

# EXPERIMENTAL STUDIES OF THE NASOPHARYNGEAL SECRETIONS FROM INFLUENZA PATIENTS.

## II. FILTERABILITY AND RESISTANCE TO GLYCEROL.

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PLATES 32 TO 34.

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In our first communication<sup>1</sup> we described the particular effects induced in rabbits by the nasopharyngeal secretions from cases of uncomplicated influenza. We now propose to define more exactly the nature of the peculiar or active substance responsible for these effects and to distinguish it from bacteria of the ordinary species.

Before presenting what are regarded as the decisive experiments, all of which were made in rabbits and guinea pigs, we desire to put briefly on record series of tests carried out on monkeys, *Macacus rhesus*, chiefly, in which the nasopharyngeal secretions from cases of uncomplicated influenza, collected from 12 to 48 hours after the onset of the symptoms, were filtered through Berkefeld V or N candles and injected intratracheally or subconjunctivally, or by both routes, into these animals.<sup>2</sup> In some instances the material was injected as it came from the filter. Occasionally it was concentrated at low temperature *in vacuo* according to the method of Amoss and Taylor.<sup>3</sup>

All these experiments resulted negatively in that no effects were observed which were not also obtained from similarly treated secretions from persons believed not to have suffered from influenza. Hence the work of Nicolle and Lebailly,<sup>4</sup> Gibson, Bowman, and

<sup>1</sup> Olitsky, P. K., and Gates, F. L., *J. Exp. Med.*, 1921, xxxiii, 125.

<sup>2</sup> All operations were performed under light ether anesthesia.

<sup>3</sup> Amoss, H. L., and Taylor, E., *J. Exp. Med.*, 1917, xxv, 507.

<sup>4</sup> Nicolle, C., and Lebailly, C., *Compt. rend. Acad.*, 1918, clxvii, 607.

Connor,<sup>5</sup> and Bradford, Bashford, and Wilson<sup>6</sup> was not confirmed by these experiments. But since in many of the monkeys employed any effect produced by the intratracheal injections might have been masked by the presence of lesions of pulmonary tuberculosis, these experiments are not regarded as conclusive. In the meantime the work with rabbits was proceeding in a promising manner and hence the latter animal was chosen for the new series of experiments.

These experiments extended our observations as follows: (1) A condition similar to that found in rabbits injected with the unfiltered nasopharyngeal secretions was obtained by employing filtrates of the lung tissue of such affected animals. (2) Filtrates of the nasopharyngeal washings from early cases of epidemic influenza also induced similar effects in rabbits. (3) When guinea pigs were used instead of rabbits, they showed clinical and pathological effects indistinguishable from those already observed. (4) The peculiar substance inducing these effects, when submitted to the action of 50 per cent glycerol, maintained its activity without alteration in its effects.

#### *Filtered Lung Tissue from Affected Rabbits.*

It was thought advisable to employ at first, for filtration experiments, material possibly more active than the patient's nasopharyngeal secretions. The active substance was therefore carried through several rabbit passages without filtration in the following manner. The nasopharyngeal washings from Case 17, described in the first communication,<sup>1</sup> had been collected 12 hours after the onset of uncomplicated influenza and injected intratracheally in rabbits. As judged by the occurrence of typical effects on the blood and on the lungs, the active material was then transmitted through two successive rabbits by means of the unfiltered lung tissue of each previous animal. The lung tissue of the last rabbit, obtained at the height of the reaction, was ground with sand in sterile saline solution and filtered.

<sup>5</sup> Gibson, H. G., Bowman, F. B., and Connor, J. I., *Brit. Med. J.*, 1919, i, 331.

<sup>6</sup> Bradford, J. R., Bashford, E. F., and Wilson, J. A., *Brit. Med. J.*, 1919, i, 127.

*Protocol 1.*—The lung tissue of the rabbit corresponding to the third passage of this series was ground with sterile sand in saline solution<sup>1</sup> and the suspension centrifuged at low speed. The supernatant fluid was removed and filtered through a tested Berkefeld candle, size N. 0.5 cc. of the filtrate gave no growth on blood agar plates.

