EXTRUSION OF NUCLEOLI FROM PRONUCLEI OF THE RAT

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ABSTRACT

Electron microscope observations of osmium tetroxide-fixed rat eggs indicate that small nucleoli are extruded from pronuclei in a sharply demarcated time period after sperm penetration. Approximately 4½ hours after sperm penetration, fine fibrous material aggregated in distinct loci along the inner surface of the nuclear envelope and condensed into small, dense bodies. The term tertiary nucleolus or extrusion body is used to designate the forming bodies. The small tertiary nucleoli form distinct protrusions from the pronuclei during the following developmental period and finally bud off into the cytoplasm, carrying with them a small portion of the double nuclear envelope. The extrusion bodies can be observed only in the vicinity of the pronuclei and have not been seen near the cell membrane. The fate of the tertiary nucleoli is not known; apparently they transform or disappear after they have passed into the cytoplasm. Eleven hours after sperm penetration, tertiary nucleoli are not present near the nuclear membrane and the extrusion activity has apparently ceased. Large and small nucleoli react similarly to cytochemical reagents: they are Feulgen negative; they are positive to the Millon, Sakaguchi, brom-phenol blue, and PAS reactions. Azure B stain combined with nuclease extraction indicates the presence of small amounts of RNA in the nucleoli.

INTRODUCTION

Extrusion of nucleoli from the germinal vesicle as observed by light microscopy has been described in different phases of oogenesis for representatives of every phyla (36). In general, the extruded nucleolar material has been implicated in controlling yolk formation but the evidence provided for this hypothesis has not been conclusive. Several observers have reported nucleolar extrusion also in mammalian eggs. Nucleoli were reported by Kremer (28) to be extruded as intact small bodies from the germinal vesicle of mice and rats, but other investigators concluded that the nucleolar material passed across the nuclear membrane in solution or in an unknown manner (3, 24). In the mouse and rat, extrusion of nucleoli also occurs from pronuclei in the form of small spherical bodies (28, 38, 39). During later embryonic development of the rat (2- to 8-cell stages), extrusion of nucleoli from cleavage nuclei has been reported (18).

Although some investigators have not observed the extrusion of nucleoli as a distinct phenomenon, extrusion of nucleolar material was inferred from the decrease of staining reaction for nucleolar material in the nucleus and the concomitant appearance of cytoplasmic granules with similar staining properties (3).

In several electron microscope studies, fragmentation of the nucleolus was reported and these fragments subsequently were thought to migrate across nuclear pores into the cytoplasm (5, 11, 33). In a very recent electron microscope study of oocytes from Thyone briareus, Kessel and Beams (27) described the morphological changes of the
nuclear membrane during extrusion of small nucleoli.

Several electron microscopists have also reported on nuclear "blebbing" (17, 23, 26) and actual emission of large bodies from the nuclei (1, 2). These processes were interpreted as the microscopically visible elements of nucleocytoplasmic interactions. One report (25) described the extrusion of nuclear material into the perinuclear space where it became membrane-bounded in an unknown manner and was distributed to the cisternae of the endoplasmic reticulum.

Shortly after formation of pronuclei in mammalian eggs, several nucleoli appear associated with the chromosomal masses. These nucleoli aggregate and fuse to form gigantic, primary nucleoli. During pronuclear growth phase the so called secondary nucleoli appear in association with the nuclear membrane. Recent review articles discuss these events in detail (7, 10, 14).

The present study on pronuclei in the rat egg provides electron microscope evidence for formation of a third, small set of nucleoli along the nuclear envelopes and for the mode of their transport into the cytoplasm. These nucleoli can be defined only by their small size and by the time of their appearance in relationship to the time of sperm penetration. Morphologically and chemically (as determined by cytochemical methods) they seem to be identical with the primary and secondary nucleoli. The term tertiary nucleoli or extrusion bodies will be used to designate these small nucleoli.

