STUDIES ON PNEUMOCOCCUS INFECTION
IN ANIMALS.

FIRST PAPER.*

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INTRODUCTION.

Lobar pneumonia is simply one phase of pneumococcus infection. Previous research has clearly established this even if it has failed to secure practical results in either prevention or cure of the disease. In order to understand adequately the conditions underlying the development, the prevention, and the cure of the disease, tradition, which has so long considered the lobar pneumonia of man to be a disease entity by itself, must be set aside and the broader conception of it as one form only of pneumococcus infection must be fully realized.

Experimental Pneumonia.—The results of the early attempts to induce lobar lesions in animals proved so unsatisfactory that in 1901, when the writer (1) first turned to the study of experimental pneumonia, the significance of the results of previous observers was not apparent. By experiments on the rabbit with the pneumococcus in which the conditions of tissue susceptibility and bacterial virulence were accurately controlled, varied, and balanced one against the other, it became evident, that it was not wholly the bacterial incitant, but the susceptibility of the host as well, that determined whether the disease processes would be bronchopneumonic, lobar, or bacteremic. In the light of these studies the failure of previous observers to obtain lobar lesions in susceptible animals, such as the mouse, guinea pig, and rabbit, became significant, and the importance of the relatively few successful experiments of Gamalka (2), in 1888, and of Tschistovitsch (3), in 1890, on more resistant animals such as the dog, were emphasized. These successful experiments Welch (4), in 1892, was unable to confirm, but they have been fully verified by the convincing results recently secured by Lamar and Meltzer (5).

The results of the researches of the writer and those of previous observers were fully recorded in 1904, and it is not necessary to review them here in detail. Let it suffice to say that this study clarified the broader conception of

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the etiological conditions underlying pneumococcus infection in general and of lobar pneumonia in particular.

In man, pneumococcus infection of the lung takes the lobar form; in very susceptible animals, such as the rabbit, the experimental infection gives rise to comparatively little lung reaction, and is chiefly bacteriemic, but when partially immunized these animals may develop extensive lesions of the lung. In man it is the exceptional conditions of an exalted virulence of the pneumococcus in the presence of a relative insusceptibility that determines the lobar form of the disease, characterized as it is by a specific or limited bacterial etiology and by distinct anatomic and symptomatic manifestations.

Recovery.—Previous study of the phenomena of recovery in the lobar pneumonia of man and in pneumococcus infection of animals has failed to reveal any fundamental differences. Even crisis, which in no other disease and in no other form of pneumococcus infection is so marked, so complete, or so frequent, is, after all, simply sudden recovery, and all attempts to discover in the crisis something apart from recovery in the ordinary sense have one and all failed. Nevertheless all of this study has contributed much valuable information to our knowledge of pneumococcus infection.

Pneumococcus Immunity.—The most significant results have been derived from the study of the protective mechanism in pneumococcus infection. Following the discovery of the relationship of the pneumococcus to pneumonia by Fraenkel (6) in Germany, Talamon (7) in France, and Salvioli (8) in Italy, these observers and many others, notably Foa and Bonome (9), Foa and Carbon (10), Behring and Nissen (11), Bonome (12), Foa and Bordoni-Uffreduzzi (13), Emmerich and Fowitsky (14), Kruse and Pansini (15), Tschistovitsch (16), and Arkharow (17), recognized and studied pneumococcus immunity. These and other more recent studies on the phenomena of immunity in general and of pneumococcus immunity in particular have revealed the several processes active during recovery although they have failed to establish their relationship to each other, or the practical significance of the protective mechanism as a whole.

A. Humoral Activities.—Theories of toxin and antitoxin neutralization were first suggested by Behring’s studies in diphtheria and Kitasato’s in tetanus, both of which studies were then new.

Behring and Nissen noted a slight bactericidal action on the pneumococcus in the blood sera of the more resistant animals after immunization, but in the blood sera of very susceptible animals this was even less marked. These observers considered pneumococcus immunity to be chiefly antitoxic and quite similar to that of tetanus and diphtheria. Similarly, by precipitation of forty-eight hour cultures of the pneumococcus, G. and F. Klemperer (17) obtained substances which were toxic in large doses. The toxic action of this “pneumotoxin” was neutralized by sera from immunized animals. These sera possessed also a curative action on mouse infections, but gave less definite results when used in the lobar pneumonias of man. Mosny (18) also concluded that the immunity in pneumococcus infections was due to antitoxins, and the recent studies of Tizzoni and Panichi (19) revert to these early interpretations of the action of pneumococcus immunity. But no true toxin has been demonstrated, and the presence of antitoxin has only been inferred. Hence, theories of toxin and antitoxin neutralization have aroused little interest in modern research.
The failure to find a soluble toxin in pneumococcus cultures has fostered the belief that the active substances were endotoxins. The endotoxins of cholera, typhoid, and many other organisms were readily demonstrated, but those of the pneumococcus have proved more elusive. In a recent and very complete summary of previous research, Kruse (20) concludes that active poisons from the pneumococcus have not been secured.

More recently, however, Rosenow (21) has described the toxic action of substances obtained at certain stages of the digestion of sera with autolyzed pneumococcus material. His results are extremely suggestive in the light of similar work by Dellezenne and Ledebt (22) on cobra venin. At certain stages of the digestion of sera with cobra venin substances were formed that proved 10,000 times as toxic as the venin alone. These observations not only open to investigation a large and extremely important field, but they broaden our conceptions of hypersusceptibility and of the action of toxin and endotoxin in the animal organism, so that in the light of future study, these may be brought into a closer and truer relation.