Apr. 17, 1919. 3.5 cc. of the filtrate were introduced intratracheally in a rabbit<sup>7</sup> whose total leucocytes were 17,500, of which 9,625 were mononuclears. Apr. 18. Total leucocytes 10,360, of which 3,936 were mononuclears. The animal had lost 100 gm. in weight; the temperature was unchanged (39.1°C.); conjunctivitis appeared. Apr. 19. Leucocyte count unchanged; temperature 40.1°C. Killed. All the organs except the lungs were normal in appearance. The lungs were voluminous, edematous, and emphysematous, and in the lower lobes diffuse hemorrhages were noted, in the upper lobes small discrete hemorrhages, especially underneath the pleura, which was apparently unaffected. On section, a blood-stained frothy fluid escaped and the hemorrhages were observed to occupy the depth of the tissues. The trachea, especially in the lower third, showed congestion and small hemorrhages and was covered with mucus.

The microscopic examination (Figs. 1 and 2) confirmed the gross appearance. There were generalized extravasations of erythrocytes into the interalveolar structures and intraalveolar spaces, localized small hemorrhages, extensive edema, and emphysema. In addition, a small amount of cellular exudate was found in the parenchyma, consisting mainly of polymorphonuclear acidophilic and of mononuclear cells, among which were larger cells of the respiratory epithelial type. The bronchi showed exfoliated epithelium, some cells of which were necrotic, and contained a mixture of red cells, coagulated serum, leucocytes, and fibrin in small amount. No growth was obtained on aerobic cultivation of the lung tissue.<sup>8</sup>

This experiment, which is typical of many, shows that the lesions of the lungs and of the circulating blood described in the previous paper as arising in the rabbit from the injection of unfiltered nasopharyngeal secretions from early cases of influenza can be produced by the injection of the filtered extract of the lungs of affected rabbits. The next step was the passage from rabbit to rabbit of the active material contained in the lung tissue by means of successively filtered materials.

*Protocol 2.*—A suspension of the lung of the previous rabbit was filtered through a Berkefeld candle, size V. The filtrate was proved sterile by aerobic cultivation tests and was introduced intratracheally into another series of rabbits. While

<sup>7</sup> Several rabbits were inoculated, but only the rabbit used for further transmission experiments is described in this paper.

<sup>8</sup> The results of anaerobic cultivation will be reported in a later paper.

tests were made with the filtrate an alternate test with unfiltered lung suspension was carried out.

This consecutive series consisted of ten rabbits, which developed in 24 to 48 hours temperature reaction, conjunctivitis, and leucopenia, involving especially the mononuclear cells. At autopsy the animals exhibited the pathological condition of the lungs described as typical of the action of the influenzal material (Fig. 3). There was no essential difference noted in the action of the filtered as compared with the unfiltered suspensions. Aerobic cultures of the lung tissue in blood and plain dextrose broth were systematically made. As a rule no growth was obtained. But in three instances common bacterial species were found. Thus the fifth passage, in which an unfiltered suspension was used, yielded an indefinite pneumococcus; the seventh and eighth passages, in which filtered suspensions were employed, *B. pyocyaneus*;<sup>9</sup> and the tenth passage made with an unfiltered suspension, an unclassified, small, Gram-negative bacillus of hemoglobinophilic nature. The variety of bacteria appearing in these cultures indicates that they were of accidental occurrence.<sup>10</sup>

The series of experiments suggests that the particular clinical reactions and pathological effects induced in rabbits by the nasopharyngeal washings from early cases of epidemic influenza are due to an active substance which passes through Berkefeld filters and survives successive animal passages of the filtrate.

The preceding experiments were made with material obtained in 1919 and passed through rabbits before filtration. The recurrence of the epidemic of influenza in 1920 afforded another opportunity for the study of material from early cases of the disease in the manner indicated, and for the direct injection into the rabbit of the filtered nasopharyngeal secretions of man.

