GRANULES IN THE CELLS OF CHICK EMBRYOS PRODUCED BY EGG ALBUMIN IN THE MEDIUM OF TISSUE CULTURES.

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PLATE 51.

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It has been shown that connective tissue cells of the chick embryo become greatly altered when placed in an abnormal environment (M. R. Lewis, 1918; W. H. Lewis, 1919; M. R. Lewis, 1920; Prigosen, 1921). These alterations in appearance can be compared with certain changes which take place in pathological conditions of adult tissue such as fatty degeneration, necrosis, autolysis, phagocytosis, etc.

The present paper is a report of the results obtained when white of egg is introduced into the medium of a tissue culture of connective tissue. This substance not only has a decidedly toxic influence upon the growth of the cells, but it also produces a marked change in the appearance of the cells comprising the growth, owing to an accumulation of large, somewhat refractive granules within the cytoplasm of these cells. These granules take the eosin stain when methylene blue and eosin are used. In this regard the connective tissue cells resemble the epithelium of renal tubules filled with colloid or hyaline droplets described by MacCallum (1916).[1] On the other hand, when the stain used is iron-hematoxylin, these cells closely resemble certain cells undergoing active secretion described by Hoven (1910), Mislawsky (1913), and Saguchi (1920).

It has been shown that explants of connective tissue from the chick embryo grow in pure egg albumin (Swezy, 1915). These cultures do not live so long or attain so extensive a growth as cultures explanted in Locke-Lewis solution. Cells from the subcutaneous tissue

[1] MacCallum (1916), p. 91.

of the chick embryo, explanted into egg albumin, become filled with large round granules of different sizes (Figs. 1 and 2). It has not been possible so far to demonstrate the exact chemical nature of these granules; therefore, in order to differentiate them from other granules observed in the cells, they will be called al. granules throughout this paper. This term is not meant to signify that these granules are or are not albumin, but only to specify that they are due to the presence of albumin in the environment of the cells.

Chick Embryo Connective Tissue in Egg Albumin.

An egg was opened under sterile conditions and a small quantity of the white removed to a sterile dish. This was then cut many times with sharp curved scissors in order to obtain a solution of egg albumin. A piece of connective tissue was removed from a chick embryo of 7 to 10 days incubation and placed on the solution of egg white where it was cut up into small pieces. Each piece, with a small quantity of egg albumin, was placed on a clean cover-glass and inverted over a vaseline ring on a depression slide. After 16 to 20 hours connective tissue cells had grown out into the medium in the same manner as in normal cultures, though by no means in such great numbers or to so great an extent. All these cells contained a number of rather large granules, usually round in outline. The granules were composed of a substance which differed in appearance from the cytoplasm. They seemed to be denser, more opaque, and possessed an index of refraction different from that of the cytoplasm. In fixed preparations they stained a much deeper tone than the cytoplasm, becoming black and gray with hematoxylin but never so pale as the cytoplasm. There was great variation in the size of the granules in a given cell, as well as in those in different cells. They were in most cases much larger than either the mitochondrial bodies or the neutral red granules.

Shipley (1919)[2] described somewhat similar granules in plasma cultures of chick embryos. He stated, however,[3] that the degeneration vacuoles described by Lewis and Lewis were identical with these

[2] Shipley (1919), p. 288.
[3] Shipley (1919), p. 289.

plasma granules, and that the differences in the two bodies were due to differences in the media. This is not true in regard to the al. granules characteristic of the albumin cultures, for the two bodies, *i.e.* the al. granule and the degeneration vacuole (W. H. Lewis, 1919), may exist side by side in degenerating cultures (Figs. 3 and 5). In such cultures the al. granules were markedly different from the degeneration vacuoles in appearance and were easily distinguishable from them, not only in the living cell but also in fixed material.

Plato (1900) injected dried and powdered white of egg into the abdominal cavity of a guinea pig. Shortly afterwards leucocytes were obtained from this region and stained with neutral red. They were found to contain large, irregularly shaped, orange-red clumps, which Plato supposed were ingested particles of white of egg taken in as foreign bodies. The structures obtained in this manner do not resemble to any great extent those described above as al. granules.

