STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

PARTS I TO III.*

BY M. C. WINTERNITZ, M.D., AND A. D. HIRSCHFELDER, M.D.

(From the Pathological Laboratory and the Physiological Laboratory of the Medical Clinic of Johns Hopkins University, Baltimore.)

PLATES 108 TO 111.

I. THE PRODUCTION OF LOBAR PNEUMONIA.

In the course of some experiments carried out with Dr. H. M. Evans upon the action of benzol on the hematopoietic system, one of the rabbits that died showed a spontaneous pneumonia, lobar in character. The inflammatory exudate in the alveoli of the lungs presented a striking appearance: the cells of the exudate were not only the usual red and white blood cells, but also granulocytes and megakaryocytes, apparently arising from the bone marrow. This finding started us on an investigation to determine whether it was possible to produce lobar pneumonia in rabbits with any degree of uniformity.

Many of the older workers upon pneumonia (Fraenkel, Gamaleia, Weichselbaum, Salveoli, and Tschistovitch) produced occasional lobular or even lobar pneumonias by intratracheal inoculations of pneumococcus cultures in dogs, cats, and rabbits, and Monti claims to have produced typical fibrinous pneumonia of one lobe or an entire lung often complicated by pericarditis and sometimes almost without any general infection. None of these observers, with the possible exception of Tschistovitch and Monti, were able to produce the lesion with any degree of uniformity. Wadsworth, in 1904, came to the conclusion that in the rabbit "the development of acute exudative pneumonia offers an especially clear example of the nice balancing of the essential conditions determining infection," and stated that that was as yet for practical purposes beyond experimental control. Later, however, Lamar and Meltzer demonstrated, in 1910, that it is possible to produce lobar pneumonia uniformly in dogs by the intratracheal injection of large quantities of broth cultures and the pneumococcus. The low mortality (16 per cent.) of the disease in dogs and the difficulty of handling a large number of large animals simultaneously in most laboratories places ob-

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stacles in the way of many forms of experimental investigations which are obviated by the use of rabbits.

The method employed in these experiments is essentially the same as that introduced by Lamar and Meltzer for the production of lobar pneumonia in dogs. In this series rabbits were used exclusively. The animals were etherized deeply, the mouth was held open by a simple instrument, and a small, soft, rubber catheter, three millimeters in diameter, open at both ends, and stretched over a silver probe to give it rigidity, was introduced through the mouth and larynx into the trachea. The silver probe was slightly bent to correspond to the curve of the larynx and was introduced with its concavity upward. The presence of the catheter in the trachea was easily determined by palpation. The procedure entails some difficulty. Under no circumstances must the slightest force be used, as injury to the tissues readily leads to hemorrhage into the trachea which usually results in the immediate death of the animal. The catheter must be inserted well down into the thoracic portion of the trachea. The probe is then removed and four to five cubic centimeters of culture fluid are injected from a syringe, after which the syringe is detached, the piston drawn out, and an equal volume of air is in turn forced through the catheter.

The culture employed was a typical pneumococcus obtained from the heart blood of a patient who had died of acute lobar pneumonia. It had been grown by Dr. P. W. Clough for several weeks and its virulence was kept high by repeated passages through white mice and rabbits. Twenty-four hours before inoculation it was transferred from blood agar to broth of double the usual strength containing one part of pig serum to twenty parts of broth, and sterilized on three successive days with moist heat. It has been found subsequently that frequent passage through animals is not necessary to maintain the virulence of the cultures, nor does it make any appreciable difference whether twenty-four or forty-eight hour cultures were employed.

One hundred and five rabbits were used. In twenty the disease was allowed to run its course uninfluenced by other experimental procedure. All the rabbits except three died; three died in less than twenty-four hours, four in twenty-four to thirty-six hours,

four between thirty-six and forty-eight hours, four between forty-eight and seventy-two hours, and two later than seventy-two hours.

