STUDIES ON BACTERIAL NUTRITION.

V. The Effect of Plant Tissue upon the Growth of Anaerobic Bacilli.

By OSWALD T. AVERY, M.D., and HUGH J. MORGAN, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research.)

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INTRODUCTION.

In previous papers of this series it has been shown that unheated vegetable tissue, when added to buffered broth, not only makes possible, in the absence of blood, the cultivation of the so called hemoglobinophilic organisms (1), but also greatly favors the growth of other entirely unrelated bacteria, as for example certain Gram-positive cocci (2).

In the case of the pneumococcus, a seeding so minute as to cause in dextrose broth a period of bacterial lag extending over 18 hours will amply suffice to induce prompt growth in plain broth to which pieces of sterile unheated potato have been added. The presence of vegetable tissue causes other changes in the configuration of the growth curve. There is a prolongation of both the stationary phase and phase of decline of the pneumococcus culture. Moreover, in plant tissue medium the zone of pH within which growth can be initiated is extended beyond the acid and alkaline limits of the range in ordinary bouillon. A study of the behavior of other Gram-positive organisms shows that the growth of *Streptococcus viridans* and *Streptococcus hæmolyticus* is greatly accelerated in vegetable tissue media.

The present communication deals with the effect of plant tissue upon the growth of anaerobic organisms. Since the exclusion of air is the prime requisite for growth of obligate anaerobes in ordinary media, it seemed of interest to determine the effect of heated and unheated vegetable tissue upon this cultural requirement.

Tizzoni, Cattani, and Boquis (3) in 1889 demonstrated that, by the use of coagulated blood medium, the tetanus bacillus could be grown in the presence of air. Kedrowski (4), employing a different method, was also able to grow anaerobes

in the presence of oxygen. An agar slant was seeded with an aerobic organism. After growth had occurred the culture was killed by chloroform vapor, and a small amount of broth added. Anaerobes seeded into this medium grew even when no precaution was taken to exclude air. Theobald Smith (5) in 1890 first grew anaerobic bacteria in media containing sterile animal tissue and found that under these conditions the rigid exclusion of air was not necessary. These facts were confirmed in 1905 by Tarozzi (6) and by Wrzosek (7).

Unheated plant tissue was first employed in bacteriology by Ori (8). This investigator and Tarozzi (9) found that the growth of anaerobes was favored by the presence of vegetable tissue in the medium. Wrzosek (10) likewise used vegetable tissue in the cultivation of this group of organisms. In his hands heated plant tissue was more effective than fresh tissue, and by the addition of it to ordinary media, he was able to grow anaerobes for one subculture in the presence of air. Wrzosek found that the "substance" contained in the plant tissue which facilitated aerobic growth was not destroyed by desiccation and was relatively thermostable, resisting freezing and a temperature of 140°C. It was not affected by light, but deteriorated rapidly in the presence of air. He also used charcoal, zinc, iron, etc., in media for the cultivation of anaerobes, and concluded that these substances, as well as animal and plant tissue, favored growth of anaerobic bacteria by virture of the reducing substances they contained. Kitt (11) also found that he could induce the rauschbrand bacillus to grow under aerobic conditions by seeding large quantities of bouillon with a large inoculum.

In the present work the anaerobic organisms studied were seeded into media exposed to air and containing heated and unheated plant tissue. In each instance two or more inocula, of different size, were used; and when growth occurred subcultures were made into media of the same type. Cultivation was continued, when possible, until the organisms had been grown in at least three subcultures under aerobic conditions.

EXPERIMENTAL.

Media.—The medium was freshly prepared. Pieces of potato[1] weighing approximately 0.5 gm. were placed in sterile tubes 15 by 1.5 cm. and 5 cc. of phosphate broth were added. Air was not excluded during incubation nor was any attempt made to exhaust the medium of air prior to inoculation. Three varieties of this medium were employed. The first consisted of plain broth containing 0.5 gm. of sterile unheated potato; the second, of broth containing 0.5 gm. of potato which had been boiled for 5 minutes in normal salt solution; the third, of

[1] The potato was obtained by the technique described in previous papers of this series.

broth containing 0.5 gm. of potato which had been autoclaved for 45 minutes at 120°C. Uninoculated samples of each variety of medium were incubated at 37°C. for 48 hours as controls.

