THE EFFECT OF THE VARIATION IN THE OSMOTIC TENSION AND OF THE DILUTION OF CULTURE MEDIA ON THE CELL PROLIFERATION OF CONNECTIVE TISSUE.*

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PLATES 11 TO 14.

In his fundamental experiments Jacques Loeb has shown the importance of the osmotic tension of water for the growth of marine organisms. Carrel and Burrows,[1] by cultivating for a few days embryonic chick spleen in diluted plasma and in plasma the osmotic tension of which had been modified, attempted to determine what factors could activate the rate of growth of the tissues of higher animals. These experiments were made before Carrel developed the technique which rendered possible the permanent life of connective tissue *in vitro*. Afterwards it became apparent that the modifications of the medium acted differently on tissues proliferating for many generations and tissues maintained for a few days in a condition of survival, and that the conclusions in the article of Carrel and Burrows needed to be modified.

Dr. Carrel gained the impression that it would be necessary, in order to know the influences on cell proliferation of the variation in the osmotic tension and of the dilution of the culture media, to observe for many passages the growth of a tissue in a specific medium. The present experiments were therefore undertaken to determine definitely to what extent strains of connective tissue, kept in a condition of active life, *in vitro,* for many generations, were influenced by the modification of the medium. During the time this work was in progress, an article by Lambert[2] was published on the effect of dilution of plasma on the growth of cells in tissue

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[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.,* 1911, xiii, 562.

[2] Lambert, R. A., *Jour. Exper. Med.,* 1914, xix, 398.

cultures. It was stated that plasma with isotonic solutions causes a more extensive migration in cultures of cells of the actively migratory type, such as those of spleen and bone marrow. Dilution with a limited quantity of distilled water produced the same effect. Less actively motile cells are influenced little or not at all by dilution; also, dilution of the plasma with either isotonic solutions or distilled water is without effect on the cell multiplication. His experiments did not differ in method from those of Carrel and Burrows and are, therefore, open to the same objection.

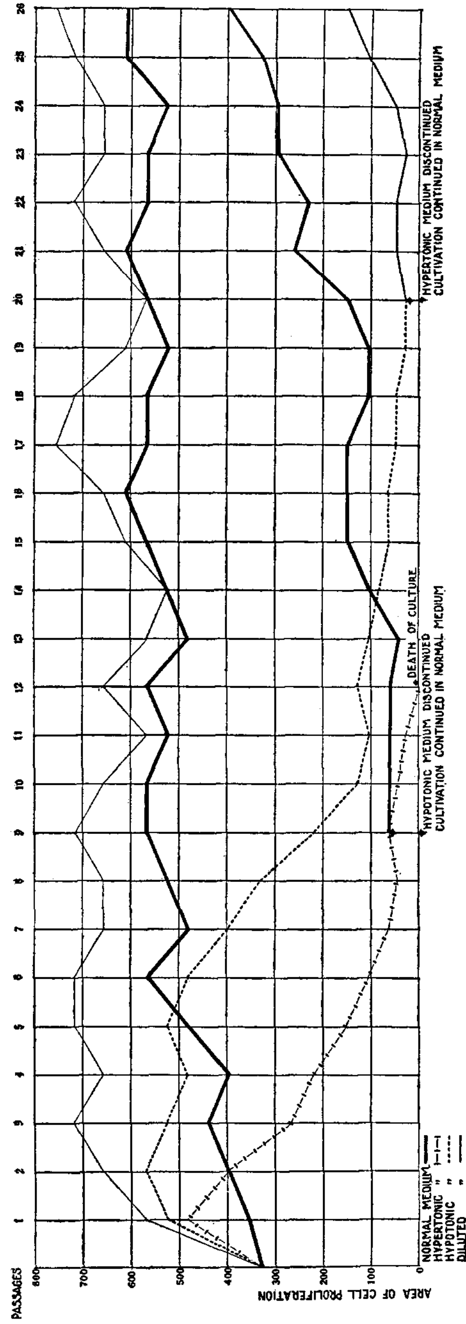
TECHNIQUE.

The strain of connective tissue used as a control was cultivated in a medium, designated normal, composed of one part of freshly prepared embryonic chick extract and of two parts of normal chicken plasma.

Hypotonic medium was prepared by adding two parts of distilled water to three parts of normal plasma and one part of fresh embryonic chick extract.

