# STUDIES ON THE MECHANISM OF RECOVERY IN PNEUMOCOCCAL PNEUMONIA

## IV. The Mechanism of Phagocytosis in the Absence of Antibody*

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Plates 17 to 22

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In the pneumonic lung of the rat the leucocytes of the alveolar exudate phagocyte virulent pneumococci in the apparent absence of type-specific antibodies (1, 2). This finding runs counter to the present teachings of immunology since numerous investigators have presented evidence that fully encapsulated pneumococci are resistant to phagocytosis except when coated with a suitable opsonin (3–5). Only Goodner and Miller (6) have reported appreciable phagocytosis in the absence of antibody and they have expressed doubt as to the significance of such phagocytosis, since the non-sensitized pneumococci appeared, in their experiments, to multiply within the phagocytes. The experiments already described in an accompanying paper indicate that the phagocytosis observed in the pneumonic lung during treatment with sulfonamide is due neither to the presence of type-specific opsonins in the lung nor to a direct effect of the drug upon the pneumococcus capsule (2). It would appear, therefore, that the destruction of pneumococci in the lungs is due to other factors hitherto not understood. The nature of these factors is defined in the present paper.[1]

It has been shown in previous experiments that pneumococci inoculated upon a resolving pneumonic lesion are quickly phagocyted and destroyed by the phagocytic cells in the alveolar exudate. Although evidence has been presented that the phagocytosis is not due to the presence of type-specific opsonins, it is still conceivable that the phagocytic reaction in the living animal is dependent upon unknown factors which (1) are concentrated in the pneumonic exudate, (2) are brought to the alveoli by the circulating blood, or (3) are present in living pulmonary tissue. The following experiments would appear to eliminate all such hypothetical "phagocytic factors" originating from any one of these three possible sources.

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[1] A preliminary report of these studies has already been published (7).

*A. Elimination of Possible Factors Peculiar to the Pneumonic Lung.*—There is some evidence, not entirely conclusive, that the exudate in the pneumonic lung is in itself injurious to pneumococci (8, 9). It has been postulated that the metabolic products of the cellular exudate create a chemical environment unsuitable for the survival of the bacteria. To determine whether or not any such factor in the pneumonic lung is responsible for the phagocytosis observed during treatment with sulfonamide, the following experiment was performed.

A mixture of rat macrophages and polymorphonuclear leucocytes, obtained from sterile peritoneal exudates,[2] was twice washed with gelatin-Locke's solution (5) and was added to washed pneumococci (4 to 6 hour culture of Type I, A-5 strain (10)) suspended in the same solution. The resulting mixture, containing 5 to 10 phagocytic cells and 25 to 30 pneumococci per oil immersion field, was inoculated in 0.5 cc. amounts into the left main bronchus of *normal* rats. The animals were sacrificed at the end of 4 hours, and the lungs were fixed for histological study by methods previously described (10).

Phagocytosis occurred in all the preparations (Fig. 1).[3] It should be pointed out that the only possible sources of factors responsible for phagocytosis (outside of the phagocytes themselves) were (*a*) the tissues of the normal rat lung and (*b*) the blood passing through it.

*B. Elimination of Possible Factors Carried by the Circulating Blood.*

The preceding experiment was repeated, using rat lungs previously perfused with gelatin-Locke's solution, removed from the thorax of the animal, and suspended on a mechanical respirator (see Text-fig. 1).[4] The respirator, which was operated in a 37°C. incubator, caused the lung to expand and contract at the rate of 80 to 100 times per minute, thus simulating the normal respiratory cycles in the intact animal. Before the perfused lung was attached to the respirator, a mixture of washed phagocytic cells and pneumococci was inoculated into the left bronchus as in the previous experiment. After 4 hours the lung was removed from the respirator and was fixed and sectioned in the usual manner for histological study (10).

Even in perfused lungs, completely devoid of a blood supply, marked phagocytosis occurred (Fig. 2). This finding proves that neither a circulating natural opsonin nor any other blood-borne factor is necessary for phagocytosis to take place in the lung.

*C. Elimination of Possible Factors Peculiar to Living Pulmonary Tissue.*—Even after phagocytosis has been demonstrated in the lung devoid of its normal blood supply, the possibility still remains that natural opsonins or other tissue

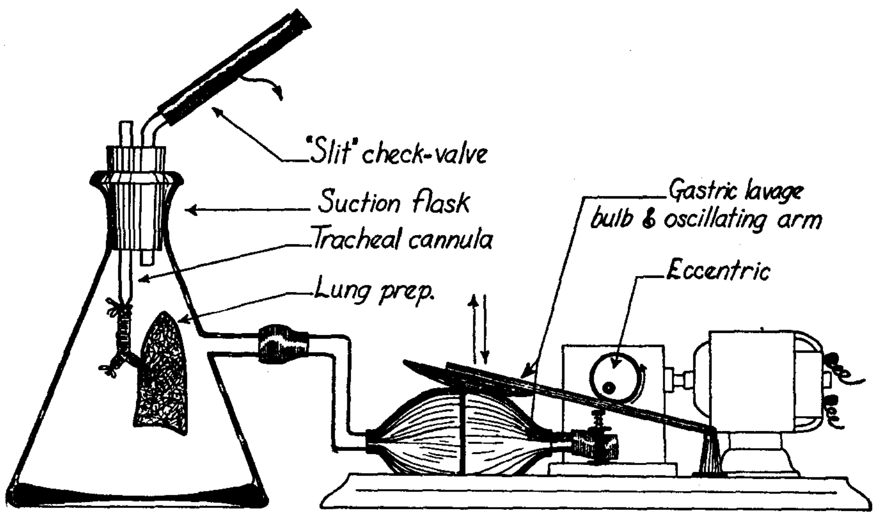
[2] Sterile peritoneal exudates were obtained from adult white rats injected 24 hours previously with 5 cc. of an aleuronat-starch mixture in tryptose broth. The aleuronat-starch mixture contained 5 gm. of aleuronat and 3 gm. of starch in 100 cc. of distilled water. The mixture was autoclaved before being added to an equal volume of sterile tryptose phosphate broth. To prevent infection of the peritoneal exudate, each rat, when injected intraperitoneally, was given 250 mg. of sulfapyridine by stomach tube.

[3] Similar results were obtained by using rabbit macrophages and polymorphonuclear leucocytes from sterile pleural exudates (gum acacia) (5).

[4] Designed with the assistance of Dr. John R. Smith.

factors may be present in the parenchyma of the normal lung and thus account for the phagocytic reaction. This possibility is ruled out by the following experiment.

Normal rat lungs were fixed for 24 hours in 10 per cent formalin. They were then washed for 5 to 7 days in tap water, and were finally rinsed with gelatin-Locke's solution before being used. Such preparations can have no living cells and no unprecipitated protein or active enzymes, except those supplied by the injected mixture of washed pneumococci and phagocytes. Into these fixed lungs phagocyte-pneumococcus mixtures were injected as in the preceding experiments, and sections were made after the lungs had been in the respirator for 4 hours.




TEXT-FIG. 1. Mechanical respirator used to cause lung preparations to expand and contract as in the intact animal.

Marked phagocytosis took place in the fixed lungs as shown in Fig. 3.

Finally, subsequent experiments revealed that it was not necessary to attach the formalin-fixed lungs to the respirator, since the same phagocytosis occurred when the fixed lungs were allowed to hang motionless in the incubator for 4 hours before being sectioned (see Fig. 4). This observation indicates that the phagocytic mechanism is in no way dependent upon the respiratory excursions of the lungs.

From these three sets of experiments it can be concluded that no factor peculiar to (*a*) the pneumonic lung, (*b*) the circulating blood, or (*c*) living pulmonary tissue is essential to the phagocytosis of pneumococci in the lung. On the contrary, it would appear that in the lungs, both polymorphonuclear leucocytes and macrophages attack virulent pneumococci directly without the aid of an opsonin or any other intermediary factor. It is of particular significance that the lung supports phagocytosis even after the tissue has been fixed in formalin, for this observation suggests that physical rather than chemical factors present in the lung enable the phagocytic cells to attack the encapsulated pneumococci directly.

