STUDIES ON THE CULTIVATION OF THE TYPHUS FEVER RICKETTSIA IN THE PRESENCE OF LIVE TISSUE

By CLARA NIGG, Ph.D., and K. LANDSTEINER, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research)

Plate 24

(Received for publication, December 29, 1931)

This investigation was prompted by the recent significant work of Mooser on the Mexican type of typhus fever. The results on the cultivation of the rickettsia of typhus fever which have already been briefly presented in a preliminary communication (1) are reported in detail in this paper.

Various attempts had previously been made by other workers (Kuczynski (2), Krontowski and Hach (3), Wolbach and Schlesinger (4), Rix (5), Zinsser and Batchelder (6) (*cf.* Zinsser and Castaneda (7))), to cultivate typhus fever rickettsiae. Some of these experiments indicated multiplication of the organisms but none led to the establishment of strains which could be maintained indefinitely *in vitro*. The methods used were practically the same; namely, the cultivation in homologous plasma of tissues (generally brain and spleen) from typhus-infected guinea pigs. In such cultures Kuczynski, Wolbach and Schlesinger, and Zinsser and Batchelder were able to demonstrate rickettsiae morphologically, and Wolbach and Schlesinger succeeded in setting up a second generation, which was done by transferring the same piece of tissue into fresh medium.

Since our preliminary article, Sato (8) reported the cultivation of the virus of typhus fever through thirteen generations, using infected, along with normal Descemet's membrane of rabbits, in a medium consisting of aqueous humor and plasma. The liquid was changed every 2 days and the tissue itself transferred to fresh medium when growth ceased, as it did after from 4 to 10 days. The virulence of such cultures was tested by injecting a suspension of cultivated tissue fragments intracardially. Such injections were followed by fever, monocytosis, and pathological changes consisting of dark red discoloration and edema of the spleen, as well as typhus nodules in the brain, liver, and heart muscle. The author was never able to demonstrate rickettsiae morphologically in his cultures, but described cell inclusion bodies (*"monokokkenförmige Körperchen"*) which he identified with the etiological agent in typhus, considering them to be a peculiar form of rickettsia which does not stain with Giemsa or at most very slightly.

Still more recently Pinkerton and Hass (9) described the cultivation of typhus rickettsiae from the testicle of an infected guinea pig. They used as explant material, small fragments of the membranous exudate imbedded in 1 drop of plasma coagulated by 1 drop of embryonic guinea pig tissue extract. The cultures were transplanted every 2 or 4 days by transferring a portion of the tissue into fresh medium. Rickettsiae were demonstrated morphologically in these cultures in histological sections. They state that in the majority of cases, the rickettsiae, whilst numerous in the first generation cultures, disappeared quite rapidly in successive transfers, but in one group of cultures rickettsiae were found in very great numbers in the fourth and fifth generations after 16 and 21 days *in vitro*.

In the following, a description is given of the technic and the media which have been used in carrying cultures of typhus fever rickettsiae for months *in vitro* without diminution either in virulence or in the number of organisms.

Technic

Typhus Virus.—The strain of typhus organisms used in these studies (unless otherwise indicated) was isolated from a case in the Southeastern United States by the U. S. Public Health Service in Washington, D. C.[1] It is in all respects quite similar to the Mexican strain of Mooser. A few experiments were also made with a strain from Nicolle's laboratory in Tunis.[2] We have carried the latter strain for some months, transferring sometimes with brain emulsions and sometimes with tunica washings. It may be noted parenthetically, in confirmation of Pinkerton's (10) observations on Wolbach's European strain, that in our hands the Nicolle strain produced, although irregularly, scrotal inflammation of slight to moderate intensity, indistinguishable from that produced by the Mexican type. Rickettsiae, although few in number, could also be demonstrated in the testicular exudate when such was present.

Media and Cultures.—Two types of tissue media have been used with equal success. One was adapted from that employed by Rivers, Haagen, and Muckenfuss (11) who used tissue cultures of rabbit cornea in coagulated plasma for the cultivation of the viruses of vaccinia and herpes. For our cultures, pieces (from 2–5 mm. square) of normal tunica from half-grown guinea pigs were soaked in the inoculum suspension, prepared as described below, for 20–30 minutes in order to insure intimate contact between virus and tissue; then, one to three

---

[1] We wish to express our gratitude for this material to Dr. G. W. McCoy, Director of the U. S. Public Health Service.

[2] This material was furnished us through the courtesy of Dr. Harry Plotz and Miss Helen Van Sant to whom we are greatly indebted.

pieces were imbedded, according to the directions given by Rivers, Haagen, and Muckenfuss (11), in large tubes (10 cm. long and 2.3 cm. wide) in a small amount of heparinized guinea pig plasma coagulated by means of Ringer solution extracts of normal guinea pig spleen.

