The Origin and Nature of the Nucleolus.

By

Mary S. Gardiner.

Associate Professor of Biology, Bryn Mawr College.

With Plate 28.

Cytoologists whose investigations have been concerned with the nucleolus have either regarded it as related to the formation of the chromosomes, or as connected actively or passively with cellular metabolism. Montgomery (1898), Ludford (1922), Wilson (1924), Fikry (1930), and Sharp (1926 and 1934) have all presented comprehensive reviews of these investigations which show little agreement in the interpretation of nucleolar function. Those investigators who relate it to chromosome formation differ among themselves in regarding the connexion either as a direct one, the nucleolus representing an active agent in the elaboration of chromatin, or as an indirect one, the nucleolus contributing non-chromatinic material to the chromosomes. Those who support the metabolic view also disagree in believing, on the one hand, that the nucleolus is an active agent in the vital processes of the cell, and on the other hand, that it is ergastic in nature, composed either of waste products of the cell's metabolism, or of material to be used in the manufacture of specific cell products.

In 1927, as a result of the study of oogenesis in Limulus polyphemus, the author suggested that the nucleolus of the oocyte was formed originally of extra-cellular material, and presented evidence for the conclusion that it contributed to the cytoplasm material which was used in the formation of yolk. The fixing and staining methods used in that study did not make possible a distinction between chromatin and other cellular constituents, and at the suggestion of Dr. E. V. Cowdry, the method of Feulgen was used in a later investigation, in order to determine whether or not the nucleolus in these oocytes is a chromatin nucleolus, or an amphinucleolus in the sense that it
contains both chromatin and plastin or other non-chromatinic substance. The study was further expanded to include an investigation of the relation of the nucleolus to vitellogenesis in the mealworm, *Tenebrio molitor*, and to the secretory activities of other cells, both plant and animal.

The epidermal gland-cells of two of the Solanaceae, *Petunia violacea* and *Nicotiana longiflora*, were selected as being comparable in their function to those animal cells which manufacture and discharge secretory products. Except for the conducting and supporting tissues, all plant-cells are essentially secretory in nature, in that they are concerned either with photosynthesis or with the accumulation and further elaboration of photosynthetic products. To this extent, the parenchyma and mesophyll cells are analogous to those animal cells which synthesize nutriment and store it for subsequent utilization.

Cells comparable to those of the epidermal glands of sticky-leaved plants like the Solanaceae are the animal gland-cells which produce and release a specific secretion, such as epidermal and intestinal mucous cells and the enzyme-secreting cells of the digestive tract. Included in this investigation, therefore, were the cells of the enteric epithelium of *Tenebrio*. The digestive tracts of both early and late larval instars were sectioned and a comparative study made of the cells lining the different regions of the gut as well as the muscle, investing connective tissue and so-called ‘regenerative cells’.

A variety of fixing agents and stains was used in order to preserve the different cell elements and so obtain a complete, if composite, picture of the various structures at all phases of their activity. The fixing and staining methods were as follows:

\textit{Limulus polyphemus.}

Gonads from young animals (about 7\text{\textfrac{1}{2}} inches in length including telson): Feulgen’s corrosive-acetic, 6–10 minutes hydrolysis, fuchsin-sulphurous acid 1\text{\textfrac{1}{2}}–1\text{\textfrac{1}{4}} hours, Lichtgrün.

Gonads from adult animals: equal parts absolute alcohol, glacial acetic acid, and chloroform with sublimate to saturation. Hydrolysis and exposure to fuchsin-sulphurous acid as above, Lichtgrün.
Tenebrio molitor.

Gonads from larvae, pupae, and beetles: Feulgen's corrosive-acetic, 6–10 minutes hydrolysis, fuchsin-sulphurous acid 1½–1¾ hours, Lichtgrün.

Bouin, haematoxylin and erythrosin.

Mann-Kopsch (6–10 minutes hydrolysis, fuchsin-sulphurous acid 1½–1¾ hours. In some preparations, Zenker Lichtgrün.

Osmic vapour, Flemming's triple stain.

Champy-Kull, Lichtgrün.

Zirkle's copper acetate and copper bichromate mixture,

Broussy's rapid haematoxylin method counterstained with 0·5 per cent. acid fuchsin.

Digestive tracts from larvae: Zenker, 6–10 minutes hydrolysis, fuchsin-sulphurous acid 1½–1¾ hours, Lichtgrün.

Feulgen's corrosive-acetic, hydrolysis, and exposure to fuchsin-sulphurous acid as above, Lichtgrün.

Petunia violacea.

Buds, young stems and leaves: Feulgen's corrosive-acetic, 6–10 minutes hydrolysis, fuchsin-sulphurous acid 1½–3 hours. Zenker, hydrolysis and exposure to fuchsin-sulphurous acid as above, Lichtgrün.

Nicotiana longiflora.

Buds, young stems, and leaves: Zenker, 6–10 minutes hydrolysis, fuchsin-sulphurous acid 1½–1¾ hours, Lichtgrün.

The basis for Feulgen's test for thymo-nucleic acid, which since 1924 has been widely used by cytologists as diagnostic of the presence of chromatin, lies in Schiff's aldehyde reaction. In Feulgen's test, the aldehyde is the carbohydrate released from the nucleic acid component of chromatin after hydrolysis with normal HCl; this carbohydrate, a d-ribodesose (Levene, 1931), combines with the active principle of fuchsin-sulphurous acid, an N-sulphinic acid with the formula R·H<SO₂H

(Weiland and Scheuring, 1921), to form a blue-red, almost purple, substance. The validity of the test obviously rests upon two conditions:
(1) the absence from the tissues of any aldehyde either produced by the cell itself, or present as a consequence of fixation, which might combine with the sulphinic acid; and (2) the prevention of oxidation of the fuchsin-sulphurous acid, whereby the fuchsin colour might be restored, and the solution act as a stain rather than a reagent.