MATERIAL AND METHODS

Eggs were flushed from oviducts of rats with Medium TC 199 (Difco Laboratories, Inc., Detroit) containing 5 or 10 per cent serum between 11 a.m. and 6 p.m. the day after mating (12, 40, 43). In most cases adult female rats in estrus were mated with vigorous males of proven fertility. In some instances ovulation was induced in 28-day old rats by an intraperitoneal injection of 30 IU pregnant mare serum (PMS) (Equinox, A. P. L. Ayerst Laboratories, Inc., New York) followed 48 hours later with a subsequent intraperitoneal injection of 30 IU of chorionic gonadotrophin (HCG) (A.P.L. Ayerst Laboratories, Inc.). The injections were administered at 2 p.m. each day. The animals were paired and checked for sperm the subsequent morning.

Abbreviations for Figures

ch, chromatin aggregates
ms, multivesiculate bodies
m, mitochondria
nch, nucleolus-associated chromatin
nm, nuclear membrane
no, primary nucleolus
p, nuclear pores
pl, perinuclear lacunae
pn, pronucleus
l, lysosome-like bodies
s, secondary nucleoli
tn, tertiary nucleoli and extrusion bodies
v, cytoplasmic vesicles
z, zona pellucida

The thin section in Fig. 6 has been stained with saturated aqueous uranyl acetate for 16 hours at room temperature. Sections in the remaining electron micrographs were stained with Millonig's alkaline lead tartrate.

**FIGURE 1** A large pronucleus (pn) approximately 4½ hours after sperm penetration is seen. The nucleus is enclosed by a double membrane (nm) demonstrating in places nuclear pores (p). In certain areas the two components of the nuclear membrane separate, forming large perinuclear lacunae (pl). Occasionally a vesicle (v) protrudes into these spaces. Within the pronucleus three large nucleoli (no), chromatin aggregates (ch), and an unidentified structure can be seen (arrow). The cytoplasm contains mitochondria (m), many single vesicles (v), multivesiculate bodies (ms) and irregular structures which may represent lysosomes (l). × 7800.

**FIGURE 2** The two components of the nuclear membrane (nm) have separated along long distances forming perinuclear lacunae (pl). In places (arrows) the inner nuclear membrane forms a small vesicle which projects into a lacuna. Fibrous, dense material accumulates in well defined loci along the inner nuclear membrane and protrudes slightly towards the cytoplasm. A mitochondrion (m), vesicles (v) and dense granules are seen in the cytoplasm. The granules are interpreted as a polysaccharide component. × 43,000.
A short region of the fallopian tube was straightened out on both ends, using watchmaker forceps. A blunted 30 gauge hypodermic needle was inserted at the isthmus and eggs were flushed out toward the fimbriated end by a gentle stream of flushing fluid. Before use, the flushing fluid and the receptacles were warmed to 37°C. Ova were quickly transferred from the flushing medium with a narrow bore pipette into cold 3.3 per cent osmium tetroxide solution, adjusted to pH 7.4 with t-collidine buffer (13) and kept in an ice bath during fixation. After 45 to 60 minutes' fixation, the eggs were washed in several changes of 70 per cent ethanol and then dehydrated by pipetting them through a series of ethyl alcohol with increasing concentrations. The eggs were then embedded in Epon 812 (31). The total infiltration time was at minimum 3 to 5 hours. The eggs were transferred into the final Epon mixture in an aluminum foil boat for flat embedding. The curing of the epoxy resin took place in three steps: 12 hours at 37°C, 24 hours at 50°C, and 24 hours at 65°C. The hardened resin was cut with a diamond dental cutting tool into small blocks which were oriented and mounted on metal chucks for sectioning.

Silver to pale gold sections were cut with a diamond knife on a Huxley ultramicrotome and the sections were placed on carbon-coated copper grids. To increase contrast of the specimen the grids were stained either with a saturated aqueous uranyl acetate solution or distilled water, respectively, at the same temperature as the enzyme solution for 16 hours at room temperature or for 12 hours with an alkaline lead stain (34). Electron micrographs were taken in either a modified RCA EMU 2C or a Siemens Elmiskop I microscope on du Pont Ortho A lithographic sheet film with Cronar (polyester) base.

