Resumido por el autor, Waro Nakahara.

Algunas observaciones sobre el crecimiento de los ovocitos
de *Perla inmarginata* Say, con especial mención del
origen y función de las estructuras nucleolares.

El núcleo del huevo ovárico de *Perla inmarginata* contiene dos clases de estructuras nucleolares, a saber: Un nucleolo único, de gran tamaño (nucleolo principal) y otro múltiple mas pequeño (accesorio). Las observaciones efectuadas por el autor indican que el primero existe en el núcleo desde los primeros estados del crecimiento, pudiendo emigrar algunas veces, fuera de él demostrando esto su origen endogénico. Los nucleolos accesorios se derivan, con toda probabilidad, de los núcleos vitelinos que se encuentran primero en el área citoplásmica y más tarde penetran dentro del núcleo. Estos hechos demuestran que las teorías endogénica y exogénica de las estructuras nucleolares, no son necesariamente contradictorias. Si consideramos las estructuras nucleolares como representación de sustancias que pasan a través del núcleo durante el metabolismo, los nucleolos endogénicos deben considerarse como pertenecientes al lado catabólico, los exogénicos al lado anabólico del proceso.

Translation by Dr. José Nonidez,
Columbia University.

SOME OBSERVATIONS ON THE GROWING OOCYTES OF THE STONEFLY, PERLA IMMARGINATA SAY, WITH SPECIAL REGARD TO THE ORIGIN AND FUNCTION OF THE NUCLEOLAR STRUCTURES¹

WARO NAKAHARA

Biological Laboratories, Cornell University, Ithaca, N. Y.

NINE FIGURES

CONTENTS

Introduction.....	203
Technique.....	204
Observations.....	204
General consideration.....	207
Review and discussions.....	210
Summary and conclusions.....	214
Literature cited.....	214

INTRODUCTION

There are two conflicting views regarding the origin of the nucleolus. According to Korschelt ('89) and Montgomery ('98), the nucleolus represents a sort of nutritive substance derived from the cytoplasm, and therefore it is extranuclear in origin; while, on the contrary, a number of recent authors, including Obst ('99), Walker and Tozer ('09), et al., maintain that it is of intranuclear origin. The general contention today that the nucleolar substance is a passive product of the nuclear activity, and hence intranuclear in origin, seems premature, in view of the fact that data cited as supporting the exogenic theory of the nucleolus have not been adequately studied.

In the course of my study of the ovarian eggs of the common stonefly, *Perla immarginata* Say, I found some facts which are

¹ In the preparation of this paper, the author is under deep obligation to Dr. Wm. A. Riley, now of the University of Minnesota, for the critical examination of the manuscripts.

directly related to the question at issue, and are of a little value, if my interpretation is correct, in showing the possibility of bringing the two conflicting views in harmony.

The following note summarizes the results so far obtained in the study, which has been carried on in the biological laboratories of Cornell University, until it was discontinued through my leaving the institution. Although there are many details remaining to be worked out, points brought out in the following lines seem clear.

TECHNIQUE

Although several other fixing fluids were employed, the one that proved to be most satisfactory and hence most used is Flemming's chromo-aceto-osmic. Ovaries, dissected out in the normal salt solution, were fixed in this fluid for twenty-four hours.

For staining, the best result was obtained by a modification of Flemming's "triple" method, which is as follows:

1. Stain in a mixture of equal parts of saturated aqueous and saturated alcoholic solutions of safranin for five minutes.
2. Rinse in water.
3. Stain in 1 per cent gentian-violet, about ten seconds.
4. Rinse in water.
5. Stain in 2 per cent solution of orange G for two or three minutes.
6. Rapidly dehydrate with absolute alcohol; differentiate with clove oil under the control of microscope; clear with xylene; mount in balsam.

Iron hematoxylin and borax carmine counterstained with Lichtgrün were also used for a few slides.

