follows on the removal therefrom.

<table>
<thead>
<tr>
<th>DEPTH OF OIL</th>
<th>ONE-QUARTER HOUR</th>
<th>ONE HOUR</th>
<th>TWO HOURS</th>
<th>SIXTEEN HOURS—(BOILED TEN MINUTES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cm.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Colorless</td>
<td>Slightly blue</td>
<td>Blue</td>
<td>Nearly colorless</td>
</tr>
<tr>
<td>3</td>
<td>Colorless</td>
<td>Slightly blue</td>
<td>Blue</td>
<td>Nearly colorless</td>
</tr>
<tr>
<td>1</td>
<td>Colorless</td>
<td>Slightly blue</td>
<td>Blue</td>
<td>Slightly blue</td>
</tr>
<tr>
<td>0</td>
<td>Slightly blue</td>
<td>Blue</td>
<td>Blue</td>
<td>Blue</td>
</tr>
<tr>
<td>Constricted</td>
<td>Above—Blue</td>
<td>Colorless</td>
<td>Colorless</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Below—Colorless</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Not boiled with other tubes—still colorless below after six days.

This representative experiment indicates that oil is much less efficacious than sometimes assumed as a means of oxygen exclusion. The progressive ease of repeated decolorization in relation to depth of oil suggests that carbon dioxide is excluded somewhat better but one must not lose sight of the fact that the maximum depth of oil in this experiment is much greater than ordinarily used.
I have noted elsewhere (1915) that, culturally, the growth of obligative anaerobes is delayed under oil except where relatively large inocula are used. In certain experiments, comparing the efficacy of the marble and oil (2.5 cm.) seals in constricted tubes with identical media, growth has been negative with the oil seal in twenty-four to forty-eight hours at a million times the dosage showing definite growth under the marble. Continued observation of the oil tubes has usually decreased the disproportion, however. Although these experiments suggest an inhibitive action of the oil this was not substantiated by comparing progressively diluted cultures under both the oil and marble with a similar set under the marble only; in this case equivalent dilutions developed in parallel order.

In this connection it was interesting to study the effect of filling a constricted tube with alkaline methylene blue solution and oil in such a way that the marble seal lay in the oil. The results of a carefully controlled experiment are summarized herewith, the solutions having been decolorized first in the usual way by heating and removed for observation.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Description</th>
<th>Ten minutes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tube 1</td>
<td>No seal</td>
<td>Solution blue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blue above</td>
</tr>
<tr>
<td>Tube 2</td>
<td>Marble in solution</td>
<td>Colorless below—remained so</td>
</tr>
<tr>
<td></td>
<td></td>
<td>for over two weeks</td>
</tr>
<tr>
<td>Tube 3</td>
<td>Marble in oil</td>
<td>Solution blue</td>
</tr>
<tr>
<td>Tube 4</td>
<td>Oil alone</td>
<td>Solution blue</td>
</tr>
</tbody>
</table>

Tube 3 in addition to showing this remarkable result also shows regularly, in such an experiment, a striking and fairly permanent emulsification of water in oil which has been made the subject of a special monograph by the writer (1917).

But it was most disconcerting to find that the marble placed in the oil fails to protect the decolorized solution from recoloration on cooling and suggests that the effect of heating a solution in contact with oil is to drive the oxygen from the solution in which it is less soluble, into and possibly to some extent through the oil, in which it is more soluble, and that on cooling there is a return of some of the oxygen from the oil to the solution.
A duplication of this experiment using phenolphthalein instead of methylene blue showed that CO₂ also is probably similarly concerned; for with the marble seal in the solution the alkalinity of that portion below the seal was protected for over twenty-one hours as against a failure above the marble but below the oil within 1 hour, while with the marble in the oil the solution was only faintly alkaline at one hour and frankly acid at three and one-half hours.

It is impossible to attribute any change in reaction directly to contact with the oil in view of an experiment with oil layered on standard buffer solutions of known acidity (pH = 5, 6, 7, 8, and 9) and colored with brom-cresol purple and cresol-red in their respective ranges for comparison with identical solutions with out oil; there was not the slightest evidence of changed reaction either after shaking together cold, or during, or immediately after heating.