The second medium was based on that used by Maitland and Maitland (12), Rivers, Haagen, and Muckenfuss (13), and others for the cultivation of vaccinia virus. This medium consists of Tyrode solution, serum, and minced tissue. For our work, cultures were prepared as follows: Minced normal tunica from half-grown guinea pigs was soaked in a few drops of inoculum for some minutes, after which Tyrode solution and guinea pig serum were added in the ratio of two parts of the former to one of the latter and the mixture distributed in amounts of about 3 cc. into 25 cc. Erlenmeyer flasks.

The tubes and flasks were closed with rubber stoppers and sealed with paraffin to prevent evaporation.

The cultures were incubated at 37.5°C. and transferred at 8–10 day intervals.

Inoculum for the Cultures.—To initiate cultures, the tunica containing rickettsiae from an infected guinea pig was scraped in a few cc. of Ringer or Tyrode solution, or ground in a heavy Pyrex 50 cc. centrifuge tube with a glass rod (inserted through a sterile gauze stopper) terminating in a ball deeply cross-hatched to make an effective grinding surface. The slightly turbid fluid thus obtained was used for inoculation. To transfer the cultures from one generation to the next, part of the tissue was removed from the medium and scraped or ground as above with a few drops of the fluid, and the cloudy suspension used to inoculate fresh tissue for several cultures. Tissue fragments were never transferred, only the supension obtained by scraping.

Stained Preparations.—Preparations for staining were made from the cultures by scraping a bit of tissue on a slide with a cataract knife, then spreading the resulting small amount of turbid liquid into a film which was allowed to dry, fixed in methyl alcohol for 2–3 minutes, again dried and stained with alkaline Giemsa in jars. Good staining was obtained in 15–20 minutes, after which time the slides were washed in running tap water, then rinsed with ethyl alcohol and xylene.

Although the Castaneda stain (14) was found to be excellent for demonstrating rickettsiae in the testicular exudate of infected guinea pigs, where the organisms are found largely within the cellular cytoplasm, it did not give as clear pictures with the scrapings of culture material where there were few or no tissue elements serving as background of contrasting color.

Tests for Virulence.—To test the virulence of the cultures, tissue was removed from the medium and ground with a small amount of liquid. This, along with the tissue debris, was injected intraperitoneally into guinea pigs. Marked scrotal swelling, characteristic temperature curve, and the subsequent demonstration of rickettsiae in the testicular exudate were used as indications of the virulence of the cultures.

Cultivation

It is apparently quite easy to establish cultures of typhus rickettsiae *in vitro* by means of either of the methods described; namely, the coagulated plasma medium or the serum Tyrode medium. All of six strains initiated by the former method and seven of thirteen by the latter were successful. With more careful selection of infectious material from lesions, the failures could doubtless be considerably reduced.

The tissue fragments imbedded in coagulated plasma began to show outgrowths within 2 or 3 days, reaching the maximum in 5–6 days, the final growth being plainly visible to the naked eye as a halo 1–1.5 mm. wide, surrounding the tissue.

Maitland and Maitland (12) originally believed that their serum Tyrode medium does not contain living cells. Rivers, Haagen, and Muckenfuss (13) have shown that the tissue, although it did not proliferate, was none the less viable for at least 5 days, and capable of proliferation when transplanted into a suitable medium. While it is uncertain whether the tissue in the typhus cultures was still viable at the end of 10 days, the incubation period which was commonly used, the rickettsiae certainly were. No systematic experiments have been made to determine precisely how long the organisms can survive without transfer.

Inasmuch as the coagulated plasma method is somewhat more arduous, it was discontinued in favor of the serum Tyrode medium after the latter was found to support growth as satisfactorily as the former. Cultures have been carried in the latter medium through twenty generations covering a period of 6 months, without diminution in numbers or virulence, and there seems to be little doubt that they can be carried indefinitely as any bacteriological culture.

Chart 1 shows the course of a characteristic infection with the cultivated rickettsiae and indicates the subsequent immunity to the passage strain.

Few to fairly numerous rickettsiae could always be demonstrated in stained preparations from the first generations in both types of cultures, their number increasing in later generations, although there was considerable variation in the number of organisms. Fig. 1 shows the characteristic microscopic picture of the rickettsiae in culture.

