FACTORS INFLUENCING ANAEROBIOSIS, WITH SPECIAL REFERENCE TO THE USE OF FRESH TISSUE.

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PLATE 2.

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Anaerobic cultivation of bacteria has in recent years developed into an important field of bacteriological research. Anaerobic methods have been extensively applied, for example, to the study of the bacterial flora of war wounds and to the investigation of diseases of unknown origin.

In these investigations the cultivation of anaerobes in tubes containing bits of fresh tissue has been extensively employed. Although study of some of the principles involved has led to improvements in method, a method for the quantitative examination of the factors involved is lacking and the establishment of practical rules and indications to meet the requirements of the more fastidious of this group of microorganisms is still to be attained. We shall attempt to show the influence of certain elements in promoting or in hindering the development of strict anaerobic conditions in culture tubes and so to indicate more exactly the value of these factors in anaerobic cultivation.

Aside from the use of mechanical methods for removing oxygen from ordinary media, much attention has been devoted in recent years to the cultivation of anaerobes in a fluid or semisolid medium containing a fragment of fresh sterile tissue, usually kidney, and overlaid with a layer of paraffin oil. The value of the tissue fragment was pointed out by Theobald Smith¹ in 1899, and rediscovered by Tarozzi² and Wrzosek³ in 1905. The use of paraffin oil is credited to Legros.⁴

In 1911 Noguchi, using tall columns of serum water overlaid with paraffin oil in narrow tubes and containing a fragment of fresh tissue, was able to grow *Spirocheta pallida* under strictly anaerobic conditions. He did not rely on the kidney tissue or the paraffin oil for the production of anaerobiosis, but for the first time employed with these a combination of hydrogen gas, vacuum, and pyrogallic acid in an anaerobic jar.

Later, in the cultivation of the globoid bodies of poliomyelitis it was found that the mechanical precautions could sometimes be omitted.

Following these reports the tissue-serum water or ascitic fluid tubes overlaid with paraffin oil came to be widely used as an anaerobic method and some investigators, seeking strictly anaerobic conditions, have disregarded the elaborate precautions which Noguchi employed. But the successful use of the tissue method alone, which appears to be simple, has been found to require considerable patience and experience, and the method has often suffered for lack of quantitative standardization and through misunderstanding of underlying principles.

A number of variables are involved. The first requisite for a study of these variables is a delicate and precise reversible indicator for the presence or absence of free oxygen in solution. The indicator should react in the presence of culture media, so that it may be added directly to the materials to be tested, and should not interfere with the reducing activity of other components of the medium or arrest the growth of test organisms. Methylene blue fulfills these requirements in a satisfactory manner.

Theobald Smith in 1896 reported the reduction of methylene blue and other indicators in the closed area of fermentation tubes by sterile peptone broth. The presence of muscle or grape sugar and the application of heat increased the speed of the reaction. Peptone and dextrose water were inert. Spina had already noted the reduction of methylene blue by nutrient gelatin, but not by agar.

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Methylene Blue as Indicator.

By the action of weak deoxidizing agents methylene blue (tetramethylthionine chloride)

\[
\text{CH}_4\text{N}(\text{CH}_3)_2\text{S} + 3\text{H}_2\text{O} \rightarrow \text{CH}_4\text{N}(\text{CH}_3)_2\text{Cl} + \text{H}_2\text{O}
\]

is converted readily to colorless leucomethylene blue, or \(\alpha\)-(p)-tetramethyldiaminothiodiphenylamine

\[
\text{NH}_2\text{CH}_3\text{N}(\text{CH}_3)_2\text{S}.
\]

The reaction is reversible, and the conversions proceed rapidly at incubator temperature in the presence of free oxygen or under the attack of the deoxidizing agent after the free oxygen has been consumed.

Experiments were made to determine the sensitiveness of the indicator and the conditions under which it reacts. Ordinarily 0.1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of 2 per cent dextrose peptone broth in a test-tube is placed in the anaerobic jar and the removal of free oxygen is indicated by the gradual decolorization of the dye. Since methylene blue is not decolorized under similar conditions in a medium of distilled water, we sought the ingredient in the dextrose broth which promotes the decolorization.