#### *Filtered Nasopharyngeal Secretions.*

Nasopharyngeal washings, obtained from two patients (Nos. 24 and 26, described in the first paper<sup>1</sup>) who had been ill 36 and 30 hours, were shaken in saline solution and filtered through Berkefeld N candles. The filtrates tested by aerobic culture were sterile. They were inoculated intratracheally into rabbits as follows:

<sup>9</sup> Subsequent tests proved that the Berkefeld candle used in these two passages was contaminated with this organism.

<sup>10</sup> The part played by these aerobic microorganisms will be described in another communication.

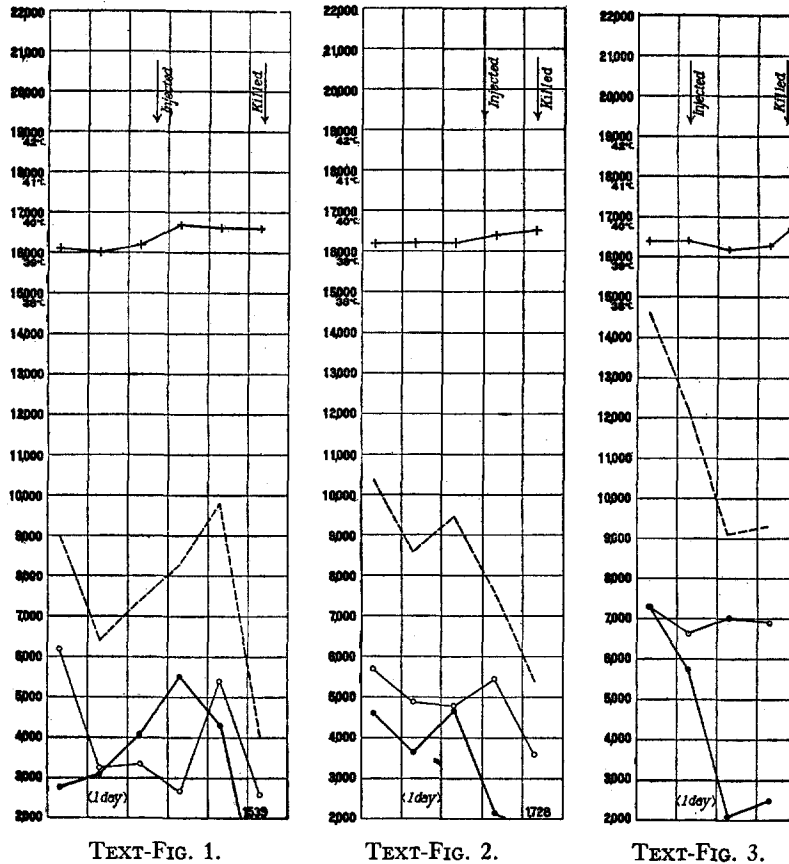
*Protocol 3.*—Jan. 21, 1920. 4 cc. of the filtered nasopharyngeal washings from Patient 24 were introduced intratracheally in a rabbit whose total leucocytes were 10,400, of which 6,760 were mononuclears. Temperature, before injection, 39.2°C. On Jan. 23, after 48 hours, conjunctivitis and a temperature rise to 39.4°C. were noted. The total leucocytes fell to 6,800, of which 4,080 were mononuclears. Jan. 24. Temperature 39.6°C.; total leucocytes 8,200, of which 2,970 were mononuclears. Killed. All the organs except the lungs were normal in appearance. The right lung was more extensively involved than the left. They were both voluminous as a result of edema and emphysema. Small discrete hemorrhages were seen only in the right lung, especially under the pleura. On section the hemorrhages were noted to occupy the depths of the tissues. The bronchi contained mucopurulent material. The microscopic appearance confirmed the gross condition. There were localized small hemorrhages, edema, and emphysema. The capillaries were filled with blood. The small amount of cellular exudate in the parenchyma consisted of polymorphonuclear cells, of respiratory epithelial cells, and, especially in the interalveolar strands, of mononuclear cells. The bronchi showed necrosis and exfoliation of the lining cells and contained coagulated serum, leucocytes, and fibrin. No growth was obtained on aerobic cultivation.

