THE CULTIVATION OF THE MICRO-ORGANISMS OF ROCKY
MOUNTAIN SPOTTED FEVER (*DERMACENTROXENUS RICKETTSI*)
AND OF TYPHUS (*RICKETTSIA PROWAZEKI*) IN TISSUE
PLASMA CULTURES *

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The outcome of the experiments reported below, while failing to realize the expectation raised by preliminary successes, furnishes evidence of a crucial character in regard to the causal relationships of the micro-organisms to the diseases with which they are respectively associated. The idea of using tissue cultures to secure multiplication of micro-organisms not cultivable upon artificial media is not new. Zinsser and Carey¹ in 1912 used the method with probable success in an endeavor to cultivate the bacillus of rat leprosy.

An endeavor to grow the organisms of Rocky Mountain spotted fever in tissue culture was made in 1917, with the assistance of Dr. N. C. Foot. The method employed was that of using oxalated guinea-pig plasma, made to clot at the time of the experiment by the addition of embryonic tissue extract and calcium chloride. A few experiments, which resulted in successful tissue cultures from portions of the testes and tunica vaginalis of infected guinea pigs, proved not to contain the virus of the disease. With the assistance of Mr. Henry Pinkerton, in the summer of 1922, the experiment was repeated, using plasma collected in chilled paraffined tubes. These experiments yielded tissue cultures which were infective at the end of fourteen days, and in which micro-organisms consistent in morphology with *Dermacentroxenus rickettsi* were found. The work was continued, with the expectation of obtaining voluminous growths of the micro-organisms. It was also extended to the study of typhus. The strain of spotted fever was obtained through the kindness of Dr. R. R. Parker of the Public

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Health Service, and has been maintained for a period of over a year in this laboratory. The strain of typhus was one brought from Poland in 1920 and maintained since that time in this laboratory.

After a few successful experiments with the strains of the two diseases, it became evident that the method as employed by us did not promise the achievement of voluminous growth of the micro-organisms; but it was equally evident that we had command of a method that would bring new proof of the causal relationship of the micro-organisms with their diseases.

The protocols of our experiments are reported in abstract form. Daily temperatures were taken of all guinea pigs used, and the criteria for the diagnosis of Rocky Mountain spotted fever and typhus in guinea pigs adhered to were those of our previous work.^{2,3} The Giemsa staining technique employed for teased preparations and for sections was as described in the same publications.

It is impossible to correlate the results we have obtained with the results reported by several European workers who believe they have cultivated the causative agent of typhus fever. Neither is it possible for us to determine whether the organisms described by the European workers are identical or represent totally different bacteria. We refer particularly to the publications of Weigl⁴ on the relationship of strains of *proteus bacillus* to *Rickettsia prowazeki*; Kuczynski,⁵ who reports the cultivation of a micro-organism consistent with *Rickettsia prowazeki* for the third time by a third method; and Barykin and Kritsch⁶ who also regard their *Microbion typhi exanthematici* as consistent with *Rickettsia prowazeki*. Our intention is merely to report the results of our only successful method of preserving the viruses of these two diseases outside of the animal body, and the evidence that the micro-organisms associated with the two diseases multiply in the tissue cultures employed for the purpose. Our results indicate that under the conditions of the experiments both micro-organisms survive only within cells and are capable of multiplying only within cells which are of endothelial origin.

Technique. — The technique used for the tissue cultivation was the simplest compatible with the requirements of the experiments. Guinea-pig tissues were used throughout. In the case of Rocky Mountain spotted fever the testis was used, and in the case of typhus, the brain, previous studies having shown us that the lesions of the two diseases were most abundant in these organs. Blood from normal half-grown guinea pigs was used as a source of plasma, and a technique was adopted which permitted the collection of blood, its centrifugalization, and separation of the plasma with as few manipulations as possible. The guinea pigs used to furnish the blood were anesthetized with ether. The skin over the abdomen and thorax was reflected. The thorax was opened with heavy scissors heated to a red heat and the pericardial sac removed from the heart with sterile cold forceps. This method gives a sterile approach to the heart. For the collection of blood, test-tubes with special head-pieces were prepared as shown in Figure 1. These test-tubes were sterilized in the autoclave, and then the inner surface of the whole was coated with a thin layer of sterile paraffin. The outer surface of the terminal canula end of the longer tube was also coated with paraffin. It was found that thorough cleanliness and a very thin coating of paraffin were essential to prevent flaking and exposure of the glass during the chilling. Just before using, the paraffin-coated tubes were thoroughly chilled by plunging into an ice-salt mixture for five to ten minutes.

FIG. 1

The shape of the longer canula tube was designed in order to reach the heart over the chest wall and still keep the tube in an inclined dependent position. In using, the tip of the canula tube is forced through the wall of the left ventricle, and the blood is collected by the pumping action of the heart. Usually the heart muscle gripped the tube firmly and there was no leakage. When leakage occurred, clotting was apt to take place

during the centrifugalization. For a successful withdrawal of blood by this method it is necessary to use care in the anesthetization of the guinea pig, in order to have it complete and yet ensure a vigorous heart-action. Unless anesthesia is complete, reflex movements of the guinea pig make it difficult to keep the canula in place. By this method it is easy to obtain 10 to 12 c.c. of blood in a half-minute. After collection of the blood, the tube was immediately placed in the ice and salt mixture, and chilled for a period of about three minutes. It was then centrifugalized at high speed for three to five minutes, surrounded by an ice and salt mixture. Immediately after centrifugalization, the tube was returned to the ice and salt bath in order again to reduce the temperature to just above the freezing point. The supernatant plasma was then collected by means of a chilled paraffined pipette, and transferred to another chilled paraffined tube, and kept in this tube in the ice and salt bath at a temperature of -5° to -7° C. We have usually succeeded by this method in keeping the plasma without clotting for several hours. Should the temperature become too low and the plasma become frozen, it can be slowly thawed out and still be serviceable.

The tissue for cultivation was obtained with aseptic precautions, and unless otherwise mentioned was kept immersed in Ringer's solution until the plasma was prepared. The tissues for the cultures were cut into pieces 0.5 to 1.0 c.mm. in size under Ringer's solution. These pieces were each transferred to a sterile coverslip and covered immediately with a drop of sterile plasma. As soon as the plasma clotted, the coverslips were inverted over hollow ground slides and sealed with sterile vaseline.

Where tissue cultures were transferred to fresh plasma, in order to prolong the period of growth, for the purpose of ascertaining the relationship between survival of the virus and living cells, the bits of tissue or fractions thereof were removed from the plasma, washed for a few minutes in sterile Ringer's solution, transferred to fresh sterile coverslips, and fresh sterile plasma added. In this way we have kept tissue cultures from the testes and brains of guinea pigs in active growth for four to six generations, over a period of four to six weeks.

For injection into guinea pigs the tissue cultures were removed from the coverslips, placed in Ringer's solution and teased into pieces small enough to pass through a number 18-gauge hypodermic needle. The material injected consisted therefore of tissue fragments, the plasma, and the whole of the Ringer's solution used in teasing out the culture.

