OBSERVATIONS ON *BACILLUS* (*HAEMOPHILUS*) *INFLUENZAE* WITH SPECIAL REFERENCE TO MORPHOLOGY AND COLONIAL CHARACTERS.

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(With Plates IV and V containing 21 figures.)

Bacillus (*Haemophilus*) *influenzae* is characterised by wide variation both in morphology of the individual organisms and in colonial type. The investigation here reported shows that such variation can be induced by altering the growth conditions, and that there is a considerable degree of correlation between individual morphological variation and colonial change. The variants obtained were examined further with respect to biochemical reactions, haemolysis and growth requirements; they were shown to retain the essential characteristics of the species.

I. Variations in morphology in strains of *H. influenzae*.

Differences in morphological appearance form one of the characteristic features of those haemophilic bacilli which are commonly grouped together under the name "influenza bacillus." Typically Pfeiffer's bacillus is a short coccobacillus, but every gradation is met with between this coccobacillary type and the very long filaments or large thick curved forms of the atypical type. The colonial appearance also varies. Kristensen (1922) described large and small colonies, rough and smooth colonies, and colonies which were darker in colour than others. He stated that on suitable media the morphologically typical and atypical bacilli showed on the whole a corresponding difference in colonial form. The typical gave smooth colonies with a butyrous consistency, while the atypical tended to give rough colonies with a dry friable consistency. The dividing line between these two types however was not sharp. He also said that the type of colony retained its character relatively unchanged through a long period of cultivation. Wollstein (1915) drew attention to the readiness with which the coccobacillary type formed long threads in artificial cultures. Dible (1924), on the other hand, found that the morphology of a number of strains remained constant enough to enable one to recognise certain distinct types such as the coccobacillary type, and the bacillary and long filamentous types both of which he excluded from the true Pfeiffer group.

Technique.

All the strains of the influenza bacillus were isolated from nasopharyngeal swabs taken from normal people.

After isolation the usual procedure of studying variation was to grow the strain in about 100 c.c. of Fildes' broth (Fildes, 1920) in a small flask at 37° C. for several weeks. Cultures were made on a Fildes' plate about every other day and the colonies examined and filmed. It was usually only after 3 to 4 weeks' incubation that variants arose. These were picked off and cultured in tubes of Fildes' broth for 24 hours and again plated to see whether the variations remained constant. Stock cultures of the different variants were always kept in tubes of Fildes' broth at 37° C.

Other media were tried such as (1) Fildes' broth to which about 5 per cent. whole blood was added; (2) Fildes' broth to which about 5 per cent. of the homologous antiserum was added. The cultures to be examined were grown in the above media for varying lengths of time and plated every alternate day as above.

There seemed to be no advantage in the use of either of the above media over that of Fildes' broth alone.

Inoculation into mice was also utilised in the attempt to produce variants. In each case the mouse was inoculated intraperitoneally with 1 c.c. of a 24-hour culture in Fildes' broth; it was killed not later than the third day and films were made from the heart blood and peritoneal exudate, and cultures inoculated on to a Fildes' agar plate and into Fildes' broth which was again plated for variant colonies.

All films were stained by Gram's method, using as a counterstain 1/10 carbol fuchsin in distilled water applied for 15 minutes.

A description of the technique employed in the testing of sugar reactions, haemolysis, growth requirements, etc., is given in Section II under the biochemical characteristics.

Definition of terms used.

"Typical": Short coccobacilli, $1\mu \times 0{\cdot}8\mu$. (Figs. 4 and 9.)

"Atypical, curly": Large curly bacilli, single or in short chains, $10–50\mu \times 2\mu$. (Fig. 17.)

"Atypical, coliform": Bacillary in form, similar in appearance to *Bact. coli communis*, but length varies from $1{\cdot}5\mu$ to 8μ. (Fig. 20.)

Strain 8/7.

This strain was first isolated from the nasopharynx on 19. iii. 29. It was then typical in morphology, showing very short bacilli and many coccobacilli; no long forms were observed. The colony after 24 hours on a Fildes' agar plate was smooth, dome-shaped and translucent, about 0·6 mm. in diameter with entire edge and butyrous consistency.

A culture was seeded into a small flask containing 100 c.c. of Fildes' broth and incubated at 37° C. Every alternate day one loop was plated on a Fildes' agar plate and the colonies and morphology examined. After 4 weeks' incubation there appeared on the plate two kinds of colony.

(1) A large smooth colony, 0·6 mm. in diameter after 24 hours, which on being filmed showed a typical morphology.

(2) A small colony, 0·1 mm. in diameter after 24 hours. This showed an atypical morphology with many long slender filaments, 40–50 μ in length.

Each type of colony was now picked off into Fildes' broth and its behaviour further examined.

The *large smooth* colony remained constant both with regard to morphology and colonial form. Repeated subcultures were made from Fildes' broth to Fildes' broth for long periods of time. (Figs. 1, 4.)

The *small* colony was very variable. It readily gave rise to two or three kinds of colonies:

Large rough, about 0·6 mm. in diameter, with a contoured surface and irregular edge. Films from this colony showed an atypical morphology with many very long curved filamentous forms, as well as shorter coliform bacilli. (Figs. 2, 5.) Where picked off into Fildes' broth and further subcultured it was found that the large rough colony always produced some small rough colonies, but very seldom gave rise to the large smooth type. On two occasions only was a large smooth type of colony obtained from a large rough culture.

