EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

IX. THE RECURRENCE OF 1922.

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PLATES 46 TO 49.

INTRODUCTION.

The presence in New York City during the early months of 1922 of an acute respiratory infection that, clinically, resembled more or less the epidemic influenza of the autumn and winter of 1918–19 and the spring of 1920 afforded us an opportunity to repeat and extend the studies of the nasopharyngeal secretions from influenza patients which had resulted in the isolation of *Bacterium pneumosintes*. The reports of the earlier experiments have formed Papers I to VIII of the present series.[1-8]

As an introduction to our observations with material obtained during the 1922 recurrence of influenza, the facts already established may be passed briefly in review, since they form the basis for a comparison of the data recently obtained with the findings in similar experiments during the past $3\frac{1}{2}$ years.

[1] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.
[2] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 361.
[3] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 373.
[4] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 713.
[5] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiv, 1.
[6] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 1.
[7] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 553.
[8] Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 813.

Summary of Earlier Observations.

Having in mind the possible presence in the nasopharyngeal secretions of influenza patients of an agent whose effects might be noted in animals, we injected the whole or the filtered nasopharyngeal washings intratracheally into rabbits. In eight instances the nasopharyngeal washings were obtained within 36 hours of the onset of the uncomplicated disease. The material from five of these patients during the epidemic wave of 1918–19 and from two during the recurrence of 1920 produced typical clinical and pathological effects in the experimental animals. On the other hand, the active agent was not obtained from twelve influenza patients, the onset of whose illness had occurred more than 36 hours previously, nor from fourteen persons free from influenza during the epidemic and interepidemic periods.[1]

The effects produced by the active agent in the experimentally infected animals bore a certain similarity to those observed in epidemic influenza in man. Clinically, the affection was typified by a rise in temperature within 24 to 48 hours, by conjunctivitis, and by a characteristic change in the blood picture—a rapidly developing leucopenia, due mainly to a critical drop in the mononuclear cells. Unless the train of events was interrupted by secondary infection, the rabbits returned to normal in the course of 2 or 3 days. Most of them were killed after 24 to 48 hours, however, for observation of the lung lesions and to obtain material for further passage, and for cultivation. In such instances a typical pathological condition was revealed, consisting of a diffuse hemorrhagic edema of the lungs, with emphysema, and with a peculiar interalveolar cellular exudate, and a similar hemorrhagic involvement of bronchi and trachea, with exfoliation of the necrotic epithelium.[1]

When lung tissue containing such lesions was ground and extracted with salt solution, the turbid suspension produced similar effects in other rabbits, and in this way the substance active in the process was transmitted for as many as fifteen passages, indicating the multiplication of a living agent rather than the mere passive transfer of some original material.

This living agent was found to have the following properties.

As it existed in the nasopharyngeal secretions in man, and in the lungs of affected rabbits, it passed through Berkefeld V and N candles, and the filtered material produced the same effects on the blood and in the lungs of rabbits and guinea pigs as the unfiltered material.[2]

When contained in bits of lung tissue, it withstood the action of a 50 per cent glycerol solution for periods up to 9 months.[2]

It produced effects upon the pulmonary tissues of rabbits which favored the invasion and infection of the lung with other bacteria, such as the pneumococcus, streptococcus, and *B. pfeifferi*.[3]

It led to the development, in experimentally infected animals, of a specific immunity against reinfection with similar material.[6]

Meanwhile, cultivation experiments had shown the presence in the active material of a hitherto undescribed microorganism. This microorganism, which was subsequently named *Bacterium pneumosintes* because of its injurious effect upon the lung, was first observed in November, 1918, in strictly anaerobic cultures of the filtered nasopharyngeal secretions of an influenza patient in the early hours of the disease. Subsequently the same bacterium was isolated from material originally derived from all the seven cases of influenza from which active material was transmitted to rabbits, and from three other cases not so transmitted. The immediate sources of the cultures were the filtered nasopharyngeal secretions of patients or, more frequently, the unfiltered or filtered lung tissue suspensions of affected rabbits. These rabbits had been intratracheally injected with whole or filtered nasopharyngeal secretions, whole or filtered lung suspensions of other affected animals, or the glycerolated lung tissue of such animals. The cultivation experiments were carefully controlled with similar materials from non-influenzal sources. All of the control materials gave negative results.[4]

The primary cultures of *Bacterium pneumosintes* were obtained under strictly anaerobic conditions in a medium composed of sterile human ascitic fluid and a fragment of fresh rabbit kidney. In this medium the microorganism developed slowly, producing a faint haze in the region of the kidney fragment that gradually extended to a depth of about 3 cm. by the 8th day and in the course of 2 weeks settled to the bottom of the tube, leaving a clear, faintly opalescent, supernatant fluid.

In stained films, *Bacterium pneumosintes* appeared as a minute bacilloid body of regular morphology, measuring 0.15 to 0.3 micron in the long axis. Usually solitary, the bacteria were often found in diplo form, and occasionally in short chains of three or four members. All of the strains decolorized uniformly by Gram's method. They were stained with some difficulty by the usual basic dyes.

The intratracheal injection, in rabbits and guinea pigs, of mass cultures of *Bacterium pneumosintes* in the earlier generations induced effects on the blood and lungs of these animals which could not be distinguished from those obtained with the active agent of the nasopharyngeal secretions of influenza patients. In addition to this identity of source and of pathogenic effect in animals, *Bacterium pneumosintes* exhibited the other qualities characteristic of the active agent. Thus the microorganism passed Berkefeld V and N filters even in remote generations. Protected by the lung tissue of rabbits affected by the injection of mass cultures, it withstood glycerolation for a period of 9 months. Infection with *Bacterium pneumosintes* reduced the resistance of the pulmonary tissues of rabbits to infection with other bacteria, such as Pneumococcus Type IV and *B. pfeifferi*.[4] Finally, by cross-immunity experiments, the antigenic identity of the various strains of *Bacterium pneumosintes* with each other and with the active influenzal agent was completely established.

Before methods had been found for the cultivation of *Bacterium pneumosintes* in simplified media, the opportunity was lost to examine for specific antibodies the blood serum of persons convalescent from influenza or of most of the animals

which had recovered from the experimental infection. Later, methods of cultivation were devised which did not interfere with the detection of specific antibodies.[8,9] Rabbits intravenously injected with such cultures produced antibodies which were recognized by precipitation, agglutination, complement fixation, and phagocytic tests, and animals injected with glycerolated lung tissue containing the active agent yielded serum that specifically agglutinated *Bacterium pneumosintes*. It is significant that the four available strains of *Bacterium pneumosintes*, three from 1918–19 and one from 1920, showed identical antigenic characters by serological and immunological tests.

