A STRAIN OF CONNECTIVE TISSUE SEVEN YEARS OLD.

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PLATES 51 TO 55.

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In July, 1914, Dr. Carrel reported the condition of a strain of connective tissue 28 months old, isolated from a fragment of heart extirpated from a chick embryo on January 17, 1912.[1] Today this strain is still alive. It has been under cultivation *in vitro* for a period of over 7 years and has undergone 1,390 passages.

The purpose of this article is to describe the technique employed in perpetuating the strain during the last 5 years and in measuring the increase of the tissue, the factors which influence the rate of growth, and the present condition of the strain.

Technique.

The technique does not differ fundamentally from the technique already reported.[2] The fragments of the old strain are allowed to grow undisturbed for 48 hours and are then divided into two parts and transferred to a fresh medium in the following manner.

The cover-glass is lifted with the heated point of a blunt cataract knife. It is then placed upon a piece of black glass. The fragment is extirpated by four clean cuts made with the blade of a sharp cataract knife within the area of new growth, and divided into two or three pieces as equal in size as possible. The pieces are transferred, with the point of the knife and a needle if necessary, to a bath of Ringer's solution. They are allowed to remain in the bath for about 45 seconds. Meanwhile the substances composing the medium are dropped upon a cover-glass and thoroughly mixed with the end of the cataract knife. Then the medium is spread over the surface of the glass, in

[1] Carrel, A., *J. Exp. Med.*, 1914, xx, 1.

[2] Ebeling, A. H., *J. Exp. Med.*, 1913, xvii, 273.

order that the depth and area of the clot may be approximately uniform in all preparations.

The fragments of tissue are transferred by means of the knife point to the medium. They must be embedded thoroughly in it, without folding or curling. This step must be carried out rapidly to guard against embedding after coagulation has set in; that is, after 15 to 20 seconds, under ordinary conditions of room temperature and moisture. Coagulation is allowed to proceed and occurs in from 45 to 50 seconds. During this period, as well as during the period of washing in Ringer's solution, the preparations are kept under a large Petri dish in order to eliminate as nearly as possible chance bacterial contamination from the dust of the atmosphere.

After coagulation the cover-glass is inverted and placed on a hollow slide and held in place by a small quantity of vaseline. When coagulation does not occur promptly, the slide is prepared with vaseline in the manner described above, but instead of inverting the cover-glass over the slide, the slide is inverted and placed over the cover-glass, care being exercised not to touch the periphery of the medium. The slide is then picked up with its adherent cover-slip and set aside until coagulation occurs. This procedure prevents undue evaporation, which occurs if the medium is exposed for any length of time to the surrounding air, even though kept under the large Petri dish. The slides are sealed with paraffin melting at 56°C. and placed in the incubator at an average temperature of 39°C.

The medium used for perpetuating the strain is composed of equal volumes of chicken plasma and chick embryo extract. This combination produces a clot firm but not dense enough to interfere with the migration of the cells. Although a mixture of one volume of extract and two volumes of plasma constitutes a satisfactory medium, it has been observed that the new growth is less extensive. The clot is firmer and contracts more closely around the tissue fragment. It seems that the central portion of the tissue is not reached by fresh medium. The cells composing it die, and the necrotic tissue appears to retard the growth of the peripheral portion of the culture.

The plasma is obtained from adult chickens. The blood is taken from the carotid artery through an oiled glass cannula, received in chilled paraffined tubes, and centrifuged. Finally, the supernatant

plasma is pipetted off and kept in tightly corked paraffined tubes in cold storage at 4°C. The best plasma is obtained from young, healthy adult chickens, not over 2 years old, which have not been fed for a period of 24 hours previous to bleeding. Blood withdrawn from more recently fed chickens gives a turbid plasma rich in fat globules. Such plasma gives a hazy clot in which the cells do not develop so well as in a clear plasma.

The tissue extract employed is obtained from 7 to 8 day chick embryos. The embryos are first washed in Ringer's solution to remove the amniotic fluid and traces of blood which adhere to them after removal from the shell. Then the solution is drawn off in order to obtain an undiluted tissue juice. The embryos are minced in a watch-glass with sharp, curved scissors. The pulp is centrifuged for 10 minutes and the supernatant fluid obtained is drawn off.

