CHARACTERISTICS OF GROWTH OF SARCOMA AND CARCINOMA CULTIVATED IN VITRO.*

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PLATES LXIII–LXXII.

In this article we shall describe the growth of rat and mouse sarcomata and of mouse carcinomata when cultivated *in vitro*. By growth we mean the performance of metabolic functions by the cells and cell multiplication.

Historical.—The essentials of the method of cultivating tissues *in vitro* we owe to R. G. Harrison (1907, 1910), who used lymph obtained from the dorsal lymph sacs of frogs as a medium for the cultivation of the tissues of frog embryos, and especially as a means of stuyding the outgrowth of nerve fibres from nerve cells. The successful work of Harrison is too well known to require detailed mention. M. T. Burrows (1910, 1911), working in Professor Harrison's laboratory at Yale University, greatly improved Harrison's original method by perfecting a technique whereby the tissues of a warm-blooded animal could be cultivated outside the body in the plasma of an animal of the same species. The modifications introduced by Burrows are scarcely second in importance and value to Harrison's original conception.

Several observers (Ljunggren, 1898, Jolly, 1903, Beebe and Ewing, 1906, Carrel, 1910, Volpino, 1911) have shown that tissues from warm-blooded animals may be kept alive for a time by employing as media blood serum, defibrinated blood, ascitic fluid, etc. Carrel and Burrows (1911) point out with justice, we think, the great difference that exists between methods which simply permit the temporary survival of cells outside the body and a method which insures the growth of such cells.

The technique employed by Leo Loeb (1902) in studying the growth of cells in foreign media occupies a position midway between procedures which permit merely the temporary survival of cells outside the body and the method of cultivation of tissues *in vitro*. In a study of the regeneration of epithelium in wounds of the skin, Loeb observed that the growing epithelium penetrated the scab covering the wound, and frequently surrounded in its growth small masses of the scab. This demonstration of the possibility of isolated and independent growth on the part of the epithelium of the skin led him to test the viability and power of growth of pieces of guinea pig skin embedded in small blocks of

* This investigation was conducted under the George Crocker Special Research Fund. Received for publication, March 7, 1911.

coagulated blood serum and agar, kept within the body of the animal. A second very ingenious method used by Loeb consisted in bringing a small undetached flap of the skin of a guinea pig's ear into contact with a piece of agar or coagulated blood-serum embedded in the deeper parts of the ear. By each of these methods Loeb was able to demonstrate that epithelium under such conditions remained alive, penetrated the foreign media, and proliferated. The many interesting details of Loeb's work must be read in his own papers; we wish simply to point out that his experimental methods, considered in their essentials, approximate very closely those of Harrison and Burrows. The defects and limitations of Loeb's methods are obvious; his procedures are in no way comparable with the refined and certain methods elaborated by Harrison and Burrows.

Burrows (1910) studied in Professor Harrison's laboratory the outgrowth of nerve fibers from nerve cells, using the tissues of chick embryos and plasma obtained from adult chickens. He also observed the growth, including ameboid movements and karyokinetic multiplication of mesenchymal tissues. He has recorded in a recent paper (1911) many important observations upon the growing tissues of chick embryos.

Carrel and Burrows (1911) in a series of notes have reported details of the growth of many adult tissues of warm-blooded animals.

Lambert and Hanes (1911) have reported briefly the results of studies upon the growth in vitro of the transplantable sarcomata and carcinomata of rats and mice. In this and the following paper the details of these studies will be presented.

Technique.—In cultivating rat and mouse tumors, we have employed the method of Harrison (1910), as modified and improved by Burrows (1911), with certain minor adaptations rendered necessary by the small size of the animals used. The diminutiveness and delicacy of the carotid artery in mice and young rats offers the chief difficulty in obtaining blood from these animals. The use of a cannula is impracticable. We have employed successfully the following technique: The carotid artery is exposed, cleaned, and ligated distally. A *serre fine* clamp is placed on the artery as far proximally as possible, and with sharp scissors the wall of the vessel is nicked near the distal ligature. The wall of the artery is picked up with fine pointed forceps, the vessel is divided distally, the *serre fine* clamp is removed and the blood allowed to flow into heavy paraffin-coated glass tubes, great care being taken to prevent contamination with tissue juices (activator). The separation of the plasma from the formed elements of the blood and the subsequent preparation of the cultures have recently been described in detail by Carrel and Burrows (1911).

CHARACTERISTICS OF GROWTH IN VITRO OF SARCOMA.

Rat and mouse sarcomata cultivated *in vitro* do not differ essentially in their manner of growth. They conform to a type of growth that would seem to be characteristic of tissues of mesenchymal origin.

If a culture of sarcoma freshly prepared *in vitro* be examined under the microscope, the edges of the piece of tissue are seen to be sharply defined and the tissue is uniformly dense and opaque. Six to twelve hours later the edges of the tissue are thickly beset with projecting cells (figure 1), and many isolated cells with extremely irregular outlines are seen to have passed out into the surrounding plasma. The tissue itself gives the impression of being less compact and more translucent than formerly. The number of cells in the plasma now increases rapidly from hour to hour (figure 2), and concomitantly the original mass of tissue becomes progressively looser in texture, and its edges pass imperceptibly into radial strands of cells lying either in contact with one another or perfectly isolated. The original mass of tissue may become resolved completely into its component cells which wander into the plasma. Serial sections (figure 3) of preparations at this stage of growth confirm very beautifully the details observed in the living cultures.