More recent experiments on methods of cultivation[8] have shown that *Bacterium pneumosintes* can be grown on media consisting of meat infusion peptone broth, or nutrient agar, as a base, enriched with fresh animal or vegetable tissue, fresh defibrinated rabbit blood, or by the growth of other organisms. Surface colonies have been obtained on blood agar plates in the Brown anaerobic jar.[10] The plates have proved especially useful for the purification of contaminated cultures and for the demonstration of viable organisms in sparse primary growths in the ascitic fluid-rabbit kidney medium. When grown on media containing nutrient broth, and especially in the presence of dextrose, *Bacterium pneumosintes* has developed larger bacillary forms up to 1 micron in length. The identity of these microorganisms with the original strains has been proved by serological reactions and by their reversion to the minute forms on transfer to the original medium.

Animal Transmission and Cultural Studies with Recent Materials.

As a result of the experiments outlined above, we had facilities for animal transmission and cultural studies at hand during the recent outbreak of clinical influenza in January and February of the present year and were able to proceed without the loss of time that was formerly spent in an empiric search for methods.

This outbreak of influenza enabled us to examine the nasopharyngeal secretions of nine persons who had within a few hours developed symptoms characteristic of the disease. The following protocol is cited as a typical example of the cases studied.

Case 36.—Adult male. Feb. 9, 1922. Illness began at night with chilly sensations, a thin, clear secretion from the nose, fever, and pains in the joints and muscles, especially those of the back and legs. Feb. 10. Remained in bed. Temperature 38.3°C., marked coryza, intense injection of conjunctivæ, dry cough. Physical examination showed no pulmonary involvement. Leucocytes

[9] Gates, F. L., *J. Exp. Med.*, 1922, xxxv, 635.

[10] Brown, J. H., *J. Exp. Med.*, 1921, xxxiii, 677; 1922, xxxv, 467.

4,000, of which 3,920 were polymorphonuclear neutrophils, and 80 were mononuclears. On this day, 12 hours after onset, nasopharyngeal washings were obtained for examination. Feb. 11. Patient's condition improved. Feb. 12. Temperature normal. Recovery uneventful.

Eight other patients whose nasopharyngeal secretions were obtained from 6 to 36 hours after onset exhibited symptoms similar to those described. The washings from one of these patients stood at room temperature for 24 hours before transmission and cultivation experiments were undertaken. Both failed. Of the remaining eight specimens, successful transmission experiments were initiated with six. The seventh failed to induce the experimental disease in rabbits. The eighth specimen was not injected into animals, but after filtration through a tested Berkefeld candle it yielded a primary growth of *Bacterium pneumosintes* in an anaerobic ascitic fluid-rabbit kidney medium.

The following protocol of a typical animal transmission experiment is reported for comparison with the recorded results of the earlier experiments.[1]

Case 28.—The unfiltered nasopharyngeal washings obtained 6 hours after onset were injected intratracheally into Rabbit A.

First Passage. Rabbit A (Text-Fig. 1).—Jan. 25, 1922. Leucocytes 12,000, of which 3,840 were polymorphonuclear cells and 8,160 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 2.5 cc. of unfiltered nasopharyngeal washings of Case 28. Jan. 26. Leucocytes 8,400, of which 3,780 were mononuclears. Temperature 39.7°C. Jan. 27. Leucocytes 5,600, of which 2,128 were mononuclears. Mild conjunctivitis. Temperature 39.9°C. Killed. All organs appeared normal except the lungs. These showed a diffuse edema and emphysema with a number of small hemorrhages scattered over the surface and on the cut sections. There was an absence of pneumonic consolidation. On microscopic examination (Fig. 1) the edema, emphysema, and the small hemorrhages were again revealed. There was a moderate degree of cellular infiltration of the interalveolar strands and about the bronchi and bronchioles, which consisted of mononuclears, polymorphonuclears containing acidophilic granules, and a few cells of the respiratory type. In addition, the interalveolar strands were distended with serum and erythrocytes. The bronchial system was also involved in a hemorrhagic and edematous process. The epithelium was necrotic in some areas and the lumen was filled with serum, leucocytes, erythrocytes, and exfoliated necrotic epithelial cells. Impression smears of the lung stained by Gram's method revealed numerous mononuclears and alveolar cells, but no bacteria. Aerobic cultures were free from growth.

In Giemsa-stained preparations of sections of the lung groups of minute organisms indistinguishable morphologically from *Bacterium pneumosintes* were noted in the bronchial exudate. Some were free and others in the different stages of phagocytosis by polymorphonuclears and monocytes (Fig. 2). In the ciliary margin of the bronchial epithelium similar clumps of these microorganisms were seen (Fig. 3).
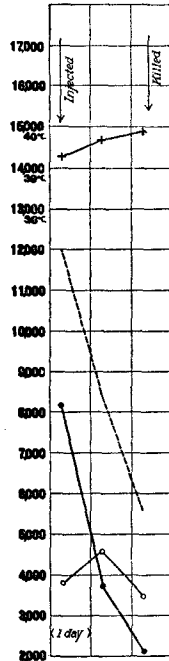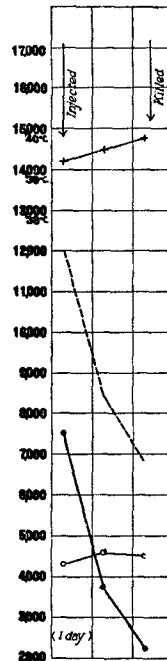
TEXT-FIG. 1.   TEXT-FIG. 2.

+———+ Temperature.
- - - - Total leucocytic count.
•———• Mononuclear cells.
o———o Polymorphonuclear cells.

TEXT-FIG. 1. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. First rabbit passage of the nasopharyngeal washings from a patient (Case 28) in the early stage of uncomplicated epidemic influenza (1922). Intratracheal injection.

TEXT-FIG. 2. Effect on the blood count and temperature. The rise in temperature and the depression in the total white blood cell count caused by a deficiency of mononuclears are shown. Rabbit passage of *Bacterium pneumosintes* from a culture, in the third generation, originally derived from a rabbit inoculated with the washings from another influenza patient (Case 36).

In the present series of experiments animal passage failed after the first transfer in one instance. The other transmission experiments were discontinued after the second to fourth transfers.

The effects produced by the intratracheal injection into rabbits of these specimens of unfiltered nasopharyngeal secretions from influenza patients appeared to us to be identical with those observed during the former epidemic waves. The active agent showed other familiar qualities also. It survived the action of 50 per cent glycerol for 4 months (the longest period yet tested) and passed Berkefeld V and N filters, producing characteristic lesions in rabbits injected with the filtrate.

