THE CULTIVATION OF HUMAN SARCOMATOUS TISSUE IN VITRO.*

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Plates 15 to 17.

The first attempt to cultivate human malignant tumor *in vitro* was made in 1911 by Carrel and Burrows.[1] Small fragments of tumor were cultivated in normal human plasma and incubated. It was observed in some cases that after a few days the fragments were surrounded by many cells; but generally liquefaction of the medium occurred. The tissues were kept in a condition of survival for a few days, but no real cultures were obtained.

Lately it became possible to keep human fetal tissue, derived from fresh cadavers, in a condition of independent life for several generations,[2] and we therefore attempted to cultivate human sarcomatous tissue in the same manner.

TECHNIQUE.

The medium employed in these experiments was composed of equal parts of normal human plasma and Ringer solution and varying quantities of extract.

The extract was prepared by cutting tissues obtained from fresh fetal cadavers into small pieces, and adding an equal quantity of Ringer solution. After forty-eight hours in cold storage the substance was centrifuged and the supernatant fluid pipetted off. This fluid was used as extract in the making of cultures.

The tissues employed were obtained from recently excised sarcomatous growths,[3] and cultures were made about one and a half hours

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[1] Carrel, A., and Burrows, M. T., *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Losee, J. R., and Ebeling, A. H., *Jour. Exper. Med.*, 1914, xix, 593.

[3] The sarcomatous growths were obtained by Dr. Carrel, through the courtesy of Dr. W. B. Coley, from some of his cases at the General Memorial Hospital, New York. Immediately after excision the tissues were carried to the Laboratories of the New York Lying-In Hospital.

after excision. The primitive cultures were made by putting small, thin fragments of this tissue into the medium. After coagulation the cultures were immediately placed in the incubator and incubated at 38° C. for 24, 48, and 72 hours, the time of passage into fresh medium being governed by conditions which developed in the culture. Before the fragments in cultures were transferred into fresh medium they were washed in Ringer solution for about one minute.

EXPERIMENTS.

Experiment 1, Series 1, Cultures 1, 2, 3, and 4.—Fragments from the periphery of an osteosarcoma[4] were cultivated in equal parts of human plasma and Ringer solution, to which one fourth part of extract was added. The cultures were made about one hour and a half after excision of the growth and incubated.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation in any of the cultures. In twenty-four hours there was still no growth to be observed and the medium had become liquefied around the fragments. The cultures were therefore washed and changed into fresh medium (first passage), to which one half part of extract was added. After twenty-four hours' incubation, growth was found to be present in culture 3, but no growth was observed in cultures 1, 2, and 4, and in forty-eight hours there was still no evidence of cell proliferation in these cultures; the medium had become liquefied, and they were therefore discarded.

Culture 3 was cultivated in the same medium (second passage), and in forty-eight hours growth was apparent. This culture was transferred into fresh medium for twelve passages, during which time (twenty-one days) growth was observed after each transfer into fresh medium. The culture was stained and photographed (figure 1).

Series 2, Cultures 1, 2, 3, and 4.—Fragments from the peripheral area of the same growth were cultivated in the same manner as series 1.

After 1, 6, and 18 hours' incubation there was no evidence of cell proliferation. The medium was still in good condition. In twenty-four hours no growth was observed, and the medium about the

[4] Pathological diagnosis: large round cell sarcoma.

pieces in cultures 1, 2, and 3 had become liquefied. Culture 4 was allowed to remain in the incubator. It was examined after forty-eight and seventy-two hours, but there was no evidence of cell proliferation, although the medium was still in good condition. The culture was discarded. After twenty-four hours cultures 1, 2, and 3 were changed into fresh medium (first passage), to which one half part of extract was added.

Culture 1 after twenty-four hours showed no growth, and the medium was completely liquefied. It was changed into fresh medium (second passage), the same proportion of extract being added as in the previous passage. In twenty-four hours a few scattered cells were observed, but after forty-eight and seventy-two hours there was no further increase in the extent of cell proliferation. The culture was discarded.

