TYPHUS FEVER

IV. Further Observations on the Behavior of Rickettsia prowazeki in Tissue Cultures

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Plate 6

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In a previous article (Paper III of this series) (1) we described a technic for the propagation and morphological study of *Rickettsia prowazeki* in tissue cultures. Although definite evidence of the multiplication of the organism was presented in this publication, we found that our cultures regularly became non-infectious and histologically *Rickettsia*-free on or before the 12th day *in vitro*, in spite of continued good growth of the cells. In one group of cultures, however, extremely heavy infection of the majority of cells present was found on the 16th and 21st days *in vitro*. Since this group of cultures was incubated at a lower temperature than the others, (30–34°C., instead of 37°C.) a systematic study of the effect of temperature on the multiplication of the organisms was undertaken. These experiments are published in a separate article (Paper V of this series) (2). They show that a temperature of approximately 32°C. is optimum for *Rickettsia* growth under these conditions, and explain our previous difficulty in carrying the infection beyond the 12th day.

Technic

In addition to the change in the temperature of incubation, the following changes from the technic described in Paper III have been adopted.

1. Extract of normal spleen from a young guinea pig (200 to 300 gm.) has been substituted for embryonic extract.

The spleen is removed aseptically, placed in about 20 cc. of Tyrode's solution and cut into ten or twelve pieces, with the scissors. Most of the blood is washed out by this procedure. The fragments are then transferred to a sterile centrifuge tube and thoroughly crushed by means of a glass rod, having a diameter about the

same as that of the narrowest part of the lower portion of the tube. 10 cc. of Tyrode's solution are then added to the tube and the pulp is thoroughly mixed with the fluid by stirring. After centrifugalization the straw-colored and very slightly cloudy fluid is drawn off and stored at low temperature.

2. The splenic extract described above has been diluted with an equal volume of distilled water before use.

Splenic extract was adopted chiefly because it is easier to make. Cultures grow more slowly than when embryonic extract is used, but remain in good condition without transfer for a longer period of time (up to 21 days when grown at 32°C.). This slowing down of the rate of growth and degeneration undoubtedly favors the multiplication of the organism.

The reduction in tonicity of the extract by adding equal parts of distilled water makes the final tonicity of the cultures about 25 per cent below normal. Evaporation before and after sealing the cultures probably compensates to a large extent for this dilution. After the air in the Petri dishes becomes saturated with moisture at the temperature of incubation, no further evaporation appears to occur.

Staining of sections was done by the Giemsa method.

Description of Cultures at Various Ages

By sectioning a large number of cultures at different intervals after planting, we have obtained data concerning the progress of the infection in cultures growing slowly at 32°C. With the elimination of variables in technic, quite uniform and consistent results have been obtained. In 81 out of 86 cultures sectioned histologically on or after the 10th day *in vitro*, the infection has been so voluminous that distended cells could be easily identified with low power magnification.

3 to 4 days.—Cultures of this age always showed less than five infected cells per section. In some instances there may not have been any increase over the number of infected cells present in the original exudate. Since, however, we have frequently found three or four infected cells per section when smears of the exudate showed only a rare infected cell after 15 to 20 minutes' search, it is probable that some multiplication occurs almost from the outset. Most of the infected cells at this stage contained diplobacillary forms like those seen in the original exudate. Cells lightly infected with the thread form of the organism were occasionally found at the growing edge of these early cultures, however. This also indicates that multiplication of the organism has begun, since these thread forms have not been found in the original exudate.

Inoculation of single cultures of this age intraperitoneally into guinea pigs almost invariably produced typical typhus with the characteristic scrotal sac and scrotal involvement. The incubation period (6 to 8 days) was about the same as that of the infections produced by injection of single fragments of exudate immediately after floating in Tyrode's fluid.