As controls for these experiments, we have negative transmission experiments with the nasopharyngeal secretions of three persons not suffering from influenza. One of these had a mild head cold which ran a typical course; the others were normal.

Because of the identity of the active agent with *Bacterium pneumosintes* in former experiments, our endeavor was directed mainly toward the demonstration of a filterable anaerobic microorganism in the material obtained during this recurrence of influenza. Filtered nasopharyngeal secretions from each of the nine influenza patients and from ten other persons were inoculated into ascitic fluid and rabbit kidney tubes under a vaseline seal, and spread upon blood agar plates in an anaerobic jar. The lung tissues of affected rabbits were likewise cultured in the fluid medium, and sometimes smeared on the anaerobic plates. When no organisms or only a few minute and questionable bodies could be demonstrated with the microscope in fluid cultures, material from these cultures was spread on blood plates for the possible observation of colony formation.

By these methods there were obtained cultures of strictly anaerobic, filter-passing, Gram-negative organisms in material derived from four of the six influenza patients from whom an active agent had been transmitted to rabbits, from a seventh influenza patient whose nasopharyngeal washings were not injected into animals, and from four of ten other persons not suffering from influenza. A survey of these microorganisms showed that *Bacterium pneumosintes* is not the only anaerobic, filter-passing, Gram-negative organism to be found in the human respiratory tract. From one of the influenza patients and from

the four non-influenzal controls which yielded positive cultures, other bacteria, not pathogenic for rabbits and not *Bacterium pneumosintes*, were obtained. A preliminary description of these other bacteria is appended to this paper.

On the other hand, we have identified as *Bacterium pneumosintes* the cultures derived from three of the six influenza patients, enumerated above, whose nasopharyngeal secretions were pathogenic for rabbits, and from the seventh patient, whose secretions were not used in a transmission experiment. No cultures of *Bacterium pneumosintes* were obtained from the controls.

It is noteworthy that only one strain of *Bacterium pneumosintes* was obtained directly from the filtered nasal washings. The others were derived from lung tissue after rabbit passage. In this connection the relative value of these two immediate sources may be indicated. Nasopharyngeal secretions require filtration before direct cultivation in fluid media and much of the active material, mixed with mucus and epithelial debris, is retained on the filter and lost. It is probable that conditions are only occasionally favorable for the primary development of *Bacterium pneumosintes* in artificial culture. As with the pneumococcus a relatively large inoculum is usually required to initiate growth. Moreover, if the first inoculations fail the entire opportunity is lost. But when the whole nasopharyngeal washings are injected into rabbits, the larger primary inoculum is further augmented by active multiplication in the pulmonary tissues, and in those favorable instances in which contaminating bacteria are suppressed, a material rich in *Bacterium pneumosintes* in pure culture is available for artificial cultivation either directly or after filtration. Moreover, through continued rabbit passage repeated opportunities are afforded for cultivation experiments and sooner or later they usually succeed. We feel that although direct cultivation of filtered nasopharyngeal secretions may be a suitable method for the demonstration of other anaerobic filter-passing microorganisms, a negative experience in the cultivation of *Bacterium pneumosintes* without the aid of animal passage is of little significance.

Identification of New Strains of Bacterium pneumosintes.

The identification of the new strains of *Bacterium pneumosintes* was made on morphology, cultural characters, filterability, typical pathogenicity for rabbits, resistance to glycerol, reduction of resistance to secondary infection, and serological and immunological reactions.

Not all of these tests have been made with each strain, but each has been definitely identified by means of a combination of them, and all

have been agglutinated specifically by sera made with the 1918–19 strains as antigen. These experiments also served as a review and check on the characters of *Bacterium pneumosintes* as formerly observed, and confirmed our reported findings. For these reasons they may be described more or less in detail.

Morphological and Cultural Characters.—The morphology and cultural characters of the new strains are in all respects identical with those of the strains obtained in 1918–19 and 1920. Photographs of Strain 16,[4] from the 1918–19 epidemic, and Strain 36, obtained in February, 1922, may be compared in Figs. 5 and 6. Colonies of these strains on anaerobic blood agar plates are shown in Figs. 8 and 9.

Filterability.—Mention has already been made of Strain 32, which was obtained by direct cultivation of the filtered nasopharyngeal washings from Case 32. Other new strains, obtained from rabbit lung tissues, have passed Berkefeld V candles and initiated growth in culture media.

Pathogenicity.—So far all of the new strains tested (first to eighth generation) have retained their pathogenicity for rabbits. With one strain the process of intratracheal injection, followed by the characteristic infection and recovery of the organism from the lung tissue, has been repeated five times. The three other strains have been recovered in this manner twice. A typical protocol illustrates the clinical and pathological results of intratracheal injection of the strain of *Bacterium pneumosintes* derived from Case 36.

Rabbit A.—Mar. 15, 1922. Leucocytes 12,000, of which 7,680 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 3 cc. of a suspension of washed mass culture[4] of *Bacterium pneumosintes*, the third generation of Strain 36. Mar. 16. Leucocytes 8,400, of which 3,780 were mononuclear cells (Text-fig. 2). Temperature 39.5°C. Mild conjunctivitis. Mar. 17. Leucocytes 6,800, of which 2,244 were mononuclear cells. Temperature 39.8°C. Mild conjunctivitis. Killed. Observable lesions were confined to the lungs and trachea. The lungs were the site of a diffuse hemorrhagic edema and emphysema without any obvious consolidation. Microscopically, the hemorrhages, edema, and emphysema were noted as well as the typical cellular exudation and bronchial lesions similar to those found in the lungs of the rabbit injected with the nasopharyngeal secretions from an influenza patient, and described above (Fig. 7).

In Giemsa-stained preparations of the lung tissue small groups of microorganisms, indistinguishable from *Bacterium pneumosintes*, were found in the ciliary layer

of the bronchial epithelium. Aerobic cultures of the lung tissue showed no growth. Anaerobic cultures in ascitic fluid-rabbit kidney medium yielded pure growths of *Bacterium pneumosintes*.