The passage of cells into the surrounding plasma, and the diminution in bulk and density of the original piece of tissue, constitute the phenomena of growth as observed under the low powers of the microscope. Seen with the unaided eye, growing preparations exhibit a filmy halo surrounding the original mass of tissue. For a closer study of the exact mechanism whereby the cells move into the surrounding plasma, and for the observance of the more intimate structure of the cells, higher powers must be used, and it is necessary to supplement the study of the living tissue by fixed and stained preparations of whole cultures and by serial sections.

The cells which have passed into the plasma, examined with the high power (dry) of the microscope, present two fairly distinct morphological types. One type is long and spindle-shaped (figure 4) with the cytoplasm forming thread-like prolongations from each end, which extend for great distances into the plasma. These attenuated processes usually terminate in several small prickles.

The nucleus lies in the middle of the cell, and is ovoid in outline or distinctly fusiform. The second type of cell, which in actively growing cultures greatly predominates, is characterized by the extreme irregularity of its shape, due to pseudopods which project from the cell in the most bizarre manner (figure 5). If one observes such cells for a time, the pseudopods are seen to undergo alterations in shape. Each pseudopod terminates in several thread-like processes, and these are thrown out and retracted from time to time. Now and then one of these delicate processes enlarges and becomes rounded as the cytoplasm streams into it. By this mechanism the cell alters its spacial relations, wandering further and further from the original tissue. Cells showing active ameboid movements assume a compact spherical form if allowed to cool.

We are inclined to regard these two types of cells as merely morphological variations, and not generically separate types. It is impossible to tell in a growing culture of sarcoma which cells were originally stroma cells and which were sarcomatous. Since, however, all the cells of the implanted tissue may show the phenomena of growth, the question is one of secondary importance. Both stroma and sarcoma cells are of mesenchymal origin, and both probably conform to the same type of growth.

Typical karyokinetic figures are not uncommon in the growing sarcoma cells, though observable only in stained preparations. Cells with two nuclei are frequently seen.

We have emphasized the ameboid wandering of the cells into the plasma. The cells lying compactly massed in the original piece of tissue, with the normal vascular food supply interrupted, are most unfavorably situated for obtaining nutriment. It is in response to the demands for food, we think, that the cells migrate into the plasma, whose fibrin meshwork is filled with an abundance of nutrient material. If this interpretation of the cell migration is correct, the cytoplasm of the cells might conceivably show recognizable signs of active metabolism. This seems to be the case. The cytoplasm of migrating cells (figure 4) exhibits very early small highly refractile droplets, which stain brilliantly with specific fat stains, such as Sudan III and Scharlach R., and otherwise respond to all the tests for neutral fats. These fat droplets increase in num-

ber with the age of the cell, until the cytoplasm is often literally packed with them. They show little tendency to fuse into large droplets, and flow with the cytoplasm into newly formed pseudopods, recalling the passive movements of foreign inclusions in amebae.

We do not, for a moment, regard these granules of fat as an evidence of degeneration, as has been suggested; they are rather the strongest proof, we think, of active anabolic function on the part of the cell. They are synthesized by the cell from the soluble elements of neutral fat present in the plasma; fat granules can not be demonstrated in the plasma itself. Cells containing numerous fat granules show active ameboid movements (figure 6) and undergo karyokinetic division. These are evidences of vitality which do not harmonize with the view that the fat is a result of "fatty degeneration" of the cytoplasm. The accumulation of fat in the cells indicates an impairment of katabolic activity through influences which are easily understood. Far from being strange or new, this process of fat accumulation, or infiltration, is seen frequently throughout the cells of the body.

In another place (1911a), we have pointed out that the power of independent ameboid movement shown by growing tumor cells offers a very reasonable explanation of the infiltrative growth of malignant tumors. The cells of a rapidly growing tumor are soon placed in a position analogous, from the standpoint of food supply, to that of a mass of tumor cells embedded in plasma. The nutritional demands of the cells may be regarded as a stimulus which results in an invasion by ameboid movement of surrounding tissues, lymph-spaces, lymph-vessels, and blood-vessels.

When suitable preparations of growing sarcoma cells are properly fixed and stained, another type of granule is seen (figure 6). These are the cell granules of Altmann. We can not enter at this time into a detailed discussion of these granules, since a proper presentation of the subject would necessitate a review of the rather specialized literature dealing with the granular structure of protoplasm. The reader whose interest leads him in this direction will find in the first volume of Martin Heidenhain's recent work, "Plasma und Zelle" a full critique of the subject. We accord to these gran-

ules more than passing mention because Beckton (1909) has stated that their absence from tumor cells is a characteristic of malignancy. Bensley (1910) has shown that Beckton is wrong with regard to human malignant tumors; the cells do contain Altmann's granules, and even in increased numbers as compared with the normal cells of the tissue from which the tumor arose. Using Altmann's fixative and stain, we have demonstrated abundant protoplasmic granules in the cells of mouse and rat sarcomata, both when growing in the animal and when cultivated *in vitro*. Furthermore, we have been able to demonstrate these granules in the living cell by a method of intra-vital staining which will now be described.

If finely powdered carmin be added to the uncoagulated plasma used for cultivating sarcoma cells, these cells take up the granules in a very actively phagocytic manner (figure 7). The carmin is partially dissolved within the cell, and the cell granules are thereby stained. This process takes place in living cells showing ameboid movements and phagocytic activity, so we can not doubt that here we have a vital demonstration of the cell granules which are also demonstrable by special methods of fixing and staining.

