NEW ANAEROBIC METHODS.*

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PLATE 8.

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The methods commonly used for the cultivation of anaerobic microorganisms are far from satisfactory. This applies to the partial anaerobes, such as the tetanus bacillus or *Bacillus botulinus*, but is particularly true of the absolute anaerobes, such as *Treponema pallidum* or the globoid bodies of poliomyelitis.

The vacuum jar described by Noguchi[1] in his method for the cultivation of *Treponema pallidum* has proved satisfactory in his hands, but requires a very strong desiccator, accurately ground, and a special type of vacuum pump. The American desiccators which I have obtained during the past 2 years, however, have either been so poorly ground that they would not hold a vacuum, or else they were so poorly made that they collapsed when subjected to vacuum.

After many trials, my difficulties were finally overcome through utilization of the suggestions of Laidlaw,[2] who used a catalyzer of oxygen and hydrogen in the preparation of anaerobic culture tubes. McIntosh and Fildes[3] developed the use of the catalyzer in making an anaerobic container, the "McIntosh bomb," but neither of these methods, useful as they are for the purposes for which they were devised, were suitable to our needs, and were therefore modified.

All the anaerobic methods to be described depend upon the catalytic action of platinized asbestos upon oxygen and hydrogen when they are brought into contact.

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[1] Noguchi, H., *J. Exp. Med.*, 1911, xiv, 99.

[2] Laidlaw, P. P., *Brit. Med. J.*, 1915, i, 497.

[3] McIntosh, J., and Fildes, P., *Lancet*, 1916, i, 768.

Methods for Anaerobic Test-Tubes.

The simplest method is used in the cultivation upon agar slants of such relative anaerobes as the tetanus bacillus or *Bacillus botulinus* and is similar to the method described by Laidlaw. Platinized asbestos is first prepared in the usual way, or it may be purchased from any laboratory supply house. A small mass of the catalyzer is firmly fixed at the end of a platinum wire by coiling the wire about it. The other end of the wire is inserted into a short glass rod, and the rod is inserted into a No. 1 one-hole rubber stopper. The apparatus is wrapped in a package and autoclaved.

The water of condensation is removed from a plain agar slant, the tube inoculated, inverted, the cotton plug removed, and the tube filled with hydrogen by means of a sterile capillary pipette. The hydrogen may be obtained from a Kipp generator, or more satisfactorily from a hydrogen tank. It should be passed through a series of wash bottles containing silver nitrate, sulfuric acid, potassium permanganate, and lead acetate to remove all impurities.

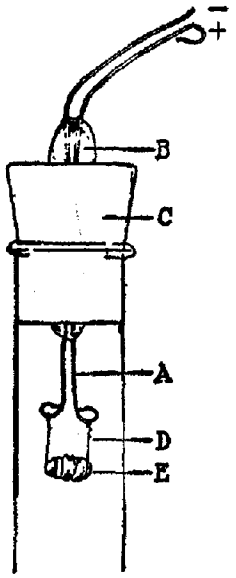
After allowing the hydrogen to fill the inverted inoculated test-tube, the platinized asbestos is heated for a moment in a free flame, the rubber stopper is inserted firmly into the inverted tube, and the end of the tube dipped into melted paraffin.

The catalyzer glows for a second or two as the hydrogen and oxygen are actively united, and the water formed is deposited upon the surface of the tube. The process is now complete, and the tube is ready for incubation.

This is essentially the method devised by Laidlaw and is very satisfactory for the growth of the usual anaerobes. The tetanus bacillus, for example, grows upon an agar slant in a thick, felt-like mass, in the profusion of its growth resembling a culture of *Bacillus subtilis*. It was necessary to devise a more strictly anaerobic method for our work, however, for Laidlaw's method could not always be relied upon to remove all traces of oxygen in the air, nor does it remove the oxygen from the surface of the media itself. The following method was devised to remove all the oxygen and has proved very satisfactory.

Two lengths of No. 22 nichrome wire, 6 cm. long, are separately

fused into a glass tube so that they are insulated (Text-fig. 1, A), and the glass tube, B, closed at each end, is passed through a one-hole rubber stopper, C. To the lower ends of the nichrome wire is attached a coil of fine (No. 31) nichrome wire, D, thus completing the circuit. In the coils of the fine wire is placed a small mass of platinized asbestos, E. The apparatus is placed in a package and autoclaved.



TEXT-FIG. 1. Sketch of anaerobic apparatus for the cultivation of absolute anaerobes in test-tubes.

