A PURE STRAIN OF THYROID CELLS AND ITS CHARACTERISTICS.

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PLATES 12 AND 13.

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The necessity of using pure cultures of tissues in physiological studies has been emphasized repeatedly, and many attempts have been made in this laboratory to obtain strains of the more important types of cells. Although pure strains of fibroblasts,[1] monocytes,[2] cartilage,[3] and pavement epithelium[4] had been isolated and maintained in active condition *in vitro* during the last few years, the attempts to cultivate the elements characterizing the endocrine glands had so far failed. However, we have recently succeeded in cultivating the epithelial cells of the thyroid in a condition of purity, and observing their multiplication *in vitro* during a period of several months. The purpose of this article is to describe the technique used in the isolation and maintenance of this strain and the cultural properties of the thyroid cells.

EXPERIMENTAL.

Preparation of the Tissues.—The tissue was obtained from the thyroid gland of 18 to 19 day old chick embryos. To facilitate the isolation of one lobe of the gland, the right side of the thorax was laid open by cutting through the chest wall and severing the right clavicle. The right lobe of the gland is located at the junction of the subclavian and common carotid arteries, just internal to the jugular vein. Under aseptic conditions the organ was dissected out carefully and placed on the surface of a glass slide. The tissue was kept moist with Tyrode

[1] Ebeling, A. H., *J. Exp. Med.*, 1922, xxxv, 755.

[2] Carrel, A., and Ebeling, A. H., *J. Exp. Med.*, 1922, xxxvi, 365.

[3] Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 379.

[4] Fischer, A., *J. Exp. Med.*, 1922, xxxv, 367. Ebeling, A. H., *Compt. rend. Soc. biol.*, 1924, xc, 562.

solution and teased out into small fragments about 0.5 sq. mm. in size. It was essential to remove as completely as possible the delicate investing connective tissue membrane. The pieces of gland substance were suspended in Tyrode solution and kept there until the actual culture was made.

Preparation of the Cultures.—Equal volumes of plasma and embryonic tissue juice were mixed on the surface of the mica slip. The fragments of fresh tissue were embedded in this medium before its coagulation had occurred. The hollow ground slide was placed over the cover-glass, and affixed by a small quantity of vaseline. After complete coagulation, the cultures were inverted and sealed with paraffin in the usual manner.

When the cultures were transferred, the fragments were extirpated, floated in Tyrode solution, and again cultivated in fresh medium. Care was required in this procedure on account of the extreme delicacy of the membranous growth which often consisted of practically only a few layers of cells, so thin as to be scarcely visible to the naked eye. It was found advisable to allow the fresh fragment of thyroid tissue to grow for 72 hours before a transfer into fresh medium was made. Sometimes, it was not technically feasible to divide a culture which had been allowed to incubate for 72 hours because the structure of the entire culture was so delicate. As a rule, in such instances the tissue could be transferred in its entirety without retraction of the clot. Generally, contamination by fibroblasts could be readily detected at the time of the first transfer and those cultures showing it were discarded, or the uncontaminated part of the culture alone was transferred. This could be done easily because the original mass separated neatly from the cells which had invaded the medium, owing probably to the partial digestion of the coagulum immediately surrounding the culture. When no fibroblasts could be detected, the excision of the central fragment, with its surrounding membrane of new cells, was made in the usual manner.

After about 10 days, during which period the fragments were transferred three or four times, the culture medium usually was found to contain epithelial cells only. Then the fragments were placed in D-5 flasks,[5] containing 1 cc. of plasma, 0.5 cc. of Tyrode solution, and 0.5 cc. of embryonic tissue juice. This medium coagulated within about 1½ minutes. The tissues began to grow in 4 to 8 hours, and after 1 or 2 days, 1 cc. of a fluid medium composed of 25 per cent embryonic juice, 5 per cent serum, and 70 per cent Tyrode was introduced into the flask. Every 48 or 72 hours, this fluid was removed and replaced by 2 cc. of Tyrode solution which was left 30 minutes, then withdrawn and replaced by the fresh fluid medium. Whenever the solid medium showed partially digested areas, they were patched with fresh plasma and embryonic tissue juice. If necessary, the coagulum and tissue were removed from the flask, the tissue was dissected out from the clot, and placed in a fresh flask.

