STUDIES ON BACTERIAL NUTRITION.

II. Growth Accessory Substances in the Cultivation of Hemophilic Bacilli.

By THEODOR THJÖTTA, M.D., and O. T. AVERY, M.D.

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

(Received for publication, April 25, 1921.)

In a preceding paper it was shown by Thjötta (1) that *B. influenzæ* will grow on hemoglobin-free medium consisting of plain broth enriched by sterile suspensions or extracts of mucoid bacteria. It was suggested that under these conditions growth may be attributable in part to the presence in the bacterial extracts of substances belonging to the class of so called vitamines. It was found that the growth-promoting action of these substances of bacterial origin was not destroyed by boiling for 10 minutes or by passage through Berkefeld filters. In the present paper are presented the facts thus far acquired in the study of the growth requirements of the so called hemophilic bacilli: the growth-stimulating action of extracts of yeast and of vegetable cells, and the importance of blood as a source of growth accessory substances in the cultivation of bacteria.

The earliest reference in literature to the importance of accessory growth factors in the cultivation of microorganisms is made by Wildiers (2) (1901). This investigator observed that watery extracts of yeast cells greatly enhance the growth of yeast in a synthetic medium.

In confirming the observation of Pasteur on the growth of yeast in a medium composed of mineral salts and sugar, Wildiers found that the size of the inoculum was of the greatest importance; a small seeding would not suffice to initiate growth, while a larger inoculum grew abundantly in this medium. This fact suggested that the growth difference lay in some intracellular factor furnished by the heavier seeding which on the death and disintegration of certain of the yeast cells was liberated, and supplied to the surviving cells a necessary growth-inducing substance. This growth-stimulating principle in yeast to which Wildiers gave the name "bios" was contained within the cell and could be extracted by boiling yeast cells in water. The extract obtained in this manner and concentrated on a water bath was a yellowish, clear, syrup-like fluid. This intracellular substance, the so called "bios," was soluble in water and 80 per cent alcohol, and insoluble in concentrated alcohol and ether. It was resistant to acids but sensitive to alkalies. It could be filtered and dialyzed through paraffined paper.

The findings of Wildiers concerning "bios" were confirmed by Amand (3) who showed that this substance disappeared from the culture medium during growth. This observation Amand interpreted as evidence that "bios" was used up by the yeast cells during growth, and was not a product of their own metabolism.

The work of Wildiers and Amand seems to have been forgotten until quite recently when several investigators have adapted the principle of these earlier workers to methods for the measurement of the vitamine content of various substances. Bachmann (4) measures the vitamines in the test material by adding the sterilized substance to yeast cultures and measuring the amount of gas evolved as an index of growth acceleration; Eddy and Stevenson (5), in determining the presence of vitamine, compare the numerical increase of cells in cultures of yeast in a medium with and without the test substance. On the other hand, from their experiments on the growth of yeast in synthetic media without growth accessory substances, McDonald and McCollum (6) conclude that yeast must be capable either of growing without vitamines or of synthesizing these substances during growth.

While Wildiers and Amand distinctly pointed out the importance of extracts of yeast in the nutrition and growth of yeast cells, they did not extend their observations to the effect of these extracts upon the growth of other microorganisms, such as bacteria. It was not until after the discovery of the value of growth accessory substances in the nutrition of animals that the question of their significance in the growth of bacteria was considered. Bottomley (7) showed the existence in peat of certain substances that have a stimulating effect upon the growth of plants and soil bacteria. These substances he called "auximones" and considered them of the same importance in the growth of plants as vitamines in the nutrition of animals. These "auximones" can be extracted from peat in the same manner as the vitamines from plants, and this fact, together with their apparently uniform action seems to indicate the close relationship between these substances.

Following this discovery it was only a question of time when the principle of growth accessory substances should be brought into full use in the cultivation of bacteria.

In 1916, Lloyd (8) showed that the meningococcus required for its growth a rich supply of vitamines in the medium. Lloyd attributes the value of blood and serous fluids in the cultivation of meningococcus to their vitamine content and believes that Gordon's pea flour and Vedder's starch medium may also contain vitamine which may partly account for the value of these media. In explanation of the fact that meningococcus after artificial cultivation tends to grow with increasing readiness on ordinary media, this author suggests that in adaptation to a more or less saprophytic existence these organisms become independent of vitamine supply.