Preliminary experiments were made to determine the concentration of the salt solution which was added to normal plasma in order to render it sufficiently hypertonic. It was found that a 2.5 per cent. sodium chloride solution was too concentrated. When employed in a culture medium it proved distinctly unfavorable to cell proliferation after one or two passages. A medium containing a 2 per cent. solution of sodium chloride in the same proportion showed that it was possible to keep strains of connective tissue cells in a condition of active life for a number of passages. 1 and 1.5 per cent. solutions were also tried, but the hypertonicity produced thereby was found to be so slight as to approach the conditions which prevailed when normal plasma was diluted with Ringer solution. The hypertonic medium was prepared by adding two parts of a 2 per cent. solution of sodium chloride to three parts of normal chicken plasma and one part of fresh embryonic chick extract.

Diluted medium was prepared by adding two parts of Ringer solution to three parts of normal chicken plasma and one part of fresh embryonic chick extract. Thus, the three modified media, hypotonic, hypertonic, and diluted, contained the same relative amount of normal plasma and fresh embryonic chick extract.



TEXT-FIG. 1. The average area of cell proliferation which developed during forty-eight-hour intervals (one passage) of incubation at 40° C. in cultures of connective tissue which were cultivated in normal, hypertonic, hypotonic, and diluted media, respectively.

Two strains of connective tissue cells were used in these experiments. One strain was derived from a fragment of heart tissue from a chick embryo seven days old, extirpated on January 17, 1912; the other from a fragment of heart tissue from a chick embryo eight days old, isolated on February 18, 1913.

Cultures were made by subdividing fragments of these two strains of connective tissue. The pieces were washed in Ringer solution for three quarters of a minute to one minute and cultivated respectively in normal, hypotonic, hypertonic, and diluted medium. After forty-eight hours' incubation the cultures were washed in Ringer solution as before, transferred into fresh medium, and again incubated. After incubation for one, six, twenty-four, and forty-eight hours, observations were made, and in some instances cultures were fixed and stained. To determine the influence of modified media on the proliferation of connective tissue cells, the growth obtained in a culture, after stated periods in normal medium, was taken as a standard of comparison. The extent of this growth was measured with an ocular micrometer and recorded. The accompanying chart shows, in curves, the relative area of cell proliferation which developed during a passage (forty-eight hours) in cultures which had been grown in modified media. Calculations were made by measuring in the different media the width of the zone of new growth which had developed during a passage. The measurements for all cultures which had been cultivated in the same medium were averaged and the area was computed. The relative density of cell proliferation was determined by observations on stained preparations, and photomicrographs of the preparations were made.

EXPERIMENTS.

Continued cultivation of connective tissue in hypertonic medium produced the following results: When a culture was transferred to hypertonic medium from a normal medium in which the width of the area of cell proliferation after forty-eight hours' incubation was equal to seven divisions of the ocular micrometer, observations showed, after one hour's incubation, no evidence of cell proliferation; after six hours' incubation a few elongated cells were observed spreading from the periphery of the central fragment into the

medium; after twenty-four hours the area of cell proliferation measured, on the average, six and a half divisions, slightly more than the area of cell proliferation in the control, which measured six divisions. Stained preparations at this stage of incubation showed the area to be denser than the control. After forty-eight hours' incubation the zone of proliferating cells measured nine divisions, and in the control seven and a half divisions. The density was greater in comparison with the normal control and the contrast could be observed in the living culture. Examination of the cells in these cultures showed the presence of many refractile globules of varying size in the cytoplasm of the cell. In the control, cells showed small refractile globules, few in number and fairly uniform in size. Preparations stained with Sudan III and hematoxylin showed these refractile globules to be fatty substances which had accumulated in the cell cytoplasm. A third passage into hypertonic medium after forty-eight hours' incubation produced a less extensive, though quite as dense an area of cell proliferation as the control. The zone measured six divisions, and the control nine and a half. The amount of fat present in the cytoplasm of the cell was observed to be apparently as great as in the previous passage. There was no increased amount of fat present in the control over that present in the new cells which developed in the previous passage. A fourth and fifth passage, after forty-eight hours' incubation, respectively, showed a decided decrease in extent of new growth. The area of cell proliferation appeared to be as dense as the area of cell proliferation in the control, but the central portion of the culture had become a thick and opaque mass. The cells were observed to be filled with large fat granules. After the sixth and seventh passages the extent of cell proliferation was decidedly less than the control, measuring two and a half divisions, whereas the extent of growth in the control measured nine and a half. The central portion of the cultures had contracted into a very dense, spherical, opaque mass. Further passages into hypertonic medium resulted in a rapid degeneration of the culture, with central necrosis and death after the eleventh or twelfth passage. In some instances tissues which had been cultivated for nine passages (eighteen days) in hypertonic medium were revived by again cultivating them in

