

THE CULTIVATION OF LENS EPITHELIUM IN VITRO.*

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PLATE 30.

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In the course of an investigation into the nature of the nutrition of the crystalline lens and the cause of cataract, it was proposed by the author that an attempt be made to cultivate the cells of the lens *in vitro*. Such a strain of tissue might be used for controlled experiments.

Since the discovery by Harrison (1) in 1907 that cells could be caused to migrate and divide outside the body, various investigators have used the method for the study of morphological and physiological problems.

Carrel (2) developed an ingenious technique by which permanent pure strains of fibroblasts could be isolated and cultivated *in vitro*. He established the procedures by which the method of tissue cultivation is becoming adapted to its main object, the study of the fundamental problems of physiology and pathology. The early technique consisting of placing a fragment of tissue in a hanging drop of culture medium, did not allow an accurate analysis of the action of a tissue upon other tissues and upon the humors. The cells were subjected to complex influences, such as those of necrotic cells of their own type, of living and dead cells of other types, and of a medium which deteriorated spontaneously in a short time (3).

Advances have been made during the last few years, chiefly through the embryonic extract-plasma clot method of Carrel by which those difficulties and

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objections are overcome, and have contributed markedly to the progress of an important method of investigation, primarily by the isolation of pure strains of cells (4), and by the development of procedures for measuring the rate of growth and for studying the functional activity of the tissues (5, 6).

Fischer (7) isolated an unmixed strain of epithelium from the anlage of the chick iris in 1922. Ebeling (8) produced cultures of the thyroid epithelium in 1925. It has been possible to carry these cultures on for long periods of time. It has been proved that the cells remain true to type, that they do not dedifferentiate, and that they produce their typical substances *in vitro*.

In the experiments about to be reported the method and technique of Carrel (2-6), were used.¹

Experiment Demonstrating the Metamorphosis of the Lens in Vitro.

The eyes of two chick embryos of about 52 hours incubation (lens vesicle stage) were removed and the anterior third of each eye placed in a homogeneous medium consisting of equal parts of adult chicken plasma and chick embryonic extract. The concave slide method of Carrel was used. The explant was oriented so that the lens vesicle in each could be plainly observed with the microscope. They were allowed to incubate for 72 hours at 37.5°C. and in two of the specimens it was observed that differentiation of the posterior portion of the lens vesicle had occurred, the cells having elongated to form lens cortex. In the other two, due to digestion of the medium, the specimens were not in proper position for observation. Microscopic sections of the specimens were not obtained. Since this work, the author has learned of the explants of chick embryo eyes by Strangeways and Fell (9). The latter workers demonstrated by microscopic sections the differentiation of the eyes which progressed *in vitro* in a surprisingly normal way.

The Cultivation of the Crystalline Lens Epithelium in Vitro.

Experiment I.—The lens and vitreous of chick embryos of from 4 to 9 days incubation at 37.5°C. were removed by incising the posterior portion of the optic vesicle and using traction on the vitreous. Usually a ring of pigmented and non-pigmented epithelium of the rim of the optic vesicle comes away with the lens and vitreous. If the vitreous be excised and the remaining explant cultivated in the manner used by Fischer (7) a growth of epithelium from the iris anlage is obtained.

This experiment demonstrates that the epithelium of the iris anlage can be cultivated *in vitro* and also that if an unmixed strain of lens

¹ Acknowledgements are gratefully made to Drs. Alexis Carrel and Albert H. Ebeling for their many courtesies.

epithelium is desired, all extraneous tissue must be removed from the lens. Strains of iris epithelium were carried along in successive subcultures, so that a ready comparison with the cultures of the lens epithelium was always available.