The significance in pneumonia of the phenomena first noted by Theobald Smith (23) and later more fully described by Rosenau and Anderson (24) as anaphylaxis has not as yet been fully determined. The experiments of Friedemann (25) and Friedberger (26), by showing that complement and immune body are consumed in anaphylaxis, suggest that this phenomenon is after all simply a phase of the intracellular chemistry of infection bearing on the action of toxins and endotoxins.

The distinction of toxin from endotoxin has been convenient and is generally although tentatively accepted, but whether or not it is fundamental will be decided when the chemistry of bacterial poisons is better known. That the differences are not necessarily fundamental is now more fully realized than it was when the distinctions were first drawn. Hence Bail's (27) attempts further to distinguish aggressins have received scant notice, despite Römer's (28) exposition of aggressin action in its relation to pneumococcus infection of the eye.

The failure to obtain significant results in the neutralization of pneumococcus poisons by the body fluids has encouraged the belief that the mechanism of recovery is chiefly bacteriolytic.

Bactericidal action was noted in certain sera by Behring and Nissen, and was considered an active factor in the destruction of the pneumococci during recovery. Foa, Carbone, Emmerich and Fowitsky, and Arkharow, all held similar views but failed to offer any very convincing experimental demonstration in support of their conclusions. Kruse and Pansini, in 1892, however, found that the pneumococcus when incubated at 37° C. developed as rapidly in immune as in normal sera. Nevertheless, Welch (29) in 1890 noted lysis of the pneumococcus within its capsule. This was described in more detail ten years later by Radziewsky (30) who used the Pfeiffer technique, and by Neufeld (31) who tested immune serum in the hanging drop.

Although the discoveries of Bordet (32) and Landsteiner (33) and the ingenious theories of Ehrlich have contributed much significant knowledge regarding the action of amboceptor and complement in the phenomena of bacteriolysis in general and of the cholera and typhoid organisms in particular,
comparatively little is known about the bacteriolysis of the pneumococcus save that it may be seen under the microscope and takes place in the tissues.

To what extent the results of tests with sera in vitro may be accepted as significant in interpreting what occurs in the tissues has never been definitely determined. Metchnikoff (34) claims that cytase does not exist free in the circulating plasma, but that it is liberated after injury or death of the leucocyte.

There is a long list of observations, which it is scarcely necessary to review. Certain of these show that lysis of foreign red cells injected into the veins of immunized animals give rise to hematuria and then suggest the presence of cytase in the plasma. Other experiments, however, indicate that cytase is not present in the aqueous humor until after injury of the eye, and the experiments of Pettersson (35) and others show that in plasma obtained from centrifuged blood, cytase is absent or present in small quantities only. None of these experiments, however, accurately represent conditions actually present in the circulating blood during infection.

Cytase may not be present in the circulating blood plasma under normal conditions, but in infectious disease there is a varying amount of injury. It may well be that in immunity, the chemical affinities between amboceptor—known to be present in the plasma—and its antigen, the bacterial cell, suffice to free the cytase which under normal conditions is held in the cells. Whether or not this liberation of cytase would constitute an injury in Metchnikoff’s sense is a question of no great practical importance at present.

In the experiments of Hoessli (36) and of Dold (37), whole blood and blood plasma were definitely more bactericidal for the pneumococcus in vitro than the corresponding sera. Whether the reactions concerned were those of specific amboceptor and complement was not determined.

Although complete experimental evidence of the action of amboceptor and complement is lacking, lysis of the pneumococcus as described by Welch, by Radziewsky, and by Neufeld, resembles very closely that of other bacteria and there is no reason to believe that a fundamental difference exists.

B. Cellular Activities.—Kruse and Pansini found that although the pneumococcus in the test-tube grew readily in immune sera, in the tissues during recovery its growth was inhibited and the pneumococcus cells were taken up by the phagocytes. In their experiments this phagocytosis was increased by the introduction of immune sera. Both Memes (38) and Issaef (39) (in Metchnikoff’s laboratory) found that immune sera favored phagocytosis. Issaef thought this was due to the action of substances stimulating the leucocytes to greater activity, a view now supplanted by the more accurate conceptions of opsonification which originated in the work of Denys and Leclef (40). These conceptions have been more fully elucidated by the work of Wright and Douglas (41), of Neufeld (42), and of Hektoen (43). These observers have shown that immune serum acts on the bacterial cell rendering it more subject to phagocytosis, that the reaction is to a certain extent specific, and that it is also complex, since complement is to some extent a factor.

Virulent strains of the pneumococcus, however, were found to be quite as insusceptible to the opsonic action of immune serum, as they were to the bactericidal action. From cultures of other organisms Van de Velde (44), Neisser and Wechsberg (45), Bail (46), von Lingelsheim (47), and others
obtained the so-called “leucocidins” which were toxic for the leucocytes. Similarly from virulent pneumococci both Rosenow (48) and Tschistovitsch (49) obtained extractions which they described independently as “virulins” and “antiplagins” and to the presence of which they ascribe the failure of the leucocytes to take up virulent strains of the pneumococcus. Non-virulent pneumococci and the virulent pneumococcus cells after extraction under certain conditions were found to be subject to the opsonic action of serum. But as might well be expected the study of phagocytosis under these conditions has proved of doubtful significance in interpreting the conditions underlying the phagocytosis of the pneumococcus in infectious processes.

Wright’s ingenious methods opened up the promising study of the opsonic activity of the serum of pneumonia patients, and with this subject research has recently had much to do. Neufeld (50), Rosenow (51), Potter and Krumwiede (52), and others have found that the opsonic activity of the blood serum and the phagocytic activity of the leucocytes were increased in pneumonia, but Seligmann and Kloftstock (53), Boettcher (54), and Strouse (55) were unable to confirm the claim that this increase is a material one.