With these limitations in mind, Feulgen (1924) cautions especially against the use of fixatives containing aldehydes and oxidizing agents, and recommends particularly alcohol, alcohol-acetic and sublimate-acetic mixtures. As these are all fixatives peculiarly destructive of cellular detail, they are undesirable from the cytological point of view, however favourable from the microchemical. Bauer (1932) has shown that other fixatives may be used provided that there is careful washing out of the reagent so that none is left uncombined in the tissues and provided also that the length of hydrolysis is appropriately adapted.

In order to check the reaction of the material used in this investigation to hydrolysis and treatment with fuchsin-sulphurous acid after fixations other than Feulgen's, test slides were made so that sections of both the plant and animal tissues in the different fixations could be subjected to subsequent identical treatment. One or two sections of the different tissues and of the differently treated tissues were mounted together on one slide, and placed so close together that it was possible to get portions of adjacent sections simultaneously in the field of vision with a high dry objective. In this way it was possible to compare the same material in different fixations, and different material in the same fixation, in respect to the effect upon it of identical periods of hydrolysis and exposure to fuchsin-sulphurous acid.

As far as the reaction is concerned, there was no difference between Tenebrio gonads fixed in Zenker and in corrosive-acetic. Similarly, Petunia tissue fixed in Zenker and in corrosive-acetic reacted typically after the 3 hours hydrolysis recommended by Feulgen for plant tissue, although after 1 and 1½ hours hydrolysis the nuclei were still colourless and the effect was produced only in the walls of the tracheal tubes and in certain of the nucleoli. A similar result from plant material
was obtained by Margolena (1932), and is to be expected since lignin, cutin, and suberin all contain furfural. A comparison between Petunia and Nicotiana material in both fixations, and between the plant and animal material in Zenker, Bouin, and corrosive-acetic showed that in each case the result was the normal one. On the other hand, the ovaries of Tenembrio fixed in Champy-Kull and in Mann-Kopsch did not give a typical reaction; the colour produced was the yellow-red of ordinary fuchsin staining and quite distinctly different from the typical blue-red of the 'nucleal' compound. It is probable that in these and similar fixatives the colour is restored to the reagent by oxidation and the effect is that of the commonly used aniline dye.

In all the following observations, the chromatinic nature of any cell component was accepted only when the colour produced was similar to that developed in the chromosomes or chromatin threads after the complete Feulgen technique.

Observations.

A. Limulus polyphemus.—In young animals from 7 to 10 inches in length, oogonia and young oocytes may be found in the developing ovarial tubules lying close to the liver tubes. In the very young oocyte, just entering the growth stage, the cytoplasm is clear and the nucleus is filled with a chromatin network. At the nodes of the net, the chromatin may be aggregated to form small lumps or spherules, but there is no plasmosome (fig. 1, Plate 28). With growth of the cytoplasm, droplets of material appear in it which stain a clear light green. These are homogeneous in appearance, and look like small drops of coagulated liquid. The chromatin of the nucleus is rather more condensed, and there is still no plasmosome (fig. 2). In older oocytes, both nucleus and cytosome increase in volume, while the amount of chromatin remains approximately constant, and within the nuclei may be found one or more spherical structures, staining light green like the cytoplasmic inclusions of younger oocytes. These are small nucleoli or plasmosomes, now evident for the first time as intranuclear elements. The chromatin is partly collected around their margins, partly
distributed through the nucleus as fine threads (fig. 3). In the cytoplasm are found small green spherules, occasionally lying in contact with, or very close to, the nuclear membrane. In the oldest oocytes found in these young animals, there may be one or more large plasmosomes, with some chromatin collected at the periphery and some scattered through the nucleus as very small threads. The plasmosomes usually show vacuolization (fig. 4). In the youngest oocytes found in the adult females, there is a single, large, greatly vacuolated nucleolus to whose periphery chromatin is attached, and several small, similarly staining spheres scattered through the nucleus and often in contact with its membrane (fig. 5). In older oocytes, it is impossible to detect any Feulgen-reacting substance; the nucleolus persists as a definite green structure, showing more and more vacuolization, until finally, in oocytes where there is a large accumulation of yolk, it appears as little more than an empty shell.

The failure to demonstrate chromatin in older oocytes has two possible explanations. As Koch (1925) has suggested, the chromatin may undergo some profound chemical change which renders it incapable of producing the reaction, or it may be so finely dispersed in the greatly enlarged nucleus that it does not give a visible reaction (Ludford, 1927). The latter seems the more plausible explanation for reasons to be presented in a later section of this paper.

B. Tenebrio molitor: 1. Gonads.—The development of the reproductive system may easily be followed in larval and pupal stages of this insect. A sheet of cells is differentiated from the genital ridge, which in the female divides into several strands, the terminal threads or germaria. Each germarium dilates posteriorly into an oval chamber packed with oocytes, which at the lower end in older pupae and young beetles differentiate into ova and follicle cells. Each ovary is thus composed of several ovarioles, at the upper end of which are found the youngest oocytes; at the lower, yolk-laden ova completely enclosed in follicle cells, and between these two extremes, oocytes in progressive stages of development.

The ovaries lie embedded in the fat-body, with the finger-
like ovarioles projecting into the fatty-mass (fig. 6). As these are solid structures, composed solely of an outer layer of epithelial cells and an inner core of germ-cells, the only external source of nutriment for the developing oocytes is the fat-body which can be seen to undergo disintegration during the pupal and early adult stages.

