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ON THE PERMANENT LIFE OF TISSUES OUTSIDE OF THE ORGANISM.*

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Plates 75 and 76.

INTRODUCTION.

It is known that the life of a tissue cultivated *in vitro* is very short.[1] Generally, in three to fifteen days after the preparation of the cultures their growth becomes less rapid and eventually stops completely. The tissues then die and the cells disintegrate.

The purpose of the experiments described in this article was to determine the conditions under which the active life of a tissue outside of the organism could be prolonged indefinitely. It might be supposed that senility and death of the cultures, instead of being necessary, resulted merely from preventable occurrences; such as accumulation of catabolic substances and exhaustion of the medium. The suppression of these causes should bring about the regeneration of old cultures and prevent their death. It is even conceivable that the length of the life of a tissue outside of the organism could exceed greatly its normal duration in the body, because elemental death might be postponed indefinitely by a proper artificial nutrition.

In the following experiments I sought not a complete solution of the problem of prolonging indefinitely the life of tissues *in vitro,* but the facts that would show in which direction we should proceed in order to secure a complete solution, if such a solution be possible.

In September, 1911, I tried to increase the duration of the life of cultures of connective tissue.[2] The cultures were washed for

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[1] Harrison, Harvey Lectures, Philadelphia and London, 1911–1912; *Jour. Exper. Zoöl.,* 1910, ix, 787; Carrel and Burrows, *Jour. Exper. Med.,* 1911, xiii, 387.

[2] Carrel, *Jour. Am. Med. Assn.,* 1911, lvii, 1611.

several minutes in Ringer's solution and were then placed in a new medium. As a consequence, the growth was observed to become more rapid. By repeated washings and passages the occurrence of senility was prevented, and the length of life was very much increased. Some cultures could be maintained in active life for fifty, fifty-five, and even for sixty days.[3] These results showed that the early death of tissues cultivated *in vitro* was preventable, and, therefore, that their permanent life was not impossible.

Subsequently, I developed other techniques by which it might be possible to obtain permanent life of tissues. The ideal method would have been to give to the cultures an artificial circulation by which they might have secured their nutrition and eliminated their waste products. It was attempted to place the tissues in a thin layer consisting of cellulose and coagulated plasma, and through this a slow stream of serum flowed. But it appeared soon that better results could be obtained by a simpler and more indirect method. Instead of establishing a circulation of the medium through the tissues, it was easier to have the cultures pass from medium to medium. The media usually employed consisted of Ringer's solution and hypotonic plasma. It was found that the resistance of a tissue placed in a non-nutritive, but preservative medium was very much stronger if the temperature was low. The cultures were, therefore, placed under conditions having two alternate phases: there was a phase of active life at 38° C. during which the tissue grew, used its medium, and was surrounded by its catabolic products; and a phase of latent life at 0° C. during which it was washed free of its products, to be subsequently placed in a new medium.

In February, 1912, this technique was modified and the phase of latent life in Ringer's solution in cold storage was replaced by a phase of active life in serum in the incubator. Instead of being interrupted, the life of the tissues was continuous and their growth more rapid.

METHODS.

The methods chiefly employed were three. They are as follows: *Technique I.*—The original piece of tissue and the surrounding

[3] Pozzi, *Bull. de l'Acad. de méd.*, 1912, lxvii, 26.

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new cells were extirpated with a cataract knife from the fragment of coagulated plasma that contained them. They were washed for several minutes in Ringer's solution at the temperature of the laboratory, and were then placed in a new medium composed of one or two parts of distilled water and of three parts of normal plasma, to which a drop of embryonal or muscle extract was added. The washings and passage were made either before the appearance in the culture of the symptoms of senility, or when these were still very slight. The passage was repeated more or less frequently according to the rate of the growth and to the conditions of the cells.

Technique 2.—In the second method the washing of the cultures was more prolonged and was performed while the tissues were in cold storage. The life of the cultures was maintained under conditions in which two phases alternated: a phase of active life in a plasmatic medium in the incubator, and a phase of latent life in Ringer's solution in the refrigerator.

