

FUNGOUS DEVELOPMENTAL GROWTH FORMS OF
BACILLUS INFLUENZÆ.

A PRELIMINARY NOTE.

BY H. W. WADE, M.D., AND C. MANALANG, M.D.

(From the Department of Pathology and Bacteriology of the College of Medicine
and Surgery, University of the Philippines, and the Bureau of Science,
Manila.)

PLATES 11 AND 12.

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The problem of the causative agent of epidemic influenza, and especially that of the importance of Pfeiffer's *Bacillus influenzae*, until recently generally believed to be the infecting organism in this disease, seems still far from solution. Of late, much attention has been directed to the search for a filterable virus, and success has been reported by a number of independent workers. Nicolle and Lebailly,¹ de la Rivière,² da Cunha, Magalhaes, and da Fonseca,³ and Gibson, Bowman, and Connor⁴ have reported transmission of the disease by filtrates, and von Angerer,⁵ da Cunha, Magalhaes, and da Fonseca, Leschke,⁶ Bradford, Bashford, and Wilson,⁷ and Gibson, Bowman, and Connor⁸ have cultivated minute filterable organisms.

The influenza bacillus has been relegated by many to the position of a mere secondary invader, with pneumococci and streptococci,

¹ Nicolle, C., and Lebailly, C., *Compt. rend. Acad.*, 1918, clxvii, 607.

² de la Rivière, R. D., *Compt. rend. Acad.*, 1918, clxvii, 606.

³ da Cunha, A., Magalhaes, O., and da Fonseca, O., *Brazil-med.*, 1918, xxxii, 376, referred to in *Med. Rec.*, 1919, xcv, 457.

⁴ Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1918, ii, 645.

⁵ von Angerer, *Munch. med. Woch.*, 1918, ii, 1280, abstracted in *Bull. Inst. Pasteur*, 1919, xvii, 161.

⁶ Leschke, E., *Berl. klin. Woch.*, 1919, lvi, 11, referred to in *Med. Rec.*, 1919, xcv, 456.

⁷ Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Brit. Med. J.*, 1919, i, 127.

⁸ Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1919, ii, 331.

though others, because of the frequency with which they have found it, or for other reasons, believe it to be in some way primarily concerned. If influenza is a virus disease, as now seems likely, it may be that the influenza bacillus has no primary relation to it. However, this cannot yet be asserted as a fact, for gradually the possibility is gaining recognition that familiar organisms, of supposedly fixed morphology, may assume a filterable phase. The work of Hort and Ingram⁹ and Hort¹⁰ on typhus fever, of Mathers,¹¹ Rosenow, Towne, and Wheeler,¹² Nuzum and his coworkers,¹³ and others with streptococci in poliomyelitis, of Hort and his associates¹⁴ in meningitis, and the uncompleted work of one of us¹⁵ on certain lesions probably due to fungi, all point in the same direction. Further, starting with pure cultures of ordinary bacteria, Löhnis and Smith¹⁶ have reported, though incompletely, the production of a filterable phase, possibly comparable with the filterable organisms mentioned, that showed no immediate tendency to revert to the bacterial form. As described, they seem as different from the parent organisms as the viruses are from Hort's bacillus of typhus fever, the streptococcus, the meningococcus, or Pfeiffer's *Bacillus influenzae*.

The possibility that the Pfeiffer organism may, in accordance with Hort's hypothesis, assume such a filterable phase has already been suggested.¹⁷ If so remarkable a transformation should be possible, and the virus so developed should prove to be the cause of influenza, it is important that this be recognized. For this reason special interest is attached to the question of the morphologic stability of

⁹ Hort, E. C., and Ingram, W. W., *Brit. Med. J.*, 1914, ii, 15.

¹⁰ Hort, E. C., *Brit. Med. J.*, 1915, i, 673, 826.

¹¹ Mathers, G., *J. Am. Med. Assn.*, 1916, lxvii, 1019.

¹² Rosenow, E. C., Towne, E. B., and Wheeler, G. W., *J. Am. Med. Assn.*, 1916, lxvii, 1202; *J. Med. Research*, 1917, xxxvi, 175.

¹³ Nuzum, J. W., and Herzog, M., *J. Am. Med. Assn.*, 1916, lxvii, 1205. Nuzum, J. W., *J. Am. Med. Assn.*, 1916, lxvii, 1437.