Organisms.—The organisms used in the experiment were *Bacterium pneumosintes*, *B. histolyticus*, *B. chauvœi*, *B. aerofœditis*, and *B. œdematiens*.[2] All five of these anaerobic organisms grew promptly and abundantly when seeded into potato broth covered by a vaseline seal. In the case of *B. aerofœtidis* and of *B. œdematiens* gas was formed in potato broth.

Procedure.—The first series of cultures were made by inoculating the three types of "aerobic" media with varying amounts of 8 hour potato broth anaerobic cultures of the test organisms. Since it was found that the results varied considerably with the size of the inocula employed, several tubes were inoculated at each subculture with varying amounts of the parent culture. The amounts used for seeding were (1) the quantity on a platinum needle which had been thrust the depth of 1 cm. in the parent cultures, (2) one 2 mm. loop, (3) 0.05 cc., and (4) 0.1 cc. When growth occurred in the "aerobic" culture a second transfer was made to medium of the same type; *i.e.*, broth containing either unheated or heated potato. If growth occurred in the second subculture, a third was made, the conditions of cultivation being kept constant throughout. These "aerobic" cultures of the various organisms will be called Subcultures 1, 2, and 3 respectively. By referring to these numerals one can determine by how many subcultures, under aerobic conditions, the organisms were removed from the original anaerobic potato broth cultures.

The final observations on growth were made after the tubes had remained in the incubator at 37°C. for 48 hours. Purity of the cultures was checked, in every instance, by examination of films stained by the Gram method.

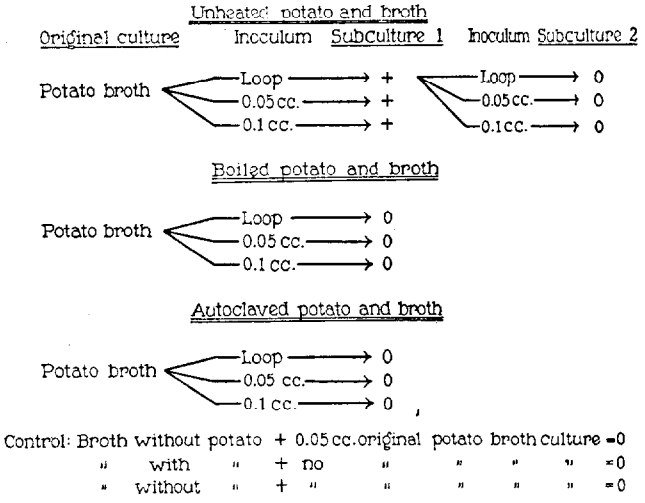
Results.—Bacterium pneumosintes (*Text-Fig. 1*): When a 2 mm. loopful of an 8 hour anaerobic potato broth culture of *Bacterium pneumosintes* was seeded into broth containing unheated plant tissue, under aerobic conditions, growth resulted. The culture showed marked clouding after 40 hours at 37°C. However, when as much as 0.1 cc. of the anaerobic culture was seeded into broth containing boiled or autoclaved potato, growth failed to occur.

When transfers were made from the aerobic Subculture 1 to Subculture 2 no growth occurred.

