RNA TRANSPORT FROM NUCLEUS TO CYTOPLASM IN *CHIRONOMUS* SALIVARY GLANDS

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ABSTRACT

The fine structure and cytochemistry of the extremely large RNA puffs, or Balbiani rings, in salivary gland nuclei of midge, *Chironomus thummi*, larvae have been investigated. The Balbiani rings are composed of a diffuse mass of electron-opaque 400 to 500 A granules, short threads about 180 to 220 A in diameter and associated fine chromatin fibrils. These components appear to be organized into brushlike elements which form the ring. Electron microscope cytochemistry has shown that the granules and short threads contain RNA. After ribonuclease digestion, only 50 to 100 A chromatin fibrils were apparent in the Balbiani ring, and the granules were no longer demonstrable. Deoxyribonuclease digestion had no apparent effect on these structures. Observations indicate that the granules are formed from the short threads and released into the nucleoplasm in which they are evenly distributed. At the nuclear envelope, many granules have been observed partially or completely within the nuclear pores. These granules become elongated and are shown to penetrate the center of the pore in a rodlike form, about 200 A in diameter. The Balbiani ring granules are not normally visible within the cytoplasm adjacent to the nuclear envelope, but have been rarely found in this region. It is suggested that the granules represent the product of the Balbiani ring, possibly a messenger RNA bound to protein, and that they regularly pass into the cytoplasm through a narrow central channel in the pores of the nuclear envelope.

INTRODUCTION

The synthesis of at least the major portion of cellular RNA occurs on DNA templates located within the nucleus. The succeeding step requires a transport of the messenger RNA from its site of synthesis in the nucleus to a polyribosome assembly in the cytoplasm. Biochemical investigations leave little doubt as to the reality of this RNA movement (Scherrer et al., 1963; Perry, 1962), and radioautographic studies of RNA synthesis clearly show a movement of the label from the nucleus to cytoplasm (Goldstein and Plaut, 1955; Prescott, 1960; Zalokar, 1961). To date, however, there has been little morphological evidence which demonstrates this RNA transfer.

One of the most prominent chromosome structures in the nucleus of *Chironomus* salivary gland cells is the relatively enormous RNA puff known as the Balbiani ring (Balbiani, 1881; Bauer 1935; Beermann, 1952, 1961, 1962). Similar to the RNA puffs observed in the polytene chromosomes from a large number of dipteran species, the Balbiani rings have a high content of RNA

and show a rapid uptake and turnover of RNA precursors (Pelling, 1964). The Balbiani ring, however, is distinguishable from other RNA puffs. It occurs only in the chironomid family of flies and is remarkable for its large size and characteristic ring shape, its presence during a greater portion of larval development, and the fact that only one to three rings are ever found in the chironomid genome. It is further identified in the salivary gland by the presence of uniform dense granules about 300 or 400 A in diameter (Beermann and Bahr, 1954; Stevens, 1964).

There is some evidence which lends support to the concept that the RNA which is rapidly synthesized at these large puffs is a messenger type. The microelectrophoretic analyses of RNA from various loci in *Chironomus tentans* nuclei by Edström and Beermann (1962) reveal that the RNA of the Balbiani ring has an asymmetrical base composition and differs significantly from that of nucleolar or ribosomal RNA. The data suggest that it may be a copy of a single DNA strand. In extensive cytogenetic studies on two species, *C. tentans* and *C. pallidivittatus*, Beermann (1961) has shown a direct correlation between the presence of an additional Balbiani ring in a few specialized cells of the salivary gland in *C. pallidivittatus* and the accumulation of a granular component in the secretion of these cells. Hybridization studies of this species with *C. tentans*, where both the Balbiani ring and the granular secretion are absent, demonstrate that at least some of the genetic information required for the production of the secretory granules resides in the locus of the additional Balbiani ring. It has thus been suggested by Beermann that the active Balbiani ring represents a specific gene locus in a functional state.