¹⁴ Hort, E. C., Lakin, C. E., and Benians, T. H. C., *Brit. Med. J.*, 1915, i, 541; *J. Roy. Army Med. Corps*, 1916, xxvi, 153. Hort, E. C., and Caulfield, A. H., *J. Roy. Army Med. Corps*, 1916, xxvii, 312. Hort, E. C., *Brit. Med. J.*, 1917, ii, 377.

¹⁵ Wade, H. W., *Philippine J. Sc., Section B*, 1918, xiii, 165.

¹⁶ Löhnis, F., and Smith, N. R., *J. Agric. Research*, 1916, vi, 675.

¹⁷ Editorial, *Brit. Med. J.*, 1918, ii, 665.

the organism. The observations to be described show that it is unstable, that the familiar bacillus is but a simple form of an organism capable of complex development. It is probably of no little significance that the most radical development seen has occurred only in association with certain bacteria, and that other bacteria have apparently the opposite, degenerative, effects. In view of the possibilities that arise from these observations, and because it will be impossible for us in the near future to extend them to the degree that seems desirable, we are led to make a preliminary report at this time.¹⁸

Our bacteriological findings during the influenza epidemic strongly indicated that the Pfeiffer bacillus plays an important part in the disease. Accordingly, we have made attempts from time to time to determine, if possible, the essential factors influencing its pathogenicity. Yanagisawa¹⁹ reported the effect of simultaneous injection of *Bacillus influenzae* and streptococci or pneumococci into white mice. Since we lacked animals necessary to duplicate the experiments, we essayed to determine the effects of cultivating these organisms together in fluid media. At the same time an attempt was made to determine whether a filterable stage might be produced in bouillons or in the synthetic media of Löhnis and Smith, with and without added blood (hemoglobin) extract. The developmental morphologic changes that appeared in certain of these first cultures were so surprising that a more extensive series of observations on this feature was made, other questions being left for future work.

Organisms Used.

The organisms used are typical strains of the influenza bacillus, obtained from autopsies. Though now relatively saprophytic in some respects, they are scarcely less exacting as to media requirements than immediately after isolation, growing not at all on non-hemoglobin agar, poorly on cool mixed (45–50°C.) blood agar, well on 58–65°C. agar, and very luxuriantly, with large, grayish colonies, on hot mixed (80–90°C.) agar. After the preliminary experiments with a single strain (Strain A) two others were chosen for parallel tests. As a

¹⁸ The more detailed report will appear in the *Philippine Journal of Science*.

¹⁹ Yanagisawa, S., *Kitasato Arch. Exp. Med.*, 1919, iii, 85.

routine precaution against possible contamination all were twice tube-seeded.

Morphologically, Strain A from blood agar tends to short plump forms, often coccobacillary, and Strains B and C to longer, rather thin, often stiff looking bacilli. However, all three may be induced to assume all the typical forms of the influenza bacillus.

The pathogenicity of these strains on subcutaneous inoculation in monkeys is now very low, much less than when first isolated. Poison production, as described by Parker,²⁰ has been determined in Strain A; the others have not been tested.

Media.

The essential media used were beef infusion bouillons made with Witte's peptone and sodium chloride in various concentrations. Synthetic media proved unsuitable. The blood extract used was made by thoroughly laking sheep or horse blood, 20 cc. per 100 cc. of distilled water, heating this to 80° or 85°C., and while hot precipitating the proteins with strong hydrochloric acid. The suspension so produced was filtered, first through gauze and then paper, and the filtrate reduced to about 1 per cent if too acid, and sterilized by filtration or repeated heating to 65°C.

Seed.

Heavy 12 to 24 hour growths on hot blood agar were used for inoculation. Material from Rothberger's neutral red agar was usually not satisfactory. The growth was removed by scraping and heavy suspensions were made in saline solution; 0.1 to 0.2 cc. of this was generally used for inoculating an ordinary tube culture. Cocci for growth in association were grown on suitable blood agar for 24 or 48 hours and suspensions made in saline solution.

Growths in Pure Fluid Cultures.

Macroscopic Growth.—Strain A has usually given heavier, more diffuse, less distinctly flocculent growths than the others. As a rule, the densities have corresponded directly to the amount of blood

²⁰ Parker, J. T., *J. Am. Med. Assn.*, 1919, lxxii, 476.

extract present and, above a certain point, inversely to the concentration of peptone and salt. In a mixture of equal parts of blood extract and normal strength bouillon growths are fairly heavy; in a similar mixture with double strength bouillon they are heavier and more diffuse. With lesser concentrations of blood extract or greater concentrations of peptone and salt they are usually progressively less.