Insuperable technical difficulties account for much variation in the determination of opsonic indices. These Wright and Hektoen have not fully recognized. But North (56), Park (57), and others have demonstrated many of the errors in the study of the opsonic index at the bedside.

The writer’s suspicions were first aroused when normal serum seemingly proved more actively opsonic for the staphylococcus than that of a highly immunized animal. By experiment, this was found to be due to the agglutinative action of the immune serum and the writer called attention to this error at the meeting of the Roosevelt Alumni Association of New York, in January, 1907. Similarly at a meeting of the Association of American Physicians in June, 1907, Baldwin noted the fact that the agglutinative action of sera was a

A very dense suspension of staphylococci was added to mixtures of normal and immune sera with the leucocytes in equal quantities and incubated at 37°C. The phagocytic index of the normal serum was 5.18 in ten minutes, and 10.86 in twenty minutes. That of the immune serum was 2.22 in ten minutes, and 23.66 in twenty minutes.

These results were due to the agglutination present in the immune sera. As shown by examination of the smears the clumping at first interfered with phagocytosis, then later greatly facilitated it, because the clumping often took place about the leucocytes.

It is not easy to estimate accurately the number of organisms the phagocytes take up. In the mixtures of leucocytes, bacteria, and immune sera, bacteria will stick to the leucocytes. This may be due to chance contact or to agglutinative action, but it is of frequent occurrence and cannot be accurately distinguished from phagocytosis. Bacteria in vacuoles of the cytoplasm and bacteria undergoing degeneration are readily recognized as intracellular, but when the leucocytes are well spread out and dried, and when the cytoplasm is faintly stained as a thin film, it is impossible to determine whether the bacteria are inside, above, or below the cell. These are real difficulties and they are here emphasized because they have received scant notice.
factor in estimating opsonic indices with tubercle bacilli. But North, at the same meeting, presented statistics showing the magnitude of the error. He sent to ten laboratories active in opsonic work sera known only to himself, and through their cooperation found that the error of these experienced workers averaged 45.5 per cent. The minimum error was 24 per cent.

Since these early investigations, the technique has been improved. This has doubtless diminished the error, but it has also rendered the method laborious without correcting the more serious difficulties. Hence the significance of the opsonic index is doubtful at best and the study of it has failed to add to our knowledge of phagocytosis in pneumonia. Study of the lung exudate, however, has given much more significant results.

Tschistovitsch (3), in 1904, found that in the experimental pneumonia of the dog phagocytosis was marked. He cites the early experiments of Patella (58) who, by paracentesis of the pneumonic lung of man, found that the pneumococci disappeared very rapidly and were rarely present after the crisis. Thus, Tschistovitsch thought the crisis was due to phagocytosis, and many of the more recent observers favor this view.

Other phases of cellular immunity more or less closely related to phagocytosis have been considered in their relation to the recovery from pneumococcus infection. Substances of a bactericidal nature have been often found in the leukocytes; for example, in Buchner's (59) early work and in the recent investigations of Pettersson (60), Wassermann (61) demonstrated the fact that of all the tissues of the immunized animal the bone marrow was the most actively protective against pneumococcus infection. Hiss (62), from his study of the protective and curative action of aqueous extracts of rabbit leukocytes, suggests that pneumococcus immunity is due to the presence of protective substances in the body cells which are liberated only under certain conditions and are not usually present in the body fluids.

After reviewing previous work Flexner (63) turns for new light to another phase of the intracellular chemistry of infection, introducing Lamar's study of the action of the unsaturated fatty acids and alkaline soaps present in exudates. Lamar (64) found that the presence of these substances in the test-tube and in the tissues during infection increased the action of immune sera on the pneumococcus, but the practical significance of this is not as yet apparent.

Up to the present, research has wavered between humoral and cellular conceptions of immunity, turning first to one then to another of the various phenomena of pneumococcus infection in the attempt to determine the conditions underlying recovery in order that practical results might be achieved in the cure of pneumonia in man. Focused on a part, this study has overlooked the protective mechanism as a whole in its broader and simpler phases, but out of this chaos some order must be established.

I have carried out many closely related experiments, but give in this paper only the ones that deal with the action of pneumococcus.
Studies on Pneumococcus Infection in Animals.

cultures on animal tissues, and the action of immune sera on the pneumococcus.

ACTION OF THE PNEUMOCOCCUS ON ANIMAL TISSUES.

The several types of tissue reaction which living cultures of the pneumococcus incite in different animals have been too fully described in the experiments of others and in those of the writer (1) to warrant further consideration.

Tissue reaction, whether in the form of local lesions or of cellular injury throughout the animal organism, is due to the poisons of the infectious agents. These poisons may be set free in soluble, diffusable form, or they may be so fixed in the bacterial cell that they become soluble only after lysis has taken place.

Dead Cultures.—In order to determine the nature of the pneumococcus poisons, attempts were made to induce the types of lesions obtained with living pneumococci by the introduction of dead culture material. Lesions of this kind have been produced by the introduction of the dead cultures of certain organisms. Roux and Yersin (65), Behring (66), and Kitasato (67) induced characteristic disease processes by the inoculation of culture filtrates free from tetanus and diphtheria organisms. With dead tubercle bacilli Prudden (68) and others obtained typical tubercular lesions in the lung.

In my experiments culture filtrates and masses of dead pneumococcus cells were injected into the veins, under the skin, intraperitoneally, and through the trachea of rabbits.

