

AN EXPERIMENTAL ANALYSIS OF THE CURATIVE ACTION OF
PENICILLIN IN ACUTE BACTERIAL INFECTIONS

I. THE RELATIONSHIP OF BACTERIAL GROWTH RATES TO THE ANTIMICROBIAL
EFFECT OF PENICILLIN*

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PLATES 21 AND 22

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That penicillin may exert a bactericidal as well as a bacteriostatic effect upon certain microorganisms is well known (1). The demonstration that the drug may kill susceptible bacteria *in vivo* just as rapidly as it does *in vitro* (2) has led to the concept that the curative action of penicillin may be unrelated to the cellular defenses of the host (3). "The recent history of bacterial chemotherapy," states a current review (4), "has so influenced our philosophy and our science, that it is usual to discuss the subject in terms of simple relations between bacteria and chemical; often it has seemed unnecessary to acknowledge the host."

The experiments upon which this oversimplified hypothesis rests fail to take into account two important factors which play critical roles in the curative process. The first is the phase of growth of the bacteria in the tissues at the time that therapy is begun; the second is the state of the cellular defenses in the lesion. Indeed penicillin has recently been shown to be bactericidal *in vivo* only during the very earliest stages of an experimental streptococcal infection (5).

The present series of investigations was undertaken in an attempt to define more precisely the relation of the cellular defenses of the host to the curative effect of penicillin. The experimental approach was based upon the following facts established during previous studies on pneumococcal pneumonia (6-16).

(a) In any well developed lesion of experimental pneumococcal pneumonia, the states of both the growth of the bacteria and the inflammatory response of the tissues vary greatly in different portions of the lesion (Fig. 1). The pneumonic process spreads

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centrifugally. In the outer "edema zone" of the spreading lesion, where the inflammatory response is in its earliest phase, the bacteria multiply rapidly in the nutritious medium supplied by the cell-free fluid of the alveoli. Inside this outermost zone, in the "area of early consolidation," the rate of bacterial growth slows as the maximum population density of the alveolar medium is approached and as the polymorphonuclear exudate increases in intensity. Where the exudate is sufficiently dense, phagocytosis and destruction of the bacteria exceed their growth rate. As a result, a sharp decline is noted in the bacterial population. In the still more centrally located and thus older "zone of advanced consolidation," bacteria are no longer visible, all of them having been destroyed by the phagocytic cells. Finally, at the center of the lesion, replacement of the polymorphonuclear exudate by macrophages indicates the beginning of resolution (6).

(b) Fully encapsulated type I pneumococci, which resist phagocytosis *in vitro* when suspended in a fluid medium devoid of antibody, are readily phagocytosed *in vivo* by surface phagocytosis (9, 11, 12). This cellular mechanism of defense, which has been shown to operate in the absence of antibody, accounts for the ingestion and destruction of pneumococci in the central portions of the spreading pneumonic lesion during the preantibody phase of the infection (9, 10, 16).

(c) Fully encapsulated type III pneumococci, in contrast to type I, possess during their logarithmic phase of growth an outer "slime layer" of polysaccharide which renders them resistant even to surface phagocytosis (13). This "super capsule," however, persists only while the organisms are multiplying rapidly; thereafter it diffuses promptly into the surrounding medium leaving behind a capsule similar in anti-phagocytic properties to that of pneumococcus I (13). The temporary presence of the slime layer merely delays surface phagocytosis. As a result, the population density of bacteria in type III lesions reaches a significantly higher maximum than occurs in analogous lesions caused by type I (14). This latter finding suggests that surface phagocytosis plays a significant role in controlling the number of pneumococci which eventually accumulate in the tissues.

(d) Unlike other types of pneumococci, many strains of type III produce lung abscesses (14). The pulmonary suppuration appears to be directly related to the excessively high population density of organisms attained in type III infections (14). The occurrence of these abscesses makes possible a valid comparison of the efficiencies of the cellular defenses in suppurative and non-suppurative pneumonic lesions. One such comparative study has revealed that significant numbers of bacteria survive sulfonamide chemotherapy indefinitely in residual pulmonary abscesses, whereas the same treatment results in rapid sterilization of non-suppurating lesions (7, 14).