Examination of the living tissue was made with low power through the thick

[5] Carrel, A., *J. Exp. Med.*, 1923, xxxviii, 407.

glass of the flask. Even photographs at a magnification of 130 or 150 diameters could be taken. For more accurate microscopical study, the cultures were fixed and stained as follows:

The cultures on mica slips were left adherent thereto and placed in Zenker's fluid containing 5 per cent acetic acid. Cultures in flasks were fixed by introducing fixing fluid. This process required from 20 to 24 hours. It was found that longer immersion appeared to interfere with the proper subsequent staining of the specimen. Then the specimens were washed in running tap water for 24 hours. After washing, the fixed coagulum of cultures on mica was removed from the slip with a safety razor blade, while the fixed clots in flasks were loosened carefully with a small flexible spatula. Both were carried through the alcohols. After the absolute alcohol, the specimens were treated with a 4 per cent tincture of iodine (U. S. P.) for 15 to 20 minutes to remove all traces of mercuric chloride. The preparations were then carried back into water, again through the alcohols and into chloroform, and finally into paraffin for sectioning. Care was exercised in the placement of the specimens at the time of embedding so that complete transverse sections could be obtained. The sections are between 3 and 5 microns thick. They were mounted on glass slides and carried through xylol, the alcohols, and into water for staining by the Kraus method. Each section individually was carried through the following steps.

It was stained with Unna's alkaline methylene blue for 10 to 20 minutes, then rinsed in water for about 45 seconds, and differentiated in a 33 per cent aqueous tannic acid solution. This procedure was carried out in a Petri dish under the microscope. The preparation was left in the solution until the nuclei had cleared. Sometimes this required from 10 to 30 minutes. The section was next placed in Unna's acid fuchsin-tannin solution for 20 minutes until the nuclei appeared blue, rinsed in water for about 45 seconds, then washed in running water for 1 minute, and rinsed in distilled water. Again it was stained for 30 seconds in a 1 per cent aqueous solution of acid fuchsin, rinsed in distilled water for 45 seconds, and differentiated in a 1 per cent aqueous solution of phosphomolybdic acid (Kahlbaum's crystals) for 3 to 4 minutes, rinsed for a few seconds in water, washed in running water for 5 to 10 minutes, rinsed in distilled water, and run through the alcohols, acetone, and xylol, into a Canada balsam mount.

During the last 2 years, several strains of thyroid cells have been obtained from chick embryos by the procedure described and kept in active condition for several months. There was no difficulty in maintaining the thyroid cells for long periods of time. Most of the observations were made on two strains which originated from fragments of thyroid gland of 19 day old chick embryos first cultivated in plasma and embryonic tissue juice. In one series of experi-

ments after 10 days cultivation on mica cover-slips, when the growth was found to be composed exclusively of epithelial cells, fragments were transferred into D-5 flasks, following which the strain was maintained in a very active state, and the cells went on multiplying for 7 months. The experiment was ended then with the cells in excellent condition and multiplying as actively as on the 1st day. There is no reason to doubt but that they could have been cultivated for a long time, probably indefinitely, as in the case of pavement epithelial cells. They had retained their individual characteristics as do cultures of fibroblasts[1] and other strains of cells.[2-4]