Relation of the pH of the Medium to Its Reducing Activity.—Beef infusion, 2 per cent peptone solution, and 2 per cent dextrose, with methylene blue, when boiled in a water bath to remove the air were not decolorized. Nor were mixtures of any two, or of all three of the ingredients. But so far in this experiment the hydrogen ion concentration of these mixtures had not been considered. The foregoing materials were retested in a solution of 0.01 N sodium hydroxide. Decolorization was immediate on heating to drive off the air, but the dye was resolved into simpler elements than the leuco form, thus destroying its value as an indicator.

2 per cent dextrose solution, colored with methylene blue, was mixed with an equal volume of buffer phosphate mixtures in \(\frac{M}{18}\) solution, to produce a range of pH concentrations from 6.6 to 7.8. Tubes of these mixtures were heated to expel the air and to increase the
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velocity of reaction. The solutions of a pH of 7.4, 7.6, and 7.8 were quickly decolorized. On cooling and exposure to air the blue color as promptly returned. Similar but less rapid effects were obtained with the 2 per cent peptone solution. The beef infusion appeared to be inert.

The reducing action of dextrose in alkaline solution is well known as the basis of the Fehling and Benedict tests in urine analysis. The same reaction is utilized here. The routine use of methylene blue in dextrose peptone broth is simply a convenient method of assembling the materials in a weakly alkaline solution.

The delicacy of the reaction depends upon the feeble reducing power of the medium at the chosen hydrogen ion concentration and at incubator temperature. An equilibrium is established between the rate of diffusion of oxygen through the medium and the activity of the reducing agent. It is only when the access of oxygen is practically completely inhibited that the weakly alkaline dextrose solution is able to fix the remaining traces of the gas and then to attack the methylene blue.

Comparison of Different Amounts of Methylene Blue.—From 0.1 to 1 cc. of a 1 per cent aqueous solution of methylene blue in 10 cc. of dextrose broth was decolorized with practically equal rapidity in less than 24 hours in a McIntosh and Fildes jar. For the purposes of this study, variations in the small quantity of the dye used in the medium were of little significance.

Effect of Temperature.—Like other chemical reactions, the rate of reduction is a function of the temperature. When access of free oxygen is prevented decolorization does not occur in 24 hours at 4°C. It proceeds slowly at room temperature (21°C.) and rapidly at 37°C. and higher temperatures.

Estimation of the State of the Medium.—It has been noted above that the state of the indicator, whether blue or colorless, depends upon the relative rate of the admission and diffusion of oxygen from the air and the activity of a reducing substance, e.g. dextrose, in the medium. The equilibrium between these two forces may be observed in a narrow test-tube by the depth below the surface at which the colorless zone begins. While methylene blue may not be decolorized in an oxygen-free liquid in the absence of a reducing substance, decolorization indicates a strictly anaerobic condition, and the return of the color is an index of the return of oxygen to the medium.

With these indications of the sensitiveness of the reaction and of the factors that control its progress, we proceeded with a study of the various elements in the technique under investigation.

Comparison of Liquid Paraffin Oil and Solid Vaseline as a Seal.

The tissue medium technique as generally employed involves the use of liquid paraffin oil as a seal or supernatant to the medium. It was presumed that the oil, besides preventing evaporation, favored deoxidation by opposing a barrier to the air. Rosenthal\textsuperscript{10} suggested the use of lanolin (melting point 42°C.) as a seal, and recently Fildes\textsuperscript{11} has stated that oil has practically no effect in preventing the return of oxygen to the medium, while solid paraffins merely delay its passage. In view of the importance of an effective seal, a quantitative comparison of oil and vaseline seemed advisable.

\textit{Experiment 1.}—Twelve tubes, 1.5 by 20 cm., each containing one-sixteenth of the kidney of a 1,600 gm. rabbit, were filled with 1 per cent dextrose broth to a height of 9.5 cm.; 2 drops of sterile 0.5 per cent aqueous methylene blue were added as indicator. Series A consisted of four tubes, not sealed (air control). Series B, four tubes overlaid with 2.5 cc. of paraffin oil. Series C, four tubes overlaid with 2.5 cc. of vaseline. All incubated at 37.5°C.

When observed after 16½ hours the tubes of Series A were decolorized 2 cm. from the bottom of the tube. Tubes of Series B decolorized 3 cm. from bottom. Tubes of Series C completely decolorized.

When observed after 42 hours, the tubes of Series A and B showed an increase of 1 cm. in the height of the decolorized column. Tubes of Series C remained completely decolorized. There was no further increase in the height of the decolorized columns in the tubes of Series A and B. Repetition with smaller fragments of kidney yielded similar results.