(1) After repeated subculture of a large rough colony in Fildes' broth for over 14 days, some smooth colonies were obtained giving typical coccobacillary morphology. This result could not be repeated.

(2) An intraperitoneal inoculation into a mouse was made from a Fildes' broth culture of a large rough colony. The mouse died on the second day. Films from the peritoneal cavity showed filamentous gram-negative bacilli similar to those in the original inoculum, and on cultivation rough colonies were obtained. On subculturing again from a rough culture and plating, large rough colonies and small smooth colonies were obtained, the former giving an atypical morphology, the latter a typical. On further subculture the rough culture became entirely smooth.

Small rough colonies, about 0·1 mm. in diameter, with rough surface and very irregular edge. The morphology was mixed, showing about two-thirds coliform and one-third long curved filaments. (Figs. 3, 6.) This type remained relatively constant, giving rise to a majority of small rough and a few large rough colonies.

There follows (p. 524) a diagrammatic scheme showing the variability of strain 8/7.

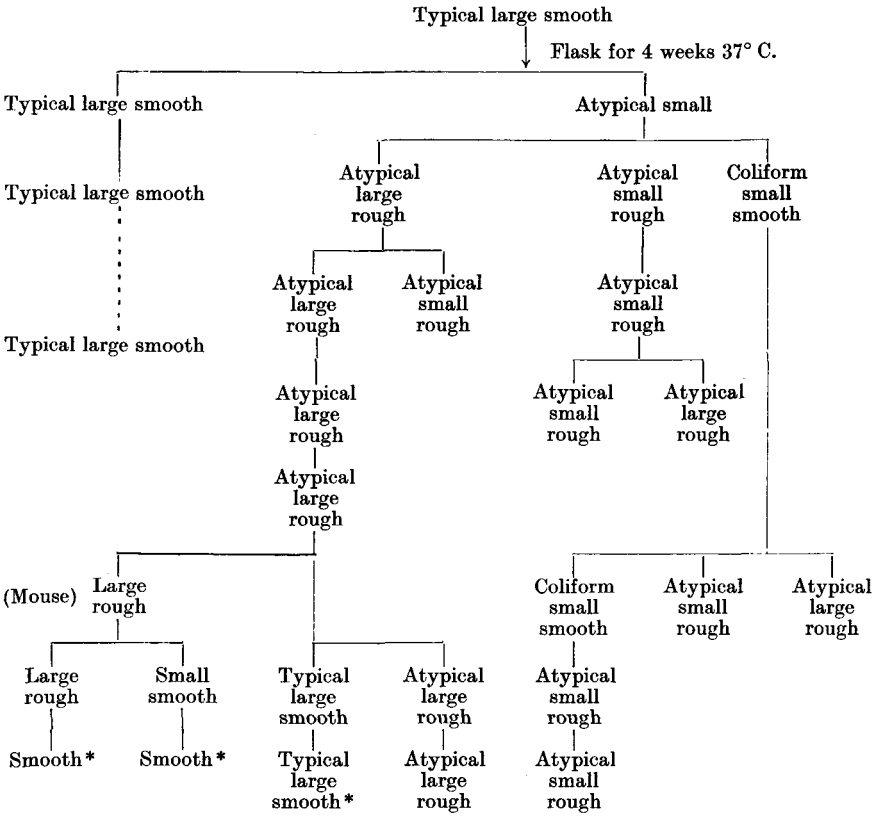
Small smooth colonies, about 0·1 mm. in diameter, with smooth surface and entire edge. These colonies were relatively few in number. The morphology was similar to that of the small rough. It is often difficult to distinguish this type of colony from the small rough. They are not very common. They have a coliform morphology and readily produce the small rough colony.

Thus in the case of this strain it was possible to demonstrate a change in the colonial form and morphology of a typical influenza bacillus into an atypical form by growing under certain conditions. Moreover, an atypical morphology was associated with a rough type of colony, whereas a typical morphology was associated with a smooth colony.

The rough and smooth strains were compared with regard to their biochemical characteristics, growth requirements, haemolysis, etc. The results are given below and show that there is no difference between any of the strains with regard to these characteristics.

	Dextrose	Maltose	Mannite	Lactose	Sucrose	Salicin	Indol	Haemolysis	Growth in XV	Growth in V	Growth in X
Large smooth	A	0	0	0	0	0	+	0	+ +	0	0
Large rough	A	0	0	0	0	0	+	0	+ +	0	0
Small rough	A	0	0	0	0	0	+	0	+ +	0	0
Small smooth	A	0	0	0	0	0	+	0	+ +	0	0

For the technique employed in these tests see Section II.



* On these occasions only.

Strain 15/9.

First isolated 15. v. 29 from nasopharynx. Typical coccobacillary in morphology. Colony smooth with entire edge and butyrous consistency, raised in centre, about 0·8 mm. in diameter.

This strain remained typical after repeated subculture in Fildes' broth tubes, on Fildes' or blood-agar plates, in flasks of Fildes' broth, or after passage through a mouse, for several months. Then after growing in a flask containing 100 c.c. Fildes' broth at 37° C. for 17 days, colonies of two different sizes appeared when plated on a Fildes' agar plate. These two colonies differed in their morphology as well as in their size.

(1) *Large colony:* similar to the original, smooth, 0·8 mm. in diameter with a typical morphology.

(2) *Small colony:* about 0·2 mm. in diameter with an atypical coliform morphology.