The appearance of the culture varied according to the mode of growth of the cells, whether at the surfaces, or inside of the coagulum. Generally after 6 or 8 hours incubation of small cultures of passage fragments, fine tube-like structures with branching processes, each one consisting of a number of cells, began to invade the medium surrounding the mother fragment. These processes resembled the structures often seen in cultures of epithelium.[6] The cells at the extremity of the processes appeared similar to fibroblasts growing in a horizontal plane, and the cytoplasm at their free ends showed delicate protoplasmic pseudopods spread out like a fan. The cells composing the body of the tube were flat and elongated. Each individual element appeared to be slightly separated from its neighbors by an interspace and this separation gave to the entire structure a mosaic appearance (Fig. 1). Often the branching extremities of the invading processes extending out into the medium joined together in such a manner as to enclose small round or ovoid spaces (Fig. 1). A few hours later, the cells surrounding such spaces appeared arranged more or less regularly, the formation suggesting that seen in the cross-section of a tubule. Generally in another few hours, the medium in such a space appeared thinned out and the cells that previously surrounded the space had grown into it and formed a single layer of typical pavement epithelium. After 24 or 36 hours, the tube-like structures could not be made out so distinctly. The peripheral zone of a culture, however, showed many cells which tended to grow in a single plane. There were also many isolated cells

[6] Ebeling, A. H., and Fischer, A., *J. Exp. Med.*, 1922, xxxvi, 285.

scattered about on the surface of the medium, or in it, or creeping along the cover-slip under the medium.

Usually, after 48 or 72 hours, a culture showed four distinct regions of cell proliferation. First, there was a central region formed by a mass of cells which had grown in many planes and included the mother fragment. Second, surrounding this mass where the medium was partially liquefied, a thin membrane had formed, composed of cells growing more or less in a horizontal plane. In many places the network of cells consisted of only a single layer adherent to the cover-slip and here the medium appeared to be almost entirely liquefied. Such spaces, filled in by cells, usually showed a number of mitoses and some dying cells (Fig. 2). These dying cells when fixed and stained showed deeply stained nuclei, with a coarsely granular cytoplasm. Their outline was characterized by many short, blunt processes. Cinematomicrographic pictures of cells at this stage showed these processes to be what Carrel has termed "pulsating hernias of the cytoplasm," because they have actually been observed to pulsate in accelerated motion pictures of fibroblasts, epithelial cells, leucocytes, and thyroid cells. The punctate thinning of the medium gave the entire membrane a moth-eaten appearance (Fig. 3). Third, in the region surrounding the membranous growth, the cells appeared piled up in many planes, forming a distinct corona. However, the medium did not look thinned out to the same degree as in the second region. Fourth, the peripheral region was composed of a zone of cells growing in practically the same formation as in the culture after 6 or 8 hours incubation. In addition, many scattered isolated cells were observed, and some of these had wandered a considerable distance from the culture proper.

The character of the growth of thyroid epithelium, like that derived from the iris, seemed to depend to some extent upon the cultural conditions, as Uhlenhuth[7] has shown in the case of frog skin epithelium. In some cultures, fusiform cells appeared, closely resembling fibroblasts. In such cases there was no digestion of the medium, and the cells invaded it as do fibroblasts. A culture that exhibited this change in the character of its growth usually prolif-

[7] Uhlenhuth, E., *J. Exp. Med.*, 1915, xxii, 76.

erated less and less actively. The central fragment became a dense, opaque mass and, after several transfers into fresh medium, failed to proliferate. At this time, the cells seemed to have lost their power of digesting the coagulum; new cells no longer invaded it, and the culture died. However, when a culture presenting this appearance survived the repeated transfer into fresh medium, the typical membrane formation of the cells again developed, as also the power to digest fibrin (Figs. 4 and 5).

When thyroid cells were cultivated in D-5 flasks, they grew at the surfaces of the coagulum, and also in the middle of it where they assumed an entirely different appearance, resuming their glandular characteristics. Cells growing like a continuous membrane on the surfaces of the coagulum and having the appearance of pavement epithelium formed acini within it similar to those of a normal gland. This phenomenon occurred a long time after the strain had been isolated from the embryonic gland. A microscopic section of a pure culture of thyroid cells, fixed after 129 days of life *in vitro* and stained by the Kraus method, showed an aggregation of thyroid cells disposed in such a way as to form about 40 to 50 acini. Many of the acini were almost completely filled with colloid substance. The cell aggregates formed a cluster which resembled the cross-section of a fresh gland of an 18 or 19 day old chick embryo. Surrounding this glandular structure, the medium appeared as an unorganized mass of fibrin in which could be seen a few isolated thyroid cells. On the upper and lower surfaces of the coagulum, a pavement layer of cells could be distinguished. These were detached in places, owing undoubtedly to mechanical injury in the course of fixation and sectioning (Fig. 6).