### *"Surface Phagocytosis"*

On reviewing the histologic sections from the previous experiment it was noted that marked phagocytosis occurred within the lumen of the large bronchi (see Fig. 5). The conditions necessary for the phagocytic reaction appeared, therefore, to be present not only in the alveoli but also in the bronchi. To test this conclusion pneumococcus-leucocyte mixtures were placed in isolated sections of formalin-fixed bronchi which were tied off with sutures above and below during a 4 hour incubation period. The fixed bronchus was opened at the end of each experiment and impression smears were made from the epithelial surface of the lumen. Marked phagocytosis was noted in all experiments. An attempt was then made to bring about the phagocytic reaction in glass tubes of approximately the same diameter as the bronchi. Even when the tubes were first lined with mucus from the bronchi of normal rats, phagocytosis failed to occur. This finding suggested that the crucial factor in the phagocytic reaction was in some way related to the surface of the bronchus.

*A. Test for Phagocytosis on Various Surfaces.*—A method was devised for carrying out phagocytic tests upon the surfaces of a variety of tissues as well as upon a number of inert materials.

The material with surface to be tested was spread flat in the bottom of a Petri dish lined with filter paper previously soaked in Locke's solution. The leucocyte-pneumococcus mixture was distributed over the test surface, and the dish was sealed and placed in the incubator (37°C.) for 1 to 3 hours. In this way the preparations were kept moist. When the Petri dishes were opened, impression smears were made from the test surfaces and were stained with methylene blue.

In Tables I, II, and III are listed the various surfaces tested by this method, together with the results obtained.

It will be noted that all tissue surfaces tested, with the exception of the acid gastric mucosa, supported phagocytosis. (Smears from the gastric preparations indicated that the cells had been injured by the acid.) Even after tissues had been boiled for 30 minutes, the phagocytic reaction took place upon their surfaces. Although the phagocytic cells failed to take up pneumococci on glass surfaces, paraffin, albumin, mucus, and cellophane (see Fig. 6), they were found to be highly active on moist filter paper, blotting paper, cloth, lens paper, fiber glass, and gelatin sponge (see Fig. 7). Phagocytosis on the inert surfaces was demonstrable as early as 5 minutes after the start of incubation and reached its maximum in less than 1 hour. From these observations it can be concluded that phagocytic cells when in contact with an inert surface possessing the proper physical properties will phagocyte virulent pneumococci without the aid of an intermediary antibody or any other tissue factor.

TABLE I

*Tests for Phagocytosis on Surfaces of Normal Rat Tissues*

| | | | |
|---|---|---|---|
| Bronchus (lumen) | + | Peritoneum (visceral) | + |
| Trachea (lumen) | + | Peritoneum (parietal) | + |
| Esophagus (lumen) | + | Liver (cut surface) | + |
| Aorta (lumen) | + | Spleen (cut surface) | + |
| Vena cava (lumen) | + | Kidney (cut surface) | + |
| Lung (cut surface) | + | Mesentery | + |
| Pleura (visceral) | + | Retina | + |
| Pleura (parietal) | + | Muscle | + |
| Pericardium (visceral) | + | Clotted plasma | + |
| Endocardium | + | Gastric mucosa | – |

TABLE II

*Tests for Phagocytosis on Surfaces of Boiled Rat Tissues*

| | |
|---|---|
| Boiled lung (cut surface) | + |
| Boiled bronchus (lumen) | + |
| Boiled liver (cut surface) | + |
| Boiled spleen (cut surface) | + |

TABLE III

*Tests for Phagocytosis on Surfaces Other Than Those of Tissues*

| | | | |
|---|---|---|---|
| Glass (coverslip) | – | Cellophane | – |
| Glass (hanging drop) | – | Filter paper | + |
| Glass (micropipette) | – | Blotting paper | + |
| Glass (test tube) | – | Lens paper | + |
| Paraffin on glass | – | Fiber glass | + |
| Albumin on glass | – | Cloth | + |
| Mucus on glass | – | Gelatin sponge | + |

*B. The Relation of Surface Phagocytosis to That Induced by Specific Opsonin.*—It is well known that type-specific antibody promotes the phagocytosis of virulent pneumococci in the test tube (11). The experiment just described indicates that such phagocytosis does not occur in the absence of antibody except when the phagocytic cells are brought into contact with a surface of suitable physical properties. The following experiments were designed to study the mechanism involved in these two apparently different types of phagocytosis.

Glass coverslips, the under surfaces of which were covered with moistened filter paper, were utilized to suspend hanging drop preparations containing phagocytes and pneumococci. Hanging drops were prepared with pneumococcus-phagocyte mixtures suspended in gelatin-Locke's solution and in gelatin-Locke's solution containing homologous type-specific antiserum. A similar test without antibody was also run with a non-encapsulated rough pneumococcus. The hanging drops were enclosed in the wells of hollow ground slides to prevent drying and were incubated for 1 hour. Smears were then made from separate samples taken from (*A*) the surface of the filter paper and (*B*) the bottom of the drop.

A second experiment was designed to test further the ability of phagocytic cells to attack pneumococci in the absence of a solid surface. The same three pneumococcus-leucocyte mixtures were suspended from the tips of small glass pipettes. The suspended drops were incubated within rubber-stoppered bottles lined with moistened filter paper to keep the surrounding air saturated with water vapor. At the end of 45 to 60 minutes of incubation, samples were drawn off carefully from the bottom of each drop and smears were prepared and stained with methylene blue in the usual manner.

In both experiments fully encapsulated pneumococci were phagocyted, in the absence of type-specific opsonin, by leucocytes which were in contact with the solid surface but were not engulfed by the phagocytic cells that were free in the fluid medium. On the other hand, in the presence of antibody, both the free leucocytes and those on the surface engulfed encapsulated organisms. Non-encapsulated pneumococci also were phagocyted by the cells in both locations. There would appear, therefore, to be a fundamental difference between the surface phagocytosis of virulent pneumococci and phagocytosis induced by the presence of specific opsonins or by the loss of the capsule.

*C. Direct Visualization of the Phagocytic Mechanism.*—An essential difference having been demonstrated between surface phagocytosis and that induced by specific opsonin, the exact mechanism involved in each was studied by direct visualization.

Hanging drop preparations, containing the same pneumococcus-leucocyte mixture used in previous experiments (smooth phase), were watched under the microscope in both the presence and absence of homologous specific antibody. Each preparation on the surface of a thin glass coverslip was sealed in the well of a hollow ground slide to prevent drying and was incubated at 37°C. No stain was used except for the purpose of photography since both the phagocytic cells and the pneumococci could be seen when the microscopic field was properly illuminated.[5]

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[5] For photography pneumococci cultured for 6 hours in beef infusion broth, containing 1 per cent dextrose and 3 per cent rabbit serum, were stained with carbofuchsin by the method described by Castaneda (12). The stain employed was a 1 to 30 dilution of Ziehl-Neelsen stain in distilled water. After the organisms had been stained and washed in saline, they were subjected to two additional washings with Locke's solution before being used in leucocyte-pneumococcus mixtures.

Repeated control observations demonstrated that formalin-killed pneumococci stained by this method behaved like living, unstained pneumococci in that they were not phagocyted when suspended in a fluid medium unless opsonized with specific antibody or trapped by the leucocytes against a suitable solid surface.

For photography fields were selected near the edge of the drop[6] where the layer of fluid was relatively shallow. Here the cells in contact with the undersurface of the coverslip frequently exhibited ameboid motion as shown in the photographs, but otherwise the behavior of the phagocytes toward the pneumococci was observed to be exactly the same as in the deeper portions of the drop where both cells and organisms were floating freely.

In the presence of specific opsonin the pneumococci, their capsules swollen from the antibody, were seen to adhere to the surfaces of the leucocytes with which they came in contact in the fluid medium (Fig. 8). After having stuck to the surfaces of the phagocytes for a few moments, they were slowly engulfed by the cells. The observed sequence of events is illustrated by photomicrographs taken at 2 minute intervals and shown in Figs. 8 to 10. When phagocytes were observed to attack large aggregations of agglutinated pneumococci, it was noted that the cells engulfed only one or two organisms at a time and did not take in sizeable clumps as postulated by Goodner and Miller (6).