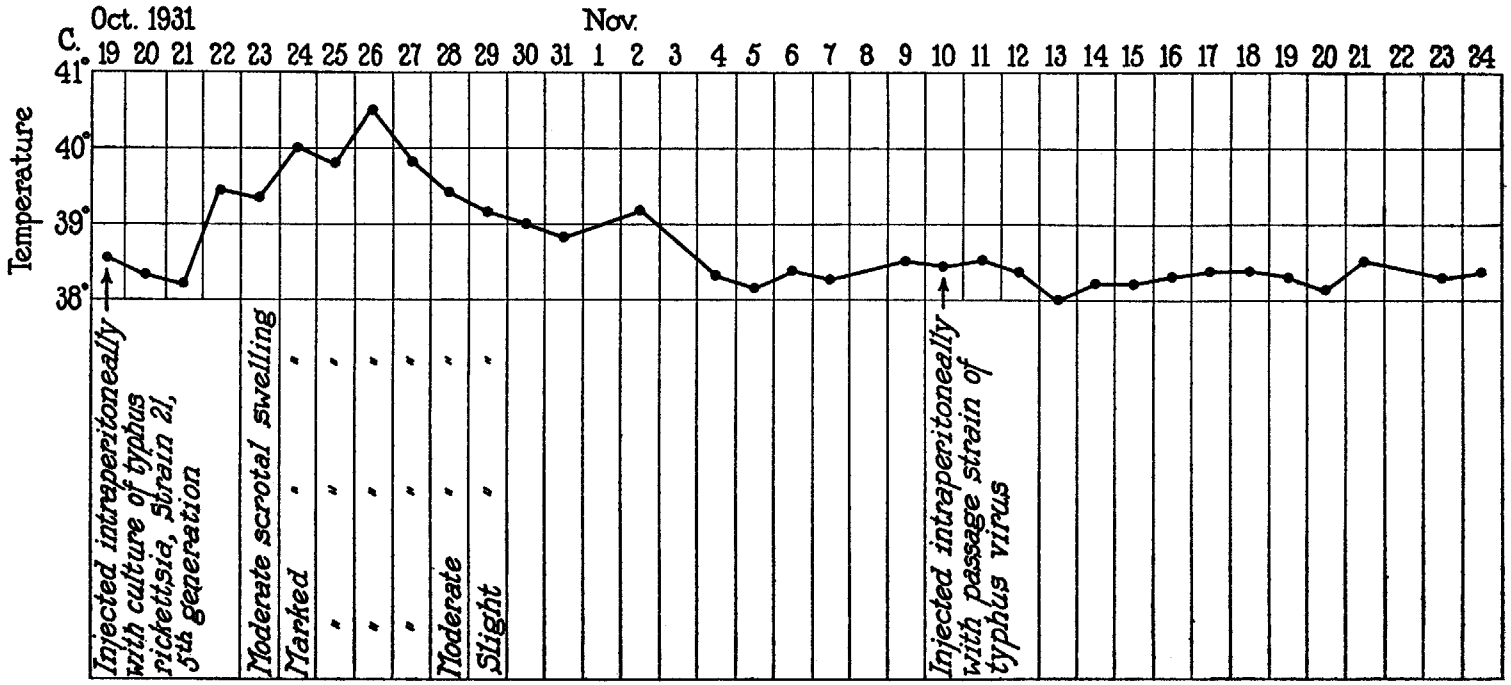


CHART 1. Temperature curve of a characteristic infection with cultivated typhus rickettsiae, showing subsequent immunity to passage strain

The rickettsiae of European typhus were also successfully cultivated from tunica scrapings of an infected guinea pig in the serum Tyrode medium. In the first generations the rickettsiae in these cultures showed a different morphology (see Figs. 2 and 3), *viz.*, a tendency to form chains of varying length, somewhat resembling minute streptococci, resulting in a picture which raises doubt as to the identity of these organisms. However, apart from the fact that there was no growth in the liquid part of the medium, slides from several of the later generations were in all respects similar to those of the Mexican type, and the strain proved to be fully and characteristically virulent on injection into guinea pigs. No growth was obtained on ordinary media with material from this or the Mexican strain.

The fact that the organisms could be demonstrated morphologically in the serum Tyrode medium only in the scrapings from the tissue fragments, and never in the supernatant liquid, would seem to indicate a parasitism of the rickettsiae for the tissue. Guinea pig tests for the infectiousness of the supernatant liquid were equivocal. In this respect the cultures differ from those of some filterable viruses grown in similar media, in that the latter can be transferred by using the liquid (Maitland and Maitland (12), Li and Rivers (15), Rivers (16)).

Experiments with Anaerobiosis and with Heated and Frozen Tissues

The significance of live tissue is indicated in the results of the following experiments in which heating, freezing, and anaerobiosis were studied as to their influence on the cultures.

The tests for virulence were made with the second generation cultures in the various media, since there was the possibility of a survival of rickettsiae in the inoculum of the first generation.

(*a*) Minced tunica, suspended in a small amount of Tyrode solution, was heated in a water bath maintained at 50°C. for 15 minutes (*cf.* Pincus and Fischer (17)). This heated tissue was subsequently inoculated and distributed in flasks in the usual manner. Appropriate controls were prepared simultaneously. The tests and controls were transferred after 10 days' incubation, the former again to heated tissue medium, and the latter to unheated tissue medium. Giemsa-stained preparations were made at the time of transfer and again at the end of the second incubation period of 10 days, at which time animals were injected. The results of these experiments are given in Charts 2 *a* and *b*. The numbers in parentheses indicate the generations of the strain.