A second flask of Fildes' broth was taken and seeded with the original typical smooth and grown at 37° C. for 17 days and then plated. Three types of colony were observed.

(1) *Large smooth:* similar to original, 0·8–0·9 mm. in diameter, smooth surface, entire edge, butyrous consistency. Morphology typical, short coccobacilli. (Figs. 7, 9.)

(2) *Large rough:* about 0·8 mm. in diameter, rough surface and irregular margin. Morphology atypical, coliform and large thick curved bacilli about 20–30 μ in length. (Figs. 8, 10.)

(3) *Small rough:* 0·2 mm. in diameter, rough surface and irregular outline. Morphology long coliform. (Fig. 11.)

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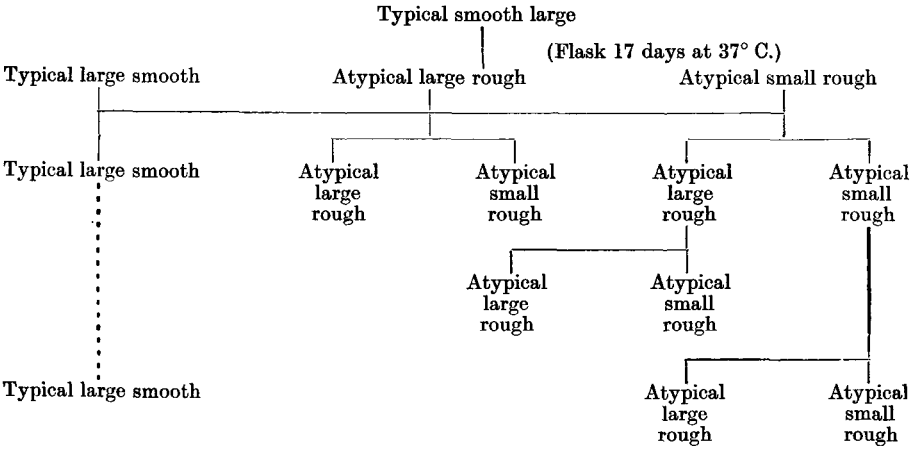
Cultures were taken from each of the above three types of colonies into Fildes' broth and further observations were made as to their variability. It was found that the large smooth colony remained constant, always giving rise to similar colonies with a typical morphology. The large rough colony gave rise to colonies of different sizes, namely, the large rough and the small rough, both atypical in morphology.

The small rough colony behaved like the large rough giving rise to rough colonies both large and small.

A rough colony whether large or small was never observed to give rise to a smooth colony.



Here, as in strain 8/7, prolonged growth in Fildes' broth tended to produce rough colonies which had an atypical morphology.

With regard to biochemical characters the smooth and rough strains appeared to behave in a similar manner.

	Dextrose	Maltose	Mannite	Lactose	Sucrose	Salicin	Indol	Haemolysis	Growth in XV	Growth in V	Growth in X
Large smooth	A	0	0	0	0	0	+	0	+	0	0
Large rough	A	0	0	0	0	0	+	0	+ +	0	0

In the three strains described below, although there was a sharp difference in morphology, the only colonial difference noticeable was that of size.

Strain 23/6.

Isolated 4. iii. 29 from nasopharynx. Morphology typical coccobacillary.

Colonial form—smooth, 0·8 mm. in diameter, entire edge, raised centre, butyrous consistency.

This strain was kept in cultivation for some weeks and grown repeatedly in flasks of Fildes' broth. Finally, 6 months after isolation, on plating from a 3 weeks' culture, two kinds of colony were obtained.

(1) *Large smooth:* 0·8 mm. in diameter.

(2) *Small smooth,* 0·1 mm.–0·2 mm. in diameter.

The morphology of both types of colony was similar, namely, typical coccobacillary.

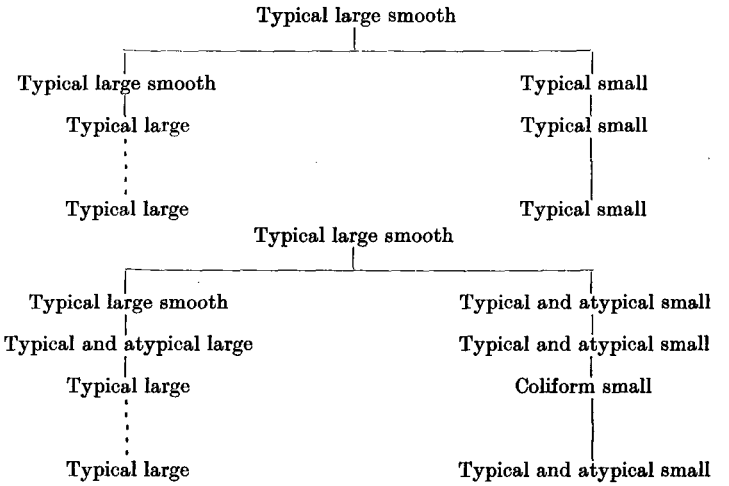
Each of the above colonies on subculturing remained constant, the large giving rise to large colonies, the small to small colonies.

Another flask was taken and seeded from the original strain. Plates were made at various intervals and again two types of colonies, large and small, were obtained, but this time the morphology differed. The large colonies still gave a typical morphology, but the

small colonies gave a mixture of small coccobacilli and very long thick curly forms. (Figs. 12, 13, 14.)