7 to 8 Days.—In cultures fixed on the 7th and 8th days, infected cells ranged from six to fifty per section and the heavily infected ones often stood out clearly under the low power lens. Under oil immersion, these heavily infected cells were found packed with organisms which stained sharply and fairly deeply, ranging in color from bluish purple to reddish purple. In undifferentiated sections, isolated groups of organisms occasionally were stained blue but usually they were purple, even when the nuclei, cytoplasm and fibrin clot were all a bright clear blue. In these heavily infected cells, the organisms were morphologically like those seen in the original exudate. Many lightly infected cells were present at this stage, however, and in these cells the organisms appeared larger and tended to grow in long chains and in what appeared to be solid threads. Chains of fifteen to twenty individual bacilli were not uncommon. These chains were often curled because they were longer than the cells in which they were enclosed and occasionally measured as much as 30 microns in length. These long chains of relatively large bacillary forms were definitely associated with lightly infected and "healthy" appearing young cells and were most numerous at the growing edge of cultures showing many mitotic figures. Inoculation of these 7 day cultures intraperitoneally into guinea pigs produced a typical reaction. Incubation periods ranged from 3 to 6 days, indicating that *Rickettsiae* were more numerous than in the 3 and 4 day cultures. (The dependence of the incubation period on the number of *Rickettsiae* injected was pointed out in Paper I of this series (3).)

13 to 18 days.—The great majority of the cultures of this age group have shown practically unrestricted multiplication of the organism. By this we mean that more than half (often as many as 99 per cent) of the cells in the cultures were infected. Of these infected cells, 10 to 15 per cent were usually so heavily infected that they stood out under low magnification as large, usually rounded, deeply stained, purple structures, ranging up to 60 micra in greatest diameter. They could be clearly seen with a lens magnifying ten diameters. The appearance of these swollen cells, tightly packed with *Rickettsiae* was quite striking (Figs. 1 and 2). In the most distended and heavily infected cells, the individual organisms often could not be resolved centrally but were clearly seen peripherally (Fig. 2). These structures often had the appearance of bacterial colonies but on close inspection it was obvious that their size was limited by the extent to which individual cells could be distended without rupturing. These colony-like structures were often spindle or irregularly star-shaped (corresponding to the shape of growing cells) and even when their position with reference to the cells might otherwise be questioned, their characteristic shape and the fact that they had sharp edges (as though bounded by cell membranes) established their intracellular location.

In cultures of this age, and to a lesser degree in the 7 day cultures, occasional masses of *Rickettsiae* were seen which did not have a sharp edge and were not apparently confined within cell membranes. We interpret these structures as ruptured or autolyzed cells from which the cytoplasm had disappeared or become disseminated, leaving the organisms in a naked mass. It is noteworthy that such free lying masses of organisms almost invariably showed morphological changes which we associate with conditions unfavorable to them. The individual organ-

isms were globular or granular (with marked variation in size but tending to minuteness) and stained more deeply. They also were somewhat more eosinophilic than the vegetative bacillary forms.

Rarely at this stage small clusters of sharply outlined bacilli were found apparently free in the fibrin clot, but such observations were infrequent and organisms were never seen in the fibrin clot at a distance from the tissue. Study of cultures at this stage led to the conclusion that the fibrin clot outside of the cells was a very unfavorable medium for the organism and that its life was brief when it lost the protection afforded by the interior of the cells.

The heavily infected cells at this stage were found chiefly in the central parts of the cultures where growth was slow and these cells usually contained minute forms of the organism with a tendency to granular degeneration in those cells which appeared to be dead or dying. Peripherally the same picture of young healthy cells, lightly infected with larger rods and long chains, was found.

Five guinea pigs inoculated with cultures of this age (13 to 18 days) all reacted typically with incubation periods of 3, 3, 4, 4 and 5 days, indicating that *Rickettsiae* were numerous.[1]

26 to 36 Days.—Cultures of these ages (twenty-eight in number) all showed about the same picture as the 13 to 18 day group. In several instances the cells were largely non-viable in appearance (pyknotic nuclei and heavily vacuolated cytoplasm) and the *Rickettsiae* were correspondingly more granular in appearance. In the majority of cultures in this age group, however, mitotic figures were abundant and healthy cells containing rod-shaped *Rickettsiae* predominated. The presence of large numbers of viable *Rickettsiae* at this stage is attested by the fact that three guinea pigs inoculated (from two different groups of cultures) on the 35th and 36th days, reacted in a typical manner after incubations of 3, 2½ and 4 days.