In a larva just before pupation, the germarium, anterior to its attachment to the ovariole, is a slender hollow tube, in which oogonia are rapidly dividing and filling the central region (fig. 7). In the oogonia, the chromatin is distributed as a network in the nucleus and there is no nucleolus (fig. 8). In the youngest oocytes, found at the junction of germarium and ovariole, the cytoplasm is clear, and the chromatin is to a great extent collected around the periphery of the nucleus, which still lacks a nucleolus (fig. 9). In the oocytes lying at the upper end of the ovariole, both nucleus and cytosome have increased in volume, and the peripheral distribution of the chromatin is even more marked (fig. 10). In the oldest oocytes in the gonads of these larvae, the chromatin takes on a very characteristic appearance, lying in broad, ribbon-like bands across the nucleus, which contains no nucleolus (fig. 11). The cytosome also is clear of granules and its volume is small compared to that of the nucleus.

Oocytes at this stage of development are found in the ovarioles of pupae of all ages and of beetles still developing ova. They may therefore be compared with older oocytes in the same animal under identical conditions of fixing and staining. In pupae just before hatching and in beetles, oocytes with these ribbon-like nuclei lie at the upper end of the gonad and by progressing posteriorly along the ovariole, a complete series of stages may be obtained from this one to that of the fully formed ova. In oocytes slightly older than those represented in fig. 11, there is an increase both in nuclear and in cytoplasmic volume, the latter increasing to a greater extent than the former. The amount of chromatin appears approximately constant, and still forms broad bands within the nucleus. In Feulgen preparations, green spheres are present in the cytoplasm, usually lying close to the nuclear membrane, and against the membrane are found...
homogeneous areas, staining green, but with none of the granular appearance of the cytoplasm (fig. 12).

The appearance suggests that a clear fluid has been coagulated in the immediate neighbourhood of the nucleus. In Bouin preparations, this liquid appearance is lacking, but fine blue granules lie in profusion on either side of the nuclear membrane (fig. 13). It is of course impossible in the Bouin material to distinguish the chromatin from other cell constituents taking haematoxylin, but the Feulgen preparations show clearly that this perinuclear substance is not chromidial. It first appears in the oocytes at the margin of the ovariole; later, the central as well as the marginal oocytes show this condition. This suggests very strongly that a liquid is diffusing into the gonad from the tissue surrounding it. Neither Bouin nor Feulgen preparations of oocytes at this stage of development show a nuclear inclusion which might be justifiably called a plasmosome.

In still older oocytes, such as those which in young beetles lie at the lower end of the ovariole and as yet have acquired no follicle, the cytoplasm shows a marked increase and the outline of the cell may be irregular, presenting an amoeboid appearance. This is probably caused by the indentation of its surface by the mass of cells, potential ova, and follicle cells, with which it is surrounded, rather than by activity of its own protoplasm. The chromatin in these oocytes gives a pronounced Feulgen reaction, and lies as disconnected threads in the nucleus. Within the nucleus also are found one or more spheres, identical in staining with those seen in the cytoplasm of younger oocytes, and similar granules may be seen in the cytoplasm lying close to the nuclear membrane (fig. 14).

In oocytes surrounded by a follicle, there is a relatively enormous increase in cytoplasm and in nucleoplasm, while the substance giving the Feulgen reaction, i.e. the chromatin, appears no greater in amount than in the smaller cells. There is as yet no 'formed yolk' in the cytoplasm; at least, in Feulgen preparations, there are no large, green-staining spheres which are found in older oocytes and in ova, but osmic preparations show fine black granules scattered through the cytoplasm which are especially numerous in the vicinity of the nucleus showing
that yolk-synthesis is in progress. In Feulgen preparations, the chromatin lies as irregular rings or rods on an amorphous mass of fairly dense green material, and near the chromatin are found several large, green spheres (fig. 15) which may occasionally be in actual contact with the chromatin masses, but by no means uniformly so. In older oocytes, in which the nucleus still occupies a central position and the cytoplasm is devoid of 'formed yolk', the green spheres are less deeply staining, often irregular in shape and sometimes vacuolated (fig. 16). The vacuolization is pronounced in oocytes in which 'formed yolk' is present (fig. 17), and the stainable green substance is greatly decreased in amount.

In oocytes whose cytoplasm is packed with yolk-spheres which have been preserved by the Feulgen technique, the nucleus has left its central position and moved to the periphery of the cell. Oocytes in this stage are still enclosed in a follicle and lie at the posterior end of the ovariole at the origin of the oviduct. The nuclei are enormous in comparison to those of the oogonia, younger oocytes and follicle cells, and a small amount of Feulgen-reacting substance is found in them, lying on a mass of nucleoplasm staining more deeply than that in the rest of the nucleus. Near this chromatin may be found one or more faint green spheres with pale centres and slightly darker rims (fig. 18). In eggs taken from the vagina of a female which had already begun oviposition, a single very faint green structure could be found in the large nucleus some distance removed from the small but intensely reacting chromatin mass (fig. 19).

The diffusion of material from the fat-body into the ovarioles is demonstrated clearly by preparations fixed in Zirkle's copper acetate-copper bichromate mixture (Zirkle, 1931) and stained in haematoxylin and acid fuchsin. This method of fixation preserves the chromatin and plastin and dissolves the mitochondria, thus eliminating the 'danger of confusing any fragment of nucleolar material extruded into the cytoplasm with the mitochondria' (Zirkle, 1928). In such preparations, droplets of material which take the acid stain are found in the ovarioles, both inside and between the oocytes. The droplets within the oocytes lie scattered in the cytoplasm and in many cases against or just inside the nuclear membrane, corresponding in size and
distribution to the green spherules of the Feulgen preparations. The acid stain is similarly taken up by the substance in the cells of the fat-body; these droplets and those dispersed inside the gonad represent the only fuchsinophil material in these preparations. The chromatin and cytoplasm of the oocytes and of the connective tissue cells surrounding the ovariole take haematoxylin and in marked contrast to their dark colour is the clear red of the nutrient globules in the fat-cells and of the material dispersed inside the ovary and collected in the nuclei of the older oocytes.