A large test-tube, 20 by 1.5 cm. is used, to which 10 cc. of media are added, sterilized, and slanted. The water of condensation is removed and the tube inoculated. The tube is inverted, the cotton plug removed, and the tube filled with hydrogen by means of a sterile capillary pipette. The platinized asbestos mass, E, is heated for a moment in a free flame, and the rubber stopper, C, is then firmly inserted into the inverted tube and the end of the tube dipped in melted paraffin. The tube may now be placed in an upright position and sufficient electric current applied to the free ends of the wire to heat the fine nichrome wire wrapped about the platinized asbestos to a red heat. The catalyzer is thus heated, and the free

oxygen and hydrogen unite to form water. The tube is set aside for ½ to 1 hour, then the platinized asbestos is reheated in order to ignite any residual oxygen. The tube may now be incubated.

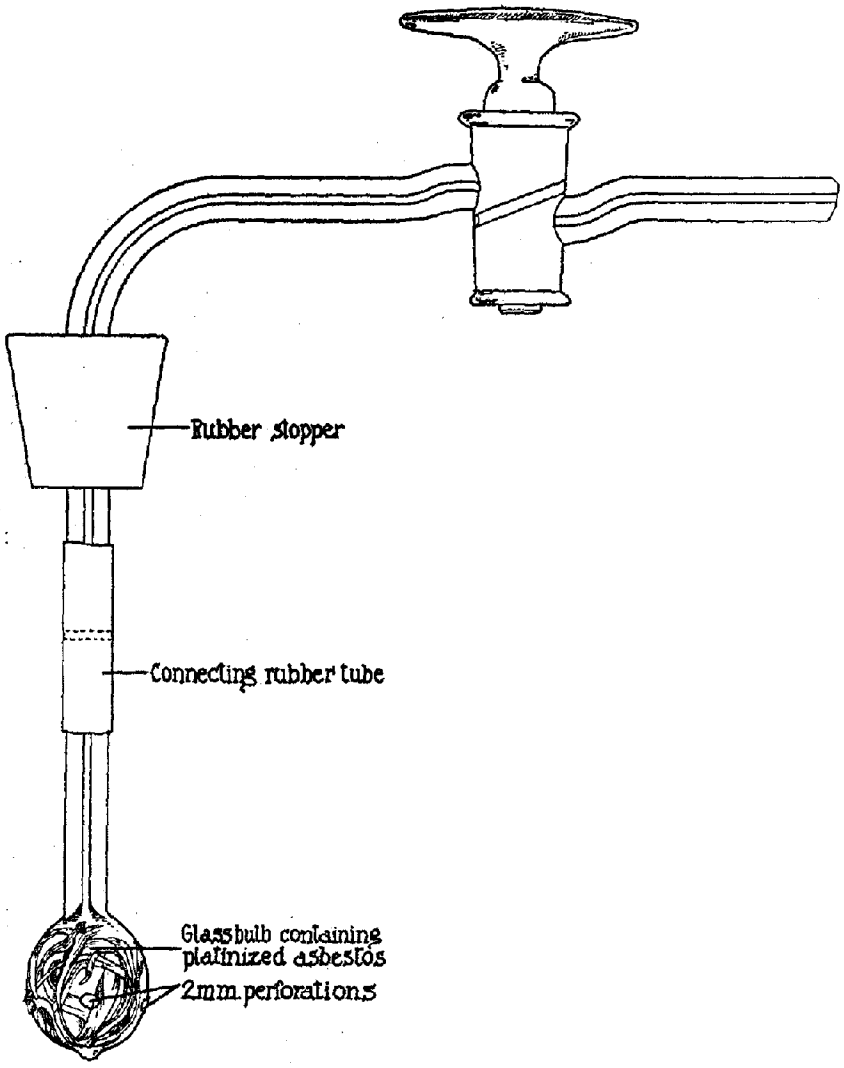
The method is very useful in growing all anaerobes, for the oxygen is always removed, whereas the Laidlaw method frequently fails. It is particularly useful for the cultivation of the stricter anaerobes. By this method the fusiform bacillus, for example, which is more strictly anaerobic than the tetanus bacillus, grows profusely in a thick, felt-like mass. By using this technique the organism of poliomyelitis grows upon ascitic agar slants, though much more slowly than other organisms; in fact, no growth is seen for 4 to 5 days, but at the end of 6 to 7 days, definite, tiny, raised, glistening colonies appear. These grow larger and larger, so that at the end of 12 to 14 days a definitely circumscribed, raised, opalescent colony is seen, some of these colonies even becoming as large as 1 mm. in diameter. In no instance was a growth of the globoid bodies of poliomyelitis obtained from the original material—brain and cord—but only from the fluid culture tubes of globoid bodies which had been growing in ascitic fluid media for several generations.