There is a variation in the manner of growth *in vitro* of sarcomatous tissue, which occurs so frequently and presents such well-defined characteristics that it demands description. This is the ring form of growth (figure 8). In this type, the growing cells do not spread uniformly in all directions from the central mass of tissue, but the plasma retracts from the tissue in an ovoid or circular fashion, leaving the tissue situated at one point of the circumference, like the setting in a signet ring. The growing cells are found to have spread out in a one-celled layer in a thin film of plasma which remains adherent to the cover glass after the retraction of the plasma, and also to have massed themselves in the edge of the plasma ring. From this secondary deposit of cells, ameboid wandering takes place into the plasma. If the remainder of the original piece of tissue be removed, and the space bounded by the ring-like deposit of cells be filled with fresh plasma, cells wander into this new supply of medium from the inner surface of the ring. Similar ring-like growths of mesenchymal tissue have been described by Harrison (1910) and Burrows (1911). Such a form of growth

might be explained by the action of a proteolytic ferment on the fibrin of the plasma, allowing the contracting plasma to retract from the embedded tissue. This ring form of growth is almost the rule when mouse sarcoma is cultivated in rat plasma. We have found the central, one-celled layer of growth exquisitely adapted to finer cytological studies.

CHARACTERISTICS OF GROWTH IN VITRO OF MOUSE CARCINOMA.

Mouse carcinoma cultivated under the same conditions as sarcoma, grows with equal readiness, although the details of growth are very different. After twenty-four hours of growth *in vitro* a culture of carcinoma presents the following appearance: The original piece of tissue has become very much thinner and more translucent, due to a spreading out of the cells on all sides. The cells, however, do not migrate into the plasma separately, as do sarcoma cells, but move in a continuous sheet or layer, which is only one cell in thickness (figure 9). The advancing edge of the growth is composed of a very delicate sheet of cytoplasm, which shows ameboid changes of form with numerous pseudopods (figure 10). The boundaries of the cells composing the sheet of growing carcinoma can not be seen with clearness, even in stained preparations. There is a heaping up of the cells just behind the advancing hyaline fringe of protoplasm (figure 9). As the cells grow out from the original mass, their nuclei increase in area, as does the cytoplasm, so that they present the picture of very large nuclei lying rather widely separated by cytoplasm.

Growing carcinoma cells, like sarcoma cells, fill themselves rather rapidly with fat granules. They also take up phagocytically carmin granules placed in the plasma, as do sarcoma cells.

Karyokinetic figures (figure 10) are frequently observed; we have seen them in preparations as old as five days.

Carcinomata are composed of epithelial cells lying in a stroma of connective tissue. We have pointed out that these two varieties of tissue vary characteristically in their manner of growth *in vitro*. It should therefore be possible to distinguish without difficulty the growth of stroma from that of carcinoma. Some of our preparations show very clearly a mixture of these two types of growth (figure 11).

CONCLUSIONS.

1. The transplantable sarcomata of rats and mice grow very readily by the method of cultivating tissues *in vitro*.

2. Sarcomatous tissue grows in conformity to a type which may be regarded as characteristic for tissues of mesenchymal origin.

3. The growth of sarcoma cells *in vitro* consists in ameboid wandering into the surrounding plasma, karyokinetic proliferation, and evidences of active metabolism on the part of the cells.

4. Mouse carcinomata can be cultivated *in vitro*. The outgrowth of carcinoma cells assumes a sheet-like form, only one cell in thickness. They migrate into the plasma by ameboid movement, the advancing edge showing numerous prolongations of the cytoplasm into pseudopods.

5. Karyokinetic figures are frequently seen in growing carcinoma cells. The cells show evidences of active metabolism.

6. Both sarcoma and carcinoma cells cultivated *in vitro* show active phagocytosis; carmin particles placed in the plasma are taken up rapidly by the growing cells.

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EXPLANATION OF PLATES.

PLATE LXIII.

FIG. 1. Eighteen hour culture of rat sarcoma, stained with iron hematoxylin and Sudan III.

PLATE LXIV.

FIG. 2. Four day culture of rat sarcoma showing the ring form of growth, stained with iron hematoxylin and Sudan III. The ameboid wandering of the cells into the plasma is well shown.

PLATE LXV.

FIG. 3. Horizontal section of a thirty-six hour culture of a rat sarcoma stained with hematoxylin and eosin. Cells have wandered from the original piece of tissue into the coagulated plasma, the fibrin meshwork of which is seen.

PLATE LXVI.

FIG. 4. High power detail from the preparation illustrated in figure 1. The cells contain numerous granules of fat.

FIG. 5. Sarcoma cells growing *in vitro,* showing many pseudopods.

PLATE LXVII.

FIG. 6. × 1,000. Altmann's fixative and stain. Two mouse sarcoma cells are shown. The fat granules are stained black by osmic acid; the protoplasmic granules are colored with fuchsin. The fat granules are discrete and are of the same size as the protoplasmic granules.

PLATE LXVIII.

FIG. 7. × 1,000. Mouse sarcoma cells. The cells marked A and B contain carmin particles which they have taken up from powdered carmin added to the plasma. In cells C and D the protoplasmic granules have been colored by the carmin. All the cells exhibit vacuoles which in the living cell contained fat; the fat has been dissolved in the process of clearing the preparation.

PLATE LXIX.

FIG. 8. Three day culture of rat sarcoma showing the ring form of growth, stained with iron hematoxylin and Sudan III.

PLATE LXX.

FIG. 9. Five day culture of mouse carcinoma, stained with iron hematoxylin and Sudan III. The cells have migrated into the plasma in a sheet which is only one cell in thickness, except in the advancing edge, where there is a heaping up of cells. The hyaline ameboid fringe of cytoplasm is seen very clearly.

PLATE LXXI.

FIG. 10. × 1,000. A high power drawing of the edge of a four day culture of mouse carcinoma, stained with iron hematoxylin and Sudan III, showing the prolongation of the cytoplasm into pseudopods. One cell contains a karyokinetic figure. Numerous granules of fat are seen in the cytoplasm. The boundaries of the cells are very indistinct, in many places indiscernible.

