CHARACTERISTICS OF FROG CARCINOMA IN TISSUE CULTURE*

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Plates 27 to 29

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The method of implanting tissue in the aqueous humor of the living eye (1) has certain similarities to the cultivation of tissue *in vitro*. In both cases, the tissue is placed in a clear medium which is confined within a closed transparent chamber; the nutrition and metabolic exchange of the implant is carried out, during the earlier stages at least, solely by diffusion, that is to say, without the mediation of blood vessels; both methods permit direct and continuous microscopic observation of living tissue, but each has certain advantages which render it complementary to the other.

The present paper is concerned with the characteristics of frog carcinoma (2) in tissue culture. The experiments afford an opportunity to compare growth and organization of the same tumor *in vitro* and *in vivo*, and, further, to compare a malignant amphibian cell with malignant cells in general.

Method

Most of the tumors which were studied by intraocular transplantation (1) were also cultivated *in vitro*; in addition to these, cultures were made from some others, 32 in all. The roller tube technique of Gey (3) and Lewis (4) as well as the simple hanging drop method was used. The former procedure consists, essentially, in planting bits of tissue in a thin layer of plasma attached to the inside of a test tube; the plasma is then coagulated by adding embryo extract. This solid medium with the explants is bathed with a nutrient liquid which is kept circulating by slow rotation of the tubes in a suitable apparatus (4).

The plasma medium for the present cultures consisted usually of equal parts of heparinized frog and chicken plasma. The nutrient liquid was a modification of that used by Lewis and Gey for culture of mammalian tumor; it was composed of 7 drops of Gey's solution, 2 of chick embryonic extract, and 4 of either human placental serum or horse blood serum. The osmotic differences between frog plasma and the other com-

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ponents of the medium were adjusted by dilution with distilled water.[1] On the average, from 10 to 20 explants were planted in each tube. The rate of rotation was from 8 to 10 times per hour.

The supernatant fluid was renewed every 3 or 4 days, at which time liquefied areas were "patched" with new plasma and embryo extract. Subcultures were made at intervals of 2 or 3 weeks, depending on the rate of growth and on the degree of liquefaction of the plasma. The cultures were kept at room temperature, between 20° and 22°C. While this temperature is below the optimum for growth it has the advantage of being more convenient, because the plasma does not become liquefied so readily and subcultures need not be made as often as at higher temperature.

For observation with the microscope and for photographing, a coverslip was attached to the tube with a drop of oil in order to flatten the field. By using objectives of long focal distance, magnifications up to 200 times were obtained. For the examination of cellular details at higher magnification the usual kind of hanging drop cultures were made directly from the tumor or as subcultures from roller tubes.

Representative cultures were fixed in Susa solution or Bouin's fluid, and either stained *in toto* with hematoxylin-eosin, or sectioned serially and then stained. Such sections gave information as to the changes within the explant.

In addition to direct observation, two cultures of the tumor were studied by cinematograph. This method not only affords a permanent record, but makes clearer the manner of outgrowth, the character of locomotion of the individual cells, as well as intercellular changes which occur so slowly as otherwise to escape detection.

Characteristics of Tumor Growth

Under the conditions stated all of the tumors grew readily. The majority of the cultures were studied for 6 to 8 weeks and then discarded; a few were maintained as long as 6 months. After this time it proved difficult to keep them in good condition; they gradually declined in vigor, degenerative changes appeared, and finally growth ceased entirely.

The outstanding characteristic of the explanted tumor is that its manner of growth closely resembles that of the transplants in the living eye (1).

Within a day, the peripheral zone of the explant becomes increasingly translucent. At the same time, bud-like projections form and promptly grow out into the solid medium. The buds at first are hemispherical, sharply outlined, slightly yellowish, and sufficiently transparent to show their component cells. These, as a rule, are tall and columnar, and so arranged as to suggest the blind end or cap of a tubule (Fig. 1). As the buds grow, they form structures more definitely resembling tubules, except for the absence of a lumen. Some of these "tubules" appear as straight slender cylinders, others are thick, curved or twisted (Fig. 2). Their rate of growth is uneven; here and there excessive local proliferation leads to the formation of irregularly shaped masses which bulge out from the tubules, and which later may develop into side branches

[1] 3 drops of water added to 10 drops of either serum, embryo extract, or Gey's solution reduce the osmotic pressure to approximately that of frog plasma.

