LIFE WITHOUT OXYGEN — THE WORLD OF THE ANAEROBES

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IN trying to evaluate the anaerobic bacteria and their need for anaerobiosis, it will be helpful at first to take an evolutionary point of view. According to Oparin, the Russian biogeologist, and others who have struggled with the problem of the origin of life on the earth, at one time all the world was anaerobic. There was almost no oxygen in the atmosphere for it was all bound as water, as sulfate, as carbonate, as carbon dioxide, and the atmosphere probably contained less than 0.01% of oxygen. It seems most likely that life first appeared during this anaerobic phase of the earth's history. If it did so, the first forms of life — our most remote ancestors — were obligate anaerobes.

We do not know what those earliest anaerobes looked like, but we can be fairly sure of the main features of their metabolism. Almost certainly, they obtained energy by the controlled transfer of electrons from one organic substance to another. And almost as certainly, they trapped energy in high-energy bonds, probably high-energy-phosphate bonds.

But all the world did not remain anaerobic. At some time during this anaerobic phase, a system of photosynthesis developed, a system of photosynthesis similar to that of the green plants, in which the oxygen of water is transformed to gaseous oxygen. When this photosynthetic system developed, when the first bubble of oxygen rose through the water of the sea, the earth started to become aerobic.

As the oxygen content of the atmosphere increased, aerobic metabolism must have developed. Basically, this differed from anaerobic metabolism in one way — oxygen became the terminal electron acceptor. This change in the terminal electron acceptor gave the aerobic organisms several advantages. First, they could obtain much more energy — 10 to 20 times as much — from the same amount of food. Second, they could use some substances, particularly highly reduced substances, as energy sources, which the obligate anaerobes could not use.

However, the aerobic metabolism suffered from one drawback — it

could proceed no faster than oxygen could dissolve in water. Oxygen is only slightly soluble in water. Water in equilibrium with the air contains only about 0.0014% oxygen — about the same solubility as calcium carbonate — and this low level of solubility is the limiting factor of aerobic metabolism.

Anaerobic metabolism, while limited in the amount of energy that can be obtained from a given amount of food, and limited in the number of kinds of substrates that can be used for energy sources, nevertheless has an important advantage. It can provide energy very quickly and continuously at a high rate so long as a fermentable substrate is present, or two compounds between which electrons can be transferred, one of the compounds being oxidized and the other reduced.

Even the higher forms of life, as they developed, retained the anaerobic form of metabolism. In man, almost all tissues can metabolize anaerobically except for the central nervous system. It is only because the central nervous system has an obligately aerobic system of metabolism that man considers himself an aerobe. Our muscular system, on the other hand, can shift smoothly and imperceptibly from aerobic to anaerobic metabolism, which it does when we need sudden or sustained muscular activity.

As evolution progressed on the earth, the aerobes, both facultative and obligate, and the anaerobes became mutually complementary until both were needed for a complete ecological system, anaerobic bacteria, for example, being required for the reduction of nitrate to gaseous nitrogen, an essential step in the nitrogen cycle.

Moreover, even as the aerobes developed the ability to use atmospheric oxygen as the final electron acceptor, they also attained an ability that the anaerobes never attained — they became tolerant of oxygen and various oxidized substances. Oxygen can be a very toxic gas for any form of life that is not equipped to handle it, and the anaerobes never developed this ability.

Consequently, we can conclude that anaerobes are anaerobes because of the toxic or inhibiting effect on them of oxygen. But further than this, we do not have any generally accepted definition of the term "anaerobe" even by the microbiologists who work with this group of organisms. One group of microbiologists define the anaerobes, rather vaguely, as "bacteria that grow better in the absence of air than they do in its presence". Another group views them as "bacteria that are unable to initiate growth from small inocula unless the oxidation-reduction potential of the medium is low", and still a third group defines them as "those bacteria that perish even on transient contact with atmospheric oxygen". All these definitions are, in some measure, satisfactory and in some measure incomplete.

One need not be surprised at this variety of views concerning these bacteria, for the anaerobes themselves form a very heterogeneous and divergent group, even in their sensitivity to oxygen and their need for anaero-

bic conditions for growth. Some, such as *Clostridium tertium* and *Clostridium carnis,* can very slowly grow on the surface of solid media exposed to air. Some others, such as *Clostridium perfringens,* are just barely anaerobes, and require only slight precautions to be isolated and grown. Others are strict anaerobes and are unable to grow if the atmosphere contains as little as 0.03 per cent oxygen. Among such strict anaerobes are *Clostridium haemolyticum, Treponema macrodentium, Butyrivibrio fibrisolvens* and several other species.