The fibrin of the clot stained a pinkish yellow. The cells stained light blue, and the nuclei dark blue or a bluish black. The colloid material in some acini stained a bluish black, with greenish pink border; in others, greenish blue with a pink undertone; and in still others, a reddish pink.

Under high magnification (2,000 diameters), part of the section showed individual acini composed of large epithelial cells more or less symmetrically arranged to form a lumen. These cells showed darkly stained, pycnotic nuclei, some with two nucleoli stained a

bluish black. The cytoplasm was stained light blue with moderately coarse, dark blue granules. In the lumen, the colloid material lay in the form of a round mass surrounded by a clear, limiting area (Fig. 7). The mass itself was stained bluish black centrally and greenish pink peripherally.

It was obvious that the thyroid cells, in spite of their long life outside of the organism, had not dedifferentiated but had conserved the property of organizing themselves in a gland, and also had retained the ability to elaborate colloid substance under certain conditions. The multiplication of the thyroid cells cultivated by means of our method is not rapid. The strain on which most of the observations were made took its origin from eight cultures; and in the space of 7 months, it was only increased to twenty-five small cultures and five or six in D-5 flasks. But the rate of multiplication at the end was as great as at the beginning of the experiment, and all the indications are that the duration of the life of the tissue is unlimited, as in the case of fibroblasts and pavement epithelium.

DISCUSSION.

Our experiments have demonstrated that strains of thyroid cells can be isolated and maintained in a pure condition, probably for an unlimited period of time. Their cultural requirements are similar to those of fibroblasts and of the pavement epithelium already studied. Embryonic tissue juice must be present in the culture medium if they are to multiply indefinitely. According to the condition of the medium, the cells assume the appearance of pavement epithelium or of fibroblasts, but they do not dedifferentiate. It has already been shown by Ebeling and Fischer[9] that pavement epithelium derived from chick embryo iris, when growing actively at the surface of the coagulum, produces very little pigment, but when it grows more slowly, it again manufactures a large amount of black pigment, like that of the original cells. An analogous phenomenon has been observed in the culture of the thyroid. When the cells are growing in thin membranes at the surface of the clot, generally no evidence of the secretion of the colloid material can be found, but when they grow within the coagulum, they again assume

a glandular appearance and form acini filled with colloid material. This change occurs spontaneously whenever the cells grow inside the clot, the presence of fibroblasts being unnecessary. Not only do the cells fail to dedifferentiate, but they keep the property of secreting growth-promoting substances for fibroblasts. In some experiments, a fragment of thyroid was inoculated into a pure culture of fibroblasts, and it was found that the proliferation of the fibroblasts was markedly increased. The growth-promoting substances are probably identical with those contained in thyroid extracts, the action of which on connective tissue was observed long ago, *in vivo* as well as *in vitro*.[8]

The behavior of this strain of thyroid cells and the persistence of its characteristics, in the absence of a supporting connective tissue, are facts which bear importantly upon the theories of Champy and Drew with regard to the dedifferentiation of tissues *in vitro*. Some years ago, Champy reported that epithelial cells migrating from pieces of kidney surviving in a drop of plasma lose most of their characteristics[9] and take on an indifferent aspect, being indistinguishable from connective tissue cells. In addition, he noted that the specific secretion of prostatic cells of the guinea pig, if cultivated in serum, soon was formed no longer.[10] In the presence of connective tissue, the epithelial cells did not dedifferentiate.[11] His conclusion was that, when epithelium is cultivated *in vitro* without connective tissue, its dedifferentiation is certain. Not long ago, Drew defended the same theory.[12] The experiments of both authors were made with the early technique and with impure cultures, which may explain how they came to this conclusion, an erroneous one as brought out by the present work and the previous observations on pavement epithelium made in this laboratory.