Further cultures from each of the above colonies showed great variability in morphology. The large smooth colonies generally showed an entirely coccobacillary morphology, but sometimes a few large thick bacilli were seen among the majority of small forms. The small colonies usually showed a mixed morphology with large numbers of thick curly forms, but occasionally the coliform type of morphology predominated, with only a few large bacilli.

The original strain was also grown in peptone water, to which 5 per cent. of an antiserum for a typical strain was added. No difference in colonial form or morphology was observed.



The biochemical characters of the large and small colonies are as follows:

	Dextrose	Maltose	Mannite	Lactose	Sucrose	Salicin	Indol	Haemolysis	Growth in XV	Growth in V	Growth in X
Large	A	0	0	0	0	0	+	0	++	0	0
Small	A	0	0	0	0	0	+	0	+	0	0

In this strain 23/6 a change from a typical to an atypical was observed with a difference in size of the colonial forms. Up to the time of writing, however, although the atypical small colony contained a preponderance of atypical forms, there were still some coccobacillary forms present, so that the change was not complete.

Strain 24/10.

Isolated 12. vi. 29. This strain was atypical when first isolated. The colonies were opaque, whitish in colour, and about 0·5 mm. in diameter. The morphology consisted chiefly of large curved forms and a few short coliform organisms.

After several subcultures large and small colonies were seen on a Fildes' agar plate (Fig. 15). Both kinds of colonies were translucent, slightly raised in the centre, and 0·8 mm. and 0·2 mm. respectively in diameter. A film made from a large colony showed very short coliform and coccobacilli (Fig. 16), while a film made from a small colony showed only large thick curly forms (Fig. 17). Cultures from both kinds of colony were picked off into Fildes' broth and remained on the whole fairly constant, although there was a tendency for the large colonies to show coliform and even a few long bacilli among the short forms (Fig. 18), and for the small colonies to show a few short forms.

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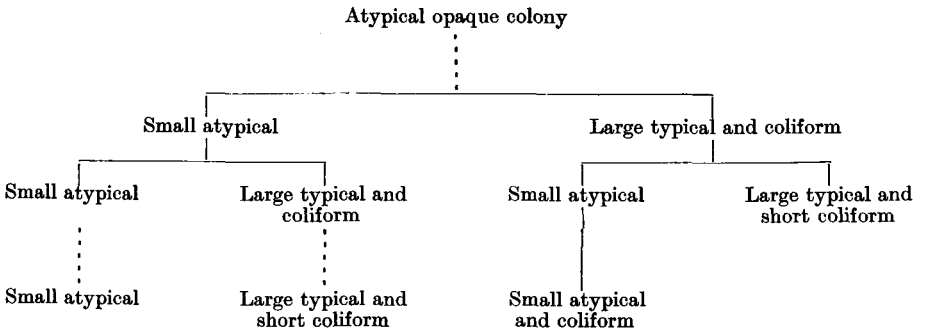
Growth in Fildes' media to which 5 per cent. whole blood had been added brought out the differences between typical and atypical morphology more clearly.



Biochemical characters are as follows:

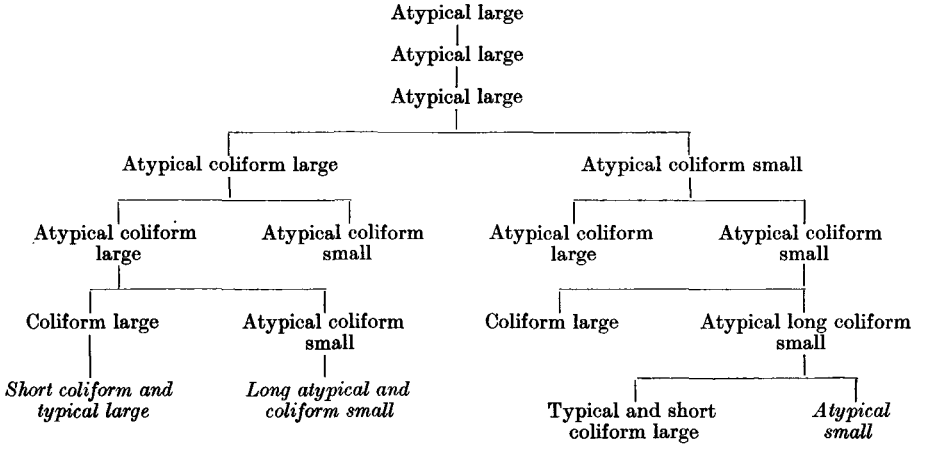
	Dextrose	Maltose	Mannite	Lactose	Sucrose	Salicin	Indol	Haemolysis	Growth in XV	Growth in V	Growth in X
Large	A	0	0	0	0	0	0	0	+	0	0
Small	A	0	0	0	0	0	+	0	+	0	0

Here the change was from an atypical to a typical with corresponding changes in colonial form. The difference in indol production between the two types should be noted.

Strain 4/5.

Isolated February 1929. Atypical, long slender bacilli 4–5 $\mu \times 0{\cdot}5\,\mu$, stained faintly. Colony translucent, butyrous, raised in centre, 0·8 mm. in diameter.