These facts dealing with the cellular defenses of the host in experimental pneumococcal infection have a direct bearing upon the curative action of penicillin. Their relevance is demonstrated in the present studies by three sets of experiments. The first, reported below, deals with the influence of bacterial growth rates (both *in vitro* and *in vivo*) upon the antimicrobial action of penicillin. The second and third, described in the two papers that follow (17, 18), concern (a) the role of phagocytic cells in destroying bacteria during penicillin

therapy and (b) the relation of suppuration to the curative effectiveness of the drug.

Methods

Bacterial Strains.—The pneumococci used throughout all the present studies were of three strains—pneumococcus type I, strain A-5 (6), and pneumococcus III, strains 8 H.C.C. (13) and A-66 (19). All three were of the same sensitivity to penicillin *in vitro* (the minimal inhibitory concentration (20) in beef infusion broth being 0.04 units per ml.). Maximum virulence of each strain was maintained by frequent mouse passage. Stock cultures were made in defibrinated rabbits' blood and were stored under vaseline at 4°C. (6).

The pneumococci added to the various culture media at the start of each *in vitro* experiment were harvested from 16 hour serum-broth cultures. The approximate dilutions for inoculation were made in tryptose phosphate broth, except in the experiment with purulent exudates in which the harvested organisms were twice washed and finally diluted in Locke's solution before being added to the pus.

Culture Media.—The *in vitro* activity of penicillin against the pneumococcus was tested in three types of media. The first was beef infusion broth containing 0.2 per cent dextrose and 10 per cent sheep serum—final pH 7.6–7.8. All three strains of pneumococcus were studied in the serum-broth mixture.

The second medium was serous pleural exudate obtained aseptically from rats dying of group A hemolytic streptococcal pneumonia (21). The pleural fluid contained a small amount of heparin, added to prevent clotting (approximately 1 mg. per ml.), and was clear in the gross. Its pH was roughly 7.5.¹ Before being inoculated with type I pneumococci (10^4 organisms) it was centrifuged at 3000 R.P.M. for 1 hour in order to remove the few leucocytes which were present and to eliminate some of the hemolytic streptococci. The number of viable streptococci remaining after centrifugation was 480,000 per ml. The minimal inhibitory concentration of penicillin for this strain of streptococcus in beef infusion broth was 0.02 units per ml.

The third type of medium studied was that provided by thick pus obtained from subcutaneous pneumococcal abscesses in albino rats. The method of producing such abscesses with the A-66 strains of type III pneumococcus is described in the third paper of this series (18). The pus used was taken from the cavities of abscesses opened on the 23rd or 24th day of infection. Although such pus usually contained between 10^8 and 10^9 viable pneumococci per ml., each sample was inoculated with approximately 10^6 washed A-66 cells from a 16 hour culture in order to insure at least this number of freshly transferred pneumococcal cells. This last step was carried out in order to approximate as nearly as possible the experimental conditions of the *in vitro* tests with broth and serum exudate.

Experimental Pneumonia.—Pneumococcal pneumonia was produced experimentally in white rats. The infecting organisms used were the A-5 strain of pneumococcus I and the 8 H.C.C. strain of pneumococcus III. The methods of producing the pneumonia, autopsying the animals, and fixing and staining the tissues have been described in a previous publication (6).

Penicillin.—Crystalline sodium penicillin G dissolved in normal salt solution was added directly to the culture medium to make a final concentration of 1 unit per ml. in each of the *in vitro* experiments. The stock solution of penicillin was made sufficiently concentrated so that the amount added to the test medium was never greater than 1 part in 20.

In the *in vivo* experiments each rat was treated with procaine penicillin in sesame oil (duracillin-Lilly). The dosage employed was 3,000 units intramuscularly every 12 hours for