While the discontinuities or pores of the nuclear envelope presumably serve in mediating nucleocytoplasmic exchanges, few such movements have been recorded and characterized at the fine structural level. Clearly, the envelope is not a passive barrier but exercises some control over materials passing through it (Bernhard, 1958). It is apparent that the circular openings are not true holes, in the physiological sense, and from recent investigations by Loewenstein and colleagues (Loewenstein and Kanno, 1963; Wiener et al., 1965), and Feldherr (1965), the presence of a barrier to free diffusion within the pores is well established. The electron-opaque material contained in the pore and composing the annular structures on either side of the envelope, forming the so called "pore complex" (Watson, 1959), is possibly involved in the regulation of nucleocytoplasmic exchanges. In many cases, this material appears to close the opening. Nevertheless, in certain cell types demonstrating an active protein synthesis, such as insect nurse cells and oocytes, electron microscope observations have been made of nuclear material passing through the pores into the cytoplasm (Anderson and Beams, 1956; Miller, 1962). These observations however, have not been able clearly to characterize the nuclear material involved in the transfer.

Earlier reports on fine structural aspects of Balbiani rings in *C. tentans* (Beermann and Bahr, 1954; Beermann, 1962) have described a diffuse, expanded region of the chromosome in which lampbrushlike elements are identified and visualized as looped projections bearing the granular product. Balbiani ring granules were observed throughout the nuclear sap, and several workers have already cited evidence that the granules regularly penetrate the pores of the nuclear envelope in an apparent nucleocytoplasmic passage (Beermann, 1962; 1963; Beermann and Clever, 1964; Stevens, 1964).

The present report offers a more detailed study of the ultrastructure and cytochemistry of the Balbiani rings in a related species, *Chironomus thummi*. The RNA synthesized at this locus is found to exist in the form of dense, 400 A granules which apparently are regularly released from the chromosome and can be observed within the nuclear pores, strongly suggesting a transport to the cytoplasm.

MATERIALS AND METHODS

Continuous cultures of *Chironomus thummi* Kieffer were maintained in the laboratory. Larvae were grown at 16°C in plastic pans containing a watery mixture of nettle powder (Penick and Co., Jersey City, New Jersey) and shredded cellulose packing material under constant aeration. When larvae reached the late fourth instar and prepupal stages, they were placed in a large breeding cage, measuring about 2 x 2 x 2 ft, at room temperature. The cage was illuminated during the day and it provided for the eclosion of the adults, mating and deposition of the eggs.

For light microscopy, 45% acetic acid squashes of salivary glands were stained in azure B at pH 4.0 and 40°C for 2 hr. For removal of RNA, the squash

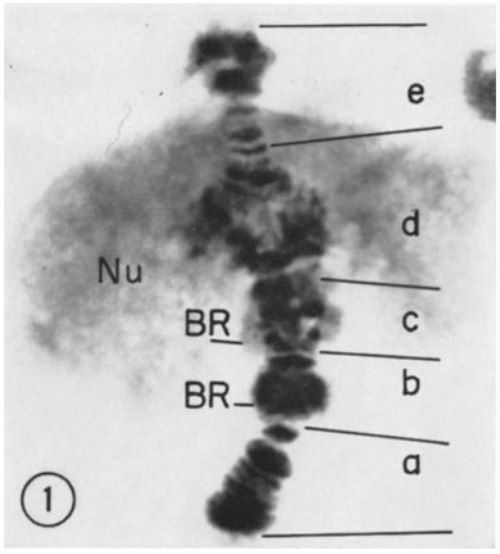


FIGURE 1 A squash preparation of the short chromosome IV from the salivary gland of a late fourth instar larva of *Chironomus thummi*. The preparation is stained with azure B, which produces a blue-green color in the DNA- and metachromatic purple in the RNA-containing areas. The chromosome regions *a* through *e* are designated according to Keyl (1957). The nucleolar organizer, with its encircling large nucleolus (*Nu*), is present in region *d*. Regions *c* and *b* each contain a Balbiani ring (*BR*); that in region *c* is more prominent at this stage. Branching of adjacent bands is visible above and below the locus in BR_c. About × 900.