42 to 52 Days.—Three cultures have been sectioned on the 42nd, two on the 45th, one on the 51st and four on the 52nd day. In the 42 day cultures, the cells were mostly non-viable in appearance and mitotic figures were very rare. (The growth energy of the cultures appears to decrease after the first 3 weeks, possibly because of the heavy infection, but we think not entirely for this reason.) In these cultures practically every cell was heavily infected but vegetative forms (sharply outlined rods) were in the minority. Many of the cells were without visible nuclei but heavily laden with *Rickettsiae*. One guinea pig inoculated with two 42 day cultures reacted positively and typically with an incubation period of about 48 hours. Smears of the scrotal sac exudate from this animal showed unusually numerous

[1] An incubation period of 3 days is achieved rarely by inoculation with scrotal sac exudate, only when the entire exudate (about 0.5 cc.) is injected and only when this exudate is unusually rich in organisms. These cultures, having a volume of about 0.3 c.mm. may be assumed to contain about the same number of *Rickettsiae* as 500 c.mm. of the most heavily infected scrotal sac exudate.

Rickettsia-filled cells and hundreds of free lying *Rickettsiae* in every field. These *Rickettsiae* appeared unusually large and stained more deeply than usual (an observation which we have repeatedly made in exudates from animals inoculated with cultures after several weeks *in vitro*). The disease was carried for three generations from this animal by inoculation with scrotal sac exudate and no change in virulence or other features was observed.[2]

The two 45 day cultures were in good condition, and mitotic figures were quite numerous. Every cell present was infected and over 50 per cent of the cells were distended with rod-shaped *Rickettsiae*. Inoculation of a guinea pig with two cultures of this age resulted in a strongly positive reaction in about 36 hours (the shortest incubation which we have recorded).

The 51 day culture was in excellent condition at the time of fixation. Sections showed extremely heavy infection of practically every cell. Peripherally, mitotic figures were present in moderate numbers and many cells were packed with long chains of *Rickettsiae*, lying parallel to one another. A guinea pig inoculated with fragments of fibrin clot cut away from the remaining cultures of this age, developed a typical reaction with an incubation period of 4 days. Unfortunately the remaining cultures in this group did not survive the transfer, although they appeared to be in good condition on the 51st day when they were transplanted. 1 week later (on the 58th day) they were found to be non-infectious and histologically *Rickettsia*-free. Apparently the *Rickettsiae* did not survive for as long as 7 days in the absence of living cells.

The four 52 day cultures sectioned all showed extremely heavy infection of practically every cell present. All cells present appeared either dead or dying and no mitoses were observed. Practically all organisms present were in the globular or granular form. Many large masses of free lying organisms were found and there was considerable spreading of the organisms through the fibrin clot. Scattered organisms were present in the fibrin clot even at the distance of a millimeter from the tissue (Fig. 4). These organisms became less numerous, however, as one passed further away from the tissue and we think that they passed out into the fibrin clot simply by diffusion after being set free from the disintegrating cells. In two instances they were heavily concentrated at the line of junction of the old and new fibrin clot but their granular nature in this location made it seem highly improbable that they had multiplied there.

No guinea pigs were inoculated from these 52 day cultures.

Virulence of Organism after in Vitro Cultivation

By fixing part of the cultures from each group on certain dates and injecting others into guinea pigs, we have been able to compare the

[2] No attempt has been made to conserve the original strain by direct transfer from animal to animal. The strain now in use has been subjected to many *in vitro* periods, ranging up to 51 days in duration. No changes in incubation period or strain virulence have been noted.

morphological picture with the virulence. The accuracy of this method depends upon the degree of variation between individual cultures of the same group, but we have never found marked differences in this respect.

The incubation period is definitely correlated with the number of visible *Rickettsiae* in sections (see Table I in Paper V (2)). In one instance (mentioned above) the injection of two cultures which had been 45 days *in vitro*, resulted in a typical reaction (with marked scrotal swelling) after an incubation period of 36 hours. Two cultures from this same group, fixed on the same day, both showed unusually heavy infection histologically.

In two instances a positive inoculation was obtained from a group which was histologically negative, but the incubation periods were 11 and 14 days. In two instances also, animals which apparently did not react were found immune to subsequent inoculation. Since this same result is occasionally obtained by inoculation with 1 or 2 cc. of blood in routine transfers, it seems reasonable to ascribe it to an insufficient dosage of organisms rather than to altered virulence.

The disease reproduced by injection of infected tissue cultures in the majority of instances differed in no way from that produced by inoculation of fresh scrotal sac exudate. The scrotal involvement was fully as severe and *Rickettsiae* were fully as numerous in the scrotal sac exudate.

