EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

IV. Anaerobic Cultivation.

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Plates 95 to 99.

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In a series of reports in this Journal\textsuperscript{1–4} and in \textit{The Journal of the American Medical Association}\textsuperscript{5,6} we have described the changes in the blood and lungs of rabbits and guinea pigs which follow the intratracheal injection of unfiltered and filtered nasopharyngeal secretions, obtained within 36 hours after onset, from patients ill with uncomplicated epidemic influenza. The activity of the injected material was traced to the presence of a substance possessing characters which could only be attributed to a living agent, not, however, of the nature of ordinary bacteria. In the earlier reports\textsuperscript{1–4} we referred to experiments on the cultivation of this living agent by anaerobic methods, and recently\textsuperscript{5} we described the nature of the characteristic, visible bodies usually found by cultural methods to be present in the nasopharyngeal secretions during the early hours of epidemic influenza in man and in the lung tissue of affected animals. The present paper describes these cultivation experiments in greater detail.

\textsuperscript{1} Olitsky, P. K., and Gates, F. L., \textit{J. Exp. Med.}, 1921, xxxiii, 125.
Sources of Material.

The characteristic bodies were 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. Since that date successful cultivation experiments have been carried out with material ultimately derived from all of the seven cases of influenza from which active material was transmitted to animals. Numerous primary cultures, directly from human sources or from the lung tissues of affected animals, have been followed by hundreds of subplants, until at present certain strains are extant in the eighteenth generation.

The materials subjected to cultivation, essentially the same as those injected in the transmission experiments, were obtained from a variety of sources. During the two epidemic waves of influenza, that of 1918–19 and that of 1920, nasopharyngeal secretions were collected from patients with the uncomplicated disease, both in the early hours of the affection and in the later stages, including convalescence. During the first epidemic filtered and unfiltered material was also obtained from the lungs, at autopsy, of patients who had succumbed to secondary or concurrent pneumonias. In the course of the various series of transmission experiments with rabbits and guinea pigs, the lungs of affected animals were cultured both aerobically and anaerobically as a routine. Usually this material was not filtered. Sometimes the tissues were ground in saline solution and filtered in order to remove bacteria of the ordinary species. As a routine also, portions of the lungs of affected animals were preserved in sterile 50 per cent glycerol. These glycerolated specimens, after preservation for periods up to 14 months, were directly cultured or injected intratracheally into rabbits, from whose lungs cultures were made at autopsy.

The control materials for cultivation experiments were likewise similar to those used in control transmission experiments in animals. They consisted of unfiltered and filtered nasopharyngeal secretions from healthy persons during the two epidemic waves, the interepidemic recession, and the postepidemic period. Nasopharyngeal washings from persons suffering from acute coryza were also cultured
during the non-epidemic intervals. The normal lungs of uninoculated rabbits, the diseased lungs of stock rabbits who fell ill of other infections, the lungs of rabbits injected with control materials, and, finally, each of the various ingredients of the culture medium were subjected to anaerobic cultivation as controls.

Methods of Cultivation.

All the methods of anaerobic cultivation employed involved the use of fresh sterile kidney tissue in tubes or flasks of human ascitic fluid or ascitic fluid agar, usually under a vaseline seal.

In the early cultivation tests sterile paraffin oil was used as a seal instead of vaseline, and the tubes were incubated in an anaerobic jar set up as described by McIntosh and Fildes. The decolorization of a tube of methylene blue in broth, included in the jar, indicated the establishment of anaerobic conditions. In later experiments the complete exclusion of oxygen by a vaseline seal permitted the rapid production and the maintenance of anaerobic conditions by the reducing substances in the medium. The use of the anaerobic jar was therefore, discontinued.

For primary isolations and routine cultivations the culture tubes were set up as follows: Relatively large pieces (0.6 to 0.8 gm.) of sterile normal rabbit kidney tissue were placed in test-tubes measuring 20 by 1.5 cm., one piece to each tube. Then 1 cc. of suspected fluid or 0.5 cm. of affected lung tissue was placed directly on the kidney tissue. These materials were covered with 8 to 10 cc. of sterile human ascitic fluid of a hydrogen ion concentration of 7.8 to 8. Ascitic fluids of a higher alkalinity were discarded. Sealed with 2 cc. of melted sterile vaseline and stoppered with cotton, the tubes were incubated at 37°C. for 8 to 12 days.