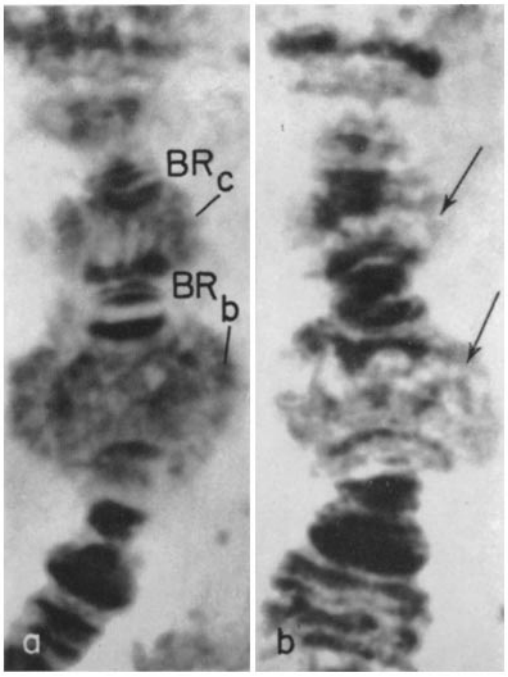


FIGURE 2 *a* A squash preparation of chromosome IV from a late prepupa, stained with azure B and showing the Balbiani rings (*BR*) of regions *b* and *c*. The purple-staining RNA is seen as material of medium density which fills these expanded regions of the chromosomes. At this stage, the Balbiani ring, BR_b, is fully expanded; that at *c* is smaller.

FIGURE 2 *b* A preparation identical to Fig. 2 *a*, but digested with ribonuclease prior to staining. The Balbiani rings show a complete loss of RNA, and only fine strands of chromatin remain in these regions. About × 2,500.

was incubated in 0.02% ribonuclease, pH 6.5, for 1 hr at room temperature, prior to staining.

For electron microscopy, salivary glands from all stages of fourth instar larvae and prepupae grown at 16°C were removed and placed in one of the following fixatives:

1. 2% osmium tetroxide, buffered to pH 7.3–7.5 with 0.1 M phosphate buffer;
2. 10% formalin, in phosphate buffer;
3. Acrolein/formalin, 5% each in phosphate buffer, followed by osmium tetroxide as in 1.

All fixations were carried out for 30 min at 0–4°C. Glands were dehydrated in ethanol and embedded in Epon.

Enzymatic digestions were performed on formalin-fixed glands by the following procedure: Glands were transferred from the fixative to 35, 50, and 70% ethanols at 4°C in 15-min changes. After 30 min or longer in 70% ethanol, the glands were rinsed briefly in water and extracted for 1 hr at room temperature in: (*a*) 0.1% deoxyribonuclease (Worthington Biochemical Corp., 1X crystallized) plus 0.003 M $MgSO_4$ at pH 6.5, or (*b*) 0.1% ribonuclease (Worthington), pH 6.5. Controls were incubated in the same solutions without enzymes. Following extraction, glands were rinsed briefly in water, washed for 30 min in cold 5% TCA, dehydrated and embedded as above.

Thin sections of the material were stained with one of the following:

1. 1% or 3% uranyl acetate for 1 hr;
2. Uranyl acetate as in 1, followed by lead hydroxide (Karnovsky, 1961) or lead citrate (Reynolds, 1963), 2 to 5 min;
3. Lead hydroxide, 5 to 30 min;
4. 10% aqueous solution of sodium tungstate, adjusted to pH 5.5 with concentrated and 1 N HCl, 1 hr (Swift and Adams, unpublished).

Sections were examined and photographed in a Siemens Elmiskop I.

OBSERVATIONS

Structure and Cytochemistry of the Balbiani Ring

The morphology of the short chromosome IV in *Chironomus thummi* (according to Keyl, 1957) can be visualized from both the squash preparation in Fig. 1 and the thin section in Fig. 3. The left end of the chromosome (*e*) bears a terminal heterochromatic band. The large nucleolus is shown traversing and encircling the chromosome at region *d*. The chromosomal and nucleolar substances here form a complex association and interdigitation which is illustrated in Fig. 3. One large Balbiani ring is situated immediately below the nucleolus at *c*, and the other one is observed at *b*. The constriction between regions *a* and *b* is visible at *a/b*, and a number of closely apposed bands form the right end at *a*.