(Fig. 3). The length which the tubular outgrowths attain also varies considerably, but many extend for a distance of about five times their diameter. Usually by the end of the 1st day, and always by the end of the 2nd day, considerable numbers of such tubular structures have extended from various parts of the explant, the contours of which have become very irregular. Sometimes, the bud-like projections remain short, become greatly distended with proliferating cells and acquire the appearance of spherical acini, but without lumen.

The character of the growth now changes. As the growing tubules make contact with the surface of the glass they become adherent; their sharp outlines are gradually lost (Fig. 4), and within a few hours, proliferating cells burst forth and spread out as thin fans of polyhedral epithelial cells (Figs. 5 and 6). These fuse with other outgrowths of similar character until the explant is entirely surrounded by a flat membrane of epithelium (Fig. 7). Sometimes such membranes grow directly from the margin of the explant; this happens where the explant is in immediate contact with glass, without interposition of plasma.

Further growth is largely a repetition of the processes described, the character of which remains unchanged throughout the life of the cultures. The explant gradually becomes translucent, its initial compact appearance is lost, its intertwined and irregularly shaped tubules and acini become more distinct and separated, and many of these become solid as proliferating cells crowd into their lumen. From the periphery new tubules continue to grow out into the plasma, but tubular differentiation is always lost and membranes are always formed when adhesion to the glass takes place (Fig. 8). The membranes rapidly increase in area as well as in thickness, consisting of several layers of cells at the edge of the explant, and tapering to a single layer of cells, which forms a thin film several millimeters in width. Sometimes two sheets of cells are present, one spreading on the surface of the glass, the other on the free surface of the plasma clot.

In most cultures stroma cells and macrophages are inconspicuous, and usually disappear entirely within a few days. The membranes consist almost exclusively of tumor cells, which show their epithelial derivation by their intimate and immediate contact with one another. The advancing margin of the membrane is generally well outlined (Fig. 9); here and there a few cells may become detached and migrate away from the margin. Such separation is particularly apt to occur when the plasma becomes soft, as usually occurs within 3 to 5 days.

The growth of the membranes is relatively rapid as shown by a representative group of photographs of a day old culture taken at intervals during a period of 4½ hours (Figs. 13 to 16).

Characteristics of Cultures of Normal Kidney

In contrast to the prompt and luxuriant growth of frog carcinoma, explants of normal kidney cultivated under identical conditions grow slowly and scantily. There are also striking differences in the quality of growth.

The first cells to wander out are numerous macrophages and lymphocytes. Next appear fibroblasts as slender, loosely coherent spikes which radiate outward as from a hub, and endothelial cells which form delicate, more compact processes. Much later, usually on the 3rd or 4th day, a thin flat sheet of kidney cells protrudes from the margin

of some of the explants. These membranes of normal cells, in contrast to those formed by malignant cells, remain relatively small, their area increases slowly; as a rule, they are not composed purely of epithelium, but contain fibroblasts and macrophages as well. The differences in character between these normal epithelial cells and the tumor cells will be discussed below. After a few days the membranes usually degenerate. Unlike the tumor cultures, explants of normal kidney produce little liquefaction of the plasma.

Characteristics of the Malignant Cells

The malignant cells of the frog carcinoma differ in many respects from their homologues, adult kidney cells, when maintained under the same cultural conditions. While both types have the same general structure, the malignant cells are usually larger and more variable.

The cytoplasm of the malignant cells is usually less transparent, of somewhat denser structure, and is more apt to contain an abundance of fine granules. The nucleus is usually larger and more variable in size than in normal cells. It is more sharply outlined. The nucleoplasm is as a rule so coarsely granular as to suggest the early prophase of mitosis (Fig. 10). Binuclear cells are common, and cells with several nuclei are not infrequently found. The nucleoli are conspicuous, and may attain very large size; usually but one or two are present; their shape varies from round to very irregular. Mitochondria are abundant, and radiate as long, coarse filaments from the central region to the periphery (Fig. 11). When the cell is viewed under the most favorable conditions, a centrosphere may be seen; it is well defined and contains radially arranged mitochondria. The centrosphere and the adjacent nucleus are surrounded by a ring of fine fat droplets and granules (Fig. 11).