Our experiments show that the study of the endocrine glands by the method of tissue culture is practicable. Since thyroid cells growing in a proper medium do not dedifferentiate, but go on secret-

[8] Carrel, A., and Burrows, M. T., *J. Exp. Med.*, 1911, xiii, 416.

[9] Champy, C., *Compt. rend. Soc. biol.*, 1912, lxxii, 987; *Presse méd.*, 1914, xxii, 87.

[10] Champy, C., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 842.

[11] Champy, C., *Compt. rend. Soc. biol.*, 1914, lxxvi, 31.

[12] Drew, A. H., *Brit. J. Exp. Path.*, 1922, iii, 20; 1923, iv, 46.

ing colloid material, our method can be used for the study of the function of this gland, at least. In the future, it should be possible to investigate with some exactitude the relation between the conditions of the cells and the character of their secretion. The main objection to the use of the method on a large scale is the minute amount of tissue which can be cultivated; but it is very probable that techniques will be developed by which the mass of the cultivated tissue can be increased appreciably, and the secretions obtained in such amounts that their physiological and chemical study will be possible.

CONCLUSIONS.

1. A pure strain of thyroid epithelium was isolated and maintained in active condition for 7 months. At the end of the experiment, the rate of cell multiplication was as great as at its beginning.

2. The thyroid cells grew at the surface of the coagulum as pavement epithelium, and within the coagulum as a glandular structure.

3. The cells did not dedifferentiate, and the lumen of the acini in cultures from a strain over 4 months old contained colloid secretion similar morphologically to that from a freshly extirpated thyroid gland.

EXPLANATION OF PLATES.

PLATE 12.

FIG. 1. Experiment 249–8. Culture of thyroid cells, fixed and stained with methylene blue after 12 hours incubation. The fragment from which the culture originated had undergone eight passages *in vitro* (21 days). The picture shows branching processes composed of flat, elongated cells; interspaces between cells give a mosaic appearance; round and ovoid spaces in the medium are formed by joining together of processes. × about 180.

FIG. 2. Experiment 99 E. Culture of thyroid cells, fixed and stained with methylene blue after 48 hours incubation. The fragment from which the culture originated had undergone 68 passages *in vitro* (166 days). The picture shows a digested area of medium filled in by cells, a mitotic figure, and a dying cell. × about 550.

FIG. 3. Experiment 92 E. Culture of thyroid cells, fixed and stained with methylene blue after 72 hours incubation. The fragment from which the culture originated had undergone 68 passages *in vitro* (165 days). One-half of the culture shows four distinct regions of cell proliferation. First, the central mass, including

the mother fragment; second, a membranous growth of cells with punctate thinning of the medium; third, piled up cells, forming a corona; fourth, fine branching processes composed of flat cells and many scattered cells. × about 22.