In gonads from beetles fixed in osmic vapour (1 ½ hours at 37° C.), small spheres of yellowish brown colour can be found both between and inside the oocytes. These give every indication of being the same structures found in the Feulgen and copper acetate-copper bichromate preparations, and like them react in this fixation as do the nutrient globules of the fat-body.

The nucleoli which eventually appear in the oocytes arise by coalescence of the smaller droplets in the nuclei. In *Tenebrio*, there may be three or four small nucleoli which have originated in this way, and which initially stain deeply and uniformly with haematoxylin, Lichtgrün, or acid fuchsin, but later show greater or less vacuolization. In copper-acetate-bichromate preparations, the vacuolization is not so marked, but there is a distinct colour difference, the newly formed nucleoli staining quite a deep red like the nutrient spherules, and those in the older germ-cells a much paler colour.

No comparable structures can be found in the developing spermatocytes. In Feulgen preparations of testes, the stainable mass described by Stevens as appearing during the condensation stage between the two meiotic divisions is not homogeneous in nature, but distinctly granular like the protoplasm (Fig. 20 and Stevens, 1905, fig. 188). The intra- and extra-nuclear spherules in the oocytes are never granular in appearance. Fig. 21 represents a young spermatid with a granular green mass in the cytoplasm below the nucleus (cf. Stevens, 1905, figs. 198 and 199), which represents the remains of the spindle. During the whole course of spermatogenesis, there cannot be demonstrated any intranuclear bodies like the plasmosomes which are so conspicuous in the yolk-forming oocytes.
2. Digestive tract.—Fig. 22 is a drawing of the digestive tract of a half-grown larva, showing the areas from which sections were made. In the youngest larvae, those about 2 mm. long, the gut was fixed as a whole and cut longitudinally; in the older and larger ones, it was cut transversely into several portions, fixed and cross-sectioned, and a comparative study made of the cells of the different regions.

Histologically, four types of cells may be recognized: (1) the epithelial cells lining the cavity, and resting on a basement membrane; (2) regenerative cells, which are found in groups at the bases of the epithelial cells; (3) muscle cells, forming a relatively thick circular layer and a much less extensive longitudinal one; and (4) the epithelial cells of the investing connective tissue.

In the region of the mid-gut, the enteric epithelium is very much folded and the cells are long and slender with granular cytoplasm, often greatly vacuolated. It is in this region that digestion for the most part takes place and the glandular cells are more or less localized. Fig. 28 is a drawing from the anterior region of the mid-gut (area 1 in fig. 22); fig. 24, one from area 2 in fig. 22, showing the elongated cells and the crypt-like indentations of the lining epithelium.

In the anterior region of the mid-gut (area 1) the cells of the enteric epithelium resemble those of the fore-gut and few of them show nucleoli (fig. 23). In the folded portion (areas 2 and 3), the nuclei of the gland-cells all contain nucleoli which stain with Lichtgrün, some more deeply than others, but rarely show vacuolization (fig. 24). In contrast to the nuclei of these cells, those of the regenerative cells lying at the bases of the gland-cells are deficient in nucleoli as are those of the investing tissue. There is also a marked diminution in the number of nucleolated cells in the posterior region of the mid-gut. In the areas 2 and 3 of fig. 22, nucleoli are found in nearly all the cells lining the gut; there are fewer cells containing them in the anterior region of 4, while they are virtually absent in the posterior region of 4, and in the whole extent of the hind-gut. None could be demonstrated in the cells of the so-called 'rectal glands'.
C. Petunia violacea.—The gland-cells of Petunia which produce the sticky substance on the stems and leaves arise from epidermal cells, certain ones of which enlarge and push out above the others (fig. 25). By a series of divisions a chain of cells is formed, the terminal one of which is the actively secreting cell, the others forming a supporting stalk. The gland-cell is readily distinguished from its fellows by its dense cytoplasm and the zymogen granules found in it.

Material fixed in Feulgen’s corrosive acetic, hydrolysed and exposed to fuchsin-sulphurous acid for 3 hours, as Feulgen recommends for plant tissue, gave a typical reaction, as did also material fixed in Zenker, hydrolysed and exposed to fuchsin-sulphurous acid for 1½ hours. Nucleoli staining a vivid green were found as constant elements of the nucleated cells; these plasmosomes lay in a clear area in the centre of the nucleus on whose surface the chromatin was distributed as a network. The nucleoli of the gland-cells are large and contain a single spherical nucleolus with a central vacuole (fig. 26). These nucleoli are larger and less deeply staining than those of the parenchyma cells, which, however, frequently show vacuolization (fig. 28).

In the prophases of mitosis, the nucleolus persists as a homogeneous structure in close association with the spireme, portions of which may partly or completely encircle it (fig. 29). In figs. 30 and 31 two cells in metaphase are shown with the nucleolus remaining as an intact structure in each. In fig. 30, one of the fourteen bivalents overlies the nucleolus, but it is impossible to establish a definite connexion between the two structures. Each one of the chromosomes seems to be surrounded or to lie very close to a green mass resembling the nucleolus in staining capacity.