Not only do the anaerobes vary among themselves, but the cells in a pure culture also vary tremendously in sensitivity. For example, *Propionibacterium acnes,* the organism that forms the major part of the microbial population of the human skin, has long been known as an easy anaerobe to cultivate, for it grows readily on surface-inoculated blood agar plates incubated in an anaerobe jar. However, if we view the situation quantitatively instead of qualitatively, and do comparative counts using a log-phase inoculum and comparing surface-inoculated plates incubated in an anaerobe jar with strictly anaerobic methods, we find that the count will be about one thousand times higher in the roll tubes than on the surface of the blood agar plates. Or to view this another way, only one cell out of a thousand in our inoculum was able to grow on the surface of a blood agar plate, inoculated in the presence of air. Obviously, not all the cells in our culture were alike. Furthermore, if we compare the sensitivity of anaerobes to oxygen at various stages of growth, we find that they are much more sensitive to the toxic effect of oxygen when they are in the logarithmic phase of growth. Older resting phase cells are much less sensitive to oxygen.

Unfortunately, this variation in sensitivity has not been generally recognized. A great confusion of ideas and much wasted work has resulted from the quite unjustified assumption that all of the anaerobes are similar in their sensitivity to oxygen and that they will all grow satisfactorily if the concentration of gaseous oxygen is reduced to some suitable low level. If the situation were this simple, we would have no trouble isolating the anaerobes, but it is not this simple. The mere absence of oxygen is not the only factor concerned.

There is no doubt that the presence of oxygen inhibits the growth of the anaerobes, but it is not clear just how oxygen does this. Oxygen could be inhibitory in several ways, directly or indirectly. First, oxygen itself could be toxic; second, it could be indirectly inhibitory by raising the oxidation-reduction potential to a level where some essential enzymes of the bacteria would become inactive; third, it might react with some component of the medium to form a toxic or inactive compound; or it might do several of these things at the same time. Let us examine each of these possibilities and see if we can get some clearer idea of the effect of oxygen, or to put it in simpler terms, let us see why anaerobes are anaerobes.

The first that we shall consider is the direct effect of gaseous or dissolved oxygen. There is no evidence indi-

cating that any essential growth factors or intermediate metabolites are converted by oxygen to inactivate or toxic compounds. We do know, however, that different enzyme systems within a single strain of anaerobe may have differing susceptibilities to oxygen. *Propionibacterium acnes.* for example, under anaerobic conditions can readily metabolize lactic acid to propionic and acetic acids. However, if the environment is only slightly aerobic, *P. acnes* is unable to metabolize lactic acid, although otherwise growth seems to take place normally. In this case, it is evident that the enzymes that are essential for the reduction of lactate are more sensitive to the effect of oxygen than are the enzymes that are essential for the growth of *P. acnes.* This apparently is not the case with the really strict anaerobes, for with them, the enzymes essential, for growth and reproduction appear to be the most sensitive.

It is possible that oxygen has an immediate toxic effect on bacterial cells by causing an increase in the number of free radicals within the cell. Although this has not, to my knowledge, been investigated with anaerobic bacteria, it has been demonstrated in freeze-dried cells of aerobic organisms exposed to the air. In investigations by two different groups of workers, when oxygen was admitted to the vials the number of free radicals increased markedly, and concurrent with the increase in free radicals, was a marked increase in the death of the bacteria. About two hundred free radicals per bacterial cell were enough to cause death. It is evident, then, that oxygen can be directly toxic, even to bacteria that are metabolically inactive.

For many years, it was thought anaerobes could not grow in the presence of air because if they did, they would produce lethal amounts of hydrogen peroxide but would not produce catalase to break down this toxic substance. While this theory of microbial suicide was attractive in some ways, there were certain facts that kept it from being accepted. First, not all the anaerobes did produce peroxide when exposed to the air, at least in detectable amount. Second, although some of the anaerobes did produce catalase, or some other enzyme which decomposed hydrogen peroxide, they could not grow in the presence of air. And finally, the addition of purified catalase to media for the anaerobes did not allow them to grow in the air. This catalase theory might hold for a few of the anaerobes; it certainly can not hold for very many.

Recently, a somewhat similar suggestion has been advanced. It has been found that almost all cells, bacterial or otherwise, that use atmospheric oxygen in their metabolism produce *superoxide.* Superoxide is a highly reactive, free-radical form of molecular oxygen — O_2^-. It can be broken down by the enzyme *superoxide dismutase.*

$$O_2^- + O_2^- \rightarrow O_2 + H_2O_2$$

The hydrogen peroxide formed in the breakdown of superoxide by the superoxide dismutase can, in its turn, be broken down by catalase This en-

zyme is found in all tissues and in the cells of aerobic bacteria but is not found in the cells of the anaerobic bacteria. Consequently, we can consider that the lack of the enzyme superoxide dismutase may be one of the factors involved in the need of the anaerobic bacteria for anaerobiosis.