PLATE LXXII.

FIG. 11. Several small groups of carcinoma cells, stained with iron hematoxylin and Sudan III, are shown, which have grown from a small detached piece of carcinomatous tissue. The growth of the carcinoma cells simulates the alveolar arrangement of carcinomatous growths in the body. The larger piece of implanted tissue is shown as a darkly stained area at one side. From this larger piece, many small, isolated cells have wandered into the plasma. These small ameboid wandering cells conform to the type of growth which we have described as characteristic of mesenchymal tissue. They are in all probability stroma cells.

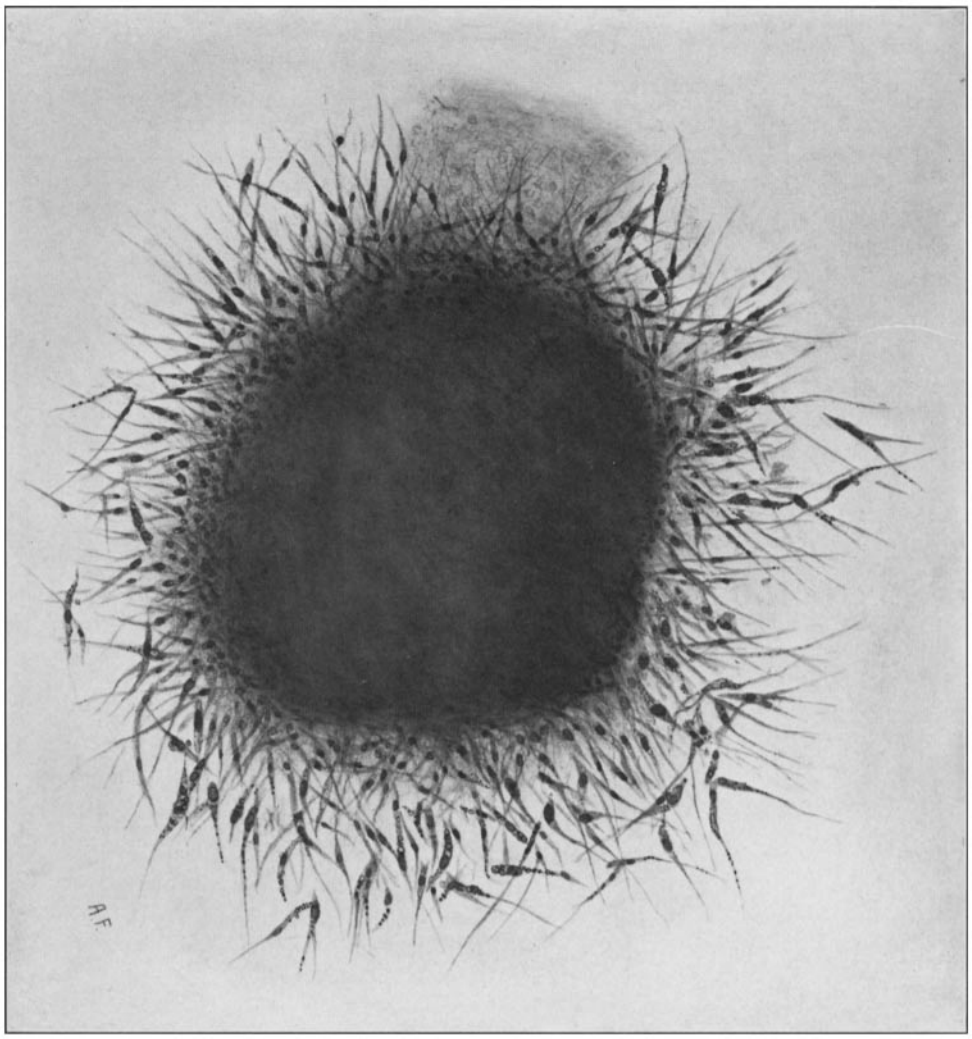


FIG. 1.