In the tissue fixed in corrosive-acetic, hydrolysed and exposed to fuchsin-sulphurous acid only 1½ hours, the nucleoli of the gland-cells, and in some cases those of the upper cells on the stalk, were definitely red. This colour was that of ordinary fuchsin-staining and not of the ‘nucleal’ compound, suggesting that the colour had been restored to the reagent by some means. The effect was a local one, restricted to these nucleoli only, and
not apparent in the nuclei or cytoplasm of these or adjacent cells. This is of interest in the light of Stauffacher's work on the chemistry of the nucleolus, which he found to contain iron in larger amounts than other regions of the cell. He presumes this iron to be concerned with cellular respiration, and regards the nucleolus as an oxidizing or oxygenating centre, in which case restoration of the fuchsin colour might be assumed to be caused by local oxidative effects. It should also be noted that Motttram (1933) reports that nucleoli in the root-tips of broad beans after gamma radiation occasionally 'stain with Feulgen's method'.

D. Nicotiana longiflora.—The gland-cells in Nicotiana arise as do those in Petunia, but the glands are compound, not single cells. A group of secretory cells is developed on the tip of a stalk which has arisen by division of an epidermal cell (figs. 32, 33, and 34). In Zenker preparations with Feulgen treatment, there are one or more nucleoli in all the cells; the chromatin in interkinesis is spread throughout the nucleus as a reticulum and the nucleoli are suspended in the meshes of the net. The cytoplasm of the gland-cells is crowded with secretory granules, and the nucleoli of these cells appear homogeneous and non-vacuolated. In the dividing cells the large number of chromosomes (2n = 48) makes a study of their relationship to the nucleolus difficult, as they are very closely massed. Fig. 35 represents an anaphase where the dense chromosomal mass surrounds nucleolar material, a condition typical of these mitoses.

DISCUSSION.

1. The 'nucleal reaction'.—Since the 'nucleal reaction' depends upon combination of the N-sulphinic acid in fuchsin-sulphurous acid with the aldehyde component released from a molecule by mild partial hydrolysis, the failure of any substance to give it may result theoretically from any of the four following causes:

   (a) The substance may not contain an aldehyde component.
   (b) The hydrolysis may be insufficient to release the aldehyde group from the molecule.
   (c) The hydrolysis may result not only in the splitting off of the aldehyde group but also in its disintegration, so
that it cannot react with the sulphinic acid to form the new compound.

(d) The aldehyde-containing substance, and consequently the aldehyde set free on hydrolysis, may be so small in quantity that, although the reaction actually occurs, the compound is too minute in amount to be visible even with high magnifications.

Typically, chromatin gives the reaction, but it does not invariably do so, nor is it the only cellular substance capable of it. Other aldehyde compounds, such as lignin, suberin, and cutin, may be present, and free aldehydes may also be found in the cytoplasm as transitory products of the cell’s metabolism. These occur in very small amounts and are temporary in their appearance, so that, except for the woody deposits in plant-cells, chromatin is the only constant cell element of which the reaction may be expected.

Chromatin does not, however, always react positively to the Feulgen test. The nuclei of the half-grown oocytes of *Limulus* fail to give the reaction, although the nuclei of the follicle cells surrounding them give it brilliantly. Similarly, Koch (1925) has not succeeded in demonstrating ‘nucleal’ chromatin in the oocytes of *Geophilus*, and has suggested, in explanation, that after the last oogonial division the chromatin undergoes a profound chemical change which renders it incapable of giving the reaction. Ludford (1927) also reports inability to obtain the reaction in the nuclei of developing oocytes (*Limnaea stagnalis*), and regards this as a consequence of the fine state of dispersion of the chromatin in the nuclei.

The generally recognized change that chromatin undergoes in developing oocytes is that from so-called basi-chromatin to oxychromatin. In the oogonial divisions, the chromosomes stain intensely with basic dyes and with haematoxylin in combination with alum, but during the growth of the oocyte they become more and more faintly staining with basic dyes and show an affinity for acid stains. This difference in their behaviour toward stains may, of course, be the result of changes in the acid-base equilibria in the nucleus, but it may also be an expression of a chemical change in the chromatin itself. Some evidence for
such a change occurring between the period of interkinesis and the early stages of mitosis is offered by the work of Wyckoff, Ebeling, and Ter Louw, who find that, in chicken macrophages and fibroblasts, the 'resting' nuclei do not absorb ultraviolet light to any greater extent than the surrounding cytoplasm, but 'as mitosis begins, intensely absorbing chromosomes make their appearance'. This increase in ultra-violet opacity, which is paralleled by an increase in intensity of reaction to the Feulgen technique, they suggest is indicative of a chemical reaction occurring during the early stages of mitosis.

In order to determine whether there was any correlation between acidophility on the part of the chromatin and failure to give the 'nucleal' reaction, a study was made of the oocytes of *Mespilia globulus*, a tropical and sub-tropical Echinoid. The material was kindly furnished me by Professor D. H. Tennent, who had collected it in Japan. Ovaries collected in July, during the spawning season, were fixed in Bouin and in corrosive-acetic and given to me the following September embedded in paraffin. The Bouin material was stained with haematoxylin and erythrosin; the corrosive-acetic treated with fuchsin-sulphurous acid after six minutes hydrolysis. There was no correlation between ability to give the Feulgen reaction and the oxyphility and basiphility of the chromatin. The chromatin net in oogonia in interkinesis stained deep blue with haematoxylin and gave a definite and characteristic 'nucleal' reaction. In young oocytes, the nucleus is filled with a diffuse acidophil reticulum which gives the 'nucleal' reaction, while in larger oocytes, the diplotene threads stain blue with haematoxylin but fail to react with fuchsin-sulphurous acid.