normal medium, and after seventeen passages (thirty-four days) the extent of cell proliferation was approaching normal. In the first few days during which a culture was cultivated in hypertonic medium the area of new growth was more extensive and apparently more dense than in the control. After that time this area decreased rapidly and eventually the culture died.

Cultivation of connective tissue in hypotonic plasma after one hour's incubation showed new cells budding out from the peripheral portions of the central fragment. The same condition was observed in the control. After six hours' incubation the area of cell proliferation was about the same as the control, but in twenty-four hours the zone of proliferating cells was more extensive than in the control, measuring about eight and a half divisions in width, whereas the width of the area in the control measured slightly over seven divisions. After forty-eight hours' incubation the increase in extent of cell proliferation was decidedly greater than the control, measuring about nine and a half divisions, as compared with seven and a half divisions in the control. The density of growth, however, was less than the control. Stained preparations showed fewer planes in which cell proliferation had taken place. After the second and third passages, respectively, the extent of growth was still observed to be greater (about nine and a half divisions) than the control (about seven and a half divisions). The accumulation of fat globules in the cytoplasm of the cells was practically the same as the control. After the fourth and fifth passages the area of proliferating cells was slightly greater than the control, but the relative density was less in the former. After the sixth and seventh passages the extent of cell proliferation decreased and was observed to be less than the control. The cells showed an increase in fat accumulation over the control and the cells appeared loosely joined, forming a large meshed network of interlacing cells. Continued cultivation in hypotonic medium up to twenty passages (forty days) showed a gradual decrease in the extent of growth. The central fragment became dense and opaque and large fat globules were observed in the cytoplasm of the cells. After twenty passages in hypotonic medium some cultures were again cultivated in normal medium. They usually recovered after five or six passages. During a period last-

ing about ten days, a culture cultivated in hypotonic medium showed that the area of cell proliferation was more extensive though less dense than the normal control. After that time the extent of growth decreased gradually and finally cell proliferation became sluggish. It was possible to revive again a culture at this stage.

The cultivation of cultures of connective tissue in diluted medium after one hour's incubation showed a number of new cells growing out into the medium from the periphery of the central fragment. In six hours an appreciable crown of new cells encircled the central portion of tissue, and after twenty-four hours the area of new growth was more extensive (about eight and a half divisions in width) than the control (about seven divisions in width). After forty-eight hours it was observed that the area of cell proliferation which had developed was decidedly more extensive (width of area eleven divisions) than the control (width of area seven and a half). Stained preparations showed this area to be less dense than the control, but there was no increase in the amount of fat globules present in the cytoplasm of the cells. A second, third, and fourth passage showed an increase in area over the control and many passages thereafter (twenty-two passages, forty-four days) showed that this increase in area of cell proliferation over the normal was fairly constant. This point is shown in the text-figure 1. The fluctuations of growth which were observed in the control were also observed at the same time in the experiment. When the extent of cell proliferation was less extensive in normal medium than in the previous passage, it was found that there was a relative decrease, on most occasions, in the extent of cell proliferation in diluted medium. The stained preparations of cultures cultivated in diluted medium for many passages showed an extensive, loosely meshed network of elongated cells. The control showed a more densely packed mass of elongated cells, but the area of cell proliferation was less. Figure 1 represents an entire culture of connective tissue forty-eight hours after passage into normal medium, which had been cultivated for about two hundred passages in this medium. Figure 2 shows an entire culture of connective tissue forty-eight hours after the last passage into diluted medium. The last passage was the twenty-fifth consecutive passage into diluted medium. Figure

3 is a higher magnification of part of a control culture after forty-eight hours' incubation. Figure 4 shows a portion of the area of cell proliferation which developed in a culture in forty-eight hours in diluted medium, after twenty-two passages into the same medium. The relative increase in the extent of cell proliferation in diluted medium, over the control, remained fairly constant through the total number of passages, although no actual increase in mass was observed (text-figure 1).