The localization of nucleic acids was carried out on eggs fixed in 10 per cent neutral formalin, buffered with 0.1 M phosphate buffer. After washing and dehydrating, the eggs were embedded either in methacrylate or in paraffin. One-μ sections were cut from the methacrylate blocks with glass knives, while the paraffin-embedded material was sectioned 4 to 6 μ thick. The preparations were stained, after removal of the methacrylate or paraffin with xylene, with an 0.5 per cent azure B solution, adjusted to pH 4.2 with aceate buffer. Alternating sections were incubated overnight at 37°C with DNase (0.2 mg/ml) solution in 0.033 M Veronal-acetate buffer at pH 7.2 containing 0.0025 M MgSO₄; RNase solution (1.0 ng/ml) in distilled water at 37°C; 4 per cent perchloric acid solution at 4°C. Some slides were incubated in both enzyme solutions in succession. Control slides were incubated for the same time periods in 0.0025 M MgSO₄ solution or distilled water, respectively, at the same temperature as the enzyme solutions.

The trichloroacetic acid–Millon reaction for the estimation of protein content (35) and the Sakaguchi reaction for the presence of arginine (20) were performed. Alkaline fast green (4) and brom-phenoI blue reactions (15) were employed for detection of basic proteins. Localization of DNA was determined by the Feulgen reaction on whole eggs fixed in alcohol vapor and posttreated with 95 per cent ethanol (30). After hydration, the eggs were hydrolyzed in saturated, aqueous picric acid at 60°C for 1 hour (15) and stained with the Feulgen reagent (29). The PAS reaction was used as a test for polysaccharides with acetylazation or amylase extraction as controls.

The time of sperm penetration in mammalian eggs cannot be determined accurately but must be estimated in a separate experiment for each strain in the particular environmental conditions. In the Sprague-Dawley rats the majority of the ovulated eggs were penetrated by spermatozoa by 7:30 a.m.; at earlier times the number of eggs penetrated was lower. This time was taken then as the average time of sperm penetration. The experimental animals were kept on a natural day-night cycle. Most experiments were carried out during the months of July and August. Sunset was between 6:50 and 8:00 p.m. (Pacific standard time) and sunrise was between 4:00 and 5:15 a.m. (Pacific standard time). Mating usually occurred between 11 p.m. and midnight. Austin and Braden (9), using a different strain of rats, found the same average time for sperm penetration under similar environmental conditions.

Detailed data on the timing of ovulation induced by PMS and HCG treatment in 28-day old rats are not available. It was found in this study that ovulation followed 10 to 12 hours after HCG injection, which is similar to that in the mouse as reported by Edwards and Gates (21). Gonadotrophins were administered so that mating and fertilization coincided approximately with the estrus period of the adult animals used in these experiments.

RESULTS

Four and one-half hours after the estimated time of sperm penetration, the pronuclei were spherical and 8 to 14 μ in diameter. They possessed a double nuclear membrane with widely dispersed nuclear pores (37, 41, 42). In Fig. 1 the female pronucleus of this developmental period can be seen with three large nucleoli. The two components of the nuclear membrane are separated in regions, forming large perinuclear lacunae. From the inner membrane small vesicles protrude into this perinuclear space.

After formation of the large secondary nucleoli along the nuclear membrane, during the pronuclear growth phase, fine fibrous electrondon- opaque material aggregated in distinct locations.
**Figure 3** Tertiary nucleoli protrude more into the cytoplasm. Both nuclear membranes can be followed along them. An unidentified very dense structure (d) is seen in the pronucleus (pn). Several mitochondria and a complex multivesiculate body are seen in the cytoplasm. × 36,500.