OBSERVATIONS

Figure 1 represents a group of early oocytes and some follicular cells. In the nucleus of the oocyte, the chromatin granules, which stain deeply with gentian-violet, are seen arranged more or less linearly. The nucleolus is very prominent, always being situated near the center of the nucleus. Before the growth

Fig 1. A portion of 'egg tube,' showing early oocytes with prominent nucleolus. $\times 670$.

Fig. 2. A portion of 'egg tube,' showing early oocytes and a growing oocyte. Two early oocytes are seen in the process of degeneration. $\times 670$.

Figs. 3 to 6. Early ovarian eggs. In figures 4 and 5 follicular cells are not shown. The yolk-nuclei are represented by black masses around the nucleus. The single nucleolus is seen near the nucleus, except in figure 5, which does not show this structure. In figure 4 a peripheral nucleolus is seen half way through the nuclear membrane. $\times 670$.

of the oocytes commences, some of them always seem to be committed to degeneration. In figure 2 two cells are shown to be in this process. The nuclear structure of the normal oocyte at this period is very much the same as before.

In its very early state (figs. 2 to 6) the ovarian egg is more or less irregularly oval. Follicular cells surrounding the eggs are few in number. The nucleus of the egg cell is usually oval or spherical, containing chromatin granules, which stain less intensely than in the oocyte. Nucleoli are of two sorts, a large principal nucleolus and a number of smaller nucleoli. The larger nucleolus, which seems doubtlessly derived from the original oocytic nucleolus, is almost always single and is situated near the center of the nucleus. Its substance is usually homogeneous, with clearly defined outline, taking acid color (orange G) when treated with Flemming's triple stain, and seldom containing a vacuole.

The smaller nucleoli vary in number; a section usually showing two or three, but sometimes more—up to six or seven. They vary also in size, although none of them were found to be even half as large as the larger nucleolus. In shape, they are in most cases spherical, or more rarely much elongated. They are very characteristic in being situated just beneath the nuclear membrane, or at least not far from the latter. The substance of the smaller nucleoli distinguishes itself from that of the larger nucleolus in choosing safranin out of Flemming's triple combination.

The most characteristic structure in the ovum of this stage is the irregular masses of dense material in the cytoplasm, always closely apposed to the nucleus. Whether the masses in question, which are apparently identical with a certain kind of yolk-nuclei, lie within or without the nucleus, is in many cases quite difficult to determine. For instance, in two cells represented in figure 3, it has been impossible for me to detect the nuclear membrane between the areas of the nucleus and yolk-nucleus. It is still more interesting that the substance of the yolk-nuclei agree with that of the smaller nucleoli in staining reaction. These facts, taken together with the peripheral posi-

tion of the nucleoli, are suggestive enough of some very close relation between these two kinds of structures.

Ova of the next stage in the growth are represented in figures 7 and 8. Here the increase in size is most marked in the nucleus, although the follicular epithelium is also attaining much development. The larger nucleolus is somewhat larger than in the earlier stage, but in other respects, it does not show any appreciable difference according to the stages. It may, however, sometimes migrate out of the nucleus in the well-known manner for the phenomenon (fig. 7). The smaller nucleoli are seen to be much increased in number, usually about twice as many as in the last stage, and not infrequently more than fifteen, being present in a single section. There is no 'yolk-nucleus' situated close to the nucleus. Sometimes, as represented in figure 8, less deeply stained and rather indistinct masses may be seen scattered near the periphery of the cell, but this is not a constant feature of the ovum at this stage. The nature of these masses is unknown, but they may be, in some way, connected with the yolk-nuclei. A few yolk-granules are seen to appear in the cytoplasm (fig. 7).

This stage of growth is followed by another, at which the volume is increased in the cell-body, rather than in the nucleus. At this stage, the follicular epithelium is attaining its full growth (fig. 9). The nucleus remains in nearly the same size as in the preceding stage and the larger nucleolus shows no special change. Most of the small nucleoli are now moved from the periphery toward the center of the nucleus, and they are somewhat less in number than in the last stage. The cell-body grows immensely. Indeed, the growth of the cell at this stage may be almost entirely attributed to that of the cell-body. The cytoplasm is rather homogeneous, and no structure resembling the yolk-nucleus is found. The yolk-granules are present in a fairly large number at this stage.