Culture 2 (first passage) after twenty-four hours' incubation showed an area of cell proliferation, with no liquefaction of the medium. In seventy-two hours a good growth had developed. The medium was slightly liquefied. It was then changed into fresh medium (second passage), to which one fourth part of extract was added. After twenty-four hours growth had developed, and in forty-eight hours the area of cell proliferation was more extensive, but liquefaction of the medium had developed. The culture was changed into fresh medium (third passage) with the same proportion of extract added as in the previous passage. After twenty-four hours a few cells were observed in the medium surrounding the central fragment. In forty-eight hours there was no increase in the extent of growth and the medium had liquefied. The culture was again transferred into fresh medium (fourth passage), one part of extract being added. In twenty-four hours the medium had become liquefied and no growth was observed. The culture was changed (fifth passage) into fresh medium, the same proportion of extract being added as in the previous passage. In twenty-four hours growth had developed, and in forty-eight hours it was more extensive, but the medium was almost completely liquefied. The culture was transferred (sixth passage) into fresh medium in the same manner as in the previous passage. After twenty-four hours cell proliferation was observed, but small colonies of bacteria had also developed. The infection was general and the culture was discarded.

Culture 3 (first passage), after twenty-four hours' incubation, showed a few scattered cells which had grown out from the original fragment. In forty-eight hours the growth of new cells had increased, but the medium was slightly liquefied. The culture was then treated (second passage) in the same manner as in the previous passage. After twenty-four hours good growth had developed and the medium was in good condition. After forty-eight hours the growth was more extensive, but after seventy-two hours there was no further increase and the medium was slightly liquefied. The third passage into fresh medium was then made, the proportion of extract being increased to one part. After twenty-four hours' incubation the growth had developed and the medium was in good condition. In forty-eight hours the culture was growing actively and the medium had become slightly liquefied. The culture was subsequently changed into fresh medium for twenty-one more passages. The extent of growth fluctuated and gradually decreased. In the fifteenth passage the culture was divided and two cultures were made; one of the cultures (figure 2) was fixed after forty-eight hours' incubation. After the twenty-fourth passage cell proliferation stopped. This culture was transferred twenty-five times, during a period of fifty-one days. The entire history is given in table I, which shows the passage, treatment, and observations that were made on culture 3.

Experiment 2.—A series of cultures, Nos. 1, 2, 3, and 4, was made from fragments of the periphery of a large round cell sarcoma,[5] and cultivated in equal parts of plasma and Ringer solution. After twenty-four hours there was no evidence of growth in any of the cultures and the medium was in good condition. In forty-eight hours there was evidence of cell proliferation in all cultures. After seventy-two hours the area of cell proliferation had increased, but the medium in cultures 3 and 4 had become liquefied. Cultures 1 and 2 were stained.

Cultures 3 and 4 were changed into fresh medium (first passage), and one part of extract was added to the medium. Both cultures developed good growth in forty-eight hours with no liquefaction of the medium. They were again transferred into fresh medium

[5] Pathological diagnosis: large round cell sarcoma.

TABLE I.

Passage.	Date (1913).	Treatment of culture.	Observations.
Experiment 206–3	Nov. 18	Culture of a fragment of the peripheral part of an osteo-sarcoma, cultivated in 1 part of human plasma, 1 part of Ringer solution, and $\frac{1}{4}$ part of extract	Nov. 19. Medium liquefied; no growth.
1	Nov. 19	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and $\frac{1}{2}$ part of extract	Nov. 20. A few scattered cells; medium in good condition. Nov. 21. Good growth; medium slightly liquefied.
2	Nov. 21	Treated in the same manner as in previous passage	Nov. 22. Good growth; medium in good condition. Nov. 23. Growth more extensive; medium in good condition. Nov. 24. No increase in extent of growth; medium slightly liquefied.
3	Nov. 24	Washed in Ringer solution for 1 minute, cultivated in 1 part of human plasma, 1 part of Ringer solution, and 1 part of extract	Nov. 25. Good growth; medium in good condition. Nov. 26. Growing actively; medium slightly liquefied.
4	Nov. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 27. Good growth; medium in good condition. Nov. 28. Growth more extensive; medium in good condition. Nov. 29. No increase in extent of growth; medium liquefied.
5	Nov. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Nov. 30. A few cells; medium in good condition. Dec. 1. A few cells; medium slightly liquefied.
6	Dec. 1	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 2. A few scattered cells; medium in good condition. Dec. 3. Good growth; medium in good condition.
7	Dec. 3	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 4. Good growth; medium in good condition. Dec. 5. Very good growth; medium in good condition.
8	Dec. 5	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 6. Good growth; medium slightly liquefied.
9	Dec. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 7. Good growth; medium in good condition. Dec. 8. More extensive growth; medium liquefied.
10	Dec. 8	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 9. Good growth; medium partially liquefied. Dec. 10. No increase in amount of growth; medium liquefied.