These observations suggest that a physical change rather than a chemical one is responsible for the 'anucleal' condition in the larger oocytes—that is, that the chromatin is so dispersed in the relatively large nucleus that it cannot be detected even at high magnifications. Evidence from the oocytes of *Tenebrio molitor* is in support of this. 'Nucleal' chromatin can be found in the nuclei of oocytes in all stages of development, but in the larger oocytes it is represented only by a mass of material which is very small in proportion to the size of the nucleus.
(figs. 18 and 19), but which actually is about the same as that in the nuclei of oogonia, younger oocytes, follicle, and somatic cells (figs. 9 and 11). These nuclei, however, are approximately one-tenth the size of the nuclei of oocytes about to pass into the oviducts. The amount of idiochromatin is constant, or very nearly so, in all cells of the same body, regardless of size, since at division the chromosomes are numerically and morphologically similar in all the cells. The same amount of reacting material is in the one case compressed into a small area; in the other it may be scattered as fine particles, or stretched as attenuated threads, over an area ten or more times as great. In Tenebrio, the idiochromatin happens to remain clumped, so that its presence can be demonstrated in all phases of oogenesis; in Limulus, the increase in nuclear size is even greater than in Tenebrio and the chromatin is scattered through the nucleus and stretched around the nucleolus (fig. 5). It seems quite conceivable that a point should be reached where the chromatin masses are too minute to produce a visible reaction; the chromatin is therefore lost to sight even after Feulgen treatment until it has again collected into masses large enough to produce a visible amount of the red-blue compound. The conditions are similar to those in bacterial preparations, where 'nucleal' chromatin cannot be demonstrated in individual organisms because of its small volume, while in smear preparations where the bacteria are sufficiently numerous a visible colour will be produced (Reichenow, 1928). Lucas and Stark (1921) in a study of the living sperm-cells of Melanoplus by means of ultraviolet microscopy determined that the 'optically empty' nuclei actually contained diffuse chromatin in 'diatene' chromosomes, thus showing that chromatin though more liquid or diffuse in some stages is actually continuous in spermatogenesis.

It is therefore concluded that the failure to obtain the reaction in the larger Limulus oocytes is due to the extreme dispersion of the reactive material, rather than to any chemical change in it.

2. Chromatin and the Nucleolus.—In none of the material studied has it been possible to establish a relationship between the plasmosome and the chromosomes. In Petunia
and in *Nicotiana* the prophase nucleus shows chromosomes lying on or close to the nucleolus (fig. 30); the nucleolus persists, in *Petunia* at least where it can be more clearly seen, through the metaphase (figs. 29 and 31) and it can be identified also in late anaphase. Zirkle is of the opinion that the nucleolus contributes indirectly to the formation of the chromosomes by providing a core of plastin which is surrounded by chromatin, and Mottram holds that its contribution is a direct one by the actual elaboration of chromatin. In many of the dividing cells of *Petunia* especially, each chromosome seems to have an attendant mass of material staining like the nucleolus (fig. 30), but it is not possible to establish a definite connexion between them. In contrast to Zirkle, Frew and Bowen (1929), Schaede (1929), Fikry (1930), and Kotliarewskaja (1932) all fail to find evidence of nucleolar-chromosomal connexion.

The absence of a nucleolus from developing spermatocytes makes it difficult to accept the view that the chromosomes are developed in connexion with it. A plasmosome never appears in the course of the development of the sperm of *Tenebrio*, and Huettner (1930) reports that in the spermatogenesis of *Drosophila*, a true plasmosome, never giving the Feulgen reaction, appears in the earliest spermatocytes when the chromatin is loose and flocculent, and disappears entirely in spermatocytes entering the leptotene stage. Prior to its disappearance, it emits heavily staining particles into the nucleus, which may pass into the cytoplasm, although this point was not definitely established. Whatever may be the fate of the nucleolar material it does not in any way contribute to the formation of the chromosomes. Its total absence from the spermatogonia and oogonia of *Tenebrio*, which undergo a series of mitoses, and its absence throughout spermatogenesis, suggest strongly that its function in the cell is other than the elaboration of chromatin or the contribution of a non-chromatinic core to the formed chromosomes.

3. Nature of the Nucleolus.—The nucleolus in the oocytes of *Tenebrio molitor* and *Limulus polyphemus* is distinctly non-chromatinic in nature. During the whole course of oogenesis in *Tenebrio* there is no trace of
'nucleal' chromatin in connexion with the plasmosome. In *Limulus*, at one stage in the development of the egg, there appears to be an association between chromatin and plasmosome which might suggest that these oocytes contained a true amphinucleolus, contributing directly to the chromatin content of the cell. In haematoxylin-eosin or erythrosin preparations, the nucleolus in oocytes of this stage is amphiphil, with a central acidophil core and a rather wide basophil rim. In Feulgen preparations of similar oocytes, the reacting substance is much less in amount than the basophil material and later disappears entirely from view, while the basophil rim of the nucleolus can be demonstrated up to the final stages of vitellogenesis. That they are not identical substances is obvious; if the plasmosome of *Limulus* oocytes is composed of two different materials, the basophil component is certainly not chromatin.

Evidence from its reaction to fixatives and stains indicates that the nucleolus is albuminoid in nature. It does not blacken in osmic vapour, but remains brownish yellow even after prolonged osmication. It stains readily with fuchsin, and it is not soluble, or at least not wholly so, in a reagent which dissolves mitochondria. The secretion granules in the cells of *Limulus* and *Tenebrio* behave in the same way toward these reagents and in Champy-Kull preparations of both *Limulus* and *Tenebrio*, the first-formed yolk-granules of the oocytes are golden brown in unstained preparations and the nucleoli of the oocytes are a similar colour. Moreover, they both stain similarly with Lichtgrün and with acid fuchsin after appropriate fixation.

