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

## II. The Role of Phagocytic Cells in the Process of Recovery*

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PLATE 23

(Received for publication, January 16, 1956)

If phagocytic cells, as indicated by the preceding study (1), play a significant role in the curative action of penicillin, invading bacteria should be more effectively destroyed by the combined actions of the antibiotic and the phagocytes than by the action of the antibiotic alone. In the following experiments the rate at which penicillin eliminates pneumococci from relatively acellular lesions has been compared to its effectiveness in the presence of a normally cellular exudate.

### *Methods*

The acellular lesions were produced by a method similar to that described elsewhere (2) in which rats were made leucopenic by exposure to x-radiation before being infected with either type I or type III pneumococci.

In preliminary experiments both normal and previously radiated rats were inoculated intrabronchially with pneumococci suspended in mucin (3). The number of penicillin-resistant contaminants encountered in the pulmonary lesions of the irradiated animals following antibiotic therapy was so great, however, as to make interpretation of the quantitative cultures difficult. Although the results obtained suggested that in fully developed pneumonia the killing of the bacteria was more rapid in the unirradiated animals with the cellular lesions, the method was eventually abandoned in favor of one in which contaminating organisms could be avoided.

The experimental technique finally adopted was a modification of that devised and extensively employed by Eagle (4). It involved producing pneumococcal myositis in the leg muscles of both normal and previously radiated

* This study was supported by research grants from the United States Public Health Service (E-662) and from the Life Insurance Research Fund.

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white mice. The infected tissue was removed *en bloc* for histologic and quantitative bacteriologic study at appropriate intervals following inoculation.

Details of the method were as follows.

Male white mice (strain "Tumblebrook") weighing approximately 20 gm. were used in all experiments. Irradiation (total dose 650 r) was performed according to a modification[1] of the method of Shechmeister (5). The irradiated mice were inoculated 72 hours after exposure to radiation. Inoculation was performed intramuscularly under light ether anesthesia, a hind leg having been previously shaved and swabbed with acetone. The inoculum, delivered through a 27 gauge needle, contained between 800 and 1000 pneumococci (either type I, strain A-5 (3) or type III, strain 8 H.C.C. (6)) in 0.1 ml. of tryptose phosphate broth. The pneumococci were of recent mouse passage and were taken from 4 hour cultures in beef infusion–dextrose broth containing 10 per cent sheep serum. At appropriate intervals after inoculation (as indicated in each experiment) groups of mice were killed with ether and the infected lower legs were aseptically removed *in toto* for either histologic study or quantitative cultures. Sections were prepared from tissue fixed in Zenker-formol solution and stained by the Gram-Weigert method (3). Cultures of the total lesions were made by the method previously described by Eagle (4), except that the tissues removed (both muscle and bone) were ground in a mortar with sterile sand rather than in a Waring blendor.[2] During the grinding 1 ml. of tryptose phosphate broth containing 5 units of penicillinase[3] was added to the mixture. Further serial tenfold dilutions were made for plating in tryptose phosphate agar containing 5 per cent rabbits' blood. Final colony counts were recorded only after the plates had been incubated for 48 hours. All mice treated with penicillin were given 3,000 units of crystalline procaine penicillin G. The drug was injected into the muscles of the uninfected hind leg. Treatment was begun at varying intervals after inoculation, as indicated in each experiment, and the injections were repeated every 6 hours for a total of 30 hours.

## RESULTS

*The Effect of Leucocytic Exudate upon the Tissue Population of Pneumococcus I.*—Curve A in Text-fig. 1 depicts the progress of bacterial growth in the myositis lesions of unirradiated mice receiving no penicillin therapy. It will be noted that the total number of type I pneumococci increased rapidly during the first 12 hours and reached a maximum of about $10^6$ organisms per lesion. Throughout the next 60 hours they remained approximately constant. In the relatively acellular lesions of the radiated mice, however, the total bacterial counts (curve B) continued to rise for 24 hours and attained a maximum of over $10^9$.[4] Thus the difference in the final maximums reached in the two types

[1] The mice were handled as described by Shechmeister, but the conditions of radiations were as follows: no portal, distance from target to skin—75 cm., filters—1 mm. aluminum and 0.25 mm. copper, voltage—200 kv., current—20 ma., dosage—28.5 r per minute (measured in air), total dose—650 r.