An attempt to make a better showing for the oil by heating the mixture of alkaline glucose methylene blue solution in the autoclave for twenty minutes at 25 pounds pressure (267°C.) gave no better results. Neither was separate heating of solution and oil either in the boiling water bath or in the autoclave, followed by immediate layering, as efficacious in preventing the return of color as heating together in the water bath.

Vigorous boiling of the solution under the oil by the cautious use of a 110°C. saturated salt solution while accelerating the rate of decolorization failed to show any material advantage in excluding the air as judged by the time in which the color returned.

Several attempts to layer oil at or near its own boiling point (about 300°C.) upon decolorized solution at 100°C. resulted disastrously in breakage of glassware and almost explosive scattering of hot oil. Cooled rapidly to about 120°C. in a few experiments I had the gratification of seeing the solution under the oil, even without the marble seal, remain completely decolorized for nearly twenty-four hours; with the marble seal in the oil it remained so for much longer. In still other experiments where the oil was heated to boiling and allowed to cool even for a few minutes, to as low as 60°C. and then immediately brought
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up to 100°C. and layered upon the hot solution the color returned to the latter in about half an hour as in the case of oil heated with the solution in a boiling water bath.

If one increases the depth of solution in a tube without oil, several times over that of a similar tube with oil, both being equally decolorized by boiling, the former may be seen to regain its color even before the latter.

These experiments lend little support to the use of oil as a means of anaerobiosis and limit the technic where it is used to layering on of freshly boiled oil quickly cooled nearly to 100°C., but even in this case it is less efficacious than the marble seal.

In none of the experiments with alkaline glucose methylene blue solution has there been any evidence of absorption of the dye by the oil. Methylene blue is insoluble in oil. A bluish tinge sometimes observed in the oil layer is really due to the dye dissolved in a film of water which separates the oil from the glass wall as I have mentioned elsewhere (1917) or, in the case of oil-dye solutions actively boiled over the free flame or in a strong salt solution bath, to emulsified water holding the dye in suspension. A suggestion that the dye might be absorbed in the form of the colorless leuco-base was proven erroneous by pipetting off the oil from the tube of decolorized dye solution into a tube of distilled water; on exposure to air the color returned at once to the original dye solution whereas the water and the oil overlying it remained quite colorless.

Experiments analogous to some of those with the liquid solution have been performed with 2 per cent neutral agar made alkaline by the addition of 1 cc. N/1 NaOH per 100 and colored with 1 part methylene blue per 100,000 as offering a roughly quantitative measure of the rate of air absorption which is indicated by the thickness of the blue band that appears at the top and deepens as exposure continues. Another advantage of this means of test is that disproportionate volumes do not introduce time differences into the observations of recoloration as they do with a liquid test solution, yet in both cases the volumes and areas exposed in different tubes have been kept identical for comparative purposes except where otherwise noted.
The importance in such observations of having a sufficient quantity of test solution is shown in the following experiment which offers a comparison of the results with equal ratios but differing absolute quantities of test solution and seal.

The liquid test solution was the usual 2 per cent glucose, with N/500 NaOH, and 1:100,000 methylene blue; the agar test solution was 2 per cent agar with N/1 NaOH and 1:100,000 methylene blue; the seal was vaseline. The mixtures were made in tubes of 10 cm. diameter and heated in a boiling water bath for a few minutes to decolorize. They were allowed to stand overnight at room temperature and then examined for recoloration. The results appear in table 5.

<table>
<thead>
<tr>
<th>Vaseline solution</th>
<th>Result</th>
<th>Vaseline solution</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
<td>cc.</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>Colorless</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>Colorless</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>2</td>
<td>Blue</td>
<td>5</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>Blue</td>
<td>10</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Agar test solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>cc.</td>
</tr>
<tr>
<td>-----</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>1</td>
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<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

The logical explanation of this result appears to lie in the assumption that sufficient oxygen or carbon dioxide is retained in the seal to recolor a small amount of test fluid previous to setting of the seal but not sufficient to recolor a larger amount.