In one experiment, however, the disease was reproduced apparently without scrotal sac involvement. A summary of this experiment is given.

Culture Group 81-10 was set out on Aug. 3 in the routine manner and incubated at 32°C. Cultures were washed and reembedded on Sept. 8, Sept. 17 and Sept. 22. Cultures on these various dates were fixed and injected always with positive results. On Oct. 9 (40 days *in vitro*) a single colony remained. The clot was partially liquefied because no transfer had been made for 17 days. The liquid portion of the clot was drawn off in a pipette and amounted to about 0.1 cc. This was divided into five equal parts, four of which were used to inoculate bacteriological culture media (with negative results). The remainder (about 0.02 cc.) was diluted with 4 cc. of Tyrode's solution and injected into a guinea pig. The temperature rose sharply to 105.2° on the 8th day and continued high for 5 days, without scrotal involvement. This animal was then killed. An enlarged spleen was found but the scrotal sac was entirely negative. The gross picture was that characteristic

of Old World typhus. Two guinea pigs inoculated with 4 cc. of heart's blood from this animal both developed typical endemic typhus, with characteristic scrotal reactions and demonstrable *Rickettsiae*.

The lack of scrotal reaction in this instance was probably the result of inoculation with a very small number of *Rickettsiae*, and does not necessarily mean that there was any alteration in virulence.

On the whole the evidence seems conclusive that loss in virulence does not take place up to the 51st day *in vitro*. It seems probable that fully virulent *Rickettsiae* would persist at this temperature as long as the cells could be kept alive. We have had no instance in which infection, once established in a group of cultures (as proven histologically or by animal inoculation) has disappeared from that group except in case of death and disintegration of the cells.

The correspondance between visible *Rickettsiae* and infectivity has been sufficiently complete to rule out the possibility of a virulent invisible form of the organism under the conditions of these experiments.

Infection of Normal Tissues in Vitro

One definite method by which the infection spreads (by mitotic division of infected cells) has been described in Paper III (1). This observation has been repeatedly confirmed and *Rickettsiae* have been found in cells in all stages of mitosis. This method does not suffice, however, to explain infection of practically every cell present (a result which has been obtained with great regularity since adopting the new technic). Since only a few of the cells are originally infected (on the average of about one in a hundred) there could be no marked increase in the percentage of infected cells unless the uninfected cells were outgrown by the infected cells. Table I, however, shows that approximately 40 per cent of the cells in mitosis were *Rickettsia*-free, while only 8.5 per cent of the cells not in mitosis were *Rickettsia*-free.

It therefore seems necessary to assume that *Rickettsiae* are set free from one cell and gain entrance to others. This must also occur *in vivo* when scrotal sac exudate is injected intraperitoneally.

In an attempt to obtain information on this point, we have cultivated normal tissues (striated muscle, lung, spleen and kidney) along with cultures of the scrotal sac exudate. In some cases the normal tissue has been in contact with the exudate originally and in others

the two tissues have been so placed that the growing cells from each would intermingle.

In the case of striated muscle which has been tested most thoroughly, we found heavy infection of what appeared to be sarcolemma cells in the late cultures (20 to 40 days *in vitro*) but no *Rickettsiae* were found in these cells in the earlier cultures. We have not been able entirely to rule out the possibility that these infected cells had grown around the muscle fibers from the exudate. The striated muscle cells do not grow but remain intact for 40 days or more. *Rickettsiae* were never found in the cytoplasm of these cells, even when they were in contact with heavily infected cells from the exudate for several weeks. Good growth of cells occurred in the cultures of lung, spleen and kidney.

TABLE I

	Degree of infection			
	Heavy	Medium	Light	Empty
Cells in mitosis	12 (9%)	16 (12%)	51 (39%)	52 (40%)
Cells not in mitosis	62 (28.5%)	75 (35%)	60 (28%)	18 (8.5%)

In the older cultures (21 to 40 days) an occasional infected cell was found along the edge of the normal tissue but we have never obtained a picture at all comparable to the unrestricted growth which occurred in the adjacent exudate.