[2] This modification in technique was shown in preliminary experiments to have no appreciable effect upon the accuracy of the final bacterial counts.

[3] Penicillinase (kindly supplied by Schenley Laboratories, Inc., Lawrenceburg, Indiana) was added only in those experiments in which the mice were treated with penicillin.

[4] Since the irradiated mice rarely survived the pneumococcal infections for as long as 36 hours, the growth of the organisms in their tissues could only be followed for 24 hours (curves *B* and *D*).

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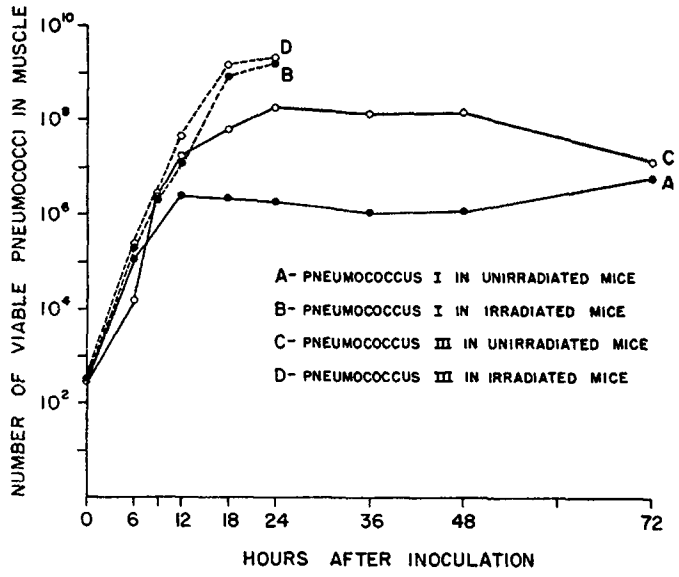
of lesions was greater than one thousandfold. This difference represents a roughly quantitative measure of the antibacterial effect of the leucocytic exudate.

*The Role of Surface Phagocytosis in Suppressing the Tissue Population of Pneumococci.*—In order to determine whether surface phagocytosis (7–12) plays a significant part in the antibacterial action of the leucocytic exudate, experiments, identical to those just described, were performed with pneumococcus III. This strain of pneumococcus had in previous studies been shown to possess, during its logarithmic growth phase, a "super capsule" which ren-




TEXT-FIG. 1. Growth curves of pneumococcus types I and III in inoculated thigh muscles of unirradiated and irradiated mice.

dered it temporarily resistant to surface phagocytosis (6). Comparison of its fate in the tissues with that of pneumococcus I, affords a rough means of estimating the part played by surface phagocytosis in destroying the bacteria (13).

Curves *C* and *D* in Text-fig. 1 summarize the data obtained with pneumococcus III. It will be noted that its growth curve *in vivo* is identical with that of pneumococcus I in radiated mice (curves *B* and *D*) but that in unirradiated mice (*A* and *C*) the two curves are quite different. The number of type III pneumococci which eventually accumulated in the normally cellular lesions was nearly one hundred times greater than occurred with type I. This difference supports the conclusion that at least part of the antimicrobial effect of the leucocytic exudate is due to surface phagocytosis.