As shown by the point at which a balance was established between the access and diffusion of oxygen and the reducing action of the kidney tissue, the addition of paraffin oil produced only a slight increase in the length of the anaerobic column, the final ratio of the oil-covered to the air-covered tubes being 4:3.


Experiments 1 and 2 are illustrated in Fig. 1.

In the McIntosh and Fildes jar a slight reduction in pressure occurs as a result of the combination of oxygen and hydrogen. The partial vacuum tends mechanically to lower the tension of oxygen dissolved in the medium and so hastens the establishment of strict anaerobic conditions through the action of a reducing agent. When large amounts of paraffin oil are employed, this favorable action is retarded. Indeed, the oil seems to serve as a reservoir for oxygen in solution. Except to prevent evaporation and to maintain anaerobic conditions after removal, the use of any seal appears to be unnecessary in a properly manipulated McIntosh and Fildes jar.

Experiments 3.—Six 20 by 1.5 cm. tubes containing 10 cc. of dextrose broth were colored with 1 drop of 1 per cent methylene blue. Series A, two tubes, unsealed. Series B, two tubes, overlaid with 4 cc. of paraffin oil. Series C, two tubes, overlaid with 2.5 cc. of vaseline. All tubes heated in a water bath until color disappeared, then plunged into cool water to bring to room temperature and to solidify the vaseline.

The blue color appeared immediately at the surface of the unsealed tubes and at the surface of the medium in contact with the oil. Streaks of blue carried by convection currents descended to the bottom of the tubes, there to be slowly decolorized again. After 20 minutes a zone of blue was established to a depth of 3.5 cm. in the unsealed tubes and to 3 cm. in the oil-covered tubes. The vaseline-covered tubes remained colorless.

After standing for 72 hours at room temperature the blue zone had descended to a depth of 4.7 cm. in the unsealed tubes and to 4.6 cm. in the oil-sealed tubes. During the following days the unsealed and the oil-sealed tubes came to an equilibrium approximately 6 cm. below the surface of the medium. The vaseline-sealed tubes remained colorless throughout.
Under air or paraffin oil, dextrose broth is unable alone to overcome the diffusion of oxygen, which is hardly retarded by the paraffin oil seal. In the depths of the tube, however, the combined reducing action of broth and kidney is able to establish and maintain an oxygen-free zone, which is slightly higher in the paraffin oil-covered tubes. Under vaseline, on the other hand, owing solely to its solid state at incubator temperature, the access of oxygen is prevented, and the dextrose and kidney tissue in the course of a few hours fix all the oxygen remaining in solution. Other aids to deoxygenation, such as a McIntosh and Fildes jar or the action of heat in reducing the oxygen solubility of the medium and increasing the chemical activity of the alkaline dextrose solution, exert a similar action in hastening the establishment of anaerobic conditions in a culture tube, provided that access of atmospheric oxygen is prevented by an impervious solid seal such as vaseline.

We conclude that the paraffin oil seal extensively used in anaerobic culture work and in gas analysis is practically valueless except to prevent evaporation. The seal itself may contain enough oxygen in solution to defeat the very object which it is used to attain. A layer of vaseline, on the other hand, is an oxygen-resisting seal that materially aids the action of deoxidizing agents in the medium.\(^\text{12}\)

**Standardization of the Kidney Tissue Component.**

Four functions have been ascribed to the fragment of kidney utilized in the tissue technique: a reducing activity, the formation of a nidus, the contribution of nutritive elements to the medium, and an effect upon the pH concentration by acid production. That the kidney tissue component is an active reducing agent has been well known since 1885 through the researches of Ehrlich and others and is demonstrated anew in Experiment 1 of this communication. But the zone of its action in the culture tube has not been subjected to quantitative estimation.

\(^{12}\)Hard, inelastic paraffin waxes may be less useful through rupture of their contact with the glass by changes of temperature in the tubes.
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Smith, 1 who was the first to advocate the use of tissue fragments, gave no indication of the size to be used. Tarozzi 2 advocated a cube of about 1 cm. Noguchi 3 suggested that the kidney of an adult rabbit should be cut into about ten pieces, each approximately the size of a split chestnut. Other original workers have not defined the tissue component so carefully and the tendency in general has been to use too small a piece, especially for primary cultivations and early transplants.

