BIOLOGICAL STUDY OF THE HEMOPHILIC BACILLI.

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In 1892 Pfeiffer[1] described a small Gram-negative bacillus which he associated with the disease influenza. Ultimately this bacillus was quite widely considered as the cause of influenza. The bacillus of Pfeiffer, or *B. influenzæ*, as it was eventually named, is a very small, non-motile, non-sporulating, faintly staining organism with rounded ends. It is irregular in form with a tendency to show bipolar staining. Coccoid forms are also frequently seen, and occasionally small chains of bacilli occur. Pfeiffer recovered the bacillus by smearing pus from bronchial secretions over serum agar, but subcultures failed to grow. He finally discovered that it was the hemoglobin in the pus which enabled the bacillus to grow. Thus this organism came to be known as a hemophilic bacillus. Since Pfeiffer's discovery, various Gram-negative hemophilic bacilli have been described, such as the pseudoinfluenza bacillus, Jochmann's bacillus, Muller's "trachoma bacillus," the Koch-Weeks bacillus, etc. The more recent studies of Wollstein[2] would seem to indicate that while there are minor morphological and cultural differences between these hemophilic bacilli, the distinctions are so slight that the various hemophilic bacilli should be considered identical with *B. influenzæ* or, at most, as varieties of the same species.

The epidemic of influenza in 1918 called to attention the lack of knowledge concerning the biology and epidemiology of the hemophilic bacilli despite the large amount of work done in connection with influenza. In a study of the occurrence of *B. influenzæ* in normal mouths, Pritchett and Stillman[3] described a hemophilic bacillus strikingly similar to, but distinguished from *B. influenzæ* by its ability to hemolyze blood. The colony of this organism cannot be differentiated from that of *B. influenzæ* on oleate agar or chocolate medium, and morphologically the differences are so slight that they cannot be relied upon. As a rule, the so called Bacillus X is slightly larger and coarser than *B. influenzæ* in stained films. The easiest method of differentiation is by growth on blood agar, upon which Bacillus X shows varying degrees of hemolysis. The majority of the strains of this organism actively hemolyze the surface of a blood agar plate and also hemolyze blood broth. An occasional strain is encountered, however,

[1] Pfeiffer, R., *Z. Hyg. u. Infectionskrankh.*, 1892, xiii, 357.

[2] Wollstein, M., *J. Exp. Med.*, 1915, xxii, 445.

[3] Pritchett, I. W., and Stillman, E. G., *J. Exp. Med.*, 1919, xxix, 259.

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whose hemolytic powers are not well developed. If good growth is obtained in plain broth enriched with 2 per cent blood extract, hemolysis may be demonstrated by the use of a 5 per cent solution of washed rabbit blood corpuscles. 1.5 cc. of an 18 hour broth culture added to 0.5 cc. of the washed blood cells and placed in a water bath at 37.5°C. for 1 hour usually cause complete hemolysis. Jordan[4] in 1919 called attention to the fact that certain strains of *B. influenzæ* produced indole. Wadsworth and Wheeler,[5] in their work with *B. influenzæ*, note the production of gas, and also the fermentation of monosaccharides by some strains. Rivers[6] reported that certain strains of *B. influenzæ* produced indole and amylase and could reduce nitrates to nitrites.

In 1906 Bordet and Gengou[7] succeeded in cultivating a small, ovoid, Gram-negative bacillus which they had observed in the sputum of children suffering from pertussis. This bacillus, although very similar morphologically to *B. influenzæ*, is less pleomorphic, slightly larger, and generally appears more ovoid. After frequent subcultures *B. pertussis* grows on ordinary media without the presence of hemoglobin. It grows much more slowly during the first 24 hours of incubation than *B. influenzæ*. On blood agar a fine film of growth is barely visible at the end of 24 hours, while at the end of 48 hours a heavy grayish growth has developed which is very different from the appearance of *B. influenzæ* grown under the same conditions. Ferry and Noble[8] have stated that there is an apparent close relation between *B. pertussis* and *B. bronchisepticus*, although the latter grows luxuriantly on ordinary media and is motile. The bacillus of rabbit septicemia, while not hemophilic, presents a striking morphological likeness to *B. influenzæ*. Because of the morphological resemblance of these various bacilli to *B. influenzæ* a few strains are included for comparison in the present study of the hemophilic bacilli.