This similarity is suggestive, but not conclusive, evidence of a relationship between the nucleolus and the secretion of material stored in the cell for its future metabolic needs. A consideration of the observations presented in the preceding section of this paper will show, however, that there is a considerable amount of evidence in respect to a connexion between the nucleolus and the elaboration not only of storage products but of those to be discharged from the cell. This evidence may be summarized under five heads:

(1) The nucleolus is absent from oogonia, and is organized first
when the germ-cell enters upon its growth and yolk-forming stage. This is true both in *Tenebrio* and in *Limulus*.

(2) In *Tenebrio*, the nucleolus is absent from the follicle cells, which are differentiated as such from cells in every visible character identical with the oocytes and future ova. In the differentiation of these hitherto similar cells, one group, the persisting oocytes, enter upon a trophic phase, in which the nuclear and cytoplasmic volumes increase, and the cytoplasm becomes loaded with nutritive material; the other, those destined to provide the follicles, enter upon a kinetic phase and undergo a series of rapid divisions, their volume becoming progressively smaller. In the first group the nucleoli remain as definite and conspicuous nuclear organs; from the second they disappear.

(3) No nucleolus ever appears in the spermatogonium or developing spermatocyte of *Tenebrio*. The development of the male and female beetle is the same; both have a larval and a pupal life of similar duration, and although mature sperm may be found in the reproductive ducts of a male beetle just out of the pupal skin, copulation does not occur until more than 36 hours later. In the same period of time the male germ-cell has undergone two complicated meiotic divisions and a metamorphosis of spermatid into mature sperm, while the female germ-cell has only begun on the prophase of the first meiotic division. In the one case, the period of development in the gonad has been one of kinetic activity, in the other of trophic. The nucleolus remains in the growing and synthesizing cell during its period of trophic activity and is lacking in the cell undergoing division.

(4) Both in *Limulus* and in *Tenebrio*, the nucleolus develops in the oocyte only at the beginning of the growth stage. It is at first homogeneous in appearance, increases in size to a certain extent, then becomes more and more vacuolated as vitellogenesis progresses, and finally disappears or becomes very much reduced in size when the process of yolk formation is complete.

(5) The nucleolus is a conspicuous nuclear structure in cells specialized for secretion. In the epidermal gland-cells of
Petunia and of Nicotiana, the nucleoli are large and often vacuolated as are those of the growing oocyte. In the glandular region of the mid-gut of Tenebrio, nucleoli are found in the gland-cells, but are lacking in the nuclei of the other cells of the gut. The number of nucleolated cells is maximum in the central portion of the mid-gut and progressively decreases from this region anteriorly to the pharynx and posteriorly to the rectum. In both the pharyngeal and rectal regions of the gut the nuclei of the cells lining the lumen are wholly deficient in nucleoli.

The constancy with which nucleoli appear in plant-cells is not without significance in this connexion. Nucleoli were found in all the cells of Petunia and Nicotiana studied, except in those of the tracheal tubes, and remained, apparently undiminished, during cell division. This is the condition one would anticipate if the relation of the nucleolus to secretion is an active and direct one, inasmuch as plant-cells are essentially secretory.

Haecker (1895) regarded the nucleolus as a useless by-product of the cell's metabolism, and Meyer (1917) has interpreted the results of his own experimental work on Galtonia candidans as evidence also of its ergastic nature. Meyer reduced the nutrition of the plants by depriving them of light, and found that after 36 days in darkness, the nucleoli of the parenchymatous cells at the base of the leaf were reduced 82 per cent in size, after two months 95.5 per cent. He concluded from these and similar experiments that the nucleolus represents an accumulation of the cell's waste, since the vitality of the nucleus was not impaired by the experimental conditions. If the nucleolus is the agent concerned in secretion, decrease in its volume might be expected if the secretory activities of the cell are checked or prevented. Lack of sunlight would result in the cessation of photosynthesis, and as a corollary the condensation of insoluble starch, protein synthesis and the production of waxes and oils in the parenchyma cells would stop. The evidence obtained from the study of developing oocytes indicates that when vitellogenesis is at an end, the nucleolus disappears or becomes markedly smaller; decrease in size is here an index of the termination of functional activity. Similarly, a decrease might be expected when cellular functions were artificially
THE NUCLEOLUS

checked, and it would seem that Meyer's experimental results might be interpreted in such a light.

In summary of these findings on the nature of the nucleolus, it may be stated that it is definitely non-chromatinic, and that chemically it is albuminoid and probably closely allied to the material of the 'formed yolk'. Functionally, it is an active agent in secretory cells, representing not an accumulation of metabolic waste but a reserve of material which is utilized in the production of secretory products.

CONCLUSION.

In the oocytes of Tenebrio molitor and Limulus polyphemus, the nucleolus is formed primarily of material which has been derived from the fat deposits of the young animals. In neither animal is a nucleolus found in spermatogonia or in spermatocytes, and it appears first as a formed structure in the oocyte nucleus when the process of yolk-synthesis is beginning. It is never formed in the follicle cells, although these have the same ancestry as the ova and are differentiated from the definitive oocytes at the time when they are starting upon their growth stage. The material from which the nucleolus is formed is derived from the fat-body; it diffuses into the ovarioles and ultimately into the cytoplasm and the nuclei of the oocytes. There it coalesces to form the single large nucleolus of the Limulus oocyte and the three or four smaller ones of the Tenebrio germ-cell.

The substance of which the nucleolus is composed is albuminoid, and is returned wholly or in part to the cytoplasm during the process of vitellogenesis. It has no chromatin component and no definite connexion with the chromosomes.