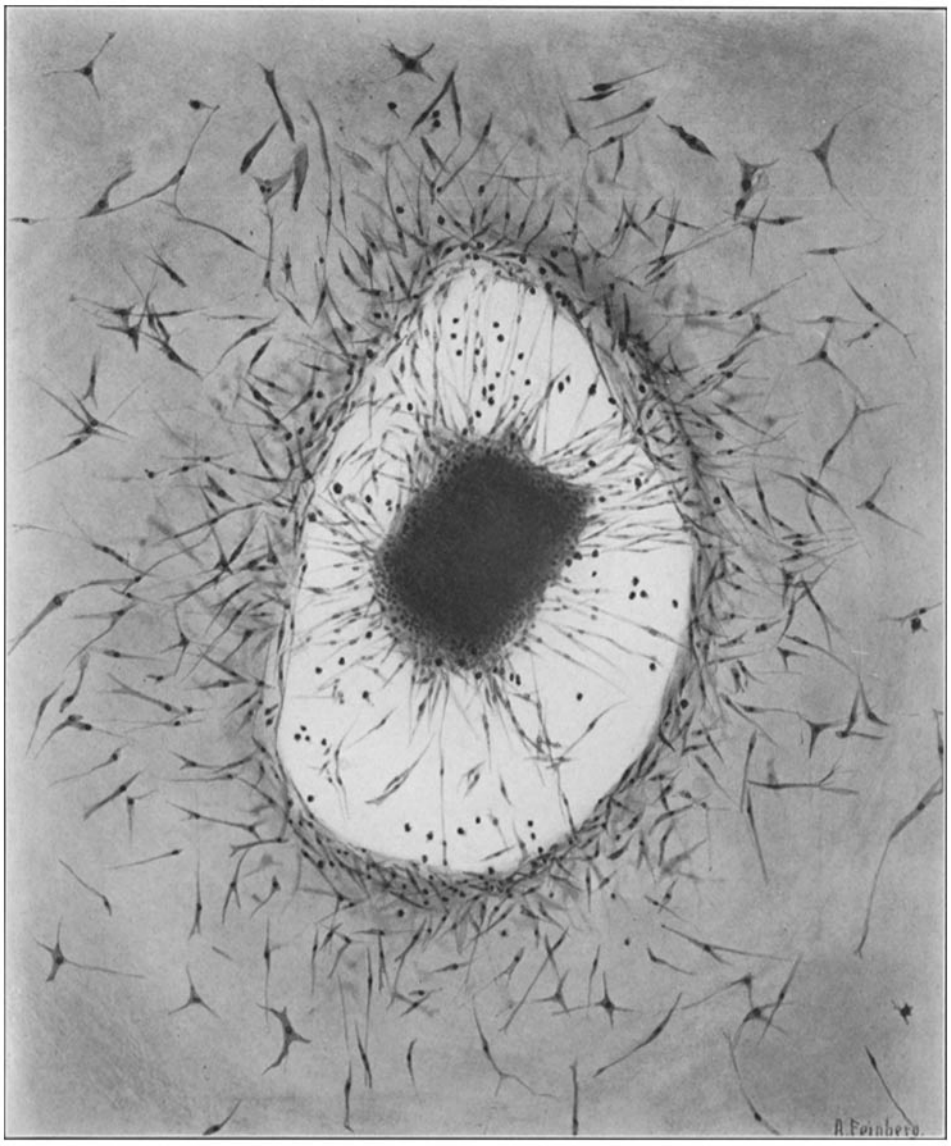
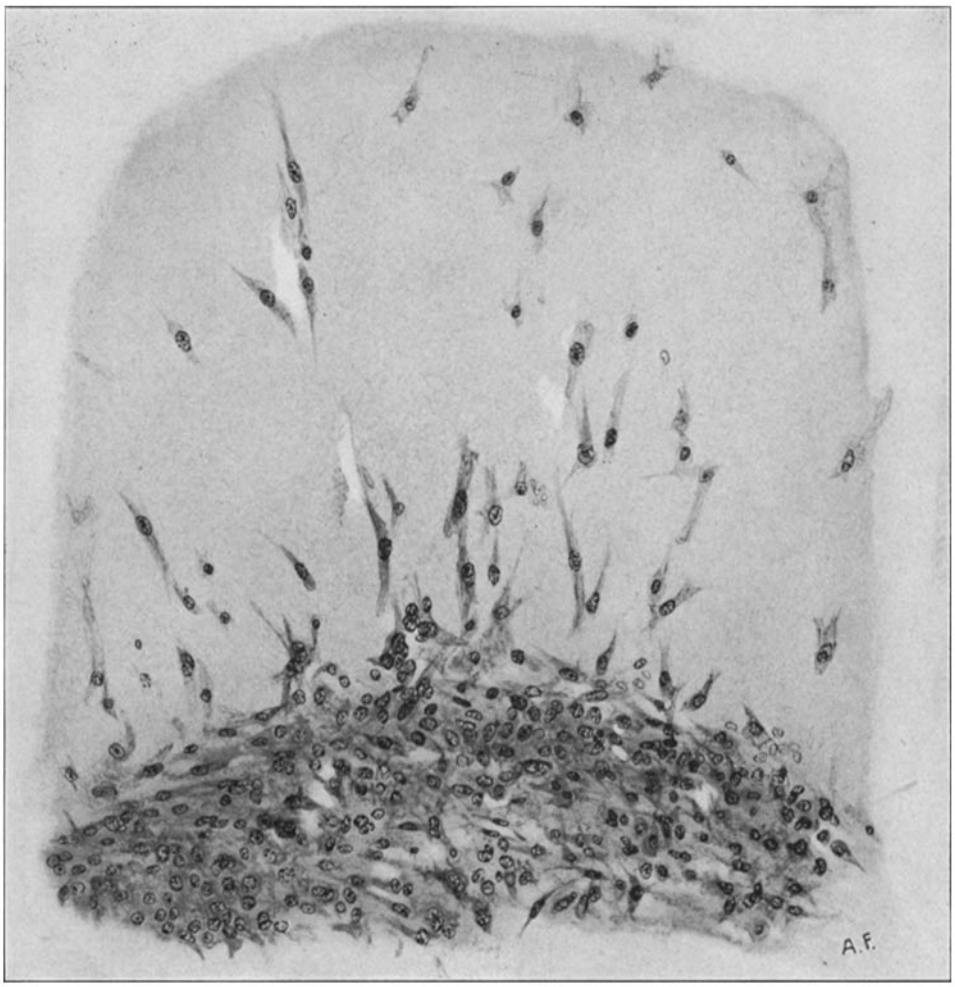


FIG. 2.