Jan. 24. A second rabbit was injected intratracheally with 3 cc. of the unfiltered lung tissue suspension from the preceding rabbit. Jan. 25. Total leucocytes fell from 11,400 to 9,800, and mononuclears from 5,358 to 4,116. There was conjunctivitis but no temperature rise. Jan. 26. Total leucocytes 9,400, of which 2,256 were mononuclears. Killed. The lesions present in the lungs at autopsy resembled those of the previous rabbit and were regarded as typical. Aerobic cultures showed no growth.

*Protocol 4.*—The typical clinical reaction and pathological effects were induced in rabbits by means of the unfiltered nasopharyngeal secretions from Patient 26. These were described in detail in the first paper.<sup>1</sup>

Feb. 5, 1920. 3.5 cc. of the filtered nasopharyngeal secretions from Patient 26, collected 30 hours after the onset of symptoms, were injected intratracheally in a rabbit whose average total leucocytic count was 7,600, of which 3,331 were mononuclears. On Feb. 8, after 72 hours, total leucocytes fell to 4,050, and mononuclears to 1,539 (Text-fig. 1). Killed. The lungs showed the lesions regarded as typical. No growth was obtained in aerobic cultures.

The next passage was effected by employing the lungs of a second filtrate-injected rabbit which reacted typically, but was killed after 48 hours instead of 72. Feb. 7. Rabbit injected with 3 cc. of the unfiltered suspension of these lungs. Feb. 8. Conjunctivitis appeared and temperature rose from 39.2° to 39.4°C. Total leucocytes fell from 9,475 to 7,600, and mononuclears from 4,643 to 2,128. Feb. 9. Temperature 39.5°C.; total leucocytes 5,400, of which 1,728 were mononuclears (Text-fig. 2). Killed. Lungs showed the lesions regarded as typical. No growth obtained in aerobic cultures.



—+— Temperature  
 — Total leucocytic count  
 — Mononuclears  
 — Polymorphonuclears

TEXT-FIG. 1. Effect on the blood count and temperature of the intratracheal inoculation of the nasopharyngeal secretions from Patient 26. First rabbit passage. The rise in the temperature, the leucopenia, and the mononuclear depression 72 hours after inoculation are shown.

TEXT-FIG. 2. Second rabbit passage of material from Patient 26. The rise in temperature, the leucopenia, and the mononuclear depression 24 hours after inoculation are shown.

TEXT-FIG. 3. First rabbit passage of glycerolated material from Patient 6. The temperature is not correspondingly raised as shown in the other text-figures.

The lung tissue of the preceding rabbit of this series of transmissions was stored in 50 per cent sterile glycerol and after 4 months was reinjected into rabbits. The characteristic clinical reaction and pathological effects were then observed in five successive rabbit passages. In all instances no growth was obtained in aerobic cultures. These experiments will be described later.

Protocols 3 and 4 indicate that the filtered nasopharyngeal washings of early cases of epidemic influenza induce in rabbits when injected intratracheally the peculiar changes in the lungs and the blood which have been previously described<sup>1</sup> and are regarded as peculiar and as related to that epidemic disease in man. The first effects of the direct inoculation of the filtrates were noted in 48 hours, but after the first animal passage they were observed at the end of 24 hours. However, it should be noted that while filtrates were employed in the first inoculations the unfiltered lung suspensions were employed in the transfer from rabbit to rabbit. With material from Patient 24 the passages were carried through only two animals, while with material from Patient 26 they were carried through seven successive animals. Moreover, the glycerolated rabbit lung from this series preserved its activity for at least 4 months.

#### *Experiments on Guinea Pigs.*

The next set of experiments was carried out on guinea pigs, the material employed for inoculation being the filtered extracts of the lungs of rabbits which showed the typical lesions.