As the hemophilic bacilli are delicate organisms which do not grow readily on artificial media special attention must be paid to minute details of technique. This fact is well exemplified by the difficulty with which *Bacillus influenzæ* was cultivated before the use of special media such as oleate agar and chocolate agar and probably in large part accounts for our lack of knowledge of the biology of this delicate organism. Freshly prepared medium adjusted to the optimum hydrogen ion concentration, pH 7.3 to 7.5, is essential for growth.

[4] Jordan, E. O., *J. Am. Med. Assn.*, 1919, lxxii, 1542.

[5] Wadsworth and Wheeler, in Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 7th edition, 1920, 457.

[6] Rivers, T. M., *Bull. Johns Hopkins Hosp.*, 1920, xxxi, 50.

[7] Bordet, J., and Gengou, O., *Ann. Inst. Pasteur*, 1906, xx, 731.

[8] Ferry, N. S., and Noble, A., *J. Bact.*, 1918, iii, 193.

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EXPERIMENTAL.

In the present paper are reported the data obtained during an investigation of the hemophilic bacilli recovered from the throats and sputum of patients suffering from acute influenza and lobar pneumonia, and from the throats and saliva of healthy individuals. The study includes the facts obtained concerning (1) final hydrogen ion concentration, (2) sugar fermentation, (3) indole production, (4) nitrate reduction, and (5) gas production. In addition to the strictly hemophilic organisms, *Bacillus influenzæ* and the so called Bacillus X, described by Pritchett and Stillman, a few strains of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* have been included for comparative study.

Upon isolation the majority of the strains of *Bacillus influenzæ* and all the strains of Bacillus X were plated on dextrose agar to which no hemoglobin had been added. In no instance was growth obtained in the hemoglobin-free medium. After prolonged artificial cultivation, in some instances over 2 years, all these strains were again plated on ascitic dextrose agar without hemoglobin. They invariably failed to grow on media which lacked hemoglobin. All media used in this study were enriched by the addition of 4 per cent defibrinated rabbit blood or 2 per cent blood extract. The latter was substituted for defibrinated rabbit blood in the case of the hemolytic Bacillus X, since the hemolysis produced by this organism might mask certain reactions. In many instances also in the work with the non-hemolytic hemophilic bacilli (*Bacillus influenzæ*) when defibrinated blood might interfere with the determination of a reaction, blood extract was used to enrich the media. The extract was made as advised by Wollstein.[9] Defibrinated rabbit blood was boiled for 2 minutes. The clot was finely broken and centrifuged. The resulting extract was added to the media in such a proportion as to give about 2 per cent enrichment. Since Winchell and Stillman[10] found that the optimum hydrogen ion concentration for growth of *Bacillus influenzæ* is between pH 7.3 and 7.5, all media used in the present study had an initial reaction of about pH 7.4 unless otherwise stated.

[9] Wollstein, M., *J. Exp. Med.*, 1919, xxx, 555.

[10] Winchell, A. I., and Stillman, E. G., *J. Exp. Med.*, 1919, xxx, 497.

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Non-Hemolytic Hemophilic Bacilli (Bacillus influenzæ).

Hydrogen Ion Concentration.—Since the final hydrogen ion concentration reached by growth of an organism in a given medium is a biological constant of differential value, this reaction was determined in the study of the hemophilic bacilli. The colorimetric method, with phenol red and brom cresol purple as indicators, was used to determine the hydrogen ion concentration, and the readings were confirmed by the electrometric method in a number of experiments.

In the usual dextrose meat infusion broth containing 0.5 per cent sodium chloride, *Bacillus influenzæ* attains a final acid reaction of about pH 6.2. If 0.2 per cent sodium phosphate is substituted for the 0.5 per cent sodium chloride in this medium, as is customary for routine purposes in these laboratories, the buffer value is so great that the changes in reaction are insignificant. Since *Bacillus influenzæ* evidently produces relatively small amounts of acid it is desirable in determining the final hydrogen ion concentration to use the medium containing less buffer. Consequently, throughout this work broth containing 0.5 per cent sodium chloride has been used.