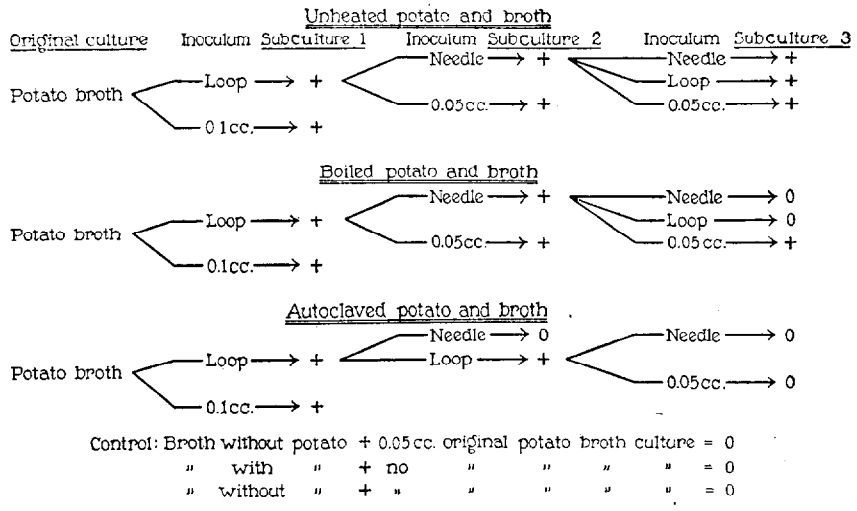
B. histolyticus (*Text-Fig. 2*): When *B. histolyticus* was transferred from an anaerobic potato broth culture into the three types of media, *i.e.* broth and unheated potato, broth and boiled potato, and broth and autoclaved potato, under aerobic conditions, growth occurred. When, in turn, organisms of Subculture 1

[2] The strain of *Bacterium pneumosintes* was furnished by Dr. P. K. Olitsky and Dr. F. L. Gates and had been grown in kidney tissue-ascitic fluid medium through many subcultures. For the other organisms we are indebted to Dr. Morton C. Kahn. Each strain was derived from a single cell culture and had been grown in cooked meat media for some time.

were transferred to a medium of the same type (Subculture 2), growth occurred in all cultures except in one of those containing autoclaved potato. The strain which



TEXT-FIG. 1. Aerobic cultivation of *Bacterium pneumosintes*.

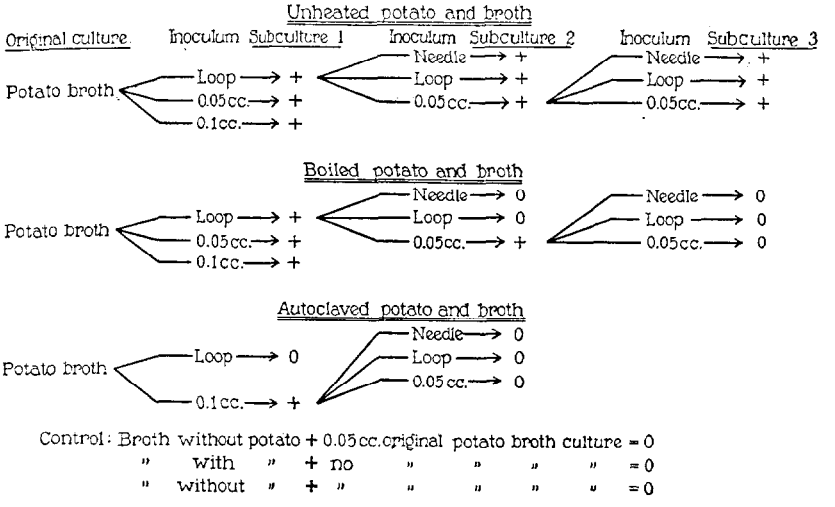


TEXT-FIG. 2. Aerobic cultivation of *B. histolyticus*.

had survived two transfers in broth containing autoclaved potato failed to grow in the third subculture even though a relatively large inoculum was used (0.05 cc.).

In the boiled potato medium growth did not occur in the third subculture when the inoculum was less than 0.05 cc., whereas the organisms carried over on the tip of a platinum needle sufficed to produce abundant growth in broth containing unheated potato.

B. chauvæi (*Text-Fig. 3*): The transfers of *B. chauvæi* in unheated potato broth media, under aerobic conditions, grew in the three subcultures. The strain seeded into boiled potato broth grew in all tubes of Subculture 1. In Subculture 2, however, growth could be initiated only when a relatively large inoculum was used (0.05 cc.), and no growth resulted in Subculture 3. When autoclaved potato was employed in the medium growth did not extend beyond Subculture 1, and there only when large inocula were used.