*Protocol 5.*—Preliminary examination of the first guinea pig gave the following results: total leucocytes 7,900, of which 2,686 were mononuclears; temperature 39.6°C.; weight 750 gm. Apr. 15, 1919, 4.30 p.m. Injected into lungs through trachea 0.65 cc. of the Berkefeld N filtrate (sterile by aerobic cultivation tests) of the suspension of rabbit lung tissue.<sup>11</sup> Apr. 16, 9.30 a.m. Animal sick; temperature subnormal; weight 725 gm.; total leucocytes 13,300, of which 1,862 were mononuclears. Killed. Only the distended lungs showed lesions. The latter consisted of hemorrhages, edema, and emphysema. The whole surfaces and, on section, the interior were mottled with extravasated blood. The microscopic sections showed, besides the escape of blood into the alveoli and bronchi, edema, emphysema, and a certain degree of polymorphonuclear exudation. Aerobic cultures and films showed no bacteria.

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<sup>11</sup> This rabbit represented the second passage of the nasopharyngeal washings from Patient 17, an influenza case described in the previous communication.<sup>1</sup>

*Second Passage.*—The lung tissue of this guinea pig was filtered through a Berkefeld V candle, and 0.5 cc. of the filtrate, sterile by aerobic cultivation tests, was introduced intratracheally into two other guinea pigs. One of the guinea pigs after 48 hours developed a leucopenia and mononuclear depression which persisted for 3 days, and was followed by a gradual return to normal. The other animal, which showed a prompt leucopenia, was killed 24 hours after inoculation. The lung lesions resembled those of the rabbits and the guinea pig of the first passage. No bacteria were obtained on aerobic culture.

The material from the lungs of this guinea pig was suspended in saline solution and injected intratracheally into other guinea pigs as shown in Table I.

TABLE I.  
*Results of Intratracheal Inoculation of Guinea Pigs.*

Guinea pig passage.	Material inoculated intratracheally.	Effect on blood.	Remarks.	Lung lesion.
3rd	0.75 cc. of lung tissue from guinea pig of 2nd passage.	Leucopenia and mononuclear depression for 4 days.	Allowed to recover.	
3rd	0.75 cc. of lung tissue from guinea pig of 2nd passage.	Leucopenia and mononuclear depression for 2 days.	Killed.	Typical.
4th	0.75 cc. of lung tissue from guinea pig of 3rd passage.	Leucopenia, 550 mononuclears, on 3rd day after inoculation.	"	"
5th	0.75 cc. of lung tissue from guinea pig of 4th passage.	Leucopenia and mononuclear depression for 2 days.	Allowed to recover.	

The foregoing experiments with guinea pigs indicate that these animals respond to the intratracheal inoculation of materials derived from cases of acute influenza and passed through the lungs of rabbits very much as rabbits do. No tests were made in guinea pigs with nasopharyngeal secretions derived directly from man.

*Effect of Glycerol on the Active Agent.*

'Since there is a class of pathogenic microorganisms (vaccinia, virus of poliomyelitis, etc.) which is resistant to glycerol, it was decided to test the action of glycerol on the active material, or agent, in the experiments described.



As a routine practice, the lung tissue derived from rabbits was cut into cubes measuring 0.5 to 1 cm., and was placed in sterile 50 per cent glycerol, which was stored in the refrigerator (4°C.). When the glycerolated tissue was to be used for inoculation it was cultured for sterility, rendered free of glycerol by washing four times in normal saline solution, and suspended as previously indicated.<sup>1</sup>

*Protocol 6.*—Total leucocytes of rabbit 12,225, of which 5,624 were mononuclears. Feb. 18, 1919. Inoculated intratracheally with 3 cc. of suspension of 5 day glycerolated rabbit lung tissue, corresponding to the ninth passage of the nasopharyngeal secretions from Case 6, an influenza patient. Feb. 19. Conjunctivitis. Leucocytes decreased to 9,125 and mononuclears to 2,099. Feb. 20. Leucopenia and mononuclear depression continued, as shown in Text-fig. 3. Killed. Lungs showed lesions regarded as typical and no growth was obtained on aerobic culture.

Feb. 20. 3 cc. of the suspension of the lung tissue from this rabbit were injected intratracheally into another rabbit whose normal total leucocytic count was 13,375, of which 6,955 were mononuclears. Feb. 21. Total leucocytes 9,750, of which 4,582 were mononuclears. A loss of 175 gm. in weight accompanied the leucopenia. This condition endured for 1 day, the animal then returning to normal.