Cole and Lloyd (9) recommend as the optimum medium for gonococci one rich in amino-acids and vitamines, or "growth hormones." These authors

believe in the existence of two different "growth hormones," a substance present in blood cells and easily absorbed from the media by filtering, which seems to induce the initial growth, and a second substance found in tissues of plants and animals which has the power of inducing luxuriant secondary growth. Davis (10) pointed out that *B. influenzæ* requires in addition to hemoglobin a vitamine substance. He compared these two factors to the fat-soluble vitamine A and the water-soluble vitamine B. Davis showed that the vitamine supply could be obtained from plant and animal tissue. By placing sterile sections of vegetables on blood agar seeded with influenza bacilli, Davis demonstrated that better growth occurred around the pieces of vegetables than on the other portion of the plates, just as Grassberger many years ago called attention to the more luxuriant growth of Pfeiffer's bacillus around colonies of staphylococci.

Davis (10) also applied this principle in the cultivation of other bacteria. As sources of vitamines Davis added to media polished and unpolished rice flour, white and whole wheat flour. If these grains were allowed to sprout the growth was more profuse. The increasing growth on the media made from sprouting grain is explained as due to enzymotic activity in the sprouting process, resulting in changes in proteins, and inversion of starch into sugars.

In the preceding paper (1) on the growth of *Bacillus influenzæ* in broth containing sterile bacterial extracts, it was pointed out that this phenomenon may be explained in part at least by the presence of growth accessory factors, or vitamines, in the bacterial extracts. In this paper an attempt is made to analyze further the growth requirements of the so called hemophilic bacilli and to indicate the significance of growth accessory substances in bacterial nutrition.

EXPERIMENTAL.

Growth Accessory Substances in Yeast and Vegetable Cells.

Although extracts from bacteria of the mucoid variety may be readily prepared, their use is not practicable. In the present study extracts were prepared from yeast cells which are known to be rich in water-soluble vitamine, and which, as previously shown by Wildiers and Amand, have a marked stimulating effect upon the growth of yeast cultures. Extracts of fresh ripe tomatoes, green peas, and green beans were also used, since these vegetables are also valuable sources of growth accessory substances.

Preparation of Extract of Yeast.—100 gm. of brewers' yeast[1] were emulsified in 400 cc. of distilled water. Since the vitamines will stand boiling better in acid than in alkaline solution, the reaction of the suspension of yeast cells was adjusted to pH 4.6, boiled over a free flame for 10 minutes, and then allowed to sediment at room temperature. The clear supernatant extract was pipetted off and tested for sterility. The clear, sterile extract, unneutralized, was stored in the ice box and added to the medium immediately before use. The extract prepared in this manner was a clear, yellowish fluid. Chemical analysis showed the following nitrogen content:[2] total nitrogen, 0.116 per cent; ammonia nitrogen, 0.011 per cent; amino nitrogen, 0.039 per cent; peptide-bound nitrogen, 0.024 per cent; undetermined nitrogen, 0.042 per cent. Before addition of the yeast extract to media, the reaction may be readjusted to optimum for growth of *Bacillus influenzæ*, pH 7.3 to 7.5.

Preparation of Extract of Tomatoes.—Ripe tomatoes were treated in the following way: The skin surface was seared with a red hot knife and through this area a sterile fork was plunged. The tomato was then dipped in alcohol and flamed, then plunged into boiling water for a minute, the skin peeled off, and the stem removed with sterile forceps. The tomatoes were placed in a sterile enamel dish, and crushed with a sterile pestle. The reaction of the tomato juice in its natural condition was pH 4.2, and therefore required no readjustment before boiling, as in the case of the yeast emulsion. The crushed tomatoes were boiled for 10 minutes and the expressed juice either filtered through a Berkefeld filter (N) or cleared by centrifugation, stored at its original acidity, and the reaction readjusted before use. This extract was a perfectly clear, slightly yellowish fluid. Nitrogen partition on this particular extract was as follows: total nitrogen, 0.14 per cent; ammonia nitrogen, 0.014 per cent; amino nitrogen, 0.079 per cent; peptide-bound nitrogen, 0.00 per cent; undetermined nitrogen, 0.047 per cent.

[1] The yeast used in these experiments was supplied through the courtesy of Mr. F. Spitzner of the Central Brewing Company of New York.