After 24 to 48 hours a difference could sometimes be detected in the appearance of the individual al. granules in a given cell. Some appeared to be of a different consistency, but whether this could be expressed as more fluid is doubtful. In the stained preparations certain of the granules were much darker than others (Fig. 2) and the changed, possibly more fluid, granules of the living cell probably correspond to the more lightly stained ones in fixed cultures.

The al. granules differed from certain other types of granules in living cells, such as pigment granules, neutral red granules, and certain secretion granules, in that they were less frequently found collected around the centrosphere (Figs. 2 and 5) than in other regions of the cytoplasm. The processes of the cells were always free from al. granules. Frequently there was an extensive ectoplasm which the granules did not enter, but occasionally this was reduced to a narrow ectosarc on one or both sides of the nucleus. These granules obscured the other granules in the living cell, except at the periphery, where the mitochondria could be seen extending out into processes of the cell. Spindle-shaped cells (Fig. 1) were more numerous than were the flat cells (Fig. 2). These elongated cells contained granules in the neighborhood of the nucleus but there were none in the ends of the cells. The cells divided by mitosis, even when full of granules (Fig. 4). This is another means of differentiating these granules from

the degeneration vacuoles, as cells containing vacuoles rarely undergo mitotic division.

As a rule, all the connective tissue cells of the growth contained some al. granules. Certain other kinds of cells in the same culture, however, did not have these granules. While the number of these bodies in a given connective tissue cell increased to some extent with the age of the cultures, most of the cells contained the maximum number at the end of 24 hours.

It was not possible to observe the fate of the granules in cultures grown in egg albumin, because degeneration took place too rapidly. Generally, after 48 hours, degeneration vacuoles began to appear. These signs of degeneration took place first in the cells along the periphery of the growth. In most of the flat cells the cytoplasm was sufficiently spread out so that it could be readily seen that the region of the centrosphere was free from al. granules and that a number of degeneration vacuoles had collected there (Figs. 3 and 5). From the location of the degeneration vacuoles it is evident that they were not formed from al. granules which had become fluid due to digestion of the substance forming them. After 72 hours vacuoles were present between some of the al. granules, as well as in the region of the centrosphere (Fig. 5), and many of the cells were so degenerate that it was impossible to determine whether the vacuoles were around the granules or between them. In some of the degenerate cultures the al. granules were small and fragmented. After the vacuoles appeared the cells rapidly assumed a rounded form so that the details of the phenomenon could not be observed clearly. The vacuoles increased in number, and degeneration took place much more rapidly than in the normal cultures. Few of the cultures in egg albumin survived longer than 3 or 4 days, while most of the control cultures grown in Locke-Lewis solution lived for over 2 weeks.

Whether the albumin was taken from a freshly laid or from an incubated egg made no difference in the appearance of the al. granules, although the hydrogen ion concentration of an incubated egg differs from that of an unincubated egg. The cells of cultures made with albumin from an incubated egg also divided by mitosis (Fig. 9) and produced a small growth around the explant. After a few days, degeneration took place and the cells died about as rapidly as in albumin from the fresh egg.

Cultures in Diluted Egg Albumin.

Much the same result in regard to the accumulation of granules in the cells was produced when the albumin was diluted one-half with Locke-Lewis solution; but when the quantity of albumin was greatly reduced the results became less constant. This may have been partly due to the difficulty of obtaining a uniform solution of the egg albumin. In media containing only a small percentage of egg albumin (1 to 5 per cent) many of the cultures exhibited no accumulation of granules in the cells (Figs. 6 and 7); in others a few of the cells contained many granules, the remaining cells being entirely free from them; while in still other cultures a few small granules were present in every cell. Some cultures in diluted egg albumin remained normal for several days but later a number of the cells exhibited typical al. granules. The smaller the percentage of albumin in the medium, the more nearly normal did the cultures appear in extent of growth and length of life; in none of the percentages of albumin used did the growths become so large or live so long as they did in the Locke-Lewis solution.

Changes Produced in Cultures When the Locke-Lewis Solution Is Replaced by Egg Albumin.