Feb. 26. A rabbit was inoculated with 3 cc. of the suspension of rabbit lung tissue corresponding to the second passage of the nasopharyngeal secretions from another case, Patient 11.<sup>1</sup> In 24 hours leucopenia and mononuclear depression occurred, and persisted for 3 days; the rabbit then returned to normal.

These experiments were preliminary and show that a short immersion for 5 days in glycerol does not affect the activity of the agent. Subsequently lung tissues immersed in the 50 per cent glycerol for longer periods, 8 and 18 days, 4, 7, 9, and 10½ months, were tested. Since there is so much similarity in the results obtained they are summarized in Table II.

From Table II it will be noted that the characteristic reaction could be obtained by the use of material immersed in 50 per cent glycerol for periods varying from 8 days to 9 months. In some instances this material was originally derived from filtrates of lung tissue. After recovery from the glycerolated lung tissue the active agent was transmitted through as many as ten successive animals. The lesions in the lungs of a rabbit inoculated with material exposed to glycerol are shown in Fig. 4. Affected lung tissue exposed to the glycerol for 10½ months failed in two series of experiments to yield the agent in an active state.

TABLE II.

*Results of Experiments with Glycerolated Lung Tissue.*

Series No.	Length of exposure to 50 per cent glycerol.	No. of successive rabbits* which showed effects.	Lung lesion.	Effect on blood.	Nature of material.
1	<i>days</i> 18	10	Typical in all.	Leucopenia and mononuclear depression: 1st passage after 72 hrs.; 2nd passage after 48 hrs.; 3rd passage and subsequently after 24 to 48 hrs.	Filtered.
2	<i>mos.</i> 9	3	" " "	Leucopenia and mononuclear depression: 1st passage after 48 hrs.; 2nd passage and subsequently after 24 hrs.	Unfiltered.
3	10½	0	None.	None.	"
4	10½	0	"	"	"
5	7	3	Typical in all.	Leucopenia and mononuclear depression after 24 to 48 hrs.	Filtered.
6 (Lung tissue of rabbit of 1st passage of Series 5 refiltered.)	7	5	" " "	Leucopenia and mononuclear depression within 24 hrs.	"
7	<i>days</i> 8	3	" " "	Leucopenia and mononuclear depression after 24 hrs.	Unfiltered.
8	<i>mos.</i> 4	5	" " "	Leucopenia and mononuclear depression: 1st passage after 48 hrs.; 2nd passage and subsequently after 24 hrs.	"
9	4	3	" " "	Leucopenia and mononuclear depression within 24 hrs.	Filtered.

\* The number represents, as a rule, the discontinuance of the experiment and not the cessation of the activity of the agent.

It will also be noted that the action on the circulating blood and lungs was detected usually only after 48 to 72 hours in the first passages, and, as a rule, within 24 hours in the subsequent passages.

#### SUMMARY.

An active transmissible agent present in the nasopharynx in early cases of influenza has been found to produce definite and characteristic clinical reactions and pathological effects in rabbits as already described in an earlier publication.<sup>1</sup>

The experiments here reported indicate that this active agent has the following properties.

1. The agent as it exists in the nasopharyngeal secretions in man, and in the lungs of rabbits injected with the human secretions, passes through Berkefeld V and N candles.

2. The filtered material produces the same effects on the circulating blood and on the lungs of rabbits as the unfiltered material.

3. The peculiar effects described as arising in the inoculated rabbit may also be induced in guinea pigs inoculated with the agent.

4. The agent responsible for the reaction on the blood and the lungs of rabbits withstands the action of glycerol in a sterile 50 per cent solution, for periods up to 9 months. The question must be left open at present whether the agent can withstand longer contact with the chemical. In two experiments after 10½ months contact the agent induced no observable changes in the blood and lungs of rabbits.