Another effect of oxygen that may be important is its action in raising the oxidation-reduction potential. This potential is a measure of the tendency of a system to give up electrons, and it undoubtedly is of importance in maintaining in an active state those enzymes with sulfhydryl groups. The initiation of growth of some anaerobic bacteria seems to be largely a function of the oxidation-reduction potential. Once anaerobes have started to grow, many of them can maintain a low oxidation-reduction potential by means of the reducing substances that they produce. The kind and amount of reducing substance varies considerably from species to species. In general, the anaerobes that are easiest to grow produce the most reducing substance. *C. perfringens* for example, is so active in producing reducing substance that even bubbling a stream of air through a growing culture will not stop its growth. Moreover, for *C. perfringens,* it is beginning to look as if part of the pathogenic effect of this organism is simply the production of reducing substance.

The hypothesis that the oxidation-reduction potential is of primary importance in the growth of anaerobes has been investigated by several workers. Unfortunately, none of them used strict anaerobes, but all worked with bacteria that have no more than a moderate need for anaerobiosis. However, when moderate anaerobes were used, such as *Clostridium sporogenes, Clostridium perfringens* and *Bacteroides vulgatus,* the hypothesis seemed to hold. These organisms could be grown in broth through which air was bubbled, if the potential was held at definite levels by electrical means. It is apparent that these organisms, under these conditions, were not inhibited by oxygen itself, nor did they form any appreciable amount of toxic substances from the oxygen. The ability of these anaerobes to grow when the oxidation-reduction potential was held low, regardless of other conditions, indicates that this may be the most important factor for these species, and that other inhibiting effects of oxygen may be insignificant for them.

All anaerobes probably have a limiting oxidation-reduction potential above which they cannot grow. Determination of such a level is a complicated problem for it varies with the hydrogen ion concentration. However, let us consider a few such findings, and compare them with the oxidation-reduction potential of the circulating blood — about 180 millivolts. The upper limit for *Clostridium sporogenes* at pH 7 is about 150 millivolts — that is, 30 millivolts below that of blood. The upper limit for *Clostridium histolyticum* is about 90 millivolts, considering more reducing. That for *Bacteroides vulgatus* is 140 millivolts. *Clostridium perfringens* has been studied most thoroughly. The limiting potential may be as low as 30 millivolts at pH 7.8, or as high as 250 millivolts at pH 6.0.

However, we must regard these values with a certain amount of reserve, because the determination of limiting potential is not simple. For example, when anaerobic organisms are inoculated into an otherwise satisfactory medium with a high oxidation-reduction potential, they either do not grow or else respond with an exaggerated lag period. When growth finally does start, it usually proceeds at the normal rate. During the long lag period, the bacteria lower the potential of their immediate surroundings by metabolizing nutrients carried over in the inoculum or occurring in their immediate vicinity. Once the potential is lowered in the immediate vicinity of the organisms and metabolism in actively started, reducing substances are produced and the whole volume of the medium becomes anaerobic.

Because material carried over in the inoculum, either within the bacterial cells or otherwise, can aid in lowering the oxidation-reduction potential, the size and metabolic state of the inoculum are important. The larger the inoculum, the higher the oxidation-reduction potential at which the organisms can start growth. The more metabolically active the cells in the inoculum, the higher the potential at which they can start growth. Consequently, limiting values for the growth of anaerobic species may not be as definite and as precise as they look.

It is not difficult to establish reducing conditions in a medium. Reducing agents are readily available and may be added before or after sterilization. It is only necessary to prevent their oxidation by the oxygen of the air. The reducing agents often used include cysteine, sodium sulfide, sodium sulfite, sodium thioglycollate, and reductone — freshly autoclaved glucose. One compound that has come into use recently, is dithio-threitol, or its close relative, dithio-erythritol. This is usually used in combination with cysteine, and they form a very effective pair. If cysteine is used alone, it is rapidly oxidized by atmospheric oxygen to the insoluble cystine, and loses its reducing ability. Dithio-threitol, however, is an active reducing agent in its own right, furnishing an oxidation-reduction potential somewhat lower than that of cysteine. It does not react with atmospheric oxygen and, moreover, it prevents cysteine from doing so. Consequently, a combination of dithiothreitol and cysteine is very useful, particularly in plating media when some of the strict anaerobes are to be isolated.

