

## STUDIES ON BACTERIAL NUTRITION.

### I. GROWTH OF BACILLUS INFLUENZÆ IN HEMOGLOBIN-FREE MEDIA.

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(Received for publication, March 8, 1921.)

Pfeiffer in 1892 first obtained growth of *B. influenza* by the use of agar slants, the surface of which was smeared with a few drops of human blood. Pfeiffer showed that this bacillus grew only in media containing blood or hemoglobin. The influenza bacillus was thus brought into the group of the hemoglobinophilic bacilli and has since always been considered an obligate hemophilic organism despite the fact that the literature shows that several investigators have been able to cultivate it in media free of blood or hemoglobin.

The first to show this fact was Cantani (1), who in 1901 obtained growth of *B. influenza* on ascites agar in symbiosis with other bacteria, such as gonococcus, diphtheria bacillus, and several large cocci. *B. influenza* grew in giant colonies in the vicinity of the other colonies. Cantani also obtained growth on the surface of agar to which had been added emulsions of bacteria killed by heat at 60°C. for 3 hours. Cantani excludes symbiosis and supposes that some factor in the bacterial cell induces the growth of *B. influenza* and that this factor is more easily liberated from the dead than from the living cell.

Neisser (2), however, demonstrated the growth of influenza bacilli in symbiosis with xerosis bacilli on plate cultures made from the inflamed conjunctivæ of a child. Impure colonies were observed which could be transferred through many generations on plain agar without the disappearance of *B. influenza*. The latter, however, failed to grow on this medium if the xerosis bacillus was not present.

Neisser also tried to cultivate *B. influenza* on media prepared by adding killed emulsions of the xerosis bacillus or the diphtheria bacillus to plain agar. A slight growth occurred for three or four generations, but the continued cultivation of *B. influenza* on this medium was impossible. He therefore considered the growth of *B. influenza* in mixed cultures as a symbiotic phenomenon.

Grassberger (3) observed that when *B. influenza* was grown on blood or hemoglobin agar plates in association with other bacteria, especially staphylococci, the colonies of influenza bacilli adjacent to the colonies of cocci were of unusually large size. He assumed that the cocci exerted some effect upon the blood medium that was beneficial for the growth of *B. influenza*.

Luerssen (4) confirmed the observations of Cantani by growing *B. influenza* on agar containing emulsions of dead staphylococci, *B. coli*, or *B. prodigiosus*. Growth of *B. influenza* did not occur in ordinary mixed cultures with these other organisms. The bacterial emulsions were sterilized by heating at 60°C. for 3 hours; if they were boiled the growth which occurred was not so good. Luerssen presumes that the bacteria contain a factor that exerts a stimulating effect upon the growth of *B. influenza*, and that this is destroyed at high temperatures. He found that this growth factor is contained in the bacterial cell, since the carefully washed cells are still active. Growth of *B. influenza* did not occur if the enriching emulsion of dead organisms was smeared on the surface of the agar; it only occurred if the emulsion was incorporated in the medium. Luerssen also observed that *B. influenza* grew sparsely in sterile filtrates of broth cultures of staphylococci, *B. diphtheria*, and *B. violaceus*.

Ghon and von Preyss (5) attempted to grow *B. influenza* in media containing no hemoglobin, but the results were negative and they concluded that the reason other investigators had been successful was that in making the inoculations small amounts of blood had been carried over. They likewise held the opinion that when *B. influenza* grows on plain agar, as occasionally happens, this agar contains traces of hemoglobin.

Recently Putnam and Gay (6) tried to confirm the experiments of the earlier investigators. They were unable, however, to obtain any growth of *B. influenza* on plain agar to which either killed or living cultures of *B. xerosis*, *B. diphtheria*, *B. coli*, or staphylococci had been added.

In spite of the observations concerning the growth of *Bacillus influenza* in media free of blood or hemoglobin, the opinion that this organism is hemoglobinophilic has not been altered, and for its cultivation media containing blood or hemoglobin in some form is always employed.

#### EXPERIMENTAL.

In a study on the growth of mucoid bacilli and the transformation of non-mucoid bacilli into mucoid ones (7) the writer undertook the cultivation of different microbes, *Bacillus paratyphosus* B and pneumococci, in broth containing mucus produced by mucoid bacilli such as Friedländer's bacillus, the ozenabacillus, and other similar organisms. An attempt was also made to cultivate *Bacillus influenza* in this medium. The development of this work led to the present studies on bacterial nutrition.