The area of new growth is measured by means of a projection apparatus. In adjusting the condenser it is essential to guard against the action of the light rays upon the tissues, since the heat developed by concentration of the radiations is injurious. Diffused light sufficiently powerful to give proper illumination without any noticeable ill effect must be used. The image of the fragment of tissue is cast upon a sheet of paper, and by means of a pencil the outline of the original fragment and that of the new tissue can be traced. Shortly after the cultures are prepared, a drawing of the outline of the piece of embedded tissue is made, which requires not more than 20 seconds. The culture can then be returned to the incubator. Subsequent tracings, after growth is well established, are made at intervals on the same sheet of paper. They must be made rapidly because, after a short time, small droplets form and settle in the concavity of the slide. These droplets, usually after a period of from $1\frac{1}{2}$ to 2 minutes, coalesce and then no longer interfere with focusing. Such a long exposure of the tissue to the light has probably a retarding effect on the growth and should be avoided.

The rate of growth is expressed in function of the initial area for a given time interval. That is, the area of the newly grown tissue is equal to n times the initial area, as, for instance, it may be said that it has doubled or tripled, or that it is 10 times larger, within the same interval of time. Then, the rates of growth of two fragments of tissue

of unequal size can easily be compared. For example, the area of a 7 sq. cm. fragment, and of a 5.8 sq. cm. fragment, became in 48 hours, respectively, 99 sq. cm., and 81.5 sq. cm. The first one increased 92 sq. cm., the second only 75.7 sq. cm. However, the relation of the size of the growth to the size of the initial area is identical:

$$\text{No. 1, } \frac{99-7}{7} = 13.1 \qquad \text{No. 2, } \frac{81.5-5.8}{5.8} = 13.1$$

Each fragment has become 13.1 times as large as it was. The relation existing between the rates of growth of two fragments can be expressed in the form of a ratio, which is equal to $\frac{13.1}{13.1} = 1$ in the above example.

Factors Which Influence the Rate of Growth.

The strain is used chiefly for measuring the influence of different factors on the rate of growth of connective tissue. The value of the method depends entirely on the property of the parts of a divided fragment of tissue to grow at the same rate when they are cultivated in identical media. Therefore it is important to know what factors may cause two pieces of the same fragment to grow at unequal rates, and to recognize the presence of these factors after the fragments have been embedded in their medium.

As a rule, when two parts of a fragment of the strain which has been growing actively for 48 hours are cultivated in identical media, they grow at the same rate. However, it was found that parts equal or unequal in size coming from the same original fragment do not always grow at the same rate. The reasons for this difference have been determined in such a manner that examination of the pieces makes it possible almost always to foresee whether they will grow at the same rate, and then to discard the useless cultures.

Fragments identical in area may differ in thickness. This may be seen easily by microscopic examination, and sometimes by the naked eye. When a region whiter than the surrounding tissue is seen, it is probable that it is thicker and possibly necrotic. Microscopic examination of the tissue then shows a spot very much darker in color. Tissues presenting these characteristics should be discarded. They

probably would not grow at the same rate as tissues of homogeneous appearance, identical in area.

The condition of the edges of the fragments is also an important factor of the rate of growth. If the incision has been made through the growing area of the tissue, the periphery of the fragments has everywhere a similar appearance. The incision can be seen easily with the microscope if it has been made in places through the plasma not yet invaded by the new cells. Very often the old plasma is slightly folded. As the rate of growth is slower under these conditions such cultures should be discarded.

The value of the growth of a piece of tissue is compared only with the value of the growth of another piece of the same tissue. Therefore the absolute value of the growth is not of great importance, and the factors which do not modify the relative value of the growth can be neglected. However, these factors exist and may increase or decrease in a large measure the extent of the growth. In measurements made on 142 cultures, it was found that the surface of a fragment may increase from 4 to 40 times the initial size. These large differences in the amount of growth are due to the quality of the plasma and of the embryo extract used for the medium, to the previous condition of the strain, to the temperature of the incubator, and to many other factors. They are of no great interest, since they act at the same time on both the fragments which are to be compared. From a practical point of view, the factors which may influence independently the rate of growth of the two halves of an original fragment are almost completely eliminated, if the cultures containing pieces of old plasma or fragments of tissue of unequal size and thickness are discarded.

Present Condition of the Strain of Connective Tissue.