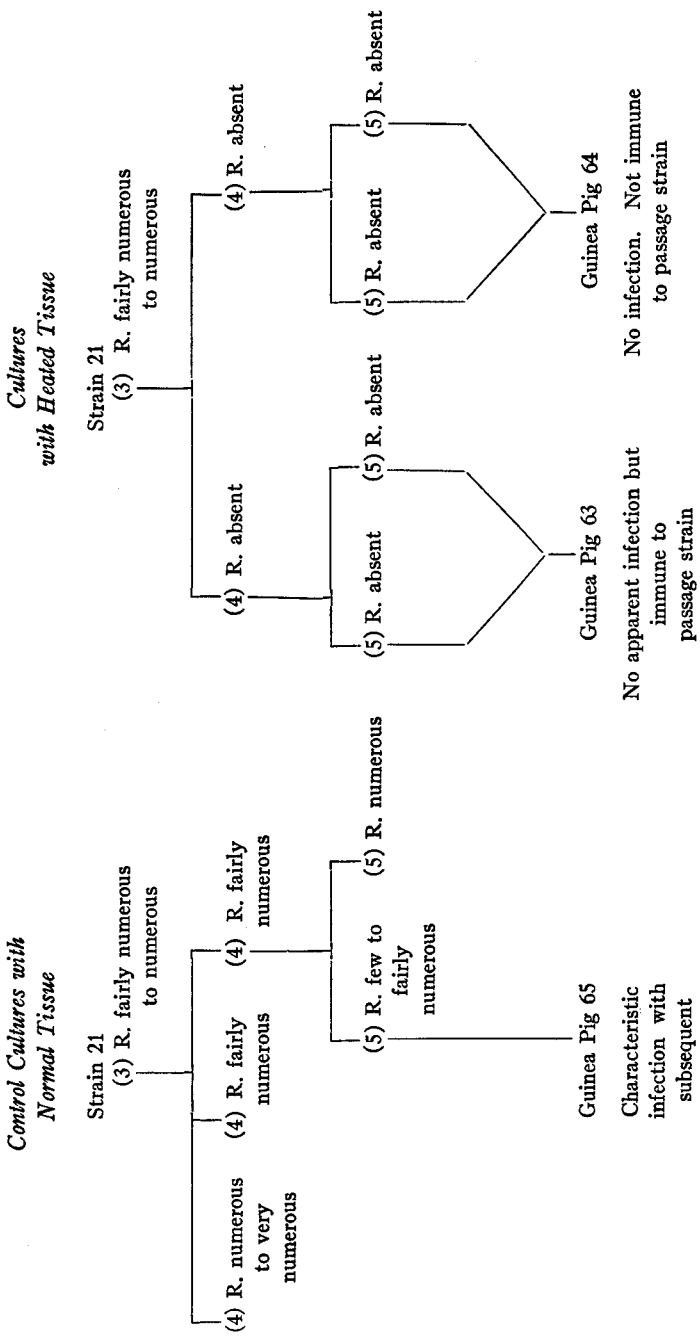
It will be noted that rickettsiae could not be demonstrated microscopically in any of the cultures prepared with heated tissue, either after the first or the second generation. However, of the three guinea pigs inoculated with material from second generations, one had a typical infection and was subsequently shown to be immune to the passage strain. A second guinea pig showed no reaction but was later found to be immune, indicating an unapparent infection, whilst only one of the three showed no signs of infection nor immunity. These results suggest two possible explanations: firstly, that heating at 50° for 15 minutes is slightly less than lethal for the tissue used; secondly, that the rickettsiae can remain viable without apparent multiplication for 20 days after being transferred into the heated tissue medium.

(*b*) Minced tunica, suspended in a small amount of Tyrode solution, was frozen with CO_2 snow and alcohol and thawed, fifteen times, after which it was inoculated and distributed in the usual manner. Appropriate controls were prepared simultaneously. Transfers, stained preparations, and guinea pig injections were carried out as in the previous experiments with heated tissue. The microscopic findings and the results of animal tests are shown in Chart 3.

It is seen that tissue killed by repeated freezing and thawing failed to support the growth of the rickettsiae.

(*c*) Normal tunica inoculated in the usual manner was divided between two series of flasks. One series was stoppered and paraffined in the usual manner to serve as controls. The flasks of the other series were identical except for a longer neck into which a two-hole rubber stopper was fitted, carrying one short and one long glass tube, the latter reaching almost to the surface of the medium. Air was replaced in these flasks by passing hydrogen gas through the long tube, the short tube serving as exit. After the air was driven out (5–10 minutes), the long tube was raised (with the stopper still in place) beyond a constriction previously made in the neck of the flask. With the hydrogen passing through, the flasks were sealed at the constriction in an oxygen flame. Transfers were made after 10 days' incubation into media subjected to anaerobiosis as described above, the controls being prepared as usual. The microscopic findings and results of animal tests are shown in Chart 4.