[2] For the nitrogen determination on both the yeast and tomato extracts we are indebted to Miss Alma Hiller of the Hospital of The Rockefeller Institute.

Preparation of Extracts of Green Peas and Beans.—Fresh green peas were prepared by flaming the surface and opening the pods with sterile forceps and crushing the separate seeds out into a sterile dish. An equal amount of sterile distilled water by weight was added and the reaction of the emulsion adjusted to pH 4.6 After boiling for 10 minutes the extract was strained through glass wool and then filtered through a Berkefeld filter (N). The resulting extract was perfectly clear and yellowish in color. Similar extracts were prepared from string beans.

Stimulating Action of Vitamine-Like Substances on the Growth of Bacillus influenzæ.—Yeast and vegetable extracts prepared in the

TABLE I.

Stimulating Action of Growth Accessory Substances in Yeast and Vegetable Extracts on the Growth of B. influenzæ.

Dilution of extract in plain broth.*	Growth-stimulating action of.		
	Yeast extract.	Tomato extract.	Extract of peas.
1:10	++	++	++
1:100	++	++	++
1:1,000	+	−	−
1:10,000	−	−	−
Plain broth.	−	−	−

* The extract-containing broth was inoculated with 0.05 cc. of the supernatant fluid of a blood broth culture of *B. influenzæ.*

++ indicates good growth; + moderate growth; ± slight growth; − no growth.

manner described were tested for their stimulating action on bacterial growth by adding them in varying concentration to plain broth of pH 7.8. This medium was inoculated with 0.05 to 0.1 cc. of the supernatant fluid of a blood broth culture of *Bacillus influenzæ.* A bacterial whirl was often visible in the cultures seeded in this manner after 6 hours incubation, and was always marked after growth over night.

Titrations of the vegetable extracts were made in infusion broth to determine the lower limit of the growth-stimulating action (Table I). Table I shows that even in high dilutions extracts of yeast and

of fresh green vegetables are able to stimulate growth of *Bacillus influenzæ* in plain broth if seeded with a small inoculum from media containing blood. When it is considered that the original extracts contain little nitrogenous matter (yeast extract 0.14 per cent) and that only one-thousandth of this amount is present in the dilution required for growth, it becomes obvious that the extracts do not serve merely as additional nutriment, but that their action is accessory in nature, similar perhaps to that of vitamines in animal nutrition. This resemblance is the more striking in that these extracts resist boiling for at least 10 minutes and are destroyed at autoclave temperatures (120°C. for 30 minutes) as shown in Table II.

TABLE II.

Relative Growth Capacity of Yeast and Tomato Extracts after Boiling and Autoclaving.

Temperature.	Tomato extract.	Yeast extract.	No extract.
	In plain broth, 1:10.*	In plain broth, 1:10.*	Plain broth.
10 min. at 100°C.	++	++	−
30 " " 120° "	−	−	−

*Inoculated with 0.1 cc. of the supernatant fluid from an 18 hour blood broth culture of *B. influenzæ*.

In the literature the statement is frequently encountered that the growth value of culture media is greatly impaired by filtration. This loss is attributed to absorption of the so called hormones during the process of filtering. In order to test the effect on the growth-stimulating value of yeast extract after absorption with bone charcoal, the following experiment was carried out.

In each of two small flasks under sterile precautions 1 gm. of bone charcoal and 5 cc. of yeast extract (pH 5.4) were mixed. One portion of the mixture was heated on a steam bath for 15 minutes in order to facilitate absorption; the other was placed at room temperature and frequently shaken. At the end of the absorption period the charcoal was removed from suspension by centrifugation and the absorbed extract tested for its growth-inducing action. Extract without charcoal was heated on a steam bath for 15 minutes to determine whether or not the additional heating affected its potency. The results are recorded in Table III.

From Table III it is apparent that the growth-promoting power of yeast extract is susceptible to absorption by bone charcoal. It is, of course, obvious that the completeness of absorption is related to the concentration of the particular extract and the length of time allowed for absorption. It is evident that under the experimental conditions described the vitamine-like principle in yeast extract is absorbed by bone charcoal and that absorption occurs more promptly under the influence of heat.

From the data presented in the preceding experiments it is evident that *Bacillus influenzæ* will grow when transferred by small inoculum from blood media to plain broth containing extracts of yeast or vege-

TABLE III.

Effect of Absorption by Bone Charcoal on the Growth-Stimulating Action of Yeast Extract.