In eighty-one animals the gross lesions were as follows: Consolidation of the entire right lung occurred nine times; of the entire left lung, nineteen times; of the upper right lung, six times; of the middle right lung, eleven times; of the lower right lung, twenty times; of the upper left lung, thirteen times; of the lower left lung, sixteen times; of both lungs, six times. Marked dry pleurisy occurred in thirty-five instances; effusion in sixteen; mediastinitis in ten; pericarditis in nine (figures 1, 2, 3 *a*, and 4 *a*). There was no consolidation in one case. This animal died thirty days after inoculation, and autopsy revealed a chronic fibrous pleurisy and encapsulated pleural and pulmonic abscesses. For five days after incubation this animal had the clinical signs of pneumonia including complete chloride retention for three days. Recovery seemed to take place by pseudocrisis.

Cultures were made in twenty-four cases, and in every case where they were made both the heart blood and lung yielded pure cultures of pneumococci. Cultures made during life from the ear vein by allowing blood to run from the ear vein on to slant agar invariably showed pneumococcemia.

Clinically the animals recovered from the operative procedure almost immediately and remained well for a variable period of time. They then began to look sick, refused food, had marked dyspnea, and in some cases they supported the affected side by resting it on the fore leg or bent the body to lessen the movement. It was sometimes possible to make out tubular breathing over the consolidated area. Pain could be demonstrated occasionally by a respiratory squeak elicited through pressure on the affected side; the white blood cells showed a persistent fall from the normal (5,000 to 8,000) to a level ranging from 2,000 to 4,600, and in one case they fell to only 760 per cubic millimeter. The temperature rose from normal (38.7° to 40° C.) to between 41° and 42° C. During the disease the chlorides were present in relatively large quantities in the first few days, curds with silver nitrate plus nitric acid having been given, but in two cases they were almost entirely absent from the third to the seventh day of the disease. In one

of the animals that recovered the chloride excretion returned to normal.

In order to demonstrate that the production of lobar pneumonia was not peculiar to the strain of pneumococcus used, another culture[1] was also tested. The results were identical with those recorded above.

II. Pneumonia in Animals Rendered Aplastic.

The experiments of Selling have shown that leucocytes may be caused practically to disappear from the circulating blood, and the bone marrow cells may be destroyed by the injection or inhalation of benzol. This method offered a ready means of studying the inflammatory exudate and the processes of phagocytosis and immunity in more or less aplastic animals. While this general problem had naturally arisen in the course of Dr. Selling's work, the present series of experiments was undertaken as a direct result of the accidental finding of an instance of spontaneous pneumonia in a rabbit in which bone marrow changes had been produced by the injection of benzol, and which was being studied for another purpose by one of the writers in collaboration with Dr. Evans. The pneumonic exudate contained, besides the usual elements, megakaryocytes and granulocytes corresponding to those formed in the regenerated hyperplastic marrow of the femur of the same animal.

From the finding in this animal it seemed probable that the nature of the cellular inflammatory exudate might depend upon what cells the marrow could supply at the time the demands of the body were made upon it, and that the cellular exudate might be varied by changing the absolute and proportionate number of the marrow cells, as may be readily accomplished by the injection of benzol. The rôle of phagocytes in the resistance of the animal, as well as many other studies, such as immunization in partially and completely aplastic rabbits, likewise suggested themselves for investigation by this method.

Several series of animals were injected with benzol. 1 c.c. of benzol per kilo of body weight, mixed with equal quantities of olive oil, were injected subcutaneously daily until the desired degree of leucopenia was attained. Pneu-

[1] This culture was sent us by Dr. J. O. Hirschfelder of San Francisco.

monia was produced in animals in many stages of aplasia and regeneration. This was accomplished by allowing varying intervals to elapse between the last dose of benzol and the production of pneumonia. It was found by Selling that animals injected with benzol after the leucocytes were 800 or less per cubic millimeter invariably died. If the injections are stopped at this stage the leucocytes will usually continue to fall for a variable period and then regeneration will ensue, provided the leucocytes have not fallen below 100 per cubic millimeter, when in our series death always ensued.

Pneumonia was produced in eight aplastic animals. In two the leucocytes were falling, in two they were stationary, and in four rising. At the time of infection the counts showed 280, 320, 400, 500, 850, 880, and 3,600 per cubic millimeter, as compared with 4,400, 6,400, and 7,500 in the controls. Counts made six hours after inoculation showed no important change in the number of white blood cells, either in the normal animals or in those treated with benzol.

