STUDIES UPON THE CHARACTERISTICS OF DIFFERENT CULTURE MEDIA AND THEIR INFLUENCE UPON THE GROWTH OF TISSUE OUTSIDE OF THE ORGANISM.*

By RAGNVALD INGEBRIGTSEN, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

PLATES 45–54.

In a previous article[1] I have called attention to the fact that heat exerts a marked influence upon serum when used as a culture medium. This was observed in cultures in serum agar of the bone marrow of adult guinea pigs. It was supposed that the influence which the heating of the serum exerted upon its value as a culture medium and upon the growth of tissue in it was indicated truly by the rapidity of the emigration of ameboid cells from the piece of tissue. I did not know then whether these cells really multiplied, or whether they only migrated. In order to reach conclusive results it was necessary to carry these experiments further.

The experiments, the results of which I shall give in this paper, were planned to investigate thoroughly the influence of heat upon serum as a culture medium, taking into consideration at the same time the value of different media and the conditions necessary for the growth *in vitro* of adult and embryonic tissue.

To distinguish between emigration and multiplication of cells in a culture may be very difficult. *In vitro* the lymphoid cells show ameboid movements, but motility is not at all limited to these cells, for it may be manifested by every connective tissue cell, even the highly differentiated cells of the cornea. The movements of the connective tissue cells, however, are very slow compared with the rapid movements of the ameboid lymphoid cells, which under the

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[1] Ingebrigtsen, R., *Jour. Exper. Med.*, 1912, xv, 397.

microscope may be seen to change from second to second. Since the first stage of growth is very often only an emigration, it is not permissible to conclude that all the cells that are seen surrounding the original fragment represent a growth of the latter.

Direct observation under the microscope of cells that are dividing is proof, of course, that they multiply, but to make these observations great patience is required, and even with patience one may fail to see cells that are dividing. Another evidence of growth is the occurrence of mitotic figures in emigrated cells that divide indirectly. But for cells that divide amitotically, such as leucocytes, this criterion cannot be used. Mitotic figures found in the original piece itself by making sections of it, as Oppel[2] did, do not prove that there is growth outside of the organism, for these figures might, of course, be present before the fragment was removed and transplanted to the plasma.

Real growth *in vitro* cannot be acknowledged as proved unless a new tissue that grows from the periphery forms around the original fragment.

This is most easily observed in cultures of certain epithelial organs (hypophysis, thyroid), which form *in vitro* strands, columns, and arches of cells that lie close together, the basal cells of which are inactive and show but little alteration, while the peripheral cells change and multiply continually. The same observation may be made in growing connective tissue (endothelial cells) (figure 13), but here it is not so marked nor so easily confirmed, because the connective tissue cells have motility and are not so liable to keep close together or to form sheets of tissues.

Such a newly formed and growing tissue proves beyond doubt that multiplication of cells has occurred, and the mitotic figures present in this tissue afford additional evidence of growth.

In these experiments I have cultivated adult and embryonic tissues in different media, that is, in autogenic and homogenic plasma, homogenic serum (heated and unheated) plus 2 per cent. agar, homogenic serum, Ringer's solution plus 2 per cent. agar, and in Ringer's solution alone.

[2] Oppel, A., *Anat. Anz.*, 1912, xl, 464.

The plasma cultures were prepared in the ordinary way described by Carrel and Burrows.[3]

The cultures in serum and agar and Ringer's solution plus agar were prepared according to the technique described by me in another article.[4]

Cultivation of tissue in serum and in Ringer's solution was carried out in drops hanging from the under surface of cover-slips that were sealed to hollow slides, care being taken that the amount of fluid surrounding each piece of tissue covered it in a very thin film.

Adult Tissue.—From several adult cats, rabbits, and guinea pigs, I cultivated spleen, bone marrow, hypophysis, and thyroid gland. Real growth, that is formation of a new tissue around the original fragment was obtained in practically all experiments when the tissues were cultivated in plasma. Morphologically the organs behaved almost exactly alike whether taken from cats, rabbits, or guinea pigs.

From thyroid and hypophysis there appeared on the second and third days solid columns of epithelial cells that represented in all probability the specific glandular cells of these organs (figures 16 and 24); in some cases long spindle connective tissue cells (figure 15) grew out and the presence of these cells apparently prevented the development of the epithelial structures.

In the first stages of growth of spleen and bone marrow there are always emigrations of ameboid cells, which start moving one or two hours after the beginning of the incubation of the culture. On the third day the ordinary connective tissue cells begin to grow and, after incubation for five or six days, form a new connective tissue that surrounds the original fragment.

While in plasma all the organs of the adult animals examined give rise to real growth, usually of their specific cells, but sometimes only of the connective tissue cells, in not one case have I found real growth of the organs of adult animals when cultivated in the other media mentioned; that is, in Ringer's solution, in Ringer's solution plus 2 per cent. agar, in serum, and in serum plus 2 per cent. agar.

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

[4] Ingebrigsten, R., *loc. cit.*

In these media the emigration of cells is very often seen, and from lymphatic tissue (bone marrow) in serum plus agar this emigration is very rapid. But the formation of new tissues has not been observed.

Embryonic Tissue.—It is quite different, however, with embryonic tissue which grows well in plasma, but just as well and even better sometimes in some of the other media.