**Figure 4** Three extrusion bodies, covered by both nuclear membranes, project clearly into the cytoplasm. A membrane-bounded membranous structure and vesicles are seen in the cytoplasm. × 44,500.
along the inner surface of the nuclear membrane, forming clumps. At these loci there are slight evaginations of the nuclear membrane toward the cytoplasm (Figs. 2, 13). In other eggs of the same rats or in eggs of different animals at a slightly later stage, the fibrous material becomes more compact, forming small dense bodies or tertiary nucleoli which form distinct protrusions from the pronucleus. Both nuclear membranes can be traced along the surface of tertiary nucleoli (Figs. 2 to 4). At a later developmental stage, the fibrous, tertiary nucleoli have protruded farther into the cytoplasm (Figs. 5 and 6), and only a narrow connection with the nucleus is visible. Both nuclear membranes can be followed around the dense tertiary nucleoli also at this stage, and in these regions nuclear pores are lacking. In the cytoplasm, in the proximity of the pronuclei, several extrusion bodies can be seen which are surrounded by two membranes (Figs. 6 to 8) and are very similar to the protruding tertiary nucleoli. Although the final separation was not seen, the observed sequence indicates strongly that the tertiary nucleoli are indeed pinched off and released into the cytoplasm.

Frequently, several tertiary nucleoli are observed projecting into a large perinuclear lacuna (Fig. 7). In these cases the outer nuclear membrane separates widely from the inner one but the inner component remains closely applied to the surface of the tertiary nucleoli.

The nuclear surface is quite smooth in eggs collected at 6 p.m. (Fig. 9). Small tertiary nucleoli are lacking completely. At this stage components of the nuclear membrane follow each other closely but the frequency of nuclear pores is low. Large secondary nucleoli lose their association with the nuclear membrane but remain at the periphery of the pronuclei. The nuclear extrusion activity apparently has ceased by this time.

Two approaches were followed in an attempt to confirm that a classification of the extrusion bodies as small nucleoli is justified: (a) morphological comparison of the small dense protruding bodies with the larger, primary and secondary nucleoli of the same pronuclei; and (b) a comparison of the cytochemical staining reactions of extrusion bodies and nucleoli.

In Figs. 10 and 11 it is clearly seen that morphologically the protruding smaller bodies are similar to the primary and secondary nucleoli. Every one of these nuclear organelles contains densely packed, thin, electron-opaque fibrils, although the fibrils found in the tertiary nucleoli seem to be more firmly packed. The granular component described in nucleoli of many animal and plant cells cannot be found. It is quite difficult to measure the width of individual fibers with great accuracy, but they seem to be about 100 A in diameter. These fibrils, thus, cannot be clearly distinguished from chromosomal fibrils on the basis of morphological criteria alone. A differentiation of these two components becomes even more difficult, because the nucleoli have no membrane surrounding them, and along their entire periphery small fibers penetrate the fibrillar nucleolar mass from the adjacent nucleoplasm. The chromosomes are in such a dispersed state at this time that they cannot be identified either in the electron micrographs or by light microscopy. No specific chromosomal region can thus be related to the site of nucleolus formation or of nucleolar attachment.

The primary and secondary nucleoli and the small extruding bodies show the same cytochemical
FIGURE 8 Portions of both pronuclei are seen with several tertiary nucleoli located in perinuclear lacunae. Three extrusion bodies are found in the cytoplasm between the pronuclei. × 18,500.