Light Microscopy

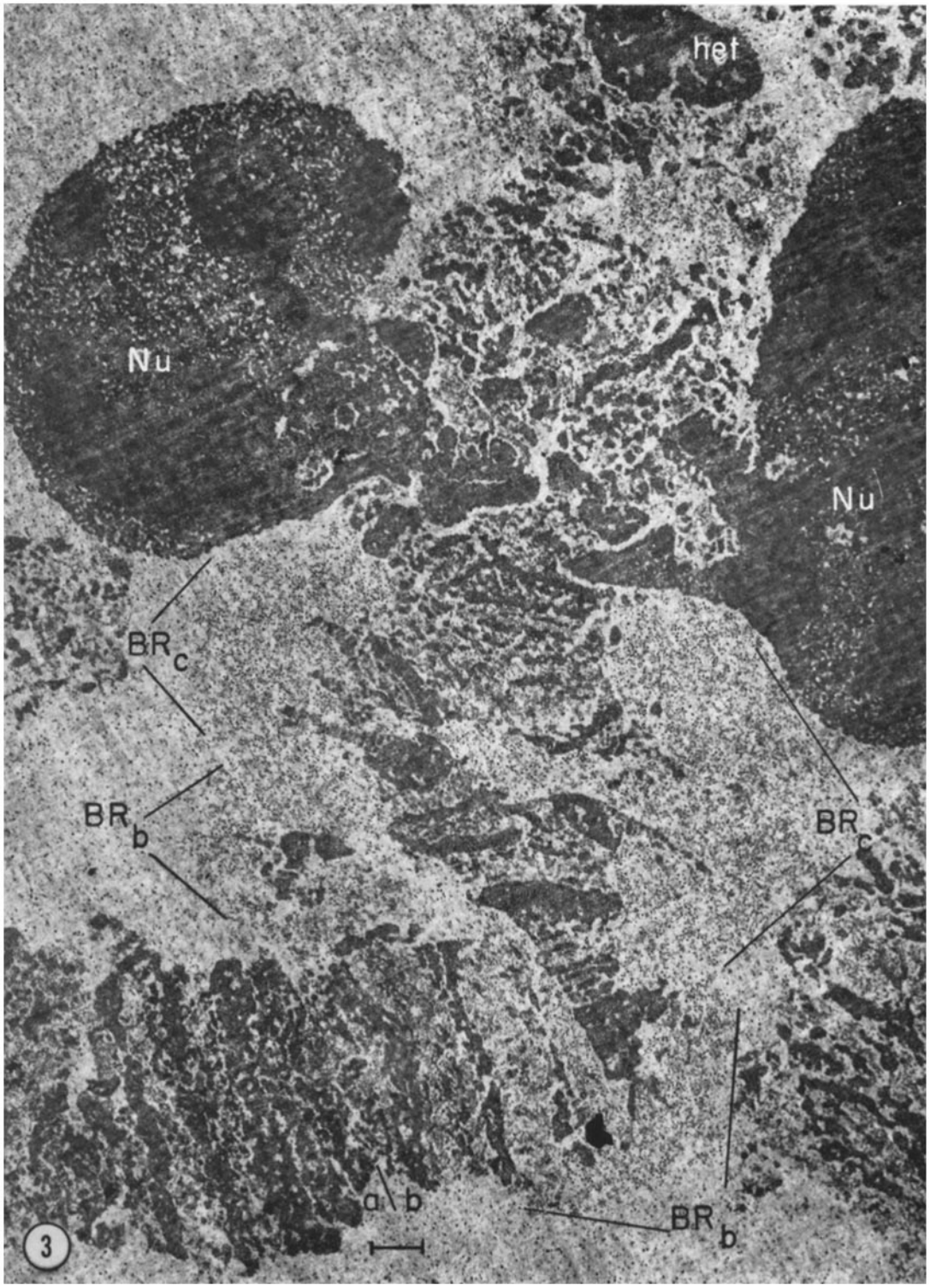
The two Balbiani rings of this species, located in regions *b* and *c*, may be easily recognized by their characteristic swollen condition and their proximity to the single nucleolus in region *d* (Fig. 1). In squash preparations it is observed that the chromatin on either side of these differentiated regions branches progressively into the expanded ring, becoming increasingly diffuse. Azure B dye produces an intensely stained purple ring, indicating a high concentration of RNA (Fig. 2 *a*). Ribonuclease digestion of a squash, followed by azure B staining, results in the complete absence of the purple ring; the Balbiani rings remain as diffuse, dilated areas of blue staining DNA (Fig. 2 *b*). Acid fast green staining has revealed a large quantity of nonhistone protein present in these and other RNA puffs (Swift, 1962).