After more than 7 years of life outside the organism, the rate of growth of the strain of connective tissue is very active. This rate seems to have increased progressively during the 7 years (Figs. 1 and 2), but it is possible that it may be only an apparent increase due to modifications of the technique. During the 1st year the growth was slow and irregular, because it was not yet known that the presence in the culture medium of certain substances contained in embry-

onic juices is essential for the permanent life of tissues *in vitro*. As soon as the tissues were washed in Ringer's solution, or in salt solution every 2 days, and embryonic juice was used in the plasma of the medium, the rate of growth increased very much and became almost constant.[2] When the strain was 28 months old, the fragments of tissue which showed the maximum speed in growth increased in 48 hours 15 times their area. The 7 year strain increases more rapidly, as the area of a fragment may become in 48 hours 40 times larger than that of the original fragment. But this difference does not prove absolutely that the amount of new tissue produced in a given time is greater than it was 5 years ago. The medium used today is composed of one volume of plasma and one volume of embryonic juice, while 5 years ago it was composed of two volumes of plasma and one volume of embryonic juice. Since the medium is less dense, the growing tissue is probably thinner, and the increase in area does not mean the production of more tissue than that produced several years ago. The morphology of the cells has not varied. The photographs made 2 years ago and a few months ago show about the same number of mitotic figures (Figs. 3 to 5).

During the last 5 years the rate of growth fluctuated under the influence of the composition of the medium and the condition of the tissues. The plasma varied according to the age and the condition of the chicken and the embryonic juices were not of constant quality. Many other factors retarded or accelerated the growth of the tissue during periods of varying lengths of time. But as soon as the strain was again cultivated in normal medium, it grew at the normal rate. As has been shown previously, since the activity of the cells is a function of the medium in which they are living, it is readily modified by altering the composition of the medium. When the rate of multiplication of cells decreased and the amount of new tissue became small, the strain could be brought to normal growth within a few weeks. It was always found that the decrease in the rate of growth was due to deficient medium, or extract, or to the presence of alkali at the surface of the slides.

CONCLUSION.

1. A strain of connective tissue is still very active after more than 7 years of life *in vitro*.

2. The rate of growth of the fragments of tissue can be measured accurately and used for testing the action of many different factors on the growth of connective tissue cells.

3. The rate of growth of the strain is at least as rapid as it was 5 years ago, and may be more active.

4. The connective tissue cells appear to have the power of multiplying indefinitely in a culture medium, as do microorganisms.

EXPLANATION OF PLATES.

PLATE 51.

FIG. 1. Culture 9211–1. Passage 1080. Stained December 31, 1917. 48 hours growth. × 16.

PLATE 52.

FIG. 2. Culture 13310–1. Passage 1367. Stained May 23, 1919. 48 hours growth. × 16.

PLATE 53.

FIG. 3. Culture 5835. Passage 732. Stained April 16, 1917. 24 hours growth. × 275.

PLATE 54.

FIG. 4. Culture 9211–1. Passage 1080. Stained December 31, 1917. 48 hours growth. × 275.

PLATE 55.

FIG. 5. Culture 12784–1. Passage 1347. Stained April 25, 1919. 48 hours growth. × 240.