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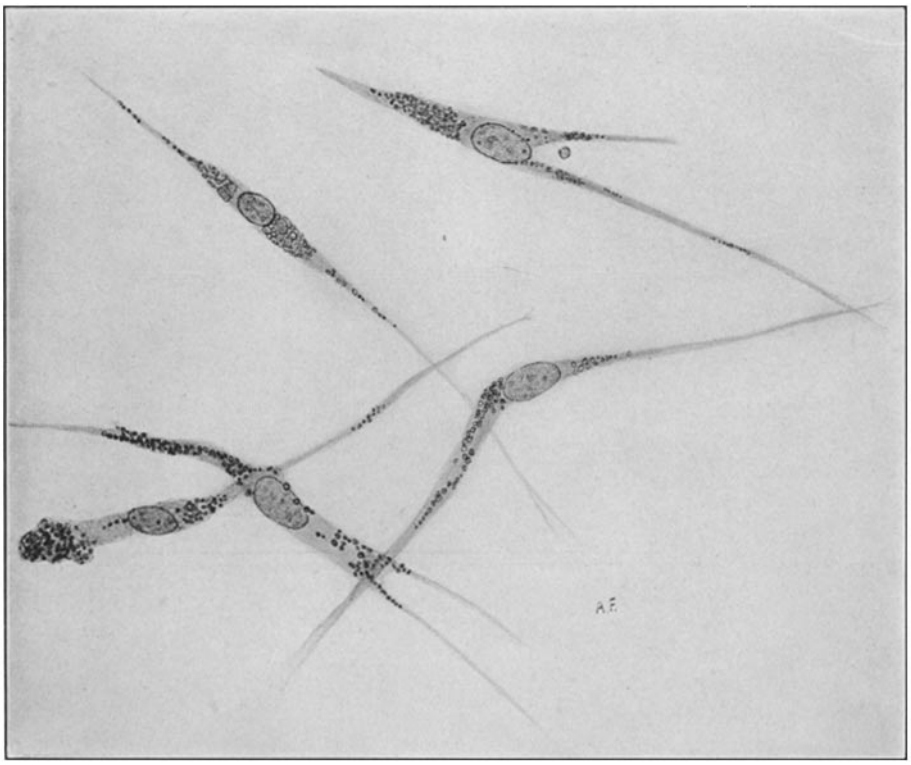

FIG. 4.

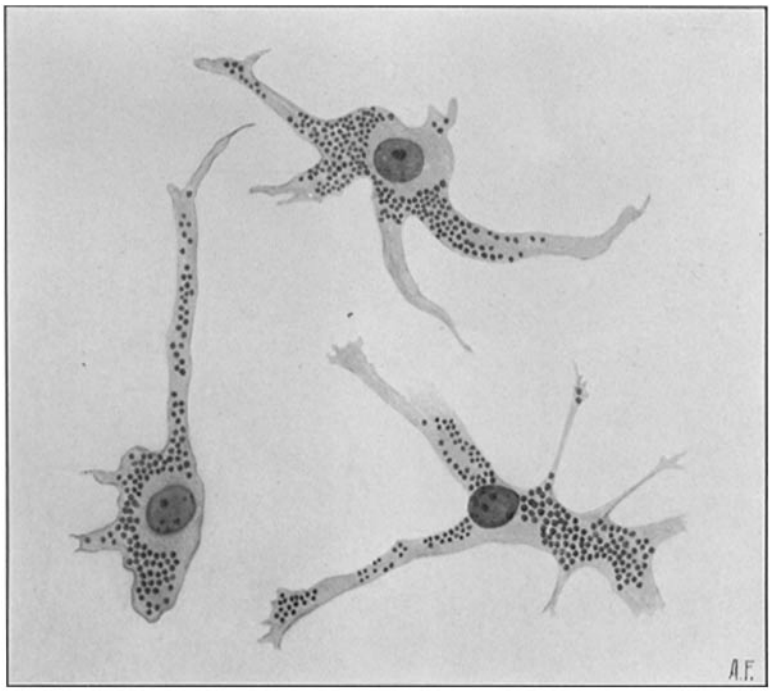

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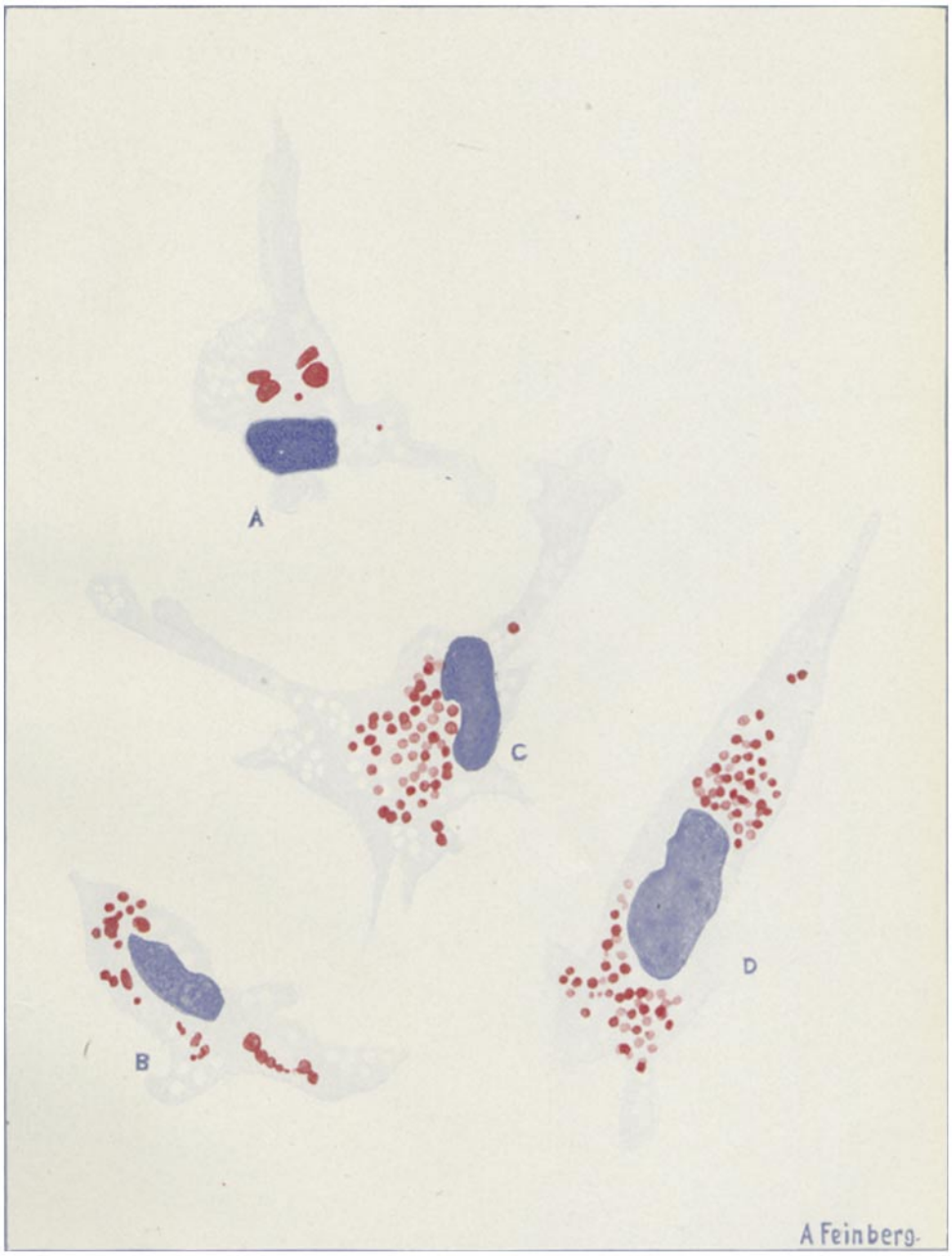
FIG. 6