In leaving the subject of the oxidation-reduction systems, we might ask the question — Is this the entire answer to the problem of growing anaerobic bacteria? If this be the case, the problem would be swiftly and easily solved. All that would be necessary would be to add a good reducing agent to all our media. Unfortunately, this works for only some species. Other factors are of more importance for other species.

Everyone who works with anaerobes finds that he must not use media that are too old, or media that have been heated too often, for they will not support the growth of the strict anaerobes although they will be en-

tirely satisfactory for the moderate anaerobes. For a sensitive anaerobe, however, such as *Clostridium haemolyticum,* blood agar medium cannot be stored even one day.

This can readily be demonstrated. If *C. haemolyticum* is streaked on the surface of a blood agar plate as soon as it is solidified and the plate immediately placed under anaerobic conditions, surface colonies can be obtained. If the plates are stored at room temperature for three or four hours before streaking, there will be no growth. If, on the other hand, blood agar plates are poured in the presence of air and then are placed under anaerobic conditions as soon as they harden, they will remain suitable for the surface growth of *Clostridium haemolyticum* for several days. Sooner or later, however, even under anaerobic storage, they will become entirely useless.

It is evident, in this experiment, that four hours exposure to the air caused some irreversible toxic changes. Even plates that were poured in the presence of air and then were immediately stored anaerobically, became inhibitory in several days. Almost certainly, an oxidative process started under the aerobic conditions while the plates were being poured and continued to progress, even after the plates were transferred to anaerobic conditions. Although the substances responsible for the inhibition of growth in this experiment have not been identified, they probably are peroxides, probably organic peroxides of some sort.

Some substances used in bacteriological culture media can react with atmospheric oxygen to form peroxides. One such substance is agar; another is yeast extract. Certain batches of agar become inhibitory to catalase-negative bacteria when they are exposed to air after autoclaving. This inhibition is probably due to hydrogen peroxide, for it is restricted to the surface of the agar, and also, the inhibiting effect may be abolished by the addition of catalase. All batches of agar seem to be potentially inhibitory, but most of them will not show this effect unless they have been autoclaved for several hours.

Another constituent of culture media that may become inhibitory on autoclaving in the presence of oxygen is yeast extract. The inhibitory agent in this commonly used nutrient may also be hydrogen peroxide, for it again is inhibited by catalase. It should be remembered, though, that hydrogen peroxide readily reacts with many compounds, including amino acids, particularly histidine and thymine to form organic peroxides. These organic peroxides may be more inhibitory and toxic than hydrogen peroxide itself. Also, they are but slowly decomposed by catalase.

Some of the common metals may also initiate peroxide formation in bacteriological media. One of the most active of these is manganese. During autoclaving in the presence of oxygen, manganese can catalyze peroxide formation with such substances as asparagine, citrate, and some reducing sugars. Trace amounts of copper may do the same thing.

Probably the worst source of trouble in peroxide formation is the reducing agents that we use in our me-

dia. We have no choice but to use them, for they are essential in poising the oxidation-reduction potential at the low values some of the anaerobes seem to require. On the other hand, it has been long known that reducing agents can yield peroxides when they react with oxygen. Usually, when peroxides are formed by the oxidation of a reducing agent, the peroxide thus formed, would be again reduced. Sometimes, unfortunately, this further reduction may not occur, or if it does occur, it may do so more slowly than the rate at which peroxide is being formed. In such case, peroxide would accumulate. Once peroxide is formed in bacteriological media, it may remain there for a long time, with a half-life of one month or more.

Let us see if we can summarize this portion of our discussion. The anaerobic bacteria may be sensitive to oxygen for any of three reasons: For some species, oxygen may be directly toxic through the formation within the bacterial cell of superoxide or other free radicals; for other species, it may be inhibitory if it raises the oxidation-reduction potential of the medium to a level that is too high; still other species may be killed by toxic peroxides in the bacteriological medium. The strict anaerobes seem to be affected by all three.

Problems in attaining anaerobic conditions are most marked when isolation of the strict anaerobes is desired. I emphasize "isolation" because this is, after all, the most rigorous test to which we put our techniques of bacterial culture. In isolation, we expect individual colonies to develop from the smallest possible inoculum — a single bacteria cell. If every isolated cell does not give rise to a colony — if, in effect, the viable count is less than the microscopic count — our technique is, in greater or lesser measure, unsatisfactory.