GENERAL CONSIDERATION

From the facts described in the last section, it is evident that the growth of the ovarian egg of *Perla* is effected first by a rather marked growth of the nucleus, and then by an immense increase of cytoplasm.

Fig. 7. An ovarian egg in the stage of nuclear growth; follicular cells are not drawn. Note the increase of the smaller nucleoli. The larger nucleolus is here seen passing out into the cytoplasm. $\times 670$.

Fig. 8. The same stage. Indistinct dark masses are seen scattered in the cytoplasm. $\times 670$.

Fig. 9. Ovarian eggs in the next stage. The cell-body is greatly increased in volume. Note the less numerous smaller nucleoli. Deposition of yolk-granules in the cytoplasm is also illustrated. $\times 670$.

The appearance of the 'yolk-nuclei' always precedes the nuclear growth. During the growing period of the nucleus, the yolk-nuclei more or less diminish and finally disappear, and the growth of the cytoplasm is rather insignificant.

It has been observed that the yolk-nucleus and the peripheral nucleoli show very similar staining reactions, and that in some cases the former is so closely apposed to the nucleus as to make it impossible to draw a line between the nuclear area and the area of the yolk-nucleus. In fact, many cases were found where the only possible interpretation was that the peripheral nucleolus and the yolk-nucleus are the same substance, and it may pass through the boundary of the nucleus when the latter is not provided with nuclear membrane. As far as these facts are concerned, it might be considered either, 1) that the nucleoli migrate into the cell-body and give rise to the yolk-nuclei, or, 2) that the latter are taken up by the nucleus and constitute the former there. However, since the yolk-nuclei appear preparatory to the growth of the nucleus, the first theory does not seem to be acceptable; therefore, the substance of the yolk-nuclei is to be considered as, at least in part, a provision for the growth of the nucleus, to be taken up by the latter to constitute the peripheral nucleoli.

In this connection, a few words might be said regarding interpretation of the 'yolk-nuclei.' The data accumulated by Hubbard ('94), Calkins ('95), Henneguy ('96), Foot ('96), Nemec ('97), Van Bambeke ('98), Munson ('98) and Crampton ('99) from various animals indicate, in a general way, that the yolk-nucleus and a certain nuclear substance are very closely allied to each other, if not exactly identical, and that later the yolk-nucleus breaks up into smaller and smaller granules, which scatter through the cell-body. Wilson ('00), in his excellent review, expresses his opinion that the yolk-nucleus may be a product of the nuclear activity, being directly or indirectly derived from the nucleus, and it many contribute to formed elements of the cytoplasm.

In his extensive comparative study on the subject, Munson ('12) speaks of the type of yolk-nuclei under discussion as

'metaplasm.' According to him, the metaplasm is a product of ferment action of karyolymph, which comes out of the nucleus, upon unassimilated, ingested food in the cytoplasm, and it is gradually absorbed as food by the sphere.

To the case of Perla egg, no one of these interpretations is applicable, because here, in all probability, the yolk-nuclei are, at least in part, provisions for nuclear growth. It may be that the yolk-nuclei in this case are derived from the degenerating cells, which are rather abundant at the time when the growth of the ovum starts, and they may be entirely different in nature from the yolk-nuclei of other forms studied. However, the whole subject obviously needs a careful re-examination. Regarding the larger nucleolus, nothing very definite can be said as to its origin. Indications as a whole, however, seem to favor the view of its intranuclear origin. It is very probable that the nucleolus was formed in the nucleus after the last oogonial division, and handed down through the stages of growth of the ovarian egg.

REVIEW AND DISCUSSIONS

Korschelt ('89), working on the ova of *Epeira*, *Dolomedes*, *Phalangium*, *Spinther*, and *Ciona*, came to the conclusion that the nucleolar substance is in close connection with the nutritive process of the cell, and probably it is derived from the cytoplasm.