*Experiment 1.*—A culture of a mucoid Gram-negative bacillus (Friedländer's) was grown on plain agar. After 24 hours in the incubator the growth from each plate was suspended in a few drops of plain broth. 0.5 cc. of this emulsion was added to 5 cc. of plain broth, then heated for 1 hour at 60°C., tested for sterility, and stored in the ice box. The reaction of the broth, pH 7.8, was not altered by the addition of the bacterial emulsion. With the medium prepared in this manner, the following tests were made.

A tube of this medium was inoculated with influenza bacilli from a blood broth culture, the blood cells of which had completely settled to the bottom of the

TABLE I.

*Growth of B. influenzae in Plain Broth Added to Emulsions of Friedländer's Pneumonia Bacillus.*

No. of transfers from original culture in emulsion broth.	Age of culture when transferred.	Growth of culture on blood agar after standing in incubator.									
		No. of days.									
		1	2	3	4	5	6	7	8	9	10
Emulsion broth 1	<i>hrs.</i> 48	++	++				0				0
	<i>days</i> 4				+						+
" " 2	<i>hrs.</i> 24	++									
" " 3	<i>hrs.</i> 48	++			++			++	+		
" " 4	<i>hrs.</i> 48	++			++						0
" " 5	<i>days</i> 5	++	++								0
" " 6	<i>hrs.</i> 24	++			++						
" " 7	<i>hrs.</i> 48	++			++						
" " 8	Not transferred.	++									

tube. The following day 0.2 cc. of this subculture was transferred to another tube of emulsion broth. Simultaneously a loopful of the first culture was streaked on the surface of blood agar to demonstrate the growth of *B. influenzae*. The last procedure was necessary since the emulsion broth in itself was too cloudy to show growth. From the first culture there were made eight successive transfers to emulsion broth, in all nine transfers from the original blood broth culture.

The result of these experiments demonstrates that the influenza bacillus grows well in the emulsion broth described. In this medium *B. influenzae* was found living and capable of multiplying after 10 days at 37°C. (Table I).

Experiment 1 was repeated in emulsion broth prepared in the manner described from the mucoid growth of an organism classified as *Bacillus ozæna*.

In this experiment the growth was washed off the surface of plain agar with 1 cc. of normal saline solution and the bacteria were killed by heating at 70°C. for 1 hour. 0.5 cc. of this sterile emulsion was then added to 5 cc. of plain broth. The emulsion broth was inoculated with 0.1 cc. of a blood broth culture of *B. influenza* after all blood cells had settled to the bottom of the tube. 24 hours later 0.1 cc. of this first emulsion broth culture was transferred to a second emulsion broth tube and simultaneously streaked on the surface of blood agar to determine growth. This series of cultures was carried on successfully for ten transfers in emulsion broth, and then was voluntarily discontinued. The ten consecutive cultures all showed typical colonies on the blood agar, and films of the last seven transfers showed typical bacilli and a few involution forms of *B. influenza*.

TABLE II.

*Determination of Smallest Amount of Emulsion Capable of Stimulating Growth of B. influenza.*

Tube No.	Amount of broth.	Amount of emulsion.	Growth.
	cc.	cc.	
1	5	0.5	+++
2	5	0.3	+++
3	5	0.1	+++
4	5	0.01	+
5	5	0	-

*Experiment 2.*—After it had been shown that an emulsion of heat-killed mucoid bacteria, when added to plain broth, is able to support growth of the influenza bacillus, it seemed desirable to learn how small an amount of the emulsion would suffice for this purpose. Accordingly, dilutions of the emulsion in broth were made and inoculated with comparable amounts of a culture of influenza bacillus. After 24 hours incubation subcultures were made on blood agar to confirm growth in the cultures containing various dilutions of the emulsion. These results are recorded in Table II.

0.01 cc. is evidently the lower limit of the growth-stimulating substance in this particular emulsion of heat-killed mucoid bacilli, since cultures containing this amount showed less growth than those in which larger quantities were used.

*Experiment 3.*—In order to determine whether the growth of *B. influenza* in the emulsion broth was more pronounced in the bottom of the tubes or at the surface of the broth, tubes were inoculated after all mucoid material had settled to the bottom. They were allowed to stand in the incubator for 24 hours and subcultures were made both from the thick residuum in the bottom of the tubes and from the superficial layers of the broth. The growth was compared with the following result.