The number of mitoses observed varies in cultures of the same tumor; in some membranes mitoses are numerous, in other membranes of approximately the same size only few are found. This difference is possibly accounted for by the rhythmic occurrence of cell division (5). Strikingly abnormal types of mitoses are uncommon. The chromosomes, as in amphibian cells generally, are large; most of them are normal in shape and arrangement, but obvious degenerative changes are occasionally encountered. Where the number of chromosomes could be counted with certainty, 26 were found. (This figure is based upon examination of fixed and stained cells.)

Cells detached from the membrane exhibit active locomotion by means of broad ruffle pseudopodia (Fig. 12). The slow and ponderous mode of locomotion can be followed far more clearly in the cinematographic films than by direct observation (Figs. 17 to 20).

Although most of the frog tumors cultured had characteristic intranuclear inclusions (2), no inclusions were observed in any of the newly formed cells; but, as determined from serial sections of explants, the inclusions persisted within the original cells. It is not known whether failure to form recognizable inclusion bodies depends on the experimental conditions, or else whether their early stages were not seen and the short time interval between

transplantations did not permit development of the mature stages. Further studies on the fate of the inclusion bodies are in progress.

The general characteristics of the malignant cells varied but little from tumor to tumor; nor were there any noticeable changes during the periods of cultivation, which ranged up to 6 months. These experiments support the view that the frog carcinoma cells, like malignant mammalian cells, are permanently altered, and do not revert to normal types during cultivation (6). The frog carcinoma cells, in appearance, structure, and habit of growth closely resemble malignant cells of mammals as recently described by W. H. Lewis (6). Thus in these widely different groups of animals, malignant cells have a strong family resemblance.

COMMENT

The character of growth *in vitro* of the frog carcinoma is similar to that of many mammalian adenocarcinomas (7). The tendency of such tumors to form tubular structures, in suitable media, has been explained on the ground that it is an intrinsic property of glandular tissue in general, while membrane formation is believed to be brought about by the modifying action of surface forces (8).

Not all glandular tissue, however, grows according to this pattern. Thus, normal adult kidney, the homologue of the frog carcinoma, does not, *in vitro*, form tubular outgrowths in any of the various media which have been employed, but from the beginning grows out as undifferentiated cellular membrane (9). Embryonic chick kidney, on the contrary, grows in a manner entirely similar to that of the adenocarcinoma of the frog's kidney (10). There is as yet no adequate explanation for these differences.

The similar character of the growth of frog carcinoma *in vitro* and in the anterior chamber no doubt depends upon the operation of similar factors. In both cases the outgrowth is at first tubular, and remains so while completely surrounded by homogeneous medium; contact with solid surfaces results in loss of differentiation and in membrane formation. Such contact is made within a few days in cultures, due partly to the thinness of the plasma clot, partly to its early digestion by the tumor cells. In the relatively abundant aqueous humor, on the contrary, tubular growth continues for long periods. Time factors probably account for the further difference of secondary tubule formation from membranes, as occurs in the anterior chamber (1), and for absence of such redifferentiation in the tissue culture: in the eye, growth of membranes continues undisturbed for many weeks or months, and thus at various points excessive cell proliferation may occur;

but in cultures, undisturbed continuous growth is prevented by the necessity of frequent transplantations. Another difference lies in the fact that *in vivo* the growth may acquire a fibrovascular stroma. These differences, however, are of minor importance as compared with the similarities of growth under the two conditions.

SUMMARY

The adenocarcinoma of leopard frogs may be cultivated with ease in plasma media. In such cultures two types of growth occur with regularity. The first is in the form of tubules which promptly grow out in the solid medium and retain their tubular form as long as they remain completely enveloped by plasma. When, however, they make contact with the surface of the glass, they adhere to it, the part in contact becomes flat, and its cells now grow no longer as tubules but as membranes.