Repeated cultivation (subculturing every week) in Fildes' broth gave no indication of variation for many months. In September, however (7 months after isolation), small colonies about 0·1–0·2 mm. in diameter were observed among the larger colonies (Fig. 19). The morphology appeared to be very similar in both types, coliform with many long slender forms and some shorter ones. After cultivation of the two kinds of colony for some weeks a much sharper difference in morphology could be seen. The large colonies now consisted of short organisms (Fig. 20), while the small colonies were almost entirely composed of long slender bacilli some 50–70 μ long. (Fig. 21.)



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Biochemical characters are as follows:

	Dextrose	Maltose	Mannite	Lactose	Sucrose	Salicin	Indol	Haemolysis	Growth in XV	Growth in V	Growth in X
Large	A	0	0	0	0	0	+	0	+ +	0	0
Small	A	0	0	0	0	0	+	0	+ +	0	0

Summarising the colonial and morphological variations among the strains studied, there seems to be some association between morphology and colonial form. Organisms having a typical morphology seem on the whole to produce a smooth colony, while an atypical morphology seems to be either associated with a rough colony, or with one very much smaller in size.

It seems possible, moreover, to produce rough variants with atypical morphology from a typical smooth strain. Smooth typical variants can also (although it is less common) be obtained from rough atypical strains.

The biochemical characters remained, on the whole, unchanged. One exception is seen in the change from a negative indol to a positive.

In view of these results it does not seem justifiable, in the present state of our knowledge, to exclude from the species *H. influenzae* those haemophilic bacilli which possess an atypical morphology, but require both *X* and *V* factors for their growth, and fail to produce haemolysis. It is, of course, quite possible that this group will be finally subdivided, either into separate species or varieties on the basis of fermentation reactions, or in some other way. Since, however, the morphology and colonial appearances may undergo marked changes under prolonged cultivation in the laboratory, while the fermentation reactions and growth requirements remain constant, it seems clear that morphology alone cannot be accepted as an adequate criterion for systematic differentiation.

It does not, of course, follow that morphological differences are devoid of significance from the pathological point of view. There is, indeed, general agreement that the coccobacillary form is that usually isolated from cases of active infection in man, with the single exception of cases of influenzal meningitis; while a considerable proportion of the haemophilic bacilli recovered from the normal mouth or throat show an atypical morphology (see Flemming and Maclean, 1930). The meaning of these facts is, at the moment, quite obscure.

II. The biochemical characters of morphologically typical and atypical strains.

The biochemical reactions of the variants obtained during this investigation, and of the parent strains from which they were derived, have been set out in tabular form above. In addition to these, a large number of other strains have been studied from this point of view.

(1) *Fermentation of sugars.*

Three types of media were tried; in each case Andrade's reagent was added as an indicator.

(*a*) Peptone water sugars, to which 5 per cent. of Fildes' tryptic blood

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digest was added. In this medium a copious growth was obtained but it had the disadvantage of having a faint reddish tinge, and thus slight traces of acid were obscured. Control tubes were always put up, and it was usually easy to detect any difference in colour between the tube fermented and the control tube. However, it was decided to discard the medium owing to this difficulty.

(*b*) Peptone water sugars to which an extract of boiled rabbits' blood was added (Stillman and Bourne, 1920). Here slight traces of acid could be easily seen, but fermentation did not always occur as readily as in the third method.

(*c*) Peptone water sugars containing yeast extract and haematin. To each tube containing approximately 5 c.c. of sugar medium about 0·1 c.c. of yeast extract and one small loop of a 1 per cent. alkaline solution of haematin were added. This last medium proved an excellent one for testing fermentation reactions. Not only was any acid produced perfectly obvious, but the results remained on the whole constant.

Table I. *Showing the percentages of the strains examined fermenting the various test substances.*

	Dextrose	Maltose	Sucrose	Mannite	Lactose	Salicin	No sugars fermented	No.
Typical	21	9	2	—	—	—	21	47
	44·8 %	19·1 %	4·3 %	—	—	—	44·8 %	—
All atypicals	51	24	21	1	6	2	32	93
	54·8 %	25·8 %	22·6 %	1·1 %	6·5 %	2·2 %	34·4 %	—
Coliform	36	11	14	1	2	—	25	67
	53·7 %	16·6 %	20·9 %	1·5 %	3·0 %	—	37·3 %	—
Atypical long and wavy	15	13	7	—	3	2	7	26
	57·7 %	50·0 %	26·9 %	—	11·9 %	7·7 %	27·0 %	—
Total { Typical, atypical coliform	72	33	23	1	5	3	53	140
	51·4 %	23·6 %	16·4 %	0·7 %	3·6 %	2·1 %	37·8 %	—

It will be seen from the above table that dextrose, maltose and sucrose are the sugars most often fermented, in fact among the "typicals" they are the only sugars to be fermented. Very small numbers of "atypicals" ferment lactose, mannite and salicin. Atypicals are definitely more active than the typicals. This is also evidenced by the lower percentage of atypicals which ferment no sugars. The coliform, as distinct from the atypical, are less active than the real atypicals, although they are more active in most cases than the typicals. These results are in general agreement with those recorded by other workers (Levinthal, 1918; Stillman and Bourne, 1920; Kristensen, 1922; Dible, 1924).

(2) *Production of indol.*

For the testing of indol, duplicate tubes were always put up, one of Fildes' broth, the other of peptone water, to which were added yeast extract and haematin. The tubes were incubated at 37° C. for 5 days, then a layer of ether was added to each tube and about $\frac{1}{2}$ c.c. of paradimethyl-aminobenzaldehyde and $\frac{1}{2}$ c.c. of potassium persulphate. There was never any doubt as to the

production of a red colour in the positive cases. The Fildes' media proved to be slightly better for the demonstration of this reaction, possibly owing to the better growth obtained in it.