The behavior of tissues in plasma cultures has been observed with considerable interest. In our experiments the fragments of tissue, probably because of their relatively large size, underwent fairly prompt degeneration, with subsequent autolysis, except at the periphery. In the case of cultures from Rocky Mountain spotted fever, where it was possible to include blood vessels of a size visible to the naked eye in each portion of tunica selected, the differences between the vessel wall at the periphery of the culture and portions lying deeper were very striking. Blood vessels of considerable size close to the surface of the tissue maintained their normal appearances for several days. Deeper-lying portions underwent post-mortem change. In the peripheral viable portions it was easy in serial sections to note the following sequence: Swelling of the endothelium, followed by the appearance of many mitotic figures, so that the lumina of vessels became filled with cells of this origin. These cells exhibited phagocytic properties and took up red blood cells, degenerated white cells, and surrounded and engulfed collagen particles in deeper-lying situations. Red blood cells were completely digested, and endothelial cells after a week or more were found containing hemosiderin granules. As far as could be determined, the smooth muscle of the blood vessels did not proliferate. Most of the radial growth at the periphery of the tissues was of fibroblastic origin. Endothelial cells tended to congregate at the peripheral portions of the tissue; many of course wandered far out into the plasma. The only blood cells that apparently survived for more than a very few days were eosinophiles; and occasionally well-preserved eosinophiles, evidently caught by the fixative in amoeboid motion, were found as late as ten to twelve days.

In the case of brain cultures employed for typhus, everything

underwent rapid degeneration except the blood vessels and meninges. As far as could be ascertained, the nervous tissues retained their staining reactions and normal appearances for about the same length of time that they would in post-mortem material kept at the same temperature. At the end of a few days all traces of ganglion cells and neuroglia cells had disappeared and the background of the surviving and proliferating blood vessels consisted of a lightly staining granular material which in the course of time became less and less evident. The blood vessels in the course of a few days became filled with large endothelial cells, and at the periphery of the tissue fibroblasts as well as endothelial cells proliferated and migrated from the blood vessels. Where the meninges were retained, very active fibroblastic growth occurred.

The endothelial cells in brain cultures after a few days were always found laden with globules of lipin material. In deeply stained sections these globules were outlined by deposits of stain (blue), causing a reticulated appearance very confusing in the search for micro-organisms. This blue reticulum, whenever desirable, was removed by further differentiation of the slide in alcohol, or by bleaching in sunlight or under the electric arc.

ABSTRACTED PROTOCOLS OF TISSUE CULTURES ROCKY MOUNTAIN SPOTTED FEVER

Experiment 1. — August 17, 1922. A number of testis tissue cultures from an infected guinea pig, eight days after inoculation on the fifth day of fever, were set up in normal plasma obtained after seven minutes' centrifugalization. The bits of tissue used did not come in contact with any liquid other than the plasma used for the medium.

On August 23, one whole culture was injected intraperitoneally into guinea pig C 22. This guinea pig developed fever on August 26, and was killed on August 30. The gross and microscopic lesions were those of Rocky Mountain spotted fever, and the micro-organisms were demonstrated in sections of the testis.

On August 30, one half of a culture was injected intraperitoneally into guinea pig C 23, the other half was carefully teased out upon a slide and stained with Giemsa's stain. The stained preparations showed short paired rods, and a few slender curved bacillary forms, always intracellular, consistent morphologically with *Dermacentroxenus rickettsi*. Guinea pig C 23 developed fever on September 2, and was killed September 6. The gross lesions were those of Rocky Mountain spotted fever. The histological material was mislaid. Two guinea pigs, C 23 A and C 23 B,

were inoculated from this guinea pig and both developed spotted fever. Guinea pigs C 23 C and C 23 D were inoculated from C 23 B, and again both developed spotted fever. C 23 C was studied histologically and showed the typical lesions of the disease and the presence of the micro-organisms in blood-vessel walls.

Summary. The virus of Rocky Mountain spotted fever survived for a period of at least thirteen days in tissue-plasma culture, and forms consistent with *Dermacentroxenus rickettsi* were found in cells of the culture in teased preparations (Figs. 1 and 2).

Experiment 2.—October 10, 1922. The testis tissue came from a guinea pig seven days after inoculation, on the fourth day of fever. An 0.8 per cent NaCl solution was used to moisten the bits while the plasma was being obtained. Four minutes was the duration of centrifugalization.

On October 17, one half of a culture was injected intraperitoneally into guinea pig C 26; the other half was teased for a smear preparation which was stained with Giemsa's stain. The stained preparation showed a few intracellular rods and paired granules consistent with *Dermacentroxenus rickettsi*. Guinea pig C 26 developed fever on October 20, and died October 25. The autopsy showed gross lesions typical of Rocky Mountain spotted fever.

On October 19, one half of a culture was injected intraperitoneally into guinea pig C 27, and the other half was used for a teased preparation. No organisms were found in this preparation, but on October 22 the guinea pig developed fever, which continued until it was killed on October 26. The autopsy showed the gross and microscopic lesions of spotted fever, and the organisms of spotted fever were found in the vascular lesions in the testis. From this guinea pig two others were inoculated, C 27 A and C 27 B; both developed typical spotted fever, verified by gross lesions.

On October 24, one half of a culture was injected intraperitoneally into guinea pig C 28 and the other half used for a teased preparation. This culture did not show very active growth, and had been showing no change for at least four days. The smear preparation showed a few intracellular rods and pairs of organisms consistent with *Dermacentroxenus rickettsi*. Guinea pig C 28 developed fever on October 27, and ran a typical course of spotted fever. It died November 3 with typical lesions of the disease, including necrosis of the genital tissues.

On October 31, one half of a culture was injected intraperitoneally into guinea pig C 29; the other half was used for a teased preparation. This culture showed but slight growth and had been stationary for at least five days. No organisms were found in this preparation. Guinea pig C 29 showed normal temperature up to October 10. On October 14 it was inoculated from one of the passage virus guinea pigs and proved susceptible, developing fever on November 17 and dying on the 28th, with lesions typical of Rocky Mountain spotted fever.

Summary. The virus of Rocky Mountain spotted fever survived for fourteen days in tissue-plasma culture. It did not survive for a period of twenty-one days in the culture selected for inoculation. Micro-organisms were found in teased preparations of cultures used for inoculation on the

seventh and ninth days, not in preparations used on the fourteenth and twenty-first days.

Experiment 3. — October 19. Twelve tissue cultures were set up from the testis and cremasteric muscle of an infected guinea pig, nine days after inoculation, on the seventh day of fever. The bits of tissue used were kept in Ringer's solution during the preparation of the plasma. Time of centrifugalization was four minutes. On November 3 one half of a culture of testis tissue was injected intraperitoneally into guinea pig C 30. This animal developed fever on November 6 and ran a typical course of spotted fever, and died November 15. The autopsy showed lesions typical of Rocky Mountain spotted fever.

