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NUCLEIC ACID STORAGE IN THE TOAD'S EGG

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From chemical analyses it has been known for some time that the unsegmented eggs of a number of different animals carry a high proportion of the nucleic acid which is present at the time larvae hatch (e.g., frog 28%, sea urchin 100%).[1] Brachet[2] has shown, in the case of the sea urchin, that this nucleic acid is initially in the ribose form (ribonucleic acid) which is partially transformed into the desoxy ribose type (thymonucleic acid or chromatin) as development goes forward. The writers have been studying the cytological mechanisms by which this nucleic acid is laid down in the egg cytoplasm.[3, 4] The present report deals with the oöcytes of the toad (*Bufo valliceps*), a form of especial interest because of the enormous germinal vesicle and the presence of the so-called "lampbrush" chromosomes. In the past, both of these features have been interpretated in various ways as contributing to the nucleic acid reserve of the egg cytoplasm.

Methods.—Fresh ovaries were preserved either in Nawaschin's fluid and the sections stained by Feulgen's Nucleal method, or fixed in Helly's fluid and stained by Unna's methyl green-pyronin mixture, both before and after treatment with a ribonuclease enzyme.

Observations.—Figure 1 is a camera lucida drawing showing in the wall of the ovary an oögonial cell in prophase and two very young oöcytes in the bouquet stage (one nucleus drawn to show the chromosomes, the other the surface of the nuclear wall). In the oögonium all of the chromatin is confined to the prophase chromosomes. In the oöcytes, on the other hand, the chromatin is found in two types of structures: (*a*) the chromosomes which have the loop form characteristic of the synizesis stage, and (*b*) free chromatin granules which lie against the inside of the nuclear wall and are most numerous on the side of the nucleus opposite that towards which the ends of the chromosome loops are oriented. This distribution of the nuclear contents makes it possible to observe that there is no connection at this

time between the chromosomes and the free granules, an important point. The subsequent history of the oöcyte is best followed by considering the behavior of the chromosomes and the free chromatin granules separately.

At the bouquet stage the chromosomes appear to be made up of granules embedded in unstained material, the matrix, which imparts a rather fuzzy outline to the whole (Fig. 1). As the egg nucleus, or germinal vesicle, grows in size the chromosomes increase in length by uncoiling and reach their maximum extension when the nucleus is about 40 or 50 μ in diameter. With a further increase in nuclear size the chromosomes occupy only a limited area within the germinal vesicle. Turning to details, when the nucleus is about double its initial diameter (10 μ) at the bouquet stage, the individual chromosomes (Fig. 2 (*a*)) are still relatively thick and granular and quite jagged in outline. Part of the latter is due to the zig-zag arrangement of the chromomeres (chromioles) and part to lateral extensions of the achromatic matrix into the nuclear sap, as we interpret it forming the side branches which characterize the lampbrush chromosomes. The chromosomes are not uniform in staining reaction. There are localized areas of different chromosomes which are thicker, because the chromatic granules are larger, as indicated in figure 2 (*a*). These heterochromatic areas persist throughout the growth period. In nuclei about 30 μ in diameter (Fig. 3 (*a*)) the chromosomes are thinner and longer and the chromomeres appear to be arranged in a zig-zag manner in a mid-focal plane due to the uncoiling of the chromomeric threads of which each chromosome is composed. The side branches, which do not color with Feulgen's stain, are more pronounced. With further extension in length the Feulgen positive chromomeres assume a linear order, a condition which is retained until a contraction sets in prior to the breakdown of the germinal vesicle wall (Fig. 4). In the later stages (from 30 μ on) it is obvious that each chromosome is made up of two threads held together by chiasmata, but we have been unable to observe any split in the two threads, which the presence of chiasmata would imply.

After Feulgen's stain the chromosomes in later stages are rather difficult to observe but the chromomeres do not lose their purple color, contrary to the report of Koltzoff[8] and others. In the toad the side branches of the chromosomes are never conspicuous and are best seen with oblique illumination. When sections are heavily overstained with haematoxylin, however, we get the classic lampbrush image except for the absence of loops which we have not seen in toad eggs fixed in Nawaschin's or Helly's fluids.