Experiment II.—After many experiments, it was found that the eyes of chick embryos incubated for 5 days at 37.5°C. were ideal for dissecting the crystalline lens free from all extraneous tissue. The eyes usually measured 3 mm. in diameter and the lens slightly less than 1 mm. in equatorial diameter. Both prior to this stage and after it, the protoplasmic adhesions are such as to make it almost impossible to express the lens free from other cells. The embryo is placed in a concave slide and the amnion removed from the eye region. A sharp, cutting needle in a holder is used in each hand, one to steady the eye and the other to incise the posterior portion of the eye and draw out the vitreous on the point of the needle, bringing with it the lens and a portion of the rim of the optic cup. This specimen is placed in Tyrode (10) solution in a clean concave slide, under the binocular dissecting microscope using oculars 2 objectives A2 with transmitted light. The needles are replaced by fresh ones. These are inserted between the equator of the lens and the investing tissue, the other needle gently expresses the lens free from all extraneous tissue. This is transferred to fresh Tyrode solution and then to the embryonic extract-plasma clot medium. After 72 hours incubation it was observed that no outgrowth of cells had occurred.

This experiment demonstrates that it is possible to dissect out the crystalline lens of a chick embryo of 5 days incubation in such a manner as to free it from all extraneous tissue, and that the lens epithelium is contained within a cuticula outside of which when uninjured the cells do not migrate. (Salzmann (11) found that the lens vesicle is soon closed off from the neighboring structures by a cuticula, later the lens capsule.)

Experiment III.—Lenses from 5 day chick embryos isolated in the manner described in Experiment II were incubated *in toto* for 24 hours at 37.5°C. The cultures were then opened and the lenses removed from the media and divided into two pieces. Outgrowths of cells were obtained from the epithelium of two of four lens incubated. One of these, No. 667-2, represented the beginning of a strain of lens epithelium which will be described in Experiment VI as Strain C.

Those incubated for a longer period apparently retained no viable cells as no outgrowths of cells were obtained after section. Some of these specimens took up and retained the neutral red vital stain (1-30,000) during the first period of incubation. The trauma of the expression of these particular lenses may have had something to do with their inability to withstand cultivation outside the

body. Undoubtedly the cuticula of this stage (later the lens capsule) has much to do as a semipermeable membrane with the process of nutrition of the embryonic lens and a slight injury to this membrane would destroy the possibility of its living *in vitro*.

24 hours is sufficient time for incubation of tissue from a 5 day embryo to exclude any growth of cells extraneous to the cuticula of the lens. In our experiments on tissue from this age embryo, growth if it is to occur, will be evident after 24 hours.

This experiment demonstrates that it is possible to rule out tissue extraneous to the cuticula of the embryonic lens by cultivation and then by section of the lens to obtain a culture of an unmixed primary culture of epithelium from the interior of the embryonic lens.

Cultivation of Lens Epithelium in Vitro.

Experiment IV.—Lenses of 5 day embryos were expressed on numerous occasions in the manner described in Experiment II. Having been placed in fresh Tyrode solution, and again replacing the needles for fresh ones, the lenses were incised anteriorly and the lens epithelium stripped off easily to the equatorial region, as there exists a potential space at this stage between the lens epithelium and the cortex. The lens epithelium is then explanted on the point of a needle directly to the embryonic tissue juice-plasma clot medium. The cortex is explanted into another slide. Incubated at 37.5°C.

After 12 to 24 hours the lens epithelium begins to migrate, divide by mitosis, and multiply. After 48 hours observations are recorded. Apparently the entire explant lives, but cells do not wander out from all parts. This depends on the particular conditions of the explant, foldings, the exposure of the cells, the trauma, and the conditions of the media. Many bizarre shapes have been observed. Small pieces of epithelium do as well as large ones. Cells apparently temporarily united move out from the explant, never losing contact, however. The walls of cells in apposition are difficult of definition in the living cultures, but apparently the cells do not form a syncytium. Both membrane and peninsula formation are observed. The explant remains transparent and the newly formed cells are transparent. The wall of the healthy cell is a geometric line. Protoplasmic processes of various lengths are seen, some being very long. The cytoplasm is clear, containing granules some of which take up neutral red in weak solution and others which do not. The mitochondria are visible in the unstained cells. When the microscope substage diaphragm is opened and slight excess light admitted, the cells become invisible. The nucleus is ovoid and usually contains one or two nucleoli. All stages of mitoses may be observed. About the entire living culture there is observed a halo of transparency of the media, indicating either, the influences of the cell products on the media or, the preparation of the media by the

cells for their nutriment. The outgrowth of the cells is apparently preceded by a process of digestion of the media.