TEXT-FIG. 3. Aerobic cultivation of *B. chauvæi*.

B. aerofœtidis: Subcultures of this organism grew through three transfers, under aerobic conditions, in broth containing unheated and autoclaved plant tissue. In autoclaved potato broth growth was slow, not abundant, and occurred only in the tube which received the largest inoculum (0.05 cc.), whereas it was quite rapid (present after 15 hours at 37°C.) and abundant in broth containing unheated plant tissue, even when the latter had received the smallest inoculum. Growth of *B. aerofœtidis* did not continue beyond the first subculture in the medium composed of broth and boiled potato.

B. œdematiens: As in the case of the preceding organism, this anaerobe grew readily, under aerobic conditions, through three subcultures in broth containing unheated and autoclaved vegetable tissue. *B. œdematiens* like *B. aerofœtidis* grew in the autoclaved potato medium, but as in the latter instance the growth under these conditions was not so rapid nor so abundant as in the medium con-

taining unheated plant tissue. Boiled potato broth supported growth through two transfers only. An inoculum as large as 0.05 cc. failed to induce growth in Subculture 3.

To summarize, it was found that under the conditions of the experiment, the five strains of anaerobic organisms tested grew under aerobic conditions in broth containing pieces of unheated potato. In the case of *Bacterium pneumosintes*, growth did not continue beyond the first subculture. *Bacillus histolyticus*, *Bacillus chauvæi*, *Bacillus aerofœtidis*, and *Bacillus œdematiens* continued to multiply through three successive subcultures under aerobic conditions in the presence of unheated plant tissue.

Attempts to cultivate these organisms aerobically were not so successful when heated instead of unheated potato was used in the medium. *Bacterium pneumosintes* failed to grow in broth containing boiled or autoclaved potato. *Bacillus histolyticus* grew through three subcultures in boiled potato broth, but a larger inoculum was required to initiate growth in the third transfer. Likewise, in the medium containing autoclaved potato growth of this organism occurred aerobically in the second transfer only when a relatively larger seeding was added, and no growth took place in the third subculture even when the maximal inoculum was used. *Bacillus chauvæi* grew aerobically in the medium containing boiled potato through two transfers only and in the second subculture growth appeared only in the medium receiving the heavier seeding. When autoclaved potato was used this organism could not be induced to grow aerobically beyond the first subculture. Cultures of *Bacillus aerofœtidis* and *Bacillus œdematiens* were successfully carried through three aerobic transfers in broth containing autoclaved potato; growth under these conditions, however, was not so prompt and abundant as in the medium in which fresh unheated vegetable was used. In broth containing boiled potato both of these organisms grew less readily than they did in the same medium containing autoclaved potato. Cultures of *Bacillus aerofœtidis* and *Bacillus œdematiens* produced gas in broth containing either heated or unheated potato.

These results indicate that fresh, unheated plant tissue (potato) possesses to a striking degree the property of supporting the aerobic growth of bacteria ordinarily considered fastidious in their anaerobic

requirements. The fact that under aerobic conditions heated plant tissue exerts a less favorable action on the growth of anaerobic bacteria than does the unheated tissue, suggests that the superior growth value of fresh raw vegetable is associated with some heat-labile quality, possibly of the nature of an enzyme or group of enzymes. The loss of the peroxidase reaction in heated vegetable tissue is evidence of the susceptibility of the vegetable oxidases to the injurious action of heat. When potato is exposed to boiling water or to steam under pressure as in the present experiments, the oxidases of the vegetable tissue are destroyed. The relative effectiveness of unheated and heated potato in supporting the aerobic growth of anaerobic bacteria so closely parallels the presence or absence of these oxidases in the tissue as to suggest that the growth differences observed are related to the activity of these oxidizing enzymes. In the absence of these active enzymes, heated vegetable tissue supports only a limited and irregular development of these air-sensitive bacteria. The heated vegetable may function owing to the presence in it of certain stable tissue constituents which act as catalytic agents in reducing oxygen tension. Moreover, the interstices of the tissue afford opportunity for nidus formation which facilitates survival and multiplication of the microorganisms. In addition the bacteria themselves, when chance thus favors their increase, contribute to further growth by autocatalysis. Whatever share these special factors may have in the mechanism of growth, they are, presumably operative regardless of whether the tissue is heated or unheated. There is, however, an additional advantage in the use of the fresh raw vegetable, and experimental evidence indicates that this advantage is associated with the presence of the active oxidases of the unheated tissues. The possible relation of the oxidase system of plant tissue to bacterial growth will be discussed later in this paper. It suffices here to point out that such a relationship may exist and the oxidizing and reducing enzymes of unheated potato may function actively in creating cultural conditions suitable for the aerobic growth of organisms otherwise incapable of development in the presence of air.