It will be noted that in these experiments the rickettsiae failed to multiply in the tissue medium under strictly anaerobic conditions. If this effect should prove to be constant, it may be attributable either to a deleterious effect on the tissue or directly on the organisms.



R. = rickettsiae.

CHART 2 *a*

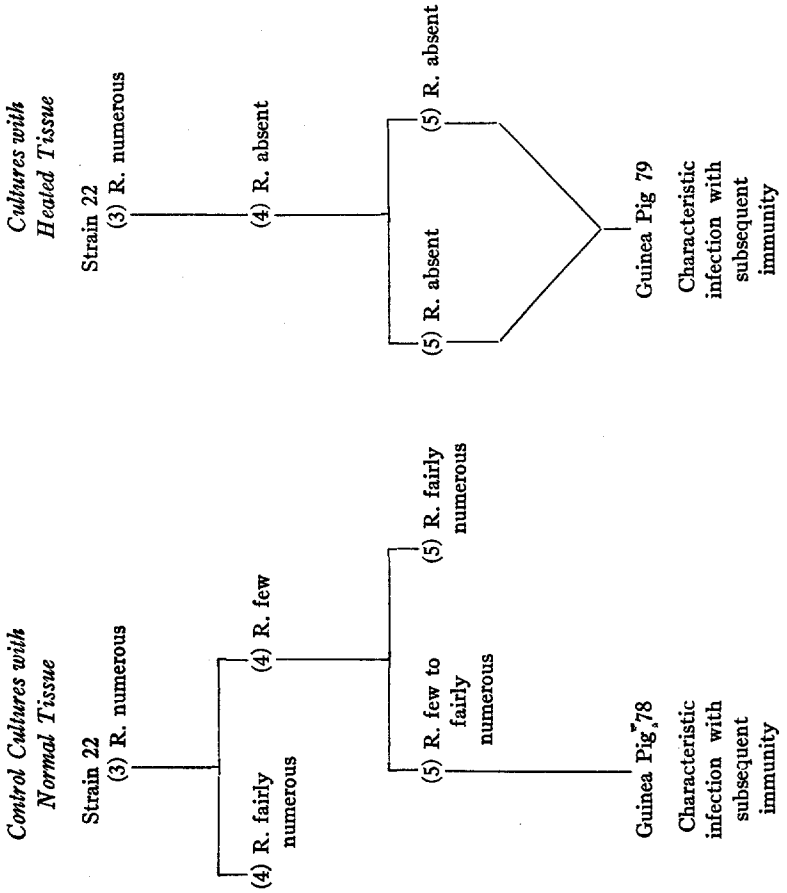


CHART 2 *b*

CHARTS 2 *a* and 2 *b*. Effect of heating the tissue in the culture medium.