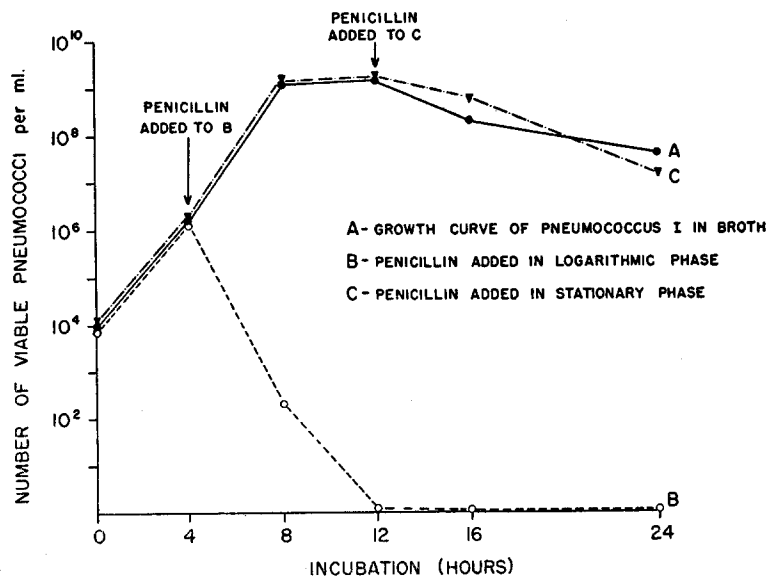
¹ Determined by means of nitrazine paper.

the first five treatments and every 24 hours thereafter until the termination of the experiment. (Expected sustained blood level of penicillin—1.0 to 10.0 units per ml. (22, 23).)

Bacterial Counts.—All bacterial counts were made by the standard dilution method employing blood agar pour plates. Whenever penicillin was present in the sample to be counted, penicillinase was added in a concentration of at least twice that of the penicillin.²

RESULTS

(a) *In Vitro Experiments.*—It is well known that penicillin exerts its bactericidal effect only upon actively metabolizing bacterial cells (24). Maximum killing occurs *in vitro* when penicillin is added to cultures in which penicillin-



TEXT-FIG. 1. Action of penicillin on type I pneumococci in broth culture.

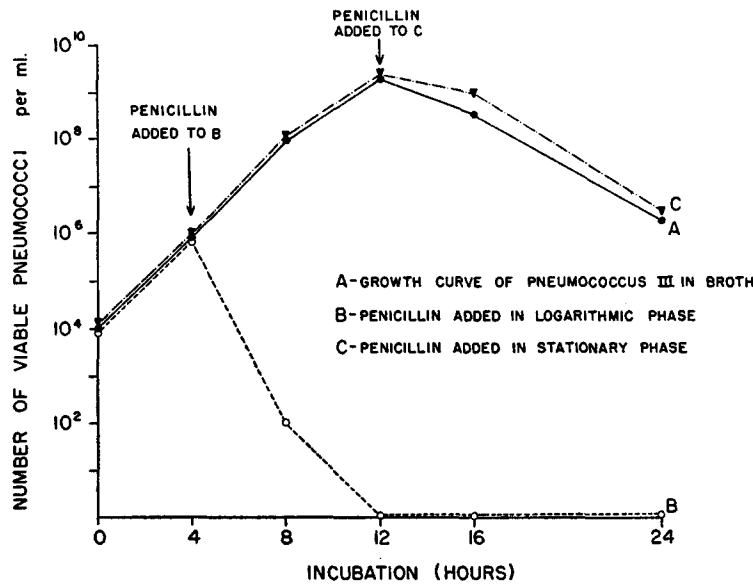
sensitive bacteria are multiplying rapidly. In contrast, sensitive bacteria which have ceased to divide and are in a “resting” metabolic state, are not killed by penicillin.

In order to be certain that the three strains of pneumococcus used in the present studies behaved *in vitro* toward penicillin in the manner just described, preliminary experiments were performed to test the relation of their destruction by penicillin to their rates of multiplication in enriched beef-infusion broth. The results obtained with two of the three strains are summarized in Text-figs. 1 and 2. It will be noted that in the case of both strains, which are

²One unit of penicillinase will inactivate one unit of penicillin in 1 hour at 37°C. The enzyme preparation was generously supplied by Schenley Laboratories, Inc., Lawrenceburg, Indiana.

equally sensitive to penicillin, the organisms were rapidly killed when penicillin was added to the medium during the logarithmic phase of growth. The same concentration of antibiotic, on the other hand, had little or no bactericidal effect when introduced during the later "stationary phase" of growth, for at this latter stage the culture had already reached its maximum population density.³ The third strain of pneumococcus also behaved in the same manner.