Many methods for obtaining anaerobic conditions have been devised, from the simple evacuation of anaerobe jars to the elimination of the oxygen by burning it out with phosphorus. However, the most widely used technique for isolating bacteria, particularly in clinical laboratories, is that of inoculating plates of solid media just as we do for aerobic bacteria, and incubating them in anaerobe jars. During this procedure, the bacteria are exposed to the air as the inoculum is spread over the surface of the agar. The anaerobe jar technique is suitable for the majority of anaerobes that are encountered in clinical specimens. It is not entirely suitable for the strict anaerobes, such as the treponemes. It does have the advantage that opaque media, such as blood agar or egg yolk agar may be used for isolation.

Obtaining isolated colonies by successive dilution of the inoculum in a series of tubes of nutrient agar is one of the oldest methods of obtaining pure cultures of anaerobic bacteria. This deep agar method is still in use, primarily at the Institute Pasteur of Paris and students of Prevot.

The deep agar method is quick, easy, does not require any special equipment, and will allow the cultivation of all the anaerobes except the treponemes. Opaque media such as blood agar and egg yolk agar cannot be used in the tube m[illegible]

there the variety of colony form that is visible on cultures on the surface of agar. Consequently, it is not as efficient for investigating a mixture of anaerobes as the use of agar plates.

One of the best methods of obtaining anaerobiosis is the roll tube procedure. This was developed by Hungate for the cultivation of the very fastidious bacteria found in the rumen of cattle and sheep. This method involves the use of "pre-reduced" media — that is, media that have been sterilized under reduced conditions and that remain reduced during storage and while they are being inoculated. This utilizes media prepared and stored in individual stoppered tubes. When the tubes are opened, a gentle