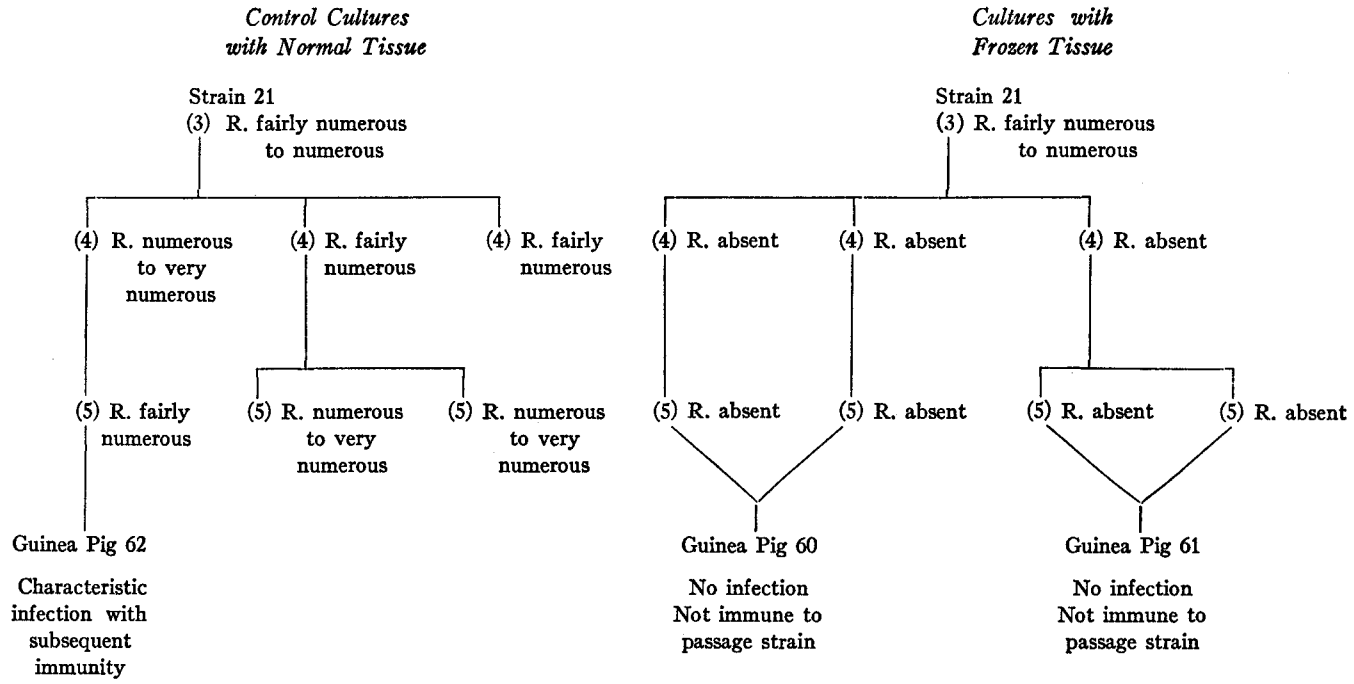


CHART 3. Effect of freezing the tissue in the culture medium. Other experiments with frozen tissue gave identical results.

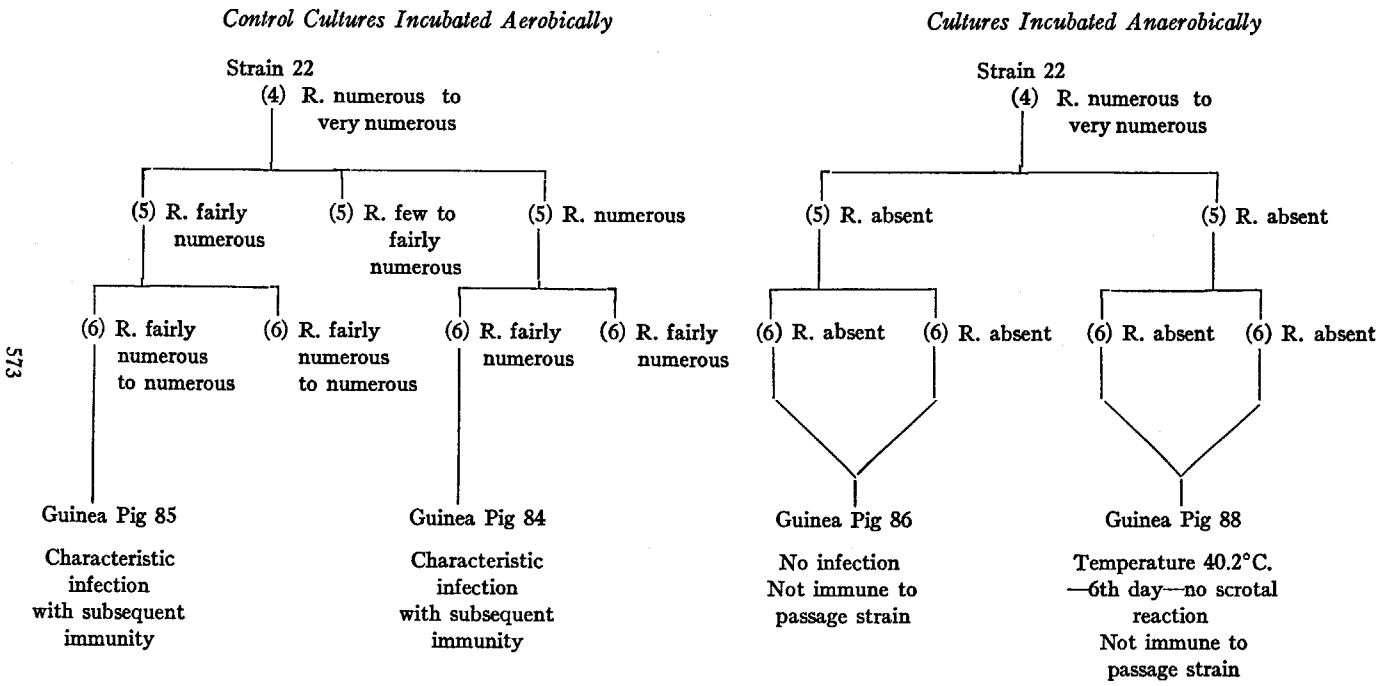


CHART 4. Effect of anaerobiosis on cultures.

Some preliminary experiments were made in order to study further the conditions necessary for cultivation.

It was found that of several tissues tested, only tunica and peritoneum gave satisfactory results in the medium described. The peritoneum, because of its greater surface, offers technical advantages in preparing rickettsia cultures on a larger scale.