Comparison of the Reducing Effect of Different Amounts of Kidney Tissue.—We tested the reducing effect of pieces of various sizes upon a medium containing methylene blue.

Experiment 4.—Two sets of three 20 by 1.5 cm. tubes were prepared, each containing 10 cc. of aerated 1 per cent dextrose broth and 1 drop of 1 per cent aqueous methylene blue in addition to the kidney fragments. The fragments were cut as uniformly as possible from 5 mm. cross-sections through the middle of the kidneys from an 1,800 gm. rabbit. In Set 1 a single piece of kidney was used. Tube A contained one-eighth, Tube B one-fourth, and Tube C one-half of a 5 mm. cross-section. In Set 2 the pieces were cut into eighths of the original cross-section. Tubes A, B, and C were the same as the corresponding tubes of Set 1, except that Tube B contained two-eighths and Tube C four-eighths of a cross-section in two and four separate fragments. No seal. Incubated at 37.5°C.

In Tubes B and C of each series decolorization began in a few moments around the kidney tissue at the bottom of the tube. Later results are shown in Table I and are illustrated in Fig. 2.

After 42 hours the tubes were removed from the incubator and allowed to stand at room temperature. After 20 hours at about 21°C. the level of decolorization had dropped 0.5 to 1 cm. in each tube.

<table>
<thead>
<tr>
<th>Set No.</th>
<th>Duration of incubation (hrs.)</th>
<th>Height of decolorized column from bottom of tube.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Tube A: small fragment of kidney.</td>
</tr>
<tr>
<td>1</td>
<td>16</td>
<td>0.4 cm.</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>1.0 cm.</td>
</tr>
<tr>
<td>1</td>
<td>42</td>
<td>1.7 cm.</td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>1.8 cm.</td>
</tr>
</tbody>
</table>

Different amounts of kidney tissue have different reducing effects. The larger the amount, the greater is the reduction. Small pieces are of little value for producing an anaerobic zone. Very large pieces do not produce a proportionately large zone of reduction. With 0.6 to 0.8 gm. of kidney tissue a zone sufficient for practical purposes is obtained. The tissue may be used in one fragment or in several. The balance between the penetration of oxygen from the surface into the medium and the reducing activity of the tissue occurs at a level determined by the temperature, other things being equal. The higher the temperature, up to 37°C., the less is the oxygen solubility and the greater the reduction.

Nature of the Reducing Substances in the Kidney Tissue.—Kidney tissue in unsealed tubes such as those of Experiment 4 exposed to the air at room temperature will maintain an anaerobic zone at the bottom of the tube for a period of weeks or months. It is not conceivable that the reduction is dependent on the maintenance of the activity of living cells. The question arises then, as to the nature of the substance responsible for the reduction. In considering this question, which properly belongs to physiology and biochemistry, we were soon led away from the subject in hand. Preliminary experiments indicated that the activity still resided in filtered extracts of kidney tissue, and that the reducing substance concerned is relatively heat-stable, so that boiled, or even autoclaved kidney is not entirely without effect, but other considerations make it seem unprofitable to attempt to modify the use of fragments of fresh sterile kidney tissue in this anaerobic technique. Thunberg refers to former researches on the reducing activity of tissues and reports his evidence of the enzymotic nature of the reaction.

Before the reducing activity of fresh tissue in anaerobic cultivation was generally recognized, it was suggested that the tissue fragment might act in an obscure and passive manner as a focus for bacterial multiplication. The demonstration of a favorable reaction on the

14 The kidneys of a medium sized rabbit, 1,400 to 1,700 gm., weigh about 6 to 6.5 gm. Eight to ten fragments may be obtained from each kidney. The large kidneys of full grown rabbits yield twelve to sixteen fragments of proper size.

surrounding medium made such an assumption unnecessary. Some investigators, however, acting on the earlier hypothesis, have advocated the use of small pieces of inorganic substances, asbestos wool or iron tacks, for example, as a nidus for anaerobic growth.

Our experiments with these substances in the presence of methylene blue need not be elaborated here. They indicate that washed asbestos wool has no effect in promoting an anaerobic zone. Indeed, in a feeble reducing medium the decolorization of the methylene blue was somewhat retarded. The oxidation of iron tacks, on the other hand, soon reduced the dye in their vicinity. It seems clear that the production of an anaerobic condition depends upon a chemical reaction rather than upon the presence of inert material.