Dilution of extract in plain broth.*	Yeast extract absorbed.		Unabsorbed.	
	15 min. on steam bath.	2 hrs. at room temperature.	Unheated.	15 min. on steam bath.
1:10	±	+	++	++
1:20	−	±	++	++
1:50	−	−	++	+

* Inoculated with 0.05 cc. of the supernatant fluid of a blood broth culture of *B. influenzæ*.

table cells but fails to grow under similar conditions in the same broth without the addition of these extracts. However, for reasons to be discussed later, continued cultivation fails in broth containing only yeast or vegetable extracts.

While the chemical nature of these growth accessory substances is not known, they are analogous in behavior to the so called vitamines. Extracts containing these substances have been prepared from fresh vegetables and from bacterial and yeast cells. It has been found that they resist boiling for 10 minutes, that they are destroyed by autoclaving, that they contain but little available nitrogen, that they pass a Berkefeld filter with little or no impairment, but are absorbed from water solution by bone charcoal.

Growth Accessory Substances in Blood.

Blood has always been considered requisite for growth of Pfeiffer's bacillus, and the inability of certain organisms to multiply in the absence of blood or blood derivatives has constituted an absolute criterion for their differentiation as hemophilic bacteria. It is important at this point to emphasize again the fact previously mentioned that although *Bacillus influenzæ* grows luxuriantly when transplanted from blood medium to plain broth containing yeast extracts, cultivation cannot be continued for more than one or two transfers in yeast broth alone. Growth deficiency under these circumstances suggests that possibly some other substance may be carried over from the original blood culture in an amount sufficient to supplement the vitamine factor in yeast broth and that growth fails in succeeding cultures in this medium because this second substance is either exhausted by growth or lost by dilution on subsequent transfer. For purposes of discussion this second substance may be referred to as the "X" factor, and the vitamine-like substance as the "V" factor. The theoretical consideration of the presence of these two essential growth factors in blood requires for its substantiation the demonstration of the dual nature of the growth-stimulating property of blood. In the following experiments the interdependence of these two substances and their distribution in the various fractions of blood, body fluids, and crystalline hemoglobin will be discussed.

It has already been pointed out in the first part of this paper that one of the substances, the V factor, can be supplied from a source other than blood, as for instance, yeast. In explanation, therefore, of failure of continued growth of *Bacillus influenzæ* in yeast broth alone, it is suggested that the X substance lacking in this medium is supplied in the first instance by the inoculum from the original blood broth, and that the amount of X furnished in this way is sufficient when supplemented by an excess of the V factor from yeast to sustain growth in the first transfer, but that in subsequent cultures the X factor is quickly lost or perhaps used up by growth of the bacilli. That this second substance, the so called X factor, is actually carried over from blood broth with the first inoculum is shown in the following experiment.

The red blood cells were sedimented from a blood broth culture of *B. influenzæ* by slow centrifugation; the supernatant culture fluid was again centrifuged and the sedimented bacteria were washed three times in large volumes of sterile salt solution to remove any trace of X substance adherent to them. The washed bacilli were resuspended in salt solution to the volume of the original culture and the relative growth capacity of the washed and unwashed bacilli from blood broth was tested in plain bouillon containing yeast extract in 10 per cent concentration.

The facts recorded in Table IV and substantiated in repeated experiments justify the assumption previously made that in addition to the V substance in blood which finds its analogue in yeast extract, there is also present another substance (X) equally essential to growth

TABLE IV.

Relative Growth Capacity of Washed and Unwashed Influenza Bacilli from Blood Culture in Yeast Extract Broth.

Inoculum.	10 per cent yeast extract broth (V) inoculated with.	
	Washed bacilli from supernatant fluid of blood broth culture (no X).	Unwashed bacilli from supernatant fluid of blood broth culture (X present).
cc.		
0.1	−	++
0.05	−	++
1 loop.	−	−

Controls: 0.1 cc. of supernatant blood broth in plain broth without yeast extract showed no growth. 1 loop of washed bacteria on blood agar yielded good growth.

of *Bacillus influenzæ*. This accessory X substance, to the lack of which in media hemophilic bacteria are peculiarly sensitive, is capable in extraordinarily small quantities, such for instance as may be carried over in a single inoculum from blood broth, of supplying the necessary growth conditions. Although this second substance, the so called X factor, can function in minute amounts, it is unable by itself to induce growth, for, as shown by the controls in Table IV, the same inoculum which yields growth in broth containing yeast extract fails to grow in plain broth alone, although in both instances the amount of X carried over in the inoculum is the same.