For special purposes this routine culture method was modified in two particulars. Occasionally tubes of a semisolid medium or mass cultures in flasks were employed, especially for subplants. The semisolid medium consisted of 1 part of beef infusion (2 per cent) agar, pH 7.4, to 2 parts of human ascitic fluid, mixed in a flask at 40°C.

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and pipetted onto the kidney tissue and inoculum in the tall test-tubes. The medium for mass cultures was slightly modified from that described by Flexner, Noguchi, and Amoss\(^8\) for the cultivation of the globoid bodies of poliomyelitis. In each Florence flask, of 50 cc. capacity, was placed one-fourth of a moderate sized rabbit kidney. The sections were cut across the entire kidney and placed with the cut surface parallel to the base of the flask. After inoculation the kidney was just covered (10 cc.) with the semisolid ascitic fluid medium described above and the agar was hardened by immersion of the flask for 15 minutes in cold water. Then the flask was filled to the neck with a mixture of 1 part of beef infusion broth, pH 7.4, and 2 parts of ascitic fluid. A seal of vaseline, 1 cm. deep, and a cotton stopper completed the preparation.

An advantage of the vaseline seal over the anaerobic jar was found in the ease with which specimens for examination or transplant could be obtained with a capillary pipette without exposure of the medium to the air. After puncture the seal was restored by gentle heat—just sufficient to melt a portion of the superposed vaseline.

All the earlier inoculations were made directly on the kidney tissue before the medium was added. More recently the incubation period has been shortened and growth facilitated by inoculating sterile preparations set up a day or two in advance, to permit the establishment of anaerobic conditions under the vaseline seal. The inoculum is placed in the vicinity of the kidney by means of a capillary pipette.

In primary cultivations and early subplants the suspected material was distributed among three to six culture tubes. Frequently only a few tubes of several in an initial cultivation showed growth. But apparent failure in the initial cultivation might be followed by success in a subplant. Hence at least two negative cultivations in succession were required before a culture series was discarded as negative.

\textit{Appearance of Positive Cultures.}

The first evidence of the multiplication of a living substance in primary cultures in fluid medium was observed in the presence of a faint haze, first visible about the 5th day, extending upward about

1 cm. from the level of the kidney. This faint even cloud gradually became denser, reaching its maximum about the 8th day, when it approximated 3 cm. in depth. At the same time the clear supernatant fluid developed a characteristic very faint opalescence, often hardly discernible. On standing at room temperature the cloud gradually settled down, in the course of 2 weeks, to the region of the kidney tissue, leaving a clear supernatant fluid.

Initial cultures in a semisolid medium usually failed to show signs of growth. Subplants from the fluid medium developed slowly in semisolid cultures with the formation of almost microscopic colonies, too small to be defined with exactness. The evidence of growth was an even clouding of the medium extending to 3 to 2 cm. from the surface.

In mass cultures of well established strains a turbidity appearing throughout the semisolid layer about the 3rd day was followed by a diffuse clouding of the fluid portion by the 5th day. On standing at room temperature the cloud gradually subsided to the vicinity of the agar, forming a dense homogeneous nebulous layer on its surface (Fig. 1).

**Morphology and Staining Reactions.**

The greatest concentration of cultivable bodies for microscopic examination was obtained from the very bottom of the culture tubes. 0.3 to 0.4 cc. of the cloudy sediment was drawn up in a capillary pipette. After removing the adherent vaseline from the pipette with gauze the first two drops of fluid were discarded and subsequent drops were spread evenly on a slide in thick films. The films were then dried in an incubator at 37°C., fixed with gentle heat—three rapid passages through the Bunsen flame—and stained by the chosen method.

Of all the stains tried, well ripened Loeffler’s alkaline methylene blue proved the most satisfactory. The preparation was flooded with the dye and steamed very gently over a flame for 2 minutes. The slide was thoroughly washed with running tap water and allowed to dry in the air.