We are unable to explain why the infection spreads and involves every cell descending from the exudate but does not (to any considerable extent) involve the cells descending from the normal tissues, in spite of prolonged contact between the two tissues. It seems possible that this fact may be explained on the basis of the experiments reported in Paper II. This work showed definitely that *Rickettsiae* did not multiply or even persist in phagocytic cells and that they did multiply voluminously in mesothelial cells. It suggested strongly that they were unable to multiply in fibroblasts. We have not been able to identify with certainty the cells present in our successful cul-

tures from the scrotal sac exudate. Phagocytic cells apparently do not persist and the cultures soon appear to be composed of cells morphologically consistent with either mesothelial cells or fibroblasts. Study of the explanted exudate fragments shows (aside from the macrophages and cells of the granulocytic and lymphatic series) only sheets of polygonal cells, closely fitted together and definitely resembling mesothelium rather than connective tissue. We believe therefore that our cultures as a rule are pure cultures of mesothelium and think that this may explain the extensive multiplication of *Rickettsiae* in them and the failure of *Rickettsiae* to grow in the cultures of normal tissues, which are presumably largely fibroblasts. This theory is strengthened by the fact that we have in several instances noted heavy infection of pleural or peritoneal lining cells covering normal lung and spleen in cultures of 20 to 25 days duration. This problem requires further study.

Behavior of Rickettsiae in Dividing Cells

Organisms are commonly found in cells undergoing mitotic division but are less numerous in such cells than in resting cells. This fact is brought out in Table I. Their behavior in dividing cells is interesting. They almost invariably assume a globular form and the cytoplasm around the dividing chromosomes is free from them. They usually become massed together along the cytoplasmic membrane, especially at the poles of the cells. Frequently they are present in clusters at each end of the cells, suggesting agglutination. Organisms in dividing cells are frequently stained blue but this is probably because of the lowered pH. These observations suggest that the dividing cell is an unfavorable medium for the organisms (probably because of its acidity) but it seems probable that a certain number of organisms usually survive the process and multiply in the daughter cells when conditions are restored to normal.

Typhus *Rickettsiae* have never been seen in nuclei, in spite of careful search. In heavily infected cells, the nucleus is often compressed or obscured, or, if visible, is recognized as a clear zone in the center of the dark purple mass of *Rickettsiae* in the cytoplasm of the cell. This fact is in contradistinction to the behavior of spotted fever *Rickettsiae* (4).

Oxygen Requirements of Typhus Rickettsiae

The voluminous multiplication of organisms described here has been obtained in cultures exposed to air within the Petri dishes. Whether oxygen gains entrance to the cells under such conditions in larger amounts than in living animals is problematical. The oxygen tension within living cells is generally believed to be practically zero.

The technic used for obtaining anaerobiosis in the tissue cultures was as follows:

> The cultures were set out in Petri dishes in the usual way. They were then placed in a glass jar and the covers were partly raised to establish free communication between the atmosphere of the Petri dishes and that of the interior of the jar. The covers were held in this position by small pyramids of plasteline. The glass jar was then sealed with the aid of wax and a brass cover having a single outlet and stop-cock. Evaporation was kept at a minimum by covering the bottom of the jar with wet absorbent cotton. Anaerobiosis was obtained by evacuating and washing out three times with hydrogen gas in contact with heated platinized asbestos. A solution made by adding 1 drop of methylene blue to 5 cc. of broth without dextrose was placed in each jar as an indicator. Decolorization of this solution was always complete in 12 hours.

Several groups of cultures proven to be heavily infected after 16 to 36 days of growth in the usual way were put under the most perfect conditions of anaerobiosis at our command. The degree of anaerobiosis was checked by the decolorization of methylene blue. These cultures remained under anaerobic conditions for 8 to 12 days at 32°C. At the end of this time they were studied histologically and by inoculating guinea pigs. The majority of the inoculated animals reacted positively but with relatively long incubation periods (6 to 10 days). In four cultures studied histologically, relatively few intact cells and no cells of viable appearance were present. *Rickettsiae* of the globular or coccoid forms were numerous in many of the intact cells, but it was obvious that they were merely surviving under adverse conditions. No organisms were found in the fibrin clot at a distance from the tissue. *Rickettsiae* were only about one-twentieth as numerous as in the aerobic control cultures, in which the growth of cells continued.

This experiment shows that factors other than oxygen tension are responsible for the multiplication of *Rickettsiae* within the cells and their lack of multiplication in the fibrin clot. (The clot presumably

contains many of the chemical constituents of cytoplasm, since cells are continually disintegrating.)