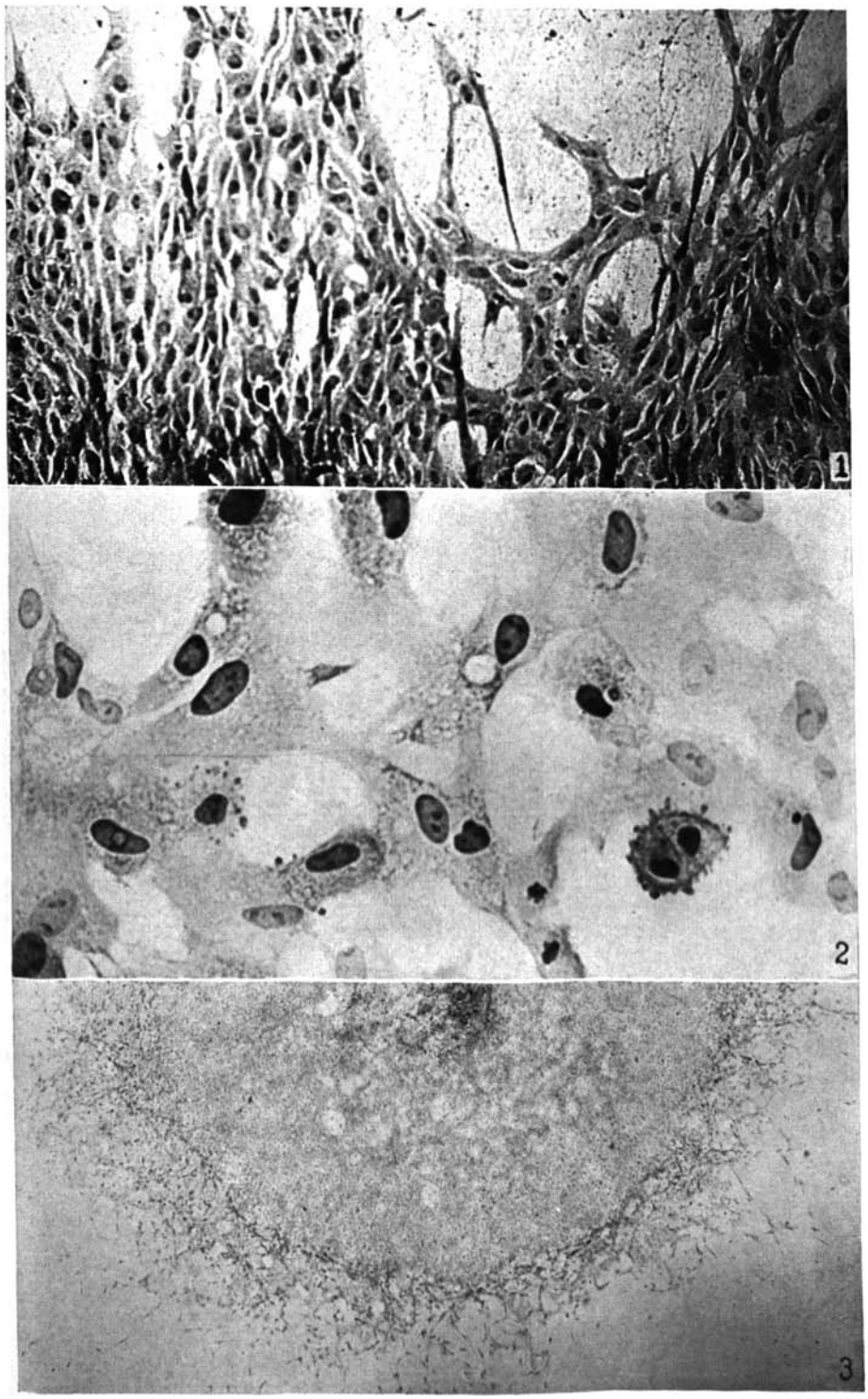
PLATE 13.

FIG. 4. Experiment 92 E-3. Pure culture of thyroid cells, fixed and stained after 72 hours incubation. The fragment from which the culture originated had been growing poorly for several passages after isolation. It then recovered and underwent 66 passages altogether *in vitro* (165 days). The photograph shows typical membrane formation with thinned out regions of the coagulum. × about 180.