In the absence of antibody the picture in the hanging drop was very different. The pneumococci were seen to rebound from the surface of the phagocytic cells with which they collided in the fluid medium and phagocytosis failed to occur (see Figs. 11 to 14).

To visualize surface phagocytosis a somewhat more complicated technique was needed. Observation of the phenomenon on inert surfaces, such as filter paper or fiber glass, was unsatisfactory because of the failure of the cells to remain active under the coverglass needed for the use of the oil immersion lens. Although phagocytosis occurred on the open lower surface of the filter paper, it failed to take place on the upper surface directly in contact with the coverslip. The phagocytic reaction, therefore, could not be visualized in such preparations owing to the limited depth of focus of the oil immersion lens. When living tissue of sufficient thickness was employed, however, phagocytosis was found to occur directly beneath the coverslip. The technique finally adopted was as follows:—

A small section about 1 centimeter square was cut from the abdominal wall of a 15 gm. mouse, after the skin had been peeled from the abdominal muscles. The peritoneal surface of the block was moistened with gelatin-Locke's solution and a small drop of the pneumococcus-leucocyte mixture was spread over the surface. A number "O" coverslip was then pressed gently upon the peritoneal surface, and the preparation was mounted on a hollow glass slide. When a 500 watt bulb was used as a source of illumination, the phagocytic cells, the pneumococci, and the peritoneal surface could all be clearly visualized just beneath the coverslip.

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[6] Microscopic fields at the extreme edge of the drop were carefully avoided because encapsulated pneumococci trapped in the shallowest portion were often phagocyted spontaneously by the leucocytes. Conditions in the extreme shallows of the drop were obviously very different from those in the rest of the preparation.

Repeated observations of the phagocytic cells on the peritoneal surface revealed that the leucocytes phagocyted only those pneumococci over which they extended on the tissue surface. The pneumococci which were not caught beneath the advancing pseudopods of the leucocytes remained free in the fluid medium and were not phagocyted. Many of the pneumococci close to the peritoneum were trapped as the leucocytes settled down on the surface of the tissue.

In order to obtain a clearer view of the actual process of surface phagocytosis, the above technique was modified as follows:—

Normal rat lungs, fixed for 24 hours in 10 per cent formalin, were washed for several days in running tap water to rid them of fixative. Tissue blocks were embedded in paraffin and were cut in sections 5 microns thick. The sections were floated onto "O" coverslips and allowed to dry. After the paraffin had been dissolved in xylol, the tissues were run through alcohol and finally were repeatedly washed in distilled water and dried. A small drop of the phagocyte-pneumococcus mixture was placed on each mounted lung section which was then inverted as a hanging drop preparation in the cavity of a hollow ground slide. The inverted coverglass was rimmed with vaseline to prevent drying of the mixture, and the preparation was observed under oil immersion lens, in the warm stage of the microscope (37°C.). In the alveoli near the margin of the drop, where the layer of fluid was relatively thin, the phagocytes and pneumococci were confined to the cavities of the alveoli and could be clearly visualized. Although the fluid layer in these alveoli was several leucocyte diameters in depth, the uppermost phagocytic cells could be seen moving about on the smooth undersurface of the coverslip. (See Figs. 15 to 20.)

Here again only the pneumococci which were trapped by the motile leucocytes against the tissue surface of the alveolar walls (or occasionally between two or more leucocytes) were phagocyted. The sequence of events observed during the phagocytic process in the alveoli is pictured in the photomicrographs of Figs. 15 to 20. As is clearly shown in the photographs, the motile leucocytes first cover the organisms lying against the alveolar walls and then phagocyte them. This phagocytic process has been repeatedly observed under direct vision.

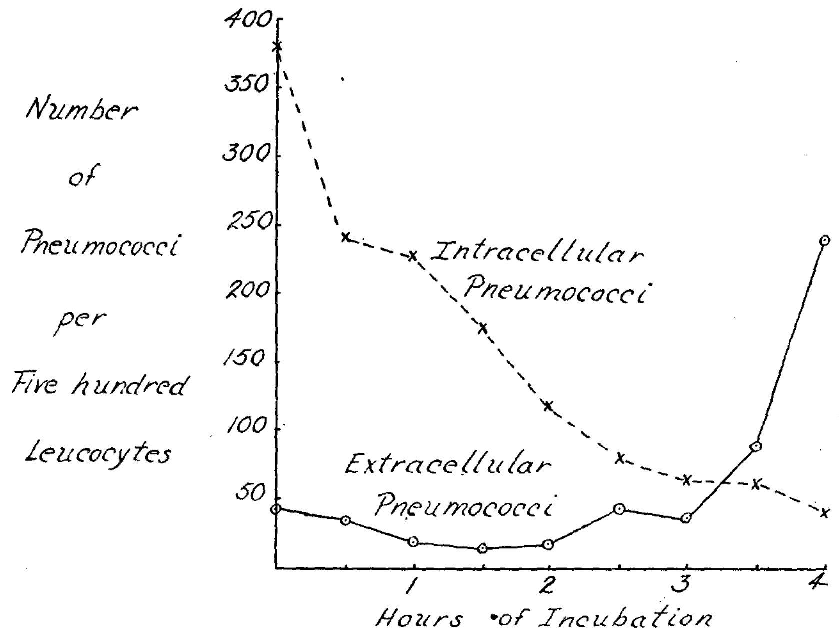
*D. The Bactericidal Effect of Surface Phagocytosis.*—Finally, to evaluate the significance of "surface phagocytosis" as a defense mechanism of the host, it is necessary to determine whether pneumococci subjected to this form of phagocytosis are killed and digested by the phagocytes. It is conceivable that bacteria thus phagocyted might survive in the cytoplasm of the leucocytes and ultimately destroy them (6). To settle this important question the following experiments were performed.

Phagocytosis was brought about in Locke's solution on the surface of filter paper in the manner previously described, and the phagocytes, many of which contained intracellular pneumococci, were washed from the surface of the filter paper and suspended in Locke's solution. To the latter was added a small amount of fresh rat serum[7] to preserve the maximum

[7] The final dilution of serum, which contained 1 drop of liquamine per 2 cc., was approximately 1 to 10. As has been previously emphasized (2), such serum contains no type-specific antibody against the A-5 strain of pneumococcus.

activity of the cells. The mixtures were then incubated at 37°C., and smears were made from the suspensions every 30 minutes over a period of several hours and were stained with methylene blue and with Gram's stain.

Poorly stained and partially digested pneumococci could be seen in the cytoplasm of the phagocytes after 30 minutes, 1, 1½, and 2 hours of incubation. (See Figs. 21 and 22.) Many of the intracellular pneumococci were also found to be Gram-negative. Careful counts of the number of intracellular and extracellular pneumococci per 500 polymorphonuclear leucocytes[8] were made




TEXT-FIG. 2. Data indicating intracellular digestion of pneumococci following surface phagocytosis.

at each 30 minute interval. As shown in Text-fig. 2, there was a rapid decrease in the number of intracellular organisms during the 2 hours of incubation without a corresponding increase in extracellular organisms. These data further support the conclusion that the bacteria were actually digested by the phagocytes and not merely freed from the cells. Single isolated phagocytes containing two or more ingested pneumococci were watched continuously under the microscope during the period of incubation. The ingested pneumococci became fainter and fainter in outline until they could no longer be

[8] Only polymorphonuclear leucocytes were included in the bacterial counts, since it was found difficult to estimate accurately the number of pneumococci within the monocytes, because of the granular nature of their cytoplasm. The same process of digestion, however, appeared to be taking place in the monocytes.

seen in the cytoplasm of the cell. Digestion was usually complete within 2 to 3 hours. Uninterrupted observation revealed that the bacteria were digested by the phagocyte, not disgorged from its cytoplasm.