Technique 3.—In the third method the cultures were maintained in a condition of continuous life by alternating passages from plasma to serum at the temperature of the organism. The cultures were prepared in the following manner: A piece of silk veil about one centimeter square was placed on a cover glass and moistened with a drop of plasma. A fragment of tissue was deposited in the centre of the square and covered by a few drops of plasma. Afterwards the culture was sealed on a hollow slide, put in the incubator, and allowed to grow. After a few days the silk veil together with the coagulated plasma and tissue, was detached from the coverglass with a knife. As the silk veil acted as a skeleton for the plasmatic jelly, the original fragment, and the new tissue cells, the culture could be handled easily without folding and retraction of the medium, and without deformation of the cells. The veil and the culture attached to it were placed in a tube containing homogenic serum, which was kept at incubator temperature. After a few days it was put again in plasma, and subsequently the two phases alternated regularly.

Experiments and Results.—A great many experiments were performed. In most of the cultures were used connective tissue of

blood vessels, heart, skin, muscles, peritoneum, and spleen, of fourteen to twenty day old chick fetuses. In other cultures were employed fragments of the Rous sarcoma, and in a few cases, tissues of adult dogs. Many cultures survived six to fourteen or fifteen passages in a condition of interrupted or continuous growth. A few cultures were transplanted seventeen, eighteen, and even twenty or more times. Some are still living and have reached the beginning of the third month of their life *in vitro*. It was possible, therefore, to study the morphological and dynamic characters of tissues cultivated for more than two months outside of the organism.

MORPHOLOGICAL CHARACTERS.

The appearance of the cultures grown by technique 1 was almost identical with that of the cultures grown by technique 2. The fragment of tissue was surrounded rapidly by cells spreading in a thin layer through the medium. After section of the plasma, the new tissue retracted around the original fragment forming an opaque crown. During the period of washing there was no modification of the culture, but as soon as the culture was put in a new medium in the incubator, elongated cells began to grow from the edges of the old plasma and to spread into the new medium. After a few passages the original fragment appeared diminished in size and surrounded by a dense envelope of a greyish tissue from which radiated a great many elongated cells, living and dead.

The original fragment was often encircled by several concentric rings, each representing a period of active life. Soon the rings were united in a homogeneous tissue. When they remained distinct, however, they might indicate the number of passages undergone by the culture, as the age of a tree can be read on a section of its trunk. Progressively the original fragment disappeared and was replaced by an amorphous yellowish substance. The form of the culture was also modified. At the beginning of the growth the culture appeared like a thin plate, but it grew progressively more and more like a flattened sphere in the center of which were accumulated a few foreign bodies and pieces of old plasma (figure 1), which were encysted by the living cells. After about forty days the

cultures of connective tissue generally took the typical appearance represented in the drawings (figures 2 and 3) of a culture fifty days old.

Cultivation of Connective Tissue (Experiment 715).—A short segment of the iliac vessels of a twenty-one day old chick fetus was extirpated on November 1, 1911, and placed in hypotonic chicken plasma and muscle extract. A large growth of connective tissue cells developed in the next few days.

On November 3 the plasma was cut and the culture was placed in Ringer's solution in cold storage from 9:52 A. M. to 10:35 A. M. Afterwards, the first passage into a new medium was made. A large growth resulted.

On November 6 the second passage was made after washing in Ringer's solution and keeping in cold storage from 9:55 A. M. to 10:58 A. M. At 2:30 P. M. fusiform cells had already invaded the new medium.

On November 9 the third passage was made after washing for six minutes in Ringer's solution, and on November 13 the fourth passage was made after washing in Ringer's solution and keeping in cold storage from 9:50 A. M. to 10:42 A. M. The iliac vessels were becoming progressively smaller, while a thick crown of cells in concentric lines surrounded the original fragment.

On November 16 the culture was washed in Ringer's solution and in cold storage from 9:20 A. M. to 10:12 A. M., and then placed in a medium composed of plasma and muscle extract. There was a large growth (fifth passage).

On November 20 the culture was washed in Ringer's solution in cold storage from 9 A. M. to 11:12 A. M., and then transferred to plasma and muscle extract (sixth passage).

On November 24 the culture was placed in a medium composed of hypotonic plasma and embryonic extract (seventh passage), after having been washed in cold storage and in Ringer's solution from 9 A. M. to 10:18 A. M.