In order to determine whether the initial hydrogen ion concentration of the medium had any effect on the final reaction, separate portions of dextrose broth, adjusted to varying hydrogen ion concentrations from pH 8 to 6.8, were inoculated with the same culture of *Bacillus influenzæ*. Text-fig. 1 shows the initial hydrogen ion concentration of the broth when inoculated and the hydrogen ion concentration of the cultures after 20, 44, and 70 hours incubation. From this it is seen that the initial reaction bears no relation to the final hydrogen ion concentration, which is pH 6.4 in each instance.

The relation of oxygen supply to growth was next tested by inoculating a series of 100 cc. Erlenmeyer flasks containing 25 cc. of dextrose broth and a set of large test-tubes containing a similar amount of broth. The initial hydrogen ion concentration of the media was pH 7.3. The test-tube cultures were incubated in an upright position. Colorimetric readings of the hydrogen ion concentrations of the cultures were made after 1, 3, 7, and 14 days incubation. After 24 hours incubation the flask cultures had attained a pH of 6.4, while the test-tube cultures did not reach this end-point until the 7th to 10th day.

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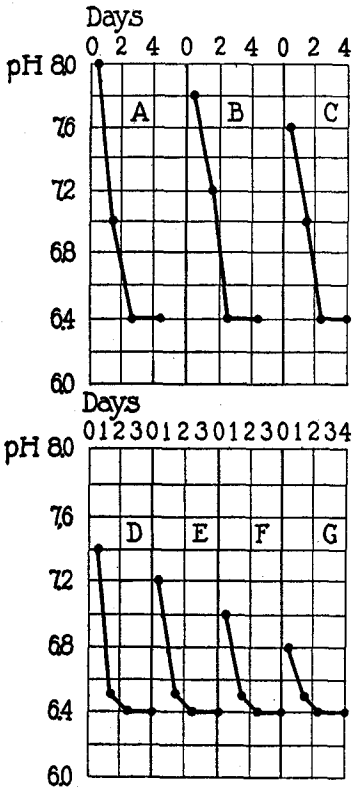
It was noted that the macroscopic appearance of the cultures is not a criterion of the hydrogen ion concentration. Cultures which are very turbid and apparently have grown well, when tested may be found not to have reached their lower limit of acid production.



TEXT-FIG. 1. Effect of different initial hydrogen ion concentrations on final hydrogen ion concentrations of *B. influenzæ*.

The final pH was determined on a large series of cultures of *Bacillus influenzæ*. It was found to lie between pH 6 and 6.4. The length of incubation necessary before the different strains, and even the same strain, reach their final hydrogen ion concentration varies. Some strains reach the final reaction at the end of 24 hours; others, at times, do not reach pH 7 even after 14 days incubation in large slanted test-tubes. This variability of growth of *Bacillus influenzæ* has been

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encountered throughout the present study. In working with this organism experiments giving negative results must be repeated, since the results may be due merely to insufficient growth.

Sugar Fermentation.—Since all the cultures of *Bacillus influenzæ* ultimately reached a final hydrogen ion concentration of at least pH 6.4 in dextrose broth, a reaction sufficiently acid to be detected by the Andrade indicator, the ability of these organisms to ferment different sugars was tested. It was found that sugar-free broth could not be used as nutrient substrate even after enrichment with blood extract and the addition of the test substance, for *Bacillus influenzæ* did not readily produce acid in this medium. Since meat infusion broth contains varying amounts of muscle sugar which might possibly modify fermentation reactions, Dunham's peptone solution was employed as a basis for the sugars, which were added in 1 per cent concentration. The peptone solution does not contain sufficient reducing sugar to give a positive test with Benedict's reagent. In Dunham's peptone solution enriched with 2 per cent blood extract *Bacillus influenzæ* grows luxuriantly. All culture tubes were incubated in a slanted position so as to expose to the air as large a surface of the medium as possible, since it has been shown that acid production is more rapid under these conditions.