Having thus demonstrated the relation of the metabolic activity of these strains of pneumococcus to their susceptibility to penicillin, it became necessary to determine whether the same relationships hold for pneumococci con-



TEXT-FIG. 2. Action of penicillin on type III pneumococci in broth culture.

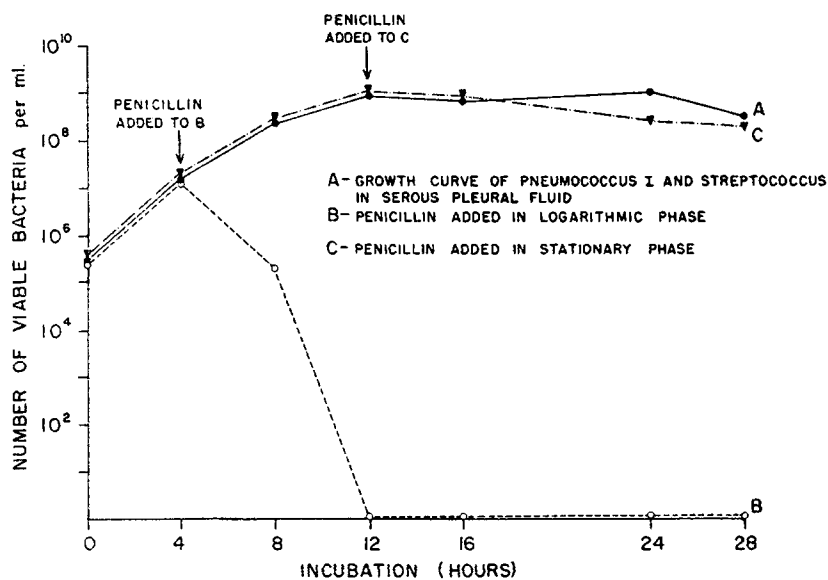
tained in infectious exudates. Accordingly, analogous experiments were performed using as supporting media for the pneumococcus (*a*) thin serous exudate obtained from the pleural cavities of rats dying of experimental streptococcal pneumonia and (*b*) thick pus from fully developed subcutaneous pneumococcal abscesses. As indicated by the data recorded in Text-figs. 3 and 4, the results obtained with the two forms of exudate were quantitatively dissimilar.

In the thin pleural fluid (Text-fig. 3), which at the start of the experiment contained both penicillin-sensitive streptococci and pneumococci, the organ-

³ When any bacterial culture reaches the maximum population density for the particular medium in which it is growing, the formation of new cells becomes greatly slowed, and the culture enters the familiar "stationary phase" of growth during which the rates of destruction and division of bacteria are approximately equal (25).

isms multiplied rapidly and were promptly killed when the penicillin was added in the logarithmic growth phase. As in the broth medium, antibiotic added during the stationary phase of growth had little or no effect.

In the thick purulent exudate from the subcutaneous abscesses, however, the behavior of the organisms was very different (Text-fig. 4). Here they failed to increase in number during incubation and accordingly were killed only slowly by the added penicillin. It will be noted that the pneumococci did not multiply significantly in the pus in spite of the fact that their population density at the start of incubation was well below the maximum attained in both



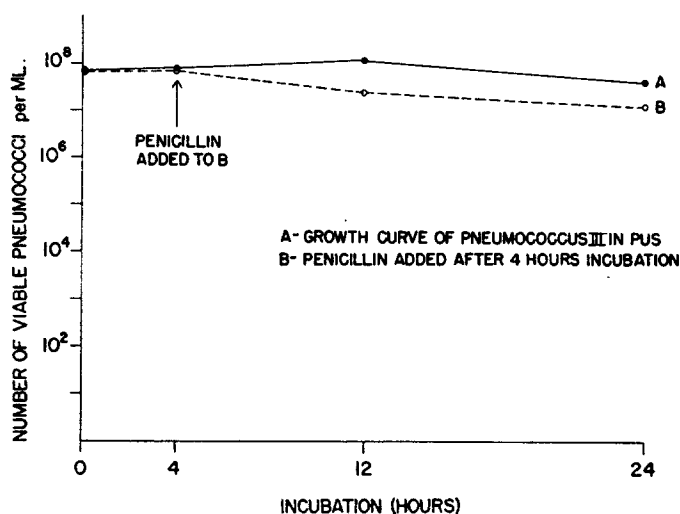
TEXT-FIG. 3. Action of penicillin on type I pneumococci and beta hemolytic streptococci contained in thin serous fluid collected from pleural cavities of rats with experimental streptococcal pneumonia. The pneumococci were added to the fluid at the start of each experiment.