#### EXPLANATION OF PLATES.

##### PLATE 32.

FIG. 1. First rabbit passage of filtered material corresponding to the fourth animal passage of the nasopharyngeal secretions from Patient 17 (Protocol 1). The extensive edema, the emphysema, the cellular exudation into the parenchyma, and localized hemorrhages are noteworthy. × 230

##### PLATE 33.

FIG. 2. Section from the same rabbit as Fig. 1. This section shows particularly the number of localized small hemorrhages and the hemorrhagic extravasation into the parenchyma. × 230.

PLATE 34.

FIG. 3. The sixth passage of the nasopharyngeal secretions from Patient 17. Rabbit inoculated with filtered material (Protocol 2). The hemorrhages and the voluminous condition of the lungs resulting from edema and emphysema are noteworthy. Natural size.

FIG. 4. Rabbit inoculated with the lung tissue shown in Fig. 3 after exposure to 50 per cent glycerol for 18 days. The hemorrhages, voluminous condition of the lungs resulting from the edema and emphysema, and the absence of pneumonic consolidation are shown. Natural size.

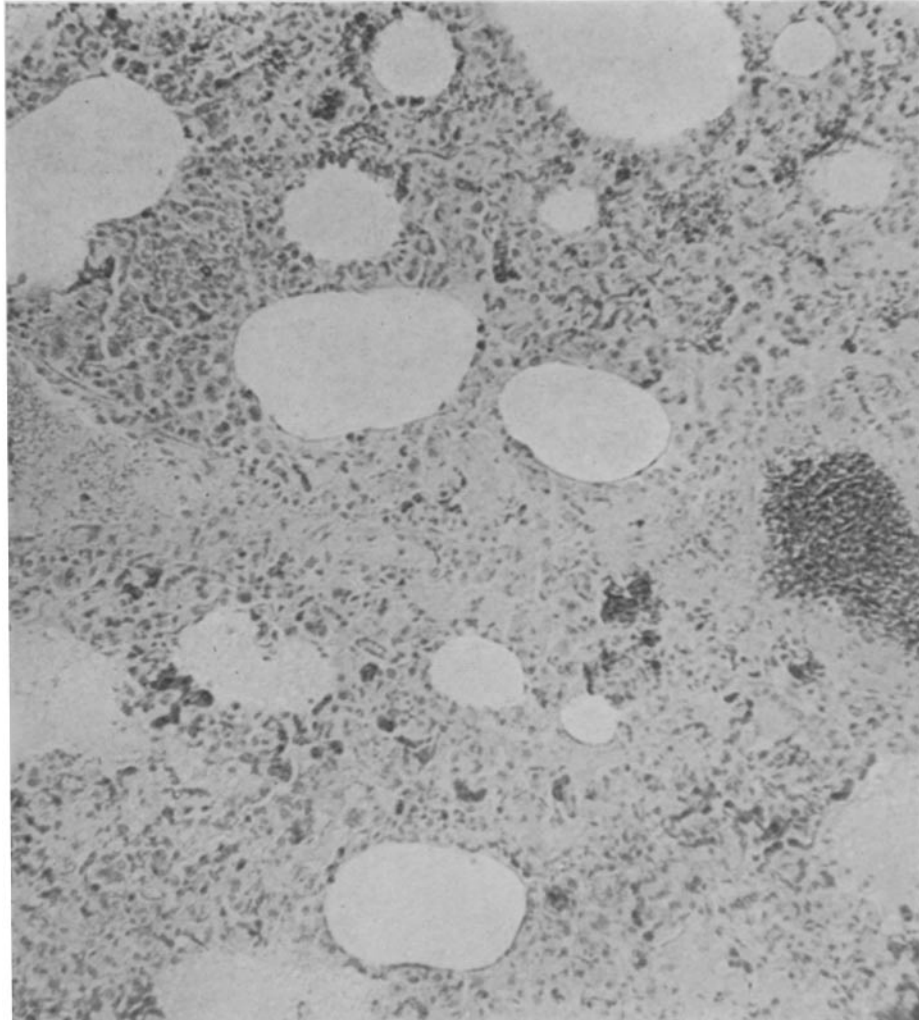


FIG. 1.