In 1890 Theobald Smith, describing for the first time the influence of sterile animal tissue on the growth of anaerobic bacteria, suggested that the term anaerobiosis in bacteriology does not imply the impor-

tance of the absence of air so much as it infers the lack of knowledge of the particular cultural conditions requisite for the aerobic growth of certain types of bacteria.

DISCUSSION.

The growth-stimulating effect of plant tissue on bacteria of such widely diverse groups as those of the hemophilic bacilli, Gram-positive cocci, and anaerobic organisms serves to emphasize the need of a fuller knowledge concerning the physiology of the bacterial cell.

The studies presented in the present series of papers were undertaken with the hope that an analysis of the factors concerned in the mechanism of growth stimulation might lead to a better understanding of the principles involved in the action of these accessory substances in complementing the cultural requirements of bacteria. The fact that the addition of sterile unheated plant tissue to an otherwise deficient medium suffices to provide conditions suitable for cell multiplication, indicates that in bacterial as in animal growth there are substances which, apart from their food value, exert a marked influence upon the metabolism of the organism.

In order to correlate the facts presented in the present study with the observations recorded in the preceding papers it seems desirable to review briefly the work as a whole. Previous study of the growth requirements of *Bacillus influenzæ* has shown that the properties of blood upon which growth of this so called hemoglobinophilic organism depends, are related to two separable and distinct substances. The combined action of both of these substances is essential, each being ineffective separately. On the basis of their relative resistance to heat these two factors can be differentiated one from the other. The so called X factor, which is associated with the pigment fraction of blood, gives the peroxidase reaction, and is not destroyed by moist heat at 120°C.; while the second or so called V factor resists boiling for short periods, but is destroyed by exposure to the higher temperature. Because of this difference in susceptibility to heat, blood can be deprived of the more labile substance (V) by autoclaving and when so treated is no longer capable of supporting growth of *Bacillus influenzæ*. The X substance under these conditions, however, remains unaffected. This is proven by the fact that the addi-

tion of extract of yeast (V) can activate autoclaved blood (X) and render it again suitable for growth. It is interesting in this connection to note that, as Hopkins, Meyerhof, and others have shown, extracts of plant and animal tissue (yeast and muscle) are of physiological importance in cell respiration. This V substance which is capable of complementing the X factor of blood has been found in extracts of green vegetable and yeast cells, and from the nature of its action it may be regarded either as a coenzyme or as a vitamin-like substance. The X factor, on the other hand, is found in hemoglobin and in certain of its derivatives (hemin) and is always associated with the fraction giving the peroxidase reaction with benzidine. Moreover, the X substance is active in such minute amounts as to suggest that it functions as a biocatalyst.

Further study has confirmed the observation that the particular system represented by the combination of the X and V factors in blood has a complete analogue in a similar system of plant tissue. Unheated vegetable, for instance potato, possesses substances having the characteristics of both the X and V factors of blood. The peroxidase and catalase of plant tissue are related in function at least to the catalytic properties of the X factor in blood; while the so called V substance which serves as activator, both in blood and plant tissue, may represent a coferment adapted to a particular oxidase system. The mechanism of the action of plant tissue in stimulating growth of the hemophilic bacteria is not wholly clear. Analysis of the factors concerned, and the application of this knowledge to the growth of other bacteria, suggest that these factors may be related to the physiological processes of cell respiration.