On November 25 a large growth of cells was seen in the medium. The original fragment had almost completely disappeared, and the culture has a tendency to take a spherical form.

On November 28 the culture was placed in Ringer's solution and in cold storage from 9:30 A. M. to 12:02 P. M. and afterwards in hypotonic plasma and embryonal extract (eighth passage), and on November 29 a large growth was observed. The culture assumed the form of a flattened sphere, the center of which was clear and amorphous.

From December 2 to December 12 the culture underwent its ninth, tenth, eleventh, and twelfth passages. Before each passage it was washed for four or five minutes in Ringer's solution at the temperature of the laboratory.

On December 14 the culture was composed of a central amorphous part of a thick ring of new tissue, and of a peripheral crown of elongated cells radiating through the culture medium.

On December 15 the culture was placed into a new medium after having been washed for four minutes, and on December 16 a large growth through the new medium was seen. The rate of growth had markedly increased.

On December 18 the culture was washed for three minutes and underwent its fourteenth passage, in which a rapid growth of elongated tissue cells was observed.

On December 20 the culture was fixed in formalin and stained with hematoxylin. Two drawings of the stained specimen were made (figures 2 and 3), showing the amorphous center and its crown of actively growing tissue.

Even after seventy-five or eighty days, cultures of connective tissue made by the first two methods had about the same appearance, all of them resembling the later stages of the culture of the iliac vessel first described.

By the same method a few fragments of adult dog tissues, such as thyroid and periosteum, were cultivated, and several generations of connective tissue cells were obtained.

Frequently the cultures of Rous sarcoma died after a few days. The plasma became liquefied and the tissue disintegrated. Nevertheless, a small fragment of it could be kept in active life for forty-six days.

Culture of the Rous Sarcoma.—On January 24, 1912, a small piece of a Rous sarcoma was cultivated in hypotonic plasma. The growth was rapid and abundant. On January 26 the fragment was surrounded by an immense number of radiating fusiform cells and by a peripheral crown of round and polygonal cells. On January 26 and 29 the culture was washed for three or four minutes in Ringer's solution and put in a new medium. On January 30 fusiform cells only could be seen in the culture. On January 30 and on February 1, 3, 5, and 7 the culture underwent its third, fourth, fifth, sixth and seventh passages. The growth was not very active. On February 7 the culture was divided into two parts. One of these was washed and underwent its eighth passage on February 9. After the ninth and tenth passages on February 10 and 12, a microbian colony appeared in the medium. Nevertheless the active part of the culture could be extirpated without being contaminated and underwent its eleventh passage on February 15.

Afterwards the growth was small but aseptic. After the twelfth passage on February 17 the tissue produced a partial liquefaction of the medium with division or dissociation of the new tissue. Nevertheless, after the thirteenth passage on February 20 and the fourteenth passage on February 22 the tissue began to grow more abundantly and regularly without liquefaction of the medium. From February 24 to March 1 the culture underwent its fifteenth, sixteenth, seventeenth, and eighteenth passages. The growth was abundant and the culture increased very much in size. On March 5 a marked retraction of the plasma occurred. After the nineteenth and twentieth passages the growth became very small and death occurred without any apparent cause.

When the tissues were cultivated according to the third method the appearance of the cultures was different. Instead of being interrupted, the growth was continuous, and as there was no sectioning of the plasma, there was, as a consequence, no retraction.

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Moreover, as the plasma was supported by its skeleton of silk, the culture could not take a spherical form, and the tissue grew in a thin layer; that is, under the best conditions for a tissue deprived of circulation. The plasmatic jelly was adherent to the silk veil, and the tissue imbedded in the plasma grew as in an ordinary culture. The cells could use the silk threads as a support, but they seldom did. The purpose of the silk was merely to give such a consistency to the medium that it would not fold over or become spherical during the passages in plasma and in serum.

Normal heart was cultivated according to this technique. Its appearance after a few passages was the same as after a few days. The size of the growth alone was modified.