Tissues from chick and cat embryo were examined. The cat fetus was almost at full term when taken from the uterus of the mother. The chick embryos used were fifteen to sixteen days old.

Peritoneum, skin, liver, and thyroid of the fetal cat grew very well in serum agar. They started to grow on the first day and formed around the original fragment during the following two or three days an extensive area of new tissue (epithelial cells from the thyroid, liver, and skin, and connective tissue cells from the peritoneum) (figures 13, 14, 17, 18, 21, and 22). In serum alone these tissues grow very little, a few connective tissue cells only spreading along the cover-glass.

In Ringer's solution and in Ringer's solution plus agar they did not grow at all.

Heart, spleen, skin, and liver were cultivated from the chick embryos fifteen to sixteen days old. These organs grew very well in serum plus agar.

The epithelial cells of the liver and skin grew much more extensively and vigorously in chick serum plus agar than in chick plasma (figures 17 and 18). In serum agar they grew usually in one layer between the serum agar clot and the cover-glass. From the heart and spleen of chick embryos connective tissue cells developed actively in serum agar (figures 1 to 12), but not as well as in plasma. After three to five days the spleen cultivated in plasma showed a wider growth of elongated connective tissue cells, and the area of emigrated cells was larger and denser in the plasma than in the serum agar (figures 11 and 12). Chick heart in plasma gave rise to a dense framework of connective tissue that surrounded the fragment evenly. In serum plus agar the same kind of cells developed but on a smaller scale.

In serum, connective tissue from chick heart grew rather exten-

sively along the cover-glass in a thin layer, but it did not grow so well as in serum agar. In serum, chick liver and spleen showed an emigration of cells, but no growth.

In Ringer's solution and in Ringer's solution plus agar some slight emigration of cells occurred from chick spleen, but these cells lost their ameboid movements within the first twenty-four hours, while the same kind of cells, emigrated from spleen in serum, showed ameboid movements for at least three days (figures 7 and 8). In Ringer's solution and in Ringer's solution plus agar, no growth of the cells from the sixteen day chick embryo has been observed.

I wish to emphasize in this place the differences found between adult and embryonic tissues. The former does not grow at all in any medium but plasma, while embryonic tissue develops vigorously also in serum plus agar.

Furthermore, in Ringer's solution and in Ringer's solution plus agar, I found no growth of the cells from the fifteen to sixteen day old chick embryos.

Even within the embryonic stage there are apparently great differences in the energy of proliferation of cells of different ages. The energy of growth *in vitro* decreases as the age of the embryo increases, and the culture experiments that succeed when performed with a chick embryo that is eight days old may be completely unsuccessful with a chick embryo sixteen days old.

Thus, in an extensive series of experiments, Lewis and Lewis[5] have cultivated several tissues of chick embryo in various salt solutions. They generally used chick embryos from seven to nine days old, and in some cases embryos that were thirteen to fifteen days old. In their experiments they observed emigration of cells as well as formation of syncytial membranes. Many of their illustrations are rather convincing as to the presence of multiplication among the cells. In a few experiments in which I cultivated in Ringer's solution connective tissue from chick embryos that were six to eight days old, I had corresponding pictures.

Lewis and Lewis, however, express it as their conviction that the

[5] Lewis, M. R., and Lewis, W. H., *Anat. Rec.*, 1911, v, 277.

salt solutions utilized by them were protective and not nutrient in action, and this view is certainly correct.

Of the cultures that they prepared, the percentage that was positive decreased as the age of the embryos utilized increased. This varying percentage shows that the very young embryonic tissue has such a tremendous energy of proliferation and is so little dependent on the constituents of the media in which the cells live that they must be considered as constituting a peculiar sort of tissue. The limits within which these salt solutions of Lewis may be varied quantitatively and qualitatively are so extensive, that they cannot be supposed to be of great importance in the metabolism of these cells. By their tremendous energy of proliferation they grow perhaps in spite of the media instead of because of them, and, as Lewis and Lewis themselves say, they grow within the limits determined by the amount of food stored up in the body of each individual cell.

They have, therefore, a comparatively short existence in salt solution, while in nutrient media they live much longer and, as shown by Carrel,[6] may even be kept in permanent life. There is, therefore, a fundamental difference between the life of very young embryonic tissue in salt solution (a preservative medium) and in plasma (a nutrient medium). If we wish to realize the relationship between these two media, we may compare the growth in salt solution with the pale and whitish buds that grow out of potatoes in the spring, if they are placed in a dark and damp cellar, while the growth in plasma and serum agar resembles the powerful, young green leaves that sprout out above the surface of the soil when the potato is permitted to draw energy and nutrition from light, air, and ground. The life and growth of these young embryonic cells in salt solution can hardly be called a culture in the true sense of this word.

Influence of Heat. Homogenic Serum.—In order to ascertain whether the influence of heat exerted upon serum is of importance, not only for the emigration and survival of ameboid cells but also for the multiplication and the growth of tissue cells, it was necessary to cultivate embryonic tissue in heated and unheated serum, because adult tissues do not grow at all in sera.