Locus of Bacterium pneumosintes in Affected Lungs.—We have not heretofore reported direct observations on the presence of *Bacterium pneumosintes* in the lungs of infected rabbits. Frequently in the course of the earlier studies, in impression smears or sections of affected rabbit lungs, stained by Gram's method, with Löffler's methylene blue, or with methylene blue and eosin, minute but questionable bodies had been made out. In view of their inconstancy and the difficulty of demonstrating them with the stains employed, we had not been willing to accord them diagnostic significance. Recently in microscopic sections stained by Giemsa's method, minute blue- or violet-stained bodies, morphologically identical with *Bacterium pneumosintes*, have been found repeatedly in scattered groups in the affected pulmonary tissues. Their usual site is deep in the ciliary margin of bronchial epithelium (Figs. 3 and 4). Less often the bodies are found in mucous and serous exudate between the cilia and in the lumen of the bronchioles, where they are in process of phagocytosis by polymorphonuclear cells and monocytes (Fig. 2). Infrequently they have been found in the interalveolar strands, where they are usually surrounded by an inflammatory mononuclear cell reaction.

These minute and characteristic bodies, which have not been observed in normal rabbit lung tissue, have been clearly made out in various sections of the pulmonary tissues of six rabbits injected with the active agent of the nasopharyngeal secretions of influenza patients. From three of these rabbits pure cultures of *Bacterium pneumosintes* were obtained. The incidence and distribution of these bodies in the lesions caused by *Bacterium pneumosintes*, and their morphological and tinctorial similarity to this microorganism lead us to suppose that they are *Bacterium pneumosintes*.

Reduction of Resistance to Secondary Infection.—A peculiar and significant property of the strains of *Bacterium pneumosintes* obtained in 1918–19 and 1920 was their effect in reducing the resistance of the lung tissues of experimentally infected rabbits to invasion by other bacteria.[5] Some of the recently isolated strains have been tested in a similar manner and with similar results. For example, after

repeated trials had shown that 3 million pneumococci of an atypical Type II strain, injected intravenously, rapidly disappeared from the blood stream and produced no lesions in rabbits, the tested dose was used in controlled experiments such as the following.

Rabbit A.—May 16, 1922. Leucocytes 6,300, of which 3,090 were mononuclear cells. Temperature 39.2°C. Injected intratracheally with 3 cc. of a saline solution suspension of *Bacterium pneumosintes*, Strain 34, third generation in mass culture.[4] May 17. Leucocytes 6,000, of which 1,200 were mononuclear cells. Mild conjunctivitis. Temperature 39.5°C. Injected intravenously with 1 cc. of a saline solution suspension containing 3 million pneumococci, atypical Type II. May 18. Leucocytes 5,600, of which 2,520 were mononuclear cells. Conjunctivitis. Temperature 41.2°C. May 19. Leucocytes 18,800, of which 11,280 were polymorphonuclear cells and 7,520 were mononuclears. Conjunctivitis. Temperature 40.7°C. Blood taken for culture. Killed. The lower right lobe was voluminous, dark, and resistant to the knife. Section revealed a dark and congested cut surface. Fibrinopurulent material exuded from the bronchi. Microscopically, the exudate was found to consist mainly of polymorphonuclears with acidophilic granules and a few mononuclear and respiratory type cells with fibrin and serum. Impression smears of the lung stained by Gram's method showed numerous polymorphonuclear cells containing phagocytosed pneumococci. Free pneumococci seen also. The blood culture and the aerobic and anaerobic cultures of the lungs yielded pure growths of the pneumococcus. Diagnosis: pneumococcus septicemia and pneumonia.

Precisely similar results were obtained with the streptococcus and *Bacillus pfeifferi*. These experiments will be described in detail in the next paper of this series.

Serological Reactions.—The first anti-*pneumosintes* rabbit serum produced was obtained by five weekly injections of a suspension of live organisms (Strain 11) and showed an agglutination titer of 1:160 to 1:320 against the homologous strain.[7] Other sera, produced with live or killed cultures of Strains 16, 17, and 26, have shown a dilution limit of 1:16 to 1:32 for complete agglutination, even after prolonged immunization. We are inclined to believe that, aside from individual variation in rabbits, the relatively low titer of immune serum to be obtained with *Bacterium pneumosintes* is characteristic of the organism.

For diagnostic purposes this low titer of the anti-*pneumosintes* serum is not objectionable, since the reactions at dilutions of 1:8 and 1:16 are sharp and specific. We have never seen *Bacterium pneu-*

mosintes agglutinate in normal rabbit serum, even undiluted, and the bacterium shows no tendency to spontaneous flocculation in salt solution, remaining in smooth suspension for 24 to 48 hours. Dr. De Kruif, who has done some unreported experiments on the acid agglutination zone of *Bacterium pneumosintes*, informs us that it flocculates out in an unusually narrow zone, lying between pH 3.0 and 3.6, and he infers from this that *Bacterium pneumosintes* is a stable microorganism, not easily agglutinable by immune serum or other chemical or physical agents.

TABLE I.

Agglutination Tests with Old (Strains 17 and 26) and New Strains of Bacterium pneumosintes.

Strain No.	Immune Serum D.				Normal rabbit serum.				Salt solution.
	Dilutions.				Dilutions.				
	1:2	1:4	1:8	1:16	1:2	1:4	1:8	1:16	
17	+++	+++	+++	+++	—	—	—	—	—
26	+++	+++	+++	+++	—	—	—	—	—
32	+++	+++	+++	+++	—	—	—	—	—
34	+++	+++	+++	++	—	—	—	—	—
36	+++	+++	+++	+++	—	—	—	—	—
40	+++	+++	+++	+++	—	—	—	—	—

In Table I are given the results of an agglutination test with old and new strains of *Bacterium pneumosintes* against immune rabbit serum produced with one of the old strains. The serum was obtained May 6, 1920, from a rabbit intravenously injected with living cultures of Strain 11. The bacterial suspensions for agglutination were grown in *Bacillus coli* broth,[8] centrifuged, taken up in salt solution, killed by heat at 56–60°C. for 30 minutes, and standardized by opacity. The tests were set up in successive dilutions in capsules,[11] incubated at 55°C. for 1 hour, and stored in the ice box over night before macroscopic examination.

[11] Gates, F. L., *J. Am. Med. Assn.*, 1921, lxxvii, 2054.

As a further test of specificity, two of the old strains derived from Cases 16 and 17[1] and three of the new strains from Cases 32, 34, and 36 were examined for their capacity to absorb specific agglutinins.

Suspensions of the absorbing strains, of ground glass opacity, were distributed into two sets of small test-tubes in lots of 1 cc. These tubes were then centrifuged and the supernatant fluid was carefully pipetted off from the compact sediment. To each of one set of tubes was added 1 cc. of immune serum (made with Strain 16 and obtained Feb. 28, 1922) in a dilution of 1:4, the bacteria were suspended by agitation, and the tubes were incubated for 1 hour at 55°C. After centrifugation, the supernatant serum from the incubated tubes (now absorbed once) was poured on to the sediment of the corresponding culture in the second set of tubes, which were shaken and incubated for the second absorption for 1 hour at 55°C. In this way dilution of the serum was avoided. The serum samples, now twice absorbed, together with an unabsorbed control which had been incubated for 2 hours at 55°C., were then used in the agglutination test shown in Table II.

