

## FURTHER OBSERVATIONS CONCERNING GROWTH REQUIREMENTS OF HEMOPHILIC BACILLI.

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Hemophilic bacilli form a heterogeneous group of bacteria. Observations concerning their growth requirements and the hemolysis caused by some strains have been reported. By means of these observations a subdivision of this group of organisms has become possible.

Pfeiffer (1), 1893, first described *B. influenzae*. He was unable to cultivate the bacillus in the absence of blood pigment. Grassberger (2), 1898, reported that Pfeiffer's bacillus in symbiosis with *Staphylococcus aureus* was able to multiply on media containing hematin. Olsen (3), 1920, showed that growth of *B. influenzae* occurred only on media giving a positive peroxidase reaction. Fildes (4), 1920, obtained satisfactory growth of Pfeiffer's bacillus on media enriched by means of a peptic digest of blood. Later, in 1921, he also showed (5) that in this digest each of two factors was essential for the growth of the organism; one was present in the clear supernatant fluid, the other occurred in the brown hematin deposit. Avery and Thjotta (6, 7), 1921, in a series of papers, reported that two accessory factors are necessary for the cultivation of influenza bacilli. These factors were designated as X and V. The V substance is relatively heat-labile and is found in blood, in cultures of many bacteria, and in yeast and vegetable cells. The X substance is heat-stable, gives a positive peroxidase test, and is found in blood and raw potatoes. Rivers and Poole (8), 1921, showed that two accessory factors are essential for the growth of influenza bacilli; one is present in filter-sterilized yeast extract, the other in autoclaved extract of blood. Rivers (9, 10), 1922, reported that Friedberger's *B. hemoglobinophilus canis* requires only the X accessory factor, and that from patients 2 strains of bacilli had been obtained that require only the V accessory factor. The strains requiring only V factor were designated *B. parainfluenzae*. Rivers and Bayne-Jones (11), 1923, obtained from throats of cats 6 strains of influenza-like bacilli that required only the V factor.

Pritchett and Stillman (12), 1919, described a Gram-negative, aerobic, non-motile, hemophilic bacillus and designated it as Bacillus "X." Stillman and Bourn (13), 1920, reported further observations concerning the characteristics of Bacillus "X." Rivers (14), 1921, suggested that these organisms be called hemolytic influenza bacilli. Rivers (10), 1922, on testing a hemolytic strain found that

it required both X and V growth accessory factors. Fildes (15), 1924, reported that 13 of 14 strains of hemolytic influenza bacilli required only the V factor. The remaining strain, however, required both X and V. The former strains grew on ordinary media in symbiosis with staphylococci, and also with *B. hemoglobinophilus canis* on media containing the X factor. This fact indicated to Fildes that *B. hemoglobinophilus canis* synthesizes the V factor.

Particular interest in the hemophilic bacilli was again aroused by the occurrence in New York during the spring of 1926 of a respiratory infection resembling mild influenza. Attempts were made to determine (1) how frequently hemophilic bacilli occur in the upper respiratory tract of patients not necessarily suffering from influenza, and (2) what variations in growth requirements exist among strains of these bacilli obtained from such sources. The results of this investigation are dealt with in the present paper.

#### *Methods and Materials.*

Autoclaved meat infusion broth was used as a basic medium. Filter-sterilized yeast extract, prepared in the manner described by Avery and Thjotta, supplied the V factor. 0.5 cc. of the extract was added to 5 cc. of broth. A solution of hematin, prepared in the manner described previously (11), supplied the X factor. Dilutions, 1 to 10,000, of this solution gave positive reactions for the presence of peroxidase. When the presence of the X factor was desired in the medium, 1 cc. of the hematin solution was added to each 100 cc. of broth. The broth or yeast extract alone never gave positive peroxidase tests.

Bacilli used in this work were obtained from the throats of patients by means of cultures on fresh 2 per cent rabbit blood agar plates. Hemolytic and non-hemolytic Gram-negative bacilli isolated from these plates were carried in stock on blood agar slants. A strain from the spinal fluid of a patient with influenzal meningitis and 1 from a pneumonic lung were also studied.

The bacilli to be tested were removed from blood agar slants by means of a platinum loop and transferred to tubes of broth containing yeast extract, hematin, and yeast extract and hematin respectively. In the case of non-hemolytic strains that grew only in broth containing both factors (X and V), and of hemolytic strains that required the addition of only the V factor, macroscopic evidence of growth was considered sufficient and no subcultures on solid media were made. Other strains, however, were tested more completely in regard to their growth requirements.