*Comparative Bactericidal Effects of Penicillin in the Presence and Relative*

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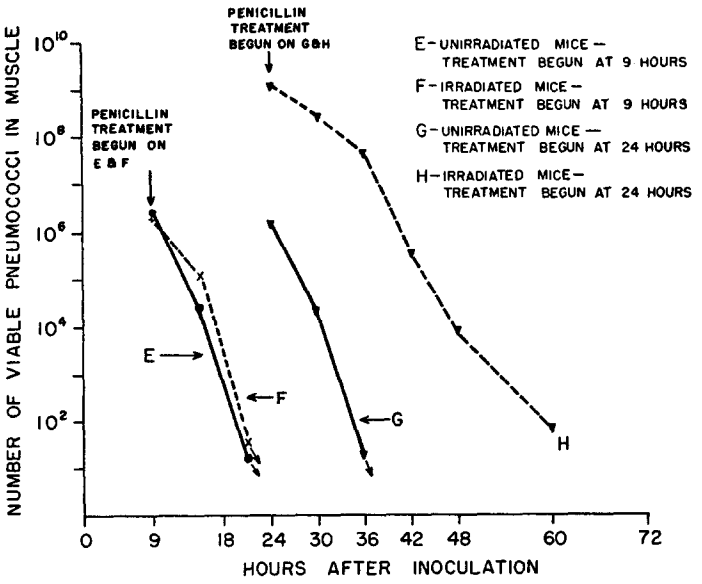
*Absence of Leucocytic Exudates.*—When in type I infections penicillin therapy was begun 9 hours after inoculation, the rate of destruction of pneumococci was approximately the same in the lesions of both the radiated and unirradiated mice (curves *E* and *F*, Text-fig. 2). This result is not unexpected for two reasons. First, as shown by the growth curves of the previous experiment (curves *A* and *B*), the pneumococci in the lesions of both groups of mice were multiplying rapidly at the time penicillin was given (9 hours), and therefore were highly susceptible to the bactericidal effect of the penicillin (1). Secondly, histologic examinations made 9 hours after inoculation revealed that at this




TEXT-FIG. 2. Comparative antimicrobial effects of penicillin therapy on pneumococcal myositis in unirradiated and irradiated mice, both treated at 9 and 24 hours after inoculation.

early stage of the infection there was no significant difference in the cellular content of the two sets of lesions (Figs. 1 and 2). In the unirradiated mice the leucocytes had not yet had time to accumulate at the site of infection.

When, on the other hand, the start of treatment was delayed until 24 hours after inoculation, the conditions were very different. By this time (as shown in curves *A* and *B* of Text-fig. 1) the bacterial counts in both groups had already reached their respective maximums. Also, the site of infection in the unirradiated mice was now teeming with leucocytes (Fig. 3), whereas that in the radiated animals was still practically devoid of phagocytic cells (Fig. 4).[5] Conse-

[5] Although a few phagocytes, mostly macrophages, could be demonstrated in the lesions of the radiated mice, they were not present in sufficient numbers to exert a phagocytic effect at all comparable to that observed in the unirradiated animals.

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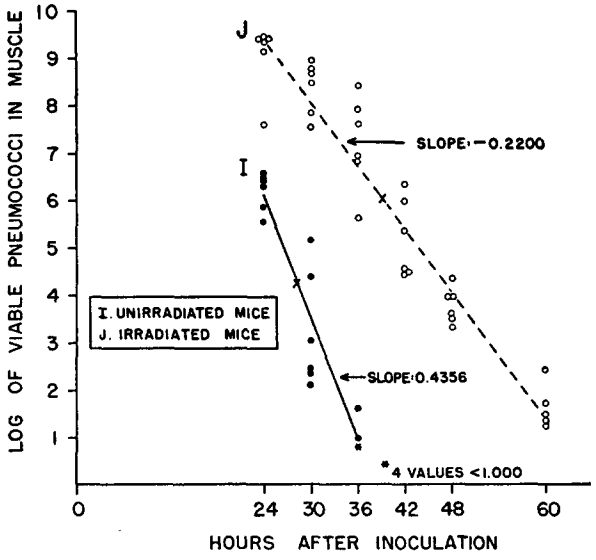
quently the pneumococci, having ceased their rapid multiplication and having thus become resistant to the killing effect of penicillin, were destroyed more promptly in the lesions with leucocytic exudates than in those without them (curves *G* and *H*, Text-fig. 2). That the leucocytes were actively phagocytic in the cellular exudates is clearly shown in Fig. 5.