Nature of the Culture Medium.

Presence of a Reducing Substance.—Methylene blue serves as an indicator of the presence or absence of free oxygen only in the presence of a reducing substance. For this reason it is not an accurate index for the removal of oxygen by physical means. Our observations led us to conclude, however, that complete deoxygenation is only very slowly accomplished by the diffusion of oxygen in solution into an oxygen-free atmosphere such as is provided by the method of Buchner, or that of McIntosh and Fildes.

In a recent communication Barber indicates that some strict anaerobes may be destroyed even by short exposures—less than an hour—to atmospheric oxygen. Under certain conditions, therefore, it may be important to attain strictly anaerobic conditions in the culture tube in the shortest possible time after inoculation or else to inoculate an already deoxygenated medium. Among the aids to such a procedure is the addition of an active reducing agent (kidney tissue, dextrose, peptone) to the culture medium.

Culture media in general may be divided into two classes, those which contain an active reducing substance, and those which are prac-

17 Buchner, H., Centr. Bakt., 1888, iv, 149.
19 Tarozzi and Noguchi undoubtedly obtained such a condition by preliminary incubation of tissue media to insure sterility.
tically inert. Artificial media containing dextrose or peptone belong in the first category. Ascitic fluid and dilute serum, widely used in anaerobic culture work, belong in the second class. Although small amounts of a copper-reducing substance may be demonstrated in these liquids, their deoxygenating action is relatively slight, and hardly to be considered of practical value. Thus, while kidney tissue was able to decolorize 10 cc. of ascitic fluid containing methylene blue under a vaseline seal in 10 days, similar tubes of ascitic fluid, without kidney tissue, still retained a pale blue color after 6 weeks observation.

The efficacy of dextrose as a reducing agent in alkaline solution suggested its addition to the ascitic fluid medium. Combinations of 0.1 to 2 per cent of dextrose were made by the addition of 10 per cent dextrose solution in isotonic saline solution to the ascitic fluid. Vaseline-sealed tubes containing ascitic fluid and methylene blue and 0.1 per cent dextrose were almost decolorized in 7 days. The larger amounts of dextrose up to 2 per cent, with the aid of a McIntosh and Fildes jar, decolorized the ascitic fluid in 5 to 6 days. Dextrose peptone broth was even more efficacious. One part of 1 per cent dextrose peptone broth with two parts of ascitic fluid decolorized methylene blue under a vaseline seal in less than 42 hours.

In the absence of a reducing agent tubes of ascitic fluid from which air is rigidly excluded may not become decolorized over a period of weeks. The addition of small amounts of dextrose, or the presence of kidney tissue renders ascitic fluid medium oxygen-free in a relatively short time. It should be understood that dextrose here is being considered solely as a reducing agent. Other effects—change of pH concentration through bacterial action, gas formation, etc.—may make its addition undesirable in certain instances.

Physical State of the Medium.—A second character of culture medium which influences the establishment of anaerobic conditions is its physical state. Fluid media suffer the disadvantages of ready diffusion of oxygen throughout the tube, of the development of convection currents on even slight changes of temperature, and of the transfer of oxygen to the depths of the tube by any agitation. These phenomena are easily observed in air or oil-covered tubes showing a decolorized zone at the bottom.
Semisolid media, formed by the addition of small amounts of agar, are not subject to these influences to the same extent. It is not surprising, therefore, to find that anaerobic conditions are more readily maintained in the depths of a semisolid culture tube. Thus, the effect of different amounts of agar on the diffusion of oxygen through dextrose broth was tested.

**Experiment 5.**—Tubes were filled with 1 per cent dextrose peptone broth and 2 per cent dextrose agar in proportions to give percentages of agar from 0 to 0.5 in volumes of 10 cc. The mixtures were colored with methylene blue and heated in the water bath to complete decolorization. They were then plunged into water to solidify the agar and incubated at 37°C. The diffusion of oxygen from the surface downward was measured in all the tubes by the advance of the returning color. After 5 and 24 hours the blue zones had descended as shown in Table II, where they remained during subsequent observations.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Amount of agar.</th>
<th>Penetration of oxygen from surface of medium.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>After 5 hrs.</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>1.0</td>
</tr>
<tr>
<td>2</td>
<td>0.02</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>0.04</td>
<td>0.9</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>0.9</td>
</tr>
<tr>
<td>5</td>
<td>0.1</td>
<td>0.8</td>
</tr>
<tr>
<td>6</td>
<td>0.2</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Even so small an amount as 0.02 per cent of agar may inhibit the diffusion of oxygen to the depths of a culture tube, or at least so retard it that dextrose broth is able to maintain anaerobic conditions below a certain level. This level occurred at 1 cm. from the surface when 0.5 per cent of agar was employed.

