STUDIES ON BACTERIAL NUTRITION.

III. Plant Tissue, as a Source of Growth Accessory Substances, in the Cultivation of Bacillus influenzæ.

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The preceding papers (1–3) in this series have dealt with the nature and action of the substances contained in blood which are of importance in the growth of *Bacillus influenzæ*.

Search for substances other than the hemoglobin of warm blooded animals, which might be capable of stimulating growth of *B. influenzæ*, has been made by several investigators. Davis (4) found that substances similar in their function to hemoglobin, namely hemocyanin, hemoerythrin echinochrom, did not sustain growth. He was unable to find any growth-inducing action in substances which readily give up oxygen, such as hydrogen peroxide and colloidal platinum, and among the various salts and organic preparations of iron he found none capable of replacing hemoglobin. Davis (5) has confirmed the observation of Ghon and von Preyss (6) that *B. influenzæ* will grow on hematin agar only in the presence of dead or living bacteria. Davis points out that while hemoglobin in plain agar yields growth, still more abundant multiplication occurs when hemoglobin is associated with bacteria or tissues, either plant or animal, and especially if these are living. However, he states that plant and animal tissues, and bacteria by themselves will not sustain growth.

Olsen (7), studying the effect of blood on growth of Pfeiffer's bacillus, tested the growth-inducing action of various constituents of blood and derivatives of hemoglobin. He found that serum and ether extracts of washed blood corpuscles were ineffective. He observed, further, that hemoglobin and methemoglobin were capable of supporting growth, while hematin and hemin, on the other hand, were effective only in association with other bacteria. This author suggests that hemoglobin acts as a catalytic agent in rendering oxygen available for the bacilli.

In a recent publication on the nature of the effect of blood pigment upon the growth of *B. influenzæ*, Fildes (8) favors the view that the iron-containing pigments function as catalysts in accelerating the transfer of oxygen to the bacilli. In interpreting the relative feebleness of growth on unchanged blood compared to that on changed blood, Fildes attributes the growth differences to the fact that the oxygen affinity of unchanged hemoglobin may, by its own avidity, divert the oxygen from the bacilli, while physiologically inactive derivatives cannot intercept the oxygen liberated by the catalytic action of the iron in these pigments. In his experiments, however, Fildes never observed the occurrence of growth of *B. influenzæ* in the total absence of blood pigment.

In preceding papers (1–3) the authors have pointed out that the hemophilic bacilli, of which Pfeiffer's bacillus serves as a type, require for their growth two distinct and separable substances, both of which are present in blood and neither of which alone suffices. These substances are (*a*) a vitamine-like substance which can be extracted from red blood corpuscles, and from yeast and vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (*b*) a so called X substance which is also present in red blood cells, is heat-stable and acts in minute amounts.

The present paper concerns itself with the nature of this X substance in blood. It will be shown that this X factor can be derived also from sources other than blood, just as in a previous paper it was shown that the V factor can be supplied apart from animal tissue. That both the V and X factors exist in combination in nature will be shown by the fact that *Bacillus influenzæ* will grow in blood-free medium provided certain plant tissues are added.

EXPERIMENTAL.

Presence of the X Substance in Blood and Blood Derivatives.

In a previous paper (3) it has been pointed out that the X factor, which constitutes one of the growth essentials of *Bacillus influenzæ*, occurs in highest concentration in the cellular fraction of blood, and that the minimal effective amount is extremely small. In fact, the quantity of the X factor carried over in a loopful of the supernatant fluid of a blood broth culture of *Bacillus influenzæ* is sufficient to induce growth in yeast extract broth; that is, in the presence of the supplementing vitamine-like substance, the V factor.

Concentration of Accessory Substances in Blood Essential for Growth of Bacillus influenzæ.—From Table I, in which is shown the minimum concentration of hemoglobin permitting growth of *Bacillus influenzæ* in broth with and without yeast extract, the relationship of the two factors, V and X, is again evident. While the source of the blood in each instance is different, the two preparations of hemoglobin illustrate in a comparative way the minimal effective ratio between these two factors in the same preparation, and the extraordinarily minute quantity of the X substance which suffices for growth in the presence of an excess of the vitamine-like factor from yeast. The hemoglobin derived from laked blood cells was physiologically active; the crystalline hemoglobin had lost its oxygen-carrying capacity, although it still retained the characteristic absorption bands on spectroscopic examination. The latter preparation was devoid of

TABLE I.