Prepared in this manner, the films from typical cultures revealed minute bodies of regular morphology, stained a deep purple, and
clearly differentiated from the background of pale blue protein precipitate. The bodies were often exceedingly numerous, and with careful focusing, they stood out in sharp relief. Usually solitary, they were often found in diplo forms, and occasionally in short chains of three or four members. Clumps occurred also, especially in older cultures, the discrete definitely stained bodies forming masses from a blood platelet to a leucocyte in size. Viewed with the highest powers of the microscope, the bodies were seen to be two to three times longer in one direction than in the other. They were, therefore, bacilloid rather than coccoid. Thus they were differentiated sharply from the globoid bodies of poliomyelitis, which they approached in size. Their long axis measured 0.15 to 0.3 microns. Occasionally longer individuals were seen, but the organisms showed little tendency to pleomorphism and were characterized by uniformity in size and shape. Irregular staining reactions have not been encountered. No granules, clubs, spores, or involution forms have been seen. The bodies in one culture in its eighteenth generation are morphologically identical with those in the initial specimen (Figs. 2 to 5).

Occasionally unmistakable clumps or colonies developed in the region of the kidney (Fig. 5), but when few in number the bodies were sometimes obscured by protein or stain precipitates (Fig. 4). Under such circumstances, the unaccustomed eye made them out with difficulty. For this reason stains which showed any tendency to precipitation on the slide were found unsuitable for their demonstration. Thus, although the bodies were died lavender with Giemsa's, Wright's, and Manson's stains, the preparations were as a rule unsatisfactory. Carbolthionine (Nicolle), carbolfuchsin, steaming safranine, and steaming fuchsin were likewise unsuitable. All the strains examined decolorized uniformly by Gram's method. The counterstain, safranine, required steaming. The Gram-negative reaction was a constant feature of young and old cultures.

The dark-field microscope has not afforded a satisfactory method of examination on account of the similarity in size of the cultivable organism to the familiar dancing bodies of control preparations.
Filterability.

The cultivable bodies, even in remote generations, have been found to pass Berkefeld V and N candles. They are, therefore, to be classed among the filter passers.

Cultural and Biological Characters.

The strict anaerobic and nutritive requirements of the cultivable bodies have necessarily limited the study of their cultural reactions. Certain conclusions have been drawn from repeated experiments.

Final Hydrogen Ion Concentration of Cultures.—The incubation of sterile kidney tissue in ascitic fluid has been shown to change the hydrogen ion concentration of anaerobic tissue cultures toward the acid side. In our experiments the final hydrogen ion concentration of growing cultures and of uninoculated controls was the same; namely, pH 7.4 to 7.8. The organisms failed to grow in media of a pH of 8 or 7, which appear to be the outside limits of the favorable range.

Action on Carbohydrates and Alcohols.—Growth took place in the presence of dextrose, maltose, lactose, saccharose, inulin, and mannitol. No observable amounts of acid or gas were produced. The media containing dextrose and maltose (1 per cent) showed a heavier cloud than usual, but this may or may not have corresponded to a more active multiplication of the organisms. No characteristic odor was detectable in any of the cultures.

Symbiosis.—In the course of the experiments certain culture tubes, inoculated with unfiltered lung tissue, yielded growths of ordinary bacteria in addition to the characteristic bodies seen in the original culture tubes and later demonstrated in pure culture with filtrates of the mixed material. It thus appears that this organism can develop in symbiosis with Bacillus pfeifferi, the pneumococcus, Streptococcus hemolyticus, Streptococcus viridans, and staphylococci. In a few experiments deliberate mixtures of these bacteria with the influenza bodies were cultivated, and the organisms were subsequently separated by plating and by filtration.
Resistance.—No growth has been obtained in subplants of cultures heated to 56°C. for ½ hour or longer. Exposure to chloroform vapor for 1 to 1½ hours apparently destroys the organism. Viable organisms have been found, however, in fluid, semisolid, and mass cultures kept at room temperature, 20–24°C. (68–76°F.) for periods up to 6 months.

In these respects the resistance of the cultivated bodies is similar to that of the active agent in glycerolated specimens of the lung tissue from affected rabbits.²

Enumeration of Positive Cultures and the Sources from Which They Were Obtained.

In the earliest experiments, before a precise technique for culturing and demonstrating the cultivable bodies was developed, it may be presumed that some active materials gave negative results. Hence the following enumeration of successful cultivations is of more significance from the positive than from the negative point of view, and cannot be regarded as an indication of the actual incidence of these cultivable bodies in epidemic influenza in man. This is particularly true in view of the fact that cultural experiments were not accepted as positive unless at least two generations of the cultivable bodies were obtained.

