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CONTRIBUTIONS TO THE STUDY OF THE MECHANISM OF THE GROWTH OF CONNECTIVE TISSUE.*

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PLATES 10 TO 18.

In previous articles[1] it has been shown that connective tissue cells can be preserved permanently *in vitro* in a condition of active life. Strains of these cells have now been proliferating rapidly outside of the organism for more than sixteen months. In the course of experiment it was observed that a constant relation existed between the rate of growth and the composition of the medium. This fact indicated that certain cell phenomena of the higher animals, such as multiplication, growth, and senility, might now be investigated profitably. Since the time of Claude Bernard it has been known that the life of an organism is the result of the interactions of the cells of which it is composed and of their *milieu intérieur*. But the nature of the interactions has not yet been ascertained; for in order to discover the laws by which they are regulated it would be necessary to modify the humors of the organism and to study the effect of these modifications on the growth of the tissues. This could not be done on account of the lack of a proper method; but this investigation is now rendered possible because of a technique which permits strains of connective tissue cells to multiply indefinitely *in vitro*, like microörganisms.

I have attempted to ascertain some of the relations which exist between the tissues and their medium, by examining the way in which the medium reacts upon the dynamic condition of the cells

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[1] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516; Pozzi, F., *Bull. de l'Acad. de méd.*, 1912, lxvii, 475; Ebeling, A. H., *Jour. Exper. Med.*, 1913, xvii, 273.

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Published September 1, 1913

of a tissue, and how, in turn, the cells modify the properties of the medium. Nearly all the experiments were carried on during the first months of 1913 with strains of connective tissue cells derived from tissues extirpated from embryonic or adult chickens during the months of January and June, 1912, and February, 1913. The fragments of tissue composed of these cells were preserved in a medium composed of two parts of adult chicken plasma and one part of juice taken from a chick embryo eight days old. Every two or three days the tissue was removed from the culture medium, washed for thirty seconds or one minute in Ringer solution, divided into two or three parts if its volume had increased, and placed in new medium at a temperature of 39° C. This fragment soon surrounded itself with a large number of cells, which formed a new tissue. After forty-eight hours the ring of new tissue was measured with a micrometer. Its width was usually 76.5 or 91.8 microns. The rate of growth remained constant as long as the composition of the culture medium was not modified.

THE INFLUENCE OF THE MEDIUM ON THE ACTIVITY OF THE CELLS.

The effect of the medium upon the rate of proliferation of the cells was studied in two series of experiments. In the first series fragments of tissue composed of cells of known activity were placed in media of different composition. In the second series strains of cells of varied conditions of activity were placed in media of the same composition. By measuring the growth of the fragments of tissue which had been living under these different conditions for several generations it was possible to ascertain in what manner the media affected the development of the tissue.

Series 1.—In the first experiments the influence of different media was studied. Normal adult chicken plasma was the basis of the medium but it was changed in many different ways by increasing or diminishing its osmotic tension, by diluting it, by modifying its reaction, and by adding certain organic or inorganic substances to it. The rate of growth of a given strain of cells cultivated in the different media varied according to their composition. The effect of a given medium was ascertained only when the tissues had been developing in it for several generations.

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Published September 1, 1913

The influence on tissues of normal adult chicken plasma was first studied. It was found that the rate of development of a tissue cultivated in plasma alone varied according to certain conditions of the animals from which the plasma was taken. The plasma of some chickens was more activating than that of others, and the greatest difference in the results was caused by the varying ages of the animals,—the younger the animal that supplied the plasma the greater the growth of the tissue. Quantitative experiments were made by employing plasma taken from chickens aged four and five months and aged five and six years. It was found that after three days the size of the ring of new tissue, in the plasma of an old animal, was 30.6 to 38.25 microns, whereas in the plasma of a young animal, it was 45.9 or 53.55 microns. When the plasma of an adult animal, whether chicken or dog, was used, the connective tissue lived for several weeks, but the mass of tissue did not increase. In the first experiments, which were performed in October, 1911, in connection with the rejuvenation of cultures, the fragments of tissue were cultivated in the plasma of an adult animal.[2] The increase in volume was so slight that the loss of cells caused by the section of the medium and the passages deprived the culture of nearly all the new tissue formed during its period of active life. After fifty-five days the fragments of connective tissue which had been growing constantly were no greater than on the first day. This proved conclusively that the plasma of an adult animal did not possess the power of producing a large increase of the mass of the tissue. It was next attempted, by modifying the conditions of the medium, to impart to the plasma of an adult animal a dynamic power analogous or superior to that possessed by the plasma of a young animal. It was found that in a medium composed of plasma and of embryonic juice the volume of tissue increased with great rapidity. The tissue grew so much that it became necessary to divide and subdivide it. Thus a large number of new cultures were made from tissues derived from the few fragments extirpated in January and June, 1912. This activating power is not peculiar to embryonic juice, for extracts taken from

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

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Published September 1, 1913

most of the organs of an adult animal possess this property in a lesser degree. When an animal is old, the juice becomes less activating. Whereas the muscle extract of a young chicken produced in two days a ring of new tissue 76.5 or 91.8 microns in width, that of an old fowl produced a ring of only 22.95 microns.

The action of media of different composition on a given strain of cells was studied in a great many experiments. A fragment of tissue composed of cells which had been living for several months *in vitro* was divided into two equal parts. One part was cultivated in normal plasma and the other in plasma containing different quantities of embryonic juice or other substances. The difference in the quantity of tissue produced in forty-eight hours around the fragments cultivated in plasma alone and mixed with embryonic juice was very large (figures 1 and 2). After several passages the growth of the tissue living in normal plasma became progressively reduced and often stopped completely. On the other hand, the growth of the tissue cultivated in a mixture of plasma and embryonic juice was so abundant that it had to be divided repeatedly. This is shown by experiments of which the following is a type.

The tissue contained in culture 5,142, which had undergone 150 passages and which was growing rapidly and regularly, was divided into two parts, A_1 and B_1. Part A_1 was placed in adult chicken plasma, while part B_1 was cultivated in a medium composed of two parts of plasma and one part of embryonic juice. After forty-eight hours the width of the ring of new tissue formed around A_1 was 30.6 microns, while around B_1 it was 91.8 microns (figure 3). A second time A_2 was cultivated in normal plasma and B_2 in plasma and embryonic juice. After forty-eight hours B_2 was surrounded by a ring 91.8 microns in width, while not a single new cell could be seen around A_2 (figure 4). Nevertheless, the tissue of A_2 was not dead, and it started to grow when it was replaced in a mixture of plasma and embryonic juice (figures 5 and 6). By increasing or diminishing the proportion of embryonic juice contained in normal plasma it became possible to regulate the quantity of new tissue produced in forty-eight hours by a group of connective tissue cells. After a few passages a strain of cells cultivated in adult chicken plasma containing a given quantity of embryonic