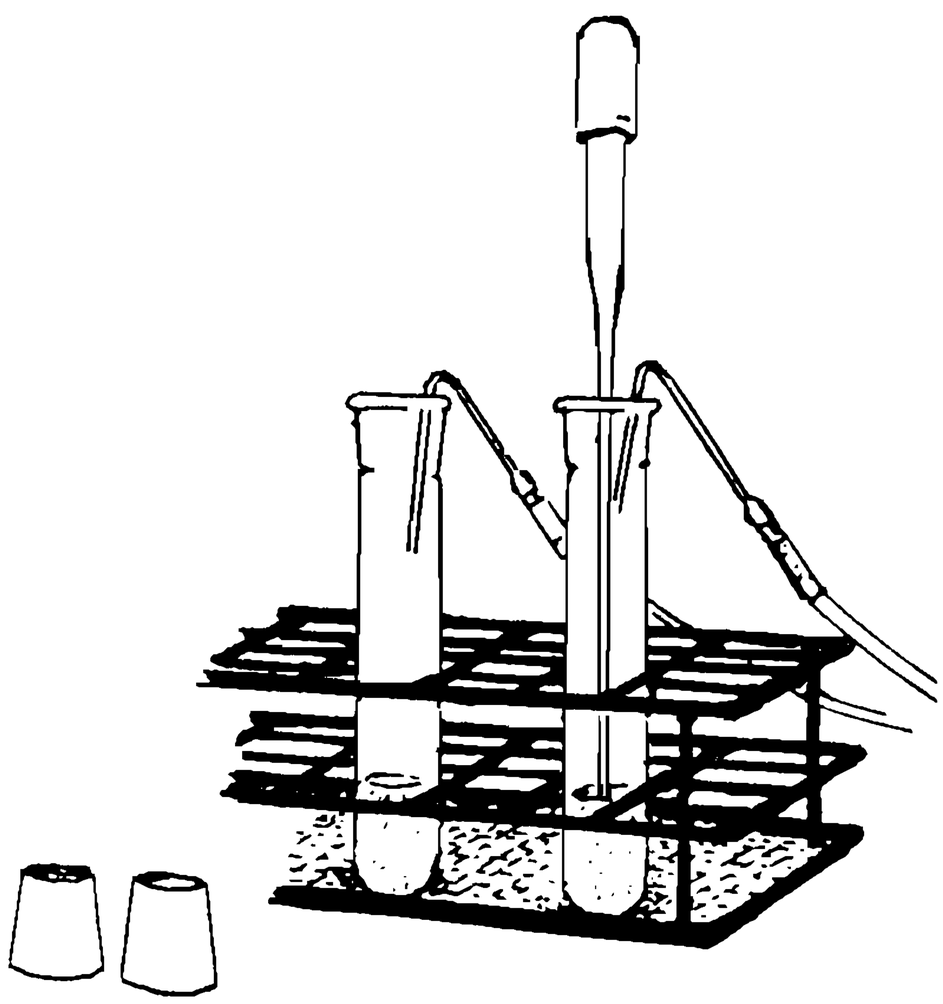


Fig. 1

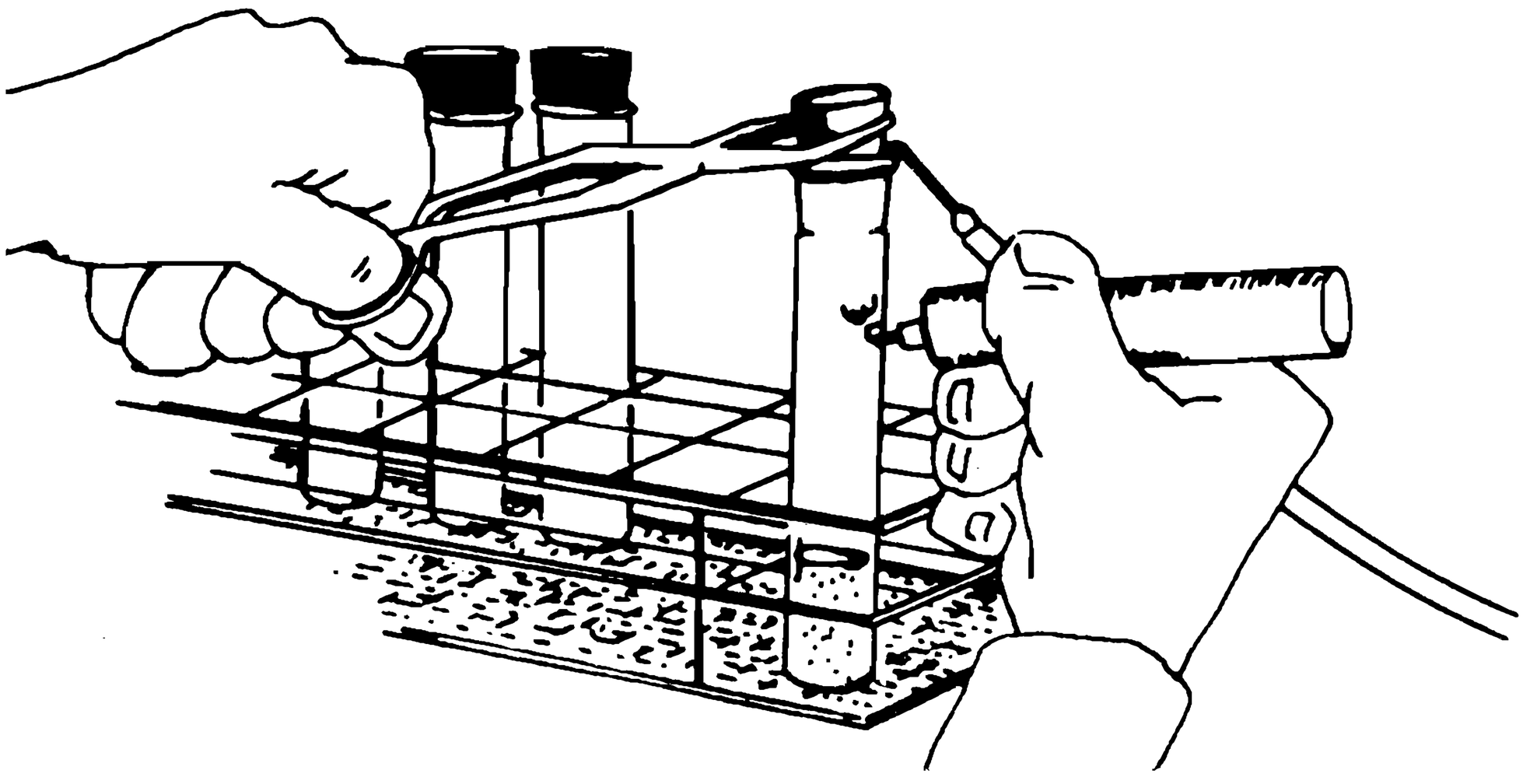


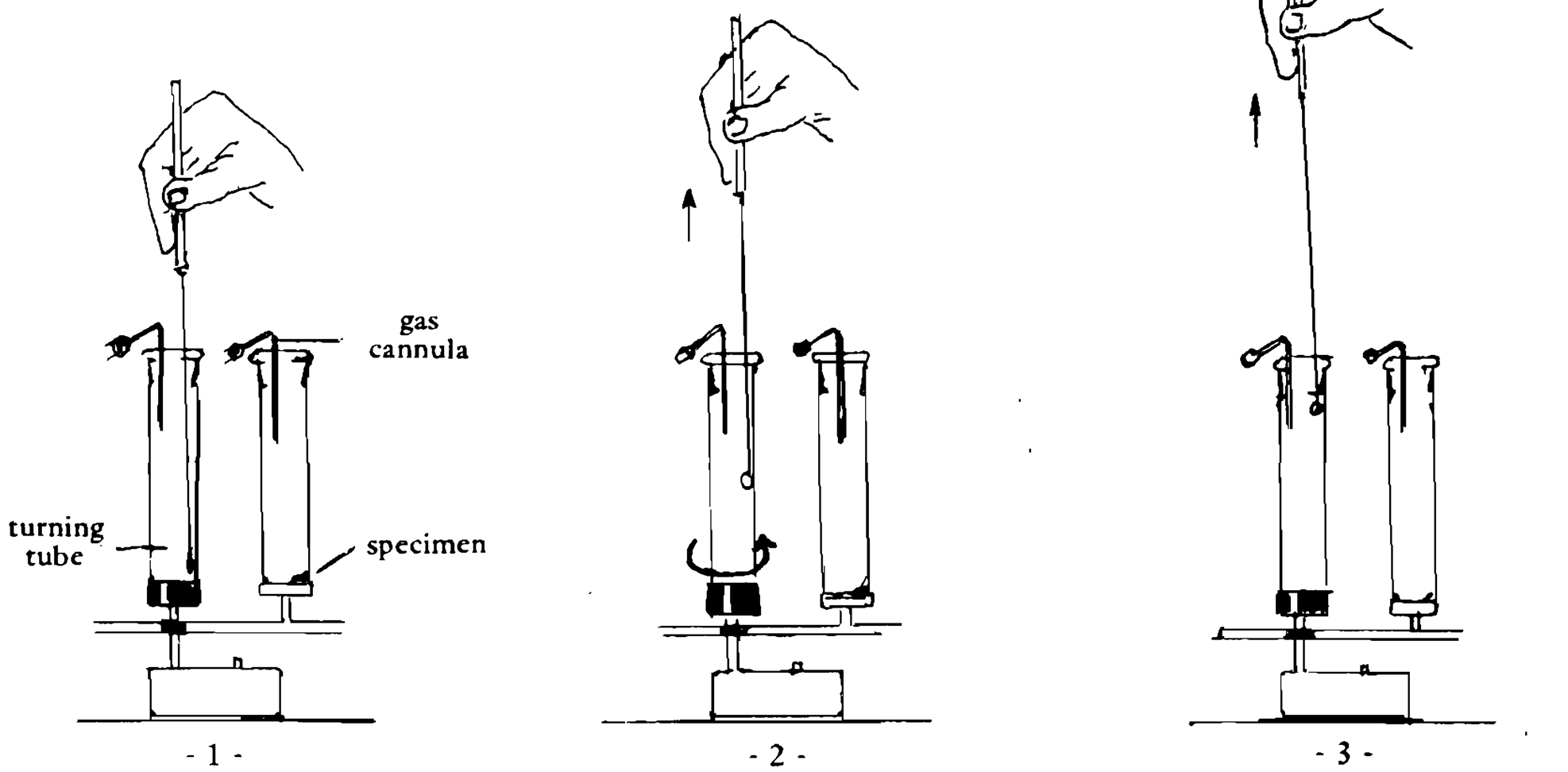
Fig. 2

stream of oxygen-free gas is led into the tubes through long, bent hypodermic needles, to prevent the entrance of air. When the stopper is replaced, the culture is ready for incubation. (Fig. 1 and 2)

Isolation is accomplished by coating the inner surface of tubes with sterile agar medium. This is accomplished by spinning the tubes in a horizontal position while the medium solidifies. Inoculation of such roll tubes is accomplished by inoculating the agar while the tube is slowly turning in a vertical position. The agar in the tube is inoculated from the bottom to the top, so that the most crowded colonies are at the bottom of the tube and those most widely separated are at the top. (Fig. 3)

The roll tube method suffers from the same disadvantages as the deep agar procedure; namely, that opaque media such as blood agar and egg

STREAKING OF AN AGAR ROLL TUBE



- 1 - loop flat against agar film

- 2 - turn loop edge to agar film

- 3 - steadily move loop toward neck of tube using decreasing pressure

Fig. 3

yolk agar cannot be used. However, even the most exacting of the anaerobic bacteria and treponemes can be grown with the roll tube method.