Functionally the nucleolus is connected with the secretory activities of the cell. In Limulus, when vitellogenesis is commencing, the nucleoli of the oocytes show vacuolization which is coincident with the appearance of granules in the lobes of the nucleus and in the perinuclear cytoplasm. In both Tenebrio and Limulus, the nucleoli first appear when yolk is being formed, and show reduction in content as vitellogenesis proceeds. Nucleoli are constant nuclear elements in cells.
specializing in secretion. They are found in the epidermal gland-cells of *Petunia* and *Nicotiana*, in the mesophyll and parenchyma cells where photosynthesis and the further elaboration of photosynthetic products are going on, and in the gland-cells of the digestive tract of *Tenebrio* larvae. They are absent in the non-secretory cells of the two plants under consideration, and also in the connective tissue, muscle, and regenerative cells of the *Tenebrio* gut. They are also lacking in the cells of the enteric epithelium in the regions of the fore- and hind-gut, in which digestion does not take place.

Evidence is presented to show that the failure to demonstrate ‘nucleal’ chromatin in the developing oocytes is the result of the physical rather than the chemical state of the chromatin. There is no correlation between the acidophilia and basiphility of the chromatin and its failure to react with fuchsin-sulphurous acid, and the absence of colour following Feulgen treatment is to be regarded as the result of the extreme dispersion of the reacting substance. The compound formed as a result of the interaction of the active principle of fuchsin-sulphurous acid and the aldehyde component of chromatin is too small in quantity at any one place in the nucleus to be visible even with high magnification.

EXPLANATION OF PLATES.

All the figures except nos. 6, 7, 22, 23, and 24 are drawn with the aid of a camera lucida and an apochromatic oil immersion lens (Zeiss, num. ap. 1-5) and a number 4 Zeiss ocular, giving a magnification of 1500 diameters. Figs. 7, 23, and 24 are drawn at a magnification of 720 diameters with a high dry objective and a number 4 Zeiss ocular. Fig. 7 is a drawing of a section through an ovary. Fig. 22 is a drawing from dissection of the gut. In all the figures of Feulgen preparations, the reacting substance is represented in black; the structures taking Lichtgrün are represented in grey.

**PLATE 28.**

Fig. 1.)

Fig. 2.)

Fig. 3.) Oocytes from a 10-inch *Limulus*. Feulgen technique.

Fig. 4.)

Fig. 5.—Oocyte from an adult *Limulus*, fixed in a saturated solution.
of corrosive sublimate in equal parts of absolute alcohol, glacial acetic acid and chloroform, with 6 minutes hydrolysis and 1½ hours in fuchsin-sulphurous acid.

Fig. 6.—Section of the ovary of a pupa of Tenebrion molitor, showing the relation of the ovarioles to the fat-body.

Fig. 7.—Longitudinal section through the germarium of a female larva of Tenebrion molitor just before pupation. Feulgen technique.

Fig. 8.—'Resting' and dividing oogonia from the germarium of the same larva.

Fig. 9.—Oogonia and very young oocytes from the posterior end of the germarium of the same larva.

Fig. 10.—Oocytes at the upper end of an ovariole of the same larva.

Fig. 11.—Oocytes from the lower end of the same ovariole.

Fig. 12.—Oocytes from the centre of the gonad of a Tenebrion beetle 74 hours after hatching. This beetle had laid no eggs. Feulgen technique.

Fig. 13.—Oocytes from the lower end of an ovariole of a 10-day Tenebrion pupa. Bouin, Heidenhain's iron haematoxylin and erythrosin.

Fig. 14.—Oocyte from the lower end of an ovariole of a 74-hour beetle. Feulgen technique.

Fig. 15.—The most advanced oocyte in the gonad of a beetle 2-3 hours after hatching. Feulgen technique.

Fig. 16.—Oocyte enclosed in follicle but containing no 'formed yolk', and with the nucleus still in a central position, from a 74-hour beetle. Feulgen technique.

Fig. 17.—Oocyte from a 74-hour beetle. The nucleus has moved to the periphery of the cell, and some 'formed yolk' appears in the cytoplasm. Feulgen technique.

Fig. 18.—Oocyte enclosed in follicle with the nucleus lying at the side and the cytoplasm containing 'formed yolk', from a 3-day beetle. Feulgen technique.

Fig. 19.—Advanced oocyte from the gonad of a beetle which had already laid at least one egg. Feulgen technique.

Fig. 20.—Spermatocyte from the testis of a male beetle just hatched. Carnoy with subsequent Feulgen treatment.

Fig. 21.—Young spermatid from the same beetle as fig. 19. Carnoy and Feulgen.

Fig. 22.—Digestive tract of Tenebrion larva, drawn from dissection.

Fig. 23.—Section of the anterior region of the mid-gut (section 1, fig. 22) of Tenebrion larva. Feulgen technique.

Fig. 24.—Section of the mid-region of the mid-gut (section 2, fig. 22) of Tenebrion larva. Feulgen technique.

Fig. 25.—Epidermal cell developing into gland from a young stem of Petunia. Zenker and Feulgen.

Fig. 26.—Gland-cell and stalk from the stem of Petunia. Zenker and Feulgen.

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Fig. 27.—Gland-cell and stalk from the stem of Petunia. Corrosive-acetic and Feulgen, fuchsin-sulphurous and 1½ hours.

Fig. 28.—Parenchyma cell from the stem of Petunia. Corrosive-acetic and Feulgen.

Fig. 29.

Fig. 30. Parenchyma cells from the stem of Petunia. Zenker and Feulgen.

Fig. 31. Parenchyma cells from the stem of Petunia. Zenker and Feulgen.

Fig. 32.—Epidermal cell developing into gland from the stem of Nicotiana. Zenker and Feulgen.

Fig. 33.—Gland-cell and stalk from the stem of Nicotiana. Zenker and Feulgen.

Fig. 34.—Glandular cells from the tip of a stalk from the stem of Nicotiana. Zenker and Feulgen.

Fig. 35.—Cells from the corolla of a very young bud of Nicotiana. Zenker and Feulgen.

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