When connective tissue was cultivated in hypertonic medium for one passage and then in hypotonic medium, observations showed no marked differences in cell proliferation, but alternate passages from hypertonic to hypotonic medium for three or four passages caused death of the culture. Alternate passages from hypertonic medium into diluted medium proved unfavorable after three or four passages, and usually resulted in death of the culture after five generations. Alternate passages from hypertonic medium into normal medium proved unfavorable after five passages, and when continued for ten passages the culture died. Alternate passages from hypotonic into normal medium were continued for ten generations. A gradual decrease in the extent and activity of cell proliferation was observed and this treatment proved unfavorable for growth in the culture. Observations showed that alternate passages from normal medium into diluted medium did not appear to affect the dynamic condition of the culture, although, relatively, there appeared to be a denser area of cell proliferation in normal medium and a less dense but more extensive area of growth in diluted medium. Alternate passages of connective tissue into modified media in most instances produced unfavorable results.

SUMMARY.

For the first few days of cultivation of connective tissue in hypertonic, hypotonic, and diluted medium, cell proliferation was stimulated. The first outgrowths of new cells in the modified media did not occur sooner than in normal medium. In hypertonic medium the density of the area of cell proliferation appeared to be greater than the control, but in hypotonic or diluted medium there seemed to be no increase in actual mass over the control. These observa-

tions confirm the conclusions of Carrel and Burrows, as well as those of Lambert.

Subsequent to the first few days of cultivation in hypertonic medium the area of cell proliferation decreased and in a short time conditions developed which were unfavorable to growth, and finally resulted in death of the culture, unless it revived before this stage. Hypotonic medium after about ten days no longer caused more extensive areas of proliferating cells; but instead, the extent of new growth gradually decreased, and the culture merely remained alive unless revived. In diluted medium the extent of the area of cell proliferation remained greater with no actual increase in mass. The area of cell proliferation which is observed during the first few days in a culture of fresh tissue recently extirpated does not indicate the actual influence of modified media. It was only after continued cultivation of strains of connective tissue in these modified media that their influence on cell proliferation was determined.

CONCLUSION.

An increase or decrease in the osmotic tension of the culture medium at first stimulates cell proliferation, but eventually retards it and proves to be unfavorable to growth. Dilution of the medium without change of the osmotic tension produces a more extensive zone of cell proliferation but no increase in the actual mass of newly formed tissue. A culture of connective tissue, which has been growing under unfavorable conditions, due to changing the osmotic tension of the medium in which it has been cultivated, is capable of being revived.

EXPLANATION OF PLATES.

Plate 11.

Fig. 1. The extent of growth obtained after forty-eight hours' incubation in normal medium. This culture had been cultivated in normal medium for about two hundred passages. Control for culture in figure 2. Stained preparation; low power.

Plate 12.

Fig. 2. The extent of growth obtained after forty-eight hours' incubation in diluted medium. This culture had been cultivated in diluted medium for twenty-five passages (fifty days). Stained preparation; same magnification as figure 1.