the broth and the serous exudate. The failure of the penicillin to kill the pneumococci promptly (*i.e.* within 8 hours) appears to be due to the fact that the metabolism of the majority of the bacteria in the purulent exudate was not sufficiently active to make them maximally susceptible to the bactericidal action of the drug.

(b) *Effect of Penicillin in Vivo.*—The above relationships which, as will be discussed below, have been shown to obtain *in vivo* (5, 17, 18) as well as *in vitro*, have a direct bearing upon the action of the antibiotic in established infections. Their relevance is evident from the following findings concerning the effect of penicillin on the pulmonary lesion of experimental pneumococcal pneumonia.

Systematic histologic examination of the lungs of infected animals killed at

intervals after treatment reveals that the penicillin exerts a direct bactericidal effect in the outer edema zone of the lesion (see Fig. 1). Here, at the advancing margin of the infection, the thin serous exudate is relatively acellular, and the uniform morphology of the invading bacteria indicates that they have been rapidly multiplying (26). Before treatment the edema-filled alveoli are teeming with bacteria of uniform appearance (Fig. 2); 12 hours after therapy they contain only scattered remnants of pleomorphic organisms (Fig. 3). Since phagocytic cells have not yet invaded these alveoli in significant numbers, it must be concluded that the rapid destruction of the pneumococci has been brought about primarily by the bactericidal action of the penicillin. The



TEXT-FIG. 4. Action of penicillin on type III pneumococci in pus obtained from 24 day old pneumococcal abscesses.

change in the morphology of the few remaining organisms adds further support to this conclusion (26).⁴

In marked contrast to these findings in the outer edema zone are the histopathologic features of the more centrally located areas of advanced consolidation. 12 hours after the start of treatment many pneumococci are still visible in the cell-packed alveoli (Fig. 4). Most of the bacteria, as would be expected from previous observations on untreated animals, are seen to be within the phagocytic cells. But many of the phagocytosed organisms are morphologically

⁴ The actual destruction of the pneumococci presumably involves not only killing of the individual bacteria by the penicillin, but also action of autolytic enzymes which are known to become activated in dying pneumococcal cells (27). No doubt, during the period of study, some of the bacteria were removed from the alveoli by lymphatic drainage (28), but the presence of partially disintegrated organisms in the alveolar fluid following treatment indicates an appreciable occurrence of bacteriolysis *in situ*.

intact and therefore presumably have only recently been ingested.⁵ That they also have not all been previously killed by the penicillin (as in the edema zone) is evident from their morphologic appearance (26). Sections made at later intervals after treatment show that the phagocytosed pneumococci are eventually destroyed within the leucocytes (Fig. 5).

DISCUSSION

From the above observations it is concluded that the curative action of penicillin in experimental pneumococcal pneumonia involves at least two distinct processes: (a) the killing of pneumococci by the penicillin itself in those portions of the lesion where the organisms are rapidly multiplying, and (b) destruction of the bacteria by phagocytic cells of the host in sites where the leucocytes are viable and the microorganisms are in a relatively inactive metabolic state. The latter condition may obtain either because the bacterial cells have already reached their maximum population density, or because the immediate chemical environment (as in thick pus) is not suitable for further multiplication even when the population density is low.

It is of interest that Abraham *et al.* (29) concluded from their early and now classical experiments with penicillin that the presence of pus does not interfere with the antimicrobial action of the antibiotic. This conclusion was based upon experiments designed to detect the presence of possible antipenicillin factors in pus. No attempt was made to study the quantitative effects of purulent exudates upon the bactericidal action of the drug. These early experiments have been interpreted by later workers (30) to indicate that "the antimicrobial activity of penicillin is not materially reduced by the presence of pus." That such a conclusion is incorrect is indicated by the results of the present studies which demonstrate that thick pus from mature abscesses, is incapable of supporting active bacterial metabolism (and growth) and thus indirectly blocks the antimicrobial action of penicillin.