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

Spleen, liver, and skin of chick and cat embryos have been cultivated in heated and unheated homogenic serum plus agar. Spleen of the fetal cat gave only a small growth in serum agar, and was, therefore, not suitable for my purpose. Spleen of a sixteen day chick embryo, on the other hand, grew very well in serum agar and was well adapted for comparative experiments. I found that the growth in unheated serum agar was quantitatively different from that in heated serum plus agar. In the former there appeared after two and three days several elongated cells that formed around the fragment a "corona radiata" that was very similar to that in plasma, while these cells did not develop in heated serum plus agar (figures 10 and 11). In the latter the area of emigrated cells was smaller than in unheated serum plus agar. In these experiments the chick serum had been heated to 52° to 53° C. only. Chick serum cannot be heated beyond that temperature without the occurrence of a precipitation with a subsequent deficit of proteins, and probably salts, in the fluid. The maintenance of the percentage of proteins was, of course, necessary.

The cultivation of epithelial tissues from cat and chick embryo in heated and unheated homogenic serum agar has not given the results that might be expected from the results obtained with chick spleen. In many of the experiments, liver and skin grew better in unheated than in heated serum agar, but in some cases they have grown as well, and in a few cases even slightly better in heated than in unheated serum (cat serum was heated to 58° to 59° C. for half an hour and chick serum heated to 52° C. for half an hour).

The epithelial cells of liver and skin grew very quickly and extensively in one or two layers between the nutrient clot and the coverglass. The epithelial cells were possibly more dependent upon mere mechanical and physical conditions, as, for example, the space available between the clot and the glass, and the degree of adhesion between these two structures, than were the connective tissue cells, which grew in many different layers through all the surrounding medium and therefore responded more quickly than the epithelial cells to changes in the medium, whatever these might be.

This possibly explains also why embryonic epithelial cells grow better in serum agar than in plasma; for the epithelial cells have a

tendency to spread rapidly along a smooth surface and find better conditions between the serum agar clot and the cover-glass than between the plasma clot and the glass, since the plasma adheres very closely to the glass.

Heterogenic Serum.—In agar plus serum from cats, dogs, and rabbits, I have cultivated in these experiments the heart of a sixteen day old chick embryo.

In the plasma from cats, dogs, and rabbits, a piece of chick heart gave rise to the growth of a connective tissue framework that surrounded the fragment evenly but not densely. In serum agar from these same animals a piece of chick heart grew somewhat differently, a thin layer of spindle connective tissue cells spreading from the fragment as a sheet of tissue or growing out in small bushes or bundles.

A comparison of the quantitative growth can easily be made. I found when comparing the growth of chick heart in unheated and heated heterogenic sera plus agar that heated heterogenic serum is a better culture medium than unheated. On the third day all the cultures examined showed more extensive growth in heated serum plus agar than in unheated serum, and the cells of the former were in a better condition.

Sera of dogs, cats, and rabbits are all hemolytic for the red blood corpuscles of chickens, and it is to be expected that the improvement as a culture medium, obtained by heating the serum, is due to the destruction of its hemolytic powers. Whether this is the whole explanation or whether there are other factors to be considered (specific cytolysins), further experiments will perhaps reveal.

In several series of experiments I have determined the relative hemolytic power of heterogenic sera against chicken and guinea pig blood cells, and then, by preparing cultures from the tissues of the animal that supplied the blood cells in the hemolysis experiments (chicken and guinea pig), I have tried to determine whether there is an inverse ratio between the hemolytic power of a heterogenic serum and the rate of growth in it. Plasma was also used.

Chick heart cultivated in agar plus serum from cats, dogs, and rabbits, after the relative hemolytic powers of these sera for

chick red cells had been determined to be 7.7 : 7.1 : 2.0, showed that the areas of growth, measured by the micrometer on the third day in the three sera had the relationship of 3 : 5 : 12. The same inverse ratio between the hemolytic powers of heterogenic serum and the growth in it was found when guinea pig spleen and bone marrow were cultivated in agar plus serum of rabbits, dogs, and cats. The limits of the growth in these cases were not determinable with exactness, and I am, therefore, unable to give figures.

In heterogenic plasma, on the other hand, this inverse ratio between the hemolytic power and the extent of the growth in it was not constant. The growth of chick heart in cat plasma, for instance, is better than in dog plasma, even if the relative hemolytic power of cat serum and dog serum for chick blood cells is indicated by 7.7 : 7.1.

This seems to indicate that the character of the fibrin framework in the plasma clot is of some importance for the growth of cells in it, and that perhaps the structure of the fibrin framework is able to modify the growth even as much as chemical and biological differences are able to.

I have, therefore, started a study of the fibrin framework in different plasmas. The results of these studies are not yet conclusive, but I wish to state that small differences between the characters of the framework of plasma from different species are present. A detailed report of these investigations will be given in another article.

CONCLUSIONS.

1. There is a great difference between embryonic and adult tissue as far as their growth outside of the organism is concerned. Adult tissue grows only in plasma. Embryonic tissue grows also very well in serum and serum plus agar. In Ringer's solution and in Ringer's solution plus agar no growth occurs, whether embryonic or adult tissue is employed; survival and emigration of cells are seen to some extent.

2. For the growth of connective tissue cells of chick embryo, unheated homogenic serum is a better culture medium than heated serum. The growth of epithelial cells is not thus influenced.

3. Heated heterogenic serum is a better culture medium for growth of embryonic connective tissue cells than unheated.

4. There is an inverse ratio between the hemolytic power of heterogenic sera and the extent of growth of tissue in them. This inverse ratio is not found in heterogenic plasmas.

EXPLANATION OF PLATES.

PLATE 45.