This theory is strongly maintained by Montgomery ('98), on the basis of his observations:

In all the cases observed by me, the nucleus appears to assimilate a substance or substances from the cytoplasm, and after this substance has entered the nucleus it apparently undergoes there a chemical change, and becomes deposited on the inner surface of the nuclear membrane in the form of masses of varying dimensions, which may be either globular or irregular in shape, according as they are fluid or viscid in consistency. In the case of the ova of the nemerteans, the substance taken up into the nucleus, and which there becomes deposited in the form of nucleoli, is sometimes exactly similar to the substance of the yolk-balls, which lie in the cytoplasm; in other cases, it is probably similar to those metabolically changed portions of or inclusions of the cytoplasm, out of which the yolk-balls are later differentiated. In *Lineus*, indeed, the yolk-balls may often be found halfway through the nuclear membrane, and their appearance is

exactly similar to that of the nucleoli. In the mesenchym cells of *Ceregratulus*, the substance of the nucleoli appears to be identical with that of the numerous nutritive granules which are dispersed in the cytoplasm; the latter globules arise in the cytoplasm before the nucleolus appears in the nucleus, and as soon as they become numerous in the neighborhood of the nucleus, peripheral nucleoli begin to appear in the latter. In the subcutical gland cells of *Piscicola*, the nucleolus, at the time of its most rapid growth, is apposed to the nuclear membrane; but when this period of volume-increase has ceased, it is never found in this position. Furthermore, the paranucleoli of *Rodalia* appear first in contact with the nuclear membrane.

The greater part of the above statements are in accordance with my observation on *Perla* egg, and in my mind there is little doubt as to the appropriateness of Montgomery's interpretation. Regarding the case of subcutical gland cells of *Piscicola* only, I am rather inclined to disagree with him, because of the fact that the nucleoli migrate into cytoplasm in great abundance at the time of the formation of secretion granules—a fact which apparently speaks against the theory of extra-nuclear origin of the nucleoli. Montgomery also cites Schwalbe's ('76) observations that in some vertebrate embryos, the nucleoli first arise as thickenings of the inner surface of the nuclear membrane. However, any literature published at as early a date as 1876 on 'nucleolus' can hardly be expected to be of much value on account of the poor technique employed and indefinite use of the term 'nucleolus,' so such early observations as these are perhaps to be intentionally excluded from our consideration.

Obst ('99) considered that the nucleoli are intranuclear in origin and, more especially, they are derived from the chromatin indirectly by some chemical change. He observed in *Unio*, *Epeira*, and other forms that in resting nuclei the basophile chromatin granules become acidophile, and then they fuse together to form nucleoli.

Page and Walker ('08), in the nerve cells of several mammals, Walker and Embleton ('08), in the cells of *Hydra*, described the multiplication of the nucleoli by regular budding. The nucleoli then pass out of the nucleus and eventually become absorbed. Walker and Tozer ('09) described similar observations in the

vegetative cells from *Spongilla*, *Planaria*, *Clepsine*, the rabbit, and the bean plant, and stated that "the regular multiplication of nucleoli within the nucleus, and their subsequent history, seems to exclude the possibility of the extra-nuclear origin suggested by Montgomery," at least in the case of the cells they considered. A similar conclusion may be reached also in the case of silk-gland cells of insects, since the numerous nucleoli in functional cells are derived from a single nucleolus of ordinary type by budding (Vorhies, '08), and they then pass out into the cell-body to form secretion granules (Maziarski, '11, and Nakahara, '17).

Medes ('04) and Dederer ('07), in the male germ cells of *Scutigera* and *Philosamia*, respectively, observed that the true nucleolus first appears in a mass of chromatin. McGill ('06), studying the ova of the dragonfly, expresses her opinion that the double nucleoli in that form are formed through the condensation of the basichromatin round the oxyphile nucleolus. Wilson ('05), Boving ('07), Randolph ('08), Stevens ('08), and Payne ('09), in the male germ cells of various insects, observed that the sexchromosomes are usually closely associated with the nucleolus, when they first appear. The idea of a close genetic connection between the nucleolus and at least of a certain chromosome thus developed has been finally put in a somewhat definite form by Goldsmith ('16), who concludes, from his study on the spermatocytes of *Pselliodes*, that "the true nucleolus is formed by the accumulation of linin about the sexchromosomes."