So far we have been unable to distinguish oöcytes earlier than the bouquet stage and have not observed just how or when the chromatic granules are separated from the meiotic chromosomes. The former lie just within the nuclear wall, are somewhat variable in size (best shown in tangential section of the lower nucleus in figure 1) and are most numerous on the side

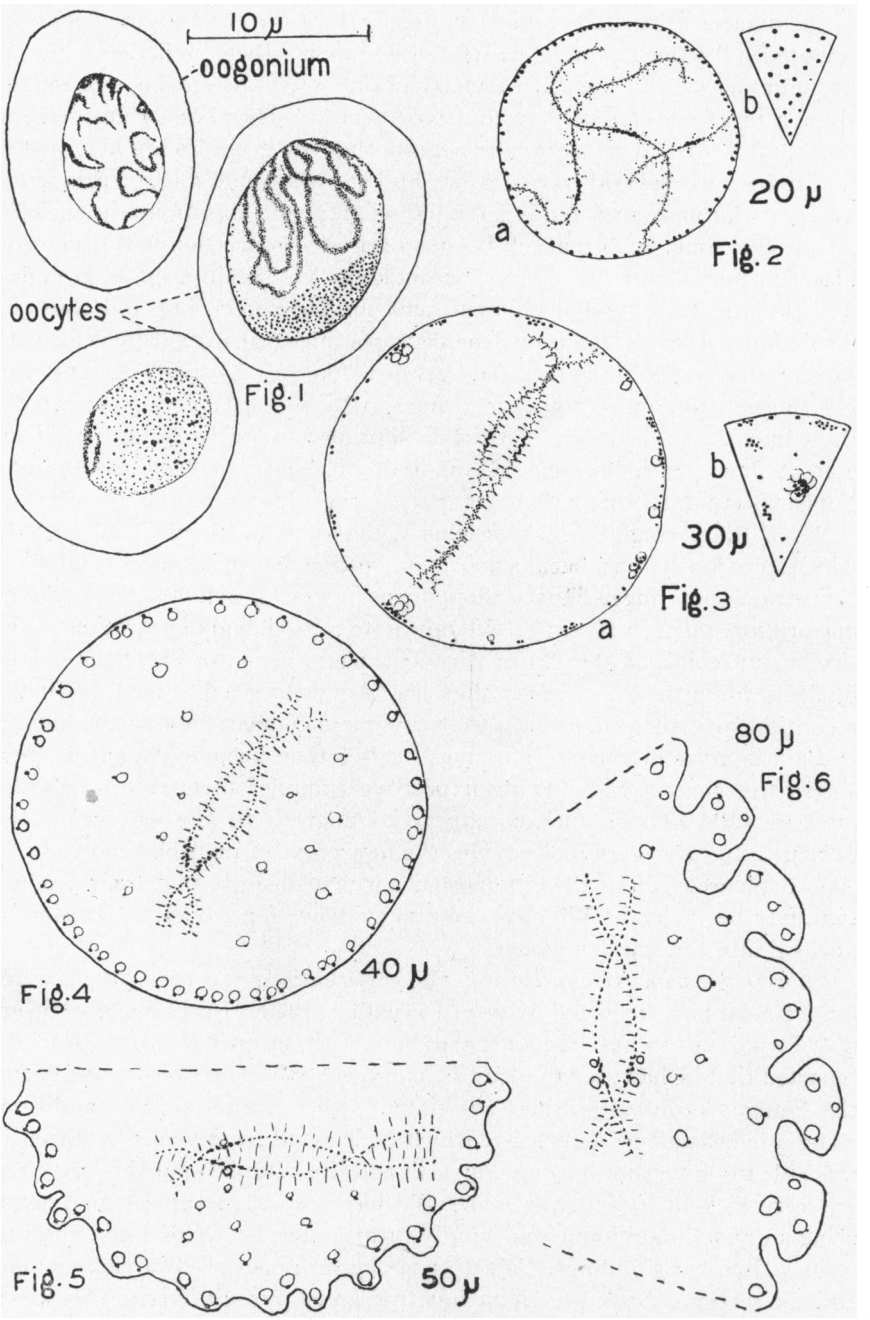

Figure 1 is a camera lucida drawing and the remaining figures are semi-diagrammatic.

of the nucleus opposite to which the free ends of the chromosome loops are oriented. As the germinal vesicle grows in size there is little change in the granules until a nuclear diameter of about 30 μ is reached when they show a pronounced tendency to associate into rather large clumps (Figs. 3 (*a*) and 3 (*b*)). This behavior suggests that the granules are heterochromatin because several workers have noted that heterochromatin from different chromosomes tends to synapse at about this point in meiosis. About this time small nucleoli begin to arise in association with the granules; clusters about the clumps, or single nucleoli against single granules. A little later the granular clumps break up and spread out and thus the whole inner surface of the nucleus becomes lined with small nucleoli with the chromatin organizers directed outward (Fig. 4). Uusually each nucleolus shows only one chromatin granule attached to it but occasionally there may be two or more. The nucleoli proper are fairly uniform in diameter at first; there are several hundreds of them and they do not take Feulgen's stain, though with haematoxylin they stain intensely.