The resistance of these aplastic animals to pneumonia was strikingly reduced. All the animals died in from thirteen to twenty-seven hours after inoculation, the average length of survival being twenty hours, while none of the six controls inoculated on the same days with the same dose of the same broth suspension of the organism died under twenty-five hours, the average being sixty-one hours.

The description of the consolidation and gross appearance of the involved portion of the pneumonic lung was exactly the same as in the normal rabbits. Pleurisy and pericarditis were not so frequent.

The histological appearances of the exudate varied with the degree of aplasia of the marrow. In the most highly aplastic animals, where there were only a few small islands of regenerating cells in the femoral bone marrow, the pneumonic exudate was likewise poor in leucocytes. It contained the usual number of red blood cells and the usual quantities of fibrin, but only occasionally polymorphonuclear leucocytes or undifferentiated mononuclear cells (figures 3 *b*, 4 *b*, 5 *a*, and 5 *b*). On the other hand, large numbers of pneumococci filled the alveoli. They were not clumped, but were diffusely scattered and were so numerous as to produce a fine stippling throughout the sections, though in the controls very few cocci

were visible (figure 5 *b*). The few white cells present, as well as the lining cells of the alveolar epithelium, contained pneumococci.

In the animals whose marrow showed more marked regeneration the exudate contained, besides the elements above noted, mononuclear cells exactly like the mononuclear cells of the marrow. Occasional large, partly degenerated cells were seen suggesting the megakaryocyte of the marrow, but, as a rule, these were so far degenerated that they could not be identified. The cell picture of the exudate corresponded to that of the marrow irrespective of the total number of white blood cells in the circulation.

This markedly decreased resistance of the aplastic rabbits to the pneumonic infection, together with the striking overgrowth of the pneumococci in the lungs of the animals seemed in harmony with Metchnikoff's theory of phagocytic immunity. The agreement was further borne out by the fact that a small series of animals treated with toluol in a similar way and in a similar physical condition, but whose marrow and blood count were normal, reacted to the pneumonic infection exactly like normal rabbits. This observation is being continued in collaboration with Dr. Kline.

III. Intra Vitam Staining.

The possibility of injecting anilin dyes into living animals and obtaining definite staining of certain tissues dates from Ehrlich's work in 1885. Ehrlich succeeded in staining most of the organs by means of indophenol and alizarin-blue. He was even able to differentiate normal areas from those which had undergone pathological changes, and states that in the hearts of animals with myocarditis, after injection of indophenol, the scars stand out blue against the unstained heart muscle. In the last few years, through the work of Ehrlich and Shiga, Nicolle and Mesnil, Bouffard, Fischel, Arnold, Michaelis, Goldmann, and others, a number of other dyes have been discovered which are also successful vital stains. In these instances the dyes are localized in the form of granules in definite cells. Until recently blood cells have not been stained *intra vitam*. Schulemann, however, demonstrated the presence of large vitally stained macrophages in the circulating blood, an observation made independently by one of the writers in collaboration with Dr. Evans.

The latter authors also showed that it was extremely rare for a polynuclear or other normal white blood cell to contain stained granules as long as it was alive, but, on the other hand, by killing the white blood cell in various ways they could be made to absorb

rapidly the vital stain. With this fact in mind, and since the pneumonic exudate consists of white blood cells, many of which are presumably injured or dead, pneumonia was induced in a few animals which were stained *intra vitam* in the course of the infection by intravenous injection of trypan-red or trypan-blue. A variety of dyes of the triphenylmethane series were used, but contrary to the statements of Morau and Germain See, they did not give rise to vital staining. The intravenous injection of trypan-blue and trypan-red gave rise to the usual diffuse staining as described by Bouffard, Goldmann, etc., but in addition to this the diseased area of lung showed a much more intense staining than any of the other tissues, while the normal lung tissue was practically normal in color. Particularly intense was the stain in the fibrinous exudates, not only over the lungs but on the pleura and pericardium and in the mediastinum. With varying amounts of the dye the intensity of the stain could be altered. With trypan-blue, for instance, minimal doses stained the fibrinous exudate a pale blue, while with larger amounts every gradation up to a royal blue could be produced. With the doses which gave the faintest stain in the exudate the tissues elsewhere were almost unstained, except the kidneys which showed about the same intensity of stain as the inflammatory area. There was thus a certain specific affinity manifested between the exudate and the dye. Histologically the stain was found to be located in the bundles and strands of fibrin of both serous and alveolar exudate, while the cells of the exudate were unstained except in a few instances. These cells showed staining of the nuclei but usually no intracellular granules, and were evidently dead cells. It is therefore evident that in these cases of early though extremely virulent experimental pneumonia almost all the leucocytes of the exudate were living. The animals studied, however, either died or were killed early in the course of the disease and quite possibly many more of the leucocytes die at a later stage in the course of more chronic infections.

