THE NUTRITION OF BACTERIA, WITH SPECIAL REFERENCE TO *BACILLUS INFLUENZAE* (PFEIFFER)[1].

By S. S. SHRI KENT, M.D., Ch.B., D.P.H., D.T.M., L.R.C.P., M.R.C.S.

John W. Garrett International Fellow in Bacteriology, University of Liverpool.

(*From the Department of Bacteriology, University of Liverpool.*)

(With Plate I.)

The experimental work on which the paper is based was undertaken with the object of investigating the factors which influence the growth of bacteria in various culture media. The *B. influenzae* was selected as the best organism because of the comparative difficulty of obtaining growth on ordinary media, and the feebleness of the growth when obtained. The importance of food accessory substances, or "Vitamins" for the proper growth and nourishment of animals is generally recognised, and the influence of the same on the nutrition of bacteria has been occupying the attention of various investigators during the last few years.

LITERATURE.

Pfeiffer (1893) said the group was haemoglobinophilic, and considered the presence of haemoglobin necessary for growth. Grassberger (1898) could not obtain growth on media containing haematin except when *B. influenzae* was grown in symbiosis with other bacteria. Cantani (1901) succeeded in growing on media enriched with spermatic fluid, and he did not consider the presence of haemoglobin to be essential. Ghon and Preyss (1902) considered haemoglobin as necessary, even though it was present in very small quantities. They also obtained growth in association with other bacteria. Neisser (1903) similarly succeeded in growing on nutrient agar in symbiosis with Xerosis bacillus for twenty generations. Luerssen (1904) was successful in obtaining growth on haematin media to which dead cultures were added, but failed to get a growth in the presence of other living bacteria, as the *B. influenzae* was always overgrown by the associated organisms. Rivers (1920) obtained growth in haemoglobin-free media in symbiosis. Olsen (1920) got growth on haematin

[1] Being a part of the thesis approved for the degree of M.D., University of Liverpool, submitted May, 1922.

https://doi.org/10.1017/S0022172400008056 Published online by Cambridge University Press

or haemin media in symbiotic relationship with other bacteria. Putnam and Gay (1920), and Stillman and Bourn (1920) failed to grow on haemoglobin-free media. Davis (1907) thought that haemoglobin was necessary, and showed that a very small amount was required. In a subsequent communication he (1907) considered two factors necessary, (1) haemoglobin which acts in very high dilutions, and (2) a factor present in animal and vegetable tissues. Fildes (1921) confirmed the observations of Davis that haemoglobin is essential but that it may be present in very high dilutions. He considered the iron in the blood pigment to act as a catalyst in accelerating the transfer of oxygen from the medium to the bacillus. He further stated that unaltered haemoglobin actually inhibits the growth. Rivers (1919) suggested the presence of some inhibitory substance in the human serum, as he obtained abundant growth on 5 per cent. fresh rabbit or cat blood, but not on human blood. Thjötta and Avery (1921) have confirmed the observations of Davis. They consider two substances essential. These are both present in blood, but also in other substances, *e.g.* vegetable material. One of these substances is resistant to heat, while the other is not. They have succeeded in growing *B. influenzae* in the complete absence of blood.

EXPERIMENTAL.

The Amount of Blood required for Growth.

Different proportions of sterile defibrinated fresh rabbit blood were added to 4·50 c.c. broth tubes (pH = 7·6). These were tested for sterility and then inoculated from a 48 hours blood broth culture of *B. influenzae*. The amount of growth developed was determined by (1) the degree of turbidity produced, (2) the relative number of bacteria in films stained with weak carbol fuchsin, and (3) the amount of growth obtained on blood agar tubes on subculturing. Table I shows that the highest dilution of rabbit blood that supported the growth was found to be 1–100,000.

One can hardly conceive that blood in such high dilutions plays the part of a nutrient material. Further experiments showed that if a watery solution, instead of nutrient agar was used no growth was obtained unless the blood was present in a dilution of at least 0·5 per cent. It seems, therefore, that the blood must be considered as an aid rather than the essential factor for growth.

Table I. *Relation of growth to concentration of blood.*

No.	Broth	Percentage of blood	Growth
1.	4·5 c.c.	0·01	+ + +
2.	,,	0·001	+ +
3.	,,	0·0,001	+
4.	,,	0·00,001	+
5.	,,	0·000,002	−

Inoculum: One drop of the supernatant blood free portion of a blood broth culture.

https://doi.org/10.1017/S0022172400008056 Published online by Cambridge University Press

Distribution of the Factors in Blood.

In order to determine in what constituent of blood or blood derivatives the accessory factor or factors are in greatest concentration a number of products were tested.

(1) Ascitic fluid (human).

(2) Blood serum (rabbit). Obtained from blood by repeated centrifugation.

(3) Blood extract (rabbit). The blood was diluted half and half with sterile saline, was raised to boiling point in a water bath. The fraction separated from the clot was employed.

(4) Solution of laked red blood corpuscles (rabbit). The corpuscles were laked with sterile distilled water.

(5) Crystalline haemoglobin (sheep).

It will be seen from Table II that the greatest concentration of the factors necessary for growth is associated with the cellular fraction of the blood.

Table II. *Concentration of Accessory Factors in Blood Derivatives.*

No.	Dilution in broth	Ascitic fluid	Serum	Blood extract	Laked R. B. C.	Cryst. Hb.	Control
1.	1–2	–	+	+ +	+ + +	+	–
2.	1–5	–	+	+ +	+ + +	+	–
3.	1–10	.	±	+ +	+ + +	–	.
4.	1–100	.	–	+ +	+ + +	–	.
5.	1–1,000	.	–	+	+ ±	.	.
6.	1–10,000	.	.	+	+	.	.
7.	1–100,000	.	.	–	+	.	.
8.	1–200,000	.	.	–	–	.	.