Concentrations of Hemoglobin Essential for Growth of B. influenzæ.

Concentration of hemoglobin in plain broth.*	Hemoglobin† from laked red blood cells.		Crystalline hemoglobin.‡	
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
1 : 10	++	++	–	++
1 : 100	++	++	–	++
1 : 1,000	++	++	–	++
1 : 10,000	+	++	–	++
1 : 100,000	–	++	–	++
1 : 1,000,000	–	+	–	++
1 : 2,000,000			–	+

* All tubes containing 5 cc. total volume were inoculated with 0.05 cc. of yeast extract broth culture of *B. influenzæ*. Plain broth without hemoglobin or yeast extract, and yeast extract broth alone served as controls and showed no growth under the same conditions of seeding.

† Hemoglobin from rabbit blood determined gasometrically by Van Slyke's method (Van Slyke, D. D., *J. Biol. Chem.*, 1918, xxxiii, 127).

‡ Crystals of hemoglobin prepared from ox blood.

++ indicates marked growth; + moderate growth; – no growth.

the more labile vitamine-like substance as shown by the fact that of itself it was incapable of supporting growth even in the highest concentration. When complemented by the V factor in yeast extract, however, a 1:2,000,000 dilution of crystalline hemoglobin sufficed to stimulate growth of *Bacillus influenzæ*. On the other hand, in the medium containing the solution of freshly laked blood cells both factors were present in amounts sufficient for growth up to a certain dilution, beyond which the native V factor was exhausted and the presence of the residual X substance could be demonstrated only by adding the vitamine principle from yeast. Under the latter circumstances the X factor was still effective in concentrations of hemoglobin as small as 1:1,000,000.

Davis found that hemoglobin from human blood was active in promoting growth of *Bacillus influenzæ* in dilutions as high as 1:180,000. Fildes (8) could not confirm the growth activity of sheep blood in these high dilutions. The sources of error suggested by Fildes, namely the carrying over of blood pigment from tube to tube, or with the inoculum from the culture itself, have been eliminated in the present study by the use of a separate pipette for each dilution, and by seeding all tubes with a small inoculum from a yeast extract broth culture known to contain only the minimal effective dose of the X substance. Furthermore, in the present experiments, broth rather than agar was used as the medium of choice. It seems not impossible, therefore, that these differences in methods, together with recognition of the need of the separation and titration of the two component factors individually, may account for the variations in question. Individual specimens of blood from the same or different species and corresponding derivatives of hemoglobin will of necessity vary in one or the other of these factors, particularly in their content of the V factor, so that comparisons of this sort express only relative values. Of more importance is the quantitative relationship, and the interdependence of these two factors in a given specimen, and the need, in testing for either substance in blood derivatives, of recognizing that the more labile vitamine-like factor may be destroyed in the preparation of the material tested, or lost by dilution without a corresponding destruction or loss of the X substance.

Heat Stability of the X Substance in Blood.—Sufficient data have been presented in the preceding papers of this series to establish the thermostability of the X substance. Unlike the V factor, the X substance in blood, blood extracts, and crystalline hemoglobin resists autoclaving at 120°C. for 45 minutes. Further evidence of the resistance of the X substance to heat is found in the fact that blood charcoal, which reacts positively to the benzidine test, retains the ability to function as the X factor in 10 per cent yeast extract broth; that is, to support growth of Pfeiffer's bacillus in the presence of the supplementing V factor.

Benzidine Reaction.—Olsen (7) states that both the guaiac and benzidine reactions go hand in hand with the ability of blood derivatives to support growth of *Bacillus influenzæ*. This color reaction for the demonstration of the so called peroxidases has been found to parallel the presence of the X substance in blood. The question as to whether the positive benzidine-reacting substances and the X substance in blood are one and the same will be discussed later in describing the occurrence of these substances in plant tissue. It need only be remarked here, that in blood at least, the benzidine-reacting substances exhibit a marked resistance to heat, and in this instance again parallel the behavior of the X substance.

TABLE II.

Absorption by Bone Charcoal of the X Substance in Crystalline Hemoglobin.