The fact that penicillin will kill only actively metabolizing bacterial cells *in vitro* was first established by Hobby, Meyer, and Chaffe in 1942 (1). The observation has since been repeatedly confirmed in connection with a variety of organisms (31).

Although the metabolic activity of a given bacterial culture does not always correspond exactly to the bacterial growth rate (24), the two variables, under most conditions, are roughly parallel. It is appreciated, for example, that metabolic activity during the lag phase of growth may be sufficient to render a sensitive organism susceptible to the bactericidal action of penicillin during a period when cell division is not occurring (24). In general, however, the susceptibility of bacteria to the killing effect of penicillin is directly proportional to the rate of bacterial growth at the time of introduction of the antibiotic.

⁵ Phagocytosed pneumococci ordinarily exhibit morphologic signs of degeneration within a relatively short period of time, *i.e.*, less than 1 hour (9).

Recently Eagle has presented clear cut evidence that this important principle of antimicrobial action applies also *in vivo* (5). In experimental streptococcal infections in mice he has shown that penicillin kills the streptococci only as long as their number is rapidly increasing in the lesions; once the organisms have reached a maximum population density, the antibiotic ceases to be bactericidal. These findings have been confirmed by the results of the analogous experiments reported in the next paper⁶ and also by the recent studies of Darnell *et al.* (32).

Such data based upon "*in vivo* growth curves," deal only with the total number of bacteria in the infected tissues. They provide no information concerning possible differences in the effect of penicillin on different parts of a given lesion. The present histologic study, on the other hand, reveals that these differences may be highly significant. In the case of experimental pneumococcal pneumonia it has been shown that in one part of the lesion, where the bacteria appear to be multiplying rapidly, they are highly susceptible to the bactericidal action of penicillin. In another part of the same lesion, on the other hand, where they have presumably reached a maximum population density and therefore have ceased to multiply, they seem to be relatively unaffected by the antibiotic.

The present histologic observations also suggest that the phagocytic cells of the inflammatory exudate play an important role in destroying the bacteria during penicillin therapy, particularly in those areas where the bacterial growth rate is slowest and where the antibiotic is therefore least effective. Evidence presented in the following paper (17) indicates that polymorphonuclear leucocytes in such areas are primarily responsible for ridding the tissues of the invading organisms.

SUMMARY

Three strains of pneumococcus (types I and III), equally sensitive to penicillin, have been shown to be killed by the antibiotic *in vitro* when grown either in enriched beef infusion broth or in a thin serous exudate. Killing of the bacteria resulted promptly when the penicillin was added during the logarithmic phase of growth but failed to occur if addition of the antibiotic was delayed until the later "stationary" growth phase. In analogous experiments with thick purulent exudates from established subcutaneous abscesses, the pneumococci failed to grow rapidly, and added penicillin exerted only a relatively slow bactericidal effect.

The relevance of these *in vitro* observations to the curative action of penicillin was demonstrated in a systematic histologic study of the antimicrobial effect of the drug in experimental (type I) pneumococcal pneumonia. Evidence was obtained that at least two distinct processes are involved. The first, the direct bactericidal effect of the penicillin itself, was shown to operate in the

⁶ See comparison of curves *F* and *H* of Text-fig. 2 (17).

outer edema zone of the spreading pneumonic lesion where the micro-organisms multiply rapidly in the thin serous exudate. The second, which predominates in the older more central portions of the lesion, was demonstrated to depend upon destruction of the pneumococci by phagocytosis. Here the bacteria, having presumably reached a relatively stationary phase of growth in the alveolar exudate, are resistant to the bactericidal action of the penicillin but are readily destroyed by the phagocytes.

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EXPLANATION OF PLATES

PLATE 21

All sections were fixed with Zenker-formol solution and were stained by a modification of the Gram-Weigert technique (6).

FIG. 1. Diagram illustrating the relationship of bacteria to inflammatory cells in the spreading pulmonary lesion of experimental pneumococcal pneumonia (type I) 24 hours after inoculation. \times 800.