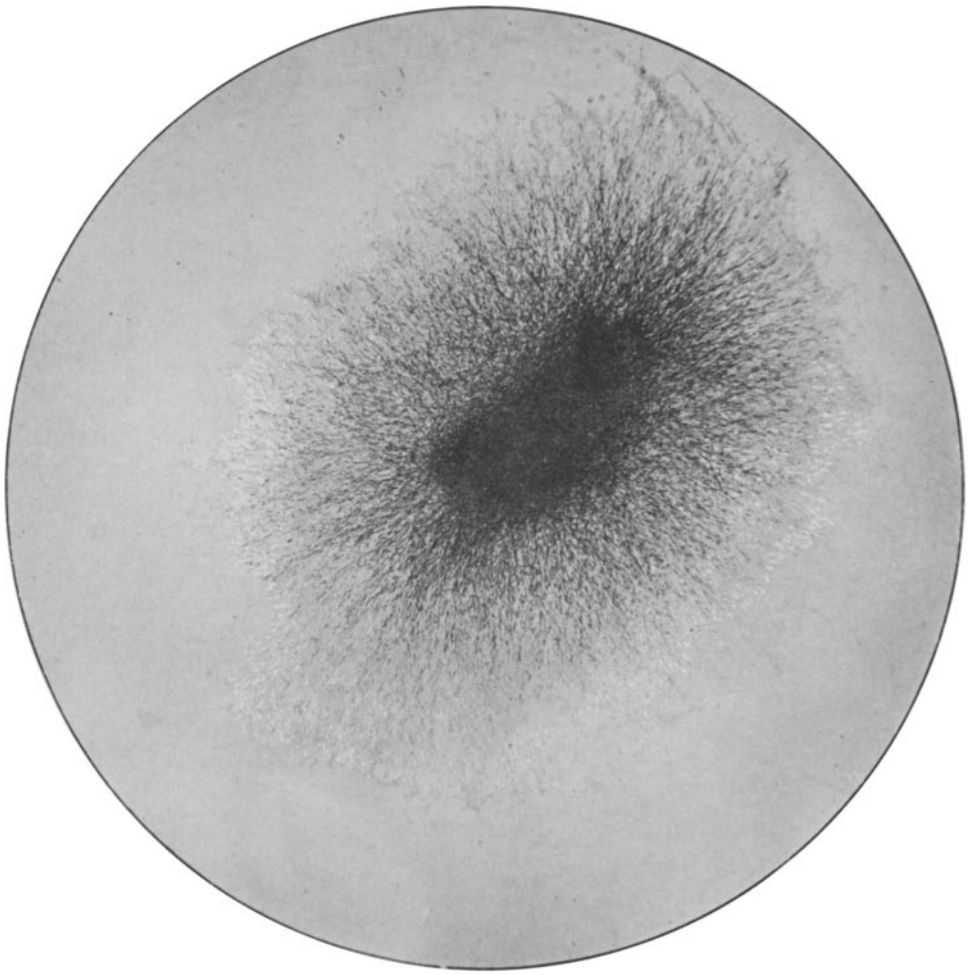

FIG. 1.

(Ebeling: Old strain of connective tissue.)

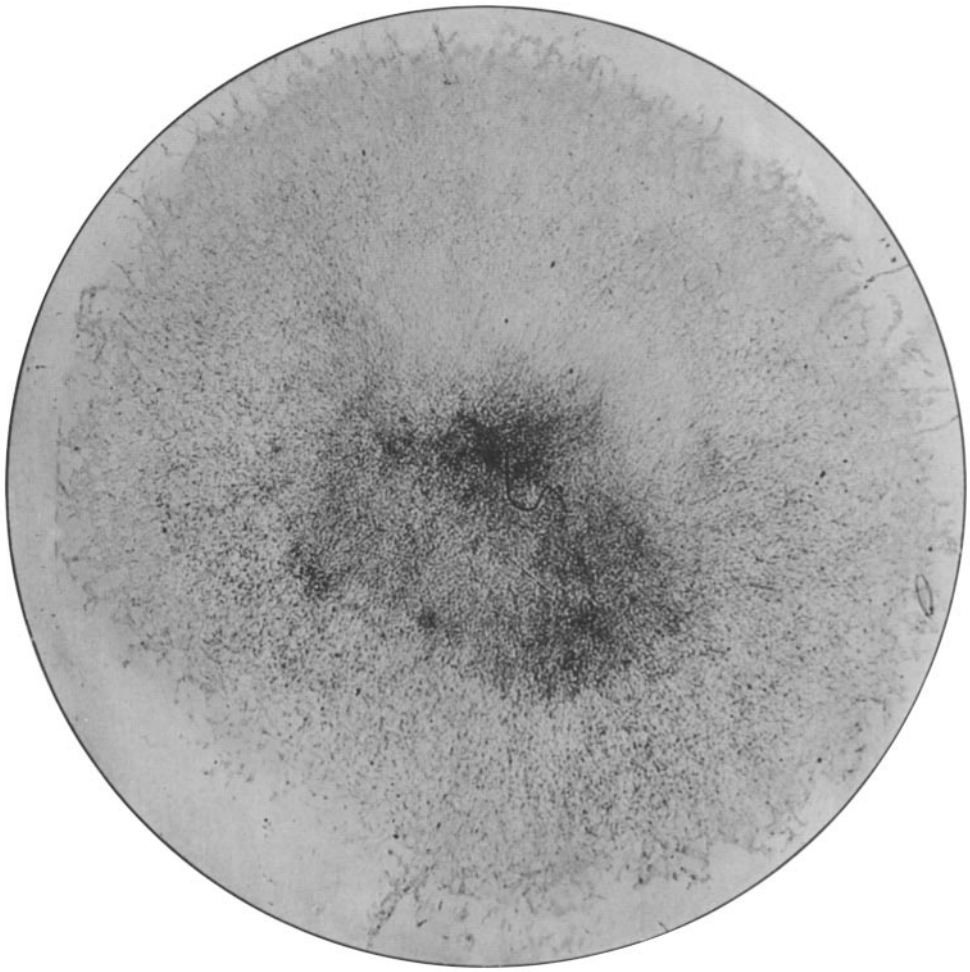

FIG. 2.