The results of the sugar fermentation are given in Table I. It is seen that almost all strains of *Bacillus influenzæ* produce acid in the monosaccharides, dextrose and galactose. Acid production is less marked and more irregular with levulose. Some strains of *Bacillus influenzæ* fermented the polysaccharides, maltose and saccharose, and to a less extent dextrin. No strains could attack mannitol or lactose. A number of strains were tested against inulin, but the results were so consistently negative that this test substance was subsequently discarded. It is evident that the sugar reactions are irregular. This irregularity is especially noticeable if the same strain is repeatedly inoculated in the same sugar. Although good growth was apparently present, a culture which had been repeatedly positive in dextrose, for instance, at times failed to produce sufficient acid to cause the Andrade indicator to change color. The same factors which sometimes prevented cultures from reaching their final hydrogen ion concentration of pH 6.4 in dextrose broth apparently were acting here.

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TABLE I.

Source, Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Non-Hemolytic Hemophilic Bacilli (B. influenzæ).

Source of strain.	Total No. of strains.	Strains fermenting sugars.																Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Acute influenza	21	20	95	20	95	13	61.9	4	19	2	9	3	14	0	0	0	0	14	66	21	100	1	4	0	0
Convalescents from influenza	11	11	100	9	82	10	91	0	0	0	0	1	9	0	0	0	0	8	72	11	100	0		0	0
Lobar pneumonia	18	18	100	18	100	8	44	2	11	1	5	1	5	0	0	0	0	12	66	18	100	1	5	0	0
Normal individuals during winter of 1918–19	33	32	97	28	85	24	72	5	15	6	18	4	12	0	0	0	0	18	54	31	94	2	6	0	0
Normal individuals during Sept., 1919	11	10	91	9	82	8	72	5	45	5	45	5	45	0	0	0	0	5	45	11	100	5	45	0	0
Normal individuals during Feb., 1920	25	25	100	24	96	24	96	15	60	16	64	14	56	0	0	0	0	6	24	25	100	7	28	0	0
Total	119	116	97	108	90	87	73	31	26	30	25	28	23	0	0	0	0	63	53	117	98	16	13	0	0

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Hydrogen ion determinations were made on a number of strains grown in media containing different sugars. The strains of *Bacillus influenzæ* which showed fermentation with the Andrade indicator had a final hydrogen ion concentration of pH 6 to 6.4 which corresponded to that obtained in dextrose broth when no Andrade indicator was present. An interesting result was the reaction of *Bacillus influenzæ* in lactose. In this sugar there was a definite increase in alkalinity, as illustrated by Table II. If plain peptone solution, peptone solution plus dextrose, and peptone solution plus lactose are inoculated with the same culture of *Bacillus influenzæ* and incubated for 10 days the dextrose culture becomes acid, but the culture in plain peptone and that containing lactose become alkaline. Bacillus X produces a similar increase in alkalinity in lactose media.

TABLE II.

Final Hydrogen Ion Concentration of the Non-Hemolytic Hemophilic Bacilli in Peptone Solution, Peptone Solution Plus Lactose, and Peptone Solution Plus Dextrose.

Solution.	pH
Peptone	8.4+
" + 1 per cent lactose	8.4+
" + 1 " " dextrose	6.0

In the course of the work on sugar fermentation Bronfenbrenner's double indicator "CR" was tested.[11] This indicator is composed of equal parts of a 0.5 per cent aqueous solution of China blue and a 1 per cent alcoholic solution of rosolic acid. China blue appears blue or bluish green in the presence of acid, and colorless in the presence of alkali. Rosolic acid, which is colorless in an acid medium, becomes pink in an alkaline medium.

Dunham's peptone solution containing 1 per cent sugar concentrations and enriched with 2 per cent blood extract was used. 1 per cent CR indicator was substituted for the Andrade indicator previously used. Only two sugars, dextrose and lactose, were tested. Representative strains of the Gram-negative bacilli under observation were used in determining the value of CR as an indicator of acid and alkali production in the presence of these two sugars.

[11] Bronfenbrenner, J., *J. Med. Research*, 1918–19, xxxix, 25.