In the table the columns grouped under A show the agglutination of the various strains in the unabsorbed control serum. In Group B each absorbed serum sample was tested against its own absorbing strain. The tests show that absorption of specific agglutinins had occurred but was not complete. In Group C the serum samples, absorbed with the various strains, were tested against the homologous strain, No. 16. All of the strains had removed specific agglutinins for the homologous strain, but again, absorption was evidently not complete with the amounts of bacteria employed. Finally, in Group D the serum was (incompletely) absorbed with the homologous strain, which removed part of the agglutinins for all of the other strains.

Numerous other agglutination tests with the new strains against serum made with the old ones, and *vice versa*, have proved the antigenic identity of the new strains with the old, and among themselves. If antigenic differences or variations exist by which the strains isolated in 1918–19 and 1920 can be differentiated from those obtained in 1922, we have so far failed to discover them.

Immunological Reactions.—Because the old strains, during long cultivation, had lost their pathogenicity for rabbits, we were able to carry out cross-immunity tests in one direction only; namely, to discover whether injections of the old strains protect against subse-

TABLE II.

Absorption Tests with Old (Strains 16 and 17) and New Strains of Bacterium pneumosintes.

Group A.					Group B.				
Absorbing strains.	Strains agglutinated.	Unabsorbed control serum.			Absorbing strains.	Strains agglutinated.	Absorbed serum.		
		Dilutions.					Dilutions.		
		1:8	1:16	1:32			1:8	1:16	1:32
Not absorbed.	16	+++	+++	+++	16	16	++	—	—
	17	+++	+++	+++	17	17	+++	+	—
	32	+++	+++	+++	32	32	+	—	—
	34	+++	+++	+++	34	34	+	+	—
	36	+++	+++	+++	36	36	++	+	—

Group C.					Group D.				
Absorbing strains.	Strains agglutinated.	Absorbed serum.			Absorbing strains.	Strains agglutinated.	Absorbed serum.		
		Dilutions.					Dilutions.		
		1:8	1:16	1:32			1:8	1:16	1:32
17	16	+++	—	—	16	17	+++	++	+
32	16	+++	+	—	16	32	++	+	—
34	16	+++	+	—	16	34	+	—	—
36	16	+++	+	—	16	36	+	—	—

quent infection with the new ones. Two such experiments have been performed and have indicated this protection, as the following proto-col shows.

Rabbit A.—Apr. 11, 1922. Injected intratracheally with 3.5 cc. of suspension of centrifuged sediment consisting of live *Bacterium pneumosintes*, Strains 16 and 26, derived from the 1918–19 and the 1920 waves, and grown in *B. coli* broth.[8] These cultures were at this time not pathogenic for rabbits.[4] Apr. 24. Leuco-cytes 12,600, of which 5,544 were mononuclear cells. Temperature 39.7°C. Bled from ear vein for agglutination test. Injected intratracheally with 3 cc. of washed sediment of live mass culture[4] of *Bacterium pneumosintes*, Strain 34, derived by rabbit passage from Case 34 of the 1922 wave, in the fourth generation. Apr. 25. Leucocytes 12,000, of which 5,760 were mononuclears. Temperature 39.5°C. Conjunctivæ normal. Apr. 26. Leucocytes 10,800, of which 5,724 were mononuclear cells. Temperature 39.5°C. Conjunctivæ normal. Killed. All organs, including lungs, normal. Aerobic and anaerobic cultures of lungs were free from growth.

Rabbit B (Normal Control).—Apr. 24, 1922. Leucocytes 9,000, of which 4,500 were mononuclears. Temperature 39.2°C. Inoculated with the recent living culture of *Bacterium pneumosintes* precisely as in the case of Rabbit A. Apr. 25. Leucocytes 4,800, of which 1,536 were mononuclear cells. Temperature 39.2°C. Conjunctivitis. Apr. 26. Leucocytes 4,600, of which 1,472 were mononuclears. Temperature 39.5°C. Conjunctivitis. Killed. Lungs showed the hemorrhagic edema, emphysema, peculiar cellular exudation, and bronchial lesions characteristic of the action of *Bacterium pneumosintes.* Aerobic cultures of lungs were free from growth; anaerobic culture yielded a pure growth of *Bacterium pneumosintes.*

We have already demonstrated[6] the presence of agglutinins against *Bacterium pneumosintes* after intratracheal injection of the active influenzal agent with which the rabbit transmission experiments were carried out. In a similar test the serum from Rabbit A, obtained 13 days after the first intratracheal inoculation with the old strains, agglutinated both the old and recent strains of *Bacterium pneumosintes.* The immunity produced by intratracheal injection, even of non-pathogenic strains of *Bacterium pneumosintes,* is therefore demonstrable by serological tests. These observations on protection and antibody formation after intratracheal inoculation agree with those of Besredka,[12] D'Aunoy,[13] and others.

SUMMARY AND CONCLUSIONS.

From the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza during the recurrence in New York City in January and February, 1922, we have again obtained an active agent, pathogenic for rabbits, and have identified this active agent as *Bacterium pneumosintes.* Four new strains of this microorganism have been isolated in pure culture and identified with the 1918–19 and 1920 strains on morphological, cultural, and serological grounds. All of the significant characteristics of the old strains, including their effect upon the resistance of the lungs of rabbits to secondary invasion with other bacteria, have been noted in the new strains, which thus have served to confirm and extend our original observations.

[12] Besredka, A., *Ann. Inst. Pasteur,* 1919, xxxiii, 882; 1920, xxxiv, 361.
[13] D'Aunoy, R., *J. Infect. Dis.,* 1922, xxx, 347.

Addendum.

Reference has already been made in this paper to the discovery of other anaerobic, filter-passing, Gram-negative microorganisms, not *Bacterium pneumosintes*, in nasopharyngeal secretions from human sources. Three of the five cultures which proved not to be *Bacterium pneumosintes* were derived from the filtered nasopharyngeal secretions of supposedly normal persons. What the importance of these microorganisms may be, or whether they have any pathogenic significance we are not prepared to suggest. They indicate, however, that the cultural methods recently employed in these studies may lead to the isolation of a group, or groups, of hitherto undescribed inhabitants of the upper respiratory tract. Our observations may therefore be reported, even though fragmentary, as an indication of interesting opportunities in this field of bacteriology.