Microscopic Growth.—Strain A has given little of the filamentous growth in these cultures; the forms developed have as a rule remained largely bacillary. In ordinary films nothing important is to be seen except an occasional element evidently branching. In Benians'²¹ Congo red films these are well demonstrated (Figs. 6, 8, and 9). The filamentous tendency is seldom as evident as in Fig. 8, segmentation usually taking place promptly.

Round, spore-like bodies that structurally are comparatively delicate are also produced. Lacking the density and rigidity that preserve the morphology of ordinary bacterial elements through the process of drying and heat fixation, they are usually greatly injured in ordinary films. As a rule, they are likely to be dismissed as shrunken involution forms. They are well demonstrated in the Congo red films, where the background evidently sets before the bodies dry out sufficiently to shrink. Here they usually measure approximately 1.5 to 3 microns, often grading down to small coccoid granules, and up to a considerable size.

Conidial bodies are apparently produced in three ways: (1) by direct transformation from short bacillary elements; (2) as terminal knobs (Figs. 1 to 9) on simple rods or on short branches that might be likened to conidiophores (Fig. 9); and (3) as simple lateral buds (Figs. 2, 3, 5, and 18). Though usually single and round, even the smaller masses are not infrequently compound and lobulate (Figs. 2 and 3).

These bodies very clearly act as fungous spores, giving rise to one (Figs. 4, 5, and 8), two (Figs. 5 and 7 to 9), and sometimes even three offshoots. The offshoots are generally elongated, but sometimes develop entirely as rounded buds (Figs. 6, 7, and possibly 9). The

²¹ Benians, T. H. C., *Brit. Med. J.*, 1916, ii, 722.

bodies are therefore analogous to the conidiospores of many species of *Discomyces*.²² So far as we know, no similar structure is produced by any strictly bacterial (non-fungous) organism. There is no evidence that these elements function as spores in the bacteriological sense.

Strains B and C have exhibited at one time or another all the forms described. However, they tend to discard quickly the more bacterial forms, developing filamentous masses that often become very complex (Figs. 10 to 12). These masses are seldom more than suggested by Strain A (Figs. 5 and 6). Similar, but extremely small, closely branching and budding complexes form the chief type of growth of all strains in high concentration.

Growths in Association with Other Organisms.

Work with the organism growing in association with other bacteria has not been so extensive as with pure cultures. However, the changes seen indicate that this phase of the problem is of greater interest. Mixed directly in ordinary bouillon with a pneumococcus, rapid degeneration occurs, the bacillus completely disappearing in a few days. On the other hand, with the strains of streptococcus used in most of the experiments a remarkable development takes place. This has been traced from day to day, with all three strains, through the forms previously described to the most extreme clusters of strictly fungous growth shown in Figs. 13 to 21. Here the long, more or less frequently branching filaments and the numbers of laterally formed conidial bodies are particularly striking.

A peculiar feature is the frequent appearance of the imperfectly defined material to be seen in Fig. 20 lying between the conidial bodies. This is usually absent but may be abundant. Lobulate growth is frequently seen in large masses (Figs. 18 and 21) evidently developed from conidial bodies. These masses sometimes attain an appearance suggestive of certain as yet unpublished observations made

²² For a discussion of the validity of this term over *Streptothrix*, *Actinomyces*, *Nocardia*, etc., see, Merrill, E. D., and Wade, H. W., *Philippine J. Sc.*, 1919, xiv, 67.

by one of us in the work that led to the formulation of the "cryptoplasm" hypothesis. It would be of interest to determine whether this type of growth could progress indefinitely.

Cultivability of Described Forms.

It need not be emphasized that none of the described forms is due to involution, but result from active growth of the organism in adaptation to influences in the medium. In this adaptation the ordinary cultural characteristics are soon lost. Repeatedly, subplants on blood agar from fluid cultures have remained sterile, though it was evident that the original cultures were not dead, since they subsequently became more turbid through further growth.