These two substances may be further differentiated by their relative susceptibility to heat. It is known that the clear fluid extracted from the coagulum of whole blood by short exposure to the temperature of boiling water supports growth of *Bacillus influenzæ*. This blood extract, therefore, presumably contains both the X and the V substances, and its addition to plain media in small amounts suffices for growth of hemophilic bacilli. Since, by boiling for several minutes, substances can be extracted from blood which still possess the growth-stimulating properties exhibited by unheated blood, it is evident that the factors concerned in promoting growth are not destroyed by

TABLE V.

Effect of Heat on the Growth Accessory Substances of Blood.

Dilution of blood extract in plain broth.	Blood extract.* 10 min. at 100°C.		Blood extract.* Autoclaved at 120°C. for 30 min.	
	Without yeast extract.	With yeast extract, 0.5 cc. (V).	Without yeast extract.	With yeast extract, 0.5 cc. (V).
1:10	++	++	−	++
1:100	++	++	−	++
1:1,000	−	+	−	+
1:10,000	−	−	−	−
1:100,000	−	−	−	−

* Prepared by placing a tube containing rabbit blood in boiling water for 10 minutes and using the clear extracted fluid after removal of coagulated material by centrifugation.

short exposure to this temperature. In studying the effect of heat on the growth-stimulating action of tomato and yeast extracts (Table II) it was found that boiling for at least 10 minutes did not appreciably impair their action, while exposure in the autoclave to 120°C. for 30 minutes greatly diminished or completely destroyed their potency. If blood, therefore, contains a substance analogous in its behavior to the vitamine-like principle extracted from yeast, then blood which has been autoclaved should fail to support growth of *Bacillus influenzæ*, since the V factor would be destroyed under these conditions. On the other hand, if the X factor of blood is stable to heat, then the autoclaved blood should be reactivated by the

addition of the fresh V substance in yeast extract. To test the validity of this assumption the experiment presented in Table V was carried out.

From Table V it is evident that the accessory substances in blood essential to growth of hemophilic bacilli are not destroyed by exposure to 100°C. for 10 minutes, since extracts prepared from boiled blood are active in promoting growth. It is further apparent that these same blood extracts after autoclaving for 30 minutes at 120°C. are no longer capable by themselves of supporting life of the bacilli. The experiment further demonstrates the interesting fact that the substance or substances in blood which are destroyed by excessive heat can be replaced by the addition of the growth-stimulating principle extractable from yeast. An analysis of these observations on the heat sensitiveness of the growth-promoting substances in blood indicates clearly that differences exist in thermostability on the basis of which it is possible to separate these two factors. The X factor, so called, is heat-stable and unaffected in action by exposure to steam under pressure at 120°C. for ½ hour. On the other hand, the so called V factor of blood, like the similarly reacting substance in extracts of yeast, is relatively more labile and is destroyed at the autoclave temperature.

Before passing to the question of the distribution of these growth accessory substances in the various fractions of blood, it is well to emphasize the peculiar sensitiveness of the hemophilic bacilli to a lack of either of these two factors in media, and the relative differences in the effective amounts of each. It has been pointed out (Table IV) that in a small inoculum from blood media, a sufficient amount of the X substance may be carried over to cause growth in blood-free broth, providing yeast extract, which supplies the V factor, is also added. In other words, mere traces of the X substance in the presence of this vitamine-like principle suffice for growth, but neither separately can function even when present in excess. This dual action, and the relative amounts of each factor necessary, particularly the minimal effective dose of X, are important in the technique of determining the presence or absence of either factor, since it is possible to carry over with the inoculum enough of the particular factor lacking in a test medium to permit growth. The nature of the seeding, therefore, must

be carefully chosen so that the culture from which it is taken shall contain only a minimum of the particular factor sought for in any given medium or extract. For example, when testing for the presence of the V factor in a given extract or medium, the seeding should be made from the supernatant fluid of a blood broth culture, since in this instance an effective amount of the X substance is carried over with little or no V factor. On the other hand, in demonstrating the presence of the X substance in blood or a derivative of blood, the medium in question should be inoculated from a yeast extract broth culture derived as above, since under these conditions a minimal and ineffective amount of X is transferred in the inoculum. In testing for the X substance an excess of the V factor should always be added in the form of yeast extract, or its equivalent.