The manner of growth *in vitro* resembles the growth of transplants of the same tumor in the anterior chamber of the living eye, thus suggesting that in each case the habit of growth is determined by the same morphogenetic factors, *i.e.* those inherent in the cells themselves, and those depending on interfacial forces.

The malignant cells of the frog carcinoma have the attributes which in general distinguish malignant cells from normal cells of corresponding type. In comparison with adult kidney cells, their normal homologues, the conspicuous properties of frog carcinoma cells are: larger and more variable size and shape of cell body, of nucleus, and nucleolus; coarser and denser structure of cytoplasm, of nucleoplasm, and of nuclear membrane; increase in number of mitochondria, and more frequent occurrence of mitosis. These cytological characteristics remain unaltered in cultures maintained for as long as six months.

Frog carcinoma is a transmissible disease due to an agent which induces inclusion bodies, and which has other attributes indicating that it is a virus. The general correspondence in character between its cells and the malignant cells of mammalian tumors of diverse origin suggests that neoplastic phenomena are essentially alike, no matter in what group of animals they occur or what their causal factors may be.

Acknowledgment.—I am indebted to Drs. Margaret and Warren Lewis of the department of Embryology, Carnegie Institution of Washington, Johns Hopkins Medical School, for much advice and generous help throughout this investigation; several of the tumors were first grown under their guidance, and the cinematographic films were made in their laboratory.

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EXPLANATION OF PLATES

All figures are unretouched photographs of living cultures or cells. Figs. 1 to 9 were photographed by Mrs. Miriam R. Barrett; Figs. 10 to 20, by Dr. Warren H. Lewis.

PLATE 27

FIG. 1. Beginning of tubule formation approximately 12 hours after explantation; a translucent bud-like projection has formed at the margin of the explant. The component cells show an arrangement similar to that in a tubule, but no lumen is present. × 80.

FIG. 2. Advanced tubule formation, approximately 24 hours after explantation. Numerous bizarrely shaped tubular structures have grown from the margin of the explant into the plasma. × 40.

FIG. 3. A more advanced stage showing lateral budding and the formation of branches. The tubule in the upper right corner has lost its sharp outline and its cells are migrating outward. A thin layer of cells, which have escaped from tubules not in focus, extends beyond the mass of coiled tubular structures. × 40.

FIG. 4. Margin of an explant, about 24 hours old. The sharp contours of the tubules are fading, and undifferentiated masses of cells are beginning to migrate. × 80.