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Published September 1, 1913

juice was growing at a constant rate. After more than sixteen months the strains of connective tissue cells were proliferating at the same rate as the original piece of embryonic tissue from which they were taken. Thus it was demonstrated that connective tissue which had been living *in vitro* for more than one year could greatly increase its mass in a short time. A fragment of tissue composed of the strain of cells of January, 1912, which had undergone 130 passages, was photographed (figure 7), then divided, and photographed again (figure 8) after forty-eight hours. These photographs show that the volume of the tissue had at least doubled during these two days. In the experiments performed with the strain of June, 1912, which had undergone eighty-seven passages, the increase in volume was almost identical. A fragment of tissue composed of these cells was photographed (figure 9), and after being divided was transferred to a new medium. Then infection occurred and the bacterial colony was extirpated together with about one fourth of the tissue; but the remaining part proliferated actively and the tissue was divided and subdivided. Other photographs (figures 10, 11, and 12) were taken during the following days. By comparing the first photograph and the photographs taken on the fourth, sixth, and tenth days it is possible to see what enormous quantities of cells were produced from the small original fragments. In ten days the volume of the tissue had increased at least fifteen times. In other experiments fragments of tissue derived from the strain of cells which had lived for more than fourteen months outside of the organism were cut in smaller pieces and suspended in Ringer solution. A drop of the suspension mixed with normal plasma and embryonic juice was placed in various test-tubes. After two days a sphere composed of elongated connective tissue cells had developed around each small fragment (figure 13). When two spheres were sufficiently close they became united by a bridge composed of chains of active tissue cells (figure 13). The increase of the mass was very large, and in one experiment the volume of the tissue augmented more than fifty times in six days. The experiments show the importance that is attached to the composition of the culture medium, since the growth of a strain of cells, the volume of which increased so rapidly in a

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Published September 1, 1913

proper medium, remained stationary when it was merely cultivated in normal adult chicken plasma. As the rapidity of growth of a tissue depends so much on the composition of the medium, it may become possible to use as a reagent of the dynamic value of the humors of an organism a strain of cells adapted to life *in vitro*. If human connective tissue cells could be preserved in a condition of permanent life, as connective tissue cells of the chicken are preserved, the value of the plasma of an individual might be appreciated by the cultivation in it of a group of these cells and by the observation of the rate of their multiplication. These observations would perhaps give some indication of certain values of the blood of an organism and possibly some clew to its age.

Series 2.—In the second series of experiments, strains of cells of varied activity were cultivated in identical media. Modifications in the rate of growth occurred and the progressive changes of the dynamic condition of the cells under the influence of the medium could be studied. It is known that the dynamic state of different tissues taken from a normal animal, or that of tissues previously cultivated *in vitro* under different conditions, varies largely. Fragments of connective tissue taken from an embryo, or from young adult and old animals and placed in normal adult chicken plasma grew at different rates. The velocity of the growth always varied in inverse ratio to the age of the animal from which the tissue had been extirpated. The various tissues of one animal, or the different parts of one tissue, are not necessarily identical as regards their dynamic condition. In one case, for instance, a small clot of blood in the subcutaneous connective tissue of an adult chicken had become encysted by connective tissue. A few fragments of the cyst wall were cultivated by the ordinary method. After twenty-four hours these fragments of connective tissue were surrounded by a large and dense area of small round and fusiform cells. During the same period of time normal connective tissue from the same region of the body produced only a few large and elongated connective tissue cells, which constituted a network. Two photographs (figures 14 and 15) show the differences in the morphology of slowly and actively growing cells and in the extent of growth after forty-eight hours. The dynamic condition of the connective tissue living

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Published September 1, 1913

in vitro varied according to the manner in which the cultures were prepared and kept. The greatest activity was observed in the old strain of cells which had been undergoing daily passages for some time past. A comparative study of the proliferation of the cells and of the connective tissue cells of an eight day old chick embryo showed that the quantity of tissue produced in forty-eight hours by the strain adapted to life *in vitro* could be twenty times more abundant than that produced by fresh embryonic tissue. When the tissues were allowed to remain in the medium for two, three, or four days without change, the rate of proliferation decreased. When a fragment of tissue was kept in its medium at a low temperature for four, five, or six days, the rate of growth and the morphological appearance of the cells were modified (figure 16). The cells became larger and their cytoplasm was darker and more abundant. The rate of proliferation had diminished and even after a few repeated passages the growth of the tissue was still slow.

An attempt was then made to determine whether the dynamic condition of the cells was a fixed character that persisted for a long time, or whether it was merely a temporary effect, produced entirely by the medium. Fragments of tissue or strains of cells of different dynamic conditions were placed in identical media, composed either of normal adult chicken plasma or of plasma containing more or less tissue juice. The rate of growth of these tissues which were originally in different conditions of activity was at first very unequal; but after a few passages it became uniform. The strains of cells which had been artificially activated through daily passages, and which in forty-eight hours produced a ring of new tissue of 153.0 or 183.6 microns in width, were, after a few passages, producing a ring of 76.5 or 91.8 microns. Again, the fragments of tissue which originally surrounded themselves in forty-eight hours with a ring of 15.3 or 30.6 microns produced after a few passages a ring of about 91.8 microns. The influence of the medium on the rate of growth was not immediate. For a few days the rate of proliferation depended in large measure upon the previous dynamic condition of the cells; then their activity increased or diminished according to the medium and finally became constant. For instance, a fragment of tissue composed of a strain of cells

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Published September 1, 1913

which had undergone more than 150 passages, and the growth of which had completely stopped, after having been left for several days in normal chicken plasma, was cultivated in a mixture composed of two parts of plasma and one part of embryonic juice. After twenty-four hours a few cells were seen in the culture medium around the original fragment. After three days the fragment was surrounded by a thin ring, the width of which was 45.9 microns. The fragment was then washed and placed in a medium of identical composition. Two days afterwards the ring of new tissue which had formed was 76.5 microns. After one more passage the rate of growth was entirely normal. The tissues which grew actively and which were placed in the plasma of adult or old animals continued for a few days to develop rapidly. Later the rate of growth diminished progressively. Therefore it seems that the dynamic condition of the cells results from certain substances supplied directly or indirectly by their medium. The activity manifested by the cells for a few days after their medium has been changed may be partly due to the reserves which they have previously accumulated. The action of the medium on the cells is not immediate, but necessarily occurs sooner or later. Hence the conclusion that the dynamic condition of a strain of cells is determined by the medium either immediately or after a short time; or in other words, it is a function of the medium in which it is cultivated.

THE INFLUENCE OF THE CELLS ON THE MEDIUM.