Distribution of the Growth Accessory Substances in Blood and Blood Derivatives.—A comparative study of the distribution of these growth accessory factors in blood and blood derivatives was made by determining the presence or absence of growth of *Bacillus influenzæ* in plain broth enriched with graduated amounts of ascitic fluid, serum, blood extract, and solutions of laked red blood cells and of crystalline hemoglobin. The sterile ascitic fluid, untinged with hemoglobin, was obtained from a patient suffering from cirrhosis of the liver and had been stored without antiseptic in the ice box for several months prior to use. The serum, blood extract, and solution of laked blood cells were prepared from freshly drawn defibrinated rabbit blood. The serum was separated from sterile defibrinated blood by repeated centrifugation, care being taken to obtain a specimen with no visible traces of blood pigment. The blood extract consisted of that fraction of whole blood expressed from the coagulum after boiling for 10 minutes, and separated from the coagulated proteins by prolonged centrifugation.

The solution of laked red blood cells was made as follows: The red cells from 20 cc. of sterile defibrinated blood were removed by centrifugation, washed three times in sterile salt solution (50 cc. each time), and taken up in sterile distilled water to the original volume of blood. After laking, the cell residue was sedimented by centrifuging and the clear supernatant fluid pipetted off and used as hemoglobin solution. By gasometric analysis 1 cc. of this solution contained 0.1 gm. of

hemoglobin. The crystalline hemoglobin[3] was prepared by the method of Welker and Williamson (11) from ox blood. The crystalline hemoglobin, as is usual with dry preparations, had lost its oxygen-carrying capacity. A water solution of the crystals (10 per cent by weight) was rendered sterile by passage through a Berkefeld filter (N).

TABLE VI.

Distribution of the Growth Accessory Substances V and X in Blood and Blood Derivatives.

Dilution in plain broth.*	Source of accessory substances.										
	Ascitic fluid.		Serum.		Blood extract.		Solution of laked red cells. †		Solution of crystalline hemoglobin. ‡		Yeast extract.
	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	Without yeast extract.	With yeast extract.	10 per cent as control.
1:2	−	++									
1:5	−	++									
1:10	−	−	−	++	++	++	++	++	−	++	−
1:100	−	−	−	+	++	++	++	++	−	++	
1:1,000			−	+	−	+	+	++	−	++	
1:10,000			−	−	−	−	−	++		++	
1:100,000			−	−				+§		++	
1:200,000										+‖	

* All tubes inoculated with 0.05 cc. of an 18 hour yeast extract broth culture of *B. influenzæ*.

† Contained 10 gm. of hemoglobin per 100 cc.

‡ Contained 10 per cent crystalline hemoglobin.

§ Represents a final concentration of 1:1,000,000 hemoglobin.

‖ Represents a final concentration of 1:2,000,000 crystalline hemoglobin by weight.

The ascitic fluid, serum, and blood derivatives prepared as described above were added to plain infusion broth in the dilutions indicated and inoculated with 0.05 cc. of an 18 hour yeast extract broth culture of *Bacillus influenzæ*. Growth was controlled by subculture on blood agar and by second transfer to yeast extract broth alone.

[3] We are indebted to Dr. J. P. Peters, Jr., of the Hospital of The Rockefeller Institute for the preparation of crystalline hemoglobin used in these experiments.

An analysis of Table VI reveals certain facts concerning the distribution of the growth accessory factors in blood and blood derivatives. In the first place, it is evident that *Bacillus influenzæ* will not grow in serum in concentration as high as 10 per cent in broth or in ascitic fluid diluted with equal volumes of broth. In the presence of yeast extract, however, growth occurred under otherwise identical conditions in ascitic fluid broth 1:5 and in serum broth 1:1,000. Secondly, blood extract and hemoglobin solution freshly prepared from laked red cells in broth were able to stimulate growth in dilutions as high as 1:100 and 1:1,000 respectively. The addition of yeast extract to broth containing these blood derivatives permitted growth in even higher dilutions; namely, 1:1,000 of blood extract, and 1:100,000 of the solution of laked cells. Finally, the addition of a solution of crystalline hemoglobin to broth in concentrations equivalent to the solution of laked red cells failed to yield growth, while in dilutions equivalent to 1:200,000 of laked cells the same solution of crystalline substance afforded excellent growth when yeast extract was added. It should be noted that for purposes of comparison the concentrations are expressed in Table VI in terms of dilution of the original body fluids or their equivalents. However, since in the solutions of laked cells and of crystalline hemoglobin the content of this substance by weight is 10 per cent, the actual dilution of hemoglobin sufficing for growth is, therefore, ten times greater than that recorded in the protocol. In terms of actual amount of crystalline hemoglobin by weight, these figures represent concentrations of 1:100 and 1:2,000,000 respectively.