It is very unfortunate that many of the recent authors are rather too skeptical toward Montgomery's theory. Goldsmith ('16) went even so far as to say that the peripheral position of the nucleoli in the nucleus was the only evidence of the theory set forth by Montgomery, and that since many nucleoli do not come in contact with the nuclear membrane throughout their entire history, the evidence does not hold. The more recent author seems to have forgotten that there are some other facts, correlated with the peripheral position of the nucleoli, pointed

out by the other author, in making his suggestion. If anyone goes over Montgomery's work very carefully he would find it impossible to escape from the conclusion that the 'extranuclear theory' is undoubtedly acceptable, at least in certain cases.

I have stated that in the ovarian egg of Perla the nucleoli are of two types, namely, a large single nucleolus and a number of smaller ones. The former increases in size with the general growth of the egg, and sometimes passes out into the cell-body, thus indicating its possible intranuclear origin. The nucleoli of the second type are absent in the early oocyte, but they are newly introduced in the nucleus at the time when the 'yolk-nuclei' make their appearance around the latter, and are in all probability directly derived from such extranuclear substance.

These facts suggest that the nuclear structures designated as nucleoli can be divided into two classes, according to whether it is derived directly from outside of the nucleus, hence probably associated with anabolism, or is secondarily produced within the nucleus from substances which have already been there, hence in probable connection with katabolism. A hypothesis might be put forth that *the nucleoli represent substances that are going through the nucleus in metabolism*. Nutritive substances taken up into the nucleus may or may not be in the form of distinct bodies, and when they are visible as such in sections, they constitute the nucleoli of extranuclear origin. For the nucleoli of intranuclear origin, the current interpretation that they represent a passive product of the nuclear, or more especially chromatin activity, may be a proper explanation. Viewed from this standpoint, the two apparently conflicting views are nothing but partial expressions of a whole truth, and recent authors' critical remarks upon Montgomery's conclusion are just as unrational as the latter author's premature generalization.

Cases where the extranuclear origin of the nucleoli is demonstrated are not abundant. Aside of those early ovarian eggs of certain animals, Conklin's ('97) figure of a cell from the dorsal wall of isopod intestine, showing nutritive substances projecting

by many processes into the nucleus, some of which apparently are being set free into the nucleus to form nucleoli, is the only published observation I know of this phenomenon.

SUMMARY AND CONCLUSIONS

1. In the ovarian egg of *Perla immarginata*, two types of nucleoli are present, namely, a large single nucleolus and a number of smaller peripheral nucleoli.

2. The larger nucleolus increases in its dimension with the general growth of the ovum, and it may sometimes pass out into the cell-body hence, possibly of intranuclear origin.

3. The smaller nucleoli are in all probability formed directly of the portions of the yolk-nucleus migrated into the nucleus—hence, extranuclear in origin.

4. Different views concerning the nucleoli, and especially the question of their origin, can be brought into harmony under the hypothesis that the nucleoli represent substances going through the nucleus in metabolism.

5. According to this hypothesis, nucleoli may be formed directly of a material taken up by the nucleus or may be produced from some substances within the nucleus in the course of metabolic processes.