## DISCUSSION

The experiments reported in the present papers demonstrate that phagocytic cells operating in the parenchyma of the lung are able to attack directly and ultimately destroy fully encapsulated pneumococci without the aid of an intermediary opsonin. It has been further demonstrated that the ability of leucocytes to perform this function in the lung depends upon the physical properties of the alveolar and bronchial surfaces. Phagocytic cells operating upon other tissue surfaces and upon relatively rough, inert materials such as filter paper and fiber glass retain their ability to phagocyte virulent pneumococci, whereas on smooth surfaces such as those of glass, paraffin, cellophane, etc., no phagocytosis takes place. This latter fact becomes of particular importance when it is considered that phagocytic tests in the laboratory are usually carried out in glass test tubes or capillary pipettes or between the surfaces of glass coverslips (13). The failure of previous investigators to describe the phenomenon of surface phagocytosis may be accounted for by this almost universal practice.

It has been repeatedly emphasized during the course of the present studies that according to the accepted concepts of immunology, fully encapsulated pneumococci (and certain other encapsulated microorganisms) are not phagocyted unless previously opsonized with specific antibody (1, 2, 10, 14). This dictum is based upon the results of *in vitro* phagocytic tests performed with standard laboratory equipment made of glass and is easily confirmed under the conditions of such experiments (15). It does not apply, however, to the conditions obtaining in the animal body where it has been shown, by the present studies, and by those of Goodner and Miller (6), that no opsonin is needed for phagocytosis on tissue surfaces. The latter authors reported phagocytosis in the peritoneal exudate of mice during experimental pneumococcal peritonitis, and in the light of the present studies their findings would appear to be explained by the occurrence of phagocytosis on the surface of the peritoneum. Since it has been shown that phagocytic cells in the absence of opsonins can engulf virulent pneumococci in the interstices of such inert fibrous materials as filter paper and fiber glass, it is possible that the cells can do likewise in the network of fibrin which so frequently occurs in areas of severe inflammation. The fallacy of assuming that phagocytic cells behave in the animal host in the same way that they behave in the test tube is self-evident.

The final phase of the study reported in this paper needs particular emphasis. For the phenomenon of surface phagocytosis to have significance in relation to recovery from pneumococcal infection, it must be proven that this form of phagocytosis is followed by destruction of the pneumococci. It is conceivable that the organisms might be taken into the leucocytes on tissue surfaces only to continue to multiply and eventually destroy the cells. Goodner and Miller (6) have in fact reported such intracellular multiplication of non-opsonized pneumococci in the leucocytes of mice, but their findings could not be confirmed in the present study. Since direct observation has revealed that non-sensitized pneumococci undergo digestion in the leucocytes of rats after phagocytosis, it must be concluded that the phagocytic process (in this animal, at least) is ultimately bactericidal.

Most previous studies of the mechanism of recovery in pneumococcal pneumonia have centered about the rôle of antibodies (16). The now classic observations of Avery, Enders, and others concerning the relation of type-specific antibodies to immunity have exerted a profound influence upon the current lines of immunological investigation. Serological studies in pneumococcal pneumonia have been based on the fundamental premise that fully encapsulated pneumococci are resistant to phagocytosis unless sensitized by specific antibody. As pointed out at the start of the present investigations (10), the results of such serological studies have failed to account for the following facts concerning the mechanism of recovery.

1. Untreated patients with pneumococcal pneumonia not infrequently recover completely from the disease long before any circulating antibody can be demonstrated in the blood (17, 18). If opsonins are needed for phagocytosis, how does it happen that the pneumococci are destroyed in the lungs of these patients in the apparent absence of antibodies?

2. Although effective chemotherapeutic agents such as the sulfonamide drugs are bacteriostatic rather than bactericidal in the concentrations usually attained in clinical practice (19, 20), pneumonia patients treated with these agents often recover without the aid of circulating antibodies (21, 22). Here again it is not clear how the pneumococci are destroyed in the apparent absence of antibody.

3. Careful histological studies of the lungs of patients (23) and experimental animals (10, 24) dying of lobar pneumonia have revealed areas of resolution in the center of pneumonic lesions which are actively spreading at the periphery. In these resolving areas the pneumococci have apparently been destroyed by phagocytosis at a time when there existed an overwhelming pulmonary infection accompanied by bacteremia, an absence of demonstrable circulating antibody (25, 26), an excess of circulating polysaccharide (27), and sufficient polysaccharide in the lung itself to neutralize tremendous quantities of antibody (28). No satisfactory explanation has been offered for the phagocytosis of pneumococci under such conditions.

To explain such apparently contradictory facts as these, Robertson (24) has postulated that immune bodies may be produced locally in the lungs in sufficient quantity to promote phagocytosis and clearing of the lesion without spilling over into the blood stream. Although this hypothesis offers an adequate explanation for each of the above observations, no direct experimental evidence has been advanced in its favor and it has not been substantiated by the present studies. On the contrary, local antibody could not be demonstrated in the pneumonic lesion, and the phagocytic reaction in the lung was found to be unrelated to the type specificity of the invading pneumococcus (2). A far more satisfactory explanation is afforded by the discovery of surface phagocytosis.

Although emphasis has been placed in the present report upon the non-antibody phagocytic mechanism described in these studies, it is not implied that type-specific antibody has no bearing upon the recovery process in pneumococcal pneumonia. When the antibodies have had time to accumulate in the blood and in the pneumonic lung, their agglutinating action tends to immobilize the pneumococci and checks the spread of the pulmonary lesion (10). Likewise their opsonizing effect undoubtedly facilitates the phagocytosis of pneumococci, particularly those that are floating freely in the edema fluid within the alveoli. The accumulation of antibodies, however, is a relatively slow process, and although immune bodies may determine the outcome of long standing pneumococcal infections, it is unlikely that they play an important rôle in the rapid recovery which so frequently results from modern chemotherapy.

From a knowledge of the pathogenesis of the pneumonic lesion it may be predicted that any therapeutic agent that will check the advance of the outer edema zone of the lesion will be effective in the treatment of pneumococcal pneumonia. Such a check will enable the leucocytes to overtake the pneumococci in the outer portion of the lesion and destroy them by the surface phagocytosis demonstrated in this study. Type-specific antibody administered parenterally in the form of antipneumococcal serum penetrates the alveoli of the pneumonic lung and causes the pneumococci to agglutinate and adhere to the alveolar surfaces (10). Since the antibody stops the spread of the lesion in this way, and also opsonizes the pneumococci, it is not surprising that adequate serum therapy usually brings about prompt recovery.

The efficacy of chemotherapy in the treatment of uncomplicated pneumonia may be explained in much the same way, although the mechanism involved is somewhat different. The chemotherapeutic agent apparently stops the spread of the pneumococci in the edema zone by its bacteriostatic action (1), and although there is usually neither circulating nor local antibody to assist the leucocytes, they are able to destroy the pneumococci by surface phagocytosis.

If, during the course of pneumonia, the invading pneumococci succeed in establishing an active focus outside of the lung, the situation is very different. In the pleura, pericardium, meninges, or joints, a pneumococcal infection quickly leads to an accumulation of pus. The leucocytes in the fluid medium of the pus do not have access to an extensive solid surface as in the lung,[9] and therefore cannot phagocyte encapsulated pneumococci in the absence of antibody. Even the introduction of antibody into the lesion seldom results in sterilization of the pus because of the failure of leucocytes to survive in the fluid pus. To cure such complications as empyema and purulent arthritis, it is necessary either to remove the pus by surgical drainage or to introduce a potent chemotherapeutic agent, such as penicillin, in sufficient concentration to destroy the bacteria (30).

Besides being of significance in the mechanism of recovery in pneumococcal pneumonia, surface phagocytosis appears to constitute an essential natural defense of the lung against invasion by encapsulated bacteria. The lung is the only major viscus that is constantly in contact with the outside air. Airborne microorganisms frequently gain access to the lower respiratory tract, and were it not for an efficient series of natural defense barriers in the lung (31), pneumonia would be an almost universal disease. The mechanisms of natural defense operating in the bronchi prevent most inspired bacteria from reaching the alveoli, but a limited number of organisms, particularly those suspended in aspirated fluid, eventually gain access to the alveolar ducts and alveoli. Once these microorganisms enter the alveoli, they give rise to pneumonia unless they are taken up by the alveolar phagocytes and either carried away by lymphatics or destroyed by intracellular digestion. Were the alveolar phagocytic cells dependent upon the accumulation of antibody, they would function much too slowly to be effective in the earliest stages of bacterial invasion. The mechanism of surface phagocytosis in the absence of antibody enables the alveolar phagocytes to operate immediately against even such highly virulent organisms as fully encapsulated pneumococci.