Inoculation from a blood broth culture, as above.

Effect of Heat on these Factors.

Two drops of fresh rabbit blood were added to tubes of broth (pH = 7·6), and these were heated up to different degrees of temperature (Table III).

Table III. *Effect of Heat on Blood and Relation to Growth.*

No.	Broth	Blood factor	Growth
1.	4·5 c.c.	Unheated tube	+
2.	,,	Heated at 60° C. for 3 hrs.	+ +
3.	,,	Heated at 100° C. for 7 mins.	+ + +
4.	,,	Autoclaved for 30 mins.	–
5.	,,	,, ,, +0·5 c.c. serum	+
6.	,,	,, ,, +autoclaved serum	–
7.	,,	+0·5 c.c. serum	–

Loop inoculation from a blood broth culture.

From these tables it is apparent that crystalline haemoglobin, or fresh blood plus plain medium is not a good menstruum for the growth of *B. influenzae*, though some growth in such media will occur. When blood is heated its value is decidedly increased, but when heated beyond a certain point the value is altogether destroyed. If fresh unheated serum be now added to this overheated blood it is reactivated, though serum by itself in the same pro-

portion does not support growth. If the serum be autoclaved its power of reactivating overheated blood is also destroyed.

This suggests that we are dealing with at least two different factors. The one present in autoclaved blood, highly resistant to heat, probably associated with the pigment, and the other heat labile present in the serum fraction of the blood.

Davis (1907) found that substances similar in their function to haemoglobin, viz. haemocyanine and haemoerythrine did not sustain growth. He was unable to find any growth inducing action in substances which readily gave up oxygen, such as hydrogen peroxide, and various organic and inorganic salts of iron, etc. Olsen (1920) tested various constituents of blood but all were ineffective. Haematin and haemin were tested in our experiments and they both failed to support the growth, but when fresh bacterial emulsions killed at 60° C. were added growth could be obtained. The inference from this is that growth inducing factors are also present in fresh bacterial emulsions.

SYMBIOTIC PHENOMENON.

It was pointed out as early as 1898 by Grassberger that close to the margin of colonies of staphylococci, the *B. influenzae* tended to form giant colonies. He also stated that if cultures of staphylococci were killed and mixed with the blood media the growth of *B. influenzae* was enhanced. We have often observed the more profuse growth of *B. influenzae* when it is cultivated on blood smeared agar in association with other organisms.

Experiments were carried out to study the phenomena with various types of organisms. A blood agar plate was inoculated with *B. influenzae*, and then at various points on the plate inoculations with other organisms were made. It is not necessary to detail these experiments as whatever type of organism was employed, similar results were noted in varying intensity. The plates showed that the colonies of *B. influenzae* close to the foreign colony are much bigger in size than those a little further away. There is seen a definite narrow area of inhibition, about $\frac{1}{2}$ to 1 mm. in size around the foreign colony. In this area none, or at the most a few colonies of *B. influenzae* develop. But beyond it is an area of augmentation of growth varying from 2 to 5 mm. in diameter, or at times more, in which the colonies of *B. influenzae* are very large and often crowded together, so as to give the appearance of "Satellitism" (see Plate I, fig. 1). Davis lays a great stress on this point; he claims to observe it in every experiment, and bases his diagnosis of suspected material on this observation. It was not found constant in our experiments, though at times it was extremely well marked.

The process is not dependent upon or due to haemolysis, and liberation of haemoglobin from the corpuscles, because it is observed equally well in blood laked or unlaked, and with haemolytic and non-haemolytic bacteria. No stimulating influence is noticed by seeding dead bacteria, but when the

dead cultures are intimately mixed with the medium the *B. influenzae* grows profusely.

It is clear that the associated bacteria during their growth alter the medium in such a way as to render it favourable for the growth of *B. influenzae*. This action may be due to:

(1) Stimulating effects of certain products of bacterial metabolic activities.

(2) Breaking up of the red blood corpuscles, and setting free the contents of the cells.

(3) Destruction of an inhibitory substance normally present in blood.

To investigate these questions further experiments were carried out as under:

I. A few tubes were inoculated with *Staphylococcus albus*, 48 hours growth from agar was washed in sterile broth, sterilized in the water bath at 60° C., and 0·5 c.c. of this sterile emulsion was added to tubes of broth 4·5 c.c. (pH = 7·6). These tubes were tested for sterility, and then inoculated with *B. influenzae* from a young blood broth culture; controls with tubes of ordinary broth were kept.

The control tubes showed no growth but in the other tubes a growth of *B. influenzae* was obtained. The small amount of blood introduced from the culture tube could have had no influence in aiding growth, and this is further established by the absence of growth in the control tubes which contained an equivalent amount of blood. Similarly other organisms were tested and the same observations made (Table IV).

Table IV. *Growth of* B. influenzae *in plain broth + Sterile bacterial Emulsion.*

No.	Broth	Emulsion	Organism	Growth
1.	4·5 c.c.	0·5 c.c.	Staphylococcus albus	+
2.	,,	,,	B. friedlander	+ +
3.	,,	,,	B. diphtheriae	+
4.	,,	,,	B. coli	+
5.	,,	,,	B. xerosis	+
6.	,,	,,	Control	−

Inoculation from a blood broth culture.