On October 31, a culture (testis) was fixed in Zenker's fluid, and subsequently embedded in paraffin and serial sections made through the whole. The slides were stained in the usual manner with Giemsa's stain, as described in a previous publication. In these sections numerous mitotic figures (fibroblasts?) were found, and numerous cells, presumably of endothelial origin, containing many minute paired micro-organisms identical in size and morphology with *Dermacentroxenus rickettsi* in the vascular lesions in man and animals. The sections showed clearly the remains of the collagen of the tunica albuginea. Large cells, containing the micro-organisms of spotted fever, were found, containing also hemosiderin granules and other foreign particles, including fragments of collagen fibril bundles. A few cells containing many micro-organisms exhibited pyknotic nuclei and acidophilic staining cytoplasm reproducing the picture of necrosis as seen in sections of organs.

On November 7, the whole of a culture from the cremasteric muscle was injected intraperitoneally into guinea pig C 31. This animal remained normal. It was tested for immunity by inoculation on December 19, from a passage virus guinea pig, and proved susceptible.

Summary. The virus of Rocky Mountain spotted fever survived in tissue-plasma culture for a period of fifteen days. It did not survive in a tissue (cremasteric muscle) plasma culture for a period of nineteen days.

Experiment 4. — On October 24, fourteen tissue cultures were set up from the testis of an infected guinea pig, the seventh day after inoculation, on the fourth day of fever. The bits of tissue were kept in Ringer's solution during the preparation of the plasma. The first attempt at securing plasma failed because of clotting. Plasma was secured in the second attempt, so that in placing the bits of tissue in plasma there was a delay of at least five minutes over previous experiments. Only four cultures showed growth.

On November 7, guinea pig C 32 was injected intraperitoneally with a whole culture. It remained normal, and on November 21 it was inoculated with passage virus and proved susceptible.

On November 11, guinea pig C 33 was injected intraperitoneally with a whole culture. It remained normal, and on November 23 it was inoculated with passage virus and proved susceptible.

Summary. The virus of Rocky Mountain spotted fever did not survive in the two cultures tested on the fourteenth and eighteenth day respectively.

Experiment 5. — This experiment was planned to test whether the survival of the virus could be prolonged by transplanting the tissue cultures or fragments thereof into fresh plasma. Microscopic studies of sections of the cultures gave indication that the virus survived but a few days after cell-division ceased, as indicated by the absence of mitoses. The next logical step was, therefore, to extend the period of growth of the cells, in order to ascertain if living cells were essential for the maintenance of the virus.

On October 31, 1922, twenty-seven tissue cultures were set up from an infected guinea pig twelve days after inoculation, on the fourth day of fever. The delayed incubation period of this animal is explained by the fact that it had recovered from infection with typhus.

On November 4, four cultures were selected for transfers. Each of the cultures, that is, the tissue with zone of growth, was cut radially into four segments. Each segment was washed for a few minutes in Ringer's solution and placed upon a sterile coverslip, and a drop of freshly prepared plasma added. These cultures were designated as second "generation" cultures.

On November 7, second generation cultures, without dividing, were cut out, washed in Ringer's solution, and fresh plasma added as above, and the subsequent growth designated as third "generation" cultures.

On November 11, the procedure of November 7 was repeated, and the subsequent growth designated as fourth "generation" cultures.

On November 14, the whole of a first generation culture was injected intraperitoneally into guinea pig C 34. This animal developed fever on November 17 and ran a typical course of spotted fever, ending in death on November 27. There was extensive necrosis of the scrotal tissues. Microscopically the lesions and micro-organisms of Rocky Mountain spotted fever were present.

On November 17, guinea pig C 35 was injected intraperitoneally with the whole of a first generation culture. This animal's temperature remained within normal limits while under observation, until November 29. On December 5 it was tested for immunity with passage virus and did not show a reaction, probably indicating immunity and therefore survival of the virus in the culture employed. The animal died seven days after the inoculation of intercurrent infection.

On November 17, guinea pig C 36 was injected intraperitoneally with the whole of a second generation culture. This animal developed fever on November 21 and ran a typical course of spotted fever. Its temperature became normal on December 30. On December 19 it was inoculated with passage virus and proved to be immune to Rocky Mountain spotted fever.

On November 23, guinea pig C 40 was injected intraperitoneally with the whole of a second generation culture. This animal maintained a normal temperature, and on December 5 was inoculated with passage virus

and developed Rocky Mountain spotted fever. This culture therefore did not contain the virus.

On November 23, guinea pig C 41 was injected intraperitoneally with the whole of a fourth generation culture. It developed fever on November 26 and ran a typical course of Rocky Mountain spotted fever, with necrosis of the scrotal tissues. It recovered, and on December 26 was tested for immunity by inoculation with passage virus. It maintained a normal temperature until January 6, 1923, and was then recorded as immune.

First generation tissue cultures were placed entire in Zenker's fluid on November 4, November 7, November 11, and November 14. They were imbedded in paraffin and serial sections made. In the preparation of November 4, there were many mitotic figures in endothelial cells lining blood-vessels and capillaries, and in fibroblasts. The blood-vessels at the periphery of the bit of tissue were well preserved; the central portions of the tissue exhibited degenerative changes and autolysis. The interesting feature was that in endothelial cells in an artery at the periphery of the section were large clumps of the micro-organisms. A rare filamentous form was also found in cells outside of blood-vessels (Fig. 4). Micro-organisms were also found in cells in the preparations of November 7 and November 11, both within vessels and in rounded cells that had migrated out into the plasma. The most striking findings were in the preparation of November 14. Here many cells that had migrated to the periphery of the culture and far out into the plasma contained clusters of the micro-organisms of spotted fever, in numbers quite unlike anything that we have seen in sections of organs. In a few endothelial cells and in a few fibroblasts, filamentous forms were found.

Summary. The virus of Rocky Mountain spotted fever survived in first generation tissue cultures for a period of fourteen to seventeen days; in second generation cultures for a period of seventeen days; in third generation cultures the period of survival was not tested. In fourth generation cultures it survived for a period of twenty-three days. The micro-organisms of Rocky Mountain spotted fever were demonstrated in first generation tissue cultures fixed at the end of four, seven, eleven, and fourteen days.

Experiment 6. — On November 7, twenty-three testis tissue cultures were set up from an infected guinea pig six days after inoculation, on the third day of fever. The plasma used in these cultures was obtained from a full-grown female guinea pig in a late stage of pregnancy. This experiment was essentially a repetition of Experiment 5. Second generation cultures were made on November 1, without dividing the initial growth. Third generation cultures were made on November 15, without dividing. Fourth generation cultures were made on November 20, without dividing. On November 21, guinea pig C 38 was injected intraperitoneally with the whole of a first generation culture. This animal developed temperature on November 23. It ran a typical course of spotted fever, exhibiting characteristic scrotal tissue reaction, but recovered. On December 1, its temperature was normal. On December 19 it was tested for immunity by the

inoculation of passage virus. It maintained normal temperatures until January 2, when it was recorded as immune to Rocky Mountain spotted fever.

On November 21, guinea pig C 39 was inoculated with a whole first generation tissue culture which exhibited no trace of growth. This guinea pig maintained normal temperatures until December 5. It was then inoculated with passage virus and developed typical Rocky Mountain spotted fever.