Electron Microscopy

FINE STRUCTURE: In the electron microscope, the Balbiani rings are identified chiefly by the large quantity of particulate material present in the region. Favorable sections of chromosome IV reveal the progressive branching of the polytene chromosome on either side of the Balbiani ring locus (Fig. 3). The greatly expanded region seemingly has forced the two members of the chromosome pair apart and consequently pulled the branchings of the neighboring bands out into the annular structure of the ring. The banding pattern of the adjacent regions remains, and can be identified in the strands of chromatin present in the ring (Fig. 4).

The Balbiani ring itself is composed of a mass of twisted fibrillar elements and electron-opaque granules. The fibrillar elements and granules fill the doughnutlike form of the Balbiani ring and can also be seen within the center of the chromosome (Figs. 3 and 4). Thin sections provide little indication of the three-dimensional organization of the ring components. Occasionally, however, segments suggesting a brushlike configuration can be identified (Figs. 5 and 6). Such segments appear to be formed by a central axis of fibrils and numerous short threads, some of which project in a perpendicular plane from the axis. Some of the short threads appear to terminate in the dense granules (Figs. 5 to 8) and many particles intermediate in shape between granules and threads can be recognized. In occasional brushlike seg-

FIGURE 3 Longitudinal medial section of a nearly entire chromosome IV from a late fourth instar larva. Region *e*, at the top, shows a small portion of the terminal heterochromatic band (*het*). The two halves of the large nucleolus (*Nu*) are continuous across the chromosome; their branches are closely intermingled with and often indistinguishable from the chromatin of the organizer. The large Balbiani ring of region *c* (BR_c) occurs immediately below the nucleolus. The smaller Balbiani ring of region *b* (BR_b) lies below, in this case at a position where the chromosome has bent along its axis. The constriction at *a·b* separates these two regions, and the right end of the chromosome lies just off the photo at left. The Balbiani rings are composed of two masses of dense particulate material on either side of the chromosome, and joined across it by a narrow band of particles. Branches of the neighboring chromosomal bands enter and disappear in the diffuse masses. Separate dense granules occur evenly distributed in the surrounding nucleoplasm. OsO_4 fixation; uranyl and lead stain. × 6,500.


het
Nu
Nu
BR c
BR b
BR c
a b
BR b
3

ments the granular forms appear to be absent. Among many electron micrographs of Balbiani ring regions, no clear cut examples have been found to demonstrate the brushlike segments in their entirety. The recognizable segments do, however, generally follow a slight curve and it is assumed that they represent portions of closed loops, as Beermann and Bahr (1954) have observed in *C. tentans*. Because of their irregularity, it is difficult to discern the exact dimensions and nature of the components of the central axis. Evidence is presented below which indicates that the axial fibrils contain DNA.

The granules in the Balbiani ring are approximately 400 to 500 A in diameter, and often appear to have a central area of lower density (Fig. 6). This observation is more clearly demonstrated in formalin-fixed tissue, stained for nucleic acid components with sodium tungstate. Under these conditions, the granules are reminiscent of round beads having a hollow core (Fig. 11 *a*).

In comparison to the typical 50 to 100 A chromatin fibrils, the short threads clearly have a greater diameter, about 180 to 220 A. Both the threads and granules are more prominent after double fixation (acrolein/formalin–osium tetroxide) and stain noticeably more intensely than the chromatin and nucleolar material with uranyl and lead staining (Fig. 7). The Balbiani ring region under these conditions appears as a mass of short dense threads, interspersed with free or attached granules. Closer inspection discloses that the threads are not uniformly dense, but apparently contain filamentous elements of higher density within (Figs. 8 and 9).