(Ebeling: Old strain of connective tissue.)

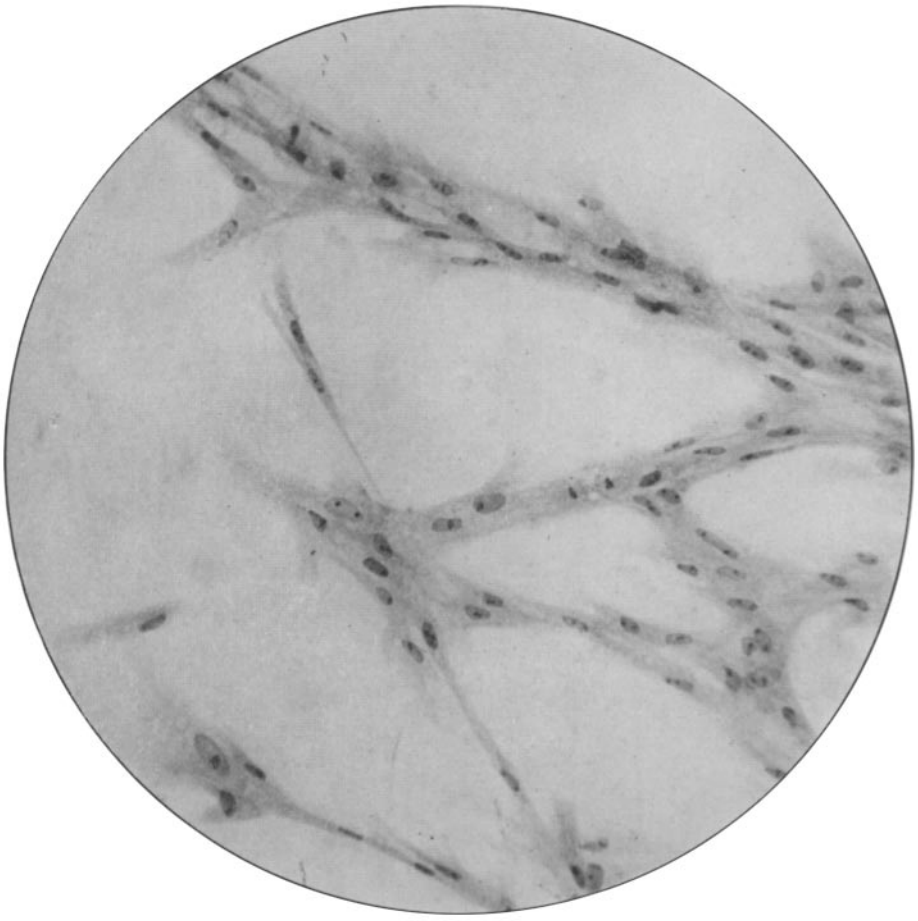

FIG. 3.

(Ebeling: Old strain of connective tissue.)