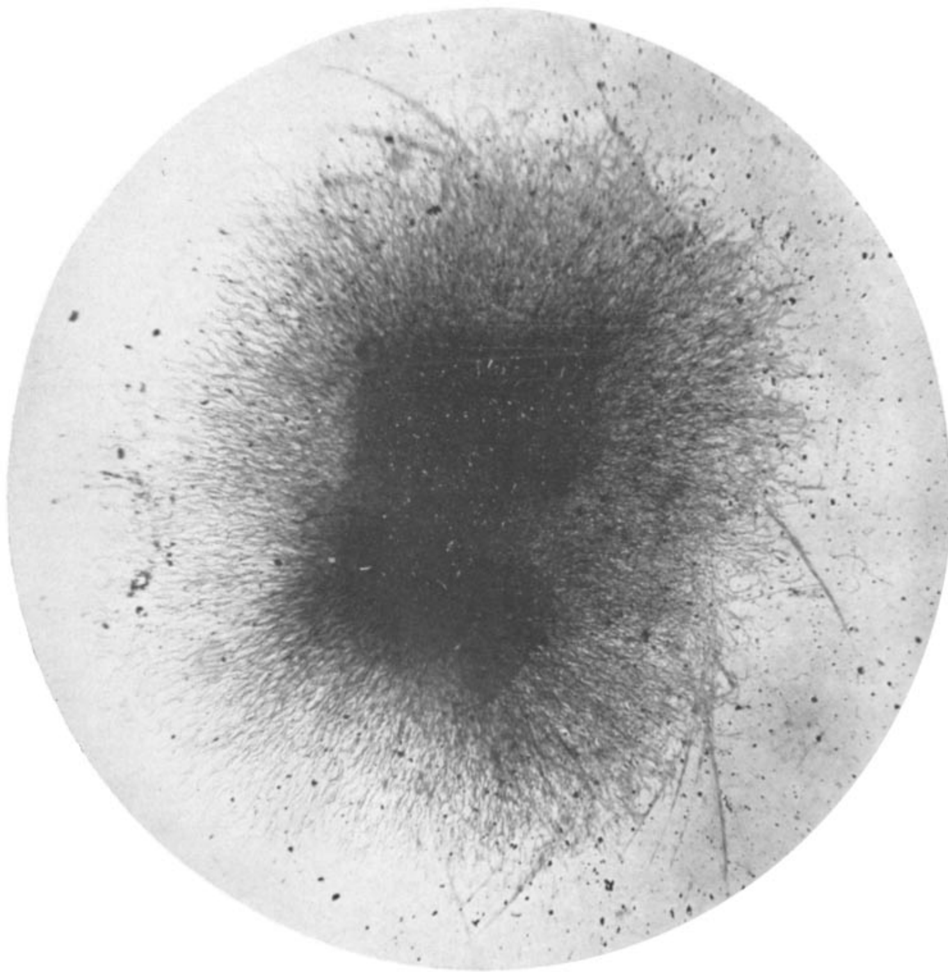


FIG. 1.

(Ebeling: Cell Proliferation of Connective Tissue.)

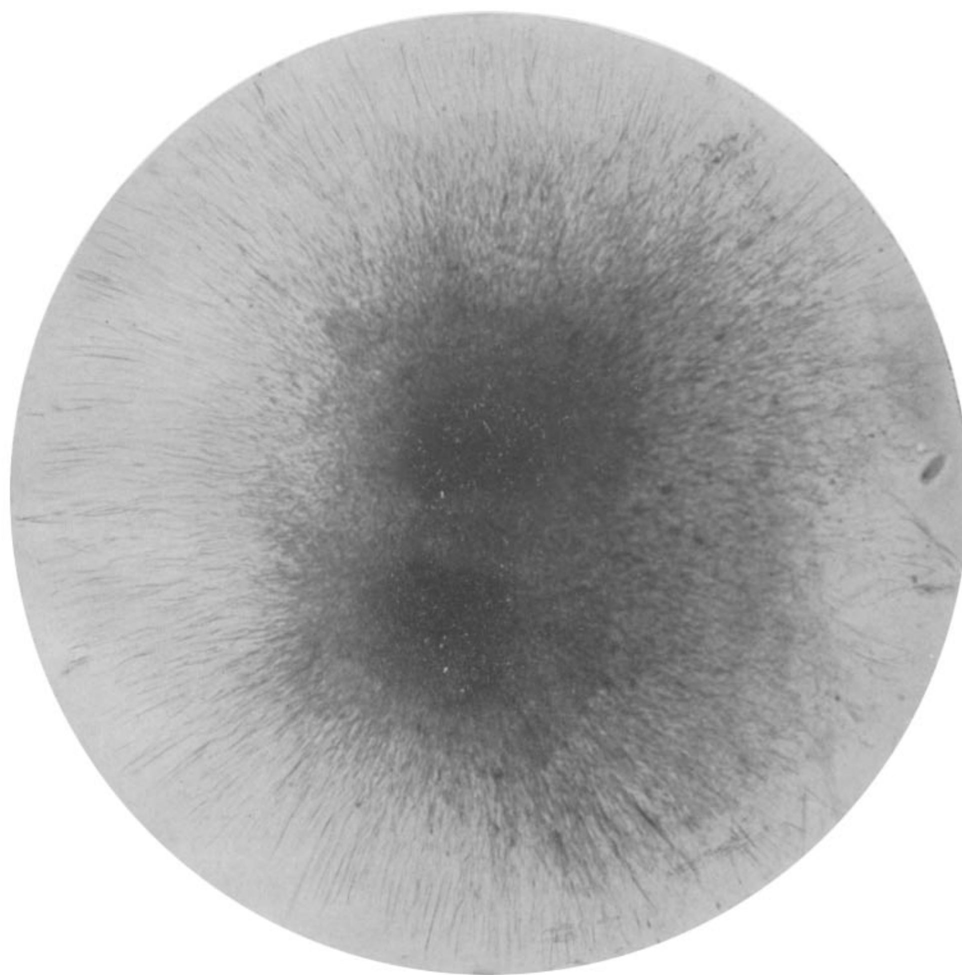


FIG. 2.