Camera lucida drawings made on the third day of incubation of cultures in different media of the spleen of a sixteen day chick embryo. The drawings show the relative extent of the emigration of cells and the growth of connective tissue cells. Growth of these cells occurred only in figures 5 and 6 and is indicated in these figures by the *dark* grey area surrounding the black central spot (the original piece of tissue). The *light* grey area represents the emigrated cells.

FIG. 1. Chick spleen in Ringer's solution.
FIG. 2. Chick spleen in Ringer's solution plus 2 per cent. agar.
FIG. 3. Chick spleen in serum.
FIG. 4. Chick spleen in heated serum plus 2 per cent. agar.
FIG. 5. Chick spleen in unheated serum plus 2 per cent. agar.
FIG. 6. Chick spleen in plasma.

PLATE 46.

Photograph of cells that had emigrated and grown out of pieces of spleen of a sixteen day old chick embryo. The pieces had been incubated in different media for three days. The relative extent of the growth is shown in figures 1 to 6.

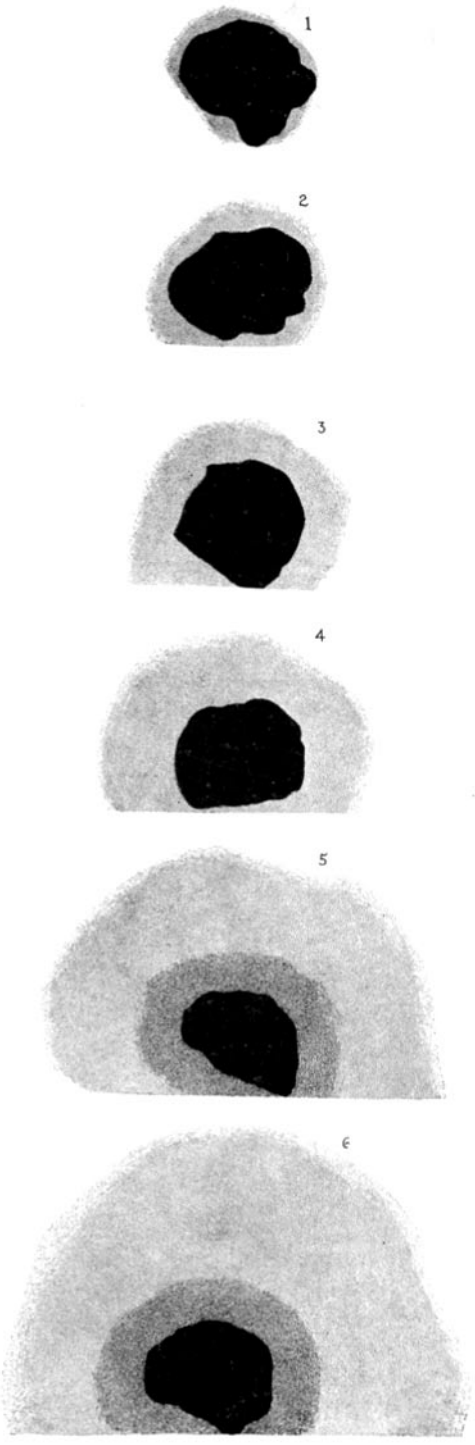
FIG. 7. Chick spleen in Ringer's solution.
FIG. 8. Chick spleen in Ringer's solution plus agar.
FIG. 9. Chick spleen in serum.
FIG. 10. Chick spleen in heated serum plus 2 per cent. agar.
FIG. 11. Chick spleen in unheated serum plus 2 per cent. agar.
FIG. 12. Chick spleen in plasma.

Notice the presence of elongated cells in figures 11 and 12 only. Further, note that ameboid movements are still present on the third day in serum (figure 9), while they stopped within twenty-four hours in Ringer's solution and in Ringer's solution plus agar (figures 7 and 8). Figures 7 to 9 are from photographs of the fresh unstained cells. Figures 10 to 12 are from cells stained with hematoxylin.

PLATE 47.

FIG. 13. A three day culture of peritoneum of fetal cat, in agar plus unheated serum.