	Growth.
(a) From bottom of tubes.....	+++
(b) From surface.....	+

The fact that this accessory substance appeared to be more concentrated in the immediate vicinity of the sedimented bacterial emulsion than in the upper portions of the culture fluid seems to indicate that the growth-stimulating factor is contained within the bacterial cell and slowly passes out into the surrounding fluid.

It therefore seemed reasonable to attempt to extract this substance from the bacterial emulsion. The addition of a clear bacterial extract to media, moreover, would have the advantage of making it possible to observe bacterial growth directly without the secondary transfer to blood agar. This was done in Experiment 4.

*Experiment 4.*—The growth of a mucoid organism was collected from agar plates and emulsified in plain broth, 1 cc. of broth being used to each plate. The emulsion was boiled for 5 minutes, and then centrifuged to separate the clear fluid extract from the bacterial bodies. This sterile extract was then tested for growth-stimulating action by the addition of decreasing amounts to plain broth. The medium prepared in this manner was inoculated with one drop from an emulsion broth culture (Table I, No. 6). The results of this experiment are recorded in Table III.

TABLE III.

*Growth-Inducing Action of an Extract of Mucoid Bacteria on B. influenza.*

Amount of extract.		Growth.
cc.	per cent	
0.3	6.0	++
0.1	2.0	++
0.05	1.0	+
0.01	0.2	—
0.001	0.02	—
0	0	—

It is evident from Table III that it is possible by simple boiling of an emulsion of mucoid organisms to obtain an extract which when added to plain broth is capable of inducing growth of *Bacillus influenzae*. That the first extraction of the bacillary emulsion does not completely exhaust it of this growth factor is shown in the following experiment.

*Experiment 5.*—An emulsion of mucoid bacilli was made as previously described. A portion of this emulsion was heated at 60°C. for 1 hour and another portion was boiled for 5 minutes and then centrifuged and the clear supernatant extract pipetted off. The bacterial residuum was then washed in normal saline solution three successive times and the following experiment carried out.

	Growth.
5 cc. of plain broth + 0.5 cc. of unboiled emulsion heated to 60°C. for an hour.....	+
5 cc. of plain broth + 0.5 cc. of extract from boiled emulsion...	++
5 cc. of plain broth + 0.5 cc. of residuum from boiled emulsion.	++
5 cc. of plain broth (control).....	—

In this experiment the extract and the residuum from the boiled emulsion, when added to broth, gave even better growth than the emulsion heated at 60°C. for 1 hour.

That extraction of the growth-inducing substance is obtained simply by allowing the bacterial cells to remain in contact with broth for some time is shown in the following experiment.

*Experiment 6.*—To two tubes of plain broth there was added 0.5 cc. of a bacillary emulsion which had been heated to 60°C. for 1 hour. The tubes were then left in the ice box for 1 week. After this time, one tube was centrifuged and the clear supernatant fluid used as culture medium, while from the other, the supernatant fluid was pipetted off without being centrifuged. The two tubes were inoculated with 0.1 cc. of an emulsion broth culture of *B. influenzae*. Good growth occurred in both tubes.

This experiment indicates that the growth-inducing substance passes from the bacterial cells into the surrounding fluid and there exists apart from the cell.

In the foregoing experiments it has been shown that it is possible to obtain good growth of *Bacillus influenzae* in plain broth to which emulsions and extracts of mucoid bacteria have been added. It seemed reasonable, therefore, to seek the same growth factors in other microorganisms, and *Bacillus proteus* was selected as an organism which normally shows an abundant growth on ordinary media.

*Experiment 7.*—Agar plates were inoculated with *B. proteus*. After 24 hours growth, to each plate 1 cc. of normal saline solution was added, and the growth washed off and collected in a sterile centrifuge tube. The emulsion was boiled for 5 minutes. The sterile emulsion was then centrifuged and the supernatant fluid pipetted off. This extract was clear, yellowish in color and had a reaction of pH 7.6. 0.5 cc. quantities of this extract were used to enrich plain broth. After the addition of this amount the beef infusion broth remained clear, so that eventual growth could be indicated by the turbidity of the medium. Control cultures, however, were always made from the extract broth on blood agar or oleate hemoglobin agar, and in plain broth.

In the fluid medium thus prepared the following experiment was made. From a 24 hour culture of *B. influenza* in blood broth 0.1 cc. was transferred to *Proteus* Extract Broth 1; from this after 24 hours growth, to No. 2, and from this to No. 3. These three cultures all gave good growth and upon transfer to blood agar showed the typical colonies of *B. influenza*. Films also showed the typical small Gram-negative bacilli.