FIGURE 9 A pronucleus approximately 12 hours after the approximated time of sperm penetration. No tertiary nucleoli nor swollen perinuclear lacunae can be seen in this pronucleus. The large secondary nucleolus lost its contact with the nuclear membrane. In the cytoplasm several mitochondria (m), vesicles of different sizes, lysosome-like bodies and a multivesiculate body are found. × 14,000.
reactions. They are negative to the Feulgen reaction (Fig. 12) even though they are frequently surrounded by a more positively reacting chromatin ring. The rest of the pronucleus stains faintly but uniformly with this reaction. The proteinaceous nature of all nucleoli is demonstrated by the trichloroacetic acid–Millon reaction (Fig. 13). The intensity of the color reaction was similar for the nucleoli and extrusion bodies, whereas the chromatin stained with a lower intensity. The Sakaguchi reaction also stained intensely the large centrally placed nucleoli and secondary nucleoli, as well as the small extrusion bodies near the nuclear membrane (Fig. 14). The results with the brom-phenol blue stain were variable. Most frequently, only the chromosomal material, corresponding to the Feulgen-positive area, stained faintly blue, but on occasion the nucleoli stained intensely instead (Fig. 15). The results were consistently negative with the alkaline fast green stain during the developmental period under investigation.

Results of azure B staining for the demonstration of RNA gave the most consistent and clearest results of the several basic dyes tried. In 4 μ paraffin sections of pronuclei the chromosomal material stained orthochromatically. In order to achieve better resolution, 1 μ sections of methacylate-embedded eggs were used for the enzyme extraction procedures. The nucleoli stained distinctly blue in the control preparations (Fig. 16). Following DNase extraction, the nucleoli stained similar to the control preparation (Fig. 17), while after RNase or perchloric acid treatment the nucleoli lost their stainability (Fig. 18). After a double extraction with DNase and RNase, only a metachromatic stain of the extracellular matrix remained between the follicle cells outside the egg.
Following formalin fixation of rat eggs, the time for the enzymatic extraction of DNA and RNA must be extended. After 1 to 2 hours' treatment, the staining intensity decreased, to be sure, but some of the stain was always still observable. All pronuclear inclusion bodies, both large and small, thus contain a small amount of RNA.

With the PAS reaction the nucleoli stain uniformly and this reaction cannot be abolished by acetylation or amylase extraction (Fig. 20). The staining, therefore, is not due to glycogen or to any other simple polysaccharide. (Table I summarizes the staining reactions.)

Both lines of evidence, the ultrastructural comparison as well as the similarities of the stain reactions, indicate that the small bodies protruding from the pronuclei are similar to the large primary and secondary nucleoli and that one is justified in regarding the extrusion bodies as small nucleoli.

Large numbers of tertiary nucleoli were found protruding from pronuclei which had reached their full size. The diameter of these nucleoli ranged between 200 and 500 mμ, but in two cases they were 1 μ or more. In one plane of section approximately forty protrusions could be counted in a single large male pronucleus. The approximate diameter of the male and female pronuclei at their maximal size was 25 and 18 μ, respectively. The duration of the nuclear surface activity lasts approximately 6 hours and, although there is no indication of the duration of the extrusion process for each individual nucleolus, the number of tertiary nucleoli extruded from both pronuclei during this interval may be very high. It is assumed that the extrusion occurs uniformly over the entire pronuclear surface in the rat, and there is evidence from examination of nearly fully serially sectioned pronuclei to support this assumption.

DISCUSSION

General Considerations

From light microscope observations of nucleolar extrusions from the germinal vesicle of different animals, four different mechanisms have been postulated for the extrusion process (36):

1. "Diffusion of nucleolar substances through the nuclear membrane in solution. . . ."
2. "Passage of nucleolar products through the membrane in fluid or semifluid state. . . ."
3. "The nucleolar extrusions are said to break bodily through the nuclear membrane. . . ."
4. "Finally, . . . the nucleolar buds first come to lie in protrusions of the nuclear membrane, after which either the membrane breaks through at these spots and the nucleoli pass into the cytoplasm or the whole nuclear evagination constricts off from the nucleolus, so that the nucleolar extrusions in the cytoplasm are at first still enveloped by the membrane, which later disappears. . . ."

The electron microscopic observations in this study indicate that, in the case of rat pronuclei, nucleoli pass into the cytoplasm, being surrounded by a double membrane which originally was an integral part of the nuclear envelope. This extrusion thus falls into the fourth category of mechanisms proposed above.