A more striking experiment is afforded by a comparison of the reducing effect of kidney tissue in peptone broth and in the same broth made semisolid by the addition of 0.25 per cent agar.
Experiment 6.—Series A; tubes contained a 9 cm. column of plain broth. Series B; the same plus kidney tissue. Series C; tubes contained a 9 cm. column of semi-solid medium (peptone broth plus 0.25 per cent agar). Series D; the same plus kidney tissue. All were colored with methylene blue and were incubated unsealed at 37.5°C. Table III shows the height of the colorless zones which developed from the bottom of the tube in each instance.

<table>
<thead>
<tr>
<th>Medium.</th>
<th>Zone of decolorization measured from bottom of tube.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>After 1 day.</td>
</tr>
<tr>
<td>Series A; plain broth without kidney tissue.</td>
<td>0 cm.</td>
</tr>
<tr>
<td>&quot; B; &quot; with kidney tissue.</td>
<td>1.5 cm.</td>
</tr>
<tr>
<td>&quot; C; semisolid medium without kidney tissue.</td>
<td>7.4 cm.</td>
</tr>
<tr>
<td>&quot; D; &quot; with kidney tissue.</td>
<td>7.0 cm.</td>
</tr>
</tbody>
</table>

Besides showing that oxygen penetrates slowly into semisolid media, this experiment demonstrates the reducing power of peptone (Series C). Comparison of Series C with Series D suggests that the reducing power of kidney tissue in a semisolid medium is confined to the zone surrounding the tissue, since its effect did not extend further than that of the peptone broth alone.

This observation is confirmed by the following experiment with a medium of much less reducing power, so that the kidney tissue alone was responsible for the production of an anaerobic zone.

Experiment 7.—Series A, two tubes, each containing one-tenth of a medium sized kidney, 7.5 cc. of dilute rabbit serum, 2.5 cc. of 2 per cent plain agar, and 1 drop of methylene blue, making a 0.5 per cent semisolid medium. Series B, two tubes, each containing the same ingredients, except that 2.5 cc. of plain broth were substituted for the agar, making a fluid control. Control tubes of each medium, without kidney tissue, were also set up. Vaseline seals. Tubes incubated at 37.5°C. for 24 hours. The tubes of Series A showed a decolorized zone 1.7 and 1.7 cm. high respectively, sharply demarcated from the deep blue agar above. Control, without kidney, deep blue. The tubes of Series B were decolorized 2.4 and 2.3 cm. from the bottom, respectively, shading off gradually to a deep blue above. Control, without kidney, deep blue. After 48 hours the semisolid tubes showed a clear-cut colorless zone, 2.7 and 2.6 cm. high; agar deep blue above. Control
deep blue. The fluid tubes showed a colorless zone approximately 4 to 4.5 cm. high, shading off so gradually that its limits were difficult to determine. The fluid above and the control were light blue.

From these experiments a semisolid medium is seen to favor the establishment of anaerobic conditions by the exclusion of oxygen from the depths of the tube. Unless a reducing agent is diffused through the medium, however, this advantage is offset by a restriction of the reducing power of the kidney tissue to the immediate vicinity of the tissue fragment. These observations may explain in part the difficulty often encountered in growing strict anaerobes in semisolid medium, especially in initial cultivation.

Length of the Column of Medium as an Aid in Deoxygenation.—It has become the standard practice in anaerobic cultivation by the tissue method to use 12 to 15 cc. of fluid, or a column 10 to 12 cm. in height in a long narrow culture tube 20 by 1.5 cm. Anaerobiosis in the depths of the tube would seem to be favored by the restricted surface exposed under paraffin oil and by the length of the column of liquid.

It was found in the present study that in a reducing medium such as dextrose broth a column length of from 8 to 16 cm., overlaid with paraffin oil, favors the decolorization of methylene blue, which occurs first at the bottom of the tube and gradually extends toward the surface. Under vaseline, on the other hand, columns of different lengths decolorize with equal rapidity. In dextrose broth the reducing action proceeds throughout the tube. When it is more localized, as with kidney tissue in a less active medium (plain broth), the effect is somewhat different.