By the same procedure many fragments of the Rous sarcoma were also cultivated. They could be kept in a condition of active and continuous development for more than fifteen days. But the medium was progressively overcrowded with dead cells and the growth of the tissue became more irregular and less abundant. The fragments could not adapt themselves to their new conditions of life and were less resistant than normal connective tissue.

The appearance of the cells growing in cultures of normal connective tissue often varied periodically. During the first few days of growth the fusiform cells were long and thin and contained clear cytoplasm. After four or five days they became larger and their cytoplasm was darker and filled with large fatty granulations. At the same time the rate of growth diminished. After being washed and transferred to a new medium, the appearance of the cells was again modified. They regained their original appearance and the rate of growth was accelerated. Instead of being periodical, the morphological modifications might be continuous. For instance, in the cultures of spleen the original fragment was surrounded during the first phases of active life by an envelope of ameboid cells. After a few days long chains of fusiform cells appeared around the fragment, while the ameboid cells died and disappeared. Ultimately the fusiform cells were replaced by small polygonal cells which spread around the fragment in a continuous layer. In the culture of the Rous sarcoma the round cells disap-

peared after a few days, while the elongated cells were still growing after forty days. In a culture of heart very large ameboid cells wandered out from the central fragment for more than two months. At the beginning of the third month of its life the culture was composed of elongated cells radiating through the old plasma. They were apparently the result of the development of the large ameboid cells.

DYNAMIC CHARACTERS.

The rate of growth could be appreciated by the rapidity with which the cells, after a passage, appeared and spread in the new medium. The nature of the medium, its osmotic tension, the way in which the plasma was cut, the amount of old plasma left around the cells, the form of the culture, and the frequence of the passages, often had a marked influence on the rate of the growth. The oscillations in the rate seemed generally to be the result of accidental causes, but it was very difficult to exclude the possibility that they might be due to causes inherent in the condition of tissues liberated from the control of its organism. Generally, after the first passage the cells appeared in the medium without a latent period. This was true even in the culture of periosteum and of other tissues of adult dogs. Two or three hours after a passage many elongated cells were observed growing out from the edges of the old plasma into the new medium, and they soon surrounded the central piece by a dense layer. The rapidity of their growth often diminished after a few passages, but afterwards increased progressively. The rate of growth of a culture forty or forty-five days old was more rapid than when it was ten or fifteen days old. It seemed that the older a culture was, the quicker it grew. The rate of growth of a fifty-four day old culture of connective tissue from the portal vein was more rapid than at the beginning of its life *in vitro*. The rate of growth of a culture of heart was greater at the beginning of the third month of its life outside of the organism than at any other period.

These accelerations of the growth were possibly functions of the age of the culture. But the number of the experiments was not large enough to exclude the possibility that they were due to other causes.

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The size of the cultures underwent also many modifications. For long periods of time, the cultures increased in size, diminished, or were not modified.

The diminutions in size were due chiefly to accidental causes, such as destruction of cells during the handling of the cultures, folding of the plasma, and concentration of the medium, but especially to microbian infection.

In a few experiments marked increase in size was observed. The tissue of a culture of portal vein had been considerably reduced after infection, and for several generations it increased but slightly. Then the growth became more active and after the fiftieth day the volume of the tissue increased rapidly. In a culture of connective tissue which had remained very small for two months, a great acceleration of growth and a marked increase in size was observed after sixty days. In another instance a culture of heart became so large after sixty-two days that it had to be divided into several parts.

It is generally difficult to ascertain whether or not the mass of the tissue actually increases, because changes in the density of the tissue can be mistaken for a change in its mass. Nevertheless, the observation of a culture of a diluted suspension of cells demonstrated that a real increase in the volume of the tissue could take place.

Culture of Cells Suspended in Ringer's Solution.—On February 1, 1912, a nine day old chick embryo was cut into very small pieces, and to the pieces an equal volume of Ringer's solution was added. The suspension was centrifugalized for five minutes, and a drop of the supernatant fluid was then added to a culture medium. On February 2 many isolated cells were seen in the peripheral part of the medium. On February 5 the number of these cells had increased very much. The part of the plasma where they had grown was resected, washed, and put in a new medium. The culture underwent its second, third, fourth, and fifth passages on February 2, 10, 13, and 16. The cells had then increased so much in number that they formed a real tissue, and had the appearance of an ordinary culture. The life of these cells afterwards was like that of other cultures.