The experiment was voluntarily discontinued after the third transfer.

This experiment showed that *Bacillus influenza* would grow on a watery extract of *Bacillus proteus* for at least three generations. An experiment was next made with the whole bacterial emulsion of *proteus* in the same manner as described in Experiment 1. Here, however, growth of *Bacillus influenza* occurred only in the first two transfers after the blood broth culture. A considerable difference between the emulsions of the mucoid bacilli and the emulsion of *proteus*, therefore, seemed to exist. The explanation of this has not been found as yet, but it is reasonable to seek this in the morphological difference between these two microbes. The large capsule of the mucoid bacilli may be a better growth-inducing factor than the capsule-free *proteus*. The possibility that the capsule may contain some nutritional reserve for the bacillus has already been put forth by Toenniessen (8), who finds that the capsule consists of a polysaccharide, galactan, and that other bacteria grow better on the surface of cultures of Friedländer's bacillus than on plain agar.

Since it had been shown that it was possible to extract a growth-inducing factor from *Bacillus proteus*, tests were made to determine the influence of this extract upon the growth of *Bacillus influenza* in various sugar solutions (1 per cent of sugar in peptone water with Andrade indicator (9)).

*Experiment 8.*—To each 5 cc. of sugar medium was added 0.2 cc. of *proteus* extract. The tubes were then inoculated with 0.1 cc. of culture (No. 2 in the foregoing experiment) and incubated. The following sugars were used: lactose, mannitol, maltose, dextrose, saccharose, raffinose, inulin, and salicin. After 12 hours incubation a heavy clouding of the medium was visible in all tubes and the dextrose culture had turned slightly red. After 24 hours the culture containing dextrose was distinctly red, while those containing the other sugars remained colorless. At this point, control cultures on blood agar from all tubes showed pure growth of *B. influenzae*. 4 days after inoculation the tubes showed the same reactions. On transfers to blood broth the cultures were all found to be living and pure.

*Experiment 9.*—It was considered of interest to determine whether or not the clear extract of *B. proteus* could be filtered through a Berkefeld filter without losing its potency. After the extract had been prepared as already described, it was passed through a Berkefeld filter N and the water-clear filtrate, after being proved sterile, was added to plain broth in the following amounts.

Broth.	Filtrate.	Growth.
cc.	cc.	
5	1.0	+++
5	0.5	+++
5	0.2	+
5	0.05	—
5	0.01	—
5	0	—

Growth was controlled by turbidity of the medium, by films, and by subcultures on blood agar and plain agar. These controls showed the growing organism to have the characters of *B. influenzae*.

This experiment shows that the bacillary extract in question can pass through a Berkefeld filter without losing its growth-inducing property.

#### SUMMARY.

From the data presented in the foregoing experiments it is evident that *Bacillus influenzae* will grow in a fluid medium consisting of plain broth to which have been added small amounts of emulsions or extracts of mucoid bacilli or of *Bacillus proteus*. The bacterial extracts may be made by simple boiling of the bacillary emulsions in broth or saline solution and centrifuging out the bacterial bodies; they may be filtered without losing their growth-inducing property.

Cultures of *Bacillus influenzae* in bacterial extract broth, if not too small doses of the extracts were employed, always showed heavier growth than the control cultures in blood broth, and growth occurred at a considerably earlier period than in blood broth. In many instances growth could be seen after 3 to 4 hours, and a bacterial whirl was always visible after 6 hours incubation.

When the nature of the culture used for seeding is not stated, this was 0.1 cc. of the supernatant fluid of a blood broth culture.

All cultures were made in fluid medium; solid medium is much more difficult to use in connection with the extracts.

In explanation of the remarkable growth of *Bacillus influenzae* in this blood-free medium, the idea is proposed that the growth-stimulating effect of the bacterial extracts is due possibly to substances of the same nature as the so called vitamins.

Further investigations on this principle of bacterial nutrition will appear in subsequent papers, together with a more thorough study of the sources and character of the growth-inducing substances.

#### CONCLUSIONS.

1. It is shown that *Bacillus influenzae* will grow profusely in hemoglobin-free media consisting only of plain broth and emulsions or extracts of mucoid bacilli and *Bacillus proteus*.
2. The emulsions and the extracts can be boiled and filtered through Berkefeld filters without losing their growth-inducing property.
3. The growth-stimulating effect of the bacterial extracts is possibly due to substances belonging to the class of the so called vitamins.

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