Method for an Anaerobic Jar.

The chief problem with which I was concerned, however, was the construction of an anaerobic jar, to be used as a container for a large number of tubes in the isolation of the organism of poliomyelitis from the infected brain and other tissues. All methods used for the cultivation of the globoid bodies failed in my hands until the jar described below was devised.

The jar used is an ordinary museum specimen jar (Fig. 1) about 30 cm. high and with an inside diameter of 12.5 cm. Two holes, 1.5 cm. in diameter, are ground in the cover, and into each hole is firmly inserted a No. 4 one-hole rubber stopper, carrying a ground glass "angle" stop-cock. To one of the stop-cocks is attached a rubber tube, at the end of which is a short piece of glass tube which reaches to the bottom of the jar. To the other stop-cock is attached, by a short rubber tube, a glass bulb, 2 cm. in diameter, which has been blown on the end of a capillary glass tube. The glass bulb is perforated with 5 to 6 holes, 2 mm. in diameter, and is filled with

platinized asbestos. The details of this apparatus are shown in Text-fig. 2.



TEXT-FIG. 2. Detail of the platinized asbestos bulb for the anaerobic jar.

Cultures are inoculated and placed in a glass tumbler, which is then placed in the jar, to which 100 cc. of a 10 per cent pyrogallic acid solution have been added.

The glass bulb containing the platinized asbestos is heated over the free flame for a few seconds, and the cover is then cemented on. A rubber ring, 0.5 cm. thick, is placed between the jar and cover, all surfaces are cemented with Major's glass cement, and the metal clamp is screwed down with thumb and forefinger. The stop-cock to which the glass bulb is connected is placed on the vacuum pump, and gentle suction is applied for 2 to 3 seconds in order to insure a good initial flow of hydrogen and thus ignite the platinized asbestos at once. The stop-cock is now closed and attached to the hydrogen apparatus, and the gas is allowed to enter. This should be done carefully at first in order that an excess of hydrogen does not enter at once; for the gas should be burned as rapidly as it enters the jar. The platinized asbestos will soon be seen to glow and from this time hydrogen and oxygen will slowly unite, and the water formed will be deposited on the sides of the jar. When all the oxygen has united with the hydrogen, the platinized asbestos will become cool, but the hydrogen will continue to enter the jar until all the space formerly occupied by oxygen is replaced by hydrogen. The result is a hydrogen-nitrogen jar under approximately atmospheric pressure. The whole process should take about 15 minutes.

In order to have an index of the completeness of anaerobiosis the second stop-cock is connected with a bottle of 20 per cent sodium hydrate, freshly washed with hydrogen. By means of slight suction through the first stop-cock, 25 cc. of the sodium hydrate solution are drawn into the jar. Both stop-cocks are now closed, the ends sealed with cement, and the jar is incubated.

If the jar is satisfactory, the mixture of sodium hydrate and pyrogallic acid will remain colorless indefinitely. This solution should not be relied upon to absorb any remaining traces of oxygen, but is simply an indicator of the presence of oxygen, and if it becomes discolored, there has been a mistake in technique, and the jar is unsatisfactory; therefore the cover should be removed, and the process repeated.

Method for Blake Bottles and Flasks.

The use of the platinized asbestos in a perforated glass bulb has been applied to mass cultures of anaerobes in flasks or Blake bottles.

The Blake bottle is useful when large amounts of an anaerobic organism, such as the tetanus bacillus, are desired, and is particularly useful when a differential anaerobic plate is desired as, for example, in the isolation of anaerobes from the pus of infected wounds.

A ground glass stop-cock of the usual type is inserted through a No. 3 one-hole rubber stopper. A heavy glass bulb, 1 cm. in diameter, is blown at the end of the stop-cock, and five or six small perforations are made in the bulb. The bulb is then filled with platinized asbestos, and the whole apparatus autoclaved.

The Blake bottle is inoculated, the platinized asbestos heated in a free flame, and the rubber stopper tightly inserted into the bottle. Slight vacuum is then produced in the bottle by gentle suction, in order to insure a good flow of hydrogen. The stop-cock is connected with the hydrogen generator and the gas allowed to enter. The catalyzer glows for a few minutes, then cools, and in 5 minutes the oxygen has been replaced by hydrogen, and the process is complete.

The use of the perforated bulb has not proved satisfactory in single test-tubes because of the small air space.