It might be assumed that cells which were actively developing in a medium markedly modified the latter, and rendered it progressively unsuited to their life. Normal death of cultivated tissues was considered as possibly brought about by the exhaustion of the nutritive substances contained in the medium and by the accumulation therein of certain catabolic substances. Some modifications of the culture medium were observed to occur when connective tissue was made to grow actively in Gabritschewski boxes or in test-tubes. The plasmatic jelly around the groups of cells which were rapidly proliferating became thinner, and serum was exuded. The atmosphere of the box became rarefied. When, in cases where tissues were

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cultivated in large tubes, the velocity of growth was great, the fibrin which is the framework of the film covering the glass wall soon disappeared and the culture medium appeared to liquefy. The modifications of the atmosphere of the boxes and of the culture medium were merely the manifestations of more profound physico-chemical changes, which will be studied later in greater detail. The only thing which was studied in the present connection was the action on a strain of cells of an extract taken from a culture medium in which normal embryonic tissue had been actively developing. The experiments consisted in adding the fluids from an old culture to the plasma in which the connective tissue cells were being cultivated. The fluids were obtained in the following manner: Normal embryonic tissue was cut into very small pieces, suspended in Ringer solution, and mixed in test-tubes with normal chicken plasma. In some experiments the plasma was allowed to coagulate at the bottom of the tubes; in others, the plasma was made to coagulate in a thin film on the glass wall, whereupon the growth of the tissue became much more rapid. Some of the tubes were placed in cold storage, and the others in the incubator. In the former no growth occurred, while in the latter the tissue grew more or less actively, according to the manner in which the culture had been prepared. After a few days the contents of the tubes were removed. The clot was cut into small pieces and centrifuged and the supernatant fluid as well as the fluid exuded during the growth of the tissue was used for the experiments, one or two parts of the fluid being mixed with one part of plasma. Extracts of cultures were used which had been kept in the incubator in active life or in the refrigerator in latent life. In these media were cultivated strains of connective tissue cells adapted to life *in vitro*. After forty-eight hours the width of the new tissue was measured with a micrometer. The results varied according to whether the cultures that supplied the extract had been kept in the incubator for a short or a long time.

When the embryonic tissues had been growing for one or two days only, the fluid extracted from their medium activated the growth of connective tissue cells. This activating power exceeded that of the fluid extracted from the cultures preserved in cold

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Published September 1, 1913

storage. Therefore it appeared that the embryonic tissues living actively for a short time had modified the culture medium and rendered it more suitable to the growth of connective tissue cells.

When the cultures of embryonic tissues were allowed to grow more extensively or for a longer time, the properties of their extracts became modified. In one form of experiment the plasma and the suspension of embryonic tissue were placed in tubes and allowed to remain in the incubator for four or five days; in a second, the plasma was distributed over the wall of the tube in a thin layer, the fragments of embryonic tissue being embedded therein at more or less regular intervals. In the latter case the growth was very rapid and in two days the plasma jelly had almost completely liquefied, while the wall of the tube had become covered with continuous and thin layers of living cells. The fluids extracted from the cultures which had been living actively acquired the property of retarding the growth of connective tissue. In a few experiments these fluids caused the proliferation of the cells to cease almost entirely. The experiments show that embryonic tissue which has been actively growing in a culture medium renders it unsuitable for the life of cells.

The results of the experiments lead one to assume that in a culture of tissue progressive diminution of the tissue's rate of growth and its ultimate death are probably brought about by similar modifications of the medium. It was this supposition which enabled me to prolong indefinitely the life of connective tissue *in vitro*. It was found that by washing the tissues of a culture in Ringer solution and placing them in a new medium the rate of their growth did not diminish and death failed to occur. This result could be obtained in several different ways. After a few days of growth in the medium the fragments of tissue were washed in Ringer solution or placed in a large quantity of fluid plasma, where it was thought that the substances which stopped the growth could easily diffuse. When the tissues were transferred to a new medium every twenty-four or forty-eight hours it was unnecessary to wash them; but when they had been allowed to grow for four or five days in a medium the washing in Ringer solution favored the rapidity of their growth. The influence of washing on the growth of tissue was

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studied particularly in connection with a strain of cells which had undergone more than 100 passages. Each fragment was divided into two parts, A and B. Part A was washed for thirty seconds or one minute, while part B was placed directly in the new medium. When the tissues were cultivated in a mixture of plasma and embryonic juice and transferred to a new medium every twenty-four or forty-eight hours, the difference in size between parts A and B was very slight and often did not exist at all. However, the new tissue was generally less abundant and less dense in the culture which had not been washed (figure 17, *a* and *b*). The differences were more marked when the tissues were transferred several times without being washed. When the tissues were cultivated in adult chicken plasma without the addition of embryonic juice the difference in growth produced by the suppression of the washing was much more apparent. In this case the tissues which had not been washed often died after a few passages.

The experiments indicate that the growing tissues modify their medium; but the nature of the modifications has not yet been determined.

CONCLUSIONS.

When connective tissue cells have been cultivated for a certain length of time in a medium which has been repeatedly changed, a definite relation arises between the rate of growth of the cells and the composition of the medium. It is possible, by adding to the culture medium a given quantity of certain substances, such as embryonic juices, to foresee the extent to which a fragment of tissue composed of a given strain of cells will increase in a given time. The rate of growth of a strain of cells can be accelerated or retarded by the addition to the medium of activating or retarding substances.

The dynamic condition of a strain of connective tissue cells, which have been living in a given medium for some time, is not a definitely acquired characteristic, but a temporary state, and is the product or function of the medium in which the cells are living, and is readily modified merely by altering the composition of the medium.

A knowledge of the characteristics of the growth of connective tissue described has led to a new result,—the indefinite proliferation of a strain of connective tissue cells outside of the organism. The

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strain of connective tissue originally obtained from a fragment of chick embryo heart, which had been pulsating *in vitro* for 104 days, was still actively alive after sixteen months of independent life and more than 190 passages. The rate of proliferation of the connective tissue sixteen months old equalled and even exceeded that of fresh connective tissue taken from an eight day old embryo. It appears, therefore, that time has no effect on the tissues isolated from the organism and preserved by means of the technique described above. During the sixteenth month of life *in vitro* the cells increased rapidly in number and were able in a short time to produce a large quantity of new tissue. This fact, therefore, definitely demonstrates that the tissues were not in a state of survival, as was the case in certain earlier experiments, but in a condition of real life, since the cells of which they were composed, like microörganisms, multiplied indefinitely in the culture medium.

EXPLANATION OF PLATES.

PLATE 10.

FIG. 1. Culture 5,200–1. March 10, 1913, fourteenth passage of connective tissue. Medium, adult chicken plasma. Photograph taken on March 12, 1913.

FIG. 2. Culture 5,200–2. March 10, 1913, fourteenth passage of connective tissue. Medium, two parts adult chicken plasma, one part embryonic juice. Photograph taken on March 12, 1913.

PLATE 11.

FIG. 3. Culture 5,166. March 10, 1913, seventy-two hours after the 151st passage. A_1, cultivated in normal plasma; B_1, cultivated in two parts normal plasma and one part embryonic juice.