A few attempts to cultivate the typhus organisms in a cell-free medium similar to that applied by Eagles and McClean (18) for vaccinia virus, were carried out with centrifuged Tyrode extracts of guinea pig kidney. So far these attempts have been unsuccessful inasmuch as no rickettsiae could be found and no infections could be induced in guinea pigs with these cultures.

Although the preceding results seem to indicate that live tissue is the significant constituent of the media described for the cultivation of the typhus rickettsiae, experiments to maintain cultures in the absence of serum have thus far been unsuccessful. In such experiments a medium was employed consisting only of tissue suspended in Tyrode solution, such as Li and Rivers (15) and Rivers (16) found to be entirely adequate for carrying cultures of vaccine virus. Actually rickettsiae were found in first generations in such a medium but usually reduced in number—and were for the most part absent in the second generations. In one such experiment rickettsiae were demonstrable in two generations, but not in the third. Although serum could not be entirely eliminated from the medium, it was found that the quantity could be reduced to at least half of that used as routine (*i.e.* one part of serum to five parts of Tyrode solution, instead of one to two parts) without damaging the cultures. It seemed to make little difference whether the serum was diluted with Tyrode, Ringer, or ordinary physiological saline. Whether the function of the serum consists merely in prolonging the viability of the tissue, has not been determined.

COMMENT

Although the etiological rôle of *Rickettsia prowazeki* in typhus fever hardly needs further confirmation, it is substantiated by the fact that guinea pigs recovered from infections, entirely typical of experimental typhus, produced by the injection of cultures as herein described, are

immune to passage virus. Moreover, rabbits infected with such cultures developed positive Weil-Felix sera.

A significant outcome of the experiments is to be found in the similarity of the growth conditions of *Rickettsia prowazeki* and filterable viruses. In general, the presence of living tissue is considered to be necessary for the cultivation of viruses. This is stressed by Rivers (19) (*cf.* Dale (20)), and was recently substantiated in a paper by Hallauer (21) on the cultivation of the virus of fowl plague (*cf.* Landsteiner and Berliner (22)). This relation of viruses to live tissues has been used as one of the arguments in favor of the view that viruses are not living organisms. It is of interest, therefore, that similar conditions for growth obtain in the cultivation of rickettsiae which, on account of their morphology, certainly must be deemed living microbes.

SUMMARY

1. *Rickettsia prowazeki* can be cultivated for many generations *in vitro*, without diminution in numbers or virulence, in media similar to those described by Maitland, Rivers, and others for the cultivation of certain viruses. In all probability, such cultures can be maintained indefinitely.

2. It has been impossible, thus far, to cultivate the typhus rickettsia without employing living tissue.

REFERENCES

1. Nigg, C., and Landsteiner, K., *Proc. Soc. Exp. Biol. and Med.*, 1930, **28**, 3.
2. Kuczynski, M. H., *Berl. klin. Woch.*, 1921, **2**, 1489.
3. Krontowski, A. A., and Hach, I. W., *Münch. med. Woch.*, 1923, **70**, 144; *Klin. Woch.*, 1924, **2**, 1625; *Arch. Zellforsch.*, 1926–27, **3**, 297; *Z. Immunitätsforsch.*, 1927–28, **54**, 237.
4. Wolbach, S. B., and Schlesinger, M. J., *J. Med. Research*, 1923–24, **44**, 231.
5. Rix, E., *Z. Hyg. u. Infectionskrankh.*, 1927–28, **108**, 103.
6. Zinsser, H., and Batchelder, A. P., *J. Exp. Med.*, 1930, **51**, 847.
7. Zinsser, H., and Castaneda, M. R., *J. Immunol.*, 1931, **21**, 403.
8. Sato, K., *Deutsch. med. Woch.*, 1931, **57**, 892, 1409.
9. Pinkerton, H., and Hass, G. M., *J. Exp. Med.*, 1931, **54**, 307.
10. Pinkerton, H., *J. Exp. Med.*, 1931, **54**, 181.
11. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1929, **50**, 665; *cf.* Carrel, A., and Rivers, T. M., *Compt. rend. Soc. biol.*, 1927, **96**, 848.