FIG. 14. A high power magnification of one of the cells in mitotic division.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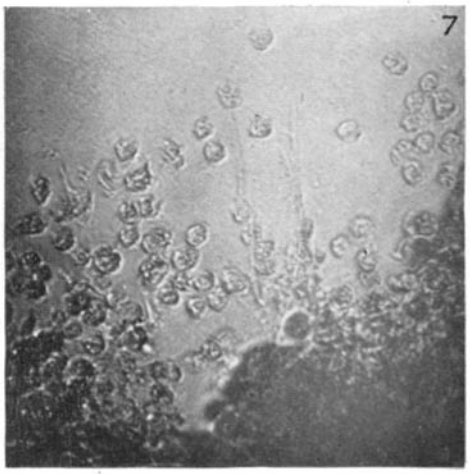
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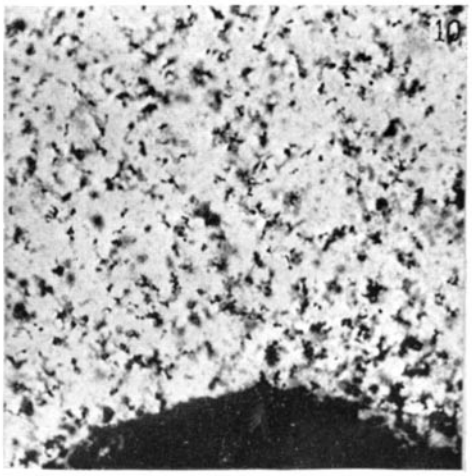
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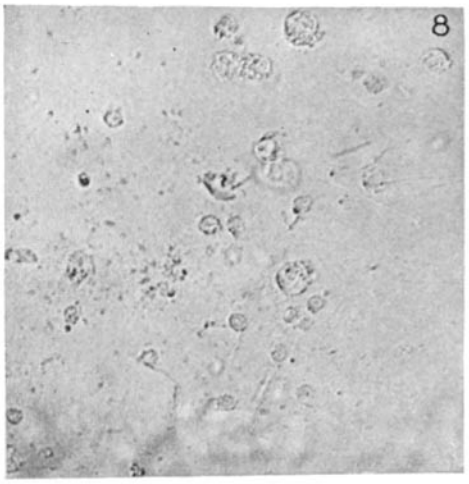
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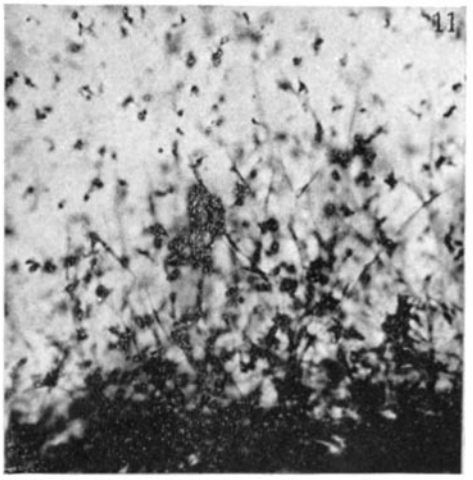
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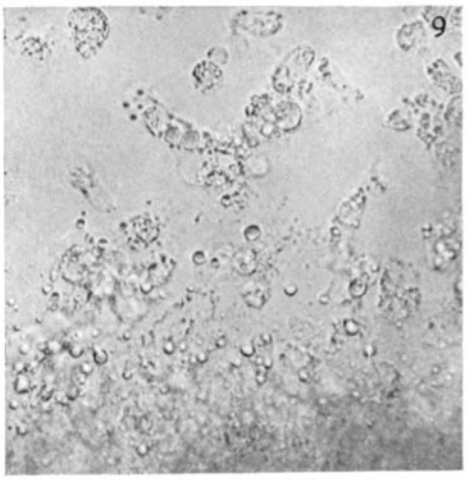
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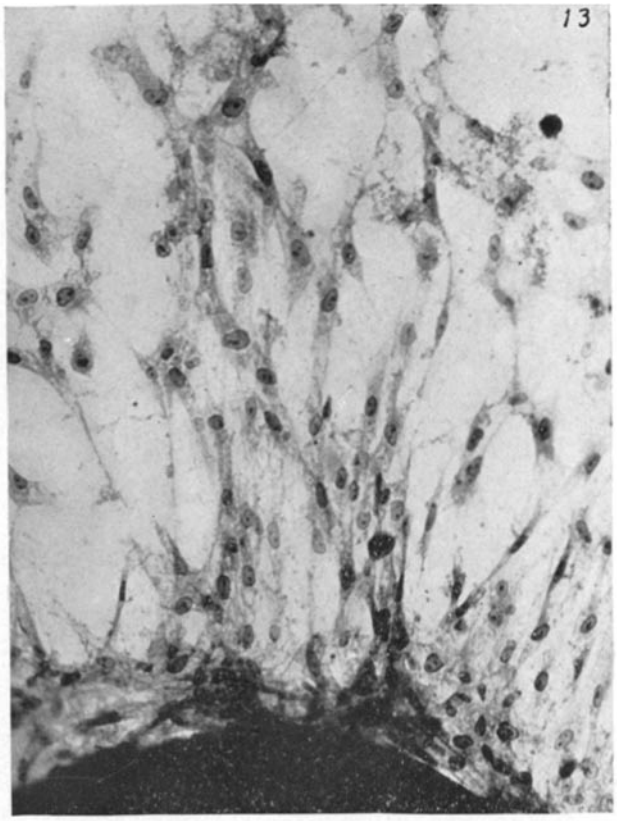