Since a few scattered cells could generate a culture similar to the culture of fragments of tissue, it is absolutely certain that the cells multiply and that they do not merely wander from the central piece.

Generally, the cultures remained very small in spite of their constant growth, and often they did not increase at all in size. It is probable that the limitation of growth is due to certain mechanical conditions. Cultures have a tendency to become spherical. The nutriment that reaches the center of such cultures is insufficient, but that which is supplied to the periphery is abundant. Hence, the cells at the periphery multiply ceaselessly. But as soon as cells are isolated from the medium by a sufficient thickness of new tissue, their nutrition probably becomes deficient and they disintegrate. While the culture increases constantly by its periphery, it diminishes in its center and therefore its volume does not increase.

A tissue deprived of circulation probably cannot grow in a nutritive medium beyond a certain volume which is a function of its permeability to the medium and consequently of the thickness of the culture. If the culture be spherical its volume will remain very small, since the nutrition of its center is insufficient. From a theoretical standpoint, on the other hand, a tissue growing in a very thin layer could increase indefinitely. It is for this reason that the tissues were cultivated on a silk veil, for the veil prevented the plasma of the culture from retracting and becoming spherical.

The attempt was also made to ascertain whether tissues after a long period of life *in vitro* could retain their normal functions. The experiments were carried out with fragments of chick fetus heart, which, as is well known, can grow and pulsate for several days when kept at a proper temperature in plasma. In December, 1911, it was observed that when the pulsations of a culture of heart stopped, they began again after the tissue had been washed and transplanted into a new medium. Then in January, 1912, the attempt was made to determine the interval during which the cardiac muscle would retain its property of contracting rhythmically. Three experiments gave positive and identical results. One of these will be described.

Cultivation of the Heart (*Experiment 720–1*).—On January 17, 1912, a small fragment of the heart of an eighteen day old chick fetus was cultivated in hypotonic plasma. The fragment pulsated regularly for a few days and grew extensively. After the first washing and passage on January 24 the culture grew again very extensively, but there were no rhythmical contractions. On

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January 29 and February 1, 3, 6, 9, 12, 15, 17, 20, 24, and 28, the culture underwent eleven washings and passages. It became surrounded by fusiform cells and many dead cells. There were no pulsations. After the twelfth passage the culture did not grow at all. Then the tissue was dissected and the old plasma was completely extirpated. A small central fragment was removed, washed, and put in a new medium. On March 1 it was pulsating at a rate that varied between 60 and 84 per minute. On March 2 the pulsations were 104 at 41° C., and on March 3, 80 at 40° C., but on March 4 the pulsations were very weak and stopped altogether at 2 P. M. On March 5 the culture underwent its fourteenth passage, and the pulsations reappeared immediately. They became weak again on March 2. On March 8 the fifteenth passage was made. On March 9 the pulsations were again 80 to 82 per minute at 40° C., and on March 12 they were 60 per minute. They then became slower and weaker. After the sixteenth passage on March 12 the pulsations were irregular, and the fragment beat for a series of 3 to 4 pulsations, and then stopped for about 20 seconds. After the seventeenth passage on March 16 regular pulsations at 52 beats per minute reappeared, and the tissue grew abundantly. After the eighteenth passage on March 19 the pulsations were irregular.

It was thus demonstrated that a fragment of chicken heart could still pulsate rhythmically at the beginning of the third month of its life outside of the organism. It showed, consequently, that a fragment of tissue living *in vitro* could retain its normal function for a long time.

The maximum age that tissues living *in vitro* can reach is still undetermined. Many cultures have died after less than two months, but a few were very active at the beginning of the third month of their life outside of the organism. Generally, the tissues seemed, after a time, to adapt themselves to their new condition, and after the fourteenth or fifteenth passage, very few cultures died spontaneously. In the handling and changes required by the passages, the cultures were exposed to many accidents, chiefly to microbian infections. In case of a local infection the part of the culture that was not yet infected was resected with a cataract knife and placed in a new medium. Often the culture recovered and produced several generations of cells that were free from microbian infection. But when infection was generalized, the tissues always died rapidly. Many cultures died of sepsis. In the description that follows is an illustration of this kind of death.