*Further Analysis of the Bacterial "Survival Curves".*—The crucial data in the above experiment concern the comparative slopes of the two bacterial "survival curves" obtained when penicillin treatment is begun at 24 hours. The validity of the difference observed was further tested in additional experiments performed under the same conditions. Again the composite results revealed




TEXT-FIG. 3. Comparative rates at which pneumococci are killed in myositis lesions of unirradiated and irradiated mice when penicillin treatment is begun 24 hours after inoculation

the slope of the curve in the unirradiated mice to be steeper than in the radiated mice (Text-fig. 3). The difference in the slopes of the two curves was shown by mathematical analysis to be statistically significant ($P = <0.001$).[6]

## DISCUSSION

Conclusive evidence has been presented that the mere presence of the leucocytic exudate in lesions of pneumococcal myositis exerts a pronounced antimicrobial effect upon the invading bacteria. This effect was demonstrable even though the infection studied was produced by encapsulated bacteria generally considered to be resistant to phagocytosis (14). In experiments with pneumo-

[6] The statistical analysis was performed by Mrs. G. D. Hixon of the Department of Mathematics of Washington University, St. Louis.

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coccus type I the maximum bacterial counts in the relatively acellular lesions of previously irradiated mice were a thousandfold higher than in comparable lesions with normal leucocytic exudates.

The factors responsible for this difference clearly include phagocytosis. Appropriate histologic studies of the normally cellular lesions revealed many phagocyted pneumococci. Since numerous intracellular organisms were demonstrable as early as 12 to 18 hours after inoculation, their ingestion was assumed to have resulted from surface phagocytosis, a mechanism of cellular defense which has been shown to operate in the early preantibody stages of acute bacterial infections (12). Further evidence in support of this assumption was provided by the comparative data obtained in experiments performed on two groups of unirradiated mice, one infected with pneumococcus type I and one with pneumococcus type III. The strain used of the latter type was known to resist surface phagocytosis during its most active growth phase (6). Therefore, the fact that the maximum bacterial counts from the type III lesions were one hundred times greater than those from the type I strongly suggests that the suppressive effect of the leucocytic exudate was due, at least in part, to surface phagocytosis (13).

It is probable also that the presence of a dense cellular exudate leads to a significant degree of bacteriostasis (15). The failure of pus to provide an "optimal" medium for the growth of pneumococci was demonstrated in the preceding experiments (1) and has been further elucidated by those reported in the next paper (16). In addition, systematic histologic study of the lesions of experimental pneumococcal pneumonia has revealed that following penicillin therapy there are many more intact pneumococci in the cellular zone of early consolidation after 12 hours of treatment than in the acellular edema zone at the periphery of the lesion (1). This difference strongly suggests that the bacteria in the cellular exudate are less susceptible to the bactericidal effect of the penicillin, presumably due to the fact that they are metabolically less active. Finally, the total bacterial counts of normally cellular myositis lesions have been shown in the present experiments to remain constant after the first 12 hours (curve *A*, Text-fig. 1). The failure of the organisms to grow beyond the comparatively low maximum of $10^6$ must be due: (*a*) to a slowing of bacterial multiplication, or (*b*) to a continuous destruction by phagocytosis of more than 100 times as many bacteria as are produced at their maximum rate of growth, or (*c*) to a combination of both bacteriostasis and the action of the phagocytic cells. That phagocytosis alone is responsible for the entire suppressive effect of the exudate seems highly unlikely in view of all of the facts at hand: bacterial growth appears also to be slowed in normally cellular lesions of more than 12 hours duration.

The foregoing findings provide a ready explanation for the further observation that pneumococci in fully established infections of muscle are more rapidly

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destroyed by the combined effect of penicillin and phagocytes than by penicillin alone. It is not surprising that this difference cannot be demonstrated in the earliest stages of pneumococcal myositis when the bacterial population is increasing most rapidly and leucocytes have not had time to accumulate in the lesion. Under such conditions the bacteria are maximally reactive to the lethal effect of the penicillin, and because of the relative absence of leucocytes, phagocytosis obviously can play no appreciable role. Accordingly, penicillin therapy brings about destruction of pneumococci just as rapidly in the 9 hour lesions of previously irradiated mice as it does in comparable lesions of unirradiated mice.