The methods are simple, rapid, clean, and efficient. They are not expensive, for only a small amount of platinized asbestos is needed, and the material may be used repeatedly without deterioration. It would at first appear that there might be some danger of explosion in the jar, but such is not the case. The hydrogen ignites as soon as it enters the jar so that there is never an excess of hydrogen in the container. As the hydrogen and oxygen unite to form water, a slight vacuum is formed and this vacuum insures a continuous gentle flow of hydrogen until all the oxygen has been replaced.

The precautions to be taken are as follows: (1) Allow the hydrogen to enter the jar slowly. (2) Be sure that the hydrogen is catalyzed as it enters, as evidenced by the glowing of the asbestos. After the glow has once appeared the remainder of the process will continue gently and completely. (3) Do not disconnect the apparatus while the process is taking place, for there is a slight vacuum in the jar which has only partially been replaced by hydrogen, and if air is allowed to rush in over the catalyzer, there is a possibility of a slight explosion.

The results that have been obtained by the use of these methods, particularly the anaerobic jar, will be published in a subsequent paper.

CONCLUSIONS.

1. Anaerobic methods have been devised which depend upon the catalytic action of platinized asbestos upon hydrogen and oxygen.

2. The methods may be utilized for the growth of anaerobes in test-tubes, upon Blake bottles, in flasks, and in a large container.

3. Because oxygen is so completely removed, the methods are of great value in the successful cultivation of absolute anaerobes.

EXPLANATION OF PLATE 8.

FIG. 1. Anaerobic jar with platinized asbestos bulb.

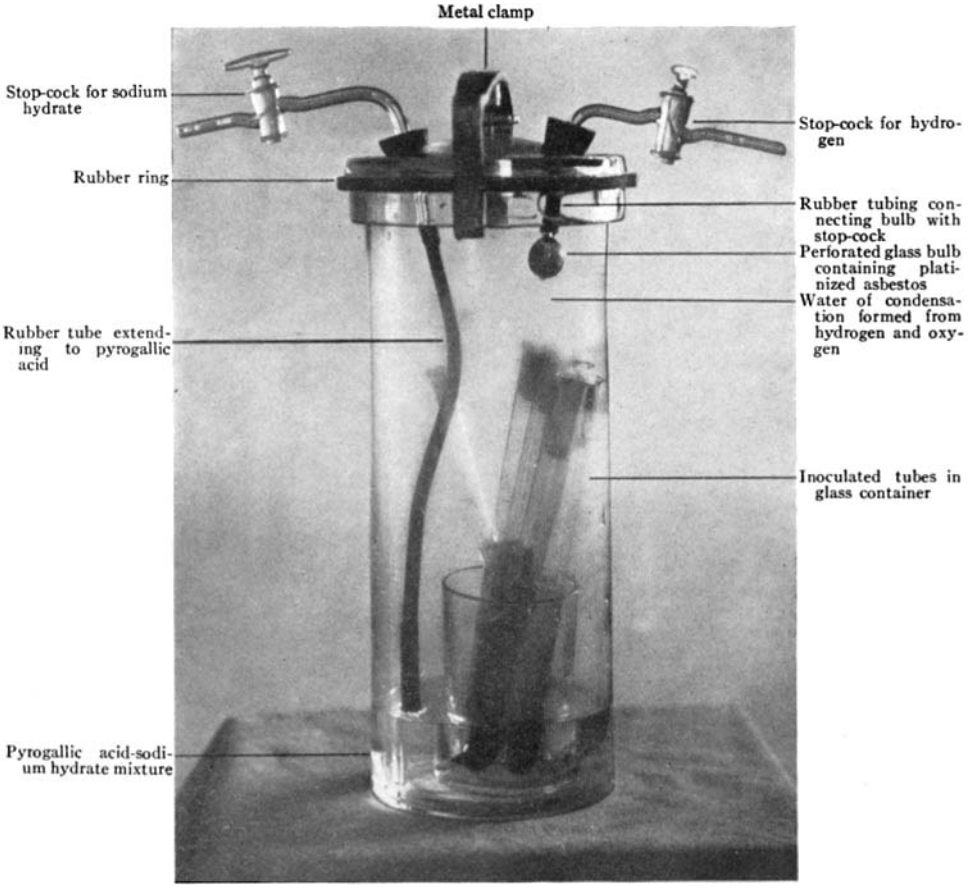

Metal clamp
Stop-cock for sodium hydrate
Rubber ring
Rubber tube extending to pyrogallic acid
Pyrogallic acid-sodium hydrate mixture
Stop-cock for hydrogen
Rubber tubing connecting bulb with stop-cock
Perforated glass bulb containing platinized asbestos
Water of condensation formed from hydrogen and oxygen
Inoculated tubes in glass container

FIG. 1.