Culture of Portal Vein (Experiment 483).—On September 27, 1911, a fragment of the portal vein of a chick fetus sixteen days old was cultivated in three

parts of chicken plasma and two parts of distilled water. The tissue grew very extensively. On October 2 the culture was divided into three parts. One was washed in Ringer's solution for five minutes and put in a new medium. On October 7 it had grown extensively and was again divided into two parts. One part was washed and transplanted into a new medium and a large growth followed. On October 9 and 12 the third and fourth washings and passages took place. On October 14 a very large growth of long, clear, slender fusiform cells was present. On October 16 the growth had stopped and the cells were large and contained very large fat granules. On October 16 the fifth washing and passage took place, and on October 17 the growth of fusiform cells, which were again clear with few granules, was very active. After the sixth passage on October 20 retraction of the medium occurred, and the growth of the tissue was slight. By October 22 the original fragment had diminished greatly in size. On October 23 and October 26 the culture underwent its seventh and eighth passages, and on October 27 the original fragment was no longer to be distinguished. The culture was composed of an amorphous central part around which a crown of active tissue had developed. The tissue was disposed in concentric circles and from its peripheral part elongated cells radiated through the culture medium (figure 1). After the ninth and tenth passages on October 30 and November 4, a great increase in the rate of the growth was observed, but after the eleventh passage on November 9 the culture assumed a spherical shape, but grew extensively nevertheless. On November 10, 13, 16, and 18, the twelfth, thirteenth, fourteenth, and fifteenth passages took place. The rate of growth was very rapid. One hour after the fifteenth passage, the fusiform cells had already invaded the new culture medium, but on November 19 a large microbian colony appeared on one side of the culture, and on November 20 the culture was invaded by a great many bacterial colonies. The death of the tissue occurred soon after.

Nearly all the cultures made in the latter part of 1911 died in the same manner after one to two months. By slight modifications of the technique, however, infection was almost completely eliminated in the experiments made in the beginning of 1912.

Of sixteen cultures of heart and blood-vessels made on January 17, 1912, five were still very active in March, 1912, and of the five active ones, two heart cultures previously described grew slowly, but pulsated, and another heart culture, which pulsated from time to time, produced a large growth of ameboid and fixed cells which covered an extensive area of the medium. In this instance after having been motionless for two months, the central part of the culture manifested strong rhythmical contractions on the sixty-fifth day of its life *in vitro*. Moreover, two cultures of connective tissue made on January 17 were growing actively at the beginning of April. The rate of growth and the increase in their volume became very much greater as they grew older.

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SUMMARY AND CONCLUSION.

In two series of experiments made at the end of 1911 and at the beginning of 1912, new techniques were developed with the view of investigating the problem of prolonging indefinitely the life of tissues isolated from the organism. These techniques are far from perfect and will doubtless be modified in the future. But they have already permitted the establishment of new facts.

Fragments of connective tissue have been kept *in vitro* in a condition of active life for more than two months. As a few cultures are now eighty-five days old and are growing very actively, it is probable that, if no accident occurs, the life of these cultures will continue for a long time.

In some cases the rate of growth of the tissues increased in direct ratio to the age of the culture.

Fragments of heart pulsated rhythmically at the beginning of the third month of their life *in vitro*.

These facts show that experiments made with these or with more perfect techniques and followed over long periods of time may lead to the solution of the problem of permanent life of tissues *in vitro*, and give important information on the characters acquired by tissues liberated from the control of the organism from which they were derived.

EXPLANATION OF PLATES.

PLATE 75.

FIG. 1. A thirty day old culture of connective tissue. The center of the culture consists of debris of old plasma. Around it is a ring of concentric layers of very active new tissue.

PLATE 76.

FIG. 2. A fifty day old culture of connective tissue. Active tissue is encircling a piece of old plasma.

FIG. 3. Peripheral part of the same culture.