CYTOCHEMISTRY: Results of enzymatic digestion and selective staining for electron microscopy give additional information on the chemical nature of the Balbiani ring components. Following fixation with formalin, or other aldehyde fixatives, staining of thin sections with sodium tungstate demonstrates a selectivity for nucleic acids. In Fig. 11 *b*, the chromosomal substance is densely stained with sodium tungstate; the nucleolar material stains with less intensity. Both the granules and the short threads of the Balbiani ring show an affinity for the stain.

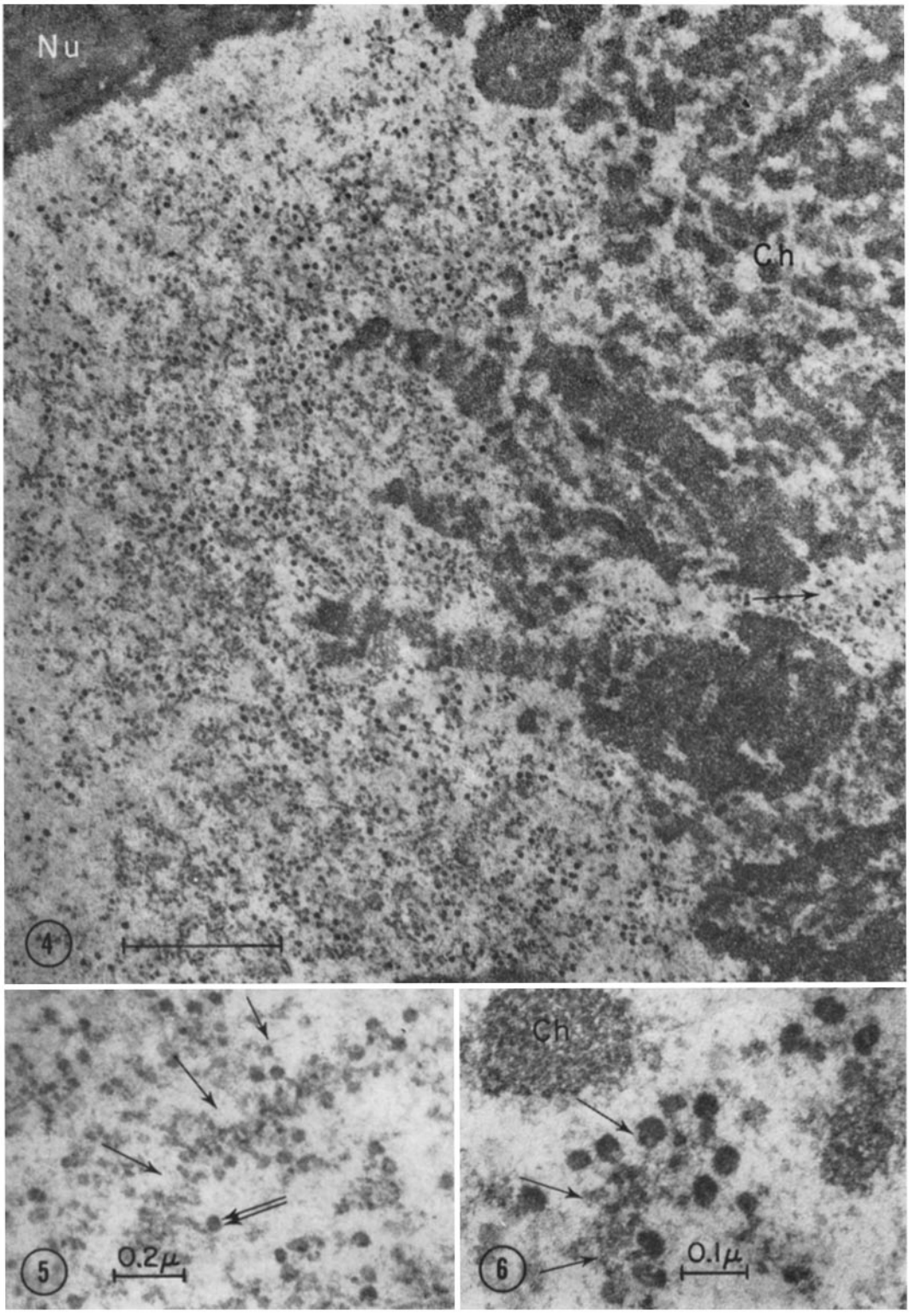
Extraction of formalin-fixed tissue with ribonuclease, followed by sodium tungstate staining, specifically demonstrates the RNA content of the granules and short threads. With this method, only the chromatin fibrils of the polytene chromosomes remain stainable. In Fig. 10, several bands can be recognized in the condensed region of the chromosome. In the encircling mass of fibrils which represents the expanded portion of the Balbiani ring, no evidence of the granular component or the short threads remains. At higher magnification (Fig. 12), the dimensions of the fibrils within the areas of compact chromatin, and the sections of fibrils found in the ring region, are shown to be similar, both having a diameter of 50 to 100 A. This is considerably less than the 180 to 220 A diameter of the irregular, RNA-containing threads.