II. In similar experiments using only Friedländer's bacillus and *B. diphtheriae* it was found that the organism could be subcultured to the twelfth generation, and live in each tube, as shown by growth on subculture, from 4 to 12 days.

III. The minimum amount of bacterial emulsion necessary for supporting the growth was next determined. The least quantity needed with the different organisms was:

1. Friedländer's bacillus ... ... 0·02 c.c.
2. B. diphtheriae ... ... 0·3 ,,
3. B. xerosis ... ... 0·2 ,,
4. B. coli ... ... 0·05 ,,
5. Staphylococcus albus ... ... 0·1 ,,

https://doi.org/10.1017/S0022172400008056 Published online by Cambridge University Press

IV. *The growth inducing substances were found to be chiefly associated with the cellular fraction of the bacterial emulsion.*

A sample of bacterial emulsion (Friedländer's bacillus) was boiled for five minutes, the product centrifuged, and the clear supernatant fluid tested for the presence of growth factors. Their presence in great abundance was demonstrated, whereas in the supernatant fluid from a similar emulsion not exposed to heat, but allowed to remain at room or incubation temperature for days —only little growth inducing substances were present. This experiment shows that the growth inducing substances are set free from the bacterial cells into the surrounding fluid, the passage being quickened by heat. One exposure for five minutes does not completely remove these bodies, and small quantities can be extracted on the second and to a still smaller extent in the third treatment.

V. Autoclaving the emulsion, or heating it at 100° C. over a prolonged period destroys the substances.

VI. The heated emulsion can be filtered through a Berkefeld filter without loss of the growth inducing properties, and through glass-wool with only slight loss. If, however, the emulsion be first filtered through a filter paper, and then sterilized by filtering through a Berkefeld candle the loss is very considerable. Similar observations have been recorded by Lloyd (1916–17).

VII. Smearing the surface of a nasgar slant with the heated bacterial emulsion does not encourage the growth. But if it be incorporated in the medium active growth is obtained. The minimum amount of bacterial emulsion required in this case is much greater than was needed for liquid media.

From these experiments it seems to be established that substances which induce the growth of Pfeiffer's bacillus and which occur in the cellular fraction of the blood, are also produced during bacterial disintegration. They are resistant to a heat of 60° C., they stand boiling for five minutes, but are destroyed by boiling for fifteen minutes or by autoclaving. They pass through a Berkefeld filter unimpaired, but are lost to a great extent in filtering through paper. To induce growth small quantities only are required in liquid media, but comparatively large amounts in solid media. Simple smearing on to the surface of a solid medium is not sufficient, and incorporation into it is essential. They are associated with the body of the bacteria, just as they were in blood with the cellular fraction, and from the cells they pass out either by disintegration of the cells or by osmosis, and reach the *B. influenzae* by diffusion. In the case of liquid media, through the constant change in surface tension and electrolytic alterations, these bodies are easily brought into contact with the growing bacillus, but there being no such factors in the case of solid media incorporation into the medium itself is essential.

These experiments show that in the phenomenon of symbiosis the supporting bacterium helps growth by producing favourable substances during its active metabolism, and that these are diffusible substances that pass out and reach the main organism (in these experiments *B. influenzae*). This seems

to offer an explanation of the picture of satellitism sometimes observed. The growth factors passing out from the central colony of the foreign organism in all directions like the rays of light from a spot; the seeded *B. influenzae* lying in the way of these products, begin to multiply at the point of contact and to form colonies around the foreign colony. These products would naturally diminish in their amount and concentration the greater the distance from the foreign colony, and consequently the augmenting effect is observed only in a limited field round the foreign colony. Probably the products which are diffused from the foreign colony also produce certain alterations in the blood on the plate.

ALTERED BLOOD AND GROWTH.

We have seen that in the growth of *B. influenzae*, haemoglobin plays the part of an accessory food factor. It has been shown that though the growth in haemoglobin media attains a certain development, it is much greater when another bacterium is at the same time allowed to grow on the plate, or if the products of metabolism of the other organisms sterilized by heating at 60° C. be added to the media. It is apparent from these experiments that at least two factors are concerned which enhance the growth of *B. influenzae*. It has been mentioned previously that these two factors behave differently towards heat, one being heat stable and the other heat labile. The foreign colony not only furnishes the heat labile substance necessary but also breaks up the haemoglobin into its derivatives haematin, and haemin, and thus act in a double capacity with material advantage to the growth of *B. influenzae*.

That changes in the haemoglobin are important are evidenced by many experiments. Avery (1918) used oleic acid and sodium oleate in 1–1000 dilution to bring about changes in the blood, and got a good growth. In our hands this was not so successful. Mathews (1918) and McIntosh (1918) both make a tryptic digest of blood and claim to obtain a copious growth. In our experience the claim has not been established. Fleming (1919) secures abundant growth by using an acid extract of blood, but this also failed in our hands. Fildes' (1920) peptic digest gives a good growth, but the colonies are pin-point in size, and in this respect it is much inferior to the so-called "chocolate medium."

This chocolate or boiled blood medium has been extensively used by various investigators. We have already shown that heat, just like a symbiotic bacterium in some way either by liberation of some substance or by destroying any inhibitory factor in the blood renders it more efficient for the growth of *B. influenzae*. In order further to illustrate this point an experiment similar to the one described at the beginning of the paper was repeated with a few additions (Table V).