FIG. 4. Same tissues. March 12, 1913, forty-eight hours after the 152d passage. A_2, cultivated in normal plasma; B_2, cultivated in two parts normal plasma, one part embryonic juice.

FIG. 5. Same tissues. March 14, 1913, forty-eight hours after the 153d passage. A_3, cultivated in two parts plasma and one part embryonic juice; B_3, cultivated in normal chicken plasma.

FIG. 6. Same tissues. March 16, 1913, forty-eight hours after the 154th passage. A_4, cultivated in two parts plasma and one part embryonic juice; B_4, cultivated in normal chicken plasma.

PLATE 12.

FIG. 7. Culture 3,579. Photograph taken on January 18, 1913, forty-eight hours after the 130th passage.

FIG. 8. Same tissue. Photograph taken on January 20, 1913, forty-eight hours after the 131st passage.

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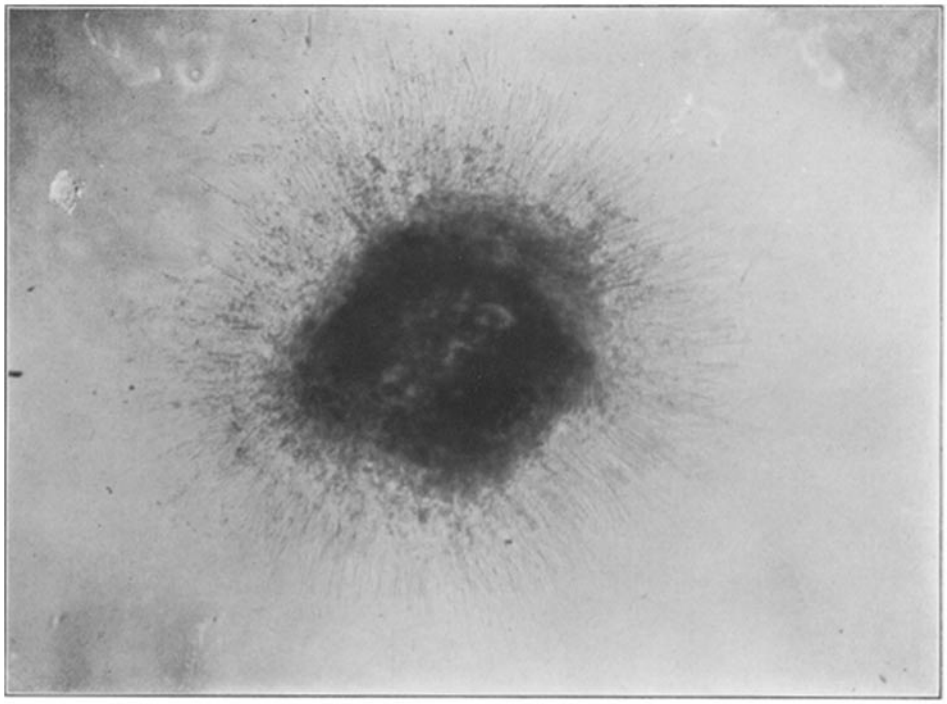

FIG. 1.

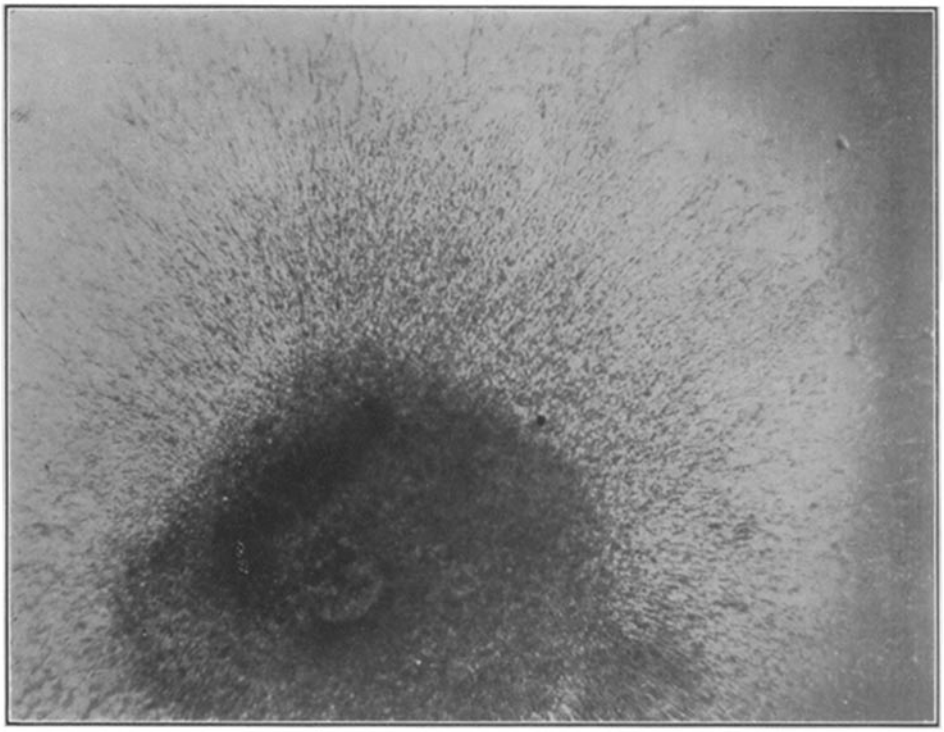

FIG. 2.