#### EXPERIMENTAL.

From 46 patients, 34 strains of so called hemophilic bacilli were isolated. In Table I the sources from which they were obtained and

the frequency with which they occurred in certain groups of cases are indicated. In some of the groups too few cases were studied to permit of a comparison between the groups. Omitting the 4 cases of influenza, however, 42 cases of other diseases were examined and the results are interesting and significant. From these 42 cases, 18 strains of non-hemolytic bacilli (43 per cent) and 11 strains of hemolytic ones (26 per cent) were obtained. Although the patients did not have influenza, 29 of them (69 per cent) harbored hemophilic bacilli in their throats. These findings are in accord with those of other workers who have shown that hemophilic bacilli are likely to be encountered very frequently during periods of epidemic respiratory infections.

TABLE I.  
*Sources of the Hemophilic Bacilli Studied.*

Type of case	Number examined	Number of non-hemolytic strains isolated	Number of hemolytic strains isolated
Chicken-pox	27	8	7
Measles	9	6	3
Influenza	4	1	4
Miscellaneous	6	4	1
Total .....	46	19	15

The 34 strains of bacilli were tested in the manner described above to determine their need of growth accessory factors, V and X. The results are summarized in Table II.

From Table II it is obvious that 17 of the non-hemolytic strains required both growth accessory factors (X and V). They were similar in every respect to organisms usually accepted as influenza bacilli (Pfeiffer). There were 2 non-hemolytic strains, however, that grew well when yeast extract (V) alone was added to the broth. These organisms were carried through 7 successive transplants in this medium. Subcultures were pure. In broth alone or in broth plus hematin, with 0.25 cc. amounts for transfers, no viable bacilli were found by means of subcultures on blood agar after the 2nd transfer. Neither strain produced indole. Both reduced nitrates to nitrites.

Each grew diffusely in liquid media. These bacilli corresponded to Rivers' *B. parainfluenzæ*.

There were 10 hemolytic strains that grew well in broth to which only yeast extract (V) was added. These bacilli were fairly uniform in type. Cultures of them in liquid media usually exhibited flocculi similar to the ones seen in streptococcus cultures. On blood agar plates, colonies of the bacilli were surrounded by large zones of hemolysed red blood cells, were slightly opaque, and were often firm enough to be pushed about intact on the surface of the medium.

TABLE II.

*Summary of Results Obtained Concerning the Growth Requirements of the Hemophilic Bacilli.*

Type and number of strains	Broth + yeast extract	Broth + hematin	Broth + yeast extract + hematin	2 per cent rabbit blood agar
17 non-hemolytic	—	—	+	+
2 non-hemolytic	+	—	+	+
10 hemolytic	+	—	+	+
3 hemolytic	—	—	+	+
2 hemolytic	—	—	—	+
1 <i>B. influenzae</i> , control	—	—	+	+
1 <i>B. parainfluenzæ</i> , control (original Rivers strain)	+	—	+	+
1 <i>B. hemoglobinophilus canis</i> , control	—	+	+	+

The + sign indicates visible growth. The — sign indicates no growth. Yeast extract supplied the V factor; hematin, the X factor.

Upon microscopic examination the bacilli were found to be exceedingly pleomorphic and usually larger than Pfeiffer's bacillus. In order to maintain stock cultures on blood agar slants it was necessary to make transfers more frequently than at weekly intervals.

Three hemolytic strains required both X and V growth accessory factors. They were carried through 7 successive transplants in broth containing yeast extract and hematin. In the later transfers, successful transplants were obtained by means of a platinum loop. In broth containing yeast extract or hematin alone no viable bacilli were present after the 3rd or 4th transfer. 2 other hemolytic strains that grew

well in rabbit blood broth were not viable after 3 or 4 transfers in broth containing both yeast extract and hematin. It is possible, however, that after a longer period of cultivation on artificial media, results with these 2 strains might have been similar to those obtained with the 3 strains described above. On blood agar plates colonies of these 5 strains were surrounded by very narrow zones of hemolysed red blood cells. They were small, transparent, moist, and soft. In fluid media the bacilli always grew diffusely. All 5 strains reduced nitrates to nitrites. One produced indole. Upon microscopic examination these organisms resembled Pfeiffer's bacillus. Weekly transplants of the organisms on blood agar were found to be sufficient for maintenance of stock cultures.