Although all the five strains of bacteria, not *Bacterium pneumosintes*, and not pathogenic for rabbits, which have been isolated, are strictly anaerobic, decolorize by Gram's method, and have passed tested Berkefeld V or N filters once or repeatedly, they differ culturally and morphologically among themselves and fall into three apparently unrelated groups.

Group I is represented by a slender vibrio which was obtained from the filtered nasopharyngeal secretions of an undoubted case of epidemic influenza from which *Bacterium pneumosintes* was not obtained.

This vibrio (Fig. 10) is approximately 0.15 to 0.2 micron in thickness, and from 0.5 to 2 microns long, with rounded ends and a smooth curve in contour which may approach a half circle. In the early generations in artificial culture media extremely minute forms were observed, but in later generations the larger forms have predominated. The culture was filtered through a tested Berkefeld V candle in the third generation. This microorganism usually takes the bacterial stain faintly and uniformly throughout. Occasionally polar staining has been observed. In fluid media, such as ascitic-kidney, dextrose broth-kidney, or *Bacillus coli* broth under a vaseline seal, it grows first in minute flakes, which soon settle out as an amorphous or flocculent sediment leaving a clear supernatant fluid. On rabbit blood plates, incubated in an anaerobic jar, it forms pin-head, dis-

crete and confluent colonies, without odor, which do not discolor or precipitate the medium (Fig. 11). The colonies are raised, convex, with an entire edge and a grayish translucency. The organism does not emulsify readily and tends to spontaneous flocculation. Serum from a rabbit immune to this organism does not agglutinate *Bacterium pneumosintes*.

Group II is represented by an extremely minute bacillus (Fig. 12) isolated from a patient with acute follicular tonsillitis. Morphologically this microorganism is not unlike *Bacterium pneumosintes*, except that it shows greater variations in length and thickness. So far we have been unable to obtain constant growths in fluid media, although the strain had survived and possibly multiplied during several weeks incubation in anaerobic blood broth. On anaerobic blood agar plates it forms extremely minute colonies, well shown in Fig. 13. In microscopic tests with the surface growth on blood agar this microorganism has failed to show any agglutination in anti-*pneumosintes* serum. It is not spontaneously agglutinable.

A third group of organisms, differing from the other two, and possibly among themselves, is found in three strains of filter passers obtained from normal throats. Morphologically these strains are also bacillary, with wide variations between the longest and the shortest forms (Fig. 14). They, too, have failed to grow consistently in fluid media, and have been maintained for many generations on anaerobic blood agar plates. The characteristic colonies of these organisms are shown in Fig. 15. A sharp cone is surrounded by a plateau with an irregular edge. These colonies are slightly brownish in color and can thus be distinguished by transmitted light. The representatives of this group flocculate in normal rabbit serum.

These fragmentary observations are reported not because of any supposed relation to *Bacterium pneumosintes*, but as an indication that other microorganisms, which must be differentiated from *Bacterium pneumosintes*, may be encountered under similar conditions. The isolation of these other bacteria has depended upon the use of anaerobic blood agar plates. The failure of all but one of them to grow in fluid media and the fact that they are not pathogenic for rabbits, under the conditions of our experiments, show why they were not encountered during the earlier periods of study, before the blood plates were used.

EXPLANATION OF PLATES.

PLATE 46.

FIG. 1. Microscopic appearance of a section of the lung of a rabbit injected intratracheally with the nasopharyngeal secretions from a patient with early influenza (Case 28). The edema, emphysema, and, in the upper portion, hemorrhage are shown. To be noted also are the distention of blood vessels with blood, the bronchial lesion (lumen filled with serum, leucocytes, and exfoliated necrotic epithelium), and the interalveolar cellular exudate. × 250.

PLATE 47.

FIG. 2. Giemsa-stained section of the lung from a rabbit injected intratracheally with the nasopharyngeal secretions from another influenza patient (Case 30). Lumen of a bronchus. Microorganisms indistinguishable from *Bacterium pneumosintes* are seen at points marked *A*, mixed with precipitate at *B*, phagocytosed by polymorphonuclear cells at *C*, and by an endothelial leucocyte, or monocyte, at *D*. The ciliary margin is indicated by *E*. × 1,000.

FIG. 3. Giemsa-stained section of the lung shown in Fig. 1. A clump of microorganisms indistinguishable from *Bacterium pneumosintes* is seen deep in the ciliary margin. Aerobic cultures of this tissue were free from growth; anaerobic cultures yielded *Bacterium pneumosintes*. × 1,000.

FIG. 4. Giemsa-stained section of the lung from a rabbit intratracheally injected with a mass culture of *Bacterium pneumosintes*, Strain 34, showing microorganisms in the ciliary layer of a bronchus. × 1,000.

FIG. 5. Stained preparation of *Bacterium pneumosintes*, Strain 16, from the 1918–19 epidemic, in fresh tissue dialysate medium.[9] Cultivated artificially for 2½ years. To be compared with Fig. 6. × 1,000.

FIG. 6. Stained preparation of *Bacterium pneumosintes*, Strain 36, from the 1922 wave, in fresh tissue dialysate medium. Cultivated artificially for 2 months. To be compared with Fig. 5. × 1,000.

PLATE 48.

FIG. 7. Microscopic appearance of a section of the lung of a rabbit injected intratracheally with a culture of *Bacterium pneumosintes*, Strain 36. To be noted are the lesions similar to those shown in Fig. 1. × 250.

PLATE 49.

FIG. 8. Colonies of *Bacterium pneumosintes*, Strain 16, from the 1918–19 wave, grown anaerobically on the surface of blood agar. × 10.

FIG. 9. To be compared with Fig. 8. Similar colonies of *Bacterium pneumosintes*, Strain 36, from the 1922 wave. × 10.