Whereas intravenous and subcutaneous inoculations of dead typhoid, dysentery, cholera, and other organisms have proved highly toxic and often fatal, pneumococcus material, in my experiments as in those of others, gave rise to little reaction in the animal organisms.

Culture filtrates of the most virulent organisms in doses of ten cubic centimeters caused little or no disturbance. The animals invariably made an immediate recovery which differed apparently in no respect from that of control animals that received similar quantities of sterile uninoculated broth. And yet, following the introduction of these culture filtrates of the pneumococcus, the ani-
mals developed an immunity to virulent pneumococci not present in the controls.

Concentrated Culture Material—Dense suspensions of pneumococcus cells proved but little more toxic than the culture filtrates. Evanescent temperature reactions were seldom noted. Continued frequent inoculation of this material caused emaciation and ultimately death, but no significant lesions were found at autopsy. Usually, however, the animals recovered rapidly after these inoculations and were then immune to virulent living cultures. Although the concentrated cultures gave rise to more disturbance in the animal organism, the culture filtrates incited more quickly and more certainly the immunity to living cultures.

In order to measure the degree of immunity incited after intravenous, subcutaneous, and intraperitoneal inoculation, the following experiment was made with dead culture material:

Six rabbits received an immunizing dose of 5 c.c. of pneumococcus culture material, two were inoculated intravenously, two subcutaneously, and two intraperitoneally. Eight days later one animal of each set was inoculated intravenously with 1 c.c. of virulent culture, the other with 0.5 c.c. At the same time two normal rabbits were similarly inoculated.

The controls died in less than twenty hours, but both of the animals immunized by intravenous inoculation survived, the virulent culture causing little or no disturbance in these animals. The animals immunized by subcutaneous inoculation died, one in three days, the other in eleven. Of the two immunized by intraperitoneal inoculation, one died and the other recovered. Intravenous inoculation was, therefore, much the most effective in producing immunity, but neither intravenous nor subcutaneous inoculation of dead pneumococcus culture material gave rise to significant lesions in the animal tissues.

Intratracheal Injection.—Owing to its structure the lung is apparently as well adapted for the accumulation of exudate as it is to its absorption. In order to determine the effect of dead pneumococcus culture material on the lung tissues intratracheal injections were made.

Five to ten cubic centimeters, depending on the size of the rabbit, is the maximum quantity that can be injected into the lung through

\[\text{This material was obtained by precipitating a twenty-four hour pneumococcus broth culture with ammonium sulphate. The precipitate after complete dialysis became partially soluble, leaving only a little suspended material in the fluid. This fluid was filtered through a Berkefeld candle and the filtrate used for the immunization.}\]
Studies on Pneumococcus Infection in Animals.

the trachea without regurgitation or great danger of a contaminating infection from the upper air passages.

In a series of rabbits, 5 c.c. of virulent culture, heated to 52° C. for thirty minutes to kill the pneumococci, failed to incite lung lesions. In fact the absorption of the foreign material and the return of the tissue to normal took place more quickly in the lung than in the subcutaneous tissues.

In another experiment (March 4, 1901) I injected intratracheally culture sediment and, for purposes of comparison, the decanted clear fluid of these cultures. The 6 rabbits receiving 10 c.c. of fluid free from pneumococcus cells, were killed on the third and fourth days. The lungs were normal. The 6 rabbits receiving 2, 2, 2, 3, 3, and 8 c.c. of the dead pneumococcus cells, were killed and autopsied two and four days after the intratracheal injection. The lungs and viscera were sterile. Irregular areas of bronchopneumonic consolidation were found about the large bronchus of the left lower lobe, to which the fluid had been directed. The extent of lung reaction depended upon the quantity injected. Microscopical examination of the lung showed atelectasis, induration with cellular infiltration of the walls of the air spaces, and some little exudation and exfoliation of cells into the air spaces; in short, a foreign body pneumonia.

In testing the power of the lungs to dispose of various solutions and suspensions, 10 rabbits (October 23, 1901) received intratracheal injections of 5 c.c. each of the following substances: sterile 3 per cent. peptone meat infusion broth, decanted culture fluid free from pneumococcus cells, culture sediment washed and suspended in salt solution, scrapings of a Berkefeld filter suspended in salt solution and sterilized; and a salt solution suspension of the scrapings of a filter that had been heated to 52° C. for thirty minutes after a liter of pneumococcus culture had passed through the filter. In forty hours the animals were killed and autopsied. The lungs and viscera were sterile and no significant lesions were found.

Neufeld (31) had found that rabbit's bile in a dilution of 1 to 20 parts of culture dissolved the pneumococcus cell. Accordingly concentrated mass cultures of pneumococcus cells were treated with bile in this dilution and injected through the trachea of 2 rabbits in doses of 7 and 8 c.c. Marked dyspnea developed rapidly in both animals and one died in twenty-four hours. The other recovered, but was killed and autopsied on the third day.

Marked lesions were found in the lungs of the animal that died. But they were also found in controls injected with dilutions of bile in sterile broth. The lungs and other organs of these animals were sterile.

Similarly extracts of the pneumococcus cell without bile were tested. A concentrated culture sediment was suspended in 17 per cent. salt solution, allowed to stand over night in the ice box, and was then diluted with 20 volumes of water and filtered. The filtrates contain specific precipitable substances as described in a former paper (69), but when injected through the trachea these extracts caused no disturbance whatever.