From the results of the experiments already described, it is evident that some hemophilic bacilli hemolyse red blood cells, while others do not, that some require both X and V growth accessory factors, while others require either X alone or only V. Fildes showed that organisms requiring only V, or both X and V, multiplied in symbiosis with *B. hemoglobinophilus canis* on media containing only X. From this observation he concluded that the latter organism synthesized V and supplied it to the bacilli needing it for growth. Since some hemophilic bacilli require the addition of only V to the media used for their cultivation, and since it has been shown that many bacteria capable of growing on ordinary media, e.g. staphylococci, give a positive peroxidase reaction, experiments were performed to determine (1) if the hemophilic bacilli capable of growing on media to which only V is added give a positive peroxidase test, and (2) if they are able to provide the X factor to bacteria needing it for growth. The results of these experiments will be described below.

In the experiments to determine whether the bacilli requiring the addition of only V to the media give a positive test for peroxidase, the following strains were employed: Rivers' original *B. parainfluenzæ*, 2 non-hemolytic strains which were described above and which will be spoken of as *B. parainfluenzæ* Nos. 2 and 3, and 1 hemolytic strain. The 4 strains of bacilli were grown in broth to which yeast extract had been added. Cultures 24 hours old were used in every instance. A portion of each culture was saved for tests. The remaining portion was centrifuged and the sedimented bacilli were resuspended in salt solution. This procedure was repeated twice. A portion of the original culture, the last supernatant salt solution, and the washed bacilli suspended in salt solution were

tested for the presence of peroxidase in the usual way by means of benzidine. The suspension of bacilli was much denser in the salt solution than in the original broth cultures. The results of the experiments are shown in Table III.

The results of the experiments summarized in Table III indicate that the hemophilic bacilli requiring the addition of only yeast extract (V) to the media give a positive test for peroxidase (X) in spite of the fact that the medium in which they are grown gives a negative one.

*B. hemoglobinophilus canis* requires the addition of X to its media, yet apparently produces V. *B. parainfluenzæ* and some of the hemolytic bacilli require the addition of V to their media, but give a positive test for peroxidase (X). In view of these facts it seemed of in-

TABLE III.  
*Results of Benzidine Tests for Presence of Peroxidase.*

Strains tested	Original culture	Supernatant salt solution	Washed bacilli
<i>B. parainfluenzæ</i> , No. 1	±	—	+
<i>B. parainfluenzæ</i> , No. 2	—	—	±
<i>B. parainfluenzæ</i> , No. 3	—	—	±
Hemolytic bacillus	—	—	±

The ± sign indicates a faintly positive reaction. The + sign indicates a positive reaction. The — sign indicates no reaction.

terest to ascertain whether the organisms requiring the addition of X alone and the ones needing the addition of only V are capable of supplementing the growth accessory factors for each other when seeded together in media to which neither X nor V has been added. The manner in which this question was investigated is described below.

Autoclaved meat infusion broth and meat infusion agar to which no growth accessory factors had been added were the media employed. *B. hemoglobinophilus canis* requiring the addition of X alone, *B. parainfluenzæ*, Nos. 1, 2, and 3, needing the addition of only V, and a hemolytic bacillus also requiring the addition of only V were the organisms studied. When a liquid medium was used transfers were effected by means of pipettes, 0.25 cc. being the size of the inoculum. Cultures were incubated 72 hours between transplants. Subcultures on blood agar plates were employed to determine the viability and the types of the bacilli in the different tubes. The ability of the bacilli to grow alone or together under the conditions mentioned is indicated in Tables IV and V.

TABLE IV.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli in Meat Infusion Broth.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. <i>B. hemoglobinophilus canis</i>	V	+	?	-				
2. <i>B. parainfluenzæ</i> , No. 1	X	+	?	?	-			
3. <i>B. parainfluenzæ</i> , No. 2	X	+	-					
4. <i>B. parainfluenzæ</i> , No. 3	X	+	?	-				
5. Hemolytic bacillus	X	+	?	-				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The - sign indicates no growth when subcultures were made on blood agar. The + sign indicates visible growth of bacilli of the type or types with which the series was started.

TABLE V.

Summary of Results of Experiments Concerning Symbiosis of Hemophilic Bacilli on Meat Infusion Agar.