Boiling an agar solution under 3 cm. of oil, in a water bath, heating to 25 pounds steam pressure (267°C.) in the autoclave for twenty minutes, or layering the separately heated oil and agar solutions, made only a slight difference in the rate of return of color at the oil-agar surface, which is almost as rapid as without the oil.
Neither heating the oil in the boiling water bath nor boiling (about 300°C.) over the free flame appeared to affect the density of color in the superficial colored layers of alkaline, 2 per cent agar containing methylene blue 1:100,000. This experiment negatives the suggestion that de-aerated oil is able to abstract oxygen and carbon dioxide from media containing them. Also, whereas melted alkaline agar colored with methylene blue and poured without further heating to decolorize into tubes, loses its color to within 1 to 2 cm. of the surface in a few hours, the immediate layering of such a solution with mineral oil makes only a barely measurable difference in the thickness of the band of color at the top.

### Table 6

A comparison of mineral oil and paraffine as seals against the recoloration of alkaline methylene blue agar

<table>
<thead>
<tr>
<th>Hours</th>
<th>0.25</th>
<th>2</th>
<th>6</th>
<th>24</th>
<th>45</th>
<th>70</th>
<th>96</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
</tr>
<tr>
<td>No seal</td>
<td>0.5</td>
<td>0.7</td>
<td>0.8</td>
<td>1.5</td>
<td>1.7</td>
<td>2.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Mineral-oil</td>
<td>0.3</td>
<td>0.5</td>
<td>0.6</td>
<td>1.0</td>
<td>1.5</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>Paraffine</td>
<td>0.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.6</td>
<td>1.1</td>
<td>1.1</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Depth of agar, 7 cm.
Depth of paraffine and oil, 2.5 cm.

An equal amount of paraffine in a similar tube, however, reduced the band of undecolorized medium to 1 cm. in sixteen hours. In a comparison of these three conditions where boiling was used as a means of immediate decolorization followed by rapid cooling and incubation at 37°C., the measurements of the blue band indicated (in centimeters) in table 6 were made.

An analogous comparison of paraffine and vaseline of approximately equal melting points (50°C.) and boiling points (about 300°C.) showed vaseline to be superior even to paraffine. Table 7 shows the actual measurements in centimeters (alkaline methylene blue agar 7 cm. deep).

The recession of the band under paraffine and vaseline was not peculiar to this particular experiment; it has frequently been
seen, but never with oil nor in media without covering except under the influence of light. It has a logical explanation, I believe in the assumption that a certain limited amount of oxygen and carbon dioxide are absorbed from the seal, thus accounting for the band observed, but failure or reduction in the supply coming through when the seal hardens permits the loss by diffusion from the lower surface of the blue band of these gases into the deeper layers of medium and their dilution thereby to a concentration insufficient to recolor the dye.

**TABLE 7**
*A comparison of paraffine and vaseline as seals against the recoloration of alkaline methylene blue agar*

<table>
<thead>
<tr>
<th></th>
<th>1.5</th>
<th>4</th>
<th>24</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
<td>cm.</td>
</tr>
<tr>
<td>No seal</td>
<td>0.7</td>
<td>1.1</td>
<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Paraffine</td>
<td>0.6</td>
<td>0.7</td>
<td>1.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Vaseline</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Tests with the liquid solution also indicate the great superiority of paraffine wax and especially vaseline over mineral oil as a means of protecting decolorized methylene blue solutions from recoloration. These compare favorably with the mechanical seals such as the marble in a constricted tube, or the cover glass in either plain or constricted tube, or sand in a Smith fermentation tube, under all of which the dye may remain water clear for days.

The efficacy of these seals is not a matter of boiling points since mineral oil has approximately the same boiling point as paraffine. Furthermore, the lower boiling hydrocarbons such as xylol (137° to 140°C.) and heptane (95° to 100°C.) are even less efficacious than mineral oil. Viscosity and consistency seem to be the essential elements; perhaps the ease with which vaseline clings to the glass on hardening explains its superiority over paraffine. The liquid oils probably permit the return of absorbed gases but more particularly operate through convection currents.
which transfer gases absorbed at the air surface to the oil-medium surface. Such convection currents are absent, of course, in the solid waxes and semisolid greases.