The hanging drop was removed from 24 to 48 hour cultures of chick connective tissue which had been explanted in Locke-Lewis solution and was replaced by egg albumin. This caused immediate changes in the appearance of the normal connective tissue cells (Fig. 11), due not only to the change in the nature of the environment from a neutral or slightly acid fluid to a markedly alkaline jelly, but also to the manipulation. The mitochondria usually assumed the form of short rods and granules instead of filaments, and the cells became somewhat rounded, showing a number of processes. In some instances many of the cells died. Al. granules did not appear for several hours, but after 20 hours these bodies were about as abundant and of the same size as those in cells of cultures explanted directly into egg albumin. It was not observed that the mitochondria stored up albumin and changed into the al. granules. They did, however, become shorter and seemed to decrease

in number as the cells became full of granules. The size of the growth of cultures under these conditions was always larger than that of cultures explanted directly into egg albumin, because the growth had already become extensive in the normal medium before the egg albumin was placed upon it. When these cultures degenerated, vacuoles appeared in the cytoplasm in the usual manner, regardless of the number or size of al. granules in the cells. Some of the cells degenerating under these conditions presented unusual appearances, especially in the region of the centrosphere. In some instances large bodies resembling a certain type of giant centrosphere described by W. H. Lewis (1920) were observed (Fig. 12).

Effect Produced by Vital Dyes.

When a solution of Janus green was placed upon a culture the cells of which contained al. granules, the mitochondria became a bright blue-green, while the al. granules remained unstained. After the cells began to die, as they soon did owing to the toxic effect of the stain, the al. granules sometimes took on the green color. There were fewer mitochondria in cells living in egg albumin than in those in Locke-Lewis solution and the filaments extending out into the processes were shorter. Scattered among the al. granules were a few, short, rod-shaped and granular mitochondria. In the region of the centrosphere the mitochondria were more like those of normal cultures.

Trypan blue dissolved in Locke-Lewis solution, when placed upon the cultures, failed to stain any body in the cell until a number of hours had elapsed; then a few small blue granules appeared but the al. granules remained colorless (Fig. 8).

Neutral red was taken up by the vacuoles and granules in these cells in about the same manner as in the normal cultures. After a few hours the al. granules were sometimes unstained, again they appeared to be pale pink, and in other cases they seemed to be outlined with red.

Fixed and Stained Preparations.

The al. granules were more successfully fixed and stained than were the mitochondria. Methods of fixation which did not preserve the mitochondria frequently afforded good results in as far as the al. granules were concerned. An easy method of fixation by which both types of granules are preserved is to wash off the albumin by means of warm Locke-Lewis solution and drop the cover-slip into Zenker's solution from which acetic acid has been omitted. With iron-hematoxylin the al. granules stained in various shades, from dense black to gray, the result probably depending upon the concentration of the material forming the granule. In appearance the cells bear a striking resemblance to the secretion cells illustrated by Hoven (1910), Saguchi (1920), Mislawsky (1913), and others. All the forms described by these investigators to show stages in the change from mitochondria into secretion granules can be found. This suggests that Scott (1916) probably was correct in his view that the granules present in certain gland cells may be material accumulated in the cytoplasm and not necessarily formed directly from any preexisting structure of the cell, such as the mitochondria.

Cultures of Fish Embryos in Egg Albumin.

A few cultures of embryos of *Fundulus heteroclitus* were made in egg albumin. The cells of these cultures grew much more slowly but almost as extensively in the egg albumin as did those in the fluid media described by Dederer.[4] Instead of all the mesenchyme cells becoming filled with al. granules, as they do in chick cultures, only a few along the edge of the growth showed the granules. This may be due to the fact, as shown by Dederer, that the mesenchyme cells grow along the cover-slip and are in most places covered by a layer of ectoderm cells which separate them from the medium. The ectoderm did not form al. granules to any appreciable extent. The peripheral mesenchyme cells, which contained al. granules (Fig. 10), appeared much the same as did those of the chick embryo.

[4] Dederer, P. H., personal communication.

DISCUSSION AND SUMMARY.