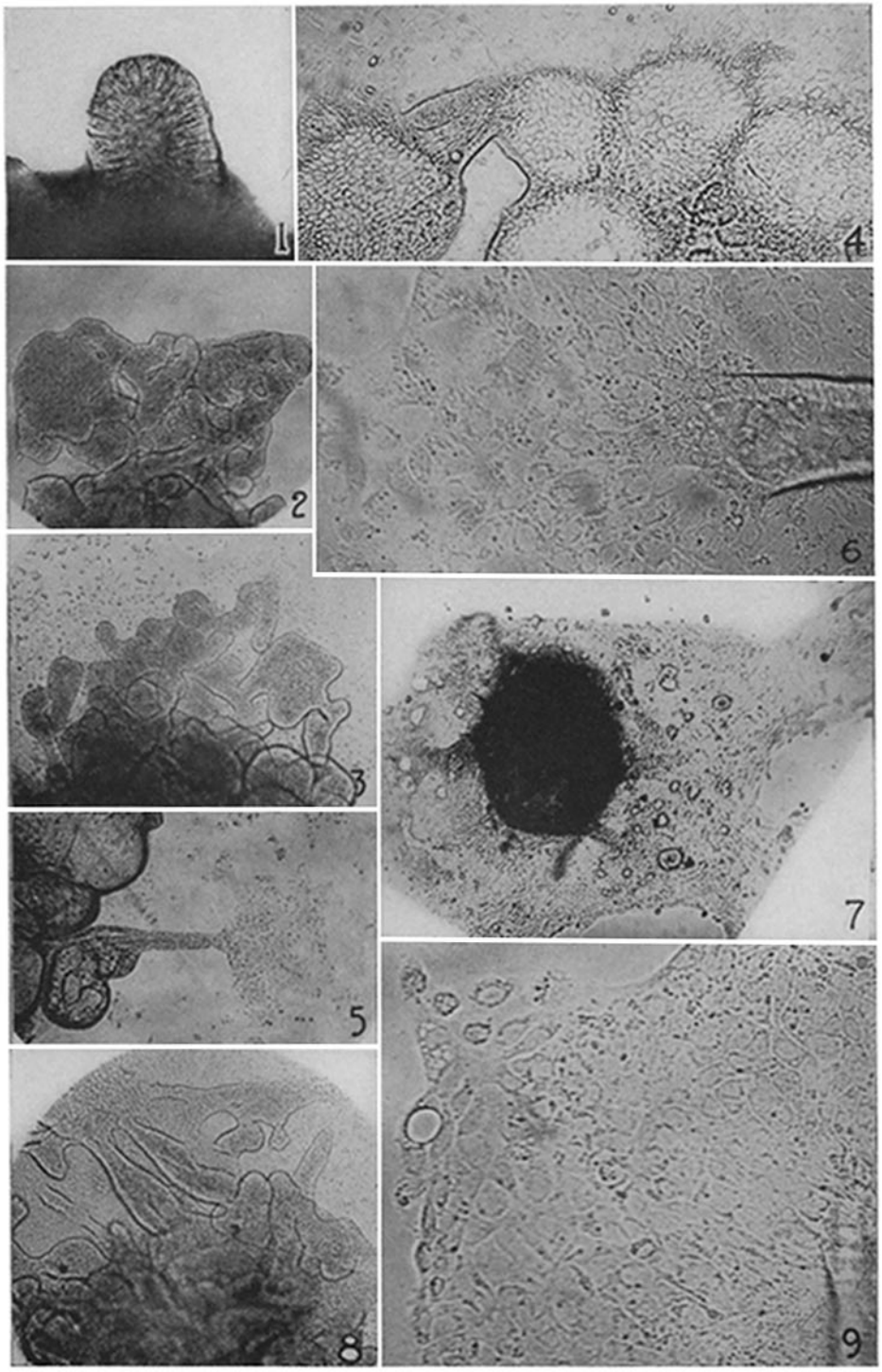
FIG. 5. A slender tubular outgrowth, from the distal end of which a fan-shaped mass of cells is spreading out on the surface of the glass. × 40.

FIG. 6. Details of the outward streaming of cells from the distal end of a tubule. The sides of the tubule are still sharply outlined; its end is split wide open. The migrating cells form flame-like processes which taper to a thin membrane. × 170.

FIG. 7. A broad undifferentiated membrane completely surrounds the explant 36 hours after culture was made. × 12.

FIG. 8. Appearance of margin 3 days after explantation. Tubular structures are continuing to grow out, becoming transformed into an undifferentiated membrane as they make contact with the glass. × 40.

FIG. 9. A membrane composed exclusively of tumor cells having the arrangement characteristic of epithelium. The margin is sharply defined. × 170.