LITERATURE CITED

- VAN BAMBEKE, C. 1893 Elimination d'éléments nucléaires dans l'oeuf ovarien du *Scorpaena scrofa*. Arch. de Biol., T. 13.
- BORING, A. M. 1907 A study of the spermatogenesis of twenty-two species of the Membracidae, Jassidae, Cercopidae and Fulgoridae, with special reference to the behavior of the odd chromosome. Jour. Exp. Zool., vol. 4.
- CALKINS, G. N. 1895 Observations on the yolk-nucleus in the eggs of *Lumbricus*. Trans. N. Y. Acad. Sci.
- CONKLIN, E. G. 1897 The relation of nuclei and cytoplasm in the intestinal cells of land isopods. Amer. Nat.
- CRAMPTON, H. E. 1899 The ovarian history of the egg of *Molgula*. Jour. Morph., vol. 15, suppl.
- DEDERER, P. H. 1907 Spermatogenesis in *Philosamia cynthia*. Biol. Bull., vol. 13.
- FOOT, K. 1896 Yolk-nucleus and polar rings. Jour. Morph., vol. 12.
- GOLDSMITH, W. M. 1916 Relation of the true nucleolus to the linin network in the growth period of *Pselliodes cinctus*. Biol. Bull., vol. 31.

- HENNEGUY, L. P. 1896 *Leçons sur la cellule*. Paris.
- HUBBARD, J. W. 1894 The yolk-nucleus in *Cymatogaster*. *Proc. Amer. Phil. Soc.*, vol. 33.
- KORSCHULT, E. 1889 Beiträge zur Morphologie und Physiologie des Zellkernes. *Zool. Jahrb., Abt. Anat.*, Bd. 4.
- MAZIARSKI, S. 1911 Recherches cytologiques sur les phénomènes sécrétoires dans les glandes filières des larves des Lépidoptères. *Arch. f. Zellforsh.*, Bd. 6.
- MCGILL, C. 1906 The behavior of the nucleoli during oogenesis of the dragonfly, with especial reference to synapsis. *Zool. Jahrb., Abth. Anat.*, xxiii.
- MEDIS, G. 1904 The spermatogenesis of *Scutigera forceps*. *Biöl. Bull.*, vol. 9.
- MONTGOMERY, T. H. 1898 Comparative cytological studies, with especial regard to the morphology of the nucleolus. *Jour. Morph.*, vol. 15.
- MUNSON, J. P. 1898 The ovarian egg of *Limulus*, etc. *Jour. Morph.*, vol. 15.
1912. A comparative study of the structure and origin of the yolk nucleus. *Arch. f. Zellforsh.*, Bd. 8.
- NAKAHARA, W. 1917 On the physiology of the nucleoli as seen in the silk-gland cells of certain insects. *Jour. Morph.*, vol. 29.
- NEMÉC, B. 1897 Über die Structur der Diplopodeneier. *Anat. Anz.*, Bd. 13.
- OBST, P. 1899 Untersuchungen über das Verhalten der Nucleolen. *Zeit. f. wiss. Zool.*, Bd. 46.
- PAGE, M. W., AND WALKER, C. E. 1908 Note on the multiplication and migration of the nucleoli in the nerve cells of mammals. *Quart. Jour. Exp. Phys.*, vol. 1.
- PAYNE, F. 1909 Some new types of chromosome distribution and their relation to sex. *Biol. Bull.*, vol. 16.
- RANDOLPH, H. 1908 The spermatogenesis of the ear-wig, *Anisolabia maritima*. *Biol. Bull.*, vol. 15.
- STEVENS, N. M. 1908 A study of the germ cells of certain Diptera, with reference to the hetero-chromosomes and the phenomena of synapsis. *Jour. Exp. Zool.*, vol. 5.
- VORHEIS, C. T. 1908 The development of the nuclei of the spinning gland cells of *Platyphylax designatus* Walker. *Biol. Bull.*, vol. 15.
- WALKER, C. E., AND EMBLETON, A. L. 1908 Observations on the nucleoli in the cells of *Hydra fusca*. *Quart. Jour. Exp. Phys.*, vol. 1.
- WALKER, C. E., AND TOZER, F. M. 1909 Observations on the history and possible function of the nucleoli in the vegetative cells of various plants and animals. *Quart. Jour. Exp. Phys.*, vol. 2.
- WILSON, E. B. 1900 *The cell in development and inheritance*. Revised ed. New York.
1905 Studies on chromosomes. The behavior of the idiochromosome in Hemiptera. *Jour. Exp. Zool.*, vol. 2.