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Fig. 3

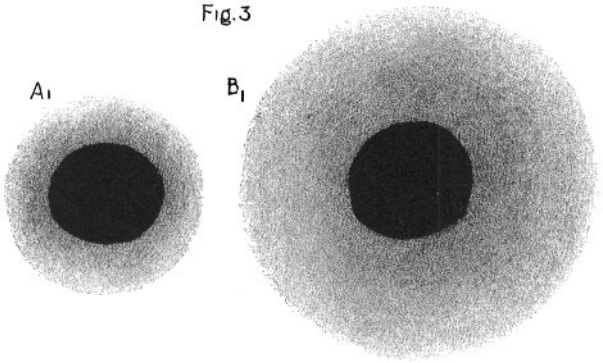


Fig. 4

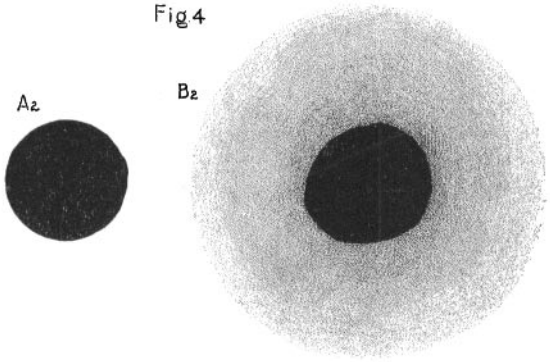


Fig. 5

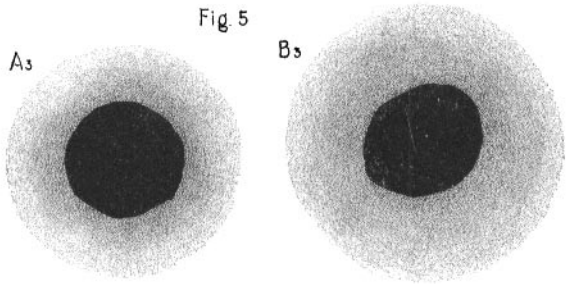
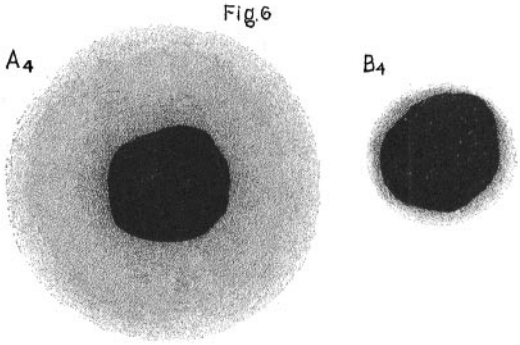


Fig. 6



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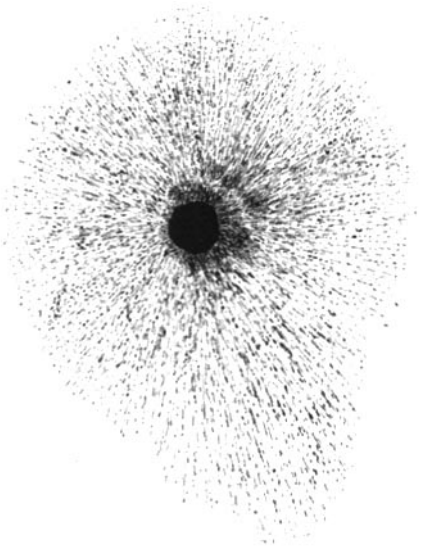

FIG. 7.

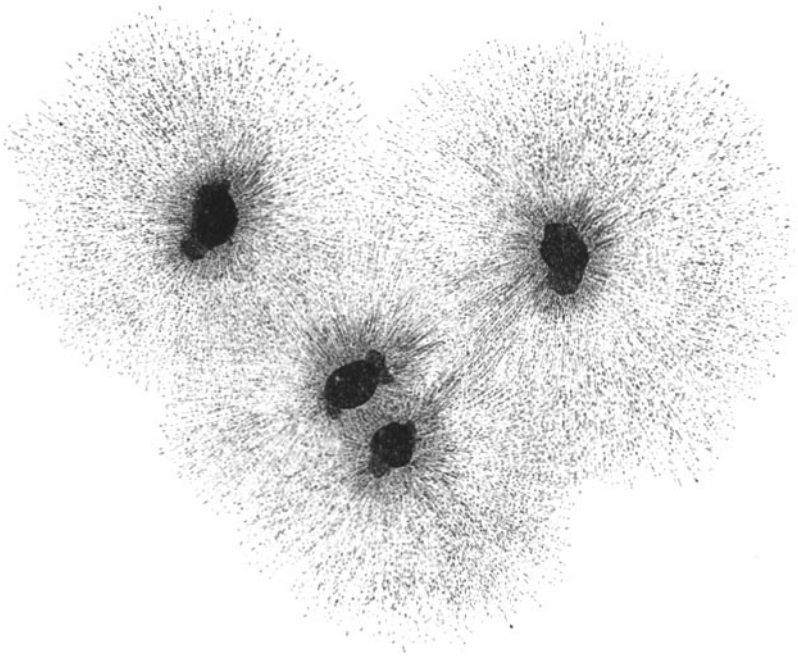

FIG. 8.

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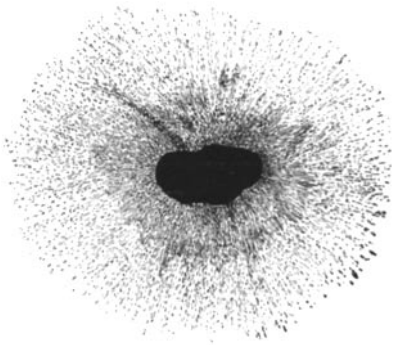

FIG. 9.

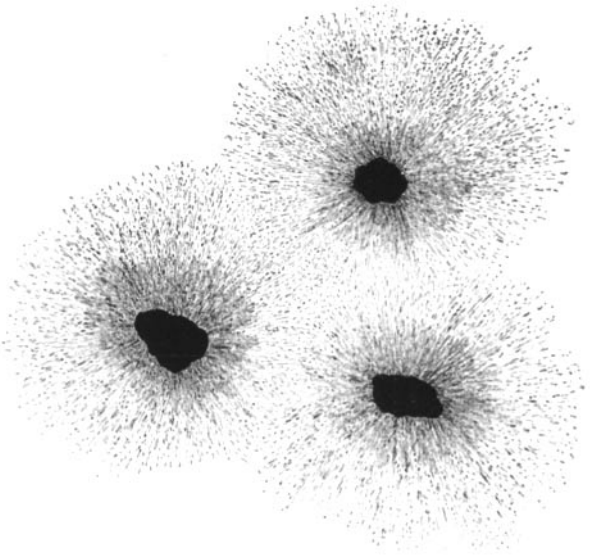

FIG. 10.