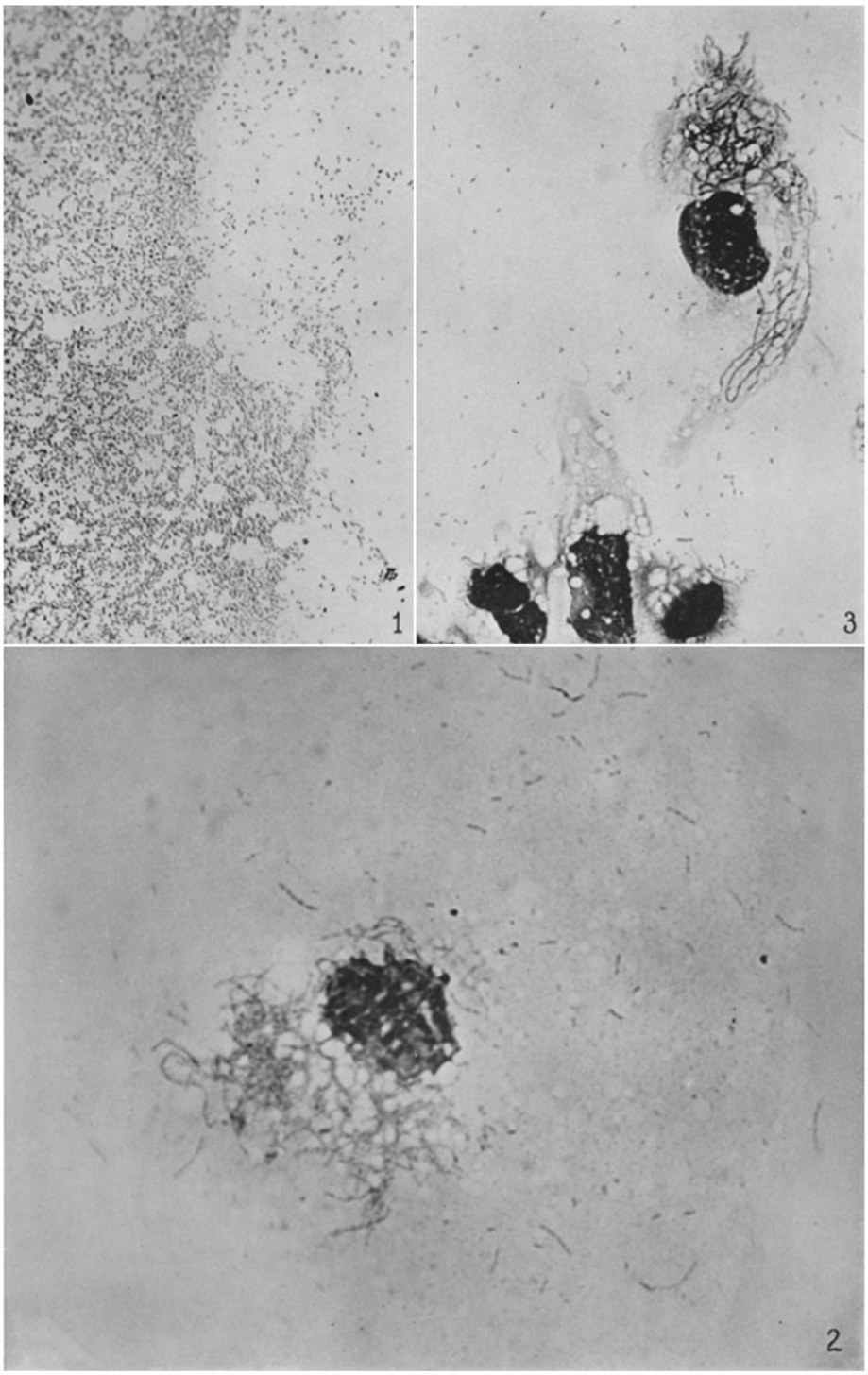
12. Maitland, H. B., and Maitland, M. C., *Lancet*, 1928, **2**, 596.
13. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1929, **50**, 181.
14. Castaneda, M. R., *J. Infect. Dis.*, 1930, **47**, 416.
15. Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.
16. Rivers, T. M., *J. Exp. Med.*, 1931, **54**, 453.
17. Pincus, G., and Fischer, A., *J. Exp. Med.*, 1931, **54**, 323.
18. Eagles, G. H., and McClean, D., *Brit. J. Exp. Path.*, 1930, **11**, 337; *Brit. J. Exp. Path.*, 1931, **12**, 97.
19. Rivers, T. M., *Physiol. Rev.*, in press.
20. Dale, H. H., *Nature*, 1931, **128**, 599.
21. Hallauer, C., *Z. Hyg. u. Infectionskrankh.*, 1931, **113**, 61.
22. Landsteiner, K., and Berliner, M., *Centr. Bakt., 1. Abt., Orig.*, 1912–13, **67**, 165.

EXPLANATION OF PLATE 24

FIG. 1. Typhus rickettsiae in culture, sixth generation. Giemsa stain. × 1000.

FIG. 2. From a culture of European typhus, fourth generation, showing chains intra- and extracellularly. Giemsa stain. × 1500.

FIG. 3. From a culture of European typhus, sixth generation, showing long chains intracellularly. Giemsa stain. × 1000.



Photographed by Louis Schmidt

(Nigg and Landsteiner: Cultivation of typhus fever rickettsia)