10 per cent yeast extract broth 5 cc.* plus	Before absorption.		After absorption.	
	Benzidine test.	Growth-inducing action.	Benzidine test.	Growth-inducing action.
Crystalline hemoglobin, 1 : 1,000........	++	++	—	—
Bone charcoal.........................	—	—	++	++

* Inoculated with 0.05 cc. of 18 hour yeast extract broth culture of *B. influenzæ*.
++ indicates positive benzidine test or marked growth.

Absorption of the X Substance.—It has been noted in the preceding paper of this series that the V factor in yeast extract can be completely removed from solution by absorption with bone charcoal. Similarly it has been found that the X substance can be absorbed from solutions of crystalline hemoglobin by this agent. The solution of hemoglobin after absorption no longer reacts positively to the benzidine test, and has suffered loss of the X substance as evidenced by the lack of any growth-inducing property.

From Table II it is evident that bone charcoal can absorb the X substance from solutions of crystalline hemoglobin. Absorption is facilitated by heat, and is related to the concentration of the X substance in solution and to the time allowed for the reaction. It is of interest to observe that the original bone charcoal failed to give the benzidine test, but, after absorption, reacted positively and had itself acquired the X substance, as shown by its growth-promoting action in 10 per cent yeast extract broth. The supernatant solution of hemoglobin after absorption had lost both its benzidine-reacting and growth-promoting properties as shown in Table II.

In view of these facts, an attempt was made to determine whether the X substance present in blood might also be found in other proteins of animal origin, such as crystalline egg albumin, or in protein split products such as peptone, erepton, and "aminoids." None of these substances, however, gave a positive benzidine test and none could be substituted for the X factor in supplying the cultural needs of this organism. In these experiments the V factor was always supplied by the addition of yeast extract to the medium, so that if the X factor were present in any test substance, the growth requirements would be complete. Furthermore, lipoidal extracts of heart muscle with and without cholesterol, as described by Noguchi, were tried under similar conditions without result.

Occurrence of the Growth Accessory Substances, X and V, in Plant Tissue.

In the course of these experiments it was observed, as already noted, that a striking parallelism seemed to exist between the presence of the benzidine reaction in blood derivatives and the ability of these same substances to promote growth of *Bacillus influenzæ*. This fact is not final evidence that the reacting substances are necessarily the same in both instances, but suggests rather that this color reaction for the so called peroxidases may serve as an indicator of the presence of the X factor in tissues other than those of animal origin. In the search for the X substance in vegetables, the potato was selected first, since it is known to contain peroxidases and catalysts and also both the fat-soluble A and the water-soluble B vitamines. For these reasons it was thought possible that raw potato would furnish both the V factor and the X substance. This was found to be the case by the luxuriant and continued growth of the bacilli which occurs in blood-free medium containing pieces of sterile raw potato.

Technique.—While no special attempt has been made to devise a precise method for obtaining pieces of sterile potato, and while modifications will naturally suggest themselves to those trained in bacteriological technique, the following procedure is the one originally adopted. It is desirable to select a potato without surface abrasions or imperfections, and for this reason an old potato is preferable because of the thicker skin protection. Possibly differences in the content of the vitamine and the X substances in new and old potatoes exist, but these have not been determined. After thorough cleansing of the outer surface in running water, the skin is dried and well charred with a red-hot searing iron in a broad band encircling the potato. Through this seared band the potato is cut or broken open and from the inner portions small pieces are removed with a sterile scalpel and placed in sterile petri dishes, care being taken to avoid touching the margins or piercing the outer surface of the potato. The sections removed in this fashion are divided into pieces of suitable size and dropped into tubes of plain broth.

Oxidizing Enzymes of Potato.—It is well known that in plant, as well as in animal tissues, substances concerned with physiological oxidation and reduction are widely distributed. Scrapings from fresh potato exposed to the air rapidly change color as a result of oxidation processes. The benzidine reagent in the presence of hydrogen peroxide gives a blue color when applied to the cut surface of potato, the so called peroxidase reaction. Hydrogen peroxide alone applied in a similar way is rapidly decomposed with the liberation of gaseous oxygen through the action of a catalase in the potato. In studying the oxidase, peroxidase, and catalase of potato and other vegetables, Falk, McGuire, and Blount (9) noted that the enzyme reactions were destroyed by heating to boiling for several minutes. They observed that there was no well defined hydrogen ion concentration for maximum action of the vegetable enzymes, but that on the average the optimal zone lay between pH 7 and 10. According to the work of Cohn, Gross, and Johnson (10) on the isoelectric points of the proteins in certain vegetable juices, the natural hydrogen ion concentration of potato is pH 6 to 7.