Extraction of formalin-fixed glands with deoxyribonuclease, followed by sodium tungstate staining, does not appear to alter the structure or staining ability of the short threads and granules (Fig. 13). The chromosomes lose their stainabil-

FIGURE 4 Higher magnification of part of Balbiani ring *c* in Fig. 3. Dense spherical granules fill the expanded region, interspersed with threadlike elements and bits of chromatin. The banding pattern of the chromosome regions on either side of the Balbiani ring remains discernible into the smallest branches. Granules are seen penetrating the center of the chromosome (arrow). A portion of the nucleolus (*Nu*) is at top; chromosome, *Ch.* × 25,000.

FIGURE 5 An arrangement of granules and short threads in a Balbiani ring which suggests a brushlike configuration (arrows). Note granule attached to thread (double arrow). OsO_4 fixation; uranyl stain. × 53,000.

FIGURE 6 Another brushlike formation (arrows) of granules and threads in a Balbiani ring region from Fig. 3. Some granules appear to have a central area of lower density. Chromatin, *Ch.* × 100,000.


Nu
Ch
4
Ch
5
0.2μ
6
0.1μ

ity and become difficult to distinguish, and the Balbiani ring components are thus more evident.

In regions of the nucleus beyond the Balbiani rings which contain quantities of the spherical 400 A granules (see below), results of cytochemical methods likewise demonstrate the RNA composition of the free granules. In Figs. 14 and 15, sections of control and ribonuclease-treated tissue are shown after uranyl staining. Chromatin, Balbiani ring granules and cytoplasm all stain intensely in the control. Following ribonuclease digestion (Fig. 15), the chromatin continues to stain, but the granules are not distinguishable and only a diffuse material in the nuclear sap, presumably proteinaceous, remains visible. The small amounts of stained material in the cytoplasm represent the secretion contained within the tubules of the endoplasmic reticulum and the Golgi saccules. This material, although basophilic, is not extractable with ribonuclease, and may contain acid polysaccharides.

Balbiani Ring Granules and Nucleocytoplasmic Transport

The granular component is found associated with salivary gland chromosomes uniquely at the Balbiani ring loci. The dense spherical granules are also found in large numbers evenly distributed in the nuclear sap, always appearing single and independent (Figs. 3 and 16). The irregular short filamentous component of the Balbiani ring, however, is never apparent in the nucleoplasm.

Individual granules have an average diameter of 400 to 500 A, with occasional examples having diameters up to 600 A. Following osmium tetroxide or acrolein/formalin–osmium tetroxide fixation, the granules are spherical and uniformly dense. Indeed, their electron opacity following uranyl and lead staining combinations slightly exceeds that of the other cellular components, such as chromatin and ribosomes (Figs. 7, 16, and 17). Often, however, and particularly in formalin-fixed tissue or in sections stained with lead hydroxide alone, the less dense central core is evident (Fig. 24).