On November 25, guinea pig C 42 was inoculated with the whole of a first generation tissue culture, which showed good growth. This animal developed a temperature on November 29. It ran a typical course of Rocky Mountain spotted fever. Its temperature became normal on December 9. On December 16 it was tested for immunity by the inoculation of passage virus. Its temperatures remained normal, and on January 4 it was recorded as immune.

On November 29, guinea pig C 45 was injected with a whole first generation tissue culture, which showed good growth. It developed a temperature on December 2, which lasted until December 8. At the end of that time the scrotum was swollen but not necrotic. On December 19 it was tested for immunity by inoculation of passage virus. Its temperature remained normal and on January 1 it was recorded as immune.

On December 5, guinea pig C 48 was inoculated with the whole of a first generation tissue culture, which showed good growth and considerable liquefaction of the plasma. It maintained normal temperatures until January 15. It was tested for immunity by inoculation with passage virus on January 19. It reacted typically, and died of Rocky Mountain spotted fever January 30.

On November 29, guinea pig C 43 was injected intraperitoneally with the whole of a second generation tissue culture, which showed very good growth. It developed temperature on December 29, ran a typical course of Rocky Mountain spotted fever, and died January 7. The gross findings were typical of Rocky Mountain spotted fever, including extensive necrosis of the scrotum.

On November 29, guinea pig C 46 was injected intraperitoneally with the whole of a second generation tissue culture, which showed good growth but which had ended by completely liquefying the surrounding plasma. This guinea pig's temperature remained within normal limits until December 13. On December 19 it was tested for immunity by the inoculation of passage virus. It developed typical Rocky Mountain spotted fever, and died January 1.

On December 5, guinea pig C 47 was inoculated with the whole of a fourth generation tissue culture, which showed good growth. It developed a temperature on December 9, ran a typical course of Rocky Mountain spotted fever, and recovered. It was tested for immunity by the injection of passage virus on December 26. It failed to react.

On December 9, guinea pig C 49 was injected intraperitoneally with the whole of a fourth generation culture, which showed slight but definite growth. This animal maintained normal temperatures until December 19. On December 26 it was tested for immunity by the inoculation of passage virus. It reacted to Rocky Mountain spotted fever.

First generation tissue cultures were fixed on November 17 and November 21. They were studied in serial sections as before. The November 17 preparations showed numerous minute paired micro-organisms and a few filamentous forms, always within cells. In this preparation, for the first time, compact masses of micro-organisms, comparable to the intranuclear masses observed in ticks, were found in endothelial cells desquamated into the lumina of blood-vessels at the periphery of the sections. In the preparation of November 21 many isolated endothelial cells at the periphery of the culture and in the plasma were found to contain masses of minute paired micro-organisms identical with those found in sections of organs. Many of these cells containing the micro-organisms also contained hemosiderin pigment (Fig. 3).

Summary. The virus of Rocky Mountain spotted fever survived in first generation cultures for a period of twenty-two days. It did not survive for a period of twenty-eight days. In second generation cultures it survived for a period of seventeen days, and in cultures showing no growth tested at the end of twenty-two days the virus was not present, C 46, although present at the same time in a first generation culture which showed good growth, C 45. In fourth generation cultures the virus survived a period of twenty-eight days. It did not survive a period of thirty-six days. The micro-organisms of Rocky Mountain spotted fever were found in cultures fixed at the end of ten and fourteen days in numbers positively indicating multiplication.

Experiment 7. — On November 28, fifty-six testis tissue cultures were set up from an infected guinea pig seven days after inoculation, on the fourth day of fever. The experiment was intended as a repetition of experiments 5 and 6, and although successful sixth generation cultures were obtained, no guinea pigs were inoculated. The entire culture was preserved in Zenker's fluid on December 1. Micro-organisms were found in large numbers in the endothelium in the surviving vessels at the periphery of the growth, in numbers indicating multiplication. No filamentous forms were found.

Experiment 8. — The object of this experiment was to ascertain whether the virus would survive in tissue cultures made with normal guinea-pig tissues and plasma from an infected guinea pig.

On December 12, 1922, forty testis tissue cultures were set up from a normal guinea pig, using infected plasma from a guinea pig seven days after inoculation, on the fourth day of fever. The plasma was obtained after five minutes' centrifugalization. In removal of the plasma the buffy coat and a few red cells were included. The plasma was mixed well before it was distributed. The tissue cultures were set up in the usual way, one drop of the infected plasma being added to the usual-size piece of normal-guinea-pig testis. Also, drops of plasma were placed on coverslips without tissue, and inverted over hollow slides and sealed, in order to test the duration of survival of the virus in the clotted plasma alone under the conditions of the experiment. One drop of the plasma injected intraperitoneally

into guinea pig P 1 on December 12, at the termination of the experiment, produced a typical fatal Rocky Mountain spotted fever reaction.

On December 15, a drop of plasma was inoculated into guinea pig P 2. This animal developed a temperature on December 20, which continued until December 27, when the animal was killed. Gross and microscopic findings failed to reveal the lesions of Rocky Mountain spotted fever. The cause of the fever was probably epidemic respiratory disease.

On December 18, a drop of plasma was injected into guinea pig P 3. This animal developed temperature on December 26, which was maintained until January 3, when it was killed. Both lungs were partially solidified, and microscopic examination of the tissues failed to reveal lesions suggestive of Rocky Mountain spotted fever.

On December 23, guinea pig P 4 was injected intraperitoneally with a drop of the coagulated plasma. It maintained normal temperatures until January 4, when it developed fever. It was killed on January 9, and the autopsy again revealed pneumonia and no evidence of spotted fever.

On December 23, guinea pigs P 5 and P 6 were inoculated with tissue cultures of normal testis in the infected plasma. Both these guinea pigs failed to develop spotted fever. Similar negative results were obtained with guinea pigs P 7 and P 8, inoculated on December 26.

Summary. The virus of Rocky Mountain spotted fever in coagulated plasma did not survive for three days. The virus of Rocky Mountain spotted fever in a tissue-culture combination of infected plasma and normal-guinea-pig testis did not survive a period of eleven days.

Experiment 9.—On March 1, twenty-two testis tissue cultures were set up from a spotted fever guinea pig, nine days after intradermal inoculation, on the fourth day of fever. The intradermal inoculation explains the long incubation period.

Guinea pigs C 50 and C 51 were inoculated on March 12. Both developed typical spotted fever, verified by propagation of the virus.

Summary. The virus of Rocky Mountain spotted fever survived in tissue cultures for a period of twelve days.

Experiment 10.—This experiment was intended as a repetition of Experiments 5 and 6. It does not seem desirable to abstract the results, because of the complete failure of the virus to survive.

On August 6, 1923, forty-six testis tissue cultures were set up from an infected guinea pig, seven days after inoculation, on the fourth day of fever. The majority of these cultures showed excellent growth. The tissue cultures were maintained into the fourth generation. One guinea pig, C 56, inoculated on August 11, developed spotted fever. This was controlled by propagation of the virus. After this date first generation cultures failed to survive. Numerous guinea pigs were inoculated from each succeeding generation. None of them reacted. The cause of the failure of the virus to survive in this experiment was not determined.