Cultivation experiments were attempted with the filtered nasopharyngeal washings of eleven patients with uncomplicated epidemic influenza during the first 36 hours of the illness. Of these cultivations, six gave positive results. Five strains were obtained from eight patients in the 1918–19 epidemic and one strain from three patients in the recurrence of 1920.

Material from twenty-eight other patients was cultured during the later stages of the disease—from the 48th hour to convalescence. Only one culture yielded a growth. This material was obtained 48 hours after onset from a patient who died 2 days later from a secondary pneumonia.

The filtered nasopharyngeal washings of four patients suffering from pneumonias secondary to epidemic influenza were apparently negative. No growth was obtained with filtrates of the lung
tissue, at autopsy, of two patients who succumbed to the secondary pneumonias.

Although the cultivation of these peculiar anaerobic bodies from the lung tissues of a large number of affected rabbits and guinea pigs in the transmission experiments already described\textsuperscript{1, 2, 3} is presumptive evidence that this organism was probably the causative agent in the lesions produced, it is nevertheless important to correlate the presence of these organisms in the human nasal washings with the pathogenicity of these washings for rabbits.

The transmission experiments were initiated with the nasopharyngeal washings of seven patients, all in the first 36 hours of illness. Three of these specimens, filtered and cultivated, yielded strains of the characteristic organism. The specimens from three other patients failed to produce a growth. The seventh specimen was not cultivated. An eighth specimen, obtained early, appeared to be negative both in cultivation and animal transmission experiments. Three strains of the cultivable bodies were obtained from specimens of nasopharyngeal washings which were not used for transmission experiments in rabbits.

Strains were ultimately derived, however, from all seven of the patients enumerated above by cultivation of the lung tissues of rabbits and guinea pigs affected in animal transmissions of the active nasopharyngeal material.

Beside the unfiltered and the filtered nasopharyngeal washings and the fresh lung tissues of affected rabbits and guinea pigs, a third source of active material, pathogenic for rabbits, was rabbit lung tissue which had been preserved in sterile 50 per cent glycerol for periods up to 9 months.\textsuperscript{3}

None of the specimens of glycerolated lung which were directly cultivated yielded growths of the specific organism. We have already reported\textsuperscript{4} the activity of certain specimens of the glycerolated material in initiating characteristic lesions when injected intratracheally in rabbits. From the fresh lung tissues of these affected animals, or their successors in the line of animal passage, the anaerobic bodies were cultivated in a number of instances. In this way the primary cultivation of rabbits' lungs from eleven series of experiments in which the active material had previously been immersed in glycerol...
for 5 days to 9 months, yielded three cultures of these bodies; in one instance the length of glycerolation was 5 days, in another 1 month, and in the third 9 months. The two original sources of active material used in the above eleven series were Case 16, representing the first epidemic period, and Case 26, representing the second.

Control Cultivation Experiments.

Control cultivation experiments were made directly with the unfiltered or filtered nasopharyngeal secretions of twenty patients free from influenza. Eight of these patients were suffering from an acute coryza in the early or late stages. The control materials were collected in the epidemic, interepidemic, and postepidemic periods. None of these specimens yielded the cultivable bodies found in six of the eleven early cases of influenza examined.

Control cultures of the following materials also uniformly failed to yield growth of these bodies: the lung tissues of six stock rabbits which died of accidental or epidemic infections such as snuffles or pneumonia; uninoculated tubes of 36 samples of human ascitic fluid and portions of all the rabbit kidneys used in the culture media; and the lung tissues of 60 rabbits, either normal or injected intratracheally with control materials in the course of the transmission experiments. The control materials injected in these rabbits included normal rabbit lung tissue, saline solution, human ascitic fluid, rabbit serum, and ordinary bacteria.

Inoculation of Rabbits and Guinea Pigs with the Cultivable Bodies.

For a study of the effects of culture injections on animals, mass cultures were generally used. Because of pressure of other experi-

9 Mass cultures were prepared for inoculation as follows: The vaseline seal was removed from the Florence flask with a sterile wire and the fluid part of the culture centrifuged for 20 minutes at 1,500 revolutions per minute or until the supernatant fluid was clear. The fluid was then decanted, and the small button-like deposit of the growth left at the bottom of the tube was resuspended in saline solution. The centrifugation was repeated, the clear, supernatant saline solution was removed, and the small amount of sediment was again suspended in 4 cc. of saline solution, of which 3 cc. were used for the experiment.
ments most of the animal inoculations had to be deferred so that they were finally done with well established cultures several generations removed from the original human source.