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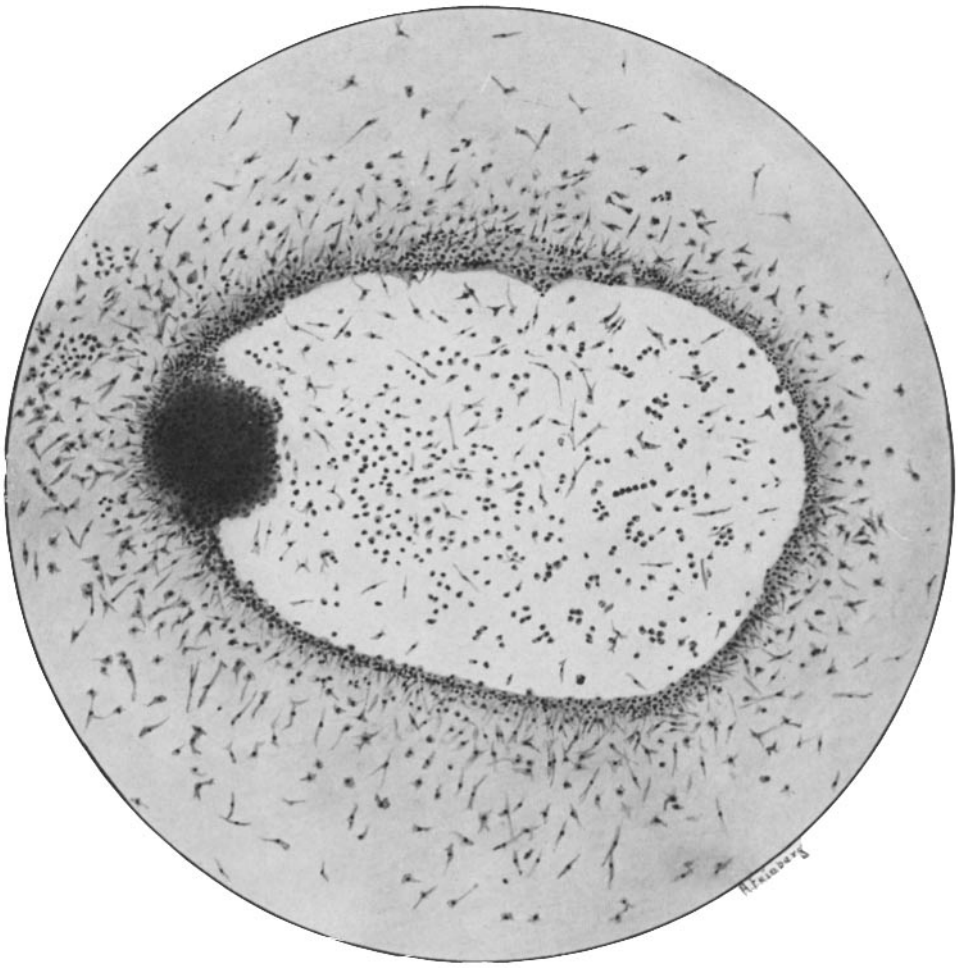