Summary of the Studies on the Action of Dead Pneumococcus Material on Animal Tissues.—From the results of these exper-
ments it is evident that dead cultures of the pneumococcus do not contain the active poisons elaborated by the organism in infection. In no instance by subcutaneous, intravenous, intraperitoneal, or intratracheal inoculation were characteristic lesions obtained. Nor was this dead culture material toxic to any demonstrable extent. And yet, the animals after inoculation acquired an immunity to pneumococcus infection, the immunity being more marked after intravenous inoculation.

From this it is apparent that pneumococcus cultures contain altered or degenerated poison, possibly comparable to the toxons or toxoids of diphtheria cultures, or substances, possibly enzymes, such as are present in snake venin, which when liberated in infection give rise to more powerful poisons, but when formed in culture are active only as antigen. These substances were present in the pneumococcus cells free from culture products, but the culture filtrates contained proportionately greater quantities. Apart from this, the results of these experiments add little to our knowledge of the pneumococcus poisons, and further light was obtained only indirectly from a study of pneumococcus immunity.

**ACTION OF IMMUNE SERA ON THE PNEUMOCOCCUS.**

That a protective mechanism brings about the recovery from pneumococcus infection is manifest, and in the crisis of lobar pneumonia in man this is particularly evident. And yet, in the previous studies of pneumococcus immunity immune sera have lacked bactericidal action, and phagocytosis was exceptional.

In the hope of determining experimentally why this is so, the following studies on agglutination, bacteriolysis, and phagocytosis of the pneumococcus were made.

*Studies on Agglutination.*—In a former paper (69) the technical difficulties and improved methods of securing agglutination of the pneumococcus in high dilution were fully recorded. By substituting fine suspensions of centrifugalized pneumococcus cells in normal salt solution for the ordinary broth cultures it was possible to obtain positive reactions even after diluting sera obtained from immunized animals and from pneumonia patients.
Forty-six cases of pneumonia were examined. These included one case complicated by empyema and one case of pneumonia in a patient with phthisis. For purposes of control, three cases of nephritis, three of gas poisoning, five of endocarditis and rheumatism, and one each of cerebrospinal meningitis, tonsillitis, and alcoholism were studied.

In all the controls except three, agglutination took place in a dilution of 1:10 or 1:15. Serum from one case of gas poisoning failed to agglutinate, but in another case and in the case of cerebrospinal meningitis, agglutination was noted in dilutions of 1:40.

In the sera from pneumonia patients the agglutination varied from 1:15 up to 1:300. The maximum agglutination was recorded in thirty-eight cases, and the average as estimated from these was 1:77. In fatal cases agglutination was occasionally low, but often it was above the average. In sera obtained after the crisis the agglutination was not significantly increased; in fact one of these agglutinated only in a dilution of 1:20. In both favorable and unfavorable cases the agglutination was below the average. Hence prognostic and diagnostic deductions could not safely be drawn from these results alone.

Pneumococcus agglutination in pneumonia, like that of the typhoid bacillus in enteric fever, varies greatly and in a similar way. It is, however, much less marked, and is, therefore, more difficult to determine accurately. But since in pneumonia bedside data usually suffice for both diagnosis and prognosis, there is little need of it for the present. But should an efficient serum therapy be secured, a specific bacterial diagnosis may be required in exceptional cases.

The results of this study of the agglutination of the pneumococcus in the blood serum of pneumonia patients were thus of comparatively little practical significance.

Studies on Bacteriolysis.—Two of the sera from the cases of pneumonia were markedly lytic. This, however, developed only in the lowest dilutions and was not present in the sera from any of the other forty-four cases. Accordingly I turned to the serum of immunized animals for a more satisfactory demonstration of the phenomena and the conditions under which it takes place. Lysis of
the pneumococcus, as described by Welch, by Radziewsky, and by Neufeld, was readily seen in the hanging drop of serum from a highly immunized rabbit. The encapsulated pneumococci swell, refract the light less and less, until finally the bacterial cell disappears within its capsule. On staining the cells with capsule stains, the capsules were found empty. This lysis, however, was rarely complete, and when studied at 37°C was invariably followed by rapid growth of the surviving pneumococci.

The activity of the different sera varied and the cultures of pneumococci differed in susceptibility. Highly virulent organisms were less susceptible. One strain, which by passage from rabbit to rabbit had been under parasitic conditions for nearly two years, was exceptionally resistant to the lytic action of sera. But no very precise estimate of this could be made.

The observer of lysis of the pneumococcus is forced to rely on impressions drawn from microscopical appearances rather than on figures. The presence of agglutination in this as in other tests of bacteriolysis is confusing when estimates are made by plating and counting colonies. But apart from this the pneumococcus often fails to grow in agar plates, and one strain, cited above, owing to its parasitic adaptation failed to grow even on the surface of agar plates, when no serum was present. Hence attempts to measure the variations in so fleeting and uncertain a phenomenon as lysis of the pneumococcus in vitro have failed.

Lysis of the pneumococcus in immune sera is slight compared with that of the organisms of cholera or of typhoid fever. This is scarcely due to structural peculiarities because in dilute rabbit's bile and even in culture the pneumococcus is exceptionally susceptible to lysis. It is in fact extremely sensitive to conditions which are in the least degree unfavorable. But in the blood sera conditions are evidently most favorable for the pneumococcus and growth takes place, whereas for the typhoid and chol-

3 In one test of the bactericidal action of different sera upon this organism, the streaks on the surface of agar plate gave growth only in the immune and normal sera, the salt solution controls being sterile. As subsequently tested, using serum agar plates, slight bactericidal action was found in certain of the immune sera, but the destruction of the pneumococci was quite as marked in the salt solution controls.
era organisms sera are not so favorable. For these two types of organisms conditions are evidently reversed, and adaptation of the cell metabolism and growth seem to be the determining factors of lysis of the pneumococcus. Thus, under the ordinary conditions of procedure, my study of the lytic action of immune sera has been barren of significance.