When the germinal vesicle reaches a diameter of about 60 μ its wall, which previously has been smooth in outline becomes wavy and soon shows numerous finger-like pseudopodia or processes each of which contains one or more nucleoli (Fig. 5). We now note a good deal of variation in the size of nucleoli and the chromatin organizers begin to disappear as the nucleoli diminish in size, as if they are being absorbed (Fig. 6). In the meantime new nucleoli are seen to be arising in connection with the heterochromatic areas of the chromosomes. After they become detached, each shows the characteristic Feulgen positive granule directed towards the nuclear wall. These nucleoli appear to migrate to the surface of the nucleus. In older germinal vesicles the new crops of nucleoli tend to lie in concentric areas about the chromosomes which suggests that their production may be cyclical. We have seen no evidence for a bodily extrusion of nucleoli into the egg cytoplasm.

Experiment with Ribonuclease.—The observations recorded above were, for the most part, reported by us in December, 1940. In the same year but unknown to us (because the journal did not reach us until in 1941), Brachet[5] reported that he had used Feulgen's technique on the eggs of frogs and found the Feulgen positive granules associated with the nucleoli. In addition, using a heat stable ribonuclease, which he isolated from pancreas, Brachet was able to prove that both the nucleoli and the cytoplasm of the frog's egg are very rich in ribonucleic acid. We have repeated these experiments using a crystalline ribonuclease kindly furnished us by Dr. Kunitz, and can confirm Brachet's findings. When toad eggs are preserved in Helly's fluid and the sections stained in Unna's methyl green-pyronin mixture both the nucleoli and the cytoplasm stain a brilliant red. Some red-staining material is present in very young oöcytes and in older eggs there is a very large

amount of it with a rather sharp gradient, the color being most intense next to the nuclear membrane and shading off towards the cortex. If, now, sections are first treated with ribonuclease for a few hours, and then stained with Unna's mixture, the red color no longer shows in either the nucleoli or the cytoplasm. In control slides treated with the inactivated enzyme the staining is the same as in untreated slides. Except for a loss of staining capacity, nucleoli are not visibly affected by ribonuclease. Brachet interprets these results to mean that the red color is due to the presence of ribonucleic acid which is removed by the ribonuclease treatment. Obviously then, the heterochromatic granules and the nucleoli associated with them are part of a mechanism for the laying down of ribonucleic acid in the cytoplasm of the toad's or frog's egg.

Discussion.—Before Feulgen's technique was developed for the identification of chromatin (thymonucleic acid) it had been commonly reported that in eggs with large germinal vesicles the chromosomes, the numerous nucleoli and various unidentified nuclear inclusions, stain deeply with basic dyes. And a number of observers, noting that the amount of basophilic material which finally entered the first polar spindle (as chromosomes) was only a small fraction of the total seen earlier in meiosis, suggested that with the breakdown of the germinal vesicle wall this excess nuclear material would be available to the developing embryo (Conklin,[6] Godlewski,[7] Koltzoff[8]). The present study, however, indicates that there is a specific cytological mechanism in the toad (and frog) which begins to deposit ribonucleic acid in the cytoplasm of the oöcyte soon after it is differentiated and continues to function through the months required to build up the mature egg. While large amounts of nuclear material are set free in the cytoplasm when the germinal vesicle breaks down, this appears to contain very little nucleic acid of either the ribose or desoxy ribose type. This conclusion is supported by the observations of Caspersson and Schultz[9] that in the sea urchin the mature germinal vesicle is relatively poor in pyrimidine bases while the cytoplasm just outside the nuclear wall is very rich in them.