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The hemolytic and non-hemolytic strains of the strictly hemophilic bacilli produced acid in the dextrose CR medium and alkali in the lactose CR medium as indicated by the definite color changes after 48 hours incubation. The intensity of the reaction increases with prolonged incubation. The bacillus of rabbit septicemia also produced acid in the presence of dextrose and alkali in the presence of lactose. The strains of *Bacillus pertussis* and *Bacillus bronchisepticus* produced alkali in both the dextrose and lactose media. These results correspond exactly with the results obtained with similar sugar media in which Andrade indicator replaced CR, as will be seen by referring to Tables I, III, and IV.

CR, in the concentration used, does not appear to be bactericidal for any of the strains tested in the fluid medium described, for good growth was obtained in each instance and the color changes which occurred were striking. In poured oleate hemoglobin agar plates to which CR and the desired sugar had been added in 1 per cent concentration, striking color changes did not occur. In the concentration used in solid medium CR did not appear to be of value as a differential indicator.

Indole Production.—Jordan has called attention to the production of indole by *Bacillus influenzæ*. The indole production by cultures included in this study was tested by Ehrlich's para-dimethylaminobenzaldehyde method. Of the 119 strains of *Bacillus influenzæ* studied, 63, or 53 per cent, produced indole. It was found that indole was present at times after only 18 hours incubation at 37°C. and was produced for as long a period as 3 weeks. Indole was produced quite regularly in plain blood broth cultures, but slightly more positive reactions were obtained if Dunham's peptone solution enriched with blood extract was used. This may be due to the fact that the defibrinated blood masked delicate reactions. The same irregularity of reaction that was noted in the fermentation of sugar by *Bacillus influenzæ* was observed in the production of indole; occasionally an indole-producing strain, which apparently had grown luxuriantly, failed to produce indole. Hence the necessity of repeated tests before a culture may be definitely classified as a non-indole producer. A possible relation exists between indole production and sugar fermentation. Only one indole-producing strain fermented the polysaccharides.

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Nitrate Reduction.—Of the 119 strains of *Bacillus influenzæ* studied, 117 were able to reduce nitrates to nitrites. This reduction as a rule occurred after 24 hours incubation. Like other reactions with these delicate organisms, at times negative results were obtained without apparent reason. In a few instances a culture which had given a positive nitrite reaction after 24 hours incubation failed to give it after 10 days incubation.

Gas Production.—The ability of the hemophilic bacilli to produce gas was first tested by making stab cultures in 1 per cent dextrose agar to which a small amount of blood extract had been added. Under these partial anaerobic conditions luxurious growth was obtained with all strains. Of the 119 non-hemolytic strains, sixteen, or 13 per cent, were found to produce gas. The gas appeared usually after 48 to 72 hours incubation, but with several strains did not appear until later. The time of incubation necessary before the appearance of gas varied on different occasions when the same strain was used. The amount of gas produced was never very great.

Stab cultures were made with blood extract dextrose medium containing agar in concentrations of 1, 1.5, and 2 per cent. 1 per cent agar seemed to be the most favorable concentration for the demonstration of gas. Gas production was demonstrated also in shake cultures of dextrose blood extract agar. With the exception of one strain, the non-hemolytic gas-producing organisms did not produce gas in Smith fermentation tubes when meat infusion broth or Dunham's peptone solution containing dextrose and blood extract was used. It appears, therefore, that a solid medium is more suitable than a fluid medium for the production of gas by these non-hemolytic strains of hemophilic bacilli.

Hemolytic Hemophilic Bacilli (Bacillus X).

The hemolytic hemophilic bacilli reach a final hydrogen ion concentration which varies from pH 6.4 to 5.8. Most strains ferment dextrose, maltose, and saccharose readily and quite regularly, and utilize galactose, levulose, and dextrin less easily and more irregularly. The same irregularities and difficulties of growth have been encountered with this organism as with the non-hemolytic type, although it is not quite so variable. Of the twenty-nine hemolytic strains studied only

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three produced indole after repeated attempts. All the strains reduced nitrates to nitrites. Only four strains, or 13 per cent, showed the production of gas in dextrose blood extract agar. With these organisms the gas appeared usually after 24 to 48 hours of incubation. With only one of the strains could gas production be demonstrated in a Smith tube with meat infusion broth containing dextrose and blood extract. Stab and shake cultures were made in a similar manner as described above in connection with the non-hemolytic organisms.