ABSTRACTED PROTOCOLS OF TISSUE CULTURES
TYPHUS

Experiment 1. — October 26, 1922. Eight cultures were set up, using bits of brain from a typhus guinea pig, sixteen days after inoculation, on the sixth day of fever. Ringer's solution was employed to moisten the bits of tissue. Time of centrifugalization was three minutes.

On November 3, guinea pigs T C 1 and T C 2 were injected intraperitoneally, each with a whole culture. Both of these guinea pigs developed fever on November 13. T C 1 died November 17. The autopsy showed massive double pneumonia. Microscopic examination, however, showed the typical lesions of typhus in the cerebrum. T C 2 maintained a temperature ranging between 103.7° and 105.0° F until November 20, when it was killed. The autopsy showed no gross lesions except a moderately enlarged spleen. Microscopic examination showed many typical typhus lesions in the cerebrum.

On November 7 a culture was fixed in Zenker's fluid, and subsequently studied in serial sections stained by Giemsa's stain. A few endothelial cells were found containing short rods morphologically consistent with *Rickettsia prowazeki*.

Summary. The virus of typhus survived for a period of eight days in brain-tissue plasma culture. Forms consistent with *Rickettsia prowazeki* were found in sections of a culture fixed after twelve days.

Experiment 2. — November 21, 1922. Twenty-five brain-tissue cultures were set up from a guinea pig fourteen days after inoculation, on the fourth day of fever. Ringer's solution was employed. Time of centrifugalization was three minutes. The plasma after separation was inadvertently frozen, but was thawed out and remained liquid until used.

On November 27, guinea pigs T C 5 and T C 6 were injected intraperitoneally, each with a whole culture.

T C 5 died December 8, of pneumonia.

T C 6 maintained normal temperatures until December 19, when it was tested for immunity by inoculation with passage virus. On December 29 it developed fever, and ran a course consistent with typhus until killed January 5, 1923. The autopsy showed a moderately enlarged spleen and a blood-tinged exudate in the pleural cavities. Microscopic examination, however, showed typical typhus lesions in the cerebrum. It was concluded that the virus of typhus was not present in the culture employed.

On December 2, guinea pigs T C 7 and T C 8 were injected intraperitoneally, each with a whole culture.

T C 7 developed fever on December 5; there was a drop on December 7 and then continuous fever until December 15. It was killed on December 16. The autopsy showed an enlarged spleen. The other organs were negative except for two walled-off abscesses in the liver (pseudo-tuberculosis). Microscopic examination showed many typical typhus lesions in the cerebrum.

T C 8 developed fever on December 9, which persisted. It was killed December 16. The autopsy showed active pseudo-tuberculosis of the

liver, a fibrinous peritonitis, and an enlarged spleen. Microscopic examination showed numerous typical typhus lesions in the cerebrum.

On December 5, T C 9 and T C 10 were injected intraperitoneally, each with a whole culture.

T C 9 developed fever on December 14, which continued until December 26. On February 23 it was tested for immunity by inoculation with passage virus. Its temperatures remained normal until March 8, when it was recorded as immune.

T C 10 failed to develop fever while under observation to December 26. On February 21 it was tested for immunity by inoculation with passage virus. It developed fever on March 2. It was killed March 9. The autopsy showed a markedly enlarged spleen and no other gross lesions. Microscopic examination showed the typical lesions of typhus in the cerebrum.

Two cultures fixed in Zenker's fluid, on November 29, and studied in serial sections stained by Giemsa, showed many mitotic figures among fibroblasts and endothelial cells. Numerous endothelial cells in each preparation contained filamentous and rod forms similar to *Rickettsia prowazeki* in lice. A culture fixed on December 2 revealed no micro-organisms.

Summary. The virus of typhus failed to survive in one brain-tissue plasma culture for eight days and in one culture for fourteen days. It survived in two cultures for eleven days and in one culture for fourteen days. Micro-organisms consistent with *Rickettsia prowazeki* were found in preparations of two brain-tissue plasma cultures fixed on the eighth day, but were not found in one culture fixed on the eleventh day.

Experiment 3. — December 6, 1922, twenty-four brain tissue cultures were set up from a guinea pig fourteen days after inoculation, on the eighth day of fever. The brain was immersed in Ringer's solution for seven to eight minutes before it was cut up and the fragments covered with plasma.

On December 14, guinea pig T C 11 was injected intraperitoneally with a whole culture. It developed fever on December 26, which continued until January 5, when it was killed. The autopsy revealed no gross lesions. Microscopic examination showed a rare typhus-like lesion in the cerebrum.

On December 15, guinea pig T C 13 was injected intraperitoneally with a whole culture. It did not react. Temperatures were taken until January 12.

On December 22, guinea pigs T C 16 and T C 17 were injected intraperitoneally, each with a whole culture. Neither animal reacted. Temperatures were taken until January 12.

On December 26, guinea pigs T C 18 and T C 19 were injected intraperitoneally, each with a whole culture. Neither animal reacted. Temperatures were taken until January 12.

Summary. The virus of typhus survived in brain-tissue plasma cultures for eight days. It did not survive in two cultures tested on the twelfth, sixteenth, and twentieth days respectively.

Experiment 4. — January 3, 1923, forty brain tissue cultures were set up from a guinea pig fourteen days after inoculation, on the ninth day of fever. The brain tissue was in Ringer's solution about thirty minutes before it was cut up and the plasma added.

On January 13, guinea pigs T C 21 and T C 22 were injected intraperitoneally, each with a whole culture.

T C 21 developed fever on January 20. It was killed January 25. The autopsy showed two small areas of consolidation in the lungs. The other organs were normal. Microscopic examination showed pneumonia. In the cerebrum there were typical typhus lesions.

T C 22 maintained temperatures within the normal range, with the exception of a rise to 103.8° F on January 24. On January 29 temperature-taking was discontinued. February 9 the animal was found dead. Autopsy showed complete consolidation of both lungs.

On January 17, guinea pigs T C 23 and T C 24 were injected intraperitoneally, each with a whole culture.

Guinea pig T C 23 developed fever on January 23. It was killed on January 29. The autopsy showed no gross lesions except a moderately enlarged spleen. Microscopic examination of the cerebrum showed many typical typhus lesions.

T C 24 developed fever on January 26. It was killed on January 31. The autopsy showed no gross lesions except a moderately enlarged spleen. Microscopic examination showed many typical typhus lesions in the cerebrum.

On January 23, guinea pig T C 25 was injected intraperitoneally with a whole culture. It developed fever on February 3 and was killed February 8. The autopsy showed no gross lesions except a moderately enlarged spleen. Microscopic examination showed many typical typhus lesions in the cerebrum.

The microscopical study of serial sections of cultures fixed in Zenker's fluid from this series resulted as follows:

One culture fixed on January 6 showed no micro-organisms. There were no wandering cells present, though the endothelial cells of blood-vessels were markedly enlarged. Red blood cells were fairly well preserved. The ganglion cells and neuroglia cells were degenerated but recognizable. One culture fixed on January 8 showed no micro-organisms. Mitotic endothelial cells were abundant, and there were wandering phagocytic cells, presumably of endothelial origin, containing myelin particles.