Experiment 8.—Three sets of tubes of plain broth colored with methylene blue, each containing one-sixteenth of a kidney of a large rabbit, the broth columns being 2, 4, 6, 8, 10, and 12 cm. long. Series A, unsealed; Series B, overlaid with 2 cm. of oil; Series C, overlaid with 1 cm. of vaseline. Control tubes of broth without kidney, column 8 cm. long, unsealed and also with oil and vaseline seals. Incubated at 37.5°C. In 16 hours decolorization had proceeded as shown in Table IV.

When oxygen is excluded (Series C) the fragment of kidney tissue is able to produce an anaerobic zone 2.1 cm. high in 16 hours. That
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it did not do so in the 2, 4, and 6 cm. tubes of Series A and B under air or oil is due to the penetration of oxygen. A column of fluid 8, 10, or 12 cm. long maintained at a constant temperature without agitation practically serves as a seal for the lower levels. But the same object is accomplished by a vaseline seal with considerable saving in medium; 7 or 8 cm. of the medium suffice.

### TABLE IV.

**Effect of the Height of the Medium Column on the Anaerobic Zone Produced by Kidney Tissue.**

<table>
<thead>
<tr>
<th>Series</th>
<th>Height of decolorized column from bottom of tube after 16 hrs.</th>
<th>Control.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length of fluid column.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 cm.</td>
<td>4 cm.</td>
</tr>
<tr>
<td>A</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>B</td>
<td>1.3</td>
<td>1.8</td>
</tr>
<tr>
<td>C</td>
<td>2.0</td>
<td>2.1</td>
</tr>
</tbody>
</table>

**Effect of Reducing Agents on the pH of the Medium.**

Although this study deals only with the deoxygenation of culture media, it is necessary to know whether the reducing agents employed have any effect upon other essential factors in anaerobic cultivation; for example, the hydrogen ion concentration of the medium. Accordingly, tubes were filled with ascitic fluid or with ascitic fluid and dextrose peptone broth, with or without kidney tissue. To each tube 5 drops of phenol red were added and the tubes incubated at 37.5°C. under a vaseline seal. When observed at intervals during the following days and weeks, the tubes without kidney tissue showed no change from the original hydrogen ion concentration of 8+. On the other hand, the kidney tissue in 20 hours had produced a clear yellow zone approximately 1.5 cm. from the bottom of the tube. The effect was the same in ascitic fluid and in the mixture of ascitic fluid and broth. The next day and thereafter, all the tubes containing kidney tissue showed a gradation from purplish pink (pH 7.8 to 8) at the surface downward in the acid direction to a clear yellow in the middle and lower portions of the column of medium. This color
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gradually became diffused throughout the tubes, bringing the medium to an orange-yellow comparable to pH 7.4 on the phenol red scale.

Ascitic fluid itself is usually alkaline, showing a pH 7.8 to 8+. This alkalinity is progressive if the fluid is allowed to stand exposed to the air but may be retarded by a layer of paraffin oil. From the foregoing experiment it is seen that the alkalinity, once established, remains unaltered for weeks under a vaseline seal. Dextrose and peptone do not in themselves produce acid and thus change the hydrogen ion concentrations. Only when they are split by bacterial activity do they have this effect. Kidney tissue produces acids; a large fragment (0.8 gm.) changes the hydrogen ion concentration in its immediate vicinity to about pH 7. This acid becomes gradually diffused throughout the medium and may ultimately bring the entire column of medium to a favorable hydrogen ion concentration (pH 7.4). The final concentration, then, depends on the ratio of the original alkalinity of the ascitic fluid to the acid production of the kidney.

SUMMARY AND CONCLUSIONS.

This study was undertaken with the object of determining the part played by the several component elements of the tissue method of anaerobic cultivation in the establishment of anaerobic conditions in the culture tube. Data have been presented to show the suitability of methylene blue as an indicator of reduction processes in culture media by which the removal of the last traces of oxygen may be demonstrated. With methylene blue as the indicator, the elements subjected to experiment included the choice of a seal for culture tubes, the activity and requisite size of the kidney tissue fragment, the chemical and physical characters of the medium which promote or retard deoxygenation, the length of the column of medium, and the advantages of external aids such as the McIntosh and Fildes anaerobic jar.