TABLE III.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of the Strains of Hemolytic Hemophilic Bacilli (Bacillus X) Isolated from Normal Individuals during the Winter of 1919–20.

Total No. of strains.	Strains fermenting sugars.																		Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
	Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
29	29	100	3	10	9	31	24	82	23	79	15	51	0	0	0	0	0	0	3	10	29	100	4	13	29	100

Relation between Indole Formation and Gas Production.

An apparent relation between indole formation and gas production can be observed. All the non-hemolytic gas-producing strains are non-indole producers and comprise strains that ferment mono- as well as polysaccharides. The hemolytic gas-producing strains, with one exception, produce indole. These hemolytic gas-producing strains do not ferment sugars so readily as the other organisms in this group.

Comparative Study of Bacillus pertussis, the Bacillus of Rabbit Septicemia, and Bacillus bronchisepticus.

Table IV shows the results of a comparative study of *Bacillus pertussis*, the bacillus of rabbit septicemia, and *Bacillus bronchisepticus* in connection with the hemophilic bacilli.

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TABLE IV.

Sugar Fermentation, Indole Formation, Nitrate Reduction, and Gas Production of Strains of B. pertussis, the Rabbit Septicemia Bacillus, and B. bronchisepticus.

Bacillus.	Total No. of strains.	Strains fermenting sugars.																		Strains producing indole.		Strains reducing nitrates.		Strains producing gas.		Strains producing hemolysis.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.		Mannitol.		Lactose.		Inulin.									
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
B. pertussis	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Rabbit septicemia bacillus	4	4	100	4	100	4	100	0	0	4	100	0	0	4	100	0	0	0	0	4	100	2	50	0	0	0	0
B. bronchisepticus	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

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Bacillus pertussis.—The four strains of *Bacillus pertussis* studied were stock cultures which had been under artificial cultivation for a considerable length of time. They had a final hydrogen ion concentration of pH 8 to 8.6 in dextrose broth and failed to produce acid in any of the sugars tested. Neither did they produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

Bacillus of Rabbit Septicemia.—The four strains of the bacillus of rabbit septicemia had a final hydrogen ion concentration of pH 6 and produced acid in dextrose, galactose, levulose, saccharose, and mannitol. They did not produce acid in maltose, lactose, dextrin, or inulin. These organisms produced indole slowly, and two strains, or 50 per cent, reduced nitrates to nitrites. In dextrose agar stab cultures there was good growth in the stab, but no gas was produced.

Bacillus bronchisepticus.—The two strains of *Bacillus bronchisepticus* studied had a final hydrogen ion concentration of pH 9.2 in dextrose broth and failed to produce acid in any of the sugars tested. They did not produce indole or reduce nitrates to nitrites. In dextrose agar stab cultures there was only a slight growth below the surface and no gas was produced.

DISCUSSION.

The small Gram-negative hemophilic bacilli which have gradually come to be considered as belonging to one group of organisms and to which the name *Bacillus influenzæ* has been given, appear in the light of the present study to be rather a group of closely allied bacilli which have demonstrable biological differences. The bacillus which Pfeiffer first described and associated with clinical influenza is now questioned as being the etiological factor in the spread of this disease. However, the percentage of cases in which the bacillus of Pfeiffer has been recovered is great enough to indicate that this organism may be at least a secondary invader. Since the first description of this hemophilic bacillus in 1892 by Pfeiffer, little has been added to our knowledge of its biological characteristics.

In this study we have found that the hemophilic bacilli observed divide themselves naturally into two large groups according to their

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ability to hemolyze whole blood. The hemolytic group comprises the organisms originally described as Bacillus X by Pritchett and Stillman, and occurs in normal mouths. Many of these hemolytic bacilli have no doubt been confused with the non-hemolytic variety due to the almost universal use of chocolate medium. On oleate agar the colonies are so similar that they cannot be distinguished, and morphological differences are so slight as not to be reliable. Organisms of the hemolytic type (Bacillus X) do not live so long in culture media as those of the non-hemolytic type. They are best preserved at a low temperature. A few strains have been found to live from 2 to 3 weeks if kept in blood broth in the ice chest, but in order to be successfully preserved in stock cultures they must be transplanted every 6 or 7 days. At room temperature Bacillus X survives about 5 days, while at 37.5°C. it remains viable about 10 days. The non-hemolytic group (*Bacillus influenzæ*), on the other hand, remains viable for a month or more at room temperature in blood broth.