Sterile raw potato possesses the property of slowly reducing methylene blue in solutions of phosphates ($\frac{M}{15}$, pH 7.5) from which atmospheric oxygen has been excluded by a vaseline seal. It has been noted that, in hemoglobin, blood, or yeast extract broth under aerobic conditions, growth appears first in the upper layers of the medium where the oxygen tension is greatest. On the other hand, in broth containing raw potato under seal, growth of *Bacillus influenzæ* occurs early in the depths of the medium about the vegetable. Although the natural reaction of potato is acid (pH 6 to 7), and despite the presence of active enzymes, the use of raw potato in well buffered broth (pH 7.8) does not necessitate further adjustment of the reaction for growth of *Bacillus influenzæ*. In the cultivation of organisms more sensitive to reaction changes, the acidity developing in potato medium requires readjustment.

TABLE III.

Effect of Heat on the Growth Accessory Substances of Potato.

Inoculum, 0.05 cc. of yeast extract broth culture of *B. influenzæ*.	Potato in plain broth.				Controls; plain broth.	
	Unheated.		Autoclaved at 120°C. for 45 min.			
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.
Type* A	++	++	—	++	—	—
" B	++	++	—	++	—	—
" C	++	++	—	++	—	—
" D	++	++	—	++	—	—

* Types A, B, C, and D refer to the biological classification of Stillman and Bourn (Stillman, E. G., and Bourn, J. M., *J. Exp. Med.*, 1920, xxxii, 665).

++ indicates marked growth; — no growth.

The nature and interaction of these vegetable enzymes are too complex and too little understood to warrant any interpretation of their possible significance in the growth of *Bacillus influenzæ* in media containing raw potato. The assumption that they may function as catalysts in facilitating the transfer of oxygen to the bacilli is perhaps justified by the fact that in potato broth, under vaseline seal, growth occurs in the depths of the culture about the vegetable tissue.

Heat Stability of the Accessory Substances in Potato.—From Table III it is evident that potato contains both the X and V factors requisite for growth of *Bacillus influenzæ*, since small pieces of the fresh sterile vegetable in plain broth suffice to stimulate rapid multiplication. Under these conditions, growth is not conditioned by the carrying over of either factor with the inoculum. Repeated experiments have amply confirmed these observations, and their validity is further established by the fact that growth in unheated potato broth is not limited merely to the first transfer, as is the case in yeast extract broth, but may be continued by loop inoculation from tube to tube.

In studying the effect of heat on the growth accessory substances of potato the interesting fact is brought out that the two factors, X and V, react to temperature in the same manner as the similar substances in blood. The vitamine-like principle is less resistant to heat than the X substance (Table III). Potato broth exposed in the autoclave to a temperature of 120°C. for 45 minutes is no longer capable of supporting growth of *Bacillus influenzæ*. That the V factor is destroyed in the heating and that the more resistant X substance is left unimpaired is shown by the fact that the autoclaved medium can be reactivated by the addition of fresh active yeast extract.

Further evidence that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood for its peculiar nutritive requirements is afforded by the fact that *Bacillus influenzæ* will grow in the complete absence of blood derivatives, meat extractives, and animal peptones. The addition of sterile raw potato to Uschinsky's synthetic medium containing asparagine and ammonium lactate suffices to support growth of *Bacillus influenzæ*. A simpler medium of unheated potato in plain buffer solutions of sodium and potassium phosphate ($\frac{M}{15}$, pH 7.5) fulfills the necessary growth requirements of this organism. It is evident then that potato contains both the V and X substances and that these factors together with the native protein and carbohydrate of potato can replace in media blood pigment and tissue derivatives from animal sources.

In the absence of knowledge of the chemical nature of the X substance and in view of the chemical complexity of the tissues in which it occurs, the identity of the X factor must remain a matter of more or less conjecture. In blood, this substance seems to be associated with the iron-containing pigment, but attempts to substitute inorganic, organic, and colloidal forms of iron have been unsuccessful. The occurrence of the X factor in potato, as well as in blood, suggests that this substance, or substances reacting similarly, may be commonly present in plant and animal tissue. Other vegetables have not been tested; banana, however, has been used and found to contain the essential growth factors. It is interesting to note that although furnishing the necessary growth accessory substances, banana fails to react positively to the benzidine tests. It possesses, however, a markedly active catalase, as evidenced by the evolution of gas when hydrogen peroxide is applied to the cut surface.