Table II. *Production of indol.*

Typicals		Coliform		Atypicals	
Indol +	Indol −	Indol +	Indol −	Indol +	Indol −
46	26	43	49	11	50
63·9 %	36·1 %	46·7 %	53·3 %	18·0 %	82·0 %

Among the typicals two-thirds are indol positive and one-third are indol negative. In the true atypicals (excluding coliform) 82 per cent. are indol negative. The coliform are intermediate between the typicals and the atypicals, with just over one-half indol negative.

(3) *Reduction of nitrates and action on litmus milk.*

Some strains were tested for the reduction of nitrates. All produced nitrites. Litmus milk was unchanged in every case.

Haemolysis. All the strains described in this paper were non-haemolytic. Any organism isolated from the nasopharynx which produced haemolysis was discarded. Haemolysis was tested in two ways:

(1) On a whole blood-agar plate, where any clearing or discoloration round the colonies was taken as an indication of haemolysis.

(2) In test-tubes of peptone water to which *X* and *V* factors had been added. After growth had occurred in this medium for 24 hours at 37° C., 1 c.c. of a 5 per cent. suspension of washed rabbit red cells was added and the mixture further incubated at 37° C. for another hour, then placed at 0° C. overnight. Rabbits' cells were found to be far better than either sheep or horse blood.

The symbiosis phenomenon.

All the strains showed this phenomenon (Grassberger, 1897) in varying degrees. A 1 per cent. whole blood-agar plate was used, spread with the strain, and then inoculated with a few isolated streaks or patches of *Staphylococcus albus*. An abundant growth occurred round the Staphylococcus.

Growth requirements. The list of workers on the growth-requirements of Pfeiffer's bacillus is a large one (Ghon and Preyss, 1904; Davis, 1917; Olsen, 1920; Fildes, 1920, 1921, 1922, 1923; Thjötta and Avery, 1921; Kristensen, 1922; to mention a few). All agree that the bacillus is dependent on a substance *X*, present in red blood cells, active in minute amounts, and thermostable. There is also required a second, vitamin-like factor, *V*, found in yeast, and certain animal and vegetable tissues.

Most of the strains studied in this work were tested with regard to their growth requirements. Three tubes of peptone water were taken, each containing 5 c.c. To the first a small loop of a 1 per cent. alkaline solution of haematin

was added, to the second about 0·1 of a yeast extract prepared according to Thjötta and Avery (1921), to the third both haematin and yeast extract were added. Thus the first tube contained the *X* factor only, the second tube contained the *V* factor only, whereas the third tube contained both *X* and *V* factors.

A 24-hour Fildes' plate of the strain of influenza bacillus to be tested was taken. A small amount of growth was emulsified in sterile saline to give an opacity of about 500×10^6 per c.c. Dilutions were then made, using tubes containing 10 c.c. sterile water and sterile dropping pipettes graduated so as to deliver fifty drops to the c.c. A fresh sterile pipette was used for each dilution. A final dilution of 50,000 organisms per c.c. was obtained. Sufficient of this dilution was then added to each of the above three tubes to give from 200 to 1000 organisms per c.c. in the tubes to be tested.

Table III. *Growth in media containing X and V.*

Strain	Amount added to 5 c.c. peptone water of a suspension containing 50,000 organisms per c.c. (drops)	*X*	*V**	*XV*
A_1	3	0	0	+
A_1 (i)	3	0	0	+
A_2	1	0	+	+
291	3	0	0	+
I 1	1	0	0	+
LPE	3	0	0	+
P_2	5	0	0	+
RIN	3	0	0	+
RIN_2	1	0	+	+
Ry	1	0	0	+
	2	0	+	+
RINP	3	0	0	+
13	3	0	0	+

* Batch D of yeast extract (see Table IV) was not used here.

The method of preparing the yeast extract was the same in the case of each of the batches used (Thjötta and Avery, 1921). One batch of extract (D, see Table IV) behaved very differently from the others, in that it allowed the growth of all strains tested without the addition of haematin. Moreover, the organisms could be sub-cultured indefinitely in media containing this extract without the addition of the *X* factor. It is probable that this extract actually contained an amount of the *X* factor sufficient for the growth requirements of *H. influenzae*. The results obtained with it must, of course, be disregarded; but it affords a useful illustration of the dangers of an undue reliance on any particular yeast extract as a source of the *V* factor for testing purposes. Growth in any tube was decided not only on the presence of turbidity or deposit, but always in addition by plating on to a Fildes' plate. Thus small amounts of growth could be observed which would otherwise be missed if opacity alone were taken as the criterion of growth.