When this part of the work was undertaken there were available for study three separate strains of cultivable bodies, two from the first epidemic and one from the second. These three strains were represented by cultures derived from nine different sources. One, a human strain, was the seventeenth generation subplant of a culture of the filtered nasopharyngeal secretion of Case 171 of the first epidemic. The others were first to seventh generation cultures of the lungs of different rabbits which had been injected with glycerolated lung tissue from earlier animals in the transmission series. Four of these cultures were thus originally derived from Case 16 and three were from Case 17 of the first epidemic. One came originally from Case 26 of the second epidemic of 1920.

Of these nine cultures with which rabbit passages were again initiated, only one, derived from rabbit lung tissue, and then in the third generation, failed to produce the effects regarded as typical for the active material in the earlier transmission experiments. The results of the intratracheal injection of the growth of mass cultures, in doses of 3 cc. were so uniform and familiar that a common description will suffice for the entire series.

On examination 24 hours after inoculation, the rabbits showed a rise in temperature and usually a conjunctivitis, varying from simple injection of the palpebral conjunctiva to a marked injection of the palpebral and ocular conjunctivæ. These signs were accompanied by a definite and often marked leucopenia, the result of a depression of the mononuclear cells (Text-figs. 1 and 2). In the animals which were kept for observation, these conditions persisted for 2 to 3 days, when the animals returned to normal. When the rabbits were killed during the reaction, a characteristic pathological picture was revealed in the respiratory organs.

Only the respiratory organs were visibly affected. The lungs (Figs. 6 and 7) were voluminous with edema and emphysema. Numerous hemorrhages were to be seen on the surface, either diffuse or discrete, and often in the form of minute petechiae. The pleuræ were not
involved. On section of the lungs, the cut surface dripped a frothy blood-stained fluid, evidence of a hemorrhagic edema. Hemorrhages similar to those which had reached the surface were scattered through the parenchyma. The trachea and bronchi showed a mucopurulent exudate covering an exfoliated and hemorrhagic epithelium.

Microscopic sections (Figs. 8 and 9) confirmed the gross observations. The hemorrhages, diffuse or discrete, were located in the interalveolar tissue, which was distended with edema and torn by emphysema. The interalveolar structures were also infiltrated to some degree with a cellular exudate consisting of mononuclear cells and some polymorphonuclear cells with large eosinophilic granules. Large cells of the respiratory epithelial type, probably desquamated bronchial epithelium, and numerous erythrocytes were seen in the parenchyma. No pneumonic consolidation was present.

The bronchi showed thickened, hyperemic walls and their lumina were partly filled with erythrocytes, leucocytes, and fragments of exfoliated and necrotic epithelium. The lung capillaries were distended with blood.

As noted above, eight series of animal transmission experiments were initiated by the intratracheal injection in rabbits of pure cultures of the anaerobic bodies. From the lung tissues of rabbits in seven of these series the anaerobic bodies were recovered in pure culture.

Both the strain obtained directly from the filtered nasal washings of Case 17, then in its seventeenth generation, and the strains derived from Cases 16, 17, and 26 after rabbit passage and glycerolation, produced the typical effects described above. The following protocols illustrate the similarity of the clinical effects produced by the human and the rabbit strains.

Protocol 1.—Preliminary observations on a rabbit for 2 days prior to inoculation gave the following results: temperature 39° and 38.9°C.; total leucocytes 15,200 and 16,000; mononuclear cells 5,168 and 7,360. Oct. 18, 1920. Inoculated intratracheally with 3 cc. of the growth of a second generation mass culture from the fifth rabbit passage of material from Case 16. Oct. 19. Temperature 39.6°C.; total leucocytes 8,000, of which 2,640 were mononuclears. Oct. 20. Temperature 39.6°C.; total leucocytes 10,400, of which 2,808 were mononuclears (Text-fig. 1). Rabbit killed. The lungs showed the hemorrhagic, edematous changes regarded as typical.
Another rabbit injected with the third generation mass culture of the same strain showed a similar picture of leucopenia and mononuclear depression. The rabbit was allowed to recover. This condition lasted for 3 days (Text-fig. 2).