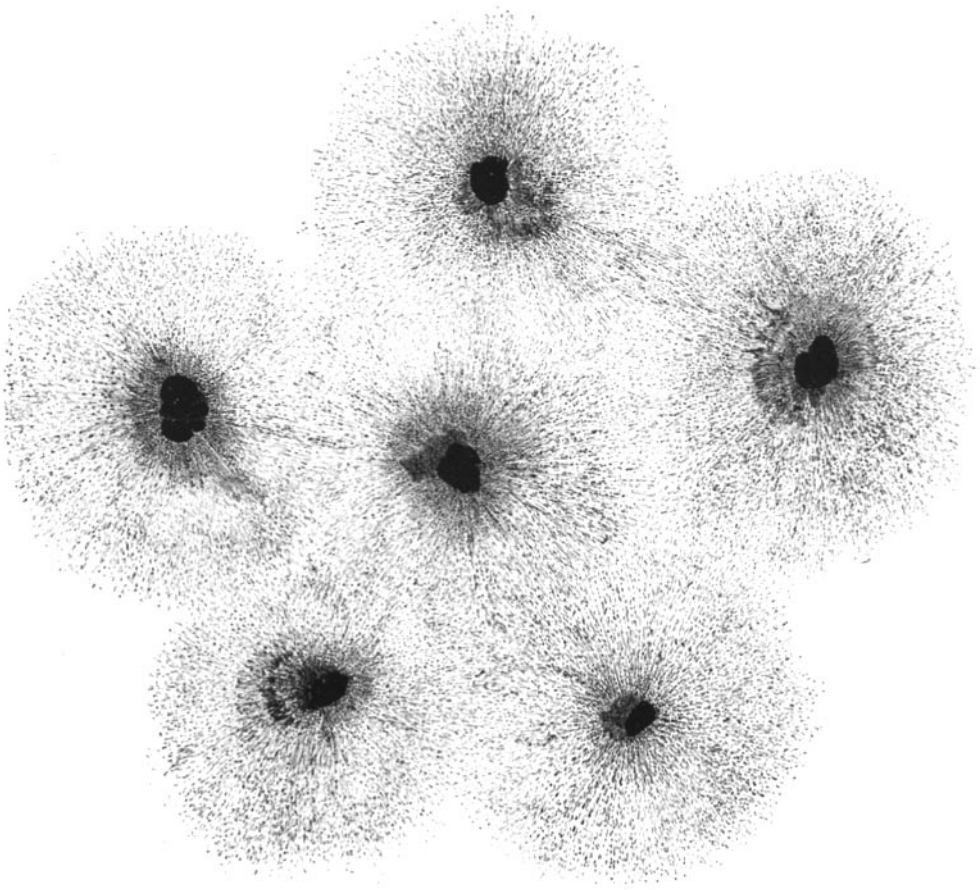

FIG. 11.

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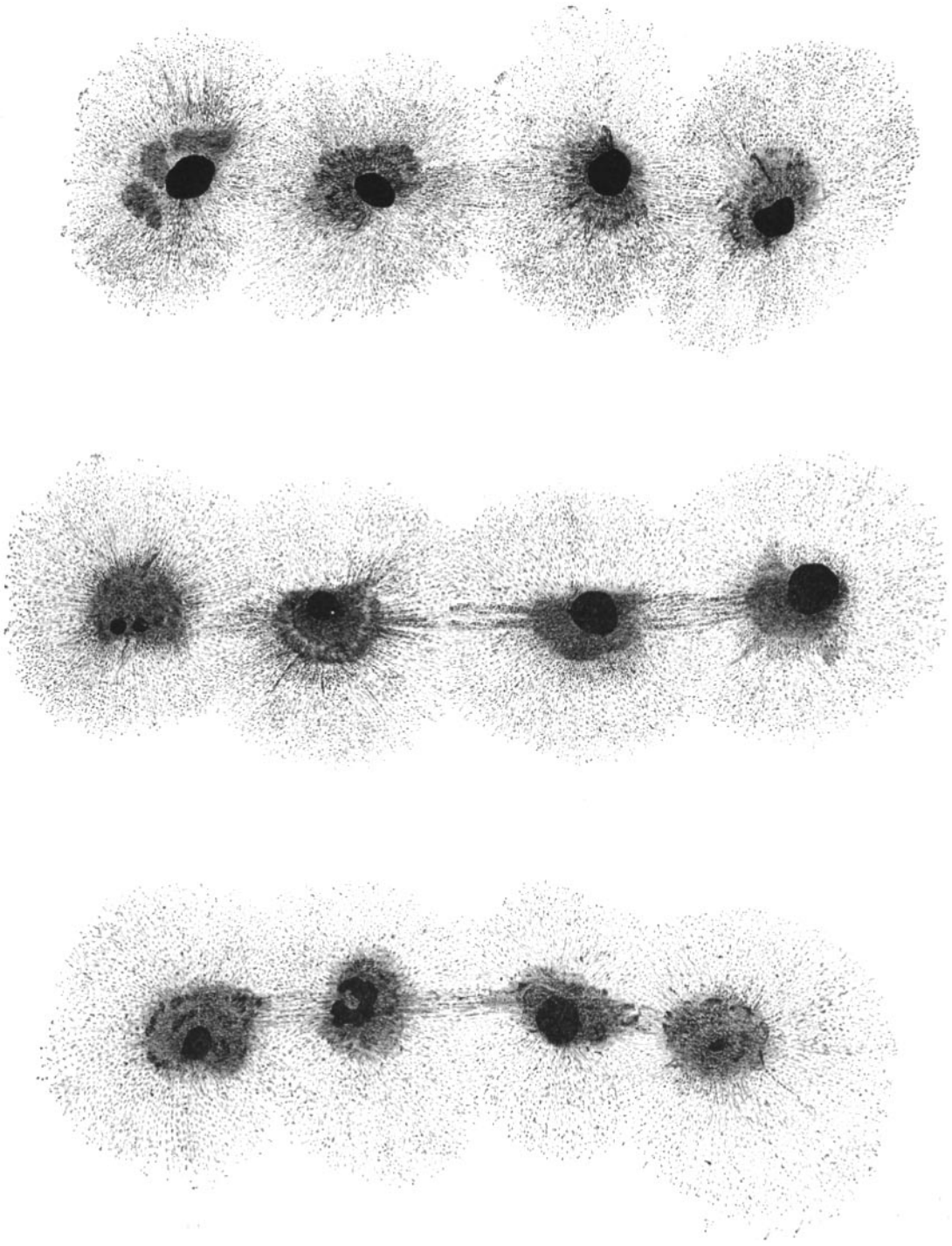

FIG. 12.