DISCUSSION.

The importance in animal nutrition of the presence in foodstuffs of growth accessory substances is now fully appreciated. There is a growing realization among biologists that this peculiar sensitiveness to the want of some particular substance in small amounts is not limited merely to the higher animals. In microbiology this principle finds its earliest expression in the work of Wildiers (11) (1901) on "bios," a substance extractable from yeast cells which exerts an accelerating influence upon the growth of yeast. Bottomley (12) has extracted from decomposing peat, substances which stimulate plant growth, and to which he has given the name "auximones."

The fact is also gaining recognition that bacteria require for growth not merely carbohydrates, proteins, and their derivatives suitably combined in a medium of optimal reaction. Many of the more difficultly cultivable microorganisms are sensitive to the lack in ordinary media of growth accessory substances allied perhaps to those of animal diet. The application of this principle to culture media has given rise to various modifications in the methods of preparation, each of which seeks to preserve or add certain substances, the presence or absence of which is recognized only by the growth-promoting value of the medium for a particular species. No group of organisms exhibits a more striking sensitiveness in this regard than the so called hemophilic variety.

The observations of Thjötta (2) on the multiplication of Pfeiffer's bacillus in hemoglobin-free medium have demonstrated that substances of bacterial origin can replace the growth-inducing factors of blood. It has been pointed out (1, 3) that there are two distinct and separable substances in blood essential to the growth of this bacillus—a vitamine-like principle, the so called V factor, and a second substance, the so called X factor. Both of these substances are found in highest concentration in the cellular fraction of blood. They are both requisite for growth of hemophilic bacilli, and each is separately inactive and without effect. They differ in character one from the other in ways suggestive of the nature of their separate functions. The vitamine-like substance, the so called V factor, can be extracted from red blood corpuscles, from yeast and vegetable cells; it is relatively heat-labile, readily absorbed from solution by charcoal, required in greater concentration than the X factor, and resembles the known vitamines in its growth-promoting action. The second substance, the so called X factor in blood, is heat-stable, present in greatest concentration in red blood cells, absorbed from solution by charcoal, and effective in such minute amounts as to suggest a catalytic nature.

This dual function of blood in fulfilling the growth requirements of *Bacillus influenzæ* has been appreciated though less fully analyzed by previous observers. Davis (5), particularly, has directed attention to the fact that for maximum development of hemophilic bacteria both pure hemoglobin and a second vitamine-like substance are essential. He suggests that these two principles in blood may be analogous to the fat-soluble A and water-soluble B vitamines. In the separation of blood pigment from the other constituents of peptic digests of blood, Fildes (8) observed that neither the clear, colorless filtrate nor the precipitated pigment alone could support growth of *Bacillus influenzæ*. Combination of these two factors in media, however, afforded conditions favorable to growth.

Bacillus influenzæ has heretofore been considered an obligate hemophile. The present studies indicate, however, that the hemophilic property of this group of organisms has been based on a lack of knowledge of their essential nutritional needs. Evidence is presented in this paper that the so called hemophilic bacillus of Pfeiffer is not dependent solely on blood or blood derivatives for its growth requirements. It has been shown that this organism will grow in the total absence of blood derivatives, meat extractives, and animal peptones. The growth accessory substances which occur in blood have been found to exist also in plant tissue. Sterile raw potato can replace blood in the cultivation of *Bacillus influenzæ*.

On the basis of the facts presented, it seems not unreasonable to assume that nutritional deficiency in the cultivation of other bacteria may be overcome by the addition to culture media of the appropriate growth accessory substances.

CONCLUSIONS.

1. Growth of *Bacillus influenzæ* depends on two distinct and separable substances.
2. These growth accessory substances are present in blood.
3. They occur in plant as well as in animal tissue.
4. Sterile, raw potato will serve as a substitute for blood in the cultivation of *Bacillus influenzæ*.

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* Since the acceptance of this paper for publication the following articles have appeared on the accessory substances concerned in the growth and symbiosis phenomenon of Pfeiffer's bacillus:

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