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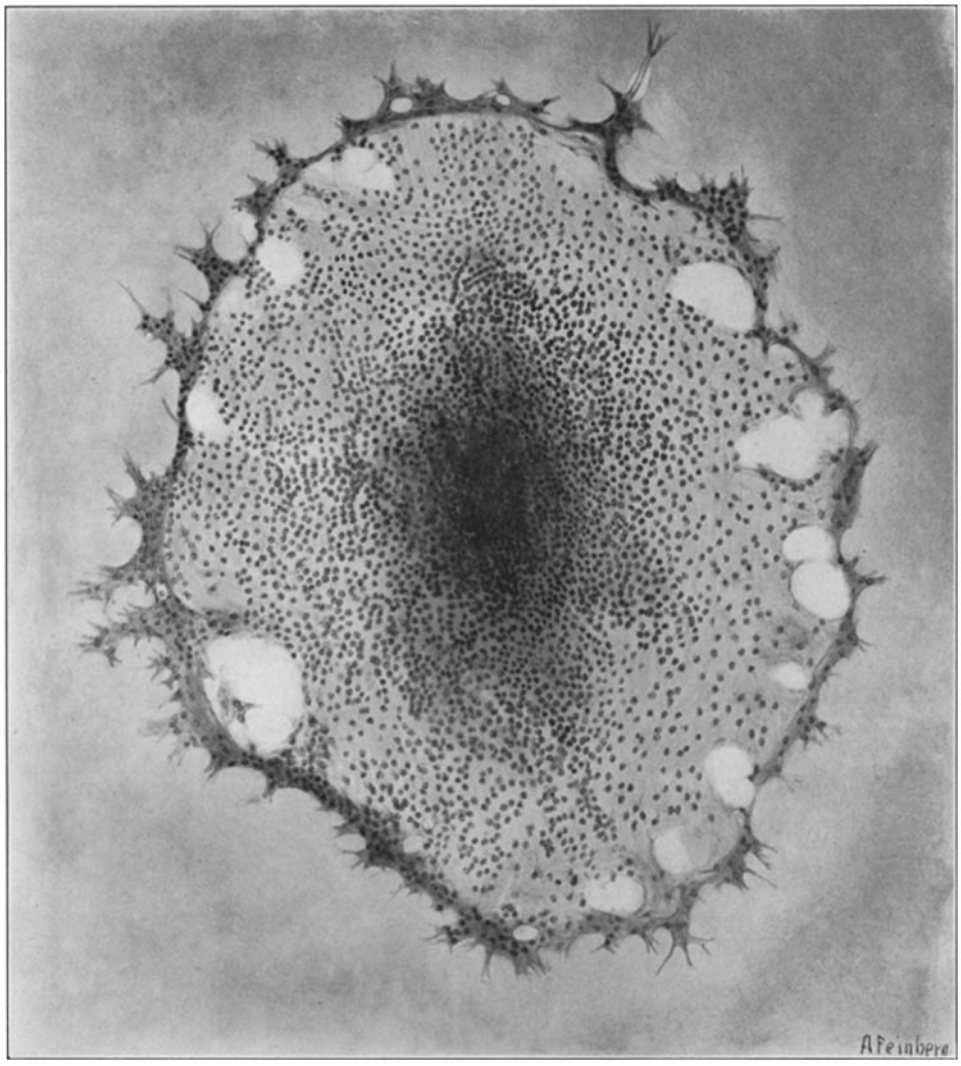

FIG. 9.

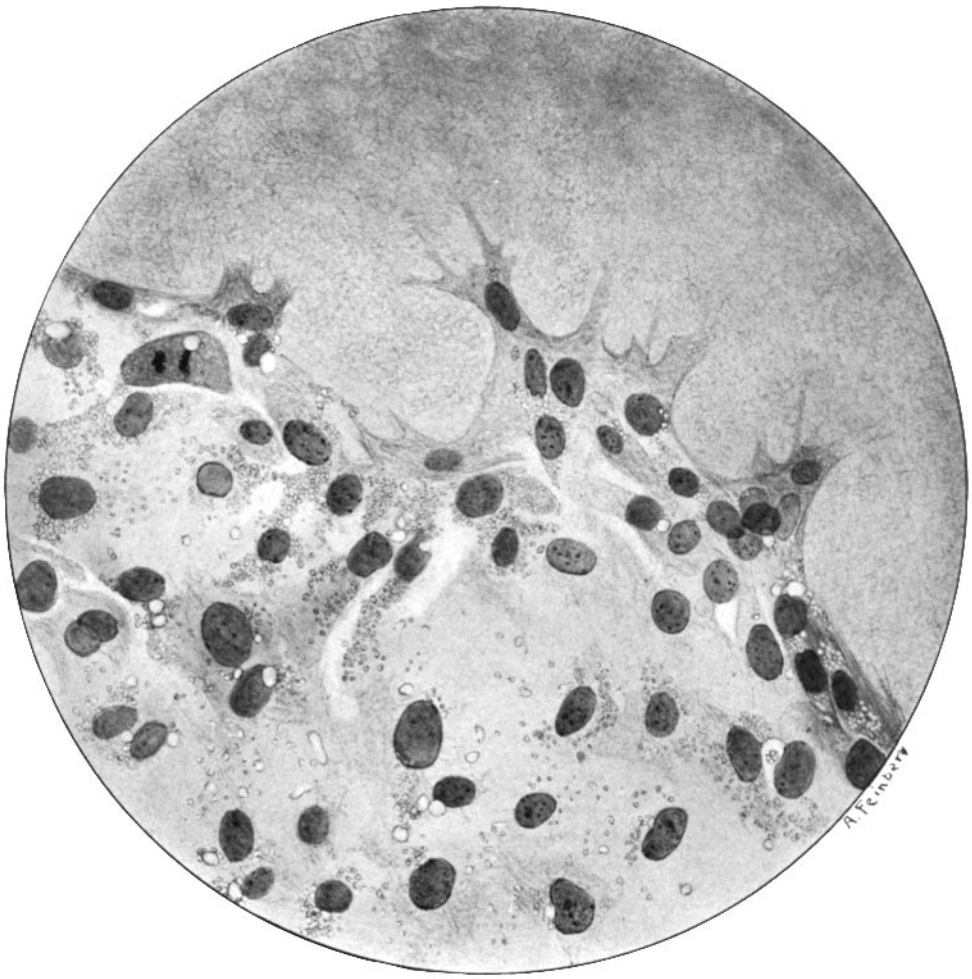

FIG. 10.

FIG. 11.