Another method used for the isolation and cultivation of the most sensitive anaerobes is that of the anaerobic glove box. This is simply a large box, of plastic or other material, that has openings in the side with long gloves in them. The interior of the box is kept anaerobic and all transfers are made within it. Probably the most widely used is one made of heavy, flexible, transparent plastic. This flexible plastic is held in shape by the pressure of the gas within it. The gas used within the glove box is usually nitrogen with 3% hydrogen. This gas passes over a

container of palladium catalyst to reduce any oxygen that diffuses in through the plastic. Using this technique, it is possible to maintain the oxygen concentration of the atmosphere within the glove box to a few parts per million, cultures or media are placed within the glove box or removed through a port that may be sealed at both ends, evacuated and filled with oxygen-free gas. An incubator may be placed within the glove box, or the whole glove box may be kept at a temperature of 35 to 37 C. The cost of such a glove box, with a continuous oxygen monitor, etc., is about two thousand dollars It has the disadvantage of taking up much table space and being somewhat subject to puncture, but is as good as the roll tube method for cultivating fastidious organisms.

Fig. 4

The ultimate in anaerobiosis is undoubtedly the anaerobic laboratory at the National Institutes of Health in Bethesda, Maryland. Here the entire laboratory is anaerobic. The walls of the laboratory are stainless steel, and the atmosphere is nitrogen with less than two parts per million of oxygen. It is self-contained with incubator, cold box, fume hoods and all the equipment necessary for laboratory operation. The microbiologists in the laboratory enter through a lock, so that air does not come in with them, and immediately put on masks similar to those used by deep sea divers so that they have air to breathe. People who work in this laboratory assure me that they became accustomed to working in masks very quickly and that they do not find them particularly inconvenient. This laboratory was designed for work not only with the anaerobic bacteria, but also for work with those enzymes of the body, particularly those of the heart, that are inactivated by exposure to the oxygen of the air. (Fig. 4 and 5).

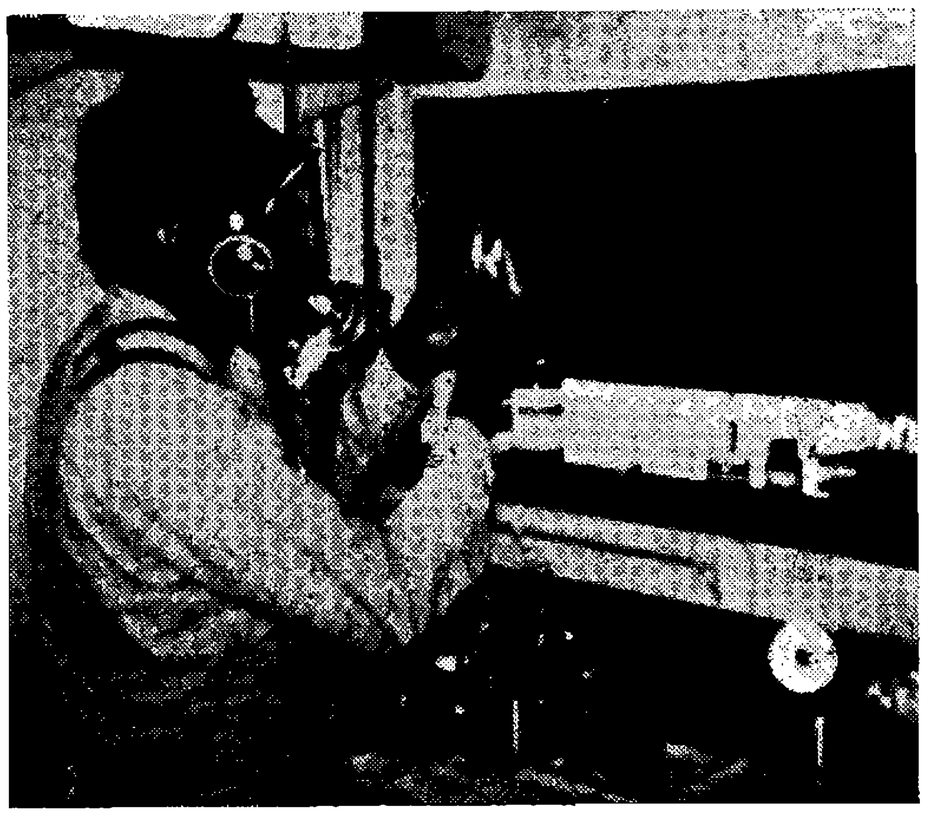


Fig. 5

From the variety of methods for obtaining anaerobiosis that have been developed in the century that has elapsed since the anaerobes were discovered by Pasteur, it is evident that these organisms are of interest to the microbiologist. He must work with the anaerobes because they are important to him. Nevertheless, there is always a certain amount of inconvenience. This is only to be expected when an obligate aerobe, such as man, attempts to work with obligate anaerobes. We cccupy two different worlds.

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