Type and combination of bacilli	Factor supplied by each bacillus or combination of bacilli	Successive transfers						
		1	2	3	4	5	6	7
1. <i>B. hemoglobinophilus canis</i>	V	?	-					
2. <i>B. parainfluenzæ</i> , No. 1	X	?	-					
3. <i>B. parainfluenzæ</i> , No. 2	X	?	-					
4. <i>B. parainfluenzæ</i> , No. 3	X	?	-					
5. Hemolytic bacillus	X	?	?	-				
6. 1 and 2	V and X	+	+	+	+	+	+	+
7. 1 and 3	V and X	+	+	+	+			
8. 1 and 4	V and X	+	+	+	+	+		
9. 1 and 5	V and X	+	+	+	+	+	+	+

The - sign indicates no growth when subcultures were made on blood agar. The + sign indicates a visible growth of bacilli of the type or types with which the series was started.

From the results shown in Tables IV and V, it is obvious that bacilli requiring the addition of X alone and those requiring the addition of only V to their media grow well in symbiosis on media to which neither X nor V has been added. This fact indicates that these organisms are capable of supplementing the growth accessory factors for each other when grown together under the conditions of the experiments.

#### DISCUSSION.

The results of experiments reported in the first part of the paper support the findings previously recorded by Rivers and Fildes concerning the growth requirements of non-hemolytic and hemolytic hemophilic bacilli. Furthermore, these results indicate that a subdivision of this group of bacilli based upon their growth requirements is not only possible but also practicable. The majority of the non-hemolytic strains require the addition of both X and V to their media, while a few need the addition of only V. On the other hand, most hemolytic strains require the addition of only V to their media, while some need the addition of both V and X. It is true that 2 hemolytic strains were encountered that were susceptible to cultivation in blood broth but not in broth containing yeast extract and hematin. In view, however, of their recent isolation and of the difficulty experienced with 3 similar strains that required both factors, it seems justifiable to consider the 5 strains as members of one group, which, in its general characteristics, is more closely related to Pfeiffer's bacillus than to the other hemolytic bacilli under discussion.

The results of the investigation reported in the latter part of the paper apparently indicate that strains of hemophilic bacilli requiring the addition of only V or X to their media are capable of supplying, or acting as, X or V respectively to an extent sufficient for the needs of other bacilli that require the addition of these factors to their media. This fact supports the view that organisms which do not require the addition of either growth accessory factor to their media only multiply on such media because they are capable of synthesizing these factors.

Discussions have arisen in regard to the number of bacilli that should be included in the hemophilic group. Some workers contend

that this group of organisms should not be regarded as hemophilic, inasmuch as all of its members under certain conditions are capable of growth in the absence of blood. There is evidence, also, that in the past similar organisms have been classified under different headings, *e.g.*, Koch-Weeks' bacillus and Pfeiffer's bacillus are identical. Because of the variety of organisms that have been placed in this group any kind of a classification will have some objectionable features. Knowledge, however, recently acquired concerning the growth requirements of the hemophilic bacilli and the ability of some of them to hemolyse red blood cells enables one to outline a relatively simple classification of this heterogeneous group of organisms. Such a classification is presented below. The growth requirements, X and V, of a few bacilli placed in the hemophilic group have not been determined. When this has been accomplished these organisms can then be given their proper place in the classification presented.

*Classification of Hemophilic Bacilli.*

- A. Requiring the addition of V and X to the media.
  - B. influenzae (Hemophilus influenzae).*
  - 1. Non-hemolytic.
  - 2. Hemolytic.
- B. Requiring the addition of only V to the media.
  - B. parainfluenzae (Hemophilus parainfluenzae).*
  - 1. Non-hemolytic.
  - 2. Hemolytic.
- C. Requiring the addition of only X to the media.
  - B. hemoglobinophilus canis (Hemophilus canis).*
- D. Requiring the addition of neither X nor V to the media.
  - 1. *B. pertussis (Hemophilus pertussis).*

SUMMARY.

1. 19 strains of non-hemolytic hemophilic bacilli were studied. 17 required the addition of V and X growth accessory factors to their media, 2 required the addition of only V.
2. Of 15 strains of hemolytic hemophilic bacilli examined, 10 were found to require the addition of only V to their media, 3 the addition of V and X in the form of yeast extract and hematin, and 2 the addition of accessory growth factors in the form of blood.

3. In media to which neither V nor X had been added true symbiosis was found to occur on growing *B. hemoglobinophilus canis*, requiring the addition of X, with *B. parainfluenzæ*, requiring the addition of V, or with hemolytic strains of bacilli, requiring the addition of only V.

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