Maturation of the infection with time, however, leads to two important changes. First, as shown by the *in vivo* growth curves, the pneumococci in the lesions reach a maximum population within the first 24 hours. As a result, after the 1st day the metabolism of a significant proportion of the bacteria is presumably slowed to the point where they are relatively resistant to the bactericidal action of penicillin. Secondly, leucocytic infiltration of the lesion increases with time, and by the end of 24 hours the exudate has become sufficiently dense to provide for active phagocytosis. Because of these two changes penicillin therapy of *mature* pneumococcal myositis is more effective in unirradiated mice than it is in previously radiated mice. In the lesions of the former, the leucocytes assist the drug in destroying the bacteria; in the lesions of the latter, the drug alone must kill the pneumococci which by this time are less than maximally susceptible to its bactericidal effect.

The importance of these temporal factors is well illustrated by recent studies of Eagle *et al.* relating to the bactericidal action of penicillin in immune and non-immune mice with streptococcal myositis (17). The rate at which the bacteria were killed in the tissues by the penicillin was found to be the same in both groups. The antibiotic, however, was given only 2 hours after the mice were inoculated with the infecting dose of streptococci. Accordingly, "there was no pronounced cellular infiltration at the focus of infection at the time of treatment with penicillin." In commenting upon this crucial point, the authors state: "It would be of interest to carry out similar studies under conditions closer to those usually operative in the natural infection of man, with the cellular host defenses already mobilized at the focus of infection" (17). And it might be added: with many of the infecting organisms already at the maximum population density in parts of the lesion.

Acute bacterial diseases such as pneumonia, tonsilitis, otitis media, etc., are rarely diagnosed in man until they have become relatively well established. Penicillin treatment is often not started until several days after the onset of infection. Certainly all the bacteria in such advanced lesions are not multiplying rapidly enough to be maximally susceptible to the bactericidal action of penicillin. Many of those that are not, are ultimately destroyed by the tissue phagocytes. When penicillin is employed in the treatment of established bacterial infections, its curative effect is manifestly due to the cellular defenses of the host as well as to the antimicrobial properties of the drug.

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SUMMARY

Type I pneumococci injected into the leg muscles of otherwise normal mice reached a maximum total population of approximately $10^6$ organisms. In mice rendered severely leucopenic by previous irradiation the maximum bacterial counts recorded were of the order of $10^9$. Since the lesions in the latter animals were relatively acellular, the thousandfold difference in the two experiments represented a rough measure of the antibacterial action of the leucocytic exudate.

The suppressive effect of the leucocytic exudate was shown by histologic studies to involve phagocytosis. The ingestion of pneumococci was clearly demonstrable within the first 12 to 18 hours. Accordingly, it was attributed to surface phagocytosis. In support of this conclusion was the finding that type III pneumococci reached a significantly higher total population in the myositis lesions than did type I. The type III strain used had been previously shown to be resistant to surface phagocytosis during active growth, whereas the type I strain was known to be susceptible throughout its growth phase. Evidence was also presented that the dense leucocytic exudate probably caused in addition a significant degree of bacteriostasis.

When penicillin therapy was begun 9 hours after inoculation, the pneumococci were cleared from the lesions with equal rapidity regardless of the presence or absence of leucocytic exudate. At this early stage the pneumococci were multiplying rapidly in the lesions of both the irradiated and unirradiated mice and therefore were promptly killed by the direct action of the penicillin. When the start of treatment was delayed, however, until 24 hours after inoculation, the bacteria in both sets of lesions had already reached their maximum counts and therefore were presumably resistant to the bactericidal effect of the antibiotic. Under such circumstances the destruction of the bacteria was found to be significantly less prompt in the acellular lesions than in those with a normal cellular exudate.

It is concluded from these findings that, in established pneumococcal myositis in mice, the curative effect of penicillin is due, not to the bactericidal action of the antibiotic alone, but rather to the combined effect of the drug and the cellular defenses of the host. The same conclusion also appears to be applicable to analogous acute infections in man, particularly when they are sufficiently advanced to be definitively diagnosed.

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## EXPLANATION OF PLATE 23

All sections were fixed with Zenker-formol solution and were stained by a modification of the Gram-Weigert technique (3).