Two cultures fixed on January 12 showed growth of fibroblasts from blood-vessels, as well as endothelial cell-growth. In one culture a few straight and curved rods morphologically consistent with *Rickettsia prowazeki* were found in endothelial cells. Two cultures fixed on January 17 showed no trace of original brain-tissue structure. They consisted of granular material surrounded by zones of fibroblasts and endothelial cells. In one of these cultures many endothelial cells were found containing filaments and rods similar to *Rickettsia prowazeki* in the louse. There were also rare cells filled with minute rods like those found in typhus-infected lice.

A culture fixed on January 23 showed a single mitotic figure. Most of

the fibroblasts were rounded off. A rare filament consistent morphologically with *Rickettsia prowazeki* was found in cells.

Summary. The virus of typhus failed to survive in one brain-tissue plasma culture for a period of ten days. It survived in one culture for ten days, in two cultures for fourteen days, and in one culture for twenty days.

Micro-organisms consistent morphologically with *Rickettsia prowazeki* were found in sections of cultures fixed on the ninth, fourteenth, and twentieth days respectively.

Experiment 5. — January 31, 1923, twenty-five tissue cultures were set up, using the brain of a guinea pig fifteen days after inoculation, on the sixth day of fever. Immediately after the conclusion of the experiment one of the cultures was injected into a guinea pig, which developed fever on February 10 and ran a course consistent with typhus.

On February 12, guinea pigs T. C. 32, 33, and 34 were injected intraperitoneally, each with a whole culture. T C 32 and T C 33 failed to react. Temperatures were taken until March 5. On March 6 these guinea pigs were injected with passage virus, and both showed typical typhus reactions.

T C 34 developed temperature on February 21, and died on February 24 of an intercurrent infection.

On February 17, guinea pigs T C 36, 37, and 38 were injected intraperitoneally, each with a whole culture. T C 36 developed fever on February 18, which continued until March 7, when it was killed. Autopsy showed pleurisy and pneumonia. Microscopic examination of the brain was negative for lesions of typhus.

T C 37 failed to react and was discarded as negative on March 9.

T C 38 developed fever on February 26, and was killed on March 1. The autopsy showed pneumonia and the microscopic examination of the brain was negative for the lesions of typhus.

Summary. The virus of typhus did not survive in brain-tissue plasma cultures for a duration of twelve days.

Experiment 6. — March 9, 1923. The guinea pig used for this experiment was guinea pig T C 10 of experiment 2, which did not acquire typhus after injection with a tissue culture fourteen days old. It responded to the passage virus, and was used for this experiment sixteen days after inoculation, on the fifth day of fever. Microscopic examination of the brain of this guinea pig showed numerous characteristic lesions of typhus.

Twenty-five cultures were set up. Immediately after the preparation of the cultures was completed, a guinea pig was inoculated, which developed a temperature on March 19 and ran a typical typhus course.

On March 24, guinea pigs T C 51, 52, and 53 were injected intraperitoneally, each with a whole culture. The only guinea pig of this series that reacted in a manner suggestive of typhus was T C 52, which developed temperature on April 2. It was killed on April 7 and the autopsy showed pneumonia. Microscopic examination of the brain showed no lesions.

On March 29, guinea pigs T C 54, 55, and 56 were injected intraperitoneally, each with a whole culture. Of these the only one that reacted

with fever was T C 55, which developed fever on April 4. It was killed on April 9. No gross lesions were found at autopsy. The spleen was not enlarged. Microscopic examination failed to reveal the lesions of typhus.

Summary. The virus of typhus did not survive for a period of fifteen days in brain-tissue plasma culture.

Experiment 7.— March 19, 1923. Twenty-five cultures were set up from the brain of a guinea pig, sixteen days after inoculation, on the fifth day of fever. Great care was used in not traumatizing the bits of brain tissue used for the cultures. The plasma was obtained from a young guinea pig. Immediately after the cultures were set up, a guinea pig was inoculated with one of them. It developed temperature on March 27 and ran a course typical of typhus.

On March 29, guinea pig T C 57 was injected intraperitoneally with a whole culture. On April 9 it developed fever. It was killed April 13. The autopsy was negative except for an enlarged spleen, on the surface of which was a thin layer of fibrin. Microscopic examination of the brain showed the typical lesions of typhus.

On April 2, guinea pigs T C 61, 62, 63, and 64 were injected intraperitoneally, each with a whole culture. T C 61 developed temperature on April 2. On April 13 it showed paralysis of the hind legs. On April 16 it died. The autopsy showed an enlarged spleen and a fibrinous exudate in the tunica vaginalis. Microscopic examination of the brain showed numerous typical lesions of typhus.

T C 62 developed temperature on April 11. On April 19 the temperature was sub-normal and there was paralysis of the hind legs. It was killed the same day, and post-mortem examination showed consolidation of one lung and an enlarged spleen. Microscopic examination of the brain showed a few typical typhus lesions.

T C 63 developed temperature on April 10. It died on April 16. The animal was discarded by mistake. No post-mortem examination was made.

T C 64 developed fever on April 10. April 14 it showed slight paralysis of the hind legs. It was killed on April 16. The autopsy showed no gross lesions other than an enlarged spleen with a fibrin coat. Microscopic examination of the brain showed numerous typical lesions of typhus.

On April 2, a culture was fixed in Zenker's fluid. Microscopic examination of the serial sections showed many large endothelial cells, chiefly at the periphery of the tissue, containing long slender straight and curved filaments, as well as many minute rod-shaped rickettsia. In the groups of endothelial cells filling the lumina of former blood-vessels were occasional endothelial cells which were distinguished in low-power examination by their deeper blue stain. These cells were packed with minute rods, reproducing with exactness the appearance of cells in the louse's intestine when filled with rickettsia. Mitotic figures in this culture were abundant. (Figs. 5, 6, and 7).

Summary. The virus of typhus survived in one culture for a period of ten days and in three cultures for a period of fourteen days. It presumably survived in the fourth culture at the end of fourteen days, but the

evidence is not complete. In a tissue culture fixed at the end of fourteen days micro-organisms were found in endothelial cells consistent morphologically with *Rickettsia prowazeki*; in fact, every form seen in lice infected with typhus was reproduced with exactness in this culture.

Experiment 8. — March 20, 1923. Twenty-five cultures were set up, using the brain of a guinea pig fourteen days after inoculation, on the sixth day of fever.

On March 29, guinea pig T C 59 was injected intraperitoneally with a whole culture. This guinea pig developed fever on April 6. It was killed on April 10. A small patch of consolidation was found in one lung. The spleen was enlarged and covered with a fibrin coat. Microscopically characteristic lesions of typhus were found in the brain.

On April 4, guinea pigs T C 65, 66, 67, and 68 were injected intraperitoneally, each with a whole culture. The only pig that reacted with temperature was T C 66, which developed fever on April 11. The temperature remained high until April 19. April 25 it was tested for immunity by inoculation with passage virus. It did not react.

The other three guinea pigs maintained normal temperatures up to May 3, when they were discarded and recorded as negative.