In interpreting conditions present in infection, the significance of tests with sera in vitro has never been satisfactorily determined. This doubtless varies in the different bacterial infections. But it is certain that during infection lysis of the pneumococcus takes place in the tissues.

Following prolonged bacteriemia the rabbit often recovers by a crisis similar to that of man. Although it is of course difficult to be sure that a sudden fall in temperature with marked betterment in the animal's condition is really crisis, this was successfully diagnosed in a few instances, and the animals were killed when the temperature fell to normal. At autopsy few pneumococci were found and often the cultures were sterile. In two animals empty capsules and other signs of lysis, but with no evidence of phagocytosis, were found in the pericardial fluid.

After intravenous inoculation when the cultures are not excessively virulent, there is for a short interval a marked destruction of the pneumococci. This destruction is extracellular and entirely bacteriolytic. At least no signs of phagocytosis are to be found.

Evidently in the tissues, conditions are present which do not exist in the test-tube, for in the tissues the activity of the pneumococcus is inhibited in varying degree and during recovery lysis takes place. An attempt was accordingly made to secure in the test-tube conditions comparable with those in infection. Inasmuch as we may conceive that the development of the pneumococcus is retarded in infection by the febrile temperatures, an attempt to secure experimental evidence of this was made. The results of these studies, however, require separate consideration and will be given in detail in another paper. Let it suffice here to note that in twenty-five strains of the pneumococcus, growth was completely inhibited between 40.5° C. and 41.2° C., temperatures often attained or exceeded in the pneumonia patient. When tests of the bacteri-
cidal action of blood sera and exudates were made at temperatures above this, lysis was immediate, marked, and often complete in a few hours. In certain of the most active sera subcultures on plates indicated that lysis was more marked at room temperature than at incubator temperature, for incubator temperature so favored growth that the fleeting lysis was quickly obscured.

In interpreting the significance of these results several factors had to be considered. The most important of these were, (1) simple autolysis of the pneumococcus cell which took place in broth and in salt solution, and (2) inhibition of growth, the latter being apparently the dominant factor.

Summary of the Studies on Bacteriolysis.—It is evident from these experiments that the blood sera of immunized animals vary greatly in their action, but that this variation is one of degree. When sera fail to cause complete lysis no criterion is left by which to measure their activity. In the presence of conditions favoring the growth of the pneumococcus, incomplete lysis permits development of the surviving organisms and whatever action the sera may possess is quickly neutralized. But when by raising or lowering the temperature, growth is prevented, the bacteriolytic action of the sera is marked and often complete. By these means, then, bacteriolysis of the pneumococcus may be demonstrated outside the body in the sera of immunized animals, but how important it is in the tissues during recovery from infection, is still to be determined.

Studies on Phagocytosis.—The destruction of the pneumococcus, however, is not entirely extracellular. Phagocytosis has been recognized and considered an important factor in the recovery of both man and animals. But phagocytosis in pneumococcus infection is apparently an extremely variable phenomenon and previous research has failed to determine the conditions under which it takes place.

Precise determination of the practical significance of phagocytosis has been difficult. Two procedures have been tried. In one of these the leucocytes were examined during infection. In this way the presence of phagocytosis was established directly as in the experiments of Kruse and Pansini (15) and of Tschistovitsch (3).
In the other procedure the opsonic action of the blood sera was studied by Wright's methods, the difficulties of which have already been pointed out in the review of previous work.

Opsonic Action of Immune Sera.—Although technical errors in Wright's methods have robbed the opsonic index of its practical significance at the bedside, under accurately controlled conditions of experiment in the laboratory important qualitative determinations have been made and certain of the quantitative estimates have proved extremely suggestive.

Phagocytosis in Vitro.—In the following experiments the pneumococci were usually grown for twenty-four hours in broth, but occasionally on agar, then centrifuged and suspended in salt solution. After centrifugation leucocytes were obtained from the blood of dogs, rabbits, and man, or from the washed pleural exudates of rabbits injected with aleuronat. The mixtures with the sera to be tested were made in equal quantities in the small agglutination tubes. Occasionally the capillary pipettes of Wright were substituted. The bath in which the capillary or other tubes containing the mixtures were immersed was especially designed to insure accurate exposure. The same culture and leucocytes were invariably employed throughout a given experiment. Counts were made of 100 cells, this being adequate for the purposes of the observation. The conditions of experiment thus afford a minimum of error.

It is scarcely necessary to give in detail the long list of my experiments by which it was definitely ascertained that virulent pneumococci are practically insusceptible to phagocytosis. Streptococci and staphylococci were readily taken up, but the pneumococci were not. Typhoid bacilli were taken up and completely digested in ten to twenty minutes, whereas in twenty-four hours the pneumococci had multiplied and none were found to be intracellular.

These facts are now well known and have been established by numerous observers since the beginning of this work. Attempts to secure more active sera failed to alter the result. In some instances agglutination about the leucocytes was noted, and pneumococci

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were seen stuck to the leucocytes, but few, if any, were inside the
cells.

Hektoen considers insusceptibility to phagocytosis an indication
of virulence and suggests the phagocytic index as a measure of
virulence. In my experiments with the pneumococcus, virulence
was lowered without an increase in susceptibility to phagocytosis,
and the phagocytic susceptibility of different strains varied more
than their virulence. Thus one species uniformly insusceptible to
phagocytosis and of high virulence for the rabbit was allowed to
deteriorate during cultivation on media until five cubic centimeters
failed to kill a normal rabbit. Up to this point these organisms
maintained their insusceptibility. Other strains of the pneumo-
coccus of moderate virulence for rabbits were found which were
taken up by the leucocytes.