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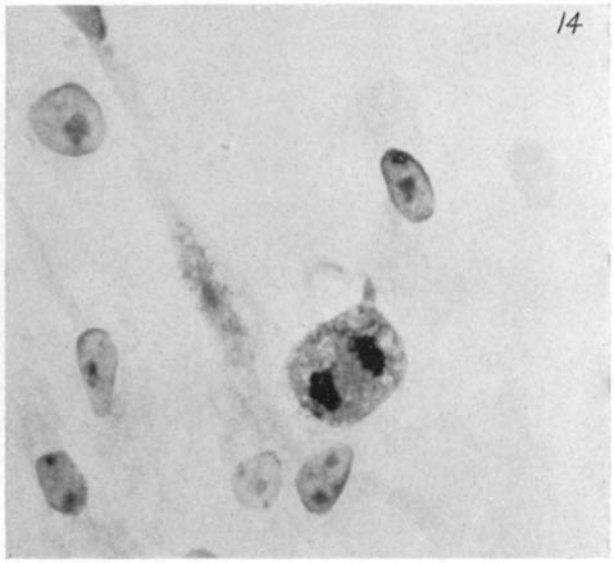
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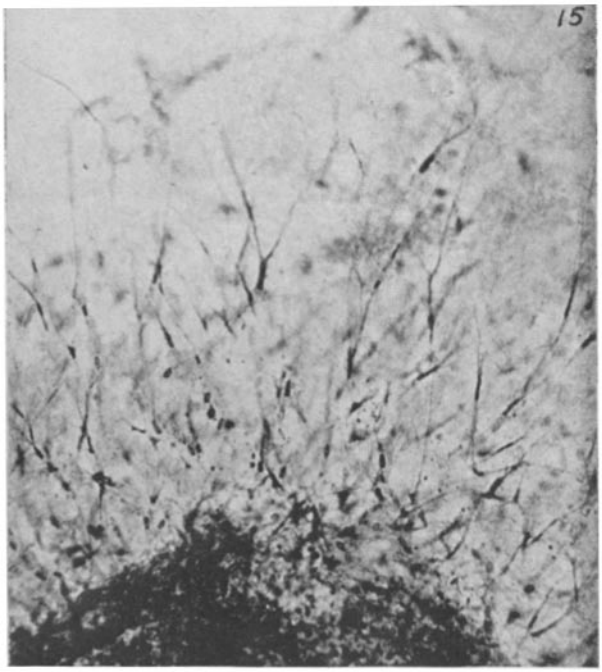
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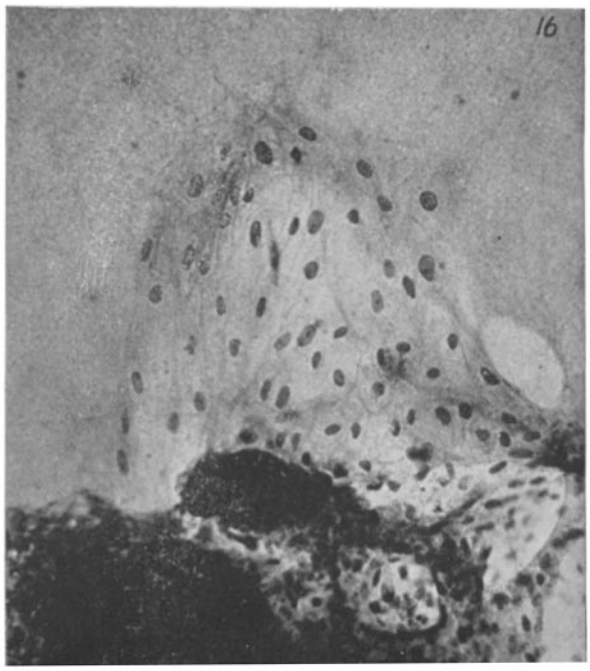
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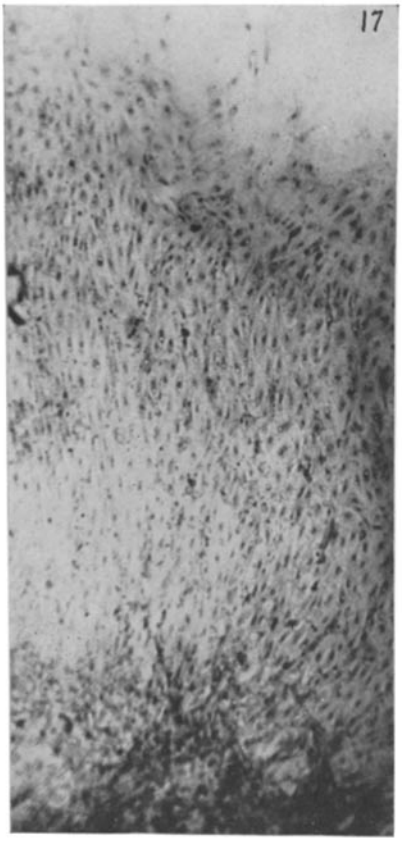
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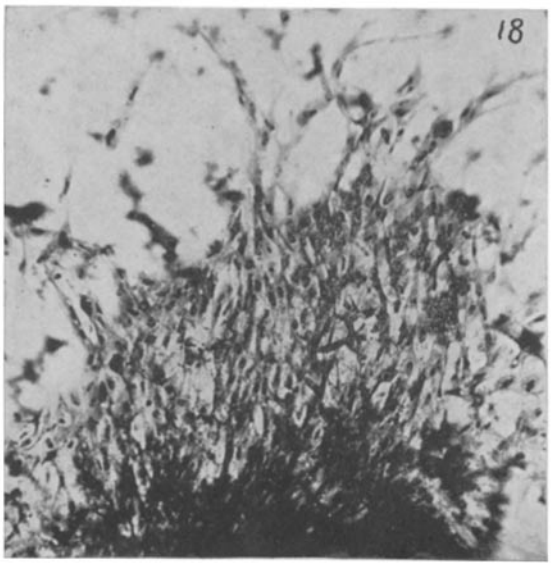
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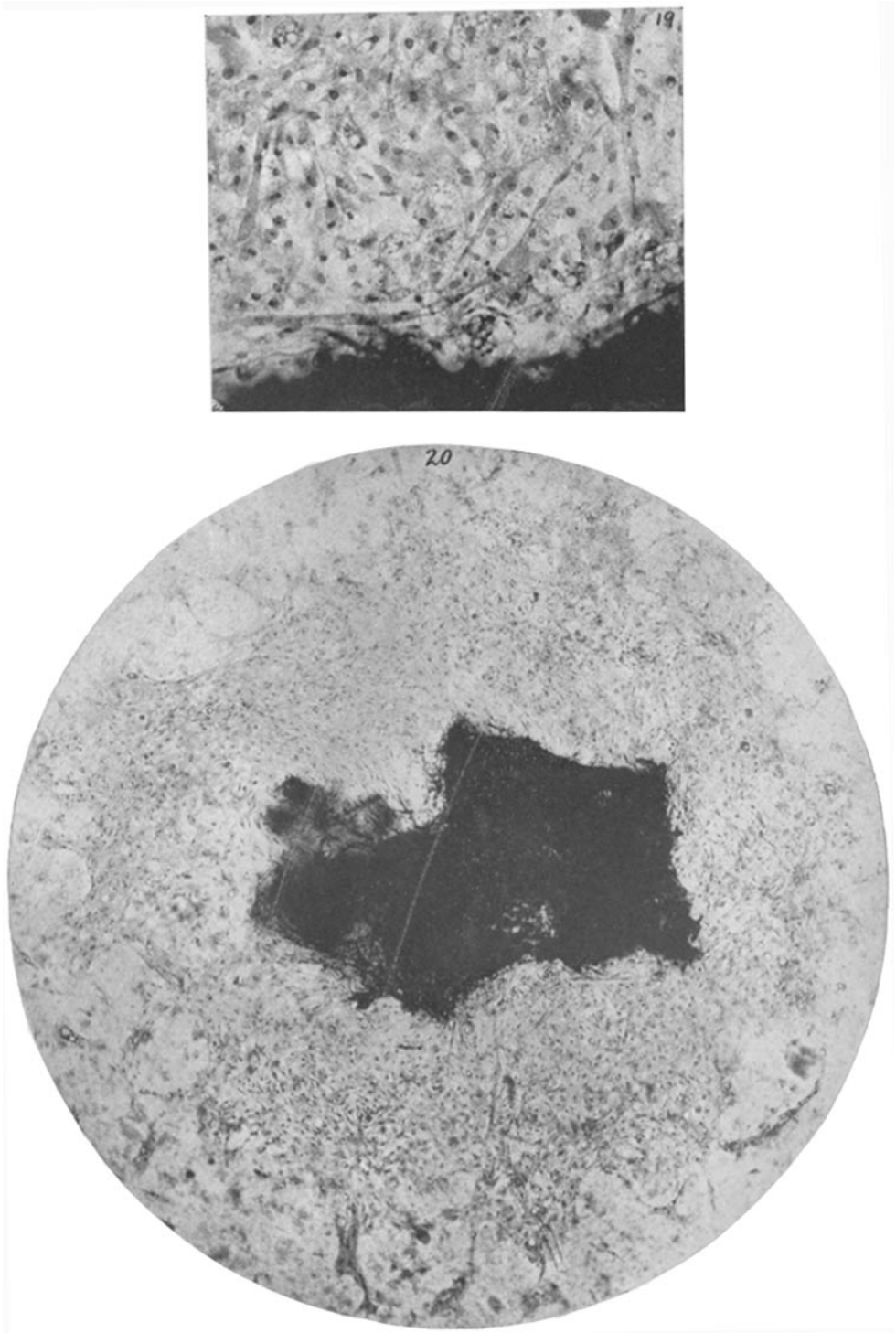
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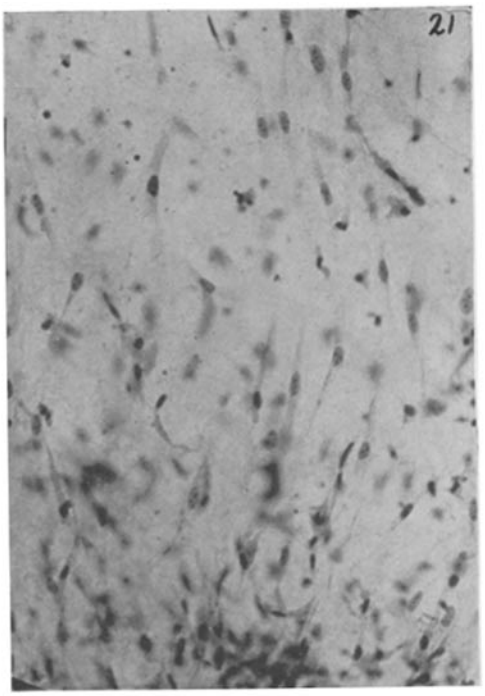
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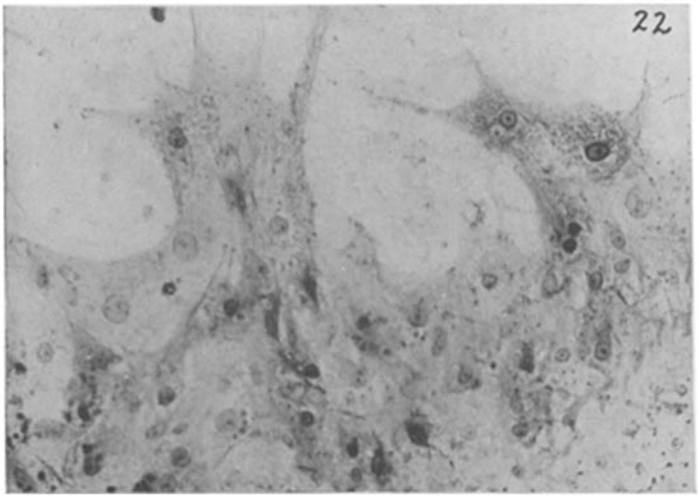