In Table III it is seen that in the case of strains A_2 and RIN_2, although only 1 drop of the saline dilution was added (giving 200 organisms per c.c. in the

Table IV. *Showing growth in V factor alone.*

Strain	Amount added to 5 c.c. peptone water of suspension containing 50,000 organisms per c.c. drops	Yeast batch: D — 1st testing: *XV*	*V*	*X*	D — 2nd testing: *XV*	*V*	*X*	D — Successive subculturing: V_1 (1 loop)	V_2	V_3	V_4	V_5	V_6	V_7	V_8	V_9	V_{10}	E — 1st testing: *XV*	*V*	*X*	E — 2nd testing: *XV*	*V*	*X*	F — 1st testing: *XV*	*V*	*X*
23/6	1	++	++	0	++	++	0	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0
20/4	1	++	++	0	++	++	0	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	.	.	.	.	.	.
8/7	1	++	++	0	.	.	.	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0
11/3	1	++	++	0	++	++	0	++	++	++	++	++	++	++	++	.	.	+	0	0	.	.	.	.	.	.
8/4	1	++	++	0	.	.	.	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	.	.	
4/5	1	++	++	0	.	.	.	++	++	++	++	++	++	++	++	.	.	+	0	0	.	.	.	.	.	.
11/6	1	++	++	0	.	.	.	++	++	++	++	++	++	++	++	.	.	+	0	0	.	.	.	+	0	0
9/7	1	++	++	0	.	.	.	++	++	++	++	++	++	++	++	++	++	.	.	.	.	.	.	.	.	.
	3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	0	0	+	0	0	+	0	0

tubes tested), growth occurred in the tube containing *V* alone. In the case of Ry, 1 drop gave no growth in *V* alone, but 2 drops (400 organisms per c.c.) gave a definite growth. In all other cases 3 drops (600 organisms per c.c.) gave no growth.

Fildes (1923) added 0·05 c.c. of a suspension containing 40,000 organisms per c.c. to 10 c.c. peptone water, thus giving 200 organisms per c.c. in the tested tubes.

It seems unlikely therefore that, in the above two cases, growth in *V* alone is due to minute traces of the *X* factor carried over from the Fildes' plate, since in most other cases larger amounts gave no growth.

With regard to the growth requirements of the influenza bacillus, by far the greater number require both an *X* and a *V* factor. In a few cases, however, it appears from the above results that organisms which in every other respect would be undoubtedly classified as true *H. influenzae* will grow in *V* alone, although even in these cases they will not grow in all batches of yeast extract.

SUMMARY.

1. Several strains of the influenza bacillus were grown under varying conditions for many months in order to ascertain whether morphology and colonial appearances could be permanently modified.

2. It was found that a modification in morphology occurred in all the strains studied.

3. Typical coccobacillary forms were changed to atypical long-curved forms.

4. The reverse change from atypical was also observed but was not so frequent, nor, when it occurred, was it so constant.

5. The change took place most readily after growth in a flask of Fildes' broth for several weeks or months at 37° C.

6. There was found to be a definite association between morphology and colonial form. The typical strains produced smooth colonies, while the atypical gave rough colonies, or colonies very much smaller than those produced by the typical.

7. When the change in morphology took place the change in colonial form occurred at the same time.

8. The biochemical characters remained on the whole unchanged.

9. One hundred and forty strains were examined for their fermentation reactions. The sugars most frequently fermented were dextrose, maltose and sucrose.

10. The atypicals were more active fermenters than the typicals.

11. 63·9 per cent. of the typicals, and 18 per cent. of the atypicals, gave a positive indol reaction.

12. Although the ability to grow on *X* or *V* alone affords an important differential criterion within this group, great care is necessary in interpreting

the results obtained, especially in the case of those strains which grow on *V* in the absence of *X*. Different yeast preparations may give very different results in this respect.

13. All the strains tested were non-haemolytic.

I wish to express my indebtedness to Prof. Topley for his advice and help throughout this investigation.

EXPLANATION OF PLATES IV AND V.

PLATE IV.

Bacillus (*Haemophilus*) *influenzae*. Colonies and film preparations.

Colonies were grown on Fildes' agar plates for 24 hours at 37° C. Films were stained by Gram's method and counterstained with a 1 in 10 dilution of carbol fuchsin.

Fig. 1. Large smooth colony of strain 8/7. (× 8.)

Fig. 2. Large rough colony of strain 8/7. (× 8.)

Fig. 3. Small rough colony of strain 8/7. (× 8.)

Fig. 4. Film preparation from a large smooth colony of strain 8/7. (× 1500.)

Fig. 5. Film preparation from a large rough colony of strain 8/7. (× 1500.)

Fig. 6. Film preparation from a small rough colony of strain 8/7. (× 1500.)

Fig. 7. Large smooth colony of strain 15/9. (× 8.)

Fig. 8. Large rough colony of strain 15/9. (× 8.)

Fig. 9. Film preparation from a large smooth colony of strain 15/9. (× 1000.)

Fig. 10. Film preparation from a large rough colony of strain 15/9. (× 1000.)

Fig. 11. Film preparation from a small rough colony of strain 15/9. (× 1000.)

PLATE V.

Fig. 12. Large and small colonies of strain 23/6. (× 8.)

Fig. 13. Film preparation from a large colony of strain 23/6. (× 1000.)

Fig. 14. Film preparation from a small colony of strain 23/6. (× 1000.)

Fig. 15. Large and small colonies of strain 24/10. (× 8.)

Fig. 16. Film preparation from a large colony of strain 24/10. (× 1000.)