TABLE I.—*Continued.*

Passage.	Date (1913).	Treatment of culture.	Observations.
11	Dec. 10	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 11. Good growth; medium in good condition. Dec. 12. Growth increased; medium in good condition.
12	Dec. 12	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 14. Good growth; medium in good condition. Dec. 15. Very good growth; medium in good condition.
13	Dec. 15	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 16. Growing; medium in good condition. Dec. 17. Very good growth; medium slightly liquefied.
14	Dec. 17	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 18. Good growth; medium in good condition. Dec. 19. Extensive growth; medium in good condition.
15	Dec. 19	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium (cultures 1 and 2)	Dec. 20. Good growth in cultures 1 and 2; medium in good condition. Dec. 21. Good growth; medium in good condition, No. 2 fixed and photographed. Dec. 22. Good growth; medium in good condition.
16	Dec. 22	Washed in Ringer solution for 1 minute, divided into 2 parts, and cultivated in the same medium	Dec. 23. Growing; medium in good condition. Dec. 24. Growing; medium partially liquefied.
17	Dec. 24	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 26. Growing; medium in good condition.
18	Dec. 26	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 27. A few cells; medium in good condition. Dec. 28. Growing; medium in good condition. Dec. 29. Growing; medium in good condition.
19	Dec. 29	Washed in Ringer solution for 1 minute and cultivated in the same medium	Dec. 30. A few cells; medium in good condition. Dec. 31. Growing; medium in good condition.
20	Dec. 31	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 2. Growing; medium in good condition.
21	(1914) Jan. 2	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 3. No growth; medium in good condition. Jan. 4. A few cells.
22	Jan. 4	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 5. A few cells; medium in good condition. Jan. 6. Slow growth; medium slightly liquefied.
23	Jan. 6	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 7. No growth. Jan. 8. A few scattered cells; medium in good condition. Jan. 9. Growing slowly; medium slightly liquefied.

TABLE I.—*Concluded.*

Passage.	Date (1914).	Treatment of culture.	Observations.
24	Jan. 9	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 10. Slight growth; medium in good condition. Jan. 11. No increase in growth; medium in good condition.
25	Jan. 11	Washed in Ringer solution for 1 minute and cultivated in the same medium	Jan. 12. No growth. Jan. 13. No growth. Jan. 14. Discarded.

(second passage). Culture 4 showed a few proliferating cells after seventy-two hours' incubation, and the medium was in good condition. The culture was discarded. After twenty-four hours' incubation in the second passage culture 3 showed growth with no liquefaction. In forty-eight and seventy-two hours growth had increased, but the medium was partially liquefied. The culture was transferred into fresh medium (third passage) and showed a few cells which had spread out into the medium from the central fragment. After seventy-two hours there was no increase in the number of cells, and the medium had liquefied. The culture was transferred into fresh medium (fourth passage), and after forty-eight hours good growth was observed. The medium was in good condition. The fifth passage into fresh medium was made in the same manner as in the previous passage. After twenty-four hours the medium was totally liquefied and no growth was observed. The fragment was transferred to fresh medium, but after 24, 48, and 72 hours no growth developed. The culture was discarded.

RESULTS.

Two experiments were made in which fragments from human sarcomatous tissue were cultivated. It was possible to keep cultures of such tissue in a condition of active life *in vitro* for several generations.