There seem to be distinct stages in the depression of cultivability. Within a day or two the organism, originally growing luxuriantly on hot blood agar, produces only small, comparatively delicate growths in subcultures. A later subplant from the fluid culture may develop only minute, almost imperceptible colonies that are found to be made up of rather short, extremely fine, irregular bacilli, usually finely beaded; these are often suggestive of the leprosy bacillus; in another day the area scraped over in getting material for the original film shows a distinct haze, also made up of these fine bacilli. Subplants from this give light growths of more typical influenza bacilli, and on further subculturing the usual heavily growing type is recovered. Both processes have been observed several times.

A few attempts have been made to perpetuate the fungous growth in the fluid media, but thus far subcultures have not developed to any great extent.

DISCUSSION AND SUMMARY.

It has been found that three different strains of an organism supposed to be *Bacillus influenzae* will, under certain conditions, abandon the usual bacillary form and grow as a frank fungus, morphologically of the *Discomyces* type. Under other conditions they show less modification, the most striking feature then being the production of conidiospores, bodies of a type not found in true bacteria. That this organism may not be the true Pfeiffer bacillus is conceivable,

of course, but considering the source, morphology, ordinary cultural characteristics, and the poison production of the one strain tested, we consider this highly improbable. Further, we are confident that the cultures do not contain any contaminating organisms, as may be suggested. In short, we believe that we have been dealing solely with the true Pfeiffer bacillus.

While these observations are of considerable interest as a contribution to the biology of the bacteria of this general type it cannot, of course, be predicted that they will prove to be of any significance as regards the true causative agent of epidemic influenza. Experimental work with this organism, apparently negative so far as reproducing true clinical influenza is concerned, has been carried out with the bacillary form exclusively. It may be found that its physiological capabilities in another phase are essentially different. This is a general biological law and there is no evident reason why it should not hold true here.

It remains to be determined whether the relatively high, complex forms described have any relation to those that occur while the organism lives among other organisms on the respiratory mucosa or acts as a tissue invader. While it seems improbable that they should develop in the animal body, that is while the organism is living as a parasite, it is at least possible that the bacillus may, under some conditions, undergo some analogous or at least similarly radical modification. If this supposition is true its cultivability might well be quite different from that of its bacillary phase, in which event it might be present in abundance and yet not be found in ordinary cultures or be recognizable in films.

But the more important problem would appear to be whether it can assume a simpler phase. If, as some believe, some of the infectious bacteria and fungi can do this, whether it be as minute, filter-passing, formed elements or as a more or less amorphous ("sympastic,"¹⁶ "cryptoplasmic,"¹⁵) substance, an organism that is capable of as remarkable a range of morphological development upward might well go to the other extreme from the mean, bacillary stage. Whether or not this occurs and if so under what conditions are questions that deserve thorough investigation.

EXPLANATION OF PLATES.

Photomicrographs of unstained organisms in Benians' Congo red films.

PLATE 11.

Figs. 1 to 8 are from a 7 day pure culture of Strain A, in blood extract bouillon. $\times 1,000$.

FIG. 1. Formation of terminal conidial bodies; one long segmenting form; ordinary bacillary forms.

FIG. 2. One element with a lobulate terminal conidial mass.

FIG. 3. Lobulate lateral mass; terminal conidium; free conidium.

FIG. 4. Sprouting conidium; terminal conidium; ordinary bacillary forms.

FIG. 5. Two adjacent conidia, one with a single offshoot, the other with two; irregular forms.

FIG. 6. Budding conidium; irregularly branching forms.

FIG. 7. Giant conidium; small conidium with two offshoots.

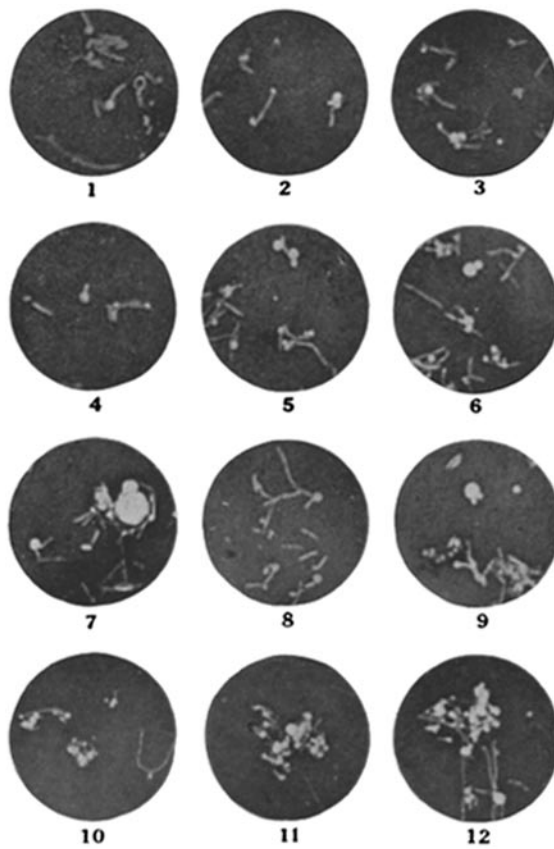
FIG. 8. Repeatedly branching growth, arising from a two-sprout conidium. Preparation for segmentation is evident.