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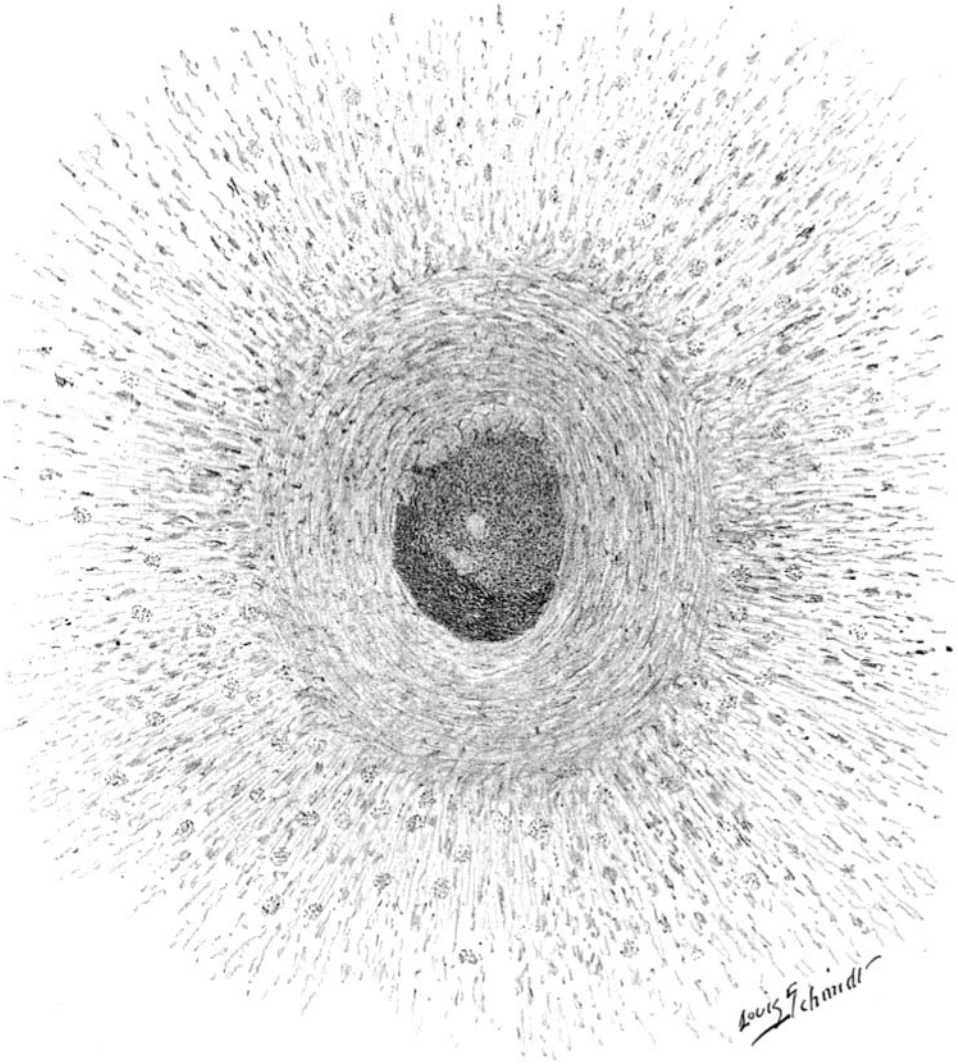

FIG. 1.