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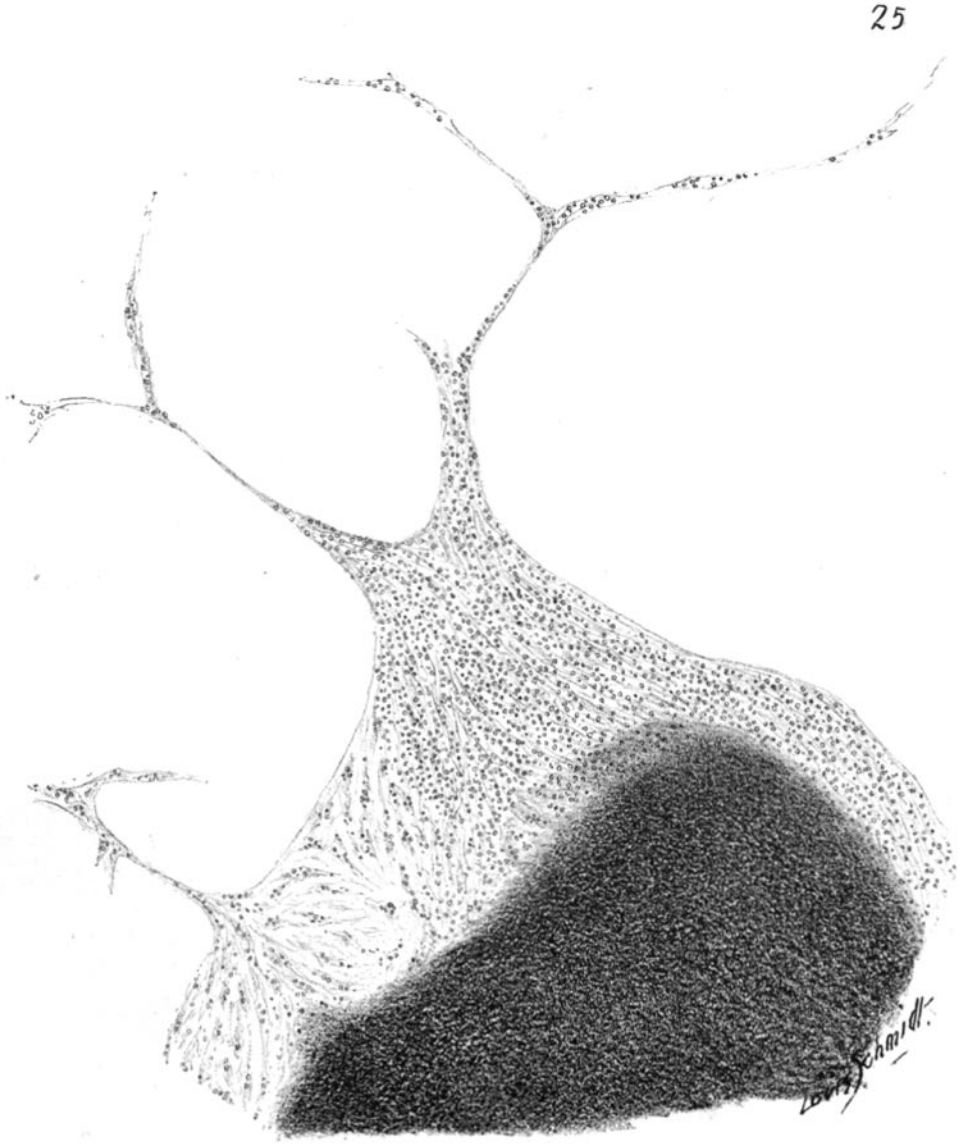
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PLATE 48.

Five day cultures in plasma of thyroid gland, from an adult cat.
FIG. 15. The development of elongated connective tissue cells.
FIG. 16. Epithelial cells (another culture). No connective tissue cells.

PLATE 49.

A three day culture of liver from a sixteen day old chick embryo.
FIG. 17. In serum agar.
FIG. 18. In plasma.

PLATE 50.

A three day culture of corium, in serum agar, from a sixteen day old chick embryo.
FIG. 19. High power magnification.
FIG. 20. Low power magnification.

PLATE 51.

FIG. 21. A five day culture in plasma of adult guinea pig bone marrow. Elongated connective tissue cells.
FIG. 22. A three day culture of liver, in serum plus agar, from a fetal cat.

PLATE 52.

FIG. 23. Connective tissue cells spreading along the cover-glass in a culture in serum of a sixteen day chick heart.
FIG. 24. A five day culture in plasma of hypophysis of an adult cat.

PLATE 53.

FIG. 25. A three day culture of chick heart (fifteen day chick embryo) in agar plus cat serum. The serum was heated previously for one half hour at 58° C.

PLATE 54.

FIG. 26. A three day old culture of chick heart (fifteen day chick embryo) in agar plus cat serum. The serum was unheated.