(Ebeling: Cell Proliferation of Connective Tissue.

Fig. 3.

(Ebeling: Cell Proliferation of Connective Tissue.)

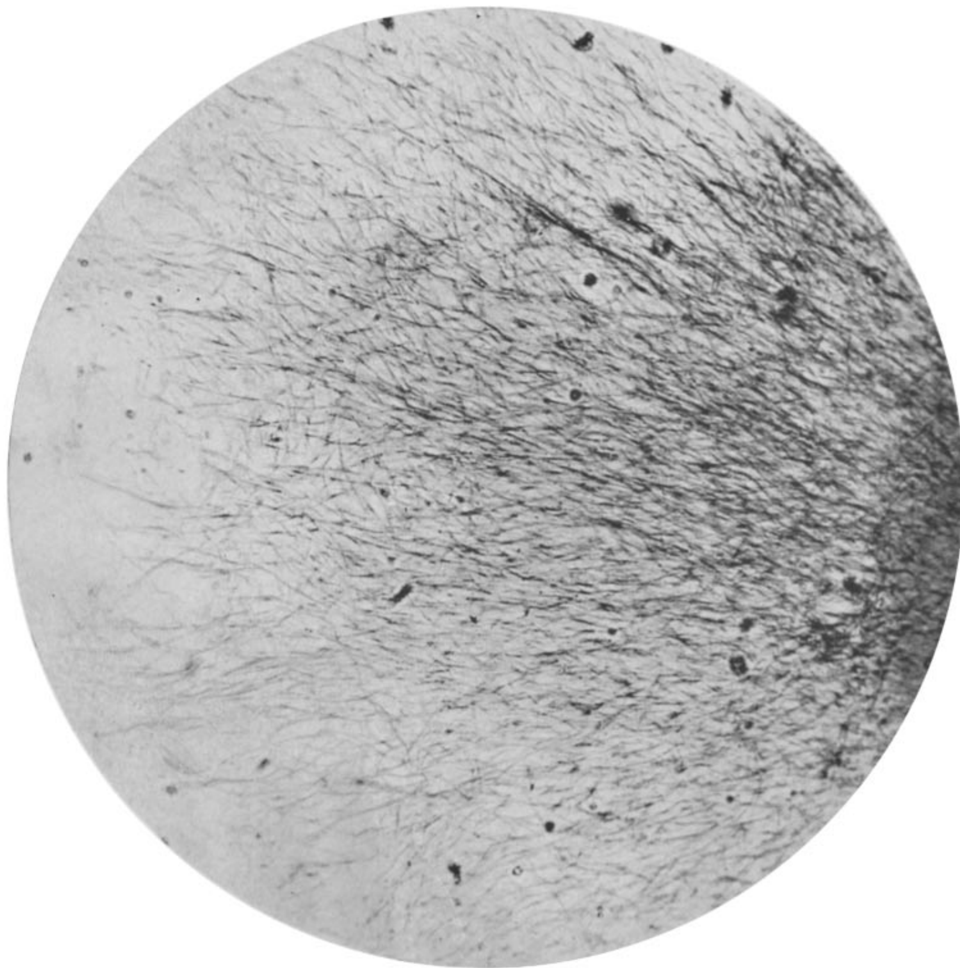


FIG. 4.

(Ebeling: Cell Proliferation of Connective Tissue.)

PLATE 13.

FIG. 3. The extent of the area of cell proliferation which developed after forty-eight hours' incubation in a normal control. Stained preparation; higher power.

PLATE 14.

FIG. 4. The area of cell proliferation which developed after forty-eight hours' incubation in diluted plasma. This culture had been cultivated in the same medium for twenty-three generations (forty-six days). Stained preparation; same magnification as figure 3.