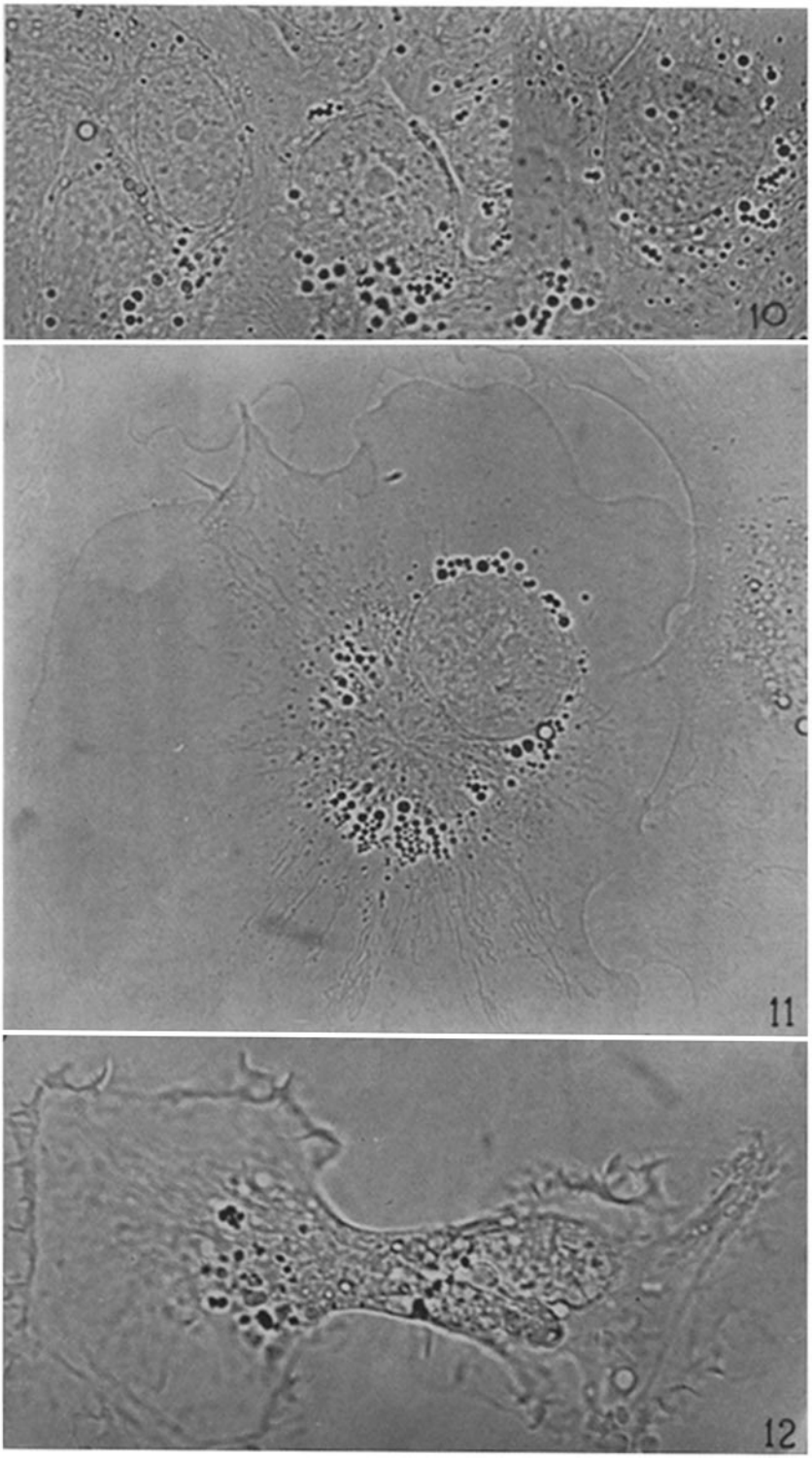
(Lucké: Frog carcinoma in tissue culture)

PLATE 28

FIG. 10. A membrane at higher magnification, showing several nuclei which have the large size and granular appearance characteristic of malignant cells; the nucleoli are conspicuous. × 1100.

FIG. 11. An isolated malignant cell which shows a conspicuous centrosphere and adjacent granular nucleus; both are encircled by fat droplets and granules. Radiating from the central region are coarsely filamentous mitochondria. × 1100.

FIG. 12. A malignant cell photographed while in active locomotion. Its peripheral zone shows broad ruffle pseudopodia. The small pale globules within the cytoplasm are droplets of fluid medium which have been engulfed by the ruffle pseudopodia; the process is known as pinocytosis (11). × 1100.