FIG. 1. Pneumococcal myositis lesion in unirradiated mouse 9 hours after inoculation. Note scarcity of cells in exudate. × 900.

FIG. 2. Analogous lesion in previously radiated mouse. Animal sacrificed 9 hours after inoculation. Note absence of inflammatory cells in exudate. × 900.

FIG. 3. Pneumococcal myositis lesion in unirradiated mouse 24 hours after inoculation. Note density of cellular exudate and relative scarcity of pneumococci as compared to Fig. 4. × 900.

FIG. 4. Analogous lesion in radiated mouse sacrificed 24 hours after inoculation. Note the myriads of pneumococci which have accumulated in the virtual absence of inflammatory cells. × 900.

FIG. 5. Phagocyted pneumococci (*pn*) still demonstrable in inflammatory cells of myositis lesion after 36 hours of penicillin therapy. Mouse had not been irradiated and was sacrificed 60 hours after inoculation. × 900.

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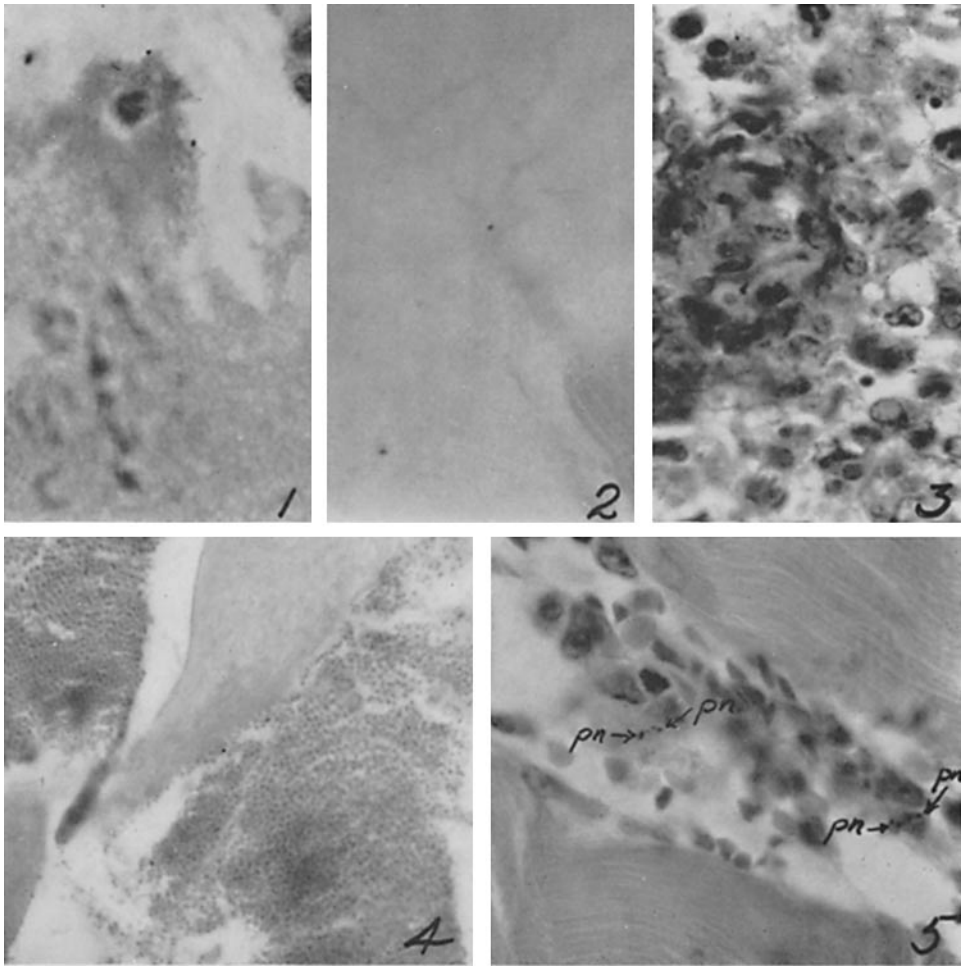


(Smith and Wood: Curative action of penicillin. II)

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