An iodin derivative of trypan-blue prepared by one of us for another purpose, whose molecule had absorbed five atoms of iodin, differed slightly from the original substance in being of a deep

lavender rather than of a blue color, but it did not possess *intra vitam* staining properties.

BIBLIOGRAPHY.[2]

Arnold, J., cited by Schulemann, W., *loc. cit.*

Bouffard, G., Injections des couleurs de benzidine aux animaux normaux; Étude expérimentale et histologique, *Ann. de l'Inst. Pasteur,* 1906, xx, 539.

Ehrlich, P., Das Sauerstoff-Bedürfniss des Organismus, Berlin, 1885.

Ehrlich, P., and Shiga, K., Farbentherapeutische Versuche bei Trypanosomenerkrankung, *Berl. klin. Wchnschr.,* 1904, xli, 329, 362.

Evans, H. M., and Winternitz, M. C., Studies on the Regeneration of the Bone Marrow after Benzol Poisoning in Vitally Stained Rabbits, Unpublished Observations.

Fischel, A., cited by Schulemann, W., *loc. cit.*

Goldmann, E., Die äussere und innere Sekretion des gesunden und kranken Organismus im Lichte der vitalen Färbung, *Beitr. z. klin. Chir.,* 1909, lxiv, 192.

Lamar, R. V., and Meltzer, S. J., A Preliminary Note upon Experimental Lobar Pneumonia with a Demonstration of Specimens, *Proc. Soc. Exper. Biol. and Med.,* 1909–10, vii, 102; Experimental Pneumonia by Intrabronchial Insufflation, *Jour. Exper. Med.,* 1912, xv, 133.

Michaelis, H., cited by Schulemann, W., *loc. cit.*

Monti, cited by Welch, W. H., *loc. cit.*

Nicolle, M., and Mesnil, F., Traitement des trypanosomiases par les couleurs de benzidine. (1re partie). Étude chimique, *Ann. de l'Inst. Pasteur,* 1906, xx, 417.

Schulemann, W., Beiträge zur Vitalfärbung, *Arch. f. mikr. Anat.,* 1912, lxxix, 233; Vitalfärbung und Chemotherapie, *Berl. klin. Wchnschr.,* 1912, xlix, 497.

See, G., and Morau, H., Note sur le pouvoir antiseptique de quelques dérives de la serie aromatique de l'analine, *Méd. mod.,* 1889–90, i, 389, 560.

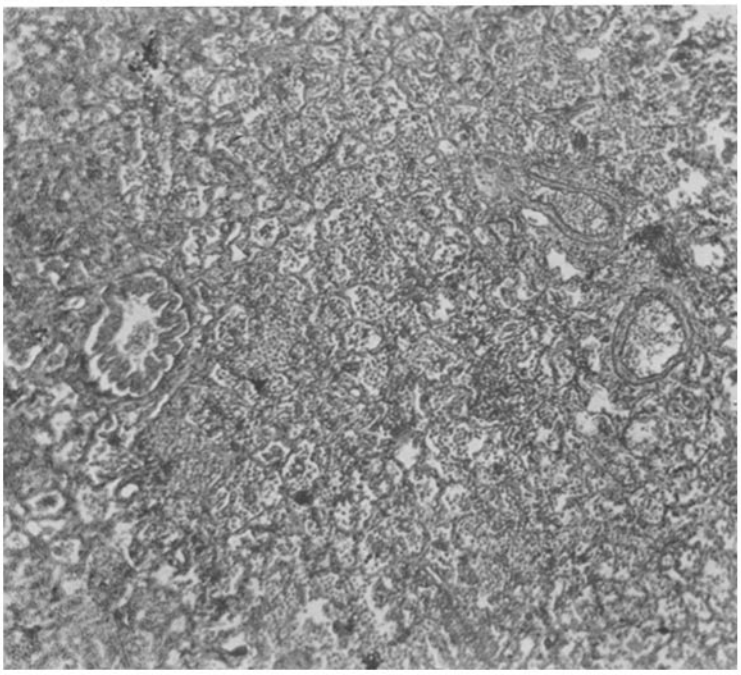
Selling, L., A Preliminary Report on Some Cases of Purpura Hemorrhagica Due to Benzol Poisoning, *Bull. Johns Hopkins Hosp.,* 1910, xxi, 33; Benzol als Leukotoxin, *Beitr. z. path. Anat. u. z. allg. Path.,* 1911, li, 576.