During the first twenty-four hours of incubation there was usually no evidence of cell proliferation, and slight liquefaction around the primitive fragments. When no liquefaction occurred, growth of new cells manifested itself after forty-eight hours. Twenty-four hours after passage into fresh medium (first passage), cell proliferation was observed in those cultures which showed no evidence of

growth when first cultivated. In comparison with human connective tissue, the rate of growth was practically the same in the beginning, but a gradual decrease in the activity and extent of cell proliferation was observed as the length of time increased during which the culture was carried through successive passages. Microscopic examination of the first outgrowth of cells showed the presence of large, round, as well as elongated and ramified cells. In subsequent passages the round cells were no longer to be identified, and the elongated, ramified variety only were observed. The morphological characteristics of these cells did not appear to differ from the cells present in cultures of normal human connective tissue. Preparations stained with Giemsa stain showed the large round cells as having a densely stained cytoplasm with from one to two nuclei and a regular outline. The elongated and ramified varieties showed no difference in comparison with those present in cultures of human connective tissue, with the exception that no mitotic figures were observed. Figure 3 shows a few of the peripheral cells in a culture of sarcomatous tissue which had been carried through twelve passages.

One culture was stained which was growing actively in its twelfth passage (twenty-one days). This culture is shown in figure 1, the area of cell proliferation being that which developed in the twelfth passage during forty-eight hours' incubation. One other culture was carried through for twenty-four passages, that is, fifty-two days. It was possible to divide this culture in its fifteenth passage, making two, and after forty-eight hours' incubation one of these cultures was fixed. Figure 2 shows almost the entire culture.

Sarcomatous tissue grew as well during a few days as normal connective tissue. Afterwards the rate of growth became less rapid and the tissue could not be kept alive for more than fifty-two days, while normal human tissue could be kept for sixty-eight days.

These differences may be due to technical factors, but they may also be the result of the nature of the tissue itself. In his attempts at keeping Rous sarcoma in a condition of permanent life *in vitro*, Carrel[6] observed that after a few generations the rate of growth became less rapid than the rate of growth of connective tissue. In

[6] Carrel, A., *Jour. Exper. Med.*, 1912, xv, 516.

other experiments with rat sarcoma and normal rat connective tissue, cultivated in guinea pig plasma, Carrel also observed the same differences. The writers observed the same phenomena when rat sarcoma and normal heart tissue of the rat were cultivated in chicken plasma.

The results obtained show that it is possible to cultivate *in vitro* fragments of human sarcomatous tissue for several generations, and that the method employed may prove of value in the study of the growth of human malignant tumor.

EXPLANATION OF PLATES.

PLATE 15.

FIG. 1. 12th passage of human sarcomatous tissue. The preparation shows the area of cell proliferation that developed during forty-eight hours' incubation. Giemsa stain.

PLATE 16.

FIG. 2. 15th passage of human sarcomatous tissue. The photograph shows the growth obtained forty-eight hours after passage into fresh medium. Fixed specimen.

PLATE 17.

FIG. 3. High power magnification of some of the peripheral cells present in the same preparation shown in figure 1.