Fresh explants of lens epithelium should be cultivated on No. 0 glass cover-slip and examined with the aid of high magnification to prove the absence of any contaminating cells. Fibroblasts can be differentiated and the living pigment epithelial cells of the eye if present will show definite completely opaque rod-shaped ovoid or rounded pigment granules. The nature of these cells from the iris anlage and their appearance when cultivated *in vitro* must be thoroughly studied by anyone attempting to cultivate lens epithelium.

The explants of cortex do not grow. No cells wander or divide. Partial solution of the explant takes place by a process of hydrolysis. Disintegration of the cells occurs and the explant has a fibrillated, washed out appearance. This process can be best observed by allowing the cortex to remain in Tyrode solution and observing it with the microscope. Hydration and swelling will occur quickly, a beaded appearance is produced by globules forming in the cells. These globules rupture, the cell bursts, and the cytoplasm goes out into the solution which is isotonic for other tissues but hypotonic for lens cortex. A solution containing 1.2 per cent NaCl is isotonic for the lens cortex in that this hydrolysis does not take place, but cell migration and division do not occur. The cells are, even at this early stage of differentiation, biologically old. They have a different osmotic coefficient than the anterior lens epithelial cells.

This experiment demonstrates that lens epithelium when cultivated *in vitro* under proper conditions will migrate, divide, and multiply, and that under these same conditions, the lens cortex cells will not do so. A distinction is drawn between lens epithelium which is limited to the anterior and equatorial regions of the lens and the lens cortex which constitutes the differentiated bulk of the lens. They are both epithelial. From the anatomic arrangement of the cells the anterior epithelium and their elongation at the equatorial region passing over into the lens cortex, it may be safe to assume that the cells of the cortex are derived from the epithelial layer, or at least from those cells which are at the equator. It may be possible to grow the cells of the cortex, as long as they retain their nuclei, under different conditions of cultivation. Alterations have been made in the media, but no successful cultures have been obtained. These facts if substantiated by further experiment would lend proof to the idea that the anterior epithelium of the lens differentiates into the cortex.

Experiment V.—Extract of the 9 day chick embryo was used in place of extract of the whole body. It was made by grinding the eyes in a Wedgwood mortar

with pestle and alkali-free ground glass. It was diluted with Tyrode solution and centrifuged. The explants grew well when this was used in place of the whole body extract, but were not free from retinal pigment rods in the cells. Now either pigment cells had been explanted with the lens, or the cells were transferred with the eye extract, or the pigment rods in the eye extract were ingested by the cells. It has already been demonstrated that the lens can be explanted free from pigment rods and these could be seen with the aid of oil immersion lens. It was therefore determined to use extract of the embryo body minus the eyes. This has been done and transparent growths free from any retina pigment rods obtained. Dr. Albert H. Ebeling has been kind enough to examine some of our cultures and has found no contaminating cells in one produced by our perfected technique.

This experiment demonstrates that lens epithelium can be cultivated in media containing extract from the embryonic eye alone and also from the embryonic body minus the eyes. In the latter media, the cells can be proved free from any contaminating cells.

Experiment VI.—To produce an unmixed strain of lens epithelium which can be carried along indefinitely and used for controlled experiments.