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

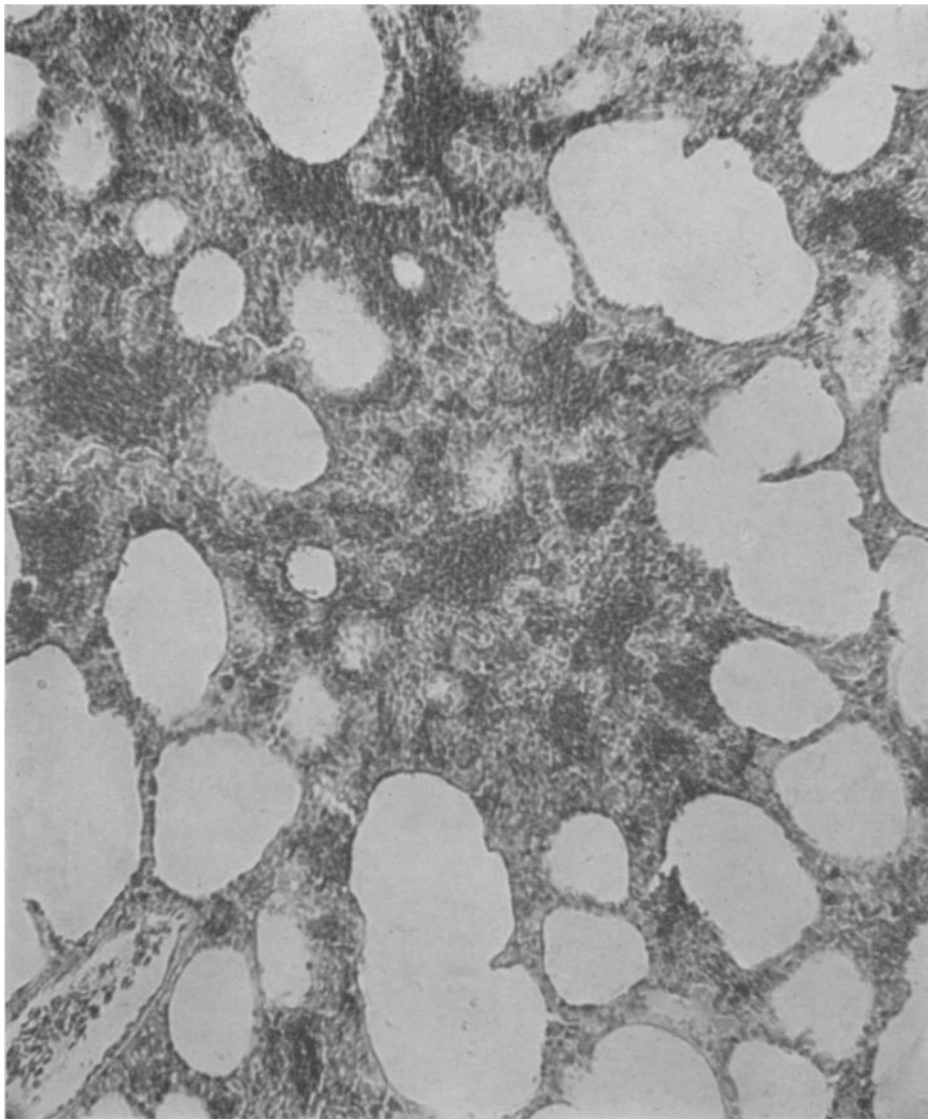


FIG. 2.

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

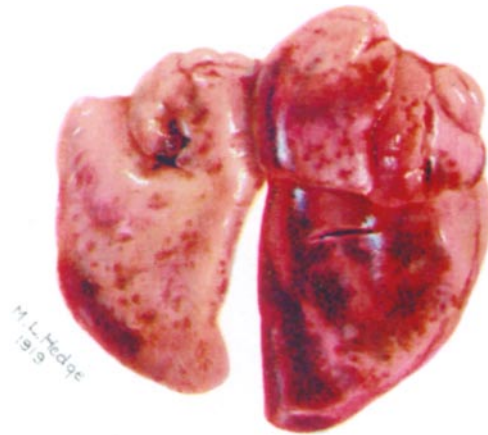


FIG. 3.



FIG. 4.

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