The passage of the nucleoli out of the nucleus as well defined bodies was not observed in living eggs by light microscopists. The appearance of structures in the cytoplasm with staining characteristics similar to those of nucleoli was taken as sufficient evidence to conclude that nucleoli or nucleolar fragments were actually extruded. The
stainability of these structures was usually lost soon after migration into the cytoplasm.

Kremer (28) was the only investigator who described previously the bodily extrusion of nucleoli from the germinal vesicle of mammalian eggs. He is the only investigator, however, who described by light microscopy the passage of nucleoli from pronuclei of any species in the form of such well defined, small chromatic bodies. He thought that the nuclear membrane breaks at a point over the protruding nucleoli so that they can pass into the cytoplasm as a distinct small chromatin granule.

The formation of tertiary nucleoli and the extrusion process can be timed exactly in pronuclei of the rat and can be studied adequately only by electron microscopy due to their small size. The first indication pointing to the appearance of tertiary nucleoli is the slight aggregation of fine fibrillar components in close apposition to the internal nuclear membrane. Further compacting of these fibrils into distinct bodies, their protrusion into the cytoplasm, and their final pinching off can be observed in detail. The probable sequence of events is summarized diagrammatically in Fig. 21 below. Extruded nucleoli were recognized only in the vicinity of the pronuclei and were never observed near the plasma membrane. They must either disperse rapidly or transform immediately into an unrecognizable form.

The electron microscopic observations do not allow at this time the drawing of any conclusions as to the site of origin of the fibrillar components of the tertiary nucleoli. The dimensions of the fibrillae are very similar to those of the dispersed chromosomal fibrils as well as to the components of the larger nucleoli. Whether this new set of nucleoli originates at any chromosomal locus or possibly from one of the secondary nucleoli (or other alternatives) must remain open at the moment. It should be stated, however, that, in contrast to some invertebrate eggs, fragmentation of the primary or secondary nucleoli was not observed (5, 11, 33).

The morphological characterization of the small extrusion bodies as nucleoli by electron microscopy seems clear cut. The localization of RNA and basic proteins at the site of tertiary nucleoli lends further support to this idea. In case of mammalian eggs, then, the extrusion bodies can be referred to justifiably as nucleoli.

**Cytochemical Characterization of Substances Extruded from Nuclei**

In most studies dealing with nucleolar extrusion, it is assumed that the nuclear inclusion bodies are nucleoli. By definition, a nucleolus should contain, among other components, ribonucleic acid. Cytochemical methods, however, often have failed to demonstrate clearly an RNA content in nucleoli of oocytes. This was the case for nucleoli of mammalian pronuclei (8, 16) and the same held true also for the snail, *Helix aspersa* (19). Cytochemical staining characteristics of the so called nucleoli and of nucleolar extrusion material were not

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**Figures 16 to 19** Sections of rat eggs stained with azure B. Fig. 16, control preparation: nucleoli of rat egg pronuclei stain strongly blue. The stain over the chromatin material is nearly imperceptible in a 1 μ section of formalin-fixed, methacrylate-embedded eggs. × 1940.

Fig. 17, DNase treatment: the chromatin of pronuclei does not stain but the nucleoli still stain with the same intensity. 1 μ section, formalin fixation, methacrylate embedding. × 1940.

Fig. 18, RNease extraction: the nucleoli have lost their stain but the chromatin stains similarly to the control preparation. 4 μ section, formalin fixation, paraffin embedding. × 2150.

Fig. 19: After a double enzymatic digestion with DNase and RNase, no stain is observed in developing rat eggs nor in the surrounding follicle cells. The intercellular matrix between follicle cells stains metachromatically. By phase-contrast microscopy, both pronuclei could be located (circles with interrupted lines). × 1870.
FIGURE 30 Nucleoli of all sizes and the zona pellucida stain with the PAS reaction. After acetylation or amylase extraction, the staining characteristics were not altered. The cytoplasm also stains faintly. X 1940.