This table shows that the ox blood broth tubes heated to 60° C. for four hours, support growth of *B. influenzae* in greater dilution than those which were not so treated. Boiling the blood broth tubes for five minutes gave similar results. Besides the amount of growth in the tubes that were either heated or

Table V. *Concentration of blood, its relation to growth, and effect of heat.*

No.	Broth	Dilution of blood, Ox	Unheated	Heated at 60° C. for 4 hrs.	Boiled for 5 mins.
1.	4·5 c.c.	1–100	+ + +	+ + +	+ + +
2.	,,	1–1,000	+ +	+ + +	+ + +
3.	,,	1–10,000	+	+ +	+ +
4.	,,	1–100,000	+	+	+
5.	,,	1–200,000	–	?	+
6.	,,	1–500,000	–	–	–

Inoculation from a blood broth culture. Results after 48 hrs. incubation.

boiled was much greater than in the unheated. With rabbit and guinea-pig bloods these results were not obtained, in fact heated tubes gave negative results in the same dilutions (higher only) in which the unheated blood gave definite growth. We cannot offer an explanation of the difference between ox and rabbit and guinea-pig bloods. There seems, however, no doubt that the growth inducing substances in rabbit and guinea-pig bloods are easily destroyed by heat. This suggests either that some inhibitory substance present in ox blood is destroyed by the heat, or that some growth inducing substance is liberated from the red cells. An explanation may be found in the inhibitory action on the growth of some bacteria by blood serum.

When graduated doses of serum were added to tubes of "chocolate" agar, it was found that the addition of large doses had a deleterious effect on the amount of growth of *B. influenzae*. It was further observed that this influence was not exerted to the same extent when the serum was previously heated for an hour at 55° C. Davis (1921) and Rivers (1919) have both observed this inhibitory phenomenon. The latter has shown that human serum is bactericidal to *B. influenzae*.

RELATION OF GROWTH TO HEAT AND TIME OF EXPOSURE.

It was found, when ordinary unheated blood was added to plain medium, the growth though definite was not abundant. When the blood was heated at 60° C. for an hour, growth was still scanty. But when heated at that temperature for a prolonged period, 4–6 hours, or at 80° C. for a shorter period, or at 100° C. for a few minutes, the growth was abundant. If submitted to a temperature below 60° C. even an exposure of many days does not render the blood suitable for growth, but generally makes it more ineffective. Exposure for half an hour to a temperature of 100° C. is still more deleterious, and autoclaving at 120° C. totally destroys the growth factors. We found that the maximum amount of growth was obtained with blood subjected to a temperature of 90°–100° C. for 10–15 minutes.

METHOD OF PREPARING A SUITABLE MEDIUM FOR *B. INFLUENZAE*.

In the preparation of this medium advantage is taken of the knowledge gained from the foregoing experiments. When blood is added to a tube of media and heat applied, the blood proteins coagulate, and the medium presents

a muddy, opaque appearance in the tube or poured plate. Filtration through ordinary filter paper by removal of the coagulated proteins produces a clear medium but one which is deficient in growth factors. Filtration through glass-wool produced a uniformly opalescent medium and this gives a luxuriant growth. The degree of opalescence can be greatly reduced by increasing the thickness of the glass-wool layer, without apparently producing any harmful effect on the medium.

After a series of experiments the following method was adopted, and in our hands it has proved of great value. Two important points came out in these experiments: (1) the reaction of the media, and (2) the degree of heat applied. In regard to the former definite growth was obtained between pH = 6·2 and pH = 8·0, but the optimum was pH 7·2–7·5. The heat applied must be just sufficient to extract all the growth factors from the blood. The nutrient agar after sterilization and addition of blood if previously adjusted to pH 7·2 gave the reaction pH 7·4–7·5.

The nutrient agar is prepared in the usual way, adjusted to pH = 7·2 and sterilized in large quantities. Blood, the source does not matter, sterile if possible, is obtained. The agar is melted in 200–500 c.c. quantities, and cooled to 80° C. While at this temperature the blood is added 1 part to 10 or 15 of media. This is well mixed and the temperature of the mixture gradually raised to 90° C., and kept at this temperature for 10 minutes. The mixture is filtered through a glass-wool filter which has been previously sterilized, the whole being kept hot. The filtrate is tubed immediately into sterile tubes, and one sterilization at 80° C. for 20 minutes is given.

One sterilization was as a rule found to be sufficient. If the temperature is raised to a higher degree the resultant blood agar is not so good. Both slant and stock tubes can be prepared. In the preparation of Petri plates care must be taken that the agar is poured into the dishes as soon as it has completely melted. If left in the bath even for a short time precipitation of the proteins takes place.

On this medium Pfeiffer's bacillus gives a slight growth in about 12 hours, and a very luxuriant growth in 48 hours. Colonies 4 mm. in diameter or even larger are sometimes seen. (See Plate I, figs. 2–4.)

In spite of this copious growth it was found that subcultures made after a week or so usually failed to give evidence of growth. This suggests either that the organisms are dead, or that during the copious growth they produce substances which are toxic to, or which at least inhibit growth even on a suitable medium. When these dead cultures and their products from the slant tube were smeared on the surface of a new slant of the same medium and this tube inoculated from a young culture, it was found that the number of colonies that developed were few whereas in a control tube the growth was abundant.

Thus it appears that on a suitable medium, the bacillus grows profusely, produces toxic matter in large quantities and itself dies. May it not be that a similar action takes place in the human body? If the soil is suitable, rapid

multiplication takes place, the toxic material possibly resulting from autolysis, is distributed throughout the body producing the severe symptoms, and the bacterium itself disappears. This view would explain the contradictory statements as to the presence or absence of *B. influenzae* in epidemics.