The hemolysin produced by the hemolytic type is quite stable, retaining its activity after being kept on ice for 6 weeks to several months. It can be demonstrated in a young broth culture after 2 hours incubation at 37°C. It is non-filterable and is destroyed by heating for $\frac{1}{2}$ hour at 56°C. Different strains vary, however, in their ability to produce hemolysis. The hemolytic bacilli are non-pathogenic for rabbits, guinea pigs, and mice.

Both the non-hemolytic and hemolytic groups of hemophilic bacilli attain a final hydrogen ion concentration of approximately pH 6.4, although the hemolytic group may reach pH 5.8. Both produce acid in dextrose, but in both groups only certain strains ferment saccharose. The greater ability of the hemolytic organisms to ferment sugars may be a basis for further differentiation.

A tentative classification, graphically illustrated below, defines a small subgroup of the hemolytic group formed by the strains which produce indole and gas but do not ferment saccharose. These strains appear to ferment sugars less readily and require further study to determine whether the indole-producing strains are also gas producers. The greater number of hemolytic strains, however, do not produce indole or gas, but ferment saccharose.

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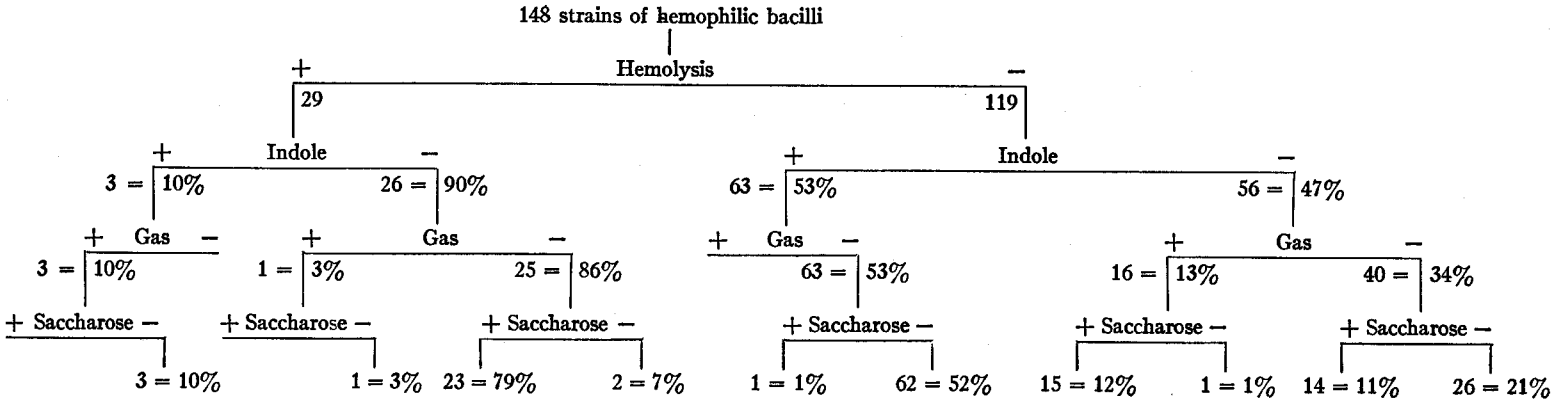
Tentative Classification of the Hemophilic Bacilli Based on Hemolysis, Indole and Gas Production, and the Fermentation of Saccharose.



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The non-hemolytic organisms are subdivided into two fairly even groups comprising indole-producing and non-indole-producing strains. None of the indole producers forms gas, in contrast with the hemolytic group. With one exception, none of the non-hemolytic indole-producing strains ferments saccharose. A large majority of the non-indole-producing organisms of the non-hemolytic type do not form gas and do not ferment saccharose. With a single exception, all the indole-negative strains which form gas also ferment saccharose.