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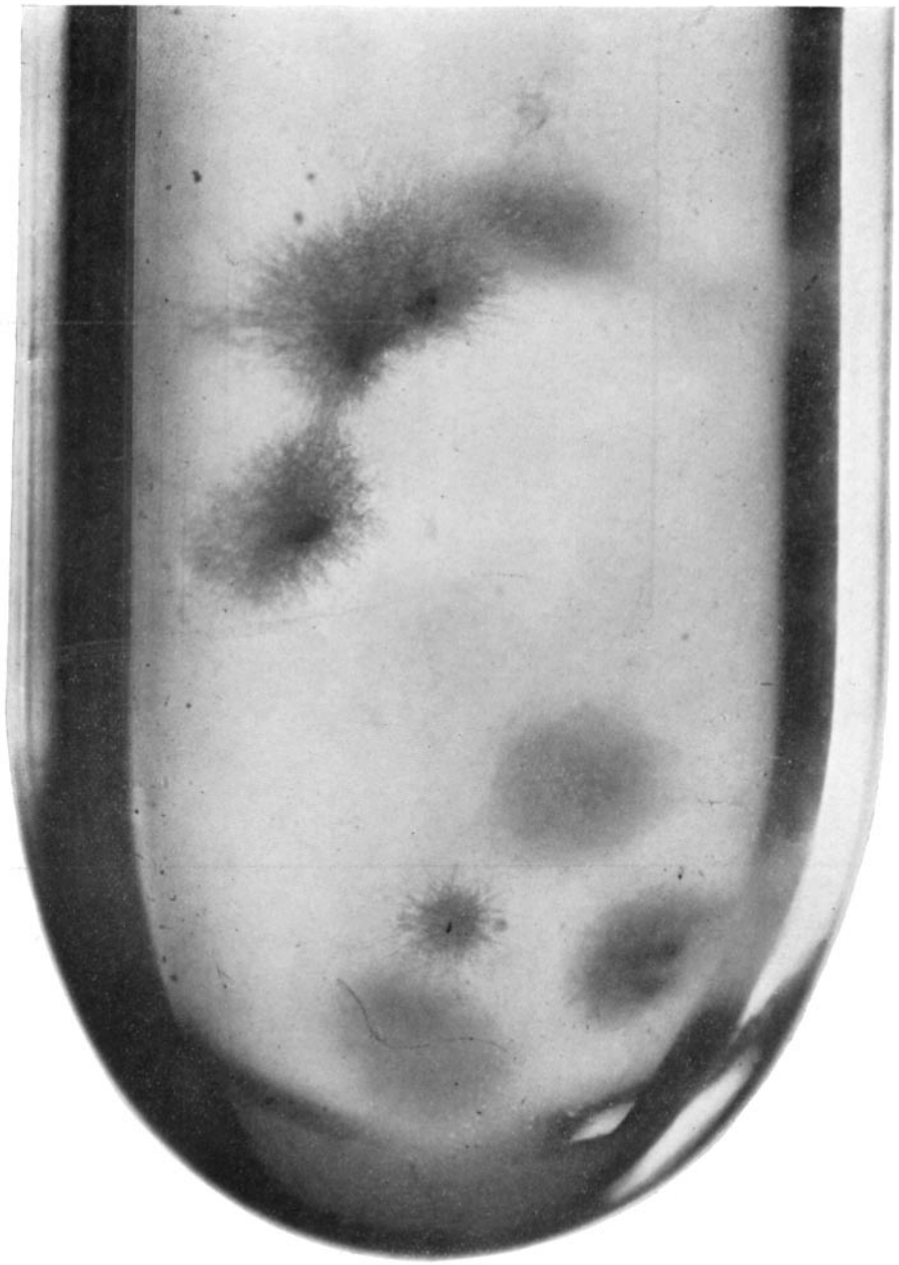

FIG. 13.

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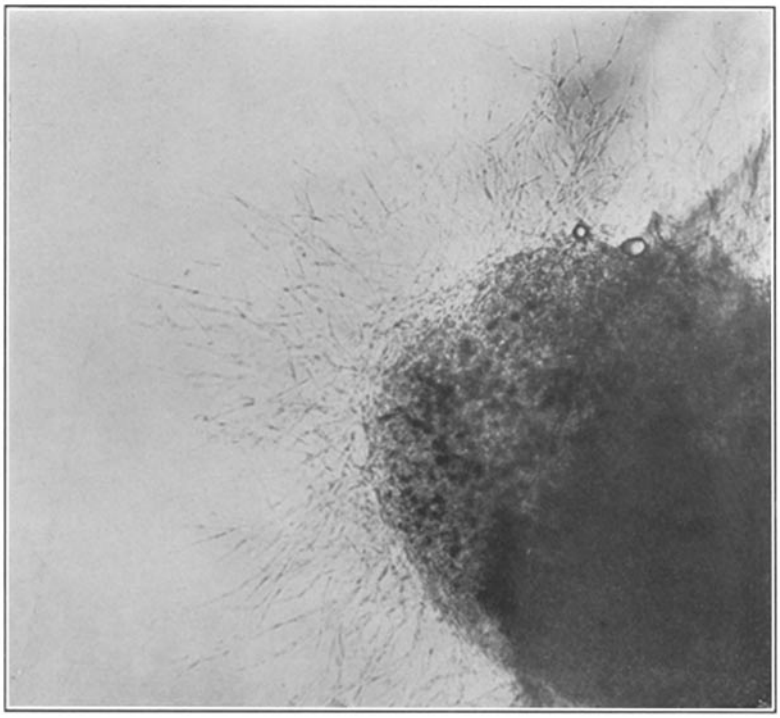

FIG. 14.

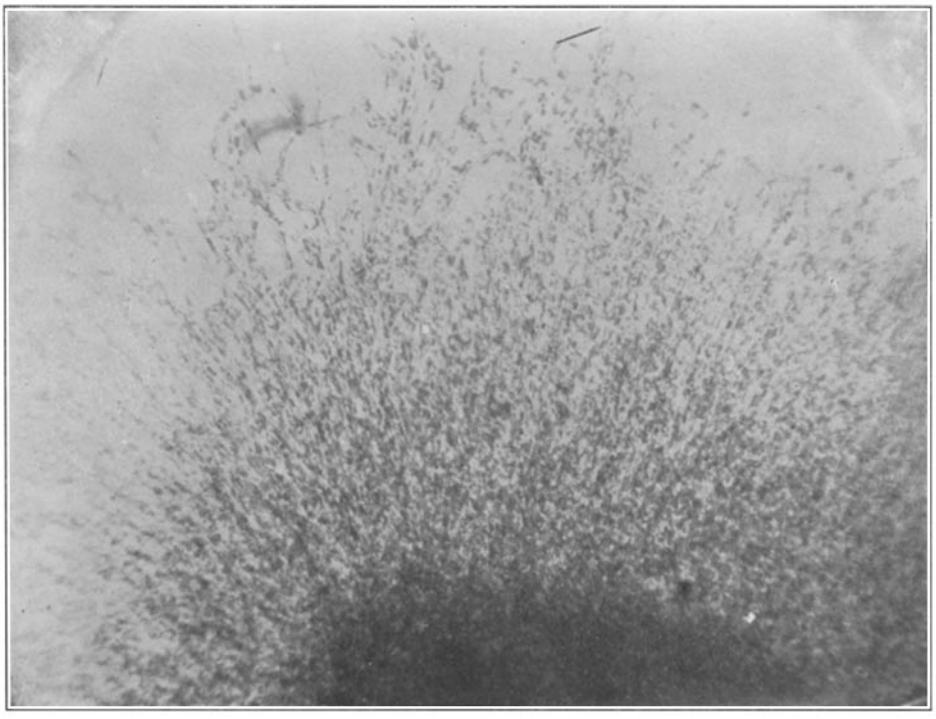

FIG. 15.

(Carrel: Mechanism of Growth of Connective Tissue.)