EFFECT OF FRESH TISSUE AS A SOURCE OF GROWTH FACTORS.

From the experiments already recorded, it is clear that the augmentative effect on the growth of *B. influenzae* in its symbiotic relationship with other bacteria, is associated with the active metabolic processes of living organisms. A drop of fresh blood cannot take the place of these living organisms, but a piece of sterile animal tissue well washed in saline was found to have this stimulating property. If the fresh tissue be autoclaved it loses its power of augmenting growth. Thus this growth factor in the fresh tissues is heat labile, and is probably allied in nature to the substance produced during bacterial symbiosis. This substance is of a diffusible nature, it is not blood, it occurs only in the fresh tissues, is heat labile, and for the growth of *B. influenzae* is needed only in small quantities.

From the clinical point of view the general statement has been made that in mixed infections *B. influenzae* is more dangerous and pathogenic to animals, and possibly to man. We have shown that living tissues will stimulate the growth of Pfeiffer's bacillus quite as actively as associated bacteria. This augmenting effect is being exerted by the tissues of the body to an unlimited extent, and in our opinion the additional influence of an associated organism in the respiratory passages would not make any very great difference in the final results, unless it be that the associated organism favours growth by removing an inhibitory or antagonistic substance in the tissues or plasma.

The same stimulating effect was demonstrated with fresh vegetable tissues. If pieces of carrot or potato were put on blood agar plates, the colonies of *B. influenzae* are crowded round these bits of vegetable tissues, and are much larger in their vicinity than the colonies in other parts of the plate. Pieces of vegetable tissues that were autoclaved or otherwise heated at a high temperature for a few minutes failed to show this augmenting effect. Fresh vegetables contain the various vitamins, which are of such vital importance in animal growth and nourishment, and probably similar substances are concerned in bacterial nutrition as well. As yeast is rich in some of these vitamin bodies, we attempted to get these growth inducing substances from it.

THE HEAT LABILE FACTOR.

Some yeast extract was prepared from a sample of dried commercial yeast. To 80 gms. of yeast were added 300 c.c. of distilled water, and 10 c.c. of $N/10$ hydrochloric acid to make it slightly acid. This was boiled over a flame for 10 minutes and the flask allowed to stand overnight. The clear supernatant fluid was pipetted off the following morning, reaction adjusted to $p\mathrm{H} = 7{\cdot}2$, and sterilized in three different portions. One portion by autoclaving at 120° C.

for half an hour, another by three days' fractional sterilization, and the third by filtering through a Berkefeld candle.

Graduated amounts of each sample were added to tubes of ordinary broth, and inoculated with *B. influenzae*. The broth tubes containing yeast extract sterilized by heating or autoclaving gave negative results, while in the third portion sterilized by filtering through a Berkefeld candle growth was obtained in slight amounts up to a dilution of 1–250. The bacillus survived for three or four days, and in such broth tubes died out in the third subculture. Similar experiments with a sample of brewer's yeast showed it to be definitely richer in the yield of growth factors. In this case growth was obtained up to a dilution of 1–1000, and subcultures up to the fifth generation.

If the primary inoculation was made from a blood broth tube, the growth survived longer than if made from a blood agar tube where the needle touched the surface only lightly. It suggests that in the former case sufficient of another factor was carried from the blood broth tube. This factor present in the blood, the "X" factor of Thjötta and Avery (1921), we have seen previously needs to be present only in very high dilutions, and is heat resistant. The other factor "V" which is heat labile as seen from absence of growth in heat sterilized yeast extract, has already been demonstrated in red blood corpuscles, bacterial extracts, and in animal and vegetable tissues.

Hopkins (1912) has observed that vitamins are substances readily absorbed from solution. Lloyd (1916–17) has also pointed this out. Thjötta and Avery (1921) found that the growth inducing substances are absorbed from yeast extract by bone charcoal, and that the process of absorption is accelerated by the agency of heat. Our results are in agreement with these observations.

Further study of the "V" factor was continued with vegetable extracts, potato, tomato, and carrot. Similar experiments as with yeast extract were carried out, and it was observed that potato, and carrot extracts were decidedly superior to the yeast extract in their content of growth inducing substances (Table VI).

Table VI. *Comparison of Yeast and Vegetable extracts.*

No.	Dilution in broth	Yeast	Potato	Carrot	Tomato
1.	1–5	+ +	+ +	+ +	+ +
2.	1–10	+ +	+ +	+ +	+ +
3.	1–100	+	+ +	+ +	+
4.	1–1,000	+	+ +	+	+
5.	1–10,000	−	+	±	−

Inoculation from a yeast extract broth culture.

THE HEAT STABLE FACTOR.

We have seen that this is present in the blood associated with the cellular fraction and the pigment. By itself it is not capable of supporting growth, but the amount needed when in association with the other factor is very very small. Olsen (1920) states that the Tincture Guiacum, and the Benzedine tests for blood, go hand in hand with the ability of blood derivatives to support

growth. Thjötta and Avery have confirmed these observations, and in our experiments we have found the same parallelism. The latter two investigators have observed a positive reaction with potato, and its capacity to support growth in the complete absence of blood. When studying the heat labile factor it was noted that potato extract was superior to the yeast extract, perhaps it is on account of the former possessing both the factors.

Its Presence in Vegetable Tissues.