In attempting to determine the distribution in blood of the so called growth accessory factors V and X, it must be borne in mind that the methods employed are not strictly comparable and that the results are merely relative and of necessity must vary with each individual specimen of serum or blood. Nevertheless, the growth differences are sufficiently great to make it apparent that the greatest concentration of both these substances is associated with the cell fraction of the blood, and that serum and ascitic fluid, on the other hand, contain little or no measurable quantity of the vitamine-like principle and only relatively small amounts of the X substance, as is evident from the fact that by themselves they are separately incapable of support-

ing growth. The quantity of X substance in pure serum is relatively slight as compared with its abundance in solutions of laked cells and crystals of hemoglobin. This fact makes it seem not unlikely that the red blood cell is the source of this substance and that the presence of X in serum or ascitic fluid is purely accidental, due to conditions which permit its escape from the blood cells. The activity of this substance in crystalline hemoglobin in dilutions as great as 1:2,000,000 by weight is strong evidence that it is some constituent of the red blood cell which may function as a catalytic agent. It must be borne in mind that the detection of the X substance by itself in solutions of crystalline hemoglobin, in ascitic fluid, and in serum is made possible only when these are used in combination with vitamine-containing extracts from yeast or from other extraneous sources, since the X substance alone is inactive and unable to support growth without the complementing V factor.

In attempting to interpret the facts brought out in Table VI, it is of further interest to observe the distribution and relative concentration of V factor, or vitamine-like principle, in the various fractions of blood and blood derivatives. Just as the X substance appears to be intimately associated with the cell fraction of blood, so the V factor is apparently found in greatest concentration in the blood corpuscles. Because of the greater susceptibility of the V factor to heat and chemical manipulation, and owing to the fact that it is present in blood in lower concentration than the X substance, this vitamine-like principle is active only when the intact red cell and solutions or extracts of these cells have not been subjected to untoward conditions. For example, the chemical procedures incident to the preparation of crystalline hemoglobin cause complete loss of this factor, while the more stable X substance remains unimpaired. Like the V factor, extractable from yeast and vegetable cells, the corresponding substance in blood extracts withstands boiling for at least 10 minutes, but is destroyed by exposure to 120°C. for 30 minutes, neither of which procedures, however, interferes with the peculiar properties of the X substance in blood. In the specimen of blood extract used in this experiment, the V factor exerted its growth-stimulating action in dilution of 1:100, and in the unheated solution of laked cells, in which the hemoglobin was physiologically active as shown by its oxygen-

carrying capacity, the V factor was present in dilutions as high as 1:10,000. On the other hand, as already noted, solutions of crystalline hemoglobin were devoid of vitamine-like property and required the addition of yeast extract to supplement the X substance present.

DISCUSSION.

As already pointed out, the importance of growth accessory substances in the cultivation of bacteria has been appreciated by numerous observers, and in the case of the hemophilic bacilli has been emphasized particularly by Davis. The foregoing experimental data are presented, therefore, not merely to direct attention to the relation of these substances to the growth of *Bacillus influenzæ*, but rather with the hope that as these studies progress, they may furnish the basis for a more accurate understanding of bacterial nutrition, and that the principles involved may find wider application in the cultivation of organisms other than those of the hemophilic group.

In preceding papers (1, 12) it has been shown that the substances requisite for growth of *Bacillus influenzæ* can be supplied from a source other than blood. The mucoid material elaborated during growth of certain bacteria, together with the dead bodies of these organisms in hemoglobin-free broth, has been found to furnish the accessory substances necessary for the cultivation of the hemophilic bacilli.