## SUMMARY

1. Evidence has been presented in previous publications that the phagocytosis of pneumococci in the pneumonic lung during chemotherapy is due neither to specific opsonins nor to capsular injury (1, 2). The present studies have shown that the phagocytosis taking place in the lung is independent of any sort of intermediary factor and results from a direct action of the phagocytic cells upon the pneumococci.

2. Phagocytosis in the absence of antibody has been demonstrated not only in the lungs of living rats but in formalin-fixed lungs, on the surfaces of a variety of tissues (both freshly removed from the animal and previously "killed" with heat), and on the surfaces of such inert materials as moistened filter paper, cloth, and fiber glass. On the other hand, smooth materials such as glass, cellophane, albumin, and paraffin have failed to support the phagocytic reaction. This latter observation indicates that the physical character of the

[9] It is estimated that there are 70 square meters of solid surface in the human lungs (29).

surface to which the leucocytes have access constitutes a determining factor in the non-antibody mechanism of phagocytosis.

3. Further experiments have defined the relationship of "surface phagocytosis" to that induced by specific opsonins. The non-antibody mechanism was found to operate only upon surfaces of suitable physical properties, whereas opsonins enabled phagocytes floating freely in a fluid medium to engulf the fully encapsulated organisms.

4. Direct visualization of the surface phenomenon in the lung revealed that leucocytes phagocyte the virulent organisms in the absence of antibody only after having trapped them against the alveolar walls. Once the encapsulated pneumococci have been ingested, they can be seen to undergo digestion within a few hours.

The discovery of the phenomenon of surface phagocytosis affords clarification of previously unanswered problems concerning the mechanism of recovery in pneumococcal pneumonia.

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## EXPLANATION OF PLATES

Sections stained by the Gram-Weigert technique (Figs. 1 to 5) and fixed smears stained with methylene blue (Figs. 6, 7, 21, 22). Figs. 1 to 7 were photographed by Mrs. Pauline Melville and the remaining photomicrographs were taken with the kind assistance of Professor Carl Lindegren and Mr. M. W. Rhoades.

### PLATE 17

FIGS. 1 to 7. The phagocytosis of virulent pneumococci on tissue surfaces and on moistened filter paper.

FIG. 1. Phagocytosis of fully encapsulated pneumococci by phagocytic cells injected into the left main bronchus of a normal rat. Lung fixed for histological study 4 hours after pneumococcus-phagocyte mixture was injected into base of left lung. × 950.

FIG. 2. Phagocytosis of pneumococci taking place in perfused rat lung attached to mechanical respirator. × 950.

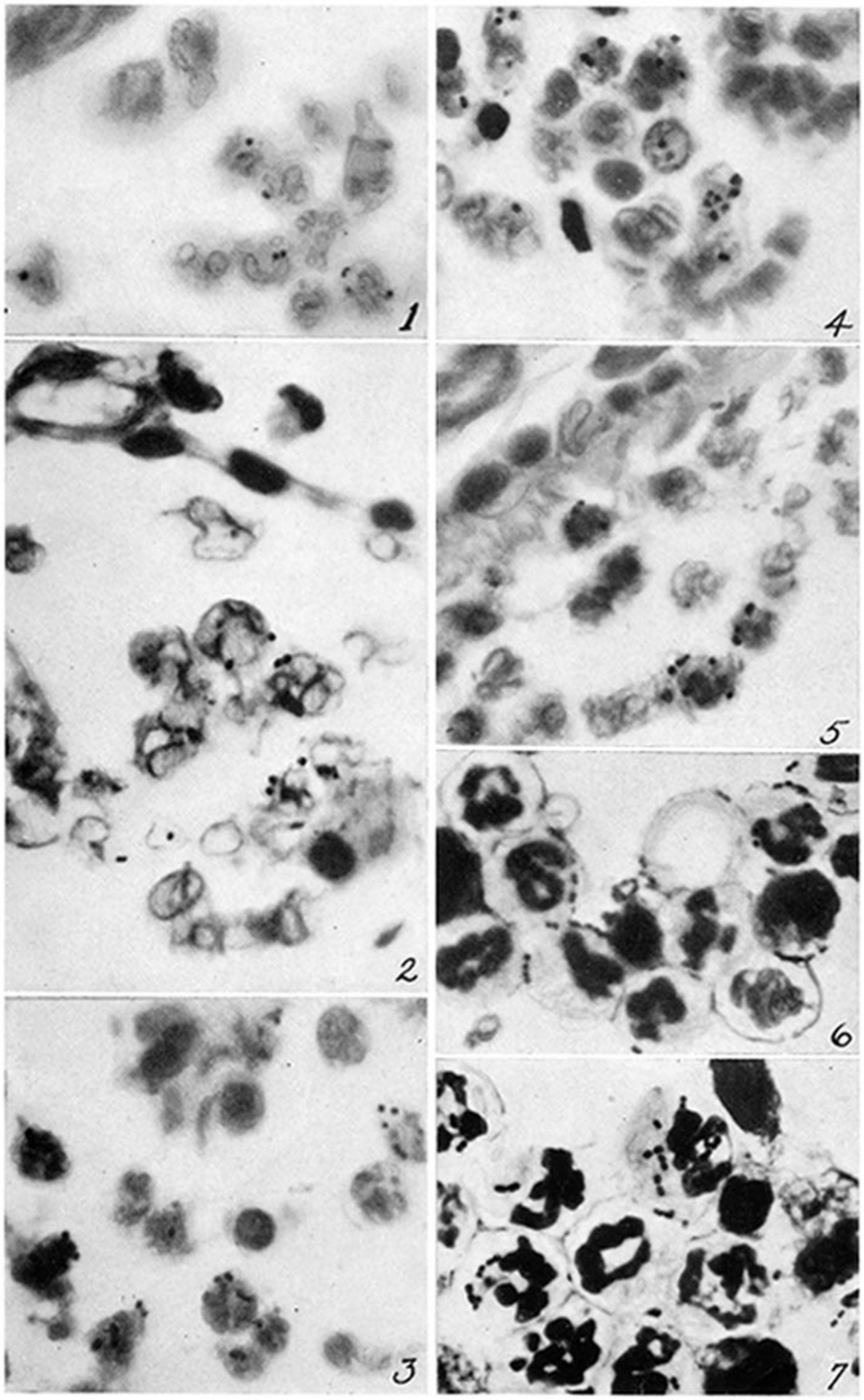
FIG. 3. Pneumococci phagocyted by cells from peritoneal exudate injected into formalin-fixed rat lung. The fixed lung was attached to a mechanical respirator for 4 hours in the incubator before being refixed for histological sectioning. × 950.

FIG. 4. Phagocytosis occurring in formalin-fixed lung suspended motionless in incubator for 4 hours. × 950.

FIG. 5. Phagocytosis of pneumococci within lumen of large bronchus in formalin-fixed lung. Note ciliated epithelium lining wall of bronchus. × 950.

FIG. 6. Failure of phagocytes to engulf encapsulated pneumococci on glass surface. Note that many of the pneumococci are in contact with the surfaces of the cells although none have been phagocyted. Methylene blue stain. × 1425.

FIG. 7. Phagocytosis of pneumococci on surface of moistened filter paper. Many of the organisms can be seen within the cytoplasm of the phagocytes. Methylene blue stain. × 1425.