Potato contains various peroxides, and the two vitamins A and B, amongst many other undetermined factors. One has often observed that scrapings of fresh potato exposed to air rapidly change colour, as a result of oxidation processes. Hydrogen peroxide is rapidly oxidised with liberation of gas bubbles, and if the benzedine reaction as used for blood is applied, a bluish-purple colour is given. If potato be heated or autoclaved the benzedine test is negative, and the potato no longer supports growth. This suggests that the enzymes concerned with the oxidising processes are more or less associated with the heat labile factor, and that the X factor of Thjötta and Avery is not connected with them. This leads one to attach less importance to the benzedine reaction, for although it is true that in the case of blood there is a parallelism between the benzedine reaction and the yield of growth, this is not the case with vegetable tissues. Moreover, we find that many other vegetable tissues which do not give this test support the growth.

An alcoholic extract of potato and carrot was prepared by triturating the vegetable with sterile sand, and alcohol was added in the proportion of an ounce to 5 gms. of the vegetable. It was left overnight, the supernatant alcoholic solution was pipetted off, the alcohol evaporated over a low heat, 45° C., and the resultant syrupy fluid taken up in sterile distilled water, and the whole sterilized by filtration through a Berkefeld filter. A watery extract was also prepared, by boiling the triturated vegetable for five minutes, the resultant fluid being sterilized by filtration through a Berkefeld filter.

Both the alcoholic and the watery extracts were found to support the growth of *B. influenzae* in the complete absence of blood. Half cubic centimetre quantities of the extracts were added to tubes of broth 4·5 c.c. There was always more growth in the tubes containing the alcoholic extract than with the watery extract, and still more in those with both the extracts. It is apparent that the growth inducing substances are extracted both by water and alcohol from its complex combination in these vegetables, alcohol being the better of the two. But it was found that all the growth requirements for a prolonged activity are not present in either of the two extracts, while the two of them in combination seem to contain most that is needed. When these extracts are added to tubes of melted agar growth is again supported. Experiments similar to those carried out with bacillary extracts were repeated. For want of space the various tables are not reproduced, and just a summary of these experiments is appended.

1.	Alcoholic extract of potato	Growth in a tube from 3–7 days	Cultivated up to 6 generations
2.	Watery extract of potato	Growth in a tube from 3–5 days	Cultivated from 4–6 generations
3.	Alcoholic extract + Watery extract of potato	Growth in a tube from 3–11 days	Cultivated from 8–10 generations
4.	Alcoholic extract of carrot	Growth in a tube from 3–6 days	Cultivated from 4–5 generations
5.	Watery extract of carrot	Growth in a tube from 3–4 days	Cultivated from 3–4 generations
6.	Alcoholic extract + Watery extract of carrot	Growth in a tube from 3–8 days	Cultivated from 6–7 generations

Although in such tubes the bacillus survived up to a number of days and could be cultivated by loop inoculation from a tube to tube to a number of generations, ultimately it died out, manifesting the want of some other product which had probably not been extracted with either of the two extractives. Thjötta and Avery use these vegetables in the raw state putting sterile pieces into tubes of broth. Their technic was followed and the experiments repeated. It was noted that in this case the bacillus could be carried from tube to tube by loop inoculation every 48–72 hours indefinitely up to, at any rate, the twenty-first generation when the experiment was discontinued. On the average in any one tube the bacillus was living from 8–18 days. The potato evidently supplies all the needs for the nourishment of *B. influenzae*.

Broth is a preparation from animal tissues, and may have various substances present in it. In order to eliminate their possible influence, tubes of Ringer's physiological solution were prepared, and to these sterile pieces of potato were added. The bacillus grows and multiplies in such a medium, and subcultures can be carried on indefinitely. But in any single tube it lives for a shorter period 8–11 days. Sterile pieces of potato added to tubes of normal saline also support growth, the bacillus living only from 5–6 days in such tubes.

The above experiments throw light on a number of interesting points, for example, it shows that potato contains all the substances needed for growth. Not only does it supply the heat labile, and the heat stable factors, but it also provides the proteins, carbohydrates, and other products of protein hydrolysis, such as aminoacids, and free nitrogen. It is interesting to note that *B. influenzae* is capable of obtaining all its nitrogen requirements from the native proteins of potato. Then, again, we notice that the bacillus lives for a longer period in tubes of broth plus potato, than in Ringer's solution plus potato, showing that the meat extracts in the broth prolong the duration of life; also the salts in Ringer's solution enable the bacillus to survive over a longer period than in saline plus potato.

Another very important point is in reference to the reaction of the medium. The optimum for growth was established at *p*H 7·2–7·5, the natural reaction of potato is acid to phenol red *p*H 6·2–6·5. The reaction of broth (*p*H 7·6) was

never readjusted after the addition of potato, and it left the final reaction slightly acid, *p*H 6·4–6·7. The bacillus grows abundantly at this *p*H value and no harmful effects of this acid reaction were noticed. It is likely in the presence of raw substances the reaction is not of great importance, perhaps on account of the presence of some "buffers" which mitigate the unfavourable effects.

ACTION OF HEAT ON POTATO.

When these tubes of potato broth were autoclaved, and tested for growth-supporting properties, no growth was obtained. But when the destroyed heat labile factor was replaced by the addition of yeast extract, which we know only contains the heat labile factor, a fair amount of growth was obtained, but it was not as luxuriant as in the original tubes. It shows that the heat stable factor was present in the autoclaved potato, and that the heat labile factor restored by the addition of yeast extract is not suitable, or else that there has been destroyed by autoclaving besides the heat labile factor, something in the potato which cannot be made good in the added yeast extract. Perhaps one of the many enzymes, but one cannot say what rôle these ferments play in the growth of the bacillus in plant tissue media.

Other Plant Tissues and Growth.