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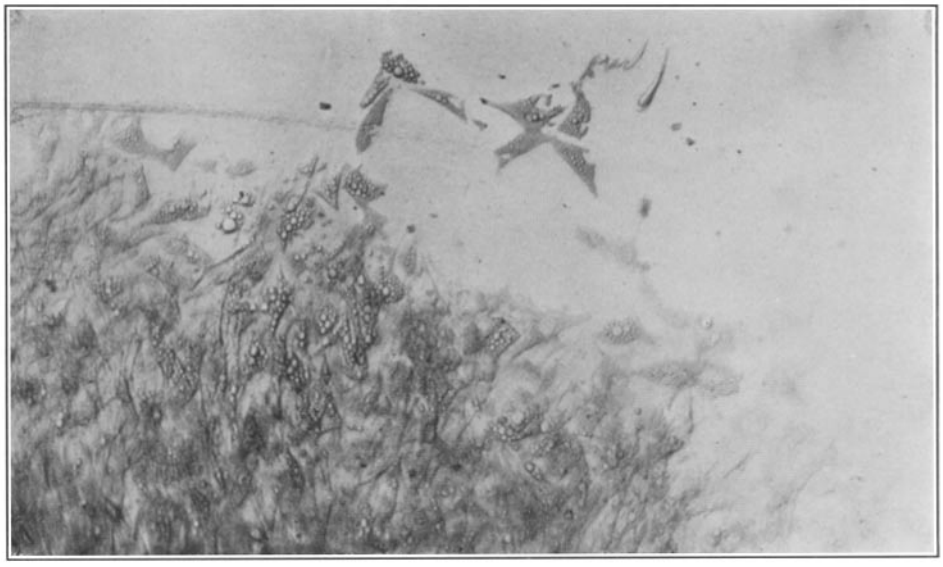

FIG. 16.

(Carrel: Mechanism of Growth of Connective Tissue.)

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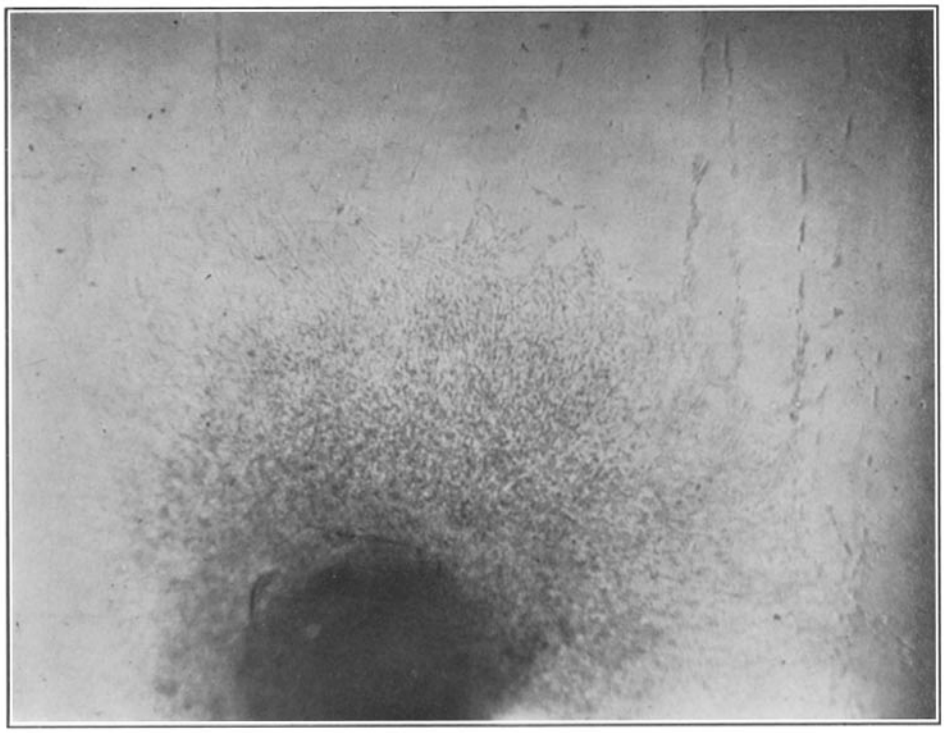
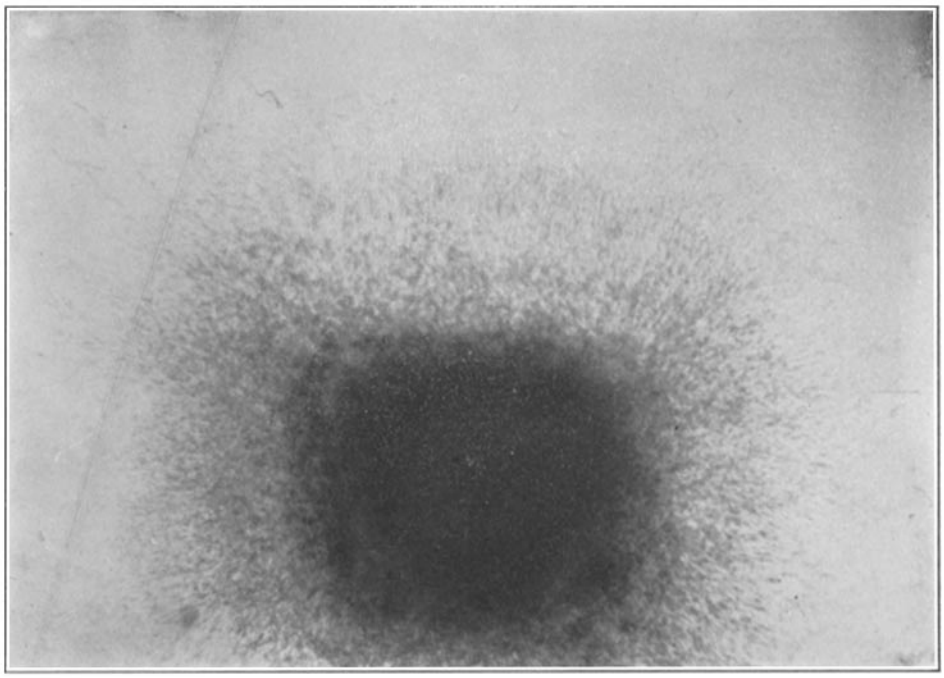


a



b

FIG. 17.

(Carrel: Mechanism of Growth of Connective Tissue.

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PLATE 13.

FIG. 9. Culture 3,575. Photograph taken on January 17, 1913, seventy-two hours after the 87th passage.

FIG. 10. Same tissue. Photograph taken on January 20, 1913, forty-eight hours after the 89th passage.

FIG. 11. Same tissue. Photograph taken on January 22, 1913, forty-eight hours after the 90th passage.

PLATE 14.

FIG. 12. Same tissue. Photograph taken on January 27, 1913, seventy-two hours after the 92d passage.

PLATE 15.

FIG. 13. Connective tissue cultivated in a test-tube after fourteen months of life *in vitro*. The photograph shows a growth of forty-eight hours.

PLATE 16.

FIG. 14. Culture of connective tissue of adult chicken, after forty-eight hours.

FIG. 15. Culture 5,200–2. Fourteenth passage of connective tissue, growing very actively.

PLATE 17.

FIG. 16. Culture of connective tissue after seven days in an incubator at a temperature of 35° C.

PLATE 18.

FIG. 17. Culture 5,192. Photograph taken on March 13, 1913, seventy-two hours after the 155th passage. *a* had been washed for one month in Ringer solution. *b* had not been washed.

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