As a result of our experiments, we have come to the following conclusions:

1. Liquid paraffin oil, used extensively as a seal for anaerobic cultures and in gas analysis, has very little value in inhibiting the access of oxygen. Solid vaseline, on the other hand, forms an effective
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oxygen-resisting seal. The difference is due to the physical states of the substances at incubator temperature.

2. Fresh kidney tissue is an active reducing agent and quickly decolorizes methylene blue in its vicinity. The reducing effect of fresh kidney tissue is relative to the amount used. As a reducing agent, at least 0.6 gm. per tube is required for the establishment of an adequate oxygen-free zone.

3. Culture media may be classified as reducing or non-reducing. Those containing dextrose or peptone in a faintly alkaline solution belong to the former class. Ascitic fluid and dilute serum belong to the latter class, for their content of reducing substances is practically insignificant. For the prompt establishment of strictly anaerobic conditions these media require the addition of reducing substances such as dextrose, peptone, or kidney tissue aided by an effective seal or an anaerobic jar.

4. Semisolid media effectively inhibit the penetration of oxygen to the depths of the tube, but they likewise limit the diffusion of reducing substances and presumably of nutrient substances from imbedded kidney tissue.

5. The length of the column of medium is of minor importance under a vaseline seal.

We clearly recognize the impracticability of standardizing a biological technique which by its very nature must be subject to wide modifications for special purposes. Such variations from a standard are especially necessary in the search for unknown organisms, and in work with hitherto uncultivated microbes in which the tissue technique has been successfully applied by Noguchi.

We wish, therefore, to present the results of our studies simply as guides in the variation and control of the elements examined and to make certain suggestions relative to the establishment of strictly anaerobic conditions in the culture tube. The numerous other factors of equal importance which must be taken into account—hydrogen ion concentration, source and character of nutritive elements, temperature, time, etc.—are outside the limited scope of the present report.

For the establishment of strictly anaerobic conditions in the culture tube, we would suggest (1) the substitution of solid vaseline for liquid paraffin oil as an oxygen-resisting seal; (2) the use of large pieces
of fresh kidney, the standard size to be upwards of 0.6 gm. unless other reducing substances are present in the medium; (3) the addition of peptone or dextrose or both in the form of peptone dextrose broth in fractional percentages to non-reducing media such as ascitic fluid or serum to aid in the prompt establishment of anaerobic conditions; (4) the use of the McIntosh and Fildes jar as a further aid to the prompt deoxygenation of the medium; (5) for reasons of economy the use of smaller amounts of culture medium, for example, 7 to 8 cc., under a vaseline seal; and (6) in dealing with anaerobes which may be injured by exposure to oxygen it might be advisable to prepare the medium a day or two in advance and to incubate it under a vaseline seal so that sterility is assured and the anaerobic conditions are already established when inoculation is made. The infected material is then introduced with a capillary pipette in the vicinity of the kidney tissue and the seal restored by gentle heating to melt a portion of the superposed vaseline.

EXPLANATION OF PLATE 2.

FIG. 1. A comparison of paraffin oil and vaseline as oxygen-resisting seals. Sample tubes from Experiments 1 and 2. Dextrose broth and methylene blue without and with kidney tissue, unsealed and under paraffin oil and vaseline. Tubes 1, 3, and 5, without kidney, were deoxygenated by the dextrose broth in a McIntosh and Fildes jar. On removal from the jar the color has returned in the unsealed and oil-covered tubes (Nos. 1 and 3), denoting the penetration of oxygen into the medium. The vaseline-covered tube (No. 5) remains colorless.

Tubes 2, 4, and 6, with kidney, show its reducing effect in the depths of the tube. The unsealed broth (Tube 2) shows almost as extensive a zone of decolorization as the oil-covered broth (Tube 4). Aided by complete exclusion of oxygen, the kidney and dextrose broth have completed the deoxygenation of the vaseline-covered tube (No. 6).

FIG. 2. A comparison of the deoxidizing value of kidney tissue fragments of different size. A, a fragment of the size ordinarily employed. B, a fragment twice the size of A. C, a fragment four times the size of A. The establishment of an oxygen-free zone is denoted by the decolorization of the medium.
(Gates and Olisky: Factors influencing anaerobiosis.)