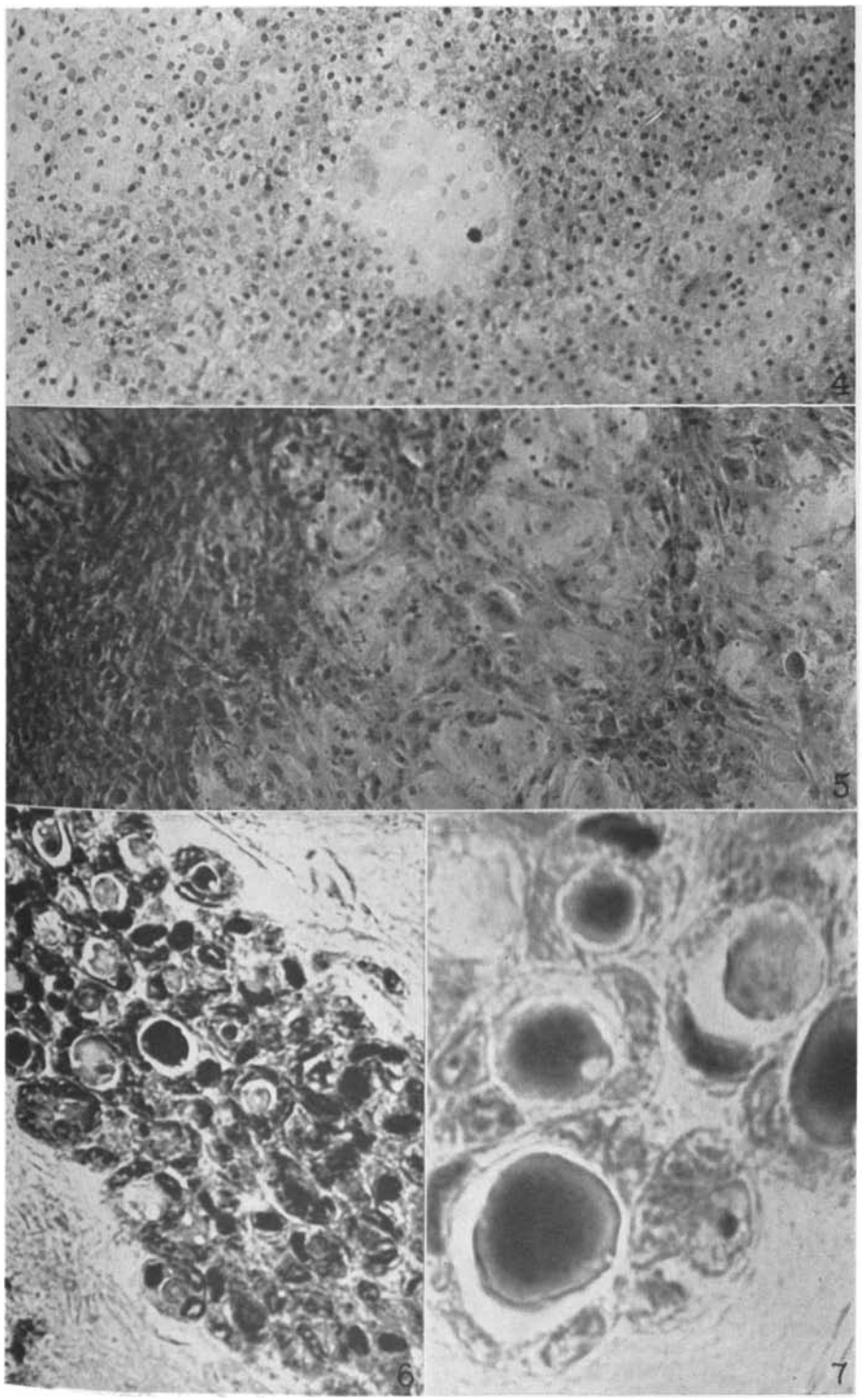
FIG. 5. Experiment 249-5. Pure culture of thyroid cells, fixed and stained with methylene blue after 72 hours incubation. The fragment from which the culture originated grew poorly for four passages. After eight passages *in vitro* (21 days), the culture had fully recovered. The picture shows part of corona of cells growing in many planes, thinned out regions in the coagulum, and membrane formation. × about 180.

FIG. 6. Experiment 173 E. Pure culture of thyroid cells, fixed, sectioned, and stained by the Kraus method after 10 days cultivation on mica cover-slips and 119 days cultivation in a D-5 flask, a total of 129 days *in vitro*. It shows a part of the cell aggregates forming a glandular structure, some of the acini of which are almost completely filled with colloid substance. The masses in the upper right and lower left corners of the picture are part of the unorganized mass of fibrin surrounding the gland and containing a few isolated cells. × about 180.

FIG. 7. Portion of same section as Fig. 6. It shows individual acini composed of large epithelial cells, more or less symmetrically arranged to form a lumen, containing colloid material in the form of round masses. × about 2,000.



(Ebeling: A pure strain of thyroid cells.)



(Ebeling: A pure strain of thyroid cells.)