[2] Since writing this article our attention has been called to a paper by Rasquin, (Études expérimentales sur la pathogénèse de la pneumonie chez le lapin, *Arch. de méd. expér. et d'anat. path.,* 1910, xxii, 804) upon the production of lobar pneumonia in rabbits. Rasquin, however, states that he was unable to produce pneumonia in more than 8 per cent. of his rabbits unless the culture injected was mixed with the serum of dogs immunized to rabbit blood, in which case he obtained pneumonia in over 90 per cent. of his animals. The serum of normal dogs had no effect. Rasquin's results are, therefore, more comparable with those of Wadsworth than with our own. The results of Neufeld and Ungermann (Ueber experimentell erzeugte Pneumonien und ihre Beeinflussung durch Antipneumokokkenserum, *Centralbl. f. Bakteriol., Ref.,* 1912, liv, Suppl., 71), who have produced experimental pneumonia in the guinea pig by the intrapulmonary (not intratracheal) method also reached us after our work was completed.

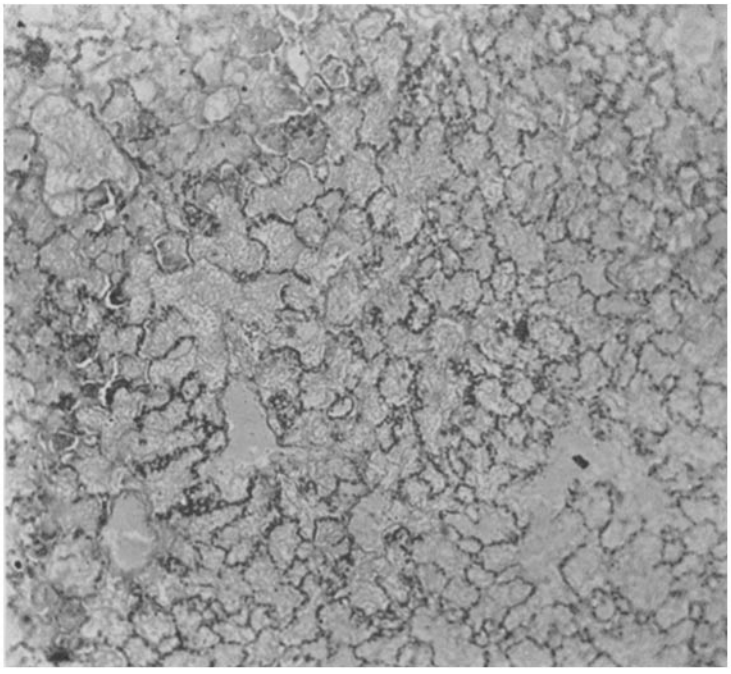
FIG. 1.

FIG. 2.