Reaction of Medium

Groups of cultures have been set out in which the pH of the medium was altered both on the acid and on the alkaline side. Sodium hydroxide and hydrochloric acid were added to the splenic extract in such amounts that the desired pH was effected when an equal volume of plasma was added. The pH of the final culture medium thus produced was determined colorimetrically. Moderate growth of cells was obtained at a pH of 6 and at a pH of 8. In both cases, no effect was noted on the number of *Rickettsiae* found in the section or on the infectivity. The cultures were incubated only for about 6 days. It seems doubtful if such changes in pH of the medium exert any marked effect on the intracellular pH so that these experiments probably are of slight value.

Relation of Typhus Rickettsiae to Metabolism of Host Cells

Upon the addition of 1 drop of 1 per cent calcium chloride to cultures, growth of cells was, as far as could be determined, completely inhibited. After 17 days *in vitro*, however, many intact cells were present and the majority of cells appeared not to have undergone necrosis. Cultures of this group were as heavily infected as those in which the cells had multiplied.

In some instances, also, heavily infected cells were found in cultures grown for 2 weeks at 26°C. in which there had apparently been no growth of cells.

As mentioned above, heavily infected cultures became non-virulent and *Rickettsia*-free in 7 days in instances in which the cells did not survive transfer. Under anaerobic conditions which eventually cause death of the cells, there was a marked diminution in the number of *Rickettsiae* seen in sections and an increase in the incubation period of the reproduced infection, as compared with the controls.

It appears therefore that typhus *Rickettsiae* require for their propagation and survival, cells in which metabolism is taking place. They grow and persist in cells which are living and multiplying and in cells which are merely kept alive. When cells die, however, they appear

to remain visible only slightly longer than the degenerating cytoplasm and nucleus of the dying cells.

SUMMARY

In tissue cultures grown at 32°C., typhus *Rickettsiae* increase rapidly within the cytoplasm of infected cells up to about the 14th day. At this time practically every cell is infected and the majority of cells are distended with organisms.

This condition remains constant as long as successful cultures of the cells can be maintained (up to 52 days).

Loss in virulence does not take place during this period *in vitro*.

The number of *Rickettsia*-filled cells found in sections and the incubation period of the infection resulting from inoculation of cultures from each age group are definitely correlated.

The behavior of typhus *Rickettsiae* in dividing cells is described and methods of spread of the infection other than by mitosis of cells are discussed.

Normal tissues do not become infected *in vitro* to any considerable extent in spite of prolonged proximity to heavily infected cultures of scrotal sac exudate.

Complete anaerobiosis and alterations in pH do not alter the intracellular location of the organism in tissue cultures.

The organisms are not seen within nuclei of infected cells. They remain intact and infective for several weeks in cells which are kept alive but not multiplying. They disappear in less than 1 week, however, when the cells undergo degeneration.

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EXPLANATION OF PLATE 6

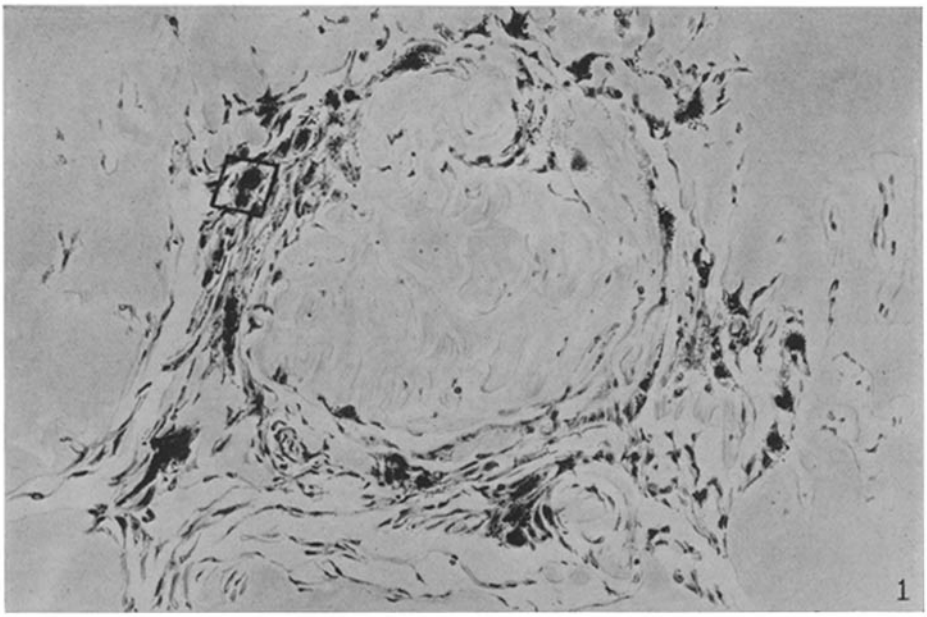
All illustrations are from paraffin sections of tissue cultures fixed in Regaud's fluid and stained by the Giemsa method.