On April 11, guinea pigs T C 69, 70, and 71 were injected intraperitoneally, each with a whole culture. T C 69 maintained normal temperatures until May 10, when it was discarded as negative.

T C 70 died on April 24, of acute enteritis. Its temperature had never been elevated. Microscopic examination of the brain showed no lesions.

T C 71 developed fever on April 21, which subsided on April 26. On April 30 it was inoculated with passage virus for the purpose of testing immunity. It died of intercurrent infection on May 6.

Summary. The virus of typhus survived in brain-tissue plasma cultures for periods of nine and fifteen days. It is questionable whether it survived in one out of three cultures for a period of twenty-two days.

Experiment 9. — April 3, 1923. In this experiment an attempt was made to ascertain whether the duration of the virus could be prolonged by sub-culture. On April 3 sixty cultures were set up, using the brain of a guinea pig thirteen days after inoculation, and the fifth day of fever. On April 10 five cultures showing good growth were halved, each half washed in Ringer's solution for a few minutes, placed upon coverslips, and fresh plasma added. The subsequent growth from these cultures will be referred to as the second "generation."

On April 18, guinea pigs T C 76, 77, 78, and 79 were injected intraperitoneally, each with a whole first generation culture. T C 76 developed temperature on May 11. It was killed May 16. The autopsy showed no gross lesions. Microscopic examination of the brain showed no lesions.

T C 77 developed fever on April 21. It maintained temperatures ranging between 103.5° and 104.5° F until May 10. May 16 it was tested for immunity by inoculation with passage virus. It did not react.

Guinea pig T C 78 developed temperature on March 24, which subsided on May 4. It was discarded on May 17 as having probably reacted for typhus. It was not tested for immunity.

T C 79 developed fever on March 24, which lasted only two days. It was observed until May 17 without further rise of temperature, and was discarded as negative.

On April 24 guinea pigs T C 80, 81, 82, and 83 were injected intraperitoneally, each with a whole first generation culture.

T C 80 did not react. Temperatures were taken until May 25.

T C 81 developed fever on May 17, when it was killed. The spleen was markedly enlarged. No other gross lesions were found. Microscopic examination of the brain was negative.

T C 82 was observed until May 26, without rise of temperature.

T C 83 was observed until May 25, without rise of temperature. Both were discarded as negative.

On May 1, guinea pig T C 88 was injected intraperitoneally with a whole first generation culture. It was observed until May 19, without rise of temperature.

On May 1, guinea pig T C 92 was injected intraperitoneally with the whole of a second generation culture. It developed fever on May 18. On May 24 it was killed. The spleen was moderately enlarged. One lung showed a small area of consolidation. Microscopic examination, however, showed numerous typical lesions of typhus.

Summary. The virus of typhus survived for a period of fifteen days in brain-tissue plasma cultures in one out of four cultures. It was probably present in another culture, but the result was not controlled. It failed to survive in four first generation cultures for a period of twenty-one days, and in one first generation culture for a period of twenty-eight days. In a second generation culture it survived for a period of twenty-eight days.

Experiment 10.—April 17, 1923. Fifty cultures were set up, using the brain of a guinea pig fourteen days after inoculation, on the seventh day of fever.

On May 1, guinea pigs T C 93, 94, and 95 were injected intraperitoneally, each with a whole culture. Guinea pigs T C 93 and 94 failed to react, and were discarded on May 31 and recorded as negative.

T C 95 developed fever on May 10. It was killed May 14. No gross lesions were found at the autopsy. Microscopically brain showed numerous typical typhus lesions.

On May 10, guinea pigs T C 96, 97, and 98 were injected intraperitoneally, each with a whole culture. T C 96 developed temperature on May 18. It was killed May 22. The autopsy showed no gross lesions. Microscopic examination of the brain was negative.

T C 97 developed fever on May 12. Temperatures ranging from 104° to 104.5° F persisted until May 31. The temperatures were normal until June 9, when it was set aside to be tested for immunity. It was found dead June 18 and discarded by mistake without post-mortem examination.

T C 98 failed to react. It was discarded on June 9 as negative.

Summary. The virus of typhus survived in one out of three cultures tested after a period of fourteen days. It probably survived in one out of three cultures after a period of twenty-three days.

Miscellaneous experiments with typhus. — Four experiments were made with tissue cultures, using the spleen of typhus-infected guinea pigs instead of brain. The results of all four of these experiments were negative. The shortest period tested for survival of the virus was eight days, the longest period twenty-one days.

SUMMARY OF THE EXPERIMENTS WITH ROCKY MOUNTAIN SPOTTED FEVER TISSUE CULTURES

The most important experiments abstracted above are shown in Table I. Comparison of our notes on the fresh examination of tissue cultures and the microscopic examination of fixed cultures indicates that the duration of the survival of Rocky Mountain spotted fever is determined by the survival of the cells of the culture. Transplanting the cultures to fresh plasma prolongs the period of survival of the virus. The finding of micro-organisms indistinguishable from *Dermacentroxenus rickettsi* in the cells of the tissue cultures in numbers greatly exceeding those found in cells in the animal body is accepted by us as proof of their multiplication.

SUMMARY OF THE EXPERIMENTS WITH TYPHUS-FEVER TISSUE CULTURES

The experiments presented in abstract form are further condensed for the purposes of summary in Table II. From these experiments we conclude that the virus of typhus survives in brain-tissue plasma cultures for a period of time corresponding to the survival of the cells of the culture — in first generation cultures a maximum period of twenty days. This period has been extended by transplantation into fresh plasma to a period of twenty-eight days. The demonstration of micro-organisms reproducing the morphological range of *Rickettsia prowazeki* in lice with great exactness and in large numbers also leads us to conclude that there is an actual proliferation of this micro-organism in the tissue cultures. As will be seen by the table, the results with typhus are much less certain than those with Rocky Mountain spotted fever. This may be explained by differences in the distribution of the virus in the tissues employed. It is quite probable that in the brain tissue the virus is re-

TABLE I. ROCKY MOUNTAIN SPOTTED FEVER

Days in Vitro		"Generation"																																					
Date	Remarks	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36		
Aug. 17, 1922	Guinea-pig, 8 days after inoculation. First day of fever.	I																																					
Oct. 10, 1922	Guinea-pig, 7 days after inoculation. Fourth day of fever.	I																																					
Oct. 19, 1922	Guinea-pig, 9 days after inoculation. Second day of fever.	I																																					
Oct. 24, 1922	Guinea-pig, 7 days after inoculation. Fourth day of fever.	I																																					
	Guinea-pig, 12 days	I																																					
Oct. 31, 1922	after inoculation. Fourth day of fever.	II																																					
	A typhus immune guinea-pig.	III																																					
		IV																																					
		I																																					
Nov. 7, 1922	Guinea-pig, 6 days after inoculation. Third day of fever.	II																																					
		III																																					
		IV																																					
																																		</					

EXPLANATION OF TABLE

The arrows indicate transfers of the tissue or fragments thereof into fresh plasma. † indicates an atypical reaction followed by immunity to subsequent inoculation with spotted fever blood.

Numbers (C 22, etc.) refer to guinea-pigs described in protocols.