(Winternitz and Hirschfelder: Experimental Pneumonia



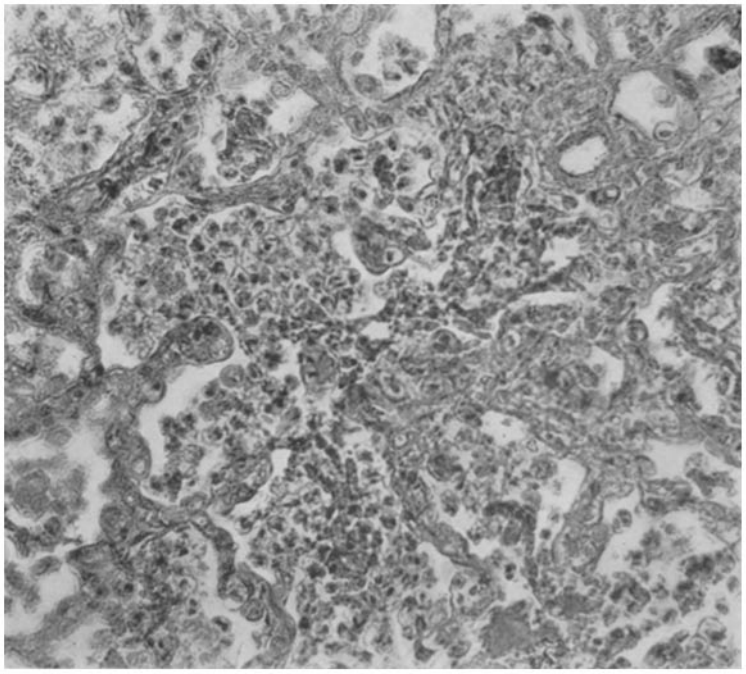
a



b

FIG. 3.

(Winternitz and Hirschfelder: Experimental Pneumonia.)



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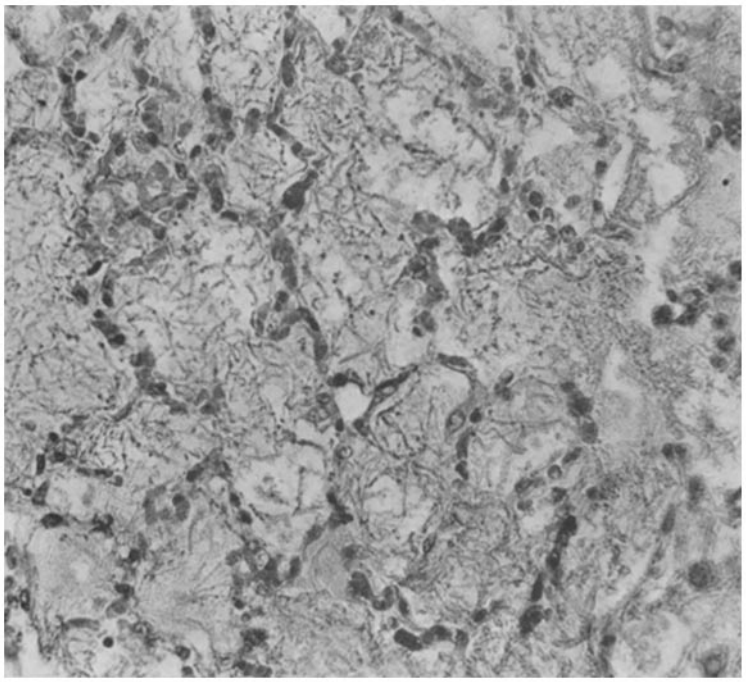
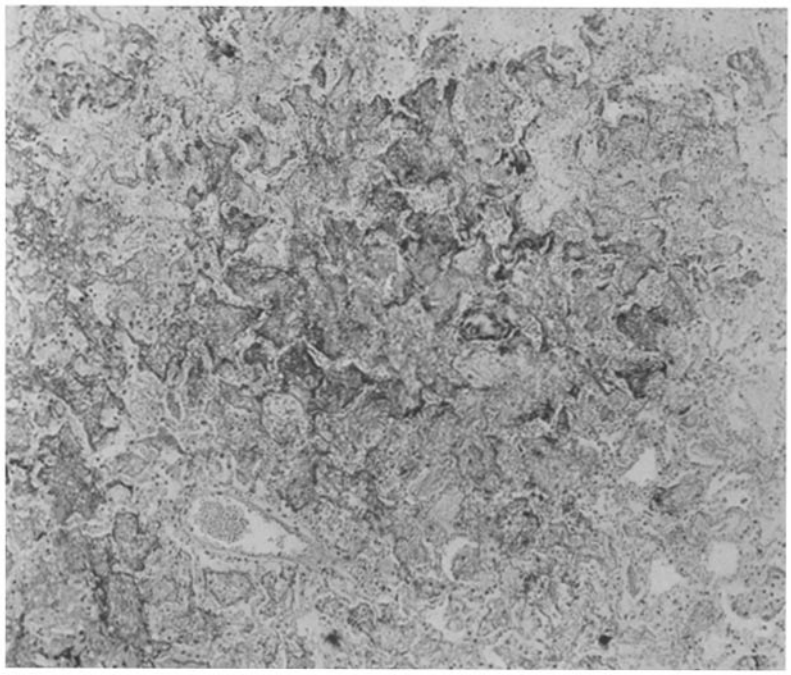