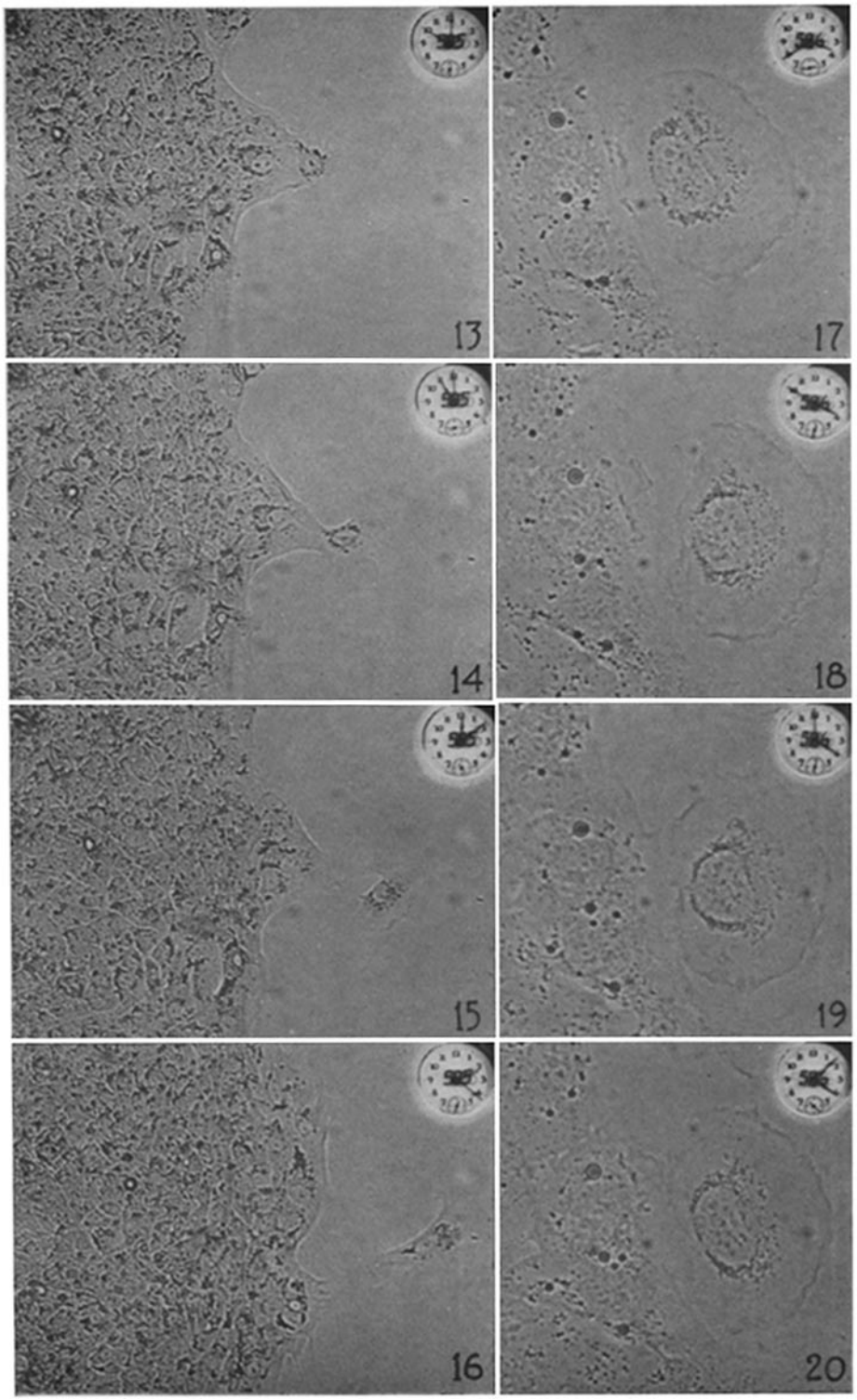


(Lucké: Frog carcinoma in tissue culture)

PLATE 29

FIGS. 13 to 16. Advancing edge of culture, approximately 1 day old, photographed over a period of 4½ hours. Note increase in width and the gradual detachment of a cell from a projection at the margin. Photographs taken from a cinematographic film. × 150.

FIGS. 17 to 20. A detached malignant cell, at intervals of 10 minutes, to show its slow rate of locomotion. Photographs are taken from a cinematographic film. × 600.



(Lucké: Frog carcinoma in tissue culture)