FIG. 10. Showing the morphology, after repeated subplants, of anaerobic, filter-passing, Gram-negative bacteria of Group I. From a culture in ascitic fluid-fresh tissue medium. × 1,000.
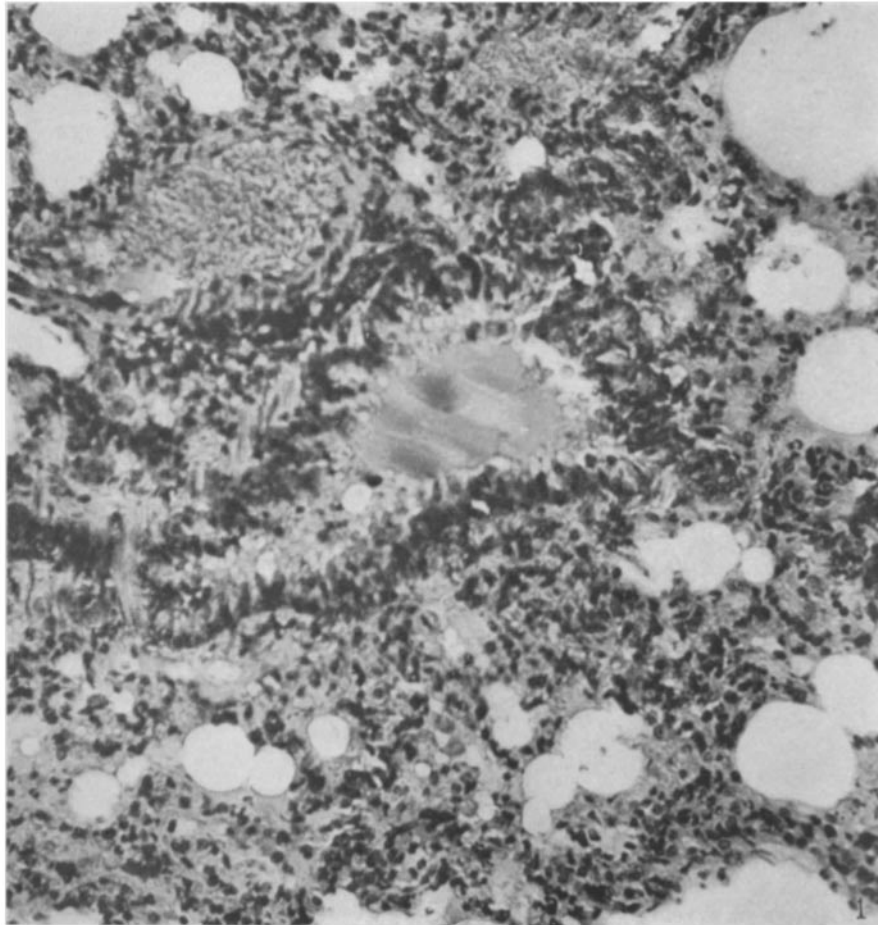
Fig. 11. Colonies of the culture shown in Fig. 10, grown anaerobically on the surface of blood agar. × 10.

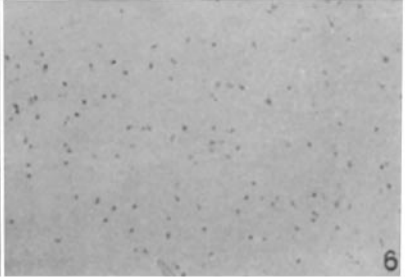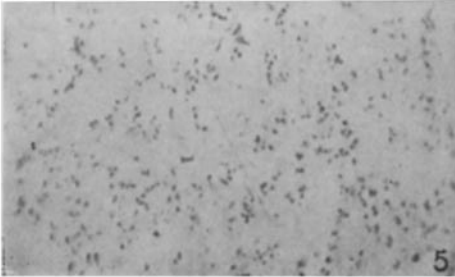Fig. 12. Morphology of anaerobic, filter-passing, Gram-negative bacteria of Group II. × 1,000.
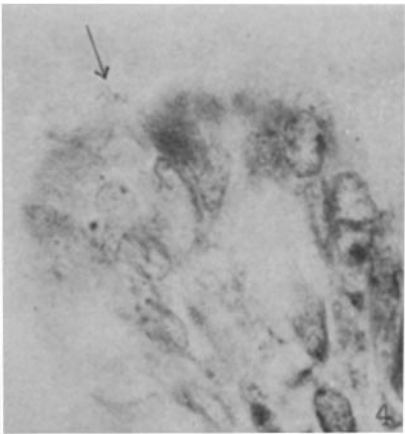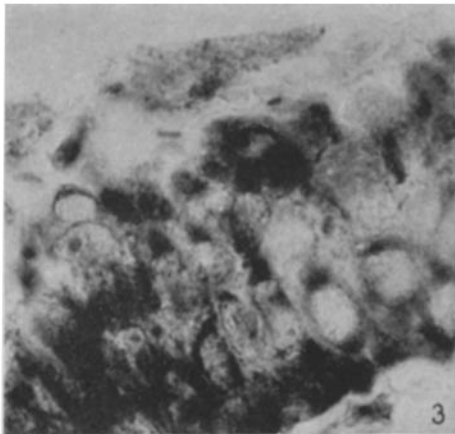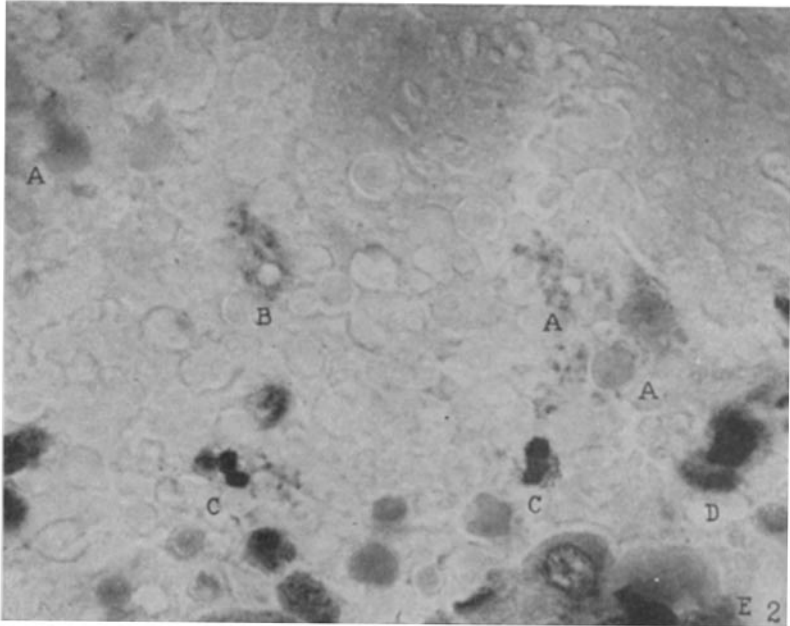
Fig. 13. Colonies of the culture shown in Fig. 12, grown anaerobically on the surface of blood agar. × 10.