**Text-Fig. 1.** Effect on blood and temperature of the intratracheal injection of the cultivable bodies in a rabbit. The rise in temperature and the leucopenia due to mononuclear depression are noteworthy.

**Text-Fig. 2.** The same as Text-fig. 1 except that in this case the animal was allowed to recover. The persistence of the effects for 3 days is seen.

The next protocol is presented to show the effect of inoculation of a strain obtained by cultivating the nasopharyngeal secretions from a human case.
Protocol 2.—Jan. 6, 1921. A rabbit with normal temperature, 39°C., total leucocytes 10,400, of which 4,472 were mononuclears, was injected intratracheally with 3 cc. of the growth in the seventeenth generation mass culture of a strain from the filtered nasopharyngeal washings of Case 17. 24 hours later the animal showed a mild conjunctivitis, temperature 39.5°C, total leucocytes 6,400, of which 1,152 were mononuclears. Jan. 8. The conjunctivitis was severe, total leucocytes 8,000, of which 2,400 were mononuclears. The lungs showed the lesions regarded as typical. The anaerobic bodies were recovered in pure culture from the lung tissues. The injection of this culture produced similar effects in a second rabbit, from the lung tissues of which the strain was again recovered.

Several series of experiments were made with guinea pigs instead of rabbits. In this species the response to the intratracheal inoculation of cultures was similar to that obtained in rabbits, and from the affected lungs of the guinea pigs, the cultivable bodies were recovered.

Among the specimens of affected lung tissue preserved in 50 per cent glycerol were several which had come from rabbits injected with cultures of the anaerobic bodies. Although subsequent direct cultivations of these preserved specimens gave negative results, the same bodies were recovered from rabbit passages of the glycerolated material containing them. We thus have evidence that the bodies themselves do withstand glycerolation. The lungs from which these bodies were recovered by animal passage were immersed in glycerol for periods up to 4 months.

**DISCUSSION.**

These experiments seem definitely to connect the cultivable bodies with the clinical effects and lesions induced in rabbits and guinea pigs by the intratracheal injections of nasopharyngeal washings from patients with uncomplicated epidemic influenza.

From the lung tissues of such affected animals the morphologically and culturally characteristic bodies have been obtained in pure culture on special media by a strictly anaerobic technique. The bodies have been cultivated in successive generations without change in character. When injected intratracheally into rabbits and guinea

10 The relation of ordinary bacteria in regard to their ability to produce concurrent or secondary infections in the presence of the cultivable bodies will form the basis of another paper.
pigs, they have given rise to pathological lesions in all respects similar to those from which they were obtained.

From the lesions the typical bodies have again been recovered in pure culture by the method employed for their primary isolation. Comparison of the strains thus derived from animal passages with those obtained directly from filtered human nasopharyngeal washings shows them to be identical in morphology and cultural characters. Finally, both the active material of the transmission experiments and the cultivable bodies obtained from similar sources withstand glycerolation and pass through Berkefeld V and N filters.

We feel, therefore, that the active material, pathogenic for rabbits and guinea pigs, found in the nasopharyngeal secretions of patients in the early hours of uncomplicated epidemic influenza has been identified in the anaerobic organism described in this paper.

It would, of course, be a simple matter to announce the inciting or etiological agent of epidemic influenza in man to be the minute, bacillloid organism here described. At present such a course does not seem desirable even though the clinical and pathological effects induced in the rabbit simulate so closely the phenomena found in epidemic influenza in man. Apparently we are at the threshold of our knowledge of a group or class of minute microorganisms which the anaerobic Smith-Noguchi technique has thrown open to exploitation. It seems wiser, therefore, to defer decision of the precise relation which the species described in this and previous communications bears to epidemic influenza until further experience is obtained.

In the meantime it is desirable to give the microorganism a name, and since a striking feature of its effect in rabbits is to diminish the resistance of the lungs to the action of ordinary pathogenic bacteria, as will be shown in a forthcoming paper, the name of *Bacterium pneumosintes* is proposed (from πνεύμα, lung, + στέρνω, injurer, or devastator).