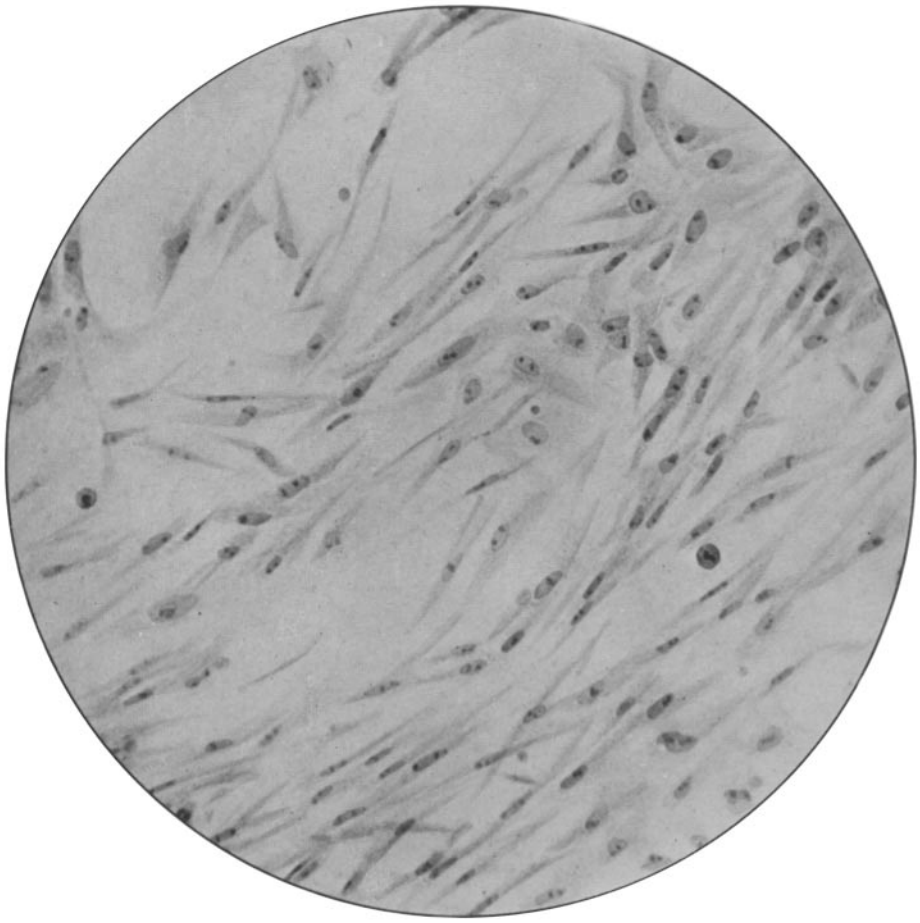

FIG. 4.

(Ebeling: Old strain of connective tissue.)

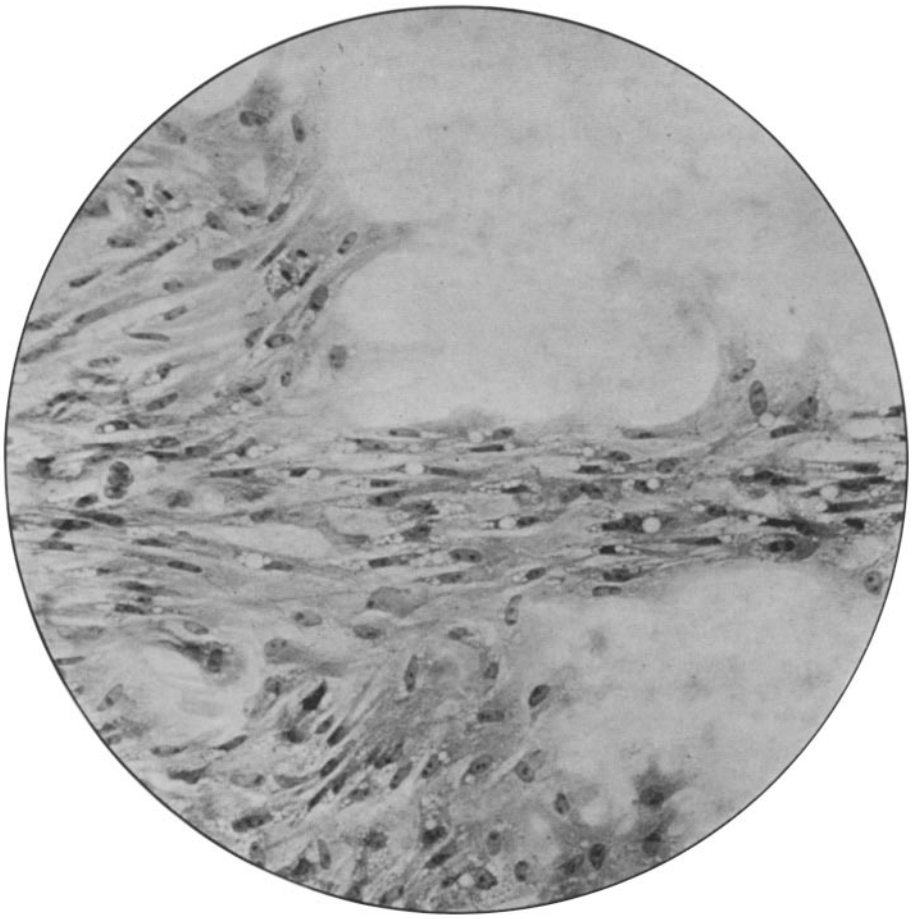

FIG. 5.

(Ebeling: Old strain of connective tissue.)