Living explants from lenses isolated and cultivated as described in Experiment IV can be divided and two subcultures obtained. These can in turn be subdivided after cultivation and thus secondary and tertiary cultures obtained. By this means a strain of lens epithelium can be propagated from an original single explant. One of our strain (C) was obtained as described in Experiment III after having incubated the entire lens proving the absence of any cells extraneous to the cuticula. The history of this strain may be reported as exemplifying the culture of lens epithelium under these conditions.

June 28, 1926 (No. 667-3). Entire lens explanted from a 5 day embryo.

June 29 (No. 669-2). The lens remains transparent. Dissected out of the media. Cortex shelled out. Found granular, partially disintegrated. Epithelium subcultivated.

July 1. Several processes. Cellular outgrowth from explant.

July 3. Small growth, vacuolated cells. Media changed.

July 6. Good growth of cells. Membrane and peninsula formation.

July 7, 9, 12, 14, 16, 19, 21, 23, 26, 28, 30, Aug. 2, 4, 6. Media changed and culture divided and subcultivated.

One-half of the culture was put on mica cover-slip and one-half on glass cover-slip. Latter examined on the ensuing working day with oil immersion lens and then fixed and stained for further study. The culture on mica was used for the propagation of the strain. Extract of the embryonic body minus eyes was used throughout. The divided cultures about doubled in size every 48 hours. The cells are transparent and remain epithelial in type. They do not dedifferentiate.

This experiment demonstrates that lens epithelium cultivated *in vitro* can be carried on for successive generations. Strain C was carried through seventeen generations over a period of 6 weeks and was thriving when the work was discontinued, August 9. It was then ready for controlled experiments and observations on the rate and character of the growth, the cellular reactions, and the substances produced by the cells.

CONCLUSIONS.

1. Differentiation of the posterior cells of the lens vesicle into lens cortex has been observed *in vitro*.
2. It is possible to dissect out the lens of the 5 day embryo chick in such a manner as to free it from all extraneous cells.
3. Lens epithelium even at a very early stage is contained within a cuticula.
4. Lens epithelium under proper conditions will live, migrate, divide, and multiply *in vitro* in primary explants.
5. An unmixed strain of lens epithelium can be propagated from a primary explant by successive subcultures. This strain can be utilized for controlled experiments on the nutrition of the cells of the lens.

BIBLIOGRAPHY.

1. Harrison, R. G., Observations on the living developing nerve fiber, *Proc. Soc. Exp. Biol. and Med.*, 1907, iv, 140.
2. Carrel, A., and Burrows, M. T., Cultivation of adult tissues and organs outside of the body, *J. Am. Med. Assn.*, 1910, iv, 1379.
3. Fischer, A., Tissue culture, Copenhagen, 1925, Introduction by A. Carrel.
4. Carrel, A., Pure cultures of cells, *J. Exp. Med.*, 1912, xvi, 165.
5. Carrel, A., Measurement of the inherent growth energy of tissues, *J. Exp. Med.*, 1923, xxxviii, 521.
6. Carrel, A., A method for the physiological study of tissues *in vitro*, *J. Exp. Med.*, 1923, xxxviii, 407.
7. Fischer, A., A three months old strain of epithelium, *J. Exp. Med.*, 1922, xxxv, 367.
8. Ebeling, A. H., A pure strain of thyroid cells and its characteristics, *J. Exp. Med.*, 1925, xli, 337.
9. Strangeways, T. S. P., and Fell, H. B., Experimental studies on the differentiation of embryonic tissues growing *in vivo* and *in vitro*. II. The de-

velopment of the isolated early embryonic eye of the fowl when cultivated *in vitro*, *Proc. Roy. Soc. London, Series B*, 1926, c, 273.

10. Fischer, A., Tissue culture, Copenhagen, 1925, 85.

11. Salzmann, B., Anatomy and histology of the human eye ball, 1912, 197.

EXPLANATION OF PLATE 30.

FIG. 1. Fresh explant of lens epithelium. Zenker acetic-Harris hematoxylin. \times about 175.

FIG. 2. Showing mitosis and membrane formation in a strain of lens epithelium. \times about 355.