The present paper concerns itself with an attempt to analyze the accessory factors in blood which have to do with the peculiar nutritional requirements of the hemophilic bacteria. It is shown that the growth accessory substances in blood involve two distinct and separable factors, neither of which alone suffices to stimulate growth. One of these factors is analogous in its behavior to substances belonging to the class of so called vitamines, and because of this similarity is referred to in the text as the V factor. The other factor is less easily defined and is spoken of as the X substance. On the basis of relative differences in susceptibility to heat, these two factors may be separated one from the other. The vitamine-like principle in blood is destroyed by exposure to a temperature of 120°C. in the autoclave, while the X substance resists heating under these conditions. These substances, however, remain unimpaired in blood sub-

jected to the temperature of boiling water for 10 minutes. Extracts expressed from the coagulum of boiled blood contain both these factors and are capable, therefore, of supporting growth of *Bacillus influenzæ*. On the other hand, autoclaved blood contains only the more stable X substance and is incapable by itself of stimulating growth unless reactivated by the addition of the V factor from another source. That the vitamine-like substance in blood can be supplied from other sources is shown by the fact that extracts of yeast, tomatoes, green peas, and beans possess the property of reactivating an otherwise inert medium containing only the X substance.

Furthermore, it is shown that the addition to plain broth of active extracts of yeast or vegetable cells in dilutions as high as 1:1,000 suffices to initiate growth of *Bacillus influenzæ* when seeded from blood media. However, the fact that cultivation cannot be continued for more than one or two transfers in yeast extract broth alone suggests that in the first instance some of the X substance is carried over in the inoculum from the original blood culture in an amount sufficient to supplement the yeast broth and that growth fails in succeeding cultures because this X factor is either exhausted by growth or lost by dilution on subsequent transfer.

Moreover, study of the relative distribution of these two factors in the constituents of blood demonstrates the fact that they are present in greater concentration in the cellular elements than in the serum or plasma. The facts so far acquired indicate that the red blood cell is the carrier of the vitamine-like principle and that this substance, like its analogue in yeast cells, is intracellular in nature and can be extracted by the methods described. While the chemical nature of this substance is not known, it is presumably analogous to the so called vitamines. It has been found that similar growth-accelerating substances capable of replacing the V factor in blood can be extracted from bacterial and yeast cells and from fresh vegetables. These extracts contain but little nitrogen, they are destroyed by autoclaving, they are water-soluble, they pass a Berkefeld filter, but are absorbed readily from heated solutions by bone charcoal.

The experimental data recorded above indicate that in blood at least the X substance is intimately associated with or a derivative of hemoglobin. The fact that the X factor in crystalline hemoglobin

can function in dilutions as high as 1:2,000,000, when supplemented by an excess of the V factor from yeast extract, suggests that it may act as a catalytic agent. Further observations on the nature of the X substance in blood and its presence in material other than animal tissue will be presented in a subsequent paper.

CONCLUSIONS.

The hemophilic bacteria of which *Bacillus influenzæ* 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, from yeast, and from vegetable cells, which is relatively heat-labile and absorbed from solution by certain agents; (*b*) a so called X substance which is present in red blood cells, is heat-stable, and acts in minute amounts.

BIBLIOGRAPHY.

1. Thjötta, Th., *J. Exp. Med.*, 1921, xxxiii, 763.
2. Wildiers, E., *La Cellule*, 1901, xviii, 313.
3. Amand, A., *La Cellule*, 1904, xxi, 329.
4. Bachmann, F. M., *J. Biol. Chem.*, 1919, xxxix, 235.
5. Eddy, W. H., and Stevenson, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1919–20, xvii, 122.
6. McDonald, M. B., and McCollum, E. V., *J. Biol. Chem.*, 1921, xlv, 307.
7. Bottomley, W. B., *Proc. Roy. Soc. London, Series B*, 1915–17, lxxxix, 102, 481.
8. Lloyd, D. J., *J. Path. and Bact.*, 1916–17, xxi, 113; *Brit. Med. J.*, 1916, ii, 143; 1917, i, 11.
9. Cole, S. W., and Lloyd, D. J., *J. Path. and Bact.*, 1917, xxi, 267.
10. Davis, D. J., *J. Infect. Dis.*, 1907, iv, 73; 1917, xxi, 392.
11. Welker, W. H., and Williamson, C. S., *J. Biol. Chem.*, 1920, xli, 75.
12. Thjötta, Th., and Avery, O. T., *Proc. Soc. Exp. Biol. and Med.*, 1921, xviii, 197.