Table I

Summary of Staining Reactions

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<th>Azure B</th>
<th>RNase</th>
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The number of extruded nucleoli may be very high. The size of the extruded nucleoli varies, but, in general, they are close to the limit of resolution of the light microscope. In Kremer's (28) drawings, many nucleoli measured between 1 and 2 μ. There is no doubt in this writer's opinion that the nucleolar extrusion described in the current study is identical with that observed by Kremer; the size difference may be due to different strains of animals used or to different preparation procedures.

Electron Microscopy of Nucleolar Extrusion

There are only a few studies by electron microscopy which would indicate extrusion of nuclear materials by budding off of membrane-bounded organelles. During oogenesis, nucleolar fragmentation within the nucleus is frequent and, in some cases, small dense masses appear in the cytoplasm. Dense materials on either side of the nuclear membrane were interpreted as evidence for an active nucleolar extrusion process (5, 11, 33). There is much greater similarity of the nucleolar extrusion, as observed in this study, to the "blebbing" of the nuclear membranes of salivary gland studied in most cases by specific staining techniques. The azure B staining experiments with RNase and cold perchloric acid extraction in the present study demonstrate the presence of RNA in the primary, secondary, and tertiary nucleoli.

The nuclear surface activity associated with extrusion of material is sharply demarcated in time. Its duration occupies the middle period of cells reported by Gay (23, 26), to the formation of "heavy bodies" in sea urchin eggs (1), and to the interesting intranuclear annulated vesicles in Noctiluca described by Afzelius (2) which are apparently transported through the nuclear membranes. Gay reported that nuclear membrane activity does not seem to be random in occurrence but seems to occur near chromosomal bands. The
sequence of events observed suggested to her that the blebs are detached from the nucleus and are liberated into the cytoplasm. Although in *Drosophila*, Feulgen-positive material was found in the blebs while still attached to the nucleus, after their detachment this was not the case. No definite statement was made about the chemical nature of the extruded material but an RNA content can be inferred from its basophilia and lack of Feulgen stain.

The formation of the heavy bodies described by Afzelius (1) in sea urchin eggs is morphologically similar to the nucleolar extrusion process found in the pronuclei of the rat. Afzelius demonstrated that they are basophilic, RNA-containing bodies. Figs. 7 and 8 in Afzelius’ paper represent different phases of heavy body formation which are very similar to phases of formation and extrusion of nucleoli from rat pronuclei but the behavior of membrane differs in later extrusion stages. Heavy body formation may be similar in general to nucleolar extrusion reported in this study but some of the details seem different. One of the most significant points of difference is that the annulated membranes surrounding the heavy bodies are not continuous around the granular matrix.

Recently, nucleolar extrusion has been described by electron microscopy to occur from oocytes of *Thyone briareus* (27). The protrusion phase of the nucleoli in this egg is very similar to that observed in rat pronuclei. Kessel and Beams suggested that the final extrusion in their material occurs by a focal dissolution of the nuclear membrane over the extruding portion of the nucleolus and that after extrusion of the nucleolus the membrane is rapidly reconstituted. The particles liberated would accordingly not be membrane-bounded. The membrane reconstitution is not documented sufficiently by electron micrographs. In case of *Thyone*, apparently the RNA content of the extruded nucleoli was assumed from the granular appearance of the nucleolar material, and the scattering of ribosome-like particles in the cytoplasm. The passage of 150 A diameter nucleolar particles through the nuclear pores was suggested for *Thyone* and other species (6, 7, 27, 33). The granular nature is, however, not convincingly documented in the presented electron micrographs. More recent studies question whether particles can pass through the nuclear pores (22). In pronuclei of mammalian eggs the granular component of nucleoli is lacking; only fibrous elements are consistently found. There are very few nuclear pores during the extrusion period and, therefore, it is not likely that this mechanism plays an important role in transport of substances between the nucleus and cytoplasm in the recently penetrated rat egg.