+ indicates presence of lesions of Rocky Mountain spotted fever. Guinea-pigs C 22, C 23, C 27 were killed.
- indicates absence of lesions. Guinea-pigs C 26, C 28, C 30, C 34 and C 43 succumbed to the infection. Guinea-pigs C 35, C 36, C 38, C 42, C 45, and C 47 survived the infection.

TABLE II. TYPHUS

Days in Vitro		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
Date	Remarks																												
Oct. 26, 1922	Pig 761, 16 days after inoculation, 6th day of fever	1+ 2+																											
Nov. 21, 1922	Pig 776, 14 days after inoculation, 4th day of fever	5+ 6-		7+ 8+		9+ 10-																							
Dec. 6, 1922	Pig 786, 14 days after inoculation, 8th day of fever	11+ 13-		14- 15-		16- 17-		18- 19-																					
Jan. 3, 1923	Pig 789, 15 days after inoculation, 9th day of fever	21+ 22-		23+ 24+		25+																							
Jan. 31, 1923	Pig 823, 15 days after inoculation, 6th day of fever			32- 33- 34+		35- 36- 37- 38-																							
Mar. 9, 1923	Pig 10, 16 days after inoculation, 5th day of fever			50- 51- 52- 53?		54- 55- 56-																							
Mar. 19, 1923	Pig 33, 13 days after inoculation, 5th day of fever	57+		61+ 62+ 63?		64+																							
Mar. 20, 1923	Pig 41, 14 days after inoculation, 6th day of fever	59 +		65- 66+ 67- 68-		69- 70- 71-																							
Apr. 3, 1923	Pig 860, 13 days after inoculation, 5th day of fever			76- 77+ 78?		80- 81- 82- 83-																							
Apr. 17, 1923	Pig 871, 14 days after inoculation, 7th day of fever			93- 94- 95+		96- 97- 98-																							

* Guinea-pig No. 92 inoculated with second generation culture, transferred from original culture on seventh day in vitro.

EXPLANATION OF TABLE: Numbers (1, etc.) refer to guinea-pigs described in protocols.

+ indicates presence of lesions of typhus.

- indicates absence of lesions.

stricted to the focal lesions; and it is of course evident that every piece of brain tissue employed for culture may not have contained a lesion. In substantiation of this we have recorded several instances where only a few of many freshly removed portions of brain from typhus guinea pigs, approximately of the size of the pieces used for tissue culture, produced typhus when injected intraperitoneally.

THE MICRO-ORGANISMS IN TISSUE CULTURES

Rocky Mountain spotted fever. — From tissue cultures fixed and sectioned at various intervals it was possible to follow the growth of the micro-organisms, and to note the increase in numbers in endothelial cells still *in situ* in vessel walls. In later periods, from seven to fourteen days, the cells containing the micro-organisms were found most abundantly at the periphery of the tissue and in the plasma immediately adjacent; with rare exceptions the only forms found corresponded to those observed in mammalian tissues. In one culture, Experiment 6, fixed on the tenth day, endothelial cells were found packed with enormous numbers of smaller forms similar to those noted within nuclei in tick tissues. Occasional filamentous forms found in teased preparations and in sections probably must be included as belonging to *Dermacentroxenus rickettsi*; but the longer of these forms as shown in illustrations 1 and 4 are unlike any forms hitherto seen in tick or mammalian tissues. We regard them as significant in indicating a closer relationship to *Rickettsia prowazeki* than we have believed.

The effect of the micro-organisms on the cells containing them seems to be deleterious. Numerous cells containing large numbers of the micro-organisms, both in the tissue and in the adjacent plasma, showed degenerative changes similar to those seen in sections of organs of infected animals — acidophilic staining reaction of the cytoplasm, and pyknosis and dissolution of the nucleus. The number of infected cells in all cultures has been disappointingly small. It is evident that cells containing the micro-organisms die, and yet we have been unable to obtain any evidence that the organisms multiplied or even

persisted in the plasma. There seems to be no mechanism for the passage of the micro-organisms from cell to cell, and we are forced to the conclusion that the cells containing the micro-organisms in tissue cultures are cells originally infected in the animal body, or descendants of such cells.

Typhus. — In the brain-tissue cultures from typhus guinea pigs it has not been possible to follow with such accuracy the growth of the rickettsia in cells *in situ*. The nearest approach has been endothelial cells within the lumina of small blood-vessels packed with the small forms of rickettsia (Fig. 5). As in the case of spotted fever, the cells containing the micro-organisms congregate at the periphery of the tissue. It does not seem necessary to go into a minute description of the morphology of the forms found in these cultures. The forms most frequently found, and illustrated in Figs. 6 and 7, are the exact replicas of the forms encountered in lice nurtured upon typhus patients and described in a previous publication.³

Tissue cultures of normal guinea-pig testes and brains were thoroughly studied as control material. Additional control material was furnished by several negative series of cultures from infected guinea pigs. In no instance did the control material reveal micro-organisms suggestive of either *Dermacentroxenus rickettsi* or *Rickettsia prowazeki*.

SUMMARY AND CONCLUSIONS

The virus of Rocky Mountain spotted fever and the virus of typhus survive in tissue-plasma cultures for a length of time corresponding to the survival of the endothelial cells multiplying in the cultures. The survival of the virus may be extended by "sub-cultures," a procedure which involves the washing of the tissue in Ringer's solution, and the addition of fresh plasma in order to permit the continuation of growth of the tissue cells.

In both instances the micro-organisms associated with the two diseases appeared in endothelial cells in the tissue cultures, in such numbers as to admit of no other explanation than that of multiplication. In the case of Rocky Mountain spotted fever a filamentous form appeared in the cultures in addition to

forms previously described in arachnid and mammalian tissues. In the case of typhus, coccoid, bacillary, and filamentous forms appeared in exact duplication of the morphology of *Rickettsia prowazeki*, as described in lice infected with typhus.

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DESCRIPTION OF PLATE XXII

Fig. 1. Rocky Mountain spotted fever. Endothelial cell, teased out preparation of tissue culture used to inoculate guinea pig C 23, Experiment 1. Duration of culture, August 27 to August 30. 1500 diameters.

Fig. 2. From the same preparation as Figure 1. 1500 diameters.

Fig. 3. Rocky Mountain spotted fever. Experiment 6. An endothelial cell at the periphery of a tissue culture, in sections. Duration of culture, November 7 to November 21. The dark masses represent hemosiderin granules. The number of organisms shown represents those visible in one focal plane. 1500 diameters.

Fig. 4. Rocky Mountain spotted fever. Experiment 5. A cell, possibly a fibroblast, at the periphery of the tissue, in section. Duration of culture, October 31 to November 14. Shows two filamentous forms. 1500 diameters.

Fig. 5. Typhus. Experiment 6. Endothelial cell in section. Duration of culture March 9 to April 2. The cell is filled with the minute paired form of rickettsia. 1500 diameters.

Figs. 6 and 7. Typhus. From the same preparation as Figure 5. Endothelial cell, showing filamentous and bacillary forms of *Rickettsia prowazeki*. 1500 diameters.

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