These researches would be barren were we not able to affirm the parallelism of cultural studies. As a matter of fact, dilution experiments with such organisms as *B. tetani*, *B. Welchii*, *B. sporogenes* and others confirm the great value of paraffine and vaseline as compared with liquid mineral oil. *B. tetani* absolutely failed to grow under either xylol or heptane, gave only delayed turbidity under mineral oil and vigorous early gas production under paraffine. Yet none of these is germicidal for *B. tetani* as shown by successful growth under the marble in a constricted tube of glucose broth covered with them.

The great objections to vaseline and paraffine are their messiness and the fact that they do not provide sufficient variety of oxygen pressures in the medium to meet the possible requirements of different organisms.

Boiling as a means of oxygen tension reduction is sometimes used in methods where the air chamber is sealed either by fusion of the glass outlet or by mechanical devices such as valves, cocks, etc. Either method is satisfactory from the standpoint of the persistence of the decolorized state of methylene blue but is obviously superfluous for deep cultures and is inapplicable to surface cultures for reasons easily apparent.

Evacuation by water pump to 58 cm. mercurial pressure with immediate sealing of a constricted portion of the tube has never sufficed to decolorize methylene blue 1:100,000 either with 2 per cent glucose and N/500 NaOH or 2 per cent agar and N/100 NaOH at ordinary room temperature in our hands. Nor have we ever been able to secure surface growths of such obligate anaerobes as *B. Welchii*, *B. tetani*, *B. oedematis* and *B. botulinus* on the surface of solidified glucose agar by this means alone. We are therefore forced to conclude that evacuation by water pumps of such efficiency is of relatively little value alone as a method of securing anaerobiosis. Higher evacuation might yield more successful results.
The literature is notably deficient in accurate data on the oxygen tension limits of anaerobic bacteria determined by volumetric methods. The limit of 35 cm. Hg pressure for the *vibrion septique* set by Rosenthal (1906) in liquid media is of doubtful value when viewed in the light of the recent observations of Harris (1919) on the apparently high tolerance of *B. sporogenes* for oxygen in liquid cultures as compared with agar slopes.

While we have tested the effect of inert gases in the case of hydrogen and carbon dioxide, it is scarcely fair to consider the results as necessarily corresponding to those obtained by cultural methods where we conceive the action to be primarily physical, since with methylene blue solutions we may also have chemical reactions. Hydrogen, indeed, did decolorize cold solutions of 0.5 per cent glucose with \( \frac{n}{500} \) NaOH and 1:100,000 methylene blue slowly, and hot solutions already decolorized remained so during fifteen minutes of active ebullition by hydrogen from a Kipp generator loaded with zinc and sulfuric acid and purified by passage through 10 per cent PhNO\(_3\) and alkaline pyrogallol. Furthermore when sealed the hot solution remained decolorized till opened on the fourth day while the cold solution showed only a trace of color while sealed.

Passage of commercial carbon dioxide through similar solutions of the dye for one hour failed to decolorize the cold solution although the hot solution remained decolorized during this part of the experiment but soon regained the blue color after sealing. Reheating these solutions failed to decolorize either of them, without further addition of alkali. Here is a situation, which, barring the known inhibitive action due to improper acidity for certain organisms, might yield satisfactory results in the case of certain others, as Pasteur found, without permitting a satisfactory degree of alkalinity for the decolorization of methylene blue.

Coming now to the matter of chemical reduction of oxygen tension, we conceive that in so far as regards reducing agents in the medium, they have been sufficiently dealt with already, and the necessity of recognizing limitations of time and space
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reduces this discussion, in so far as it relates to chemical reduction by means of an agent in the air chamber, to the most valuable agent with which we have to deal, i.e., alkaline-pyrogallol.