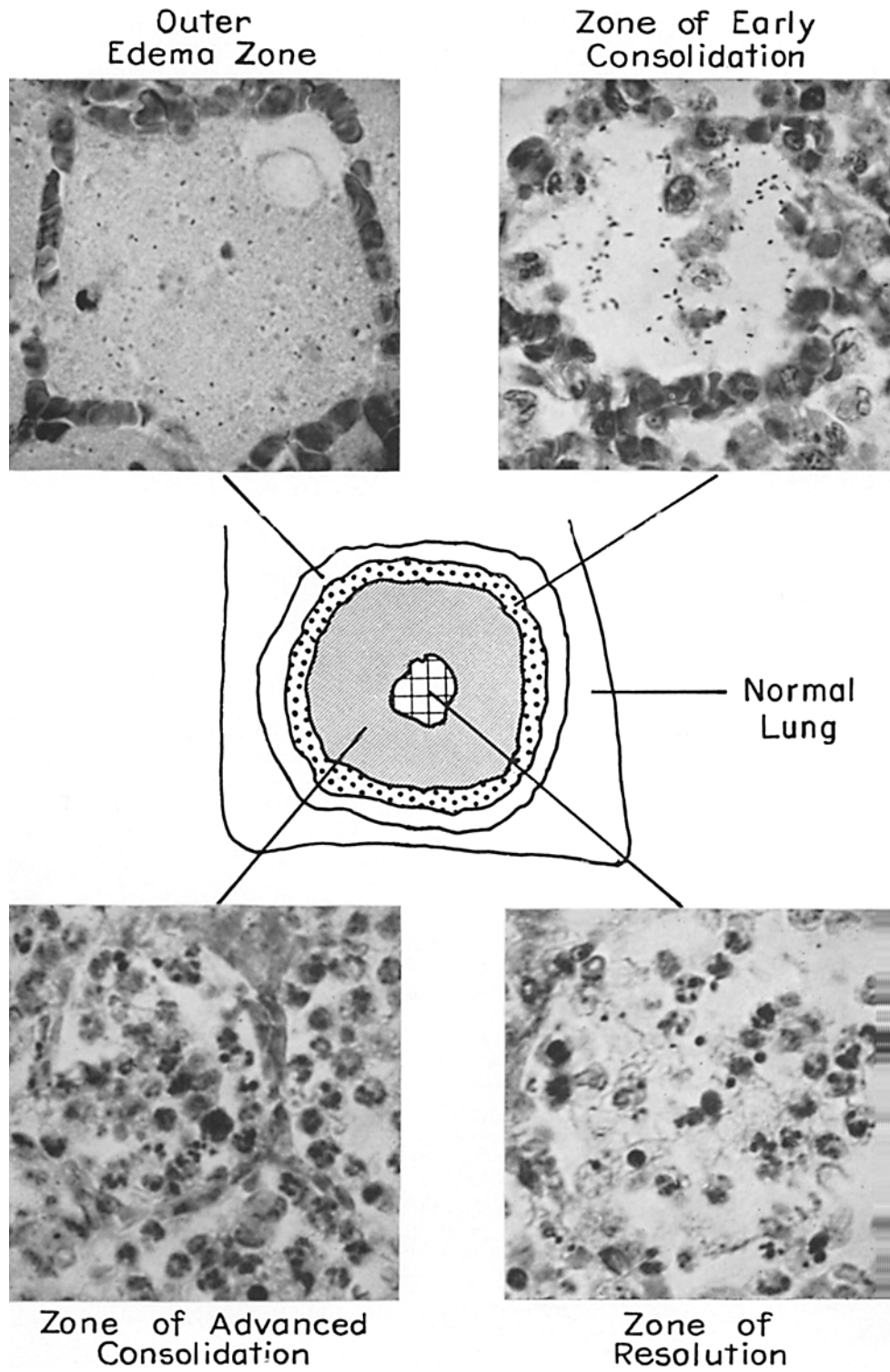


Fig. 1

(Wood and Smith: Curative action of penicillin. I)