It is of interest to correlate these facts with the effect of unheated vegetable tissue upon the growth of pneumococcus. It is, of course, well recognized that if a fluid medium be inoculated with an insufficient number of pneumococci little or no growth will occur, although the culture fluid may be optimal for growth if larger numbers of the organisms are introduced. However, if to this medium a piece of unheated potato is added, the minimal inoculum amply suffices to initiate prompt and abundant growth. Furthermore, the presence of plant tissue in the bouillon effects a remarkable alteration in the growth curve of pneumococcus. The period of lag, as pointed out

in the preceding paper (2), is eliminated, the maximum rate of cell division is promptly initiated, and growth reaches its full development in the first 8 hours of incubation. Moreover, sensitive as pneumococcus is to the initial reaction of the medium, the zone of hydrogen ion concentration within which growth can be initiated is considerably extended both on the acid and alkaline side in broth containing unheated vegetable tissue (potato). While the same mechanism which operates to facilitate growth of the hemophilic bacilli in plant tissue medium may be involved in the growth acceleration of pneumococci, in this latter instance another fact has been observed which may be of significance in explaining the action of these accessory factors. Whenever pneumococci are grown in bouillon to which there is free access of air, hydrogen peroxide is formed in readily demonstrable amounts (12, 13). This peroxide is toxic and accumulates in the culture fluid in concentrations which are bactericidal. On the other hand, under identical conditions of oxygen exposure, hydrogen peroxide is not demonstrable in cultures of pneumococci containing unheated plant tissue (potato). The absence of peroxide under these conditions is presumably due to the action of the catalase and peroxidase of the potato which possess the property of decomposing this substance. The presence of these enzymes, therefore, prevents the accumulation of hydrogen peroxide in the medium. The peroxidase in plant tissue is capable of acting upon the peroxide with the liberation of active oxygen which in turn can bring about further oxidative reactions. The results of studies on the formation of peroxide by pneumococcus and on the growth-inhibiting action of this substance on bacteria is described in another paper (13); it suffices here to point out that in the mechanism of growth acceleration of bacteria, plant tissue may exercise the dual function of providing accessory growth factors, and of destroying deleterious growth products—peroxides.

Finally, as demonstrated in the present paper, plant tissue makes possible the aerobic cultivation of anaerobic bacteria. The present study on the cultivation of anaerobes under these conditions was completed without knowledge of previous work on the use of plant tissue in the aerobic cultivation of anaerobic bacteria. Subsequent search of the literature revealed the interesting fact that 18 years ago

Italian bacteriologists of the Sienna school found that anaerobic bacilli of the obligate type grow aerobically in bouillon containing plant tissue. This phenomenon was attributed at that time to the action of reducing substances in the vegetable tissue.

The mechanism of aerobic growth of anaerobic bacteria in media containing pieces of unheated vegetable may, as already pointed out, be related to the oxidation-reduction system present in the plant tissue. Recent studies on the formation of peroxide by pneumococci on exposure to air and of the absence of this product when the organisms are grown anaerobically suggested the following assumption which finds some justification in the facts presented. Although experimental proof is not as yet available, it seems not unlikely that anaerobic organisms fail to grow in the presence of air not because atmospheric oxygen as such is a direct poison to the cell, but because of toxic peroxides which are produced whenever the oxygen of the air combines with autoxidizable substances of the bacterial cell. This process of autoxidation gives rise finally to the formation of hydrogen peroxide, which is known to be poisonous to living protoplasm. Since these peroxides are formed only by the action of molecular oxygen on some autoxidizable substance of the cell, the absence of air precludes their formation. Under anaerobic conditions, therefore, bacteria are not exposed to the injurious action of these products of autoxidation. If this assumption is correct, then the aerobic growth of obligate anaerobes in the presence of plant tissue finds partial explanation at least in the fact that the peroxide formed by autoxidation is rapidly broken up by the catalase and peroxidases of the plant tissue. Under these cultural conditions the sensitive cell is protected almost as effectively as if it were growing under anaerobic conditions. Not only is the catalase of the plant tissue capable of protecting the bacterial cell in this manner from the readily diffusible peroxide, but the peroxidase in splitting this substance liberates active oxygen which in turn is capable of bringing about oxidation of substances not readily oxidized by molecular oxygen. The peroxidase may further assist then in facilitating the transport of oxygen to other substances, and possibly to the bacteria themselves.