It is difficult to understand what factors may be concerned in the formation of the al. granules. The phenomenon may be concerned with changes in the cell membrane due to an abnormal environment; that is, material which would otherwise be excluded may be permitted to enter the cell, or, on the other hand, certain substances may be prevented from passing out of the cells. Previous investigators have shown that mesenchyme cells sometimes engulf certain foreign bodies, and it is possible that the solution of white of egg is ingested in the same manner. When a solution of peptone was placed on the cells instead of egg white, the phenomenon did not occur (Fig. 13); the cell remained normal and degenerated in the usual manner (Fig. 14). This would seem to indicate that the al. granules are not formed from peptone. Regardless of the factors involved, it is evident that egg albumin in the medium of tissue cultures of chick embryos causes the formation of numerous large granules in the cytoplasm of the connective tissue cells. This phenomenon is associated with unfavorable conditions for the life of the cells and results in the rapid death of the cultures.

BIBLIOGRAPHY.

Hoven, H., Contribution à l'étude du fonctionnement des cellules glandulaires. Du rôle du chondriome dans la sécrétion, *Anat. Anz.*, 1910, xxxvii, 343.

Lewis, M. R., The formation of the fat droplets in the cells of tissue-cultures, *Science*, 1918, xlviii, 398.

Lewis, M. R., The formation of vacuoles due to *Bacillus typhosus* in the cells of tissue cultures of the intestine of the chick embryo, *J. Exp. Med.*, 1920, xxxi, 293.

Lewis, W. H., Degeneration granules and vacuoles in the fibroblasts of chick embryos cultivated *in vitro*, *Bull. Johns Hopkins Hosp.*, 1919, xxx, 81.

Lewis, W. H., Giant centrospheres in degenerating mesenchyme cells of tissue cultures, *J. Exp. Med.*, 1920, xxxi, 275.

MacCallum, W. G., Textbook of pathology, Philadelphia, 1916.

Mislawsky, N., Ueber das chondriom der pankreaszellen, *Arch. mikr. Anat., Ite Abt.*, 1913, lxxxi, 394.

Plato, P., Ueber die "vitale" Färbbarkeit der Phagocyten des Menschen und einiger Säugethiere mit Neutralroth, *Arch. mikr. Anat.*, 1900, lvi, 868.

Prigosen, R., The formation of vacuoles in connective tissue cells due to abnormal conditions, *Bull. Johns Hopkins Hosp.*, 1921, xxxii, No. 362 (in press).

Saguchi, S., Studies on the glandular cells of the frog's pancreas, *Am. J. Anat.*, 1919–20, xxvi, 347.

Scott, W. J. M., Experimental mitochondrial changes in the pancreas in phosphorus poisoning, *Am. J. Anat.*, 1916, xx, 237.

Shipley, P. G., The physiological significance of the reaction of tissue cells to vital benzidene dyes, *Am. J. Physiol.*, 1919, xlix, 284.

Swezy, O., Egg albumen as a culture medium for chick embryo tissue, *Biol. Bull.*, 1915, xxviii, 47.

EXPLANATION OF PLATE 51.

All the figures are camera lucida drawings of cells from the subcutaneous tissue of chick embryos; oil immersion lens and No. 6 ocular.

FIG. 1. A spindle cell containing al. granules from a 48 hour culture of an 8 day embryo in egg albumin.

FIG. 2. A flat cell containing al. granules from a 48 hour culture of a 7 day embryo in egg albumin.

FIG. 3. A flat cell containing al. granules; a few degeneration vacuoles may be seen in the region of the centrosphere. 48 hour culture of an 8 day embryo in egg albumin.

FIG. 4. Mitosis of a cell containing al. granules.

FIG. 5. A cell from a 24 hour normal culture which had egg albumin on it for 72 hours.

FIG. 6. A cell from a 4 day culture in diluted egg albumin. This cell does not contain al. granules.

FIG. 7. A degenerating cell from a 4 day culture in diluted egg albumin.

FIG. 8. A cell from a culture of an 8 day embryo in egg albumin which had been stained with trypan blue.

FIG. 9. A cell undergoing mitotic division from a culture of an 8 day embryo in albumin from an egg incubated for 12 days.