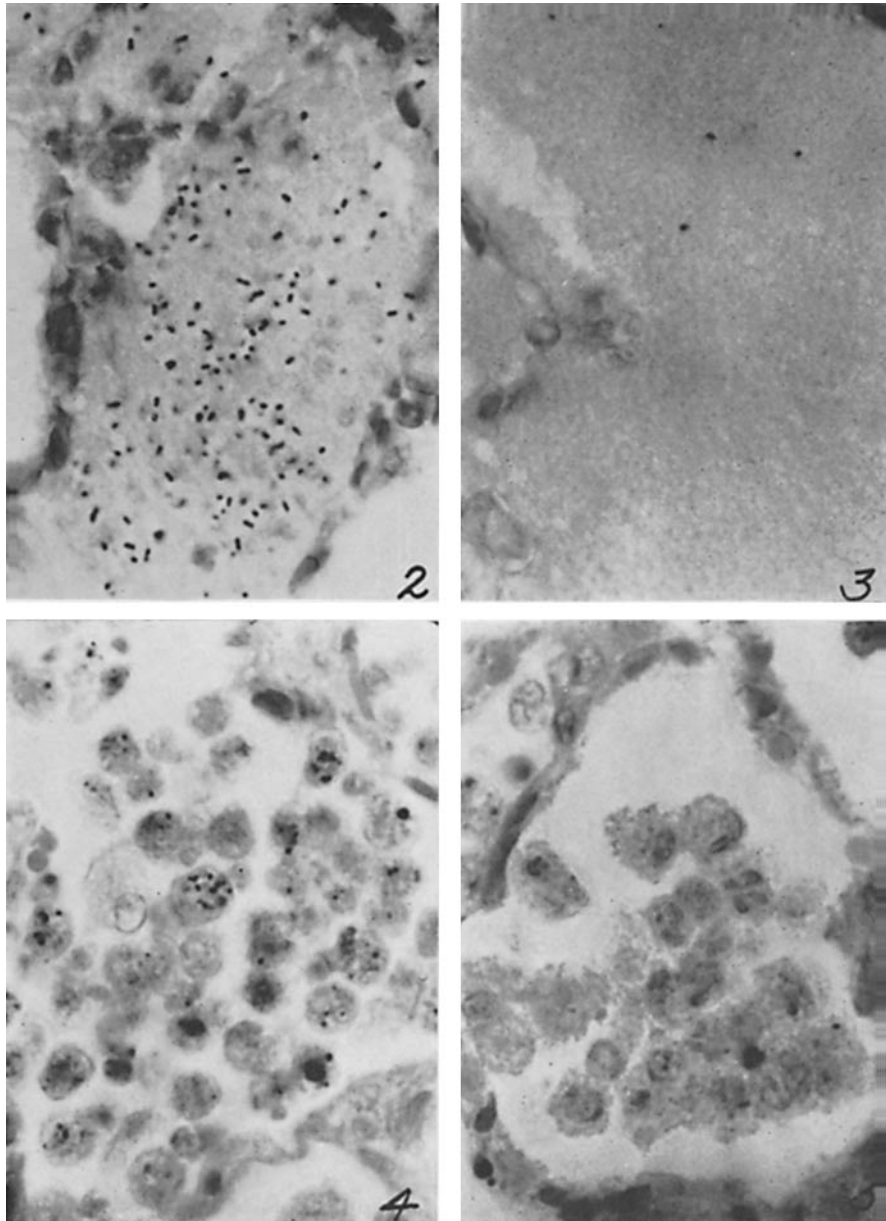
PLATE 22

FIG. 2. Alveolus in edema zone of pneumonic lesion 18 hours after inoculation. Note the large number of pneumococci (type I) in the edema fluid. $\times 900$.

FIG. 3. Similar alveolus after 12 hours of penicillin therapy (animal sacrificed 30 hours post inoculation). Note absence of all but a few pleomorphic pneumococci, in spite of fact that serous exudate contains practically no phagocytic cells. $\times 900$.

FIG. 4. Alveolus in zone of advanced consolidation 30 hours after inoculation and 12 hours after start of penicillin treatment. Note relatively large number of pneumococci (as compared to Fig. 3) virtually all of which are seen to be within phagocytic cells. $\times 900$.

FIG. 5. Similar alveolus in zone of consolidation after 30 hours of penicillin therapy (48 hours post inoculation). Most of phagocytosed pneumococci have been "digested" by the leucocytes. $\times 900$.



(Wood and Smith: Curative action of penicillin. I)