Other vegetable tissues were similarly tested for growth-supporting properties. Banana, apple, and white turnip were employed. All the three gave good growth and supported the growth in subsequent generations. There are slight differences in the amount of growth yielded, thus apple is not as good as banana, and this not so good as turnip, whilst they are all inferior to potato. But these results are not strictly comparable, as they do not refer to any weight relation of the various vegetables used, yet they are of interest to prove that *B. influenzae* can be grown in the complete absence of blood and blood derivatives. Besides they are of importance in emphasizing the relation of plant tissues to bacterial growth and nutrition.

ADDITION OF PLANT TISSUES TO BLOOD MEDIA.

On addition of watery or alcoholic extract of these vegetables to melted blood agar no stimulating effect was noticed. If, however, solid pieces of the raw tissues were added to tubes of blood broth there was a definite augmentative effect on the growth. The growth in such cases is first observed in the close proximity of the tissue pieces in the depths of the tube, rather than at the surface where the supply of oxygen is most abundant. This is of interest and importance; the organism grows where there is an abundant supply of the various enzymes, and growth factors, probably there is also a transference of oxygen from the tissue cells.

DISCUSSION.

It has been demonstrated that in the growth of *B. influenzae* haemoglobin in the high dilutions necessary to induce growth, does not play the part of a nutritive substance, but acts in some way as an accessory factor. Thus, it may

act as a catalyst, quickening the reaction velocity of proteolytic metabolism, and rendering an easy and ready supply of nitrogenous substances. The fact, however, that it acts irrespective of the nature of the medium seems to negative this suggestion. We found, for example, that Tryptamine agar supposed to be rich in free aminoacids was not superior to ordinary agar. It may, however, be supposed that haemoglobin acts as a catalyst, and that this action may be dependent in some way upon the iron in the haemoglobin molecule, but the iron itself cannot be said to be more essential than any other element, as various organic and inorganic compounds of iron tested by Davis were valueless. One may consider that the function of haemoglobin in the body, as a carrier of oxygen, gives to it the power of supporting growth. But this is evidently not so, since heating destroys its usefulness as a carrier of oxygen, but increases its capacity to stimulate growth. Again haemocyanin performs the similar function of carrying oxygen but does not support growth.

It has been shown that there are at least two distinct factors in blood which by their combined action stimulate the growth of *B. influenzae*, either of them alone being insufficient. Probably one of them is haematin, which contains the pigment and is associated with iron. The second factor is a heat labile substance, which is readily absorbed from solution, and passes a Berkefeld filter unimpaired, but its nature we do not know. Fildes raises the question as to the possibility of the second factor being a peroxide of such a nature, that through the catalytic action of haematin the transference of oxygen to the bacillus from the peroxide and the medium is accelerated. We observed in tubes of blood broth to which pieces of raw potato were added, that the growth was extensive in the vicinity of these tissues. The fresh potato contains peroxides, and various other enzymes, but it does not necessarily follow that it is the transference of oxygen by the catalytic action of haematin which aids the growth. There is sufficient evidence against this assumption in the fact that heated blood gives luxuriant growth, and that the temperature to which it is subjected alters if it does not actually destroy the peroxides. Further, autoclaved potato, which does not give the peroxide reaction, plus yeast extract, in which the peroxides are absent, supports growth, again in synthetic media in which there is no haematin to exercise a catalytic action growth is also obtained.

In the raw state the vegetable tissues contain the peroxides and various enzymes and ferments. They also contain the fat soluble A, the water soluble B, and the C vitamins, in greater or less proportions. We know these three vitamins, but probably there are many more whose value we have not so far appreciated, and which may play an important part in bacterial nutrition. One thing is certain that the growth inducing substances, like the vitamins, are associated with living and active tissues, both plant and animal. A heat labile factor corresponding to the one in blood, has been demonstrated in bacterial metabolic products, yeast, and in fresh animal and vegetable tissues. The second factor which is heat stable has been found to exist in various plant tissues as well as in blood.

Thus it seems that these growth inducing bodies comprise a large group of substances, occurring in blood, in bacterial products, in animal and in vegetable tissues. Probably the nature varies with the source, but they all seem to have certain properties in common, and they all act as substances accessory to the other food constituents. Their mode or mechanism of action is not understood, and their chemical nature not known, the criteria for their identification being so inadequate. It is suggested that the growth stimulating properties are related to the presence of certain oxidising, and reducing enzymes in fresh plant tissues, as well as to the presence of vitamins.

CONCLUSIONS.

1. Rabbit blood in 1–100,000 dilution supports growth.
2. Haemoglobin in such high dilutions acts as a catalyst in some essential process, rather than as a nutritive agent.
3. The growth depends upon two distinct and separate factors, a heat labile, and a heat stable, which occur in highest concentration in the cellular fraction of the blood.
4. Other organisms distinctly favour the growth of *B. influenzae*.
5. Growth can be obtained up to a number of generations in plain broth plus sterile bacterial emulsion, in the absence of blood.
6. The emulsions can be heated at 60° C. for many hours, or boiled for a few minutes, and filtered through a Berkefeld filter, without impairment.
7. The bacillus grows profusely on a heated blood medium.
8. The augmentative effect is also exercised by fresh animal and vegetable tissues.
9. Sterile raw potato and many other plant tissues, apple, banana, turnip, and carrot, contain both the factors and can replace blood and animal tissue extracts in culture media.
10. The bacillus is not essentially an haemophilic organism but requires certain bodies to support its growth. These are of the nature of hormones, but the way in which the different factors operate to promote the growth is not understood.