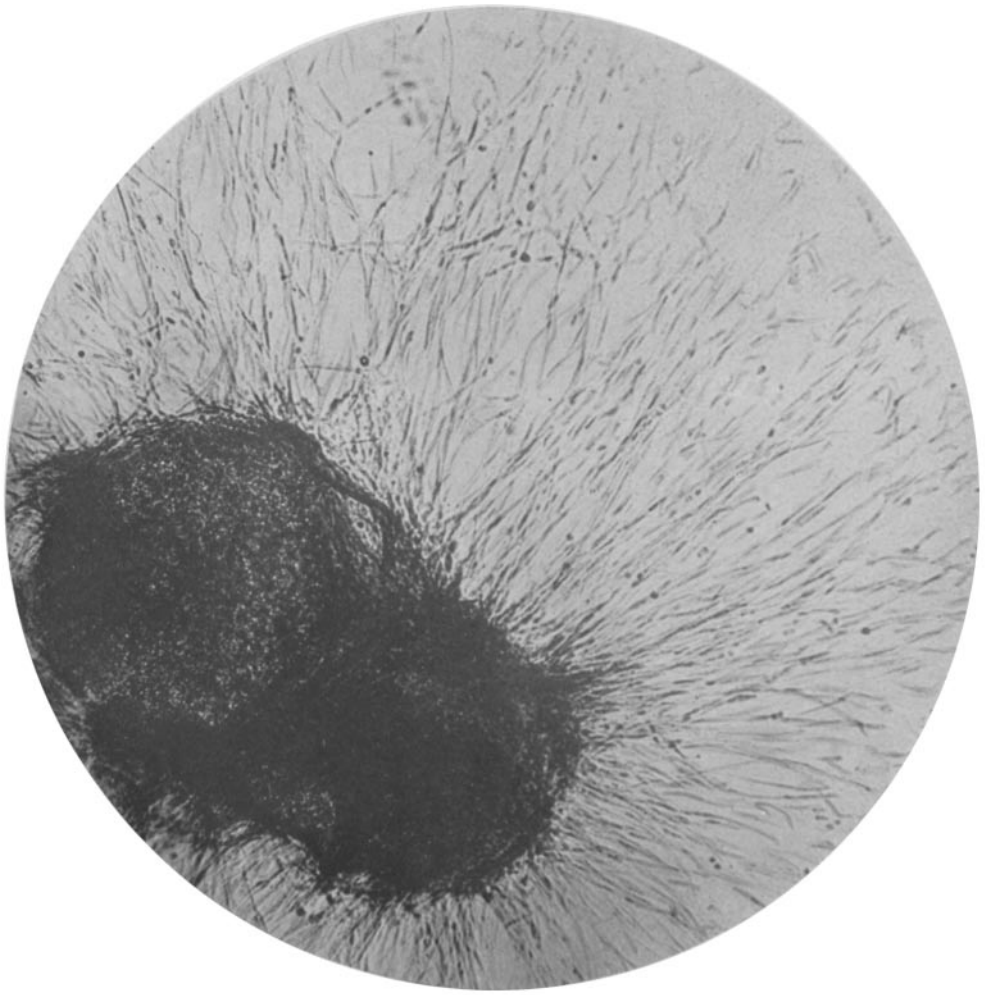

FIG. 1.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

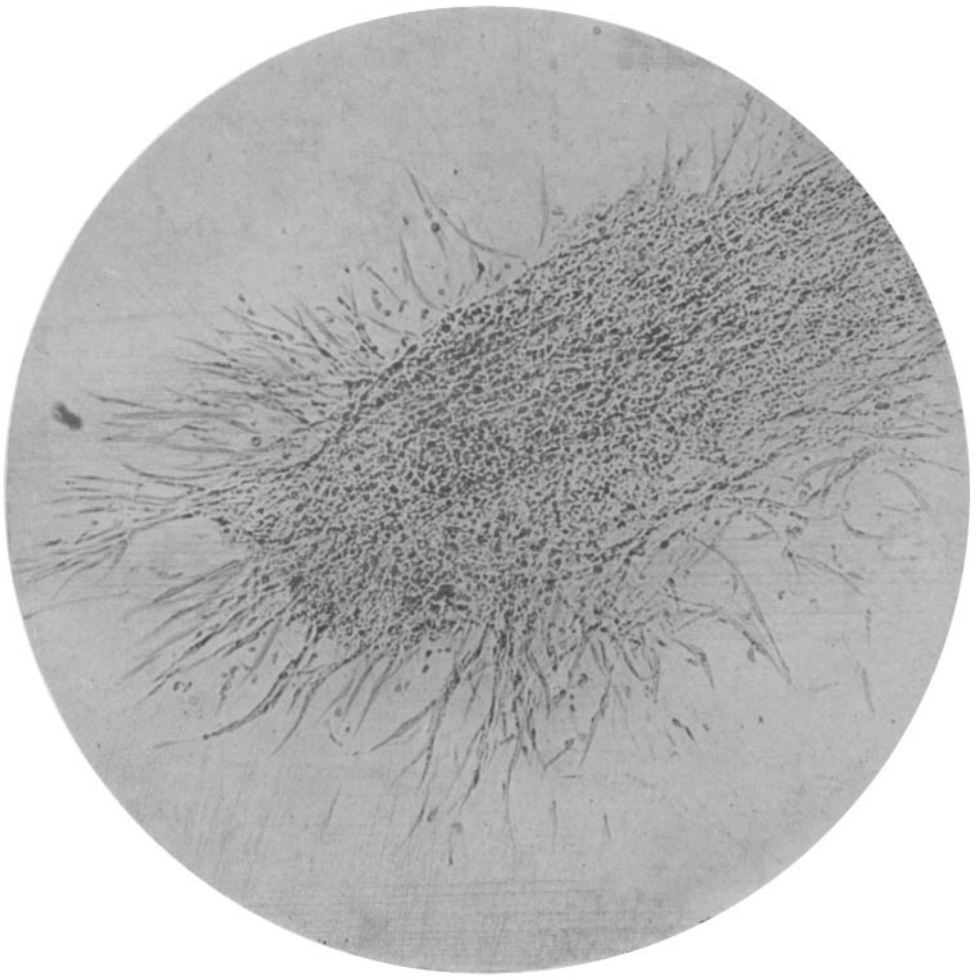