One of the most striking features of this classification may be best illustrated by Table V, which represents a comparison of three factors differentiating the hemolytic and non-hemolytic groups of the hemophilic bacilli. Here it is seen that the majority of the strains of the hemolytic type do not produce indole or gas, but ferment saccharose,

TABLE V.

Three Differential Factors of the Hemolytic and Non-Hemolytic Groups of the Hemophilic Bacilli.

Differential factors.	Hemolytic group.		Non-hemolytic group.	
	Positive.	Negative.	Positive.	Negative.
	per cent	*per cent*	*per cent*	*per cent*
Saccharose	79	21	25	75
Gas	13	86	13	87
Indole	10	90	53	47

while the reverse is true of the non-hemolytic type; that is, the majority of the non-hemolytic organisms do not produce gas but also do not ferment saccharose. It will be noted that the non-indole and indole-producing strains of the non-hemolytic type fall into almost even groups.

The classification made in this study is merely a tentative one. Undoubtedly when the technique of the reactions is more nearly perfected and a larger number of hemophilic bacilli has been studied, the group differentiations will be more striking and regular.

Although the number of strains of *Bacillus influenzæ* employed is too small to warrant any definite conclusions, it would seem that the non-hemolytic bacilli isolated from individuals suffering with and recovering from respiratory infections and those isolated from normal mouths during the epidemic period differ biologically in certain re-

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spects from the strains recovered from normal individuals during the winter of 1919–20. This point is illustrated by Table VI. It is seen that the group of non-hemolytic hemophilic bacilli recovered from normal mouths during the winter of 1919–20 shows a higher percentage of strains which ferment the polysaccharides, maltose, saccharose, and dextrin, and more strains which produce gas, but fewer indole-producing strains.

TABLE VI.

Comparison of the Strains of Non-Hemolytic Hemophilic Bacilli Recovered from Respiratory Infections and Normal Mouths during Epidemic Period of 1918 and Strains Recovered from Normal Individuals during the Winter of 1919–20.

Source of strain.	Total No. of strains.	Strains fermenting sugars.												Strains producing indole.		Strains producing gas.	
		Dextrose.		Galactose.		Levulose.		Maltose.		Saccharose.		Dextrin.					
		No.	Per cent.	No.	Per cent.	No.	Per cent.	No	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.	No.	Per cent.
Respiratory diseases and normal mouths during epidemic of 1918, and respiratory diseases during 1919–20.	83	81	97	75	90	55	66	11	13	9	10	9	10	52	62	4	4
Normal mouths during winter of 1919–20.	36	35	97	33	91	32	88	20	55	21	58	19	52	11	30	12	33

The Gram-negative bacilli which are not hemophilic and which have been studied because of their morphological similarity can be easily differentiated from the hemolytic and non-hemolytic hemophilic bacilli of the influenza type. The bacillus of rabbit septicemia shows a striking similarity to members of the non-hemolytic hemophilic group in the limiting hydrogen ion concentration, indole production, and nitrate reduction. On the contrary, *Bacillus pertussis* and *Bacillus bronchisepticus*, while resembling each other in certain reactions, do not simulate the strictly hemophilic group. These organisms have a markedly alkaline final hydrogen ion concentration, and do not produce indole or reduce nitrates.

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CONCLUSIONS.

1. The hemophilic bacilli can be divided into two large groups according to the ability of certain strains to produce hemolysis.

2. Both the hemolytic and the non-hemolytic groups may be further subdivided according to the ability of some strains to produce indole, to form gas, and to ferment certain carbohydrates.

3. The hemophilic bacilli of both the hemolytic and the non-hemolytic varieties when grown in meat infusion broth containing 1 per cent of dextrose reach a final hydrogen ion concentration of about pH 6.4. In addition, practically all the strains possess the power to reduce nitrates to nitrites.

We wish to express our thanks to Dr. N. S. Ferry for a culture of *Bacillus bronchisepticus*, to Dr. Martha Wollstein for cultures of *Bacillus pertussis* and the bacillus of rabbit septicemia, to Dr. Anna Williams for cultures of *Bacillus pertussis*, to Dr. F. S. Jones for a culture of *Bacillus bronchisepticus*, and to the American Museum of Natural History for a culture of *Bacillus pertussis*.

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