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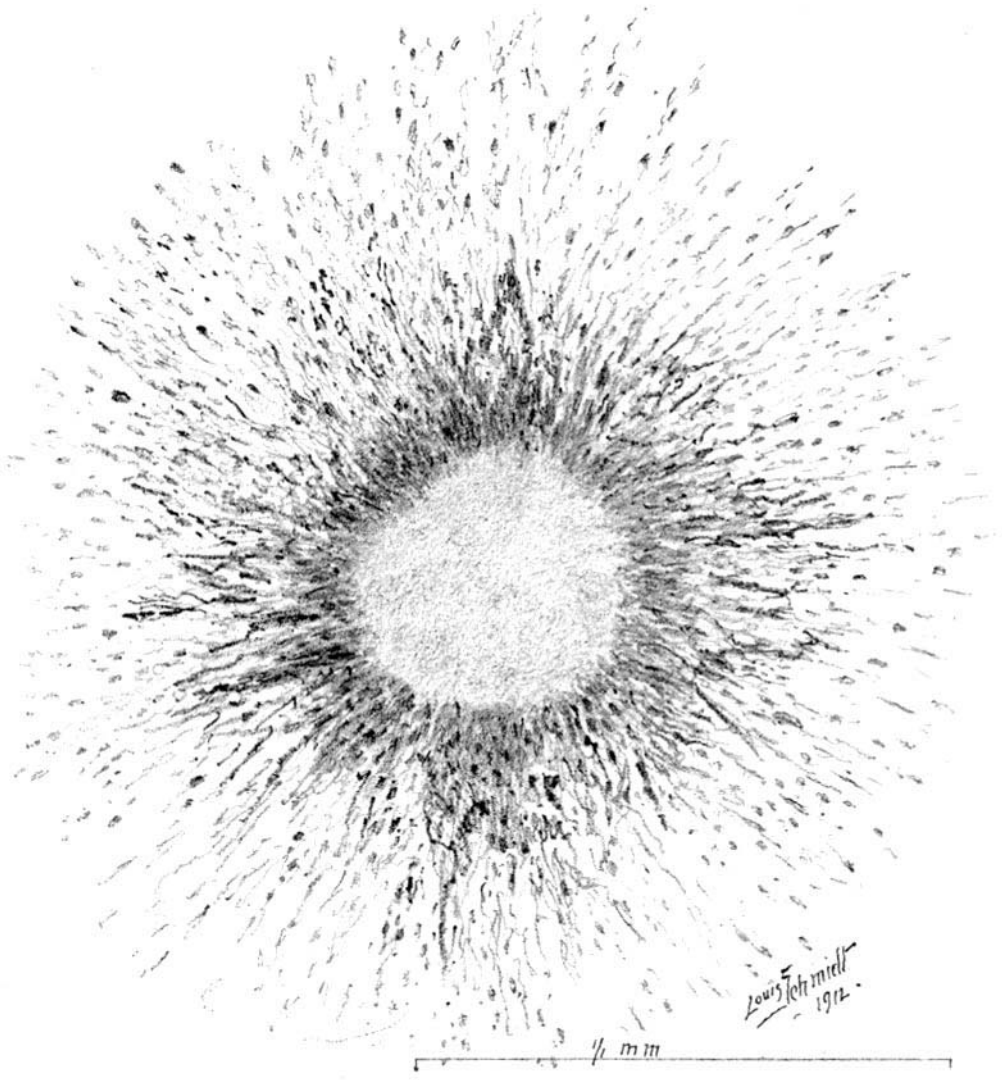

FIG. 2.

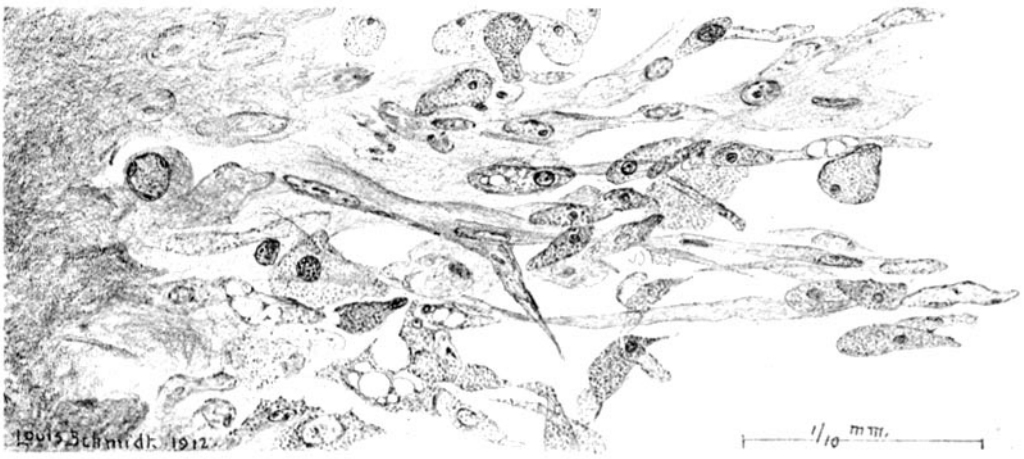

FIG. 3.