In trophoblast cells of the 6-day old rabbit embryo (as well as several other cell types), Hadek and Swift (25) described membrane-bounded intracisternal bodies which are quite similar to the extruded nucleoli reported in the present study. The interpretation offered by these investigators is somewhat different and involved the extrusion of nuclear material through “annuli” which penetrate only the inner, but not the outer, nuclear membrane. The annuli observed in their study may represent the final stages of the pinching off process of nucleoli shortly before the membrane closes over the extruding nucleolus (similar to Figs. 5, 6, and 7 in this study). In rabbit trophoblast cells, the gradual formation and protrusion of the nucleoli and the evagination of the nuclear membrane could not be followed nor timed easily and, thus, the details may have escaped these investigators.

**Functional Considerations of the Extruded Nuclear Material**

In the literature there are only vague suggestions as to the function of nucleolar extrusion. Because the extrusion process usually precedes or coincides with vitellogenesis, the extrusion material was thought to control yolk formation. The proposed functional schemes, however, could not be substantiated by subsequent studies. A generalized statement by Kremer (28) concerning the significance of the extruded nucleoli foreshadows the importance of the chromatic substances (which

\[\text{Figure 31} ~ \text{The aggregation and fate of tertiary nucleoli; a diagrammatic representation.}\]
we would call RNA today) in the economy of the egg. He postulated that the periodic accumulation and dispersion of chromatic substances and their exchange between nucleus and cytoplasm reflects the morphological expressions of the metabolic transformations in the egg.

A similar functional scheme was developed by Dalcq (18) who suggested that nucleolar materials pass into the cytoplasm in the form of small nucleoli and influence the synthesis of mucopolysaccharides and plasmalogens in the cytoplasm. After the latter substances reach a certain concentration in the cytoplasm, they become precursors for DNA synthesis for the daughter nuclei. “Thus, there would be two linked cycles of major activity; one directed outward from the nucleus and leading to the manufacture of the precursors, and the other directed toward the nucleus and leading to the synthesis of DNA . . .”

The function of nucleolar extrusion remains obscure. It is of great interest, however, that RNA and basic proteins are apparently localized in them. It should be pointed out in this respect that only a few ribosomes are present in the cytoplasm of tubal eggs of several mammals (Szollosi, unpublished studies) although enzyme-extractable RNA can be demonstrated by cytochemical methods. Ribosomes are present, however, in large numbers in trophoblast cells of rabbits (25) and rats (Szollosi, 1964, unpublished studies). It has been proposed that ribosomes may either increase slowly in numbers during the successive cleavage divisions (32) or that they may appear suddenly in the cells of the morula or blastocyst.

It is tempting to suggest that the extruded nucleoli mediate the influence or control of the nucleus over certain cytoplasmic events. The low RNA content and the high content of basic proteins of the extruded nucleoli indicate that we are dealing with the extrusion of a large and complex molecular system. Extruded material from the pronuclei could be of importance in establishing the machinery for the future protein synthetic activity of blastomeres. Genetically, it may be of importance that both male and female pronuclei extrude materials simultaneously. Nucleolar extrusion observed in the cleavage stages by light microscopy (18) indicates further that nucleolar extrusion is of general and continued importance to the developing rat embryo.

It is possible that the nucleolar extrusion or the transfer of nucleolar materials observed from germinal vesicles and pronuclei is an extreme case of a phenomenon found generally in cells. The extrusion of particles from pronuclei may be limited to certain functional periods of cell life in which large particles are being extruded. In somatic cells nuclear membrane bubbling occasionally observed may suggest the transport of nuclear or nucleolar material into the cytoplasm. The fate of the extruded nucleoli has not been determined as yet either by light or by electron microscope studies and all suggestions offered are speculative.

Some aspects of these studies represent part of the author’s doctoral dissertation presented to the Zoology Department of the University of Wisconsin. Many thanks are due to Dr. Hans Ris for his interest, supervision and stimulus during my student days. The technical assistance of Miss Sandra Hastings is gratefully acknowledged.

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