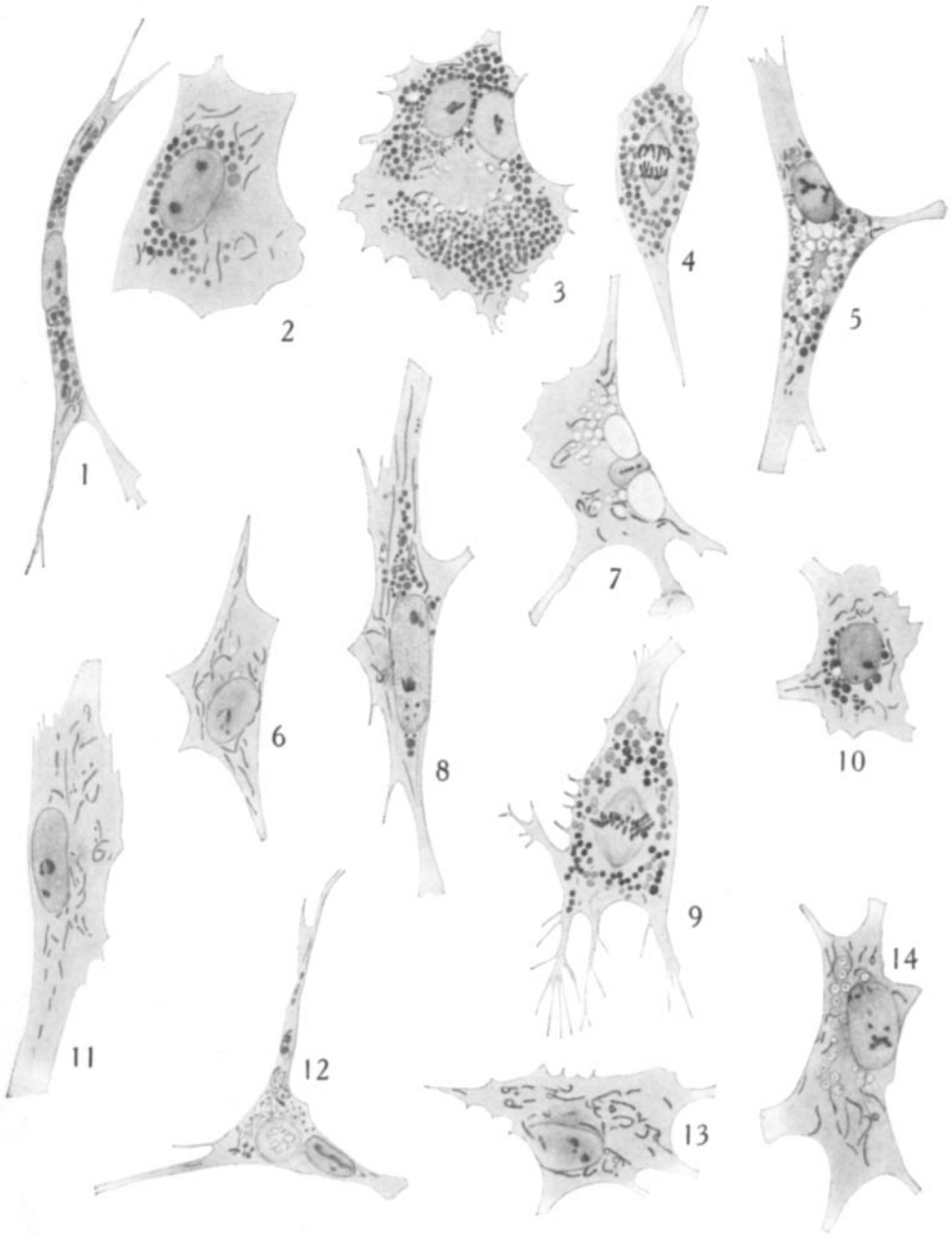
FIG. 10. A cell from a culture of *Fundulus* embryo in egg albumin.

FIG. 11. A cell from a 10 day culture of an 8 day embryo in Locke-Lewis solution.

FIG. 12. A cell containing a giant centrosphere from a culture which had the Locke-Lewis solution replaced by egg albumin for 24 hours.

FIG. 13. A cell free from al. granules which had 1 per cent Bacto peptone on it for 24 hours.

FIG. 14. A cell from a culture which had 5 per cent Bacto peptone placed upon it. This cell contains no al. granules but has degeneration vacuoles.



(Lewis: Tissue culture of chick embryos.)