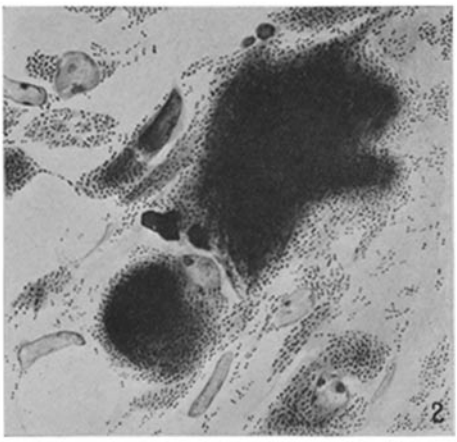
FIG. 1. Low power view of a tissue culture heavily infected with typhus *Rickettsiae*, on the 45th day *in vitro*. The dark structures (one of which is outlined) are colony-like masses of *Rickettsiae* within distended cells. ×90.

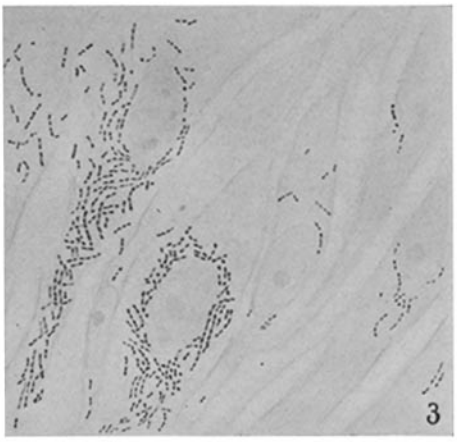
FIG. 2. High magnification of the field within square outlined in black ink in Fig. 1. The individual organisms in the center of the distended cells cannot be resolved but definite organisms are seen peripherally. ×1200.

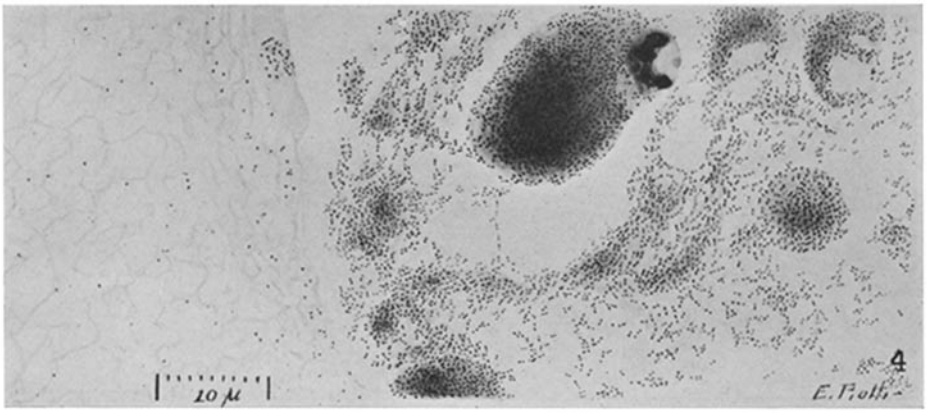
FIG. 3. Young growing cells lightly infected with thread forms of *Rickettsiae*. From a culture 51 days old. ×1200.

FIG. 4. From an infected culture on the 52nd day *in vitro*. The cells are largely disintegrated. *Rickettsiae* lie free in the plasma but are still concentrated in the remaining cytoplasm of intact cells. Organisms have diffused into the surrounding fibrin clot, but are present there in relatively small numbers and are evidently not multiplying there. ×1200.









(Pinkerton and Hass: Typhus fever. IV)