Inasmuch as this reagent finds a legitimate use only in connection with attempts to secure surface colonies on solid media, it is to this that we have limited our attention. Buchner’s (1888) original technic involving the use of a small tube containing slanted culture medium (2 per cent agar, methylene blue 1:100,000, N/100 NaOH) in a longer one containing the usual alkaline pyrogallol mixture gave satisfactory results when the cotton stopper was left out of the inner tube, but otherwise did not, unless the tube were inverted. Decolorization begins, as does growth of obligate anaerobes on suitable media, in the thinnest portion of the slant. In Wright’s (1901) modification, which involves the saturation of the plug with the mixture, sealing by rubber stopper, and inversion, even better results were obtained. In either case partial decolorization appeared at the surface in less than twenty-four hours at 37°C. and continued to completion within two to three days. Both these methods, but notably the last, have given quite satisfactory results in the surface cultivation of B. tetani, B. welchii, B. sporogenes, B. chauveauii, B. botulinus, and many unidentified anaerobes. Wright’s method has been relied upon largely for repeated surface colony isolation of the writer’s cultures.

Two plating methods for surface culture have been studied in connection with the methylene blue test, namely Lentz’s (1910) pyrogallic acid saturated cardboard, and McLeod’s (1913) divided plate. Both showed the dye-agar decolorized at 37°C. within eighteen hours. The latter method has refused consistently however to yield us surface colonies of well known anaerobes on either blood or glucose agar identical with that used in controls by Wright’s method. Dr. Oskar Klotz at the University of Pittsburg has stated his belief that aromatic substances in the clay are sufficiently germicidal to explain the failure of bacterial growth and has overcome the difficulty through the use of a special cement containing paraffine and some other substances. At any rate, McCleod’s device seems constructed
according to our best conceptions of the requirements. In contrast, the method of Lentz is open to the technical objection that considerable absorption may occur before the seal is completed. We have not tested the latter sufficiently from a bacteriological standpoint to justify any conclusion as to its real worth. The dye test points to its suitability.

In conclusion it is suggested that further studies are required to show exactly to what degree of oxygen tension reduction the decolorization of methylene blue under stated conditions corresponds. Harvey’s (1919) recent demonstration of the direct relation between oxygen content and time of decolorization of methylene blue in Schardinger’s test and the interesting parallelism between methylene blue reduction and luciferin formation (1920) is significant in this regard, but one must not overlook the great importance of the hydrogen ion concentration of the test solution, and the effect upon this of atmospheric carbon dioxide.

Similar studies are equally needed in the investigation of the oxygen relations of obligate anaerobic bacteria.

SUMMARY

This paper reviews the literature on chemical criteria of anaerobiosis, and undertakes a critical experimental survey of the subject in so far as it relates to the use of methylene blue for this purpose. The mechanism of the decolorization of methylene blue is studied in detail and shown to depend upon the interaction of alkali and certain organic substances, notably carbohydrates. A correspondence between Fehling’s test and the decolorization of methylene blue in alkaline solutions of various carbohydrates is pointed out.

It is shown that while there is a direct relation between the amount of dye decolorized by heating and the amounts of alkali and glucose, there is an inverse relation between the last two factors in the test, so that an increase in one permits a decrease in the other for the same result.

Carbon dioxide, as well as oxygen, is shown to be an effective factor in the recoloration of decolorized methylene blue.
Various methods of anaerobiosis are viewed critically in the light of experimental tests with carefully balanced solutions of alkaline glucose methylene blue in comparison with cultural tests with *B. Welchii*, *B. tetani*, *B. botulinus*, and other obligate anaerobes.

A detailed study of the decolorization of methylene blue by plant and animal tissues is described, showing the important role of adsorption as a means of decolorization by these and other porous substances. The extraction from plant and animal tissues of reducing substances for methylene blue, active in alkaline solution, is described.

The efficacy of deep culture methods for anaerobes is shown while the inefficacy of insoluble liquid (i.e., oil) seals is contrasted with the reliability of semi-solid waxes and greases, and that of mechanical seals.

The shortcomings of certain methods of surface culture of obligative anaerobes are exposed and the value of a modification of Wright's method upheld by these studies.

Finally, the desirability is indicated of determining exactly to what degree of oxygen tension reduction the decolorization of methylene blue corresponds, and whether decolorization occurs at a definite hydrogen ion concentration irrespective of the sugar content of the solution.

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