b

FIG. 4.

(Winternitz and Hirschfelder: Experimental Pneumonia.)



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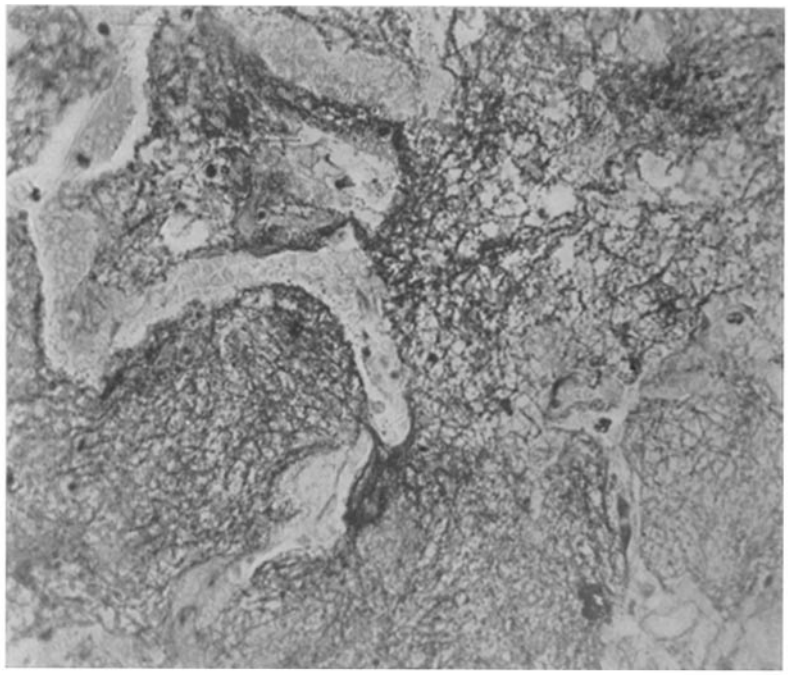

b

FIG. 5.

(Winternitz and Hirschfelder: Experimental Pneumonia.)

Wadsworth, A., Experimental Studies on the Etiology of Acute Pneumonitis, *Am. Jour. Med. Sc.*, 1904, cxxvii, 851. This article contains references to the older literature.

Welch, W. H., The *Micrococcus lanceolatus*, with Especial Reference to the Etiology of Acute Lobar Pneumonia, *Bull. Johns Hopkins Hosp.*, 1892, iii, 125.

EXPLANATION OF PLATES.

PLATE 108.

FIG. 1. Experimental lobar pneumonia and fibrinous pleurisy in a rabbit's lung, showing fibrinous pericarditis as well.

FIG. 2. Experimental lobar pneumonias in a series of rabbits, the result of one day's experimentation, showing lobar consolidation in every case.

PLATE 109.

FIG. 3 *a*. Section of lung of a normal animal with experimental lobar pneumonia. Low power.

FIG. 3 *b*. Section of lung of an aplastic animal with experimental lobar pneumonia, showing the paucity of cells in the exudate.

PLATE 110.

FIG. 4 *a*. Section of lung of a normal animal with experimental lobar pneumonia. High power.

FIG. 4 *b*. Section of lung of an aplastic animal with experimental lobar pneumonia. The pneumococci are shown within the alveoli. High power.

PLATE 111.

FIG. 5 *a*. Fibrin stain of a section of lung of an aplastic animal with experimental lobar pneumonia. Low power.

FIG. 5 *b*. Fibrin stain of lung of an aplastic animal with experimental lobar pneumonia. High power.