The evidence in favor of the hypothesis of peroxide formation during the processes of bacterial autoxidation is strong. It is not to

be inferred, however, that hydrogen peroxide is the only active agent resulting from these oxidation reactions. Previous studies have shown that hydrogen peroxide is demonstrable only in cultures in which the bacteria are devoid of catalase or in which the medium contains no efficient catalyst. If this compound were the only toxic peroxide formed during autoxidation, then the assumption of its deleterious action upon anaerobic bacteria when exposed to oxygen could only find support in the case of those organisms which possess no catalase. But it is generally recognized that a great variety of unstable peroxides may be formed as intermediary products in autoxidation. And so far as known, these other organic peroxides are not affected by catalase. It is possible that bacteria which are peculiarly air-sensitive contain autoxidizable substances which are markedly reactive with molecular oxygen and that as a result of this union there are formed toxic peroxides of an order unaffected by bacterial catalase.

The protective action of the vegetable oxidases under these circumstances can be explained by assuming that some efficient enzyme of this system, as peroxidase, brings about the decomposition of these inhibitory products. Bacteria of this type can grow, therefore, only when oxygen is excluded; that is, under conditions which render peroxide formation impossible, or when in the presence of air some mechanism is provided which inhibits the formation of these substances or renders them inert once they have been formed.

The views expressed in this paper are stated with full realization of their inadequateness to explain the complex nature of the phenomena of oxidation in the living bacterial cell; they are advanced, however, with the hope that they may serve to stimulate further work on this important phase of the physiology of bacteria.

In the present state of knowledge it seems reasonable to assume that the growth-promoting properties of fresh plant tissue may be referable to the action of the oxidation-reduction system of the tissue as well as to the presence of other growth accessory substances which together provide cultural conditions more suitable for normal functioning of bacterial cells than are ordinarily furnished in bacteriological media.

Although the views expressed in this paper concerning the mechanism of anaerobiosis were arrived at independently, they are in striking accord with and indeed lend support to the studies recently published by McLeod and Gordon (14) and by Callow (15) on the oxygen intolerance of anaerobic bacteria. Callow made comparative estimations of nine anaerobes and of twelve aerobes and found that none of the anaerobic organisms contained catalase, while all of the aerobic bacteria, with the exception of the streptococci, showed the presence of this enzyme. In no instance, however, was she able to obtain conclusive evidence that anaerobes are able to grow aerobically in broth containing catalase derived from bacteria, fat, and yeast. Moreover, repeated attempts to detect hydrogen peroxide in cultures of anaerobes which had been subsequently exposed to oxygen were negative. McLeod and Gordon, following their original investigations on the catalase production and the formation of hydrogen peroxide by bacteria, have studied the influence of liver catalase on the development of anaerobes in the presence of oxygen. Although all attempts to cultivate anaerobes aerobically on plates heavily charged with liver catalase failed, these authors do not consider that this invalidates their theory that "anaerobes cannot tolerate more than very slight concentrations of oxygen because they produce H_2O_2 as soon as oxygen is available and being very sensitive to this substance they die." The failure of the English investigators to effect aerobic growth of anaerobes by addition of catalase when contrasted with successful results obtained by the use of fresh plant tissue, suggests that possibly the complete oxidation-reduction system in the latter is able to decompose toxic peroxides of an order unaffected by catalase alone.

CONCLUSIONS.

1. Certain anaerobic bacteria grow under aerobic conditions in plain broth in the presence of sterile unheated plant tissue.

2. Aerobic growth of anaerobic bacteria under the above conditions may be related to the presence of growth-accessory substances, and to the action of oxidizing-reducing systems of plant tissue in the destruction of toxic peroxides of bacterial origin.

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