Within the past five years a good deal of evidence has come to light which indicates that there is an intimate and causal relationship between heterochromatin, nucleolar formation and the synthesis of ribonucleic acid in the cytoplasm of the cell.[9, 10] Broadly speaking, wherever cells undergo rapid protein synthesis, as in growth or secretory activity, there is usually an abundance of ribonucleic acid in the cytoplasm and prominent nucleoli in highly basophilic nuclei. In the toad this cytological and dynamic relationship stands out very clearly because the heterochromatin, which is the main source of the basophily in growing or secretory cells, is not inextricably mixed with the chromosomes but is segregated for the most part into discrete granules entirely removed from the chromosomes, and instead of one

or several nucleoli there are hundreds of them. Here each nucleolar complex consists of one or more heterochromatin granules, the main body of the nucleolus probably protein in nature,[9] and some ribonucleic acid the removal of which with ribonuclease does not morphologically affect the body of the nucleolus. It is clear that ribonucleic acid can be synthesized, or converted (from desoxy ribose) deep within the nucleus, and with the migration of the nucleoli to the nuclear wall and their absorption this is somehow transferred to the cytoplasm. But the localization of the heterochromatic granules initially on the inner wall of the nucleus, the invariable orientation of the granules towards the cytoplasm and the sequence of changes which occur at this site all suggest that most of the synthesis or conversion is occurring at the interface of the nucleus and cytoplasm where an abundance of surface energy is available.

Our observations show that the lampbrush chromosomes of the toad are normal and typical meiotic chromosomes in structure and behavior. This fact is stressed because earlier one of us (Painter[3]) postulated that lampbrush chromosomes were the result of some sort of reduplication process. Ordinarily, a great increase in nuclear volume is accompanied by the growth and division of the contained chromosomes, forming the giant chromosomes in the salivary glands of Diptera or highly polyploid nuclei as in the larval cells of many insects which grow by endomitosis. Koltzoff[8] thought that the side branches of lampbrush chromosomes represent reduplications of the primary "genonema" which are thrown off before polar spindle formation and which merge with the cytoplasm when the germinal vesicle breaks down. But since the granules which the side branches appear to enclose contain neither thymonucleic acid, as shown by Feulgen's stain, nor ribonucleic acid, as shown by Unna's stain, there is little to support this concept. On the other hand, with the increase in nuclear volume in the toad's egg there is a great increase in the amount of heterochromatin and in the number of nucleoli which form in association with the heterochromatic granules. If these nucleolar organizers are genetically the same as those which form nucleoli in ordinary somatic cells, then we may say that the germinal vesicle of the toad is highly polyploid in nucleolar organizers but otherwise lampbrush chromosomes are normal meiotic structures.

Perhaps the most important aspect of the present study is the striking demonstration that chromatin can exist and function within the nucleus apart from the chromosomes. And thus we are able to add another characteristic to the already long list of attributes of heterochromatin recently summarized by Darlington,[11] and the rôles this plays in the dynamic activities of cells.

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ON THE PERSEUS CLUSTER OF NEBULAE

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The Perseus cluster of nebulae (R.A. $3^h\ 15^m$, Dec. $+41°\ 15'$, 1930; gal. long. 118°, lat. $-13°$) according to Hubble and Humason[1] contains about 500 nebulae scattered over an area nearly 2° in diameter and lies at a distance of 11 million parsecs. As is the case with most clusters investigated, counts of nebulae made on photographs which were obtained with the 18-inch Schmidt telescope show that the Perseus cluster is considerably larger than was originally derived from the photographs taken with the large reflectors whose very severely restricted fields make them unsuitable for the efficient analysis of objects subtending large angles. For quite a different reason the analysis of the spatial distribution of the nebulae in the Perseus cluster with the 18-inch Schmidt telescope also presents considerable difficulties. The cluster is projected on a field of the Milky Way so rich in stars, that, because of the small scale of the telescope, the blurred images of close pairs and groups of stars may easily be mistaken for extragalactic nebulae. This error can partly be avoided by taking a number of well-focused photographs while the telescope is being drifted slightly in a different direction for each photograph. Groups of stars are likely to betray themselves by an image containing sharp streaks such as should not be expected in a drifted image of a nebula. Also, the precaution was taken to identify all of the nebulae involved several times through a repeated analysis conducted during several years and making use of many photographs taken on various emulsions. It is therefore felt that the results presented here can be viewed with more confidence than might have been originally hoped for.

In figure 1 the distribution of nebulae brighter than about the photographic magnitude $m_p = 16.5$ over a field of approximately 9° in diameter around the Perseus cluster is shown.