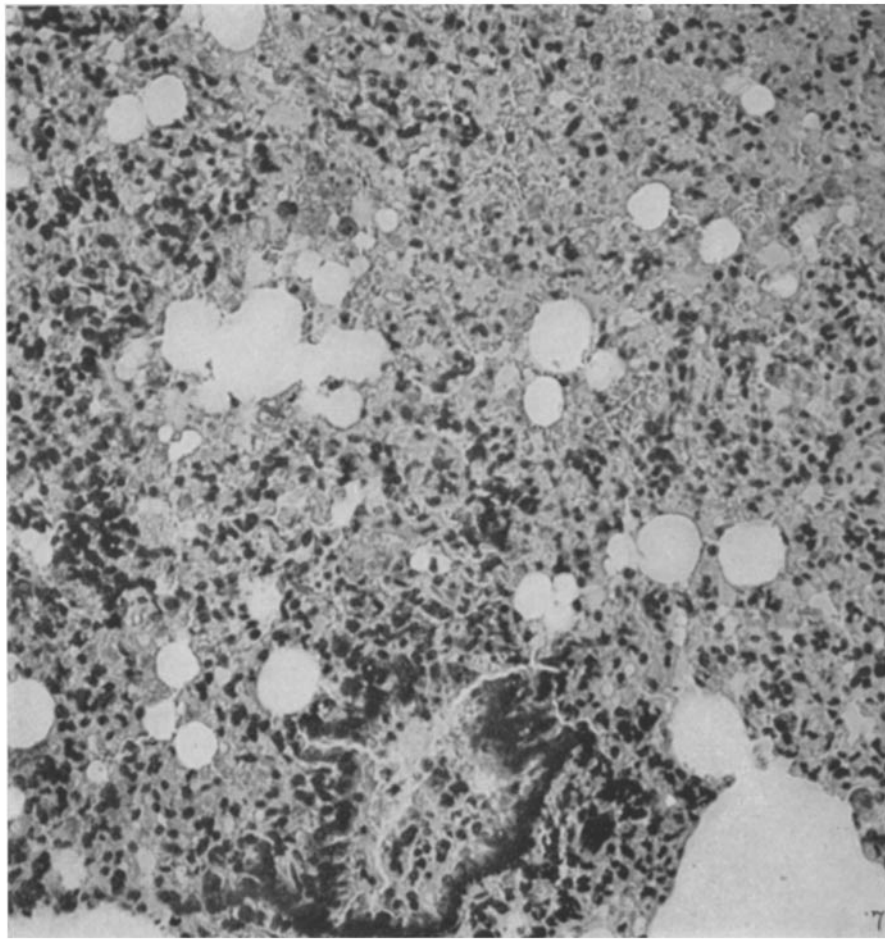Fig. 14. Anaerobic, filter-passing, Gram-negative bacteria of Group III. × 1,000.

Fig. 15. Colonies of the culture shown in Fig. 14, grown anaerobically on the surface of blood agar. × 10.
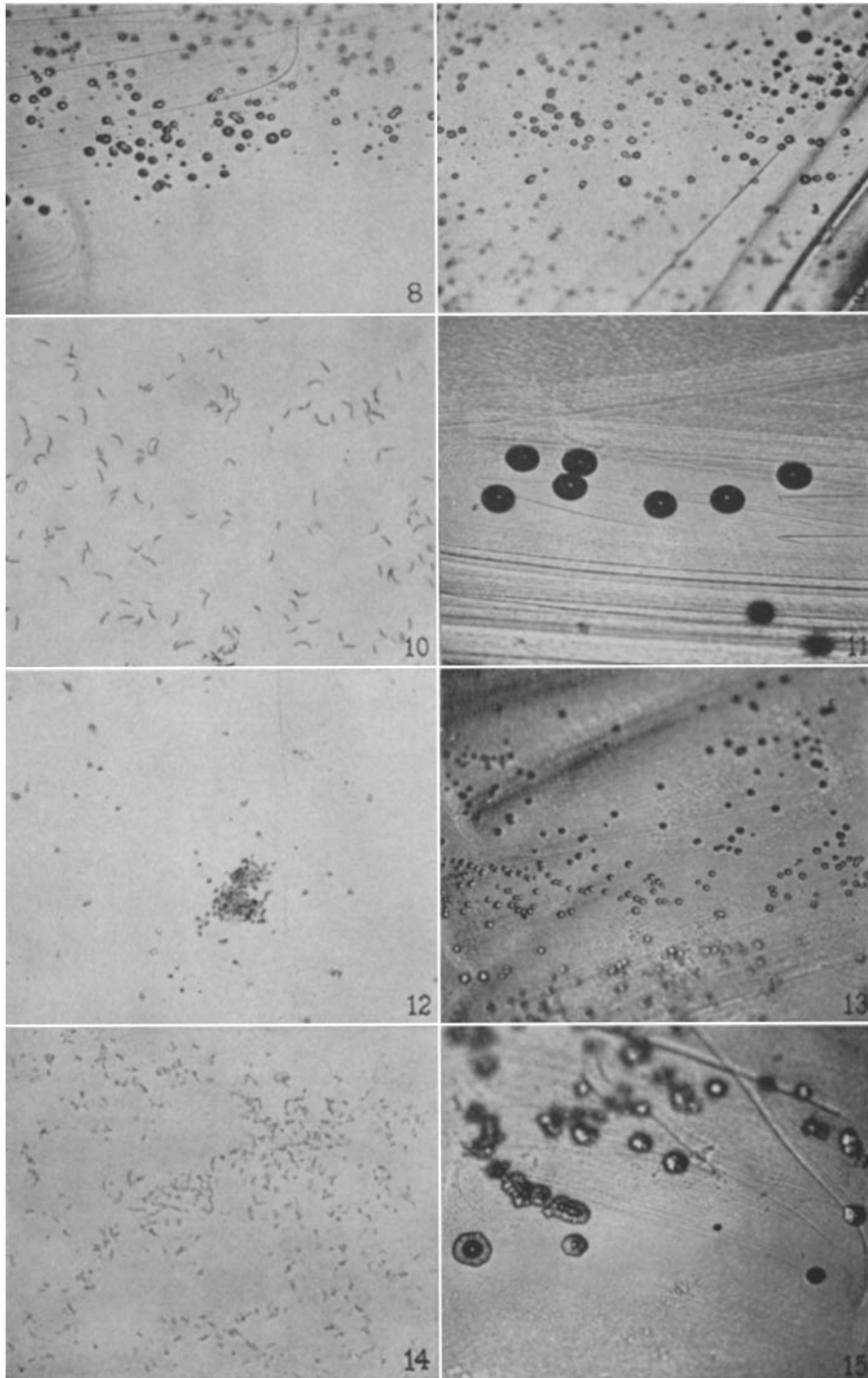
(Olitsky and Gates: Nasopharyngeal secretions from influenza. IX.)

(Olitsky and Gates: Nasopharyngeal secretions from influenza.   IX.)

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