(Wood *et al.*: Mechanism of recovery in pneumococcal pneumonia. IV)

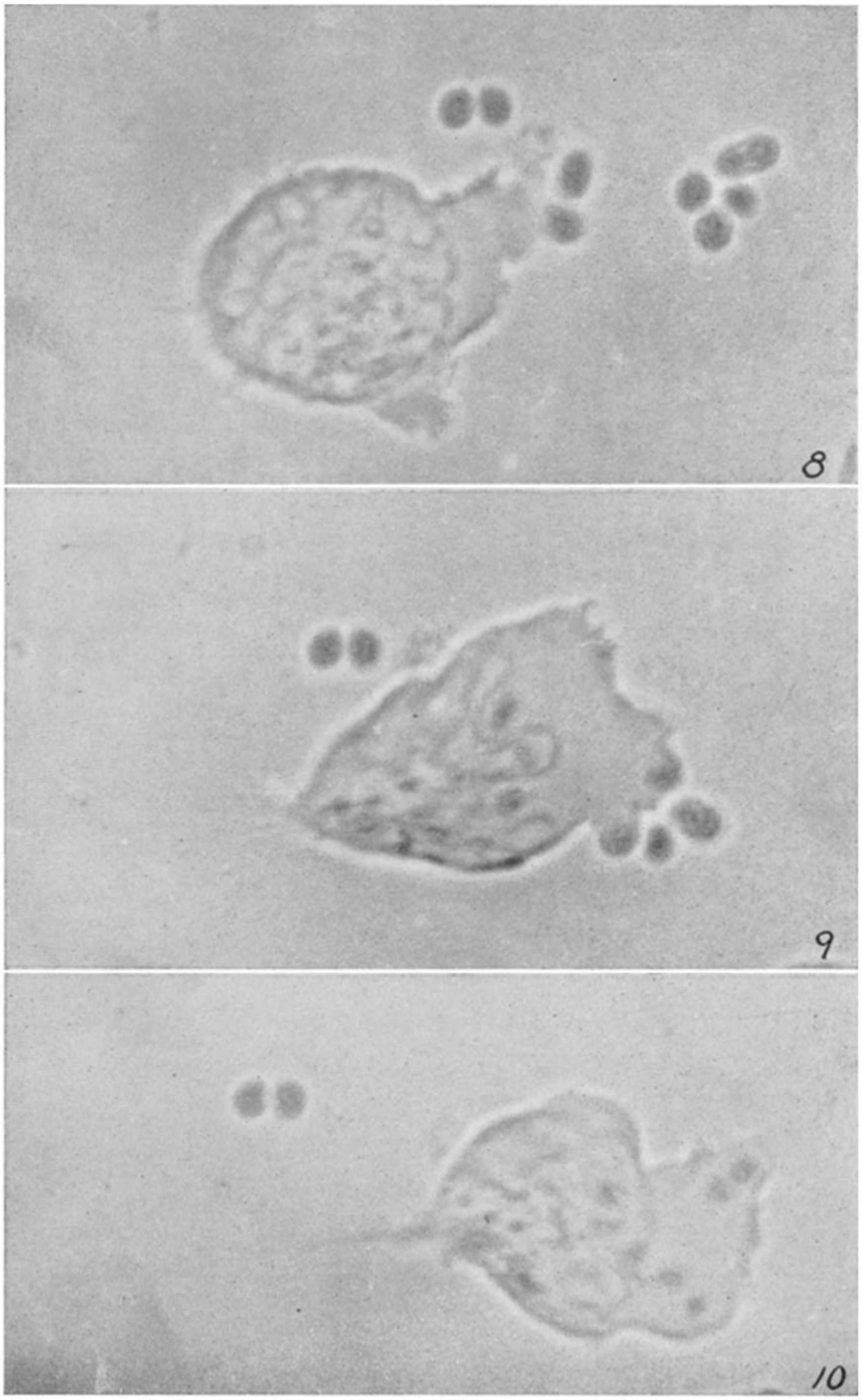
PLATE 18

FIGS. 8 to 10. The phagocytosis of pneumococci in hanging drops of gelatin-Locke's solution containing type-specific antibody.

FIG. 8. Pneumococcus-leucocyte mixture in hanging drop containing type-specific antibody. Swollen capsules of eight pneumococci in the field are clearly visible. Two are seen adhering to the pseudopod of the leucocyte. Time, 3.51 p.m. $\times$ 3350.

FIG. 9. Two minutes later the phagocyte has moved laterally on the surface of the coverslip and has left the uppermost pair of organisms untouched. The cluster of four organisms originally to the right of the cell has now been reached and two are in the process of phagocytosis. The two pneumococci first encountered are visible in the center of the cell, one on each side of a lobule of the nucleus. Time, 3.53 p.m. $\times$ 3350.

FIG. 10. After 4 minutes all six of the pneumococci reached by the phagocytes have passed into its cytoplasm. Only the two pneumococci which the leucocyte failed to touch remain outside of the cell. Time, 3.55 p.m. $\times$ 3350.

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## Plate 19

Figs. 11 to 14. Failure of leucocytes in a hanging drop to phagocytize encapsulated pneumococci in the absence of opsonin.

Fig. 11. Pneumococcus-leucocyte mixture in hanging drop containing no antibody. Motile phagocyte can be seen at edge of drop surrounded by pneumococci. Time, 4.02 p.m. × 3350.

Fig. 12. The phagocyte which is in contact with the surface of the coverslip, has changed shape and in so doing has pushed aside the adjacent pneumococci. Several dark granules are visible in the cytoplasm of the phagocyte. These granules are smaller than phagocyted organisms (compare with intracellular pneumococci in Figs. 10 and 20). No phagocytosis has occurred. Time, 4.03 p.m. × 3350.

Fig. 13. Phagocyte has extended a long pseudopod toward the edge of the drop and is plowing its way through the closely packed pneumococci. Although the cytoplasm of the cell is in direct contact with many of the pneumococci, none of them have been phagocyted. Time, 4.04 p.m. × 3350.

Fig. 14. Finally the pseudopod has pushed its way through the field of pneumococci without having phagocyted a single organism. Only the small granules originally seen in the cell are visible in the cytoplasm. Time, 4.05 p.m. × 3350.