In concluding I wish to record my sincere thanks to Professor Beattie for his kind help, valued criticism, and his permission for publication.

REFERENCES.

Avery, O. T. (1918). A selective medium for *B. influenzae*, Oleate haemoglobin agar. *Journ. Am. Med. Assoc.* LXXI. 2050.

Cantani, A. (1901). Ueber das Wachsthum der Influenzabacillen auf hemoglobinfreien Nährböden. *Zeitschr. f. Hyg.* XXXVI. 29.

Davis, D. J. (1907). Haemophilic Bacilli—Their morphology and relation to respiratory pigment. *Journ. Inf. Dis.* IV. 73.

—— (1917). Food accessory factors (vitamins) in bacterial culture with especial reference to haemophilic bacilli. I. *Ibid.* XXI. 392.

—— (1921). Food accessory factors (vitamins) in bacterial culture. The "satellite" or symbiosis phenomenon. *Ibid.* XXIX. 178.

FILDES, P. (1920). A new medium for the growth of *B. influenzae*. *Brit. Journ. Exp. Path.* I. 129.

—— (1921). The nature of the effect of blood pigment upon the growth of *B. influenzae*. *Ibid.* II. 16.

FLEMING, A. (1919). On some simply prepared culture media for *B. influenzae*. *Lancet*, I. 138.

GHON, A. and PREYSS, W. (1902). Studien zur Biologie des Influenzabacillus. *Centralbl. f. Bakteriol.* XXXII. Orig., p. 90.

GRASSBERGER, R. (1898). Zur Frage der Scheinfäden in Influenzakulturen. *Ibid.* XXIII. 353.

HOPKINS, F. G. (1912). Experiments illustrating the importance of accessory factors in normal children. *Journ. of Phys.* XLIV. 425.

LEURSSEN, A. (1904). Beiträge zur Biologie des Influenzabacillus. *Centralbl. f. Bakteriol.* XXXV. 434.

LLOYD, D. J. (1916–17). On vitamins, aminoacids, and other chemical factors involved in the growth of Meningococcus. *Journ. Path. and Bact.* XXI. 43.

MCINTOSH, J. (1918). The incidence of *B. influenzae* (Pfeiffer) in the present Influenza epidemic. *Lancet*, II. 695.

MATHEWS, J. (1918). On a method of preparing medium for the culture of Pfeiffer's Influenza bacillus. *Ibid.* II. 104.

NEISSER, M. (1903). Ueber die Symbiose des Influenzabacillus. *Deutsche med. Wochenschr.* XXIX. 462.

OLSEN, O. (1920). Ueber die Bedeutung des Blutes für das Wachstum des Pfeifferschen Influenzabacillus. *Centralbl. f. Bakteriol.* LXXXV. 12.

PFEIFFER, R. (1893). Die Aetiologie der Influenza. *Zeitschr. f. Hyg.* XIII. 357.

PUTNAM, J. J. and GAY, D. M. (1920). Behaviour of the Influenza bacillus in mixed culture on haemoglobin-free media. *Journ. of Med. Research*, XLII. 1.

RIVERS, T. M. (1919). The effect of different bloods on the growth of *B. influenzae*. *Bull. Johns Hopkins Hosp.* XXXI. 129.

—— (1920). The biological classification of Influenza bacillus. *Ibid.* XXXI. 50.

STILLMAN, E. G. and BOURN, J. M. (1920). Biological study of the haemophilic bacilli. *Journ. Exp. Med.* XXXII. 665.

THJÖTTA, T. and AVERY, O. T. (1921). Studies on Bacterial nutrition. I. Growth of *B. influenzae* in haemoglobin-free media. II. Growth accessory substances in the cultivation of Haemophilic bacilli. III. Plant tissue as a source of growth accessory substances in the cultivation of *B. influenzae*. *Ibid.* XXXIII. 763, XXXIV. 97, 455.

DESCRIPTION OF PLATE I.

Fig. 1. Symbiotic phenomenon. 54 hours' growth on blood smeared agar plate, with a foreign colony of *Staphylococcus albus*. ×6.

Fig. 2. 54 hours' growth of *B. influenzae* on ordinary blood smeared agar plate. ×6.

Fig. 3. 24 hours' growth of *B. influenzae* on blood agar, prepared as described in the text. ×6.

Fig. 4. 5 days' growth on the same medium as in Fig. 3. ×6.

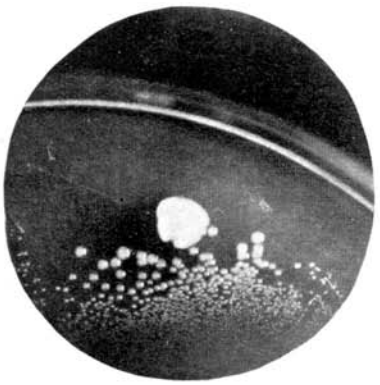

Fig. 1

Fig. 2

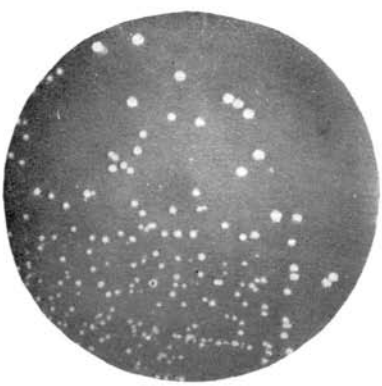

Fig 3

Fig. 4

https://doi.org/10.1017/S0022172400008056 Published online by Cambridge University Press