**SUMMARY AND CONCLUSIONS.**

From the filtered nasopharyngeal washings of patients in the first 36 hours of uncomplicated epidemic influenza and rarely in later stages of the disease, we have cultivated a minute bacillloid body,
Bacterium pneumosintes, 0.15 to 0.3 microns in length, of constant cultural characters and capable of indefinite propagation on artificial media. This organism, not of the nature of ordinary bacteria, was also recovered in pure culture from the unfiltered and filtered lung tissue of rabbits and guinea pigs inoculated with unfiltered and filtered nasopharyngeal washings of early influenza cases, both from the first epidemic of 1918–19 and from the second one of 1920. The organism grows only under strictly anaerobic conditions, passes Berkefeld V and N filters, and withstands the action of sterile 50 per cent glycerol for a period of months.

It has been recovered from cultures contaminated with a variety of ordinary bacteria such as Bacillus pfeifferi, pneumococci, streptococci, and staphylococci, and has been experimentally cultivated in symbiosis with them.

Similar cultivation of control materials uniformly failed to yield growths of this organism. The materials tested consisted of the unfiltered and filtered nasopharyngeal washings of persons free from influenza, some of whom were suffering from acute coryza, the lung tissue of normal rabbits and of rabbits with bacterial respiratory infections, and the uninoculated media.

The intratracheal injection in rabbits and guinea pigs of mass cultures of this organism has induced effects on the blood and lungs of these animals which are not to be distinguished from those obtained with the nasopharyngeal secretions of patients in the early hours of epidemic influenza. From the pulmonary lesions thus induced the same organism has been recovered in pure culture, and has been found to cause similar lesions on subsequent animal passage. Its pathogenicity is not lost by prolonged artificial cultivation.

Our experiments indicate that the cultivable bodies obtained directly from human nasopharyngeal washings and from affected rabbit lungs are strains of the same organism. This organism appears to be the source of the reactions which occur in experimental animals—rabbits and guinea pigs—as a result of the intratracheal injection of nasopharyngeal washings obtained during the early hours of uncomplicated epidemic influenza in man.
EXPLANATION OF PLATES.

PLATE 95.

Fig. 1. The construction of mass culture medium, to the left, and the growth of the cultivable bodies therein, to the right. The growth is derived from a rabbit's lungs after 8 days incubation. The turbidity of the semisolid and the haziness of the fluid layers are noteworthy. Actual size.

PLATE 96.

Fig. 2. The cultivable bodies in the eighth generation. Culture obtained from a rabbit's lung into which was injected material, after 9 months immersion in glycerol, originally derived from the filtered nasopharyngeal secretions from a case of epidemic influenza. Stained with ripened Loeffler's alkaline methylene blue. × 1,000.

Fig. 3. The bodies in Fig. 2 highly magnified. Stained similarly. × 3,000.

Fig. 4. Comparative size of the cultivable bodies. An aerobic streptococcus and a chain of poliomyelitis globoid bodies, indicated by an arrow, have been superimposed. The cultivable bodies are very minute, uniform, and lightly stained, and are to be differentiated from the small irregular masses, deeply stained, which are protein precipitates. All × 1,000.

Fig. 5. The cultivable bodies in colony formation. × 1,000.

PLATE 97.

Fig. 6. The gross lesions of the lungs of a rabbit inoculated intratracheally with the first generation of the cultivable bodies. The hemorrhages, edema, emphysema, and absence of pneumonic consolidation are noteworthy. Natural size.

Fig. 7. These lungs were obtained from a rabbit which was inoculated with the lungs pictured in Fig. 6. The more intense lesions of this second passage of the cultivable bodies are seen, especially the hemorrhagic edema of the right lung. Natural size.

PLATE 98.

Fig. 8. Microscopic section of the lung lesions in a rabbit, caused by injecting the cultivable bodies intratracheally. The edema, the emphysema, the hemorrhages, and the cellular exudate are noteworthy. × 240.

PLATE 99.

Fig. 9. Another field of the same section shown in Fig. 8, demonstrating particularly the bronchial lesions. The necrotic and exfoliated bronchial epithelium and the edema of its walls are to be seen. The general edema and the vessel filled with blood may be observed. × 240.
Fig. 1.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)
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Fig. 6.

Fig. 7.

(Olitsky and Gates: Nasopharyngeal secretions from influenza. IV.)
Fig. 8.

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