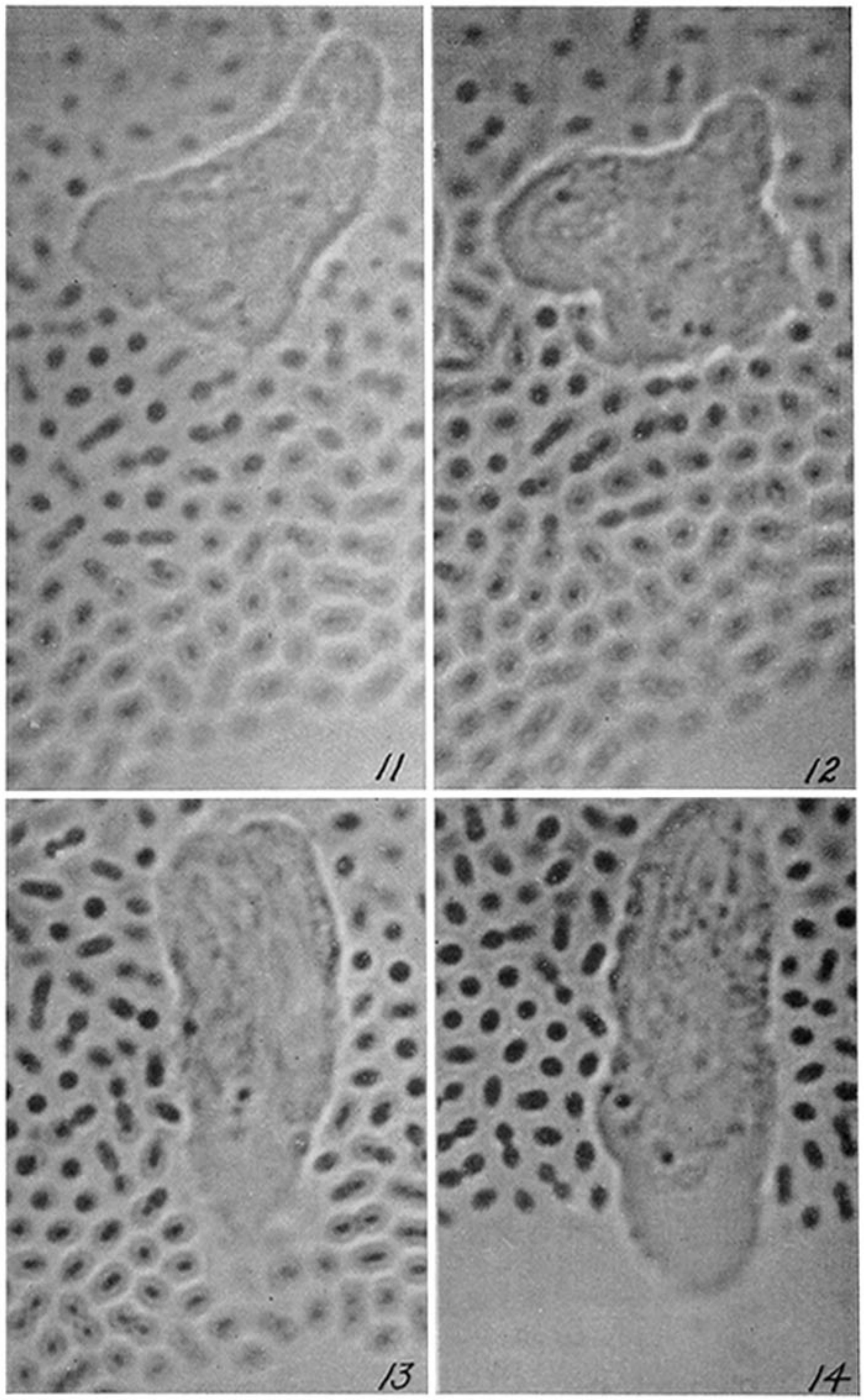


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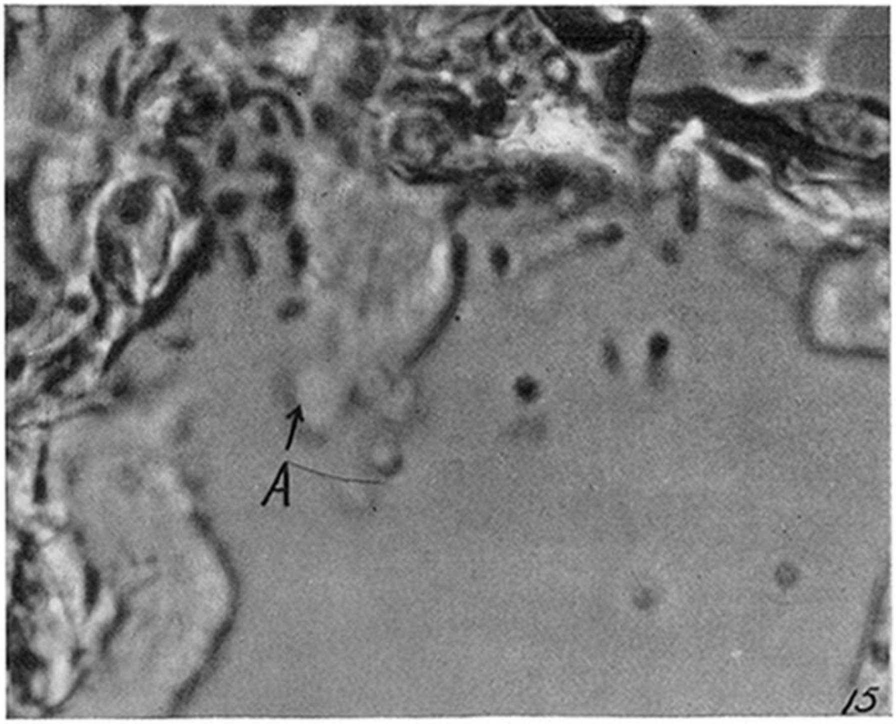
PLATE 20

FIGS. 15 and 16. "Surface phagocytosis" in lung.

FIG. 15. Direct visualization of surface phagocytosis in alveolus of fixed lung. Motile phagocytes and pneumococci are seen in lumen of alveolus. Cell *A* has already attached itself to the upper alveolar wall. Time, 3.50 p.m. × 3350.

FIG. 16. Cells *B* and *C* have migrated into field and have almost reached upper alveolar wall. They are beginning to trap pneumococci against alveolar surface. Cell *A* has closed over pneumococci in corner of alveolus and is pressing them into the extreme corner. Time, 3.53 p.m. × 3350.