Streptococci are subject to phagocytosis, and intermediate forms
of pneumococci and streptococci doubtless exhibit varying degrees
of susceptibility and insusceptibility to phagocytosis just as they
do in other characters and reactions. In my experiments it is cer-
tain that different strains of pneumococci varied in their insuscep-
tibility to phagocytosis and that this variation was not wholly de-
pendent on virulence as tested on rabbits.

In an attempt to learn something of the nature of this insuscepti-
bility of virulent pneumococci to phagocytosis, the effect upon
staphylococcus phagocytosis of the presence of cultures of the pneu-
 mococcus was studied.

After twenty-four hours at 37°C broth cultures of virulent
pneumococci taken from a dead rabbit were centrifugalized. The
clear culture, and for comparison uninoculated sterile broth, were
added to dog leucocytes and incubated for two hours at 37°C.,
when, after centrifugalizing and decanting, the leucocytes were
added to mixtures of the staphylococcus in its immune sera. In
ten minutes the leucocytes that had been exposed to sterile broth
gave a phagocytic index of 1.44, as compared with 0.76 for the leu-
cocytes exposed to pneumococcus culture fluid.

These were often seen equatorially placed outside the cell limits so that the
extracellular position was easily made out.

Atypical pneumococci of little virulence for the rabbit were found later
which to some extent were taken up by the leucocytes.
Similarly in another experiment, to three tubes, each containing sediment of pneumococcus broth cultures and dog leucocytes, were added (always in equal quantities), in one instance salt solution, in another normal rabbit serum, and in another the serum of a rabbit immunized to the pneumococcus. After an hour's incubation at $37^\circ$ C. the leucocytes were obtained by centrifugalizing and decanting the supernatant fluid. The leucocytes from these three tubes were then mixed in equal quantities with staphylococci and staphylococcus immune serum. In ten minutes the leucocytes exposed to pneumococcus sediment and salt solution gave an index of 0.6; those exposed to sediment and normal rabbit serum, 1.5; and those exposed to sediment and pneumococcus immune serum, 2.14.

Again, in still another experiment dog leucocytes were placed in three tubes. To one tube was added a salt solution suspension of a pneumococcus culture from agar and to the second a pneumococcus culture in pneumococcus immune serum. The first two tubes were heated to $37^\circ$ C. for four hours. The third tube was not heated. Then tubes 1, 2, and 3 were tested with staphylococci and normal rabbit serum, and gave indices of 0.43, 0.96, and 3.08, respectively.

From these experiments it is evident that there are substances in pneumococcus cultures which act on the leucocyte diminishing its phagocytic activity. These substances are present in the culture fluid free from the cells, and in the pneumococcus cells suspended in salt solution. The action of these substances is, in part at least, neutralized by serum of the rabbit immunized to the pneumococcus. If the determination of phagocytic indices were accurate, these experiments would offer an indirect way of measuring the activity of immune sera in neutralizing pneumococcus leucocidins, but the technical error is too great.

Evidence of the toxic action of the pneumococcus growth was also found in the marked degeneration of the leucocytes in the mixtures of pneumococci, leucocytes, and sera. In these the degeneration was less marked when homologous immune sera were present. Later the disintegration of the leucocytes in infected rabbits was noted.

Although immune sera had partially neutralized the toxic action of pneumococcus cultures for leucocytes when these were tested
by staphylococcus phagocytosis, it failed to bring about the phagocytosis of the virulent pneumococci. It was thought that this might be due to growth of the pneumococci in the test mixtures or to the fact that the toxic substances retained in the pneumococcus cell were not neutralized. Accordingly, the study of phagocytosis was conducted at elevated temperatures which not only inhibited growth of the pneumococcus, but, as determined by tests with the staphylococcus phagocytosis, increased materially the activity of the leucocytes. Nevertheless, phagocytosis of virulent pneumococci failed to take place even under these conditions.

Since virulent pneumococci killed by heat or suspended in salt solution were not taken up by the leucocytes, and since elevation of temperature preventing growth failed to bring about phagocytosis, it is evident that the insusceptibility of these pneumococci is due to the retention in the bacterial cell of substances which are to a certain extent thermostabile and not completely neutralized by immune sera.

Under certain conditions in the tissues of infected animals, however, the action of these substances is neutralized and phagocytosis takes places as was early recognized by many other observers in the study of pneumococcus infection.

Phagocytosis in Vivo.—In experiments on pneumonic infection I injected pneumococci of varying grades of virulence through the trachea of normal and immunized rabbits. Varying degrees of lung reaction were incited, but in no stages of the lesions were signs of phagocytosis seen. In the earliest stages pneumococci were present in large numbers, but none were intracellular. In later stages no bacteria were seen. In the animals that recovered, the pneumococci disappeared with astonishing rapidity, suggesting an extremely efficient protective mechanism in the local lesion, adequate in itself, and yet wholly independent of phagocytosis.

The lung lesion, however, marks only a part of the infectious process in pneumonia, and possibly a minor part. The bacteriemia is rarely absent in man. In order to determine the presence or absence of phagocytosis in the bacteriemic processes, the technique had to be modified.

It was found that the leucocytes of the circulating blood might
very easily be examined and that many of the difficulties and errors in the methods of study in vitro were here obviated. The blood was drawn from the ear vein into citrate solution and centrifuged. This was done at low speed to avoid throwing down the pneumococci on the leucocytes.