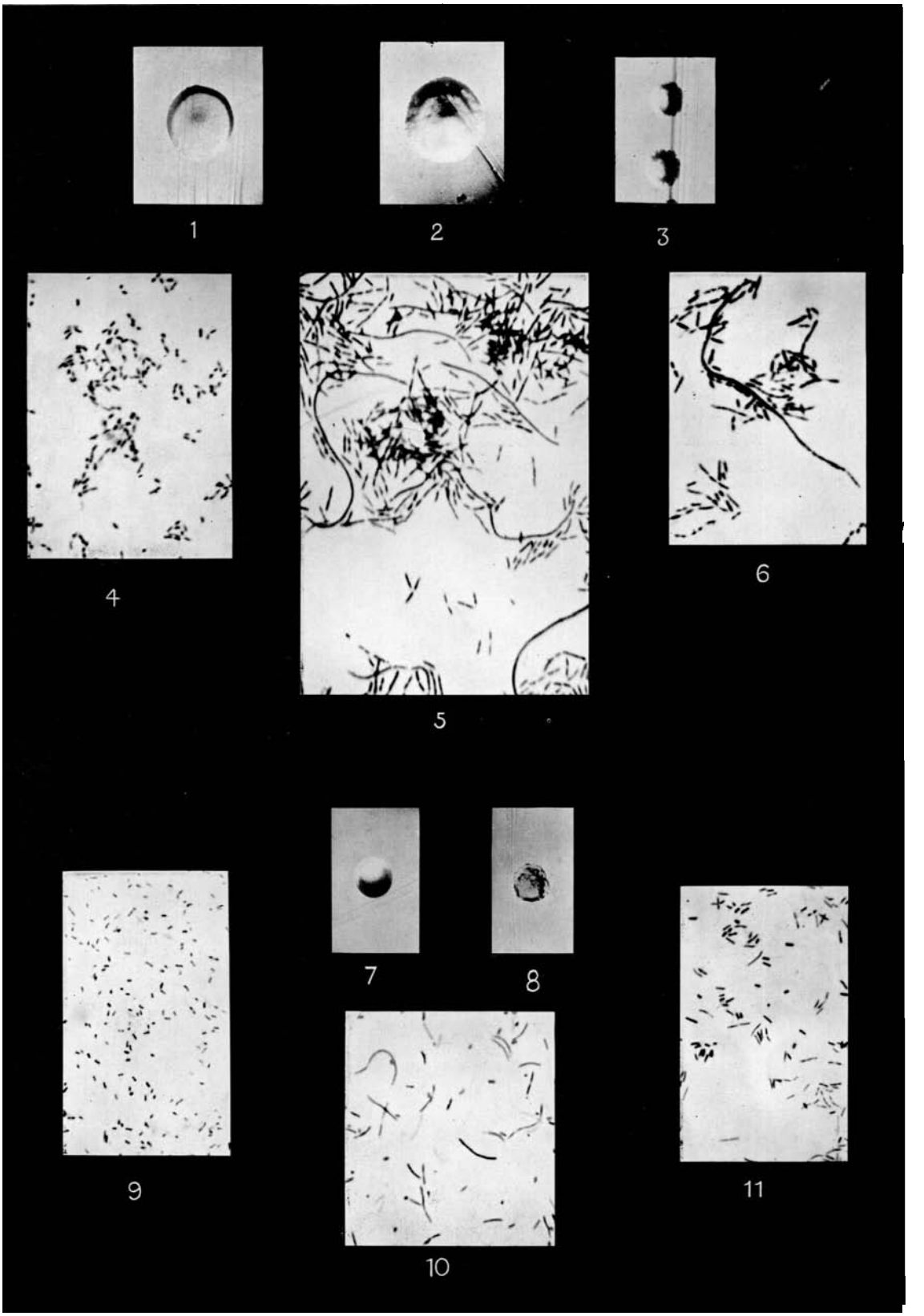
Fig. 17. Film preparation from a small colony of strain 24/10. (× 1000.)

Fig. 18. Film preparation from a large colony of strain 24/10 showing a mixture of long and short forms. (× 1000.)

Fig. 19. Large and small colonies of strain 4/5. (× 8.)

Fig. 20. Film preparation from a large colony of strain 4/5. (× 1000.)

Fig. 21. Film preparation from a small colony of strain 4/5. (× 1000.)



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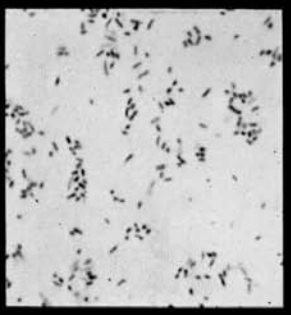

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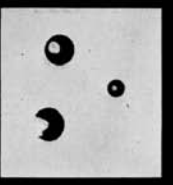

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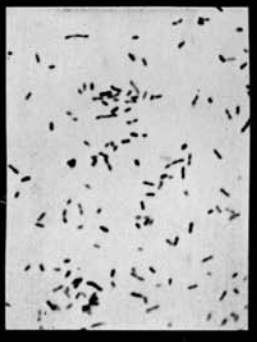

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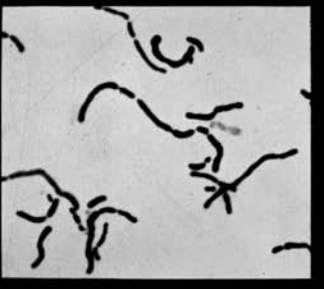

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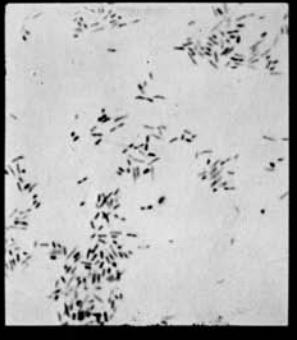

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