FIG. 2.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)

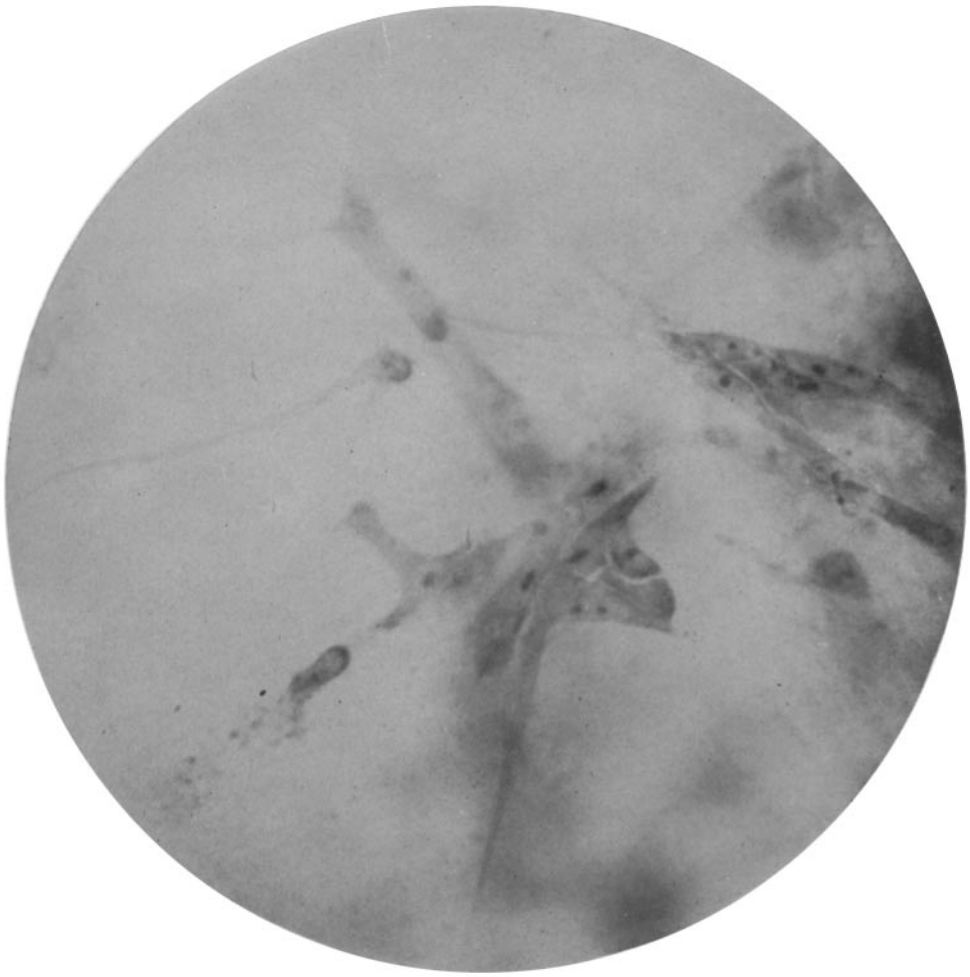

FIG. 3.

(Losee and Ebeling: Cultivation of Human Sarcomatous Tissue *in Vitro*.)