FIG. 3. Cells from tip of peninsula in a strain of lens epithelium showing fine protoplasmic processes. \times about 495.

FIG. 4. Cells from tip of peninsula in a strain of lens epithelium. Herniations of cytoplasm as first stage of degeneration of these cells. \times about 495.

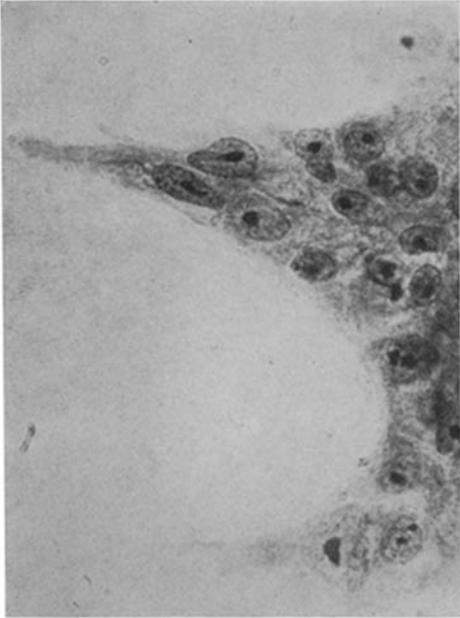


FIG. 1.



FIG. 2.

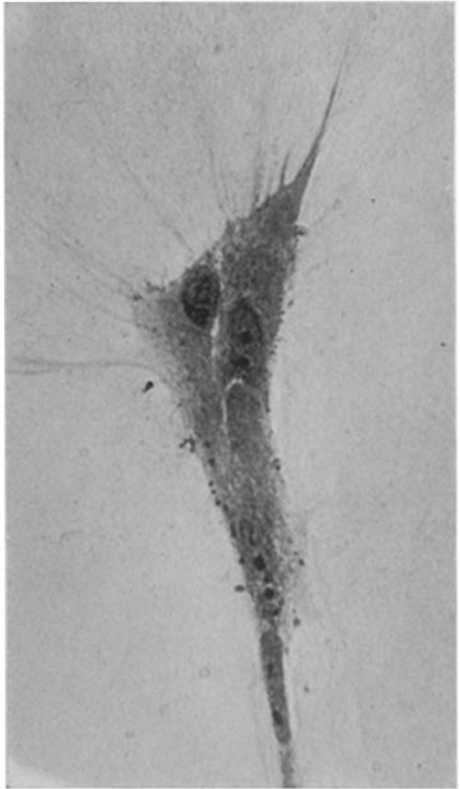


FIG. 3.

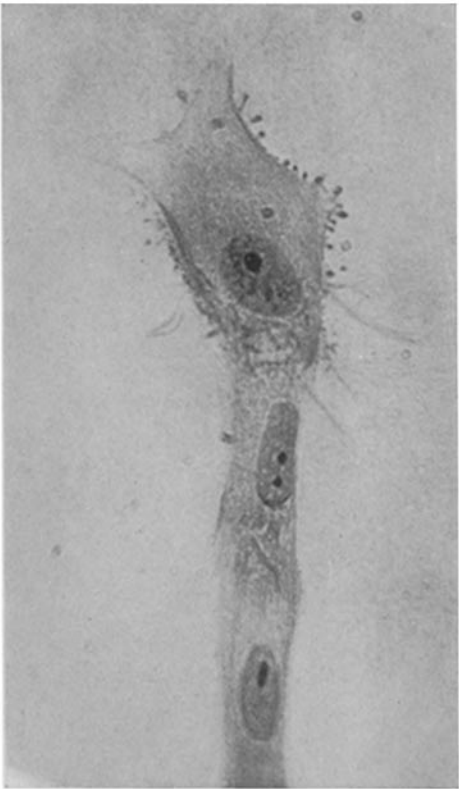


FIG. 4.
(Kirby: Cultivation of lens epithelium.)