FIG. 9. Strain A, from a 3 day culture with streptococcus, in ordinary meat infusion bouillon. Conidium with two small buds; branching form, one portion being in effect a conidiophore; associated streptococci present. $\times 1,000$.

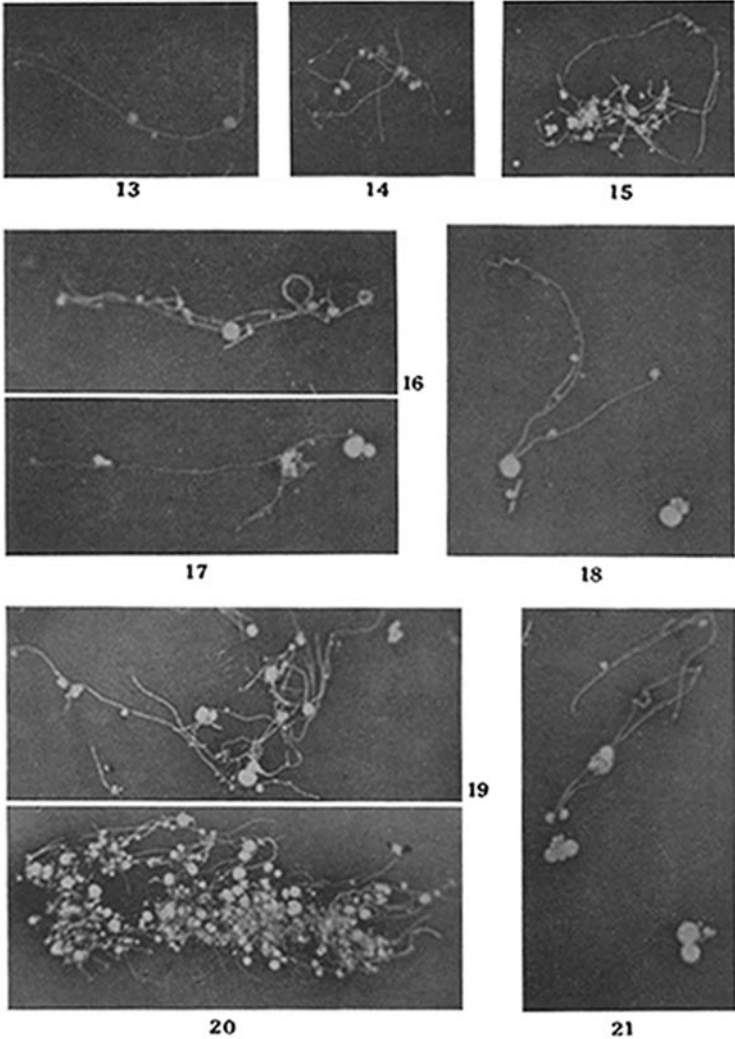
FIGS. 10 to 12. Strain B, from a 7 day pure culture in favorable blood extract bouillon. Irregular compact growth-complexes, with short filamentous development. $\times 750$.

PLATE 12.

FIGS. 13 to 21. Strain B, from a 7 day culture in plain bouillon, in symbiosis with a streptococcus. Various stages of filamentous growth and the development of conidia. In Fig. 16 two filaments have become intertwined at one point. In Fig. 17 is seen a growth-complex on the branch from the main filament. In Fig. 18 the contrast between the mother conidium and the nearby lateral bud is striking. The mass in Fig. 20 is unusual, illustrating extreme filamentous growth with numerous conidial bodies and a peculiar indefinite growth element. Figs. 14, 15, 17, and 19 to 21, $\times 750$, Figs. 13, 16, and 18, $\times 1,000$.



(Wade and Manalang: *Bacillus influenzae*.)



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