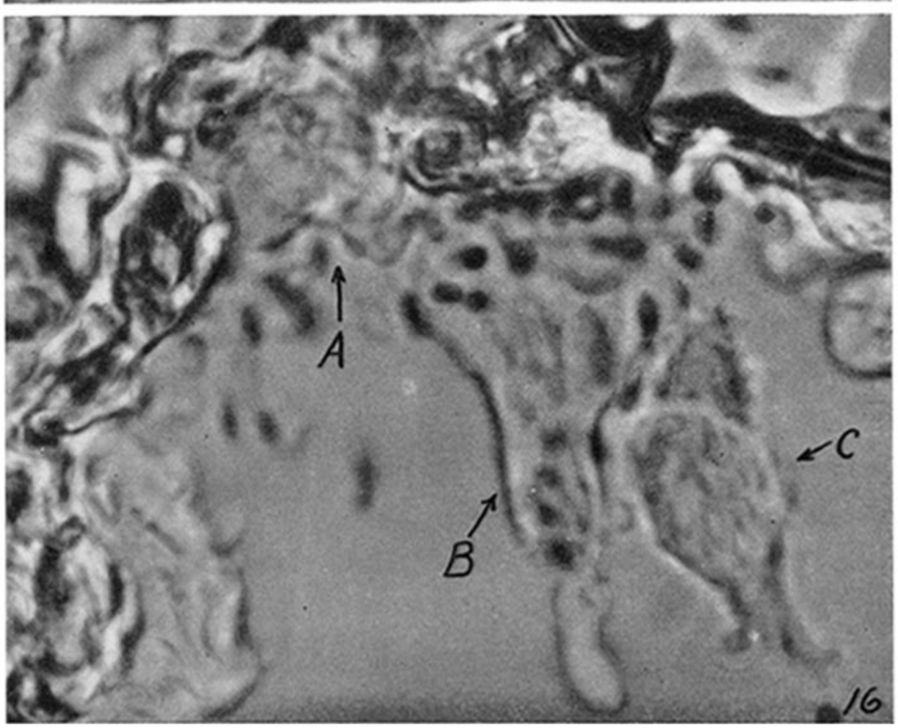




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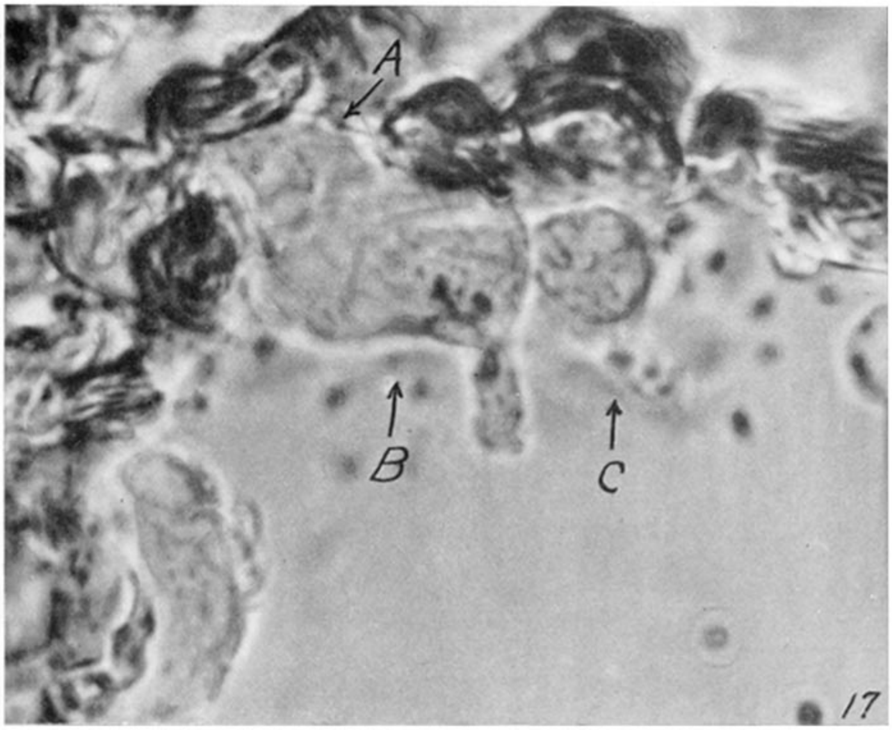
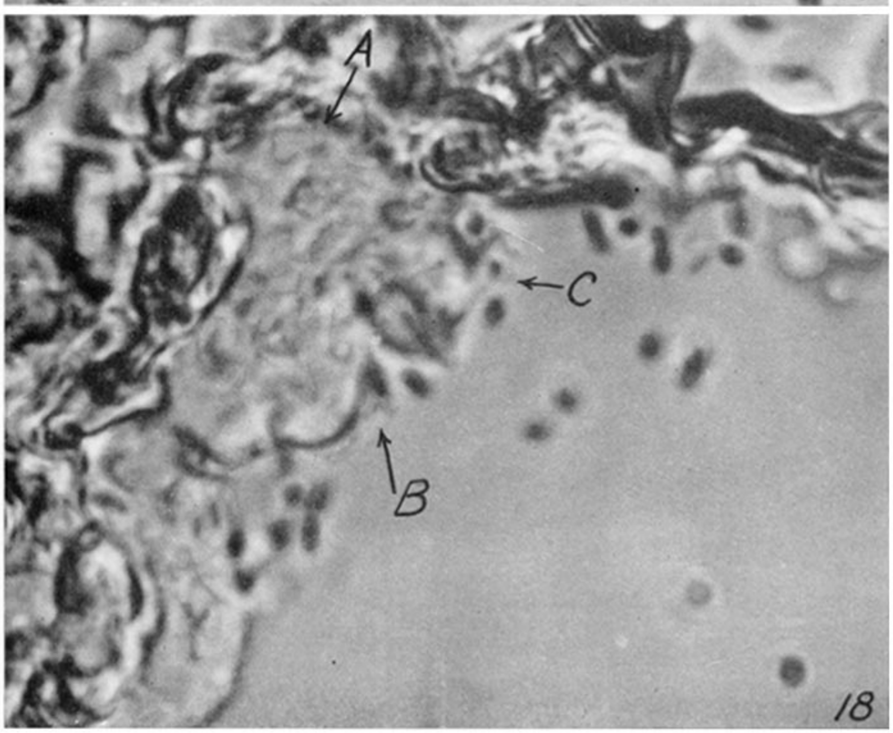
PLATE 21

FIGS. 17 and 18. "Surface phagocytosis" in lung (continued).

FIG. 17. Cells *B* and *C* have now attached themselves to upper wall and are migrating toward the corner still occupied by Cell *A*. They have trapped a large number of pneumococci against the alveolar wall. Time, 3.55 p.m. × 3255.

FIG. 18. All three cells are now crowded into the corner, and their respective cell outlines are no longer distinguishable. One pneumococcus can be seen already in the cytoplasm of Cell *C*. (Note halo about phagocyted organism.) Time, 3.57 p.m. × 3255.

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## PLATE 22

FIGS. 19 and 20. "Surface Phagocytosis" in lung (continued).

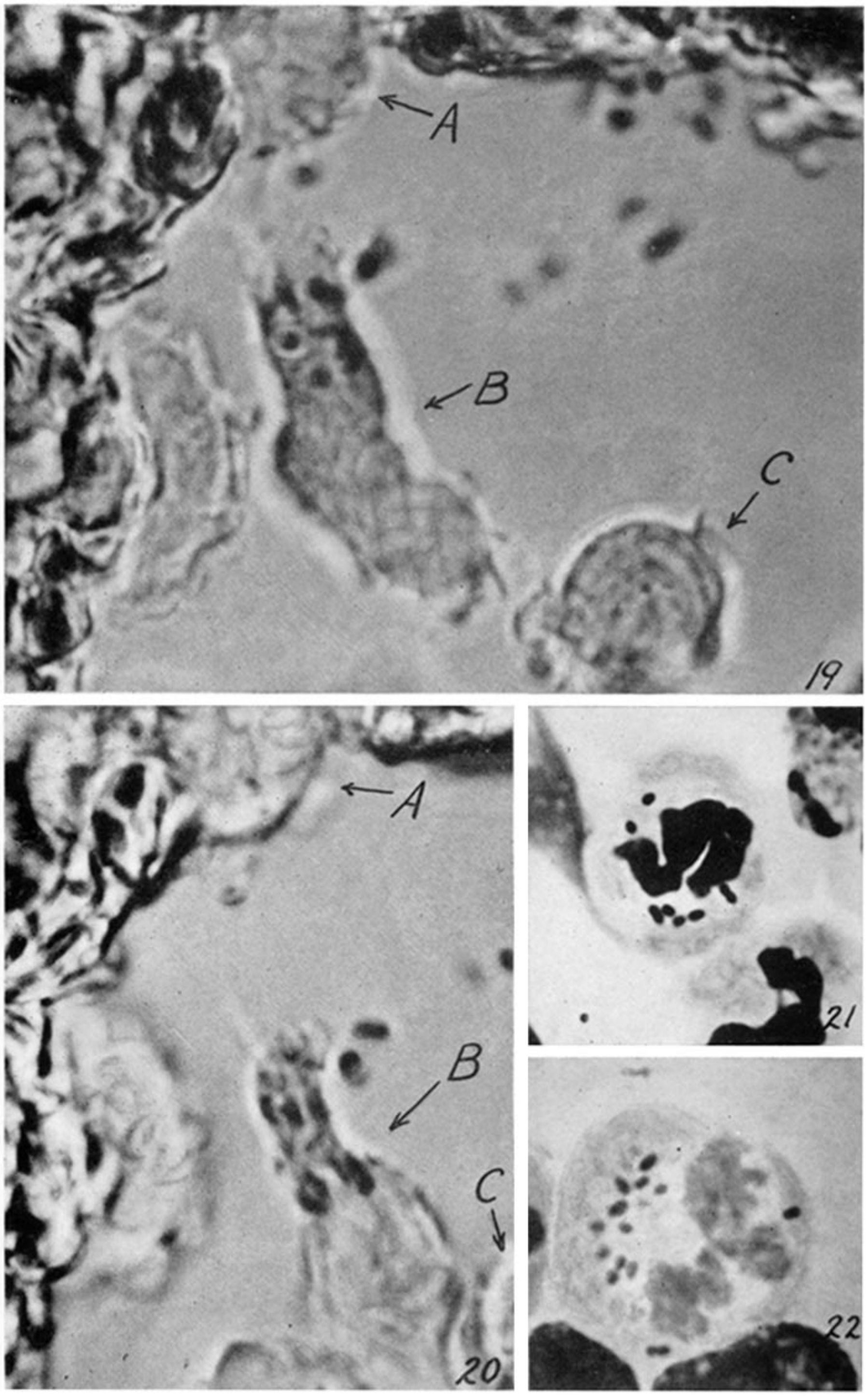
FIGS. 21 and 22. Intracellular digestion of pneumococci.

FIG. 19. Phagocytes *B* and *C* have broken away from the corner and are again migrating across the lumen of the alveolus. Four or five pneumococci previously trapped against the alveolar wall are now clearly visible in the cytoplasm of cell *B* and at least one organism is seen in cell *C*. Time, 4.01 p.m. $\times$ 3350.

FIG. 20. Phagocyte *B* continues to move downward across the alveolus carrying the phagocyted pneumococci in the hindmost portion of the cell. Time, 4.02 p.m. $\times$ 3350.

FIG. 21. Recently phagocyted pneumococci in cytoplasm of leucocyte. The organisms were phagocyted by the surface mechanism a few minutes before the preparation was fixed and stained with methylene blue. The pneumococci are clearly outlined in the cytoplasm of the cell and as yet show no signs of being digested. $\times$ 2375.

FIG. 22. Intracellular pneumococci having been phagocyted by surface mechanism 2 hours previously. Many of the organisms are only faintly outlined and have apparently been partially digested by the phagocyte during the period of incubation. Note shadow forms. $\times$ 2375.




(Wood *et al.*: Mechanism of recovery in pneumococcal pneumonia. IV)