The leucocytes were pipetted into salt solution, centrifuged again, and examined in smears for signs of phagocytosis.

To prove that the presence of phagocytosis in the circulating blood could be demonstrated by this method, dense suspensions of the staphylococcus in salt solution were injected into an ear vein of the rabbit. In the blood taken from the other ear ten and twenty minutes later, the staphylococci were found to be intracellular. There were no signs of agglutination present, and the bacteria did not adhere to the leucocytes.

Employing this method for the study of pneumococcus bacteremia in the rabbit, a large number of the examinations were made at various stages of the infection, early and late, but these revealed no signs of phagocytosis. Moreover, virulent organisms concentrated and suspended in salt solution, or in normal or immune sera, or killed by heat at 52° C. and injected into the ear vein, were found not to be intracellular. Phagocytosis was absent and apparently plays no part in the recovery of the rabbit from pneumococcus infection.

Since Tschistovitsch found phagocytosis in the lung lesions of both dogs and man my studies were repeated on dogs. An active phagocytosis was found in the circulating blood and in the lung lesions, thereby confirming Tschistovitsch's observation, but not necessarily his conclusion, that phagocytosis is the determining factor in the crisis of pneumonia.

Phagocytosis in Man.—Since phagocytosis in pneumococcus infection was shown to vary so markedly in different animals, further study was needed to confirm the claims of other observers and to determine the extent to which it occurs in man. Unfortunately the only available material was that obtained at autopsy.

Twenty-five pneumonic lungs were sectioned, stained for bacteria by Gram's method, and examined for phagocytosis. The results were discouraging. Very few bacteria were found in the exudate,
and there was very little evidence of phagocytosis, and this only
in scattered areas. Cases having early lesions, for the most part
red hepatization, were selected because previously, in making cultures
from gray hepatization, few pneumoccci grew. Yet in the earliest
case of red hepatization, with death on the third day of the disease,
the exudate in the air spaces contained surprisingly few pneumo-
cocci. The pneumococci were relatively more numerous in the
blood-vessels and it was probably from these that the cultures were
obtained.

Bezzola (70) describes the lobulated structure of lobar pneu-
monia and states that the pneumococci were most numerous in
the fresh exudate on the periphery of the lobule and less numerous
in the older exudates. Babes (71), Weichselbaum (72), and
recently Rosenow (73), note the disappearance of the pneumococci
in the exudate. These observers describe it as coincident with
fibrinization of the exudate.

Whether these findings are to be attributed to post-mortem
changes or to the efficiency of the protective mechanism in the alve-
olar exudate is not clear. But if they are due to post-mortem
changes, it is difficult to understand why the differences in distribu-
tion are so constant and why the bacteremic organisms survive.
If in these fatal cases, changes so marked take place, it is difficult
to conceive of the protective action of the lung failing to be far
more efficient in recovery.

Apart from the study of the material aspirated from the pneu-
monic lung, practiced under special conditions, there is no way of
determining whether the pneumococci are destroyed by phagocytosis
or by lysis outside the cells.

Summary of the Studies on Phagocytosis.—It is evident from
these experiments that virulent pneumococci are extremely insus-
ceptible to phagocytosis. This is due to the presence of substances
which are partly retained by the pneumococcus cell even after it is
killed by heat. These substances inhibit the phagocytic activity of
leucocytes for the staphylococcus, but are neutralized at least in
part by pneumococcus immune serum. Substances set free from
the pneumococcus cell are rendered inert by the immune serum;
but substances retained by the pneumococcus cell are not acted on
Studies on Pneumococcus Infection in Animals.

—the virulent organisms, whether living or dead, being in varying degree insusceptible to phagocytosis despite the presence of immune serum. This accounts for the fact that on virulent pneumococci the opsonic action of the blood serum of immunized animals is even less effective than its lytic action.

Non-virulent strains, however, are subject to phagocytosis. On these, immune sera are actively opsonic. But owing to the lack of reliable means of measuring this action, study of the fluctuations in the opsonic index at the bedside of the pneumonia patient have little or no practical value. Study of the phagocytosis of non-virulent pneumococci has thus contributed little to our knowledge of the phagocytosis of virulent organisms in infection, save that, under certain conditions in the tissues, the virulent cells are so altered that they become susceptible, like the non-virulent strains.

In the tissues of some animals, such as the dog and man, phagocytosis is active during recovery; but in others, such as the rabbit, it is absent. Why phagocytosis is absent in one animal, how it is brought about in another, and how important it is, are points still to be determined.

SUMMARY AND CONCLUSIONS.

In summing up the results of these investigations attention is called in particular to the following facts.

Dead pneumococcus culture material does not contain the active poisons formed in infection by living pneumococci. Characteristic lesions are not induced by dead cultures. But substances are present in the pneumococcus cells, and especially in culture filtrates free from pneumococcus cells, that give rise to an immunity in which the poisons of virulent pneumococci are inactive.

In immune sera specific agglutinative, precipitative, lytic, and opsonic activities are present. But to the action of immune sera, virulent pneumococci are singularly insusceptible.

This is due chiefly to qualities acquired by the organisms during their propagation through animals. In the test-tube this insusceptibility is overcome only under exceptional conditions which destroy these qualities or neutralize their effects. Lysis may be brought about by inhibition of growth, and phagocytosis by loss of virulence.
In the tissues inhibition of growth and resistance to the poisons of the pneumococcus are brought about, but in ways more subtle if less exceptional, for both lysis and phagocytosis are active factors in the recovery of certain animals from infection.

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