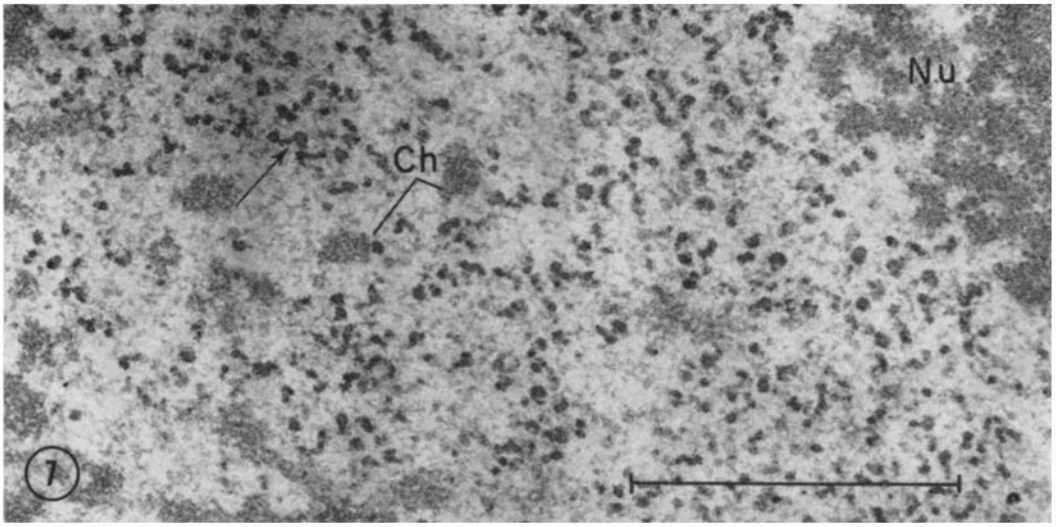
Careful inspection of any section passing through a nucleus will reveal a number of granules in close association with the nuclear envelope (Fig. 16). It can be estimated that, per section of any nucleus, at least two granules can be found which are clearly engaged in nuclear envelope pores viewed transversely. As many as 15 have been observed in nuclei having a circumference of the order of 120 μ. A total of 102 examples have been recorded photographically from a variety of stages and fixations, and many others have been observed.

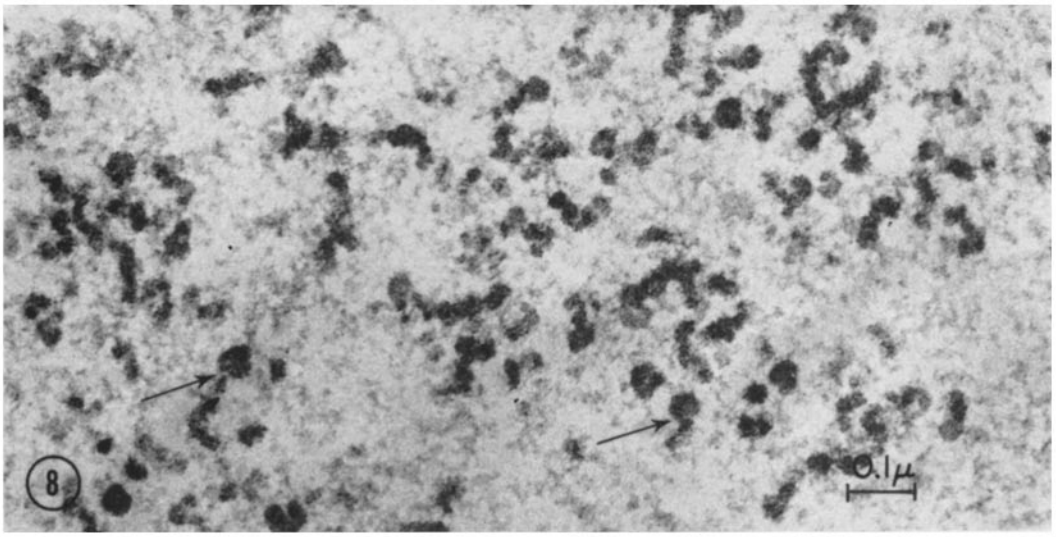
The nuclear envelope shows the typical bilamellar structure common to most nuclei. In transverse sections, the pores have a diameter of approximately 550 to 650 A (Figs. 18 and 24). In sections tangential to the envelope (Fig. 29), annuli are numerous and appear in roughly hexagonally spaced arrays. The center-to-center spacing is 1000 to 1100 A. The pores never have an "empty" appearance but contain a diffuse material which extends across the opening and covers

FIGURE 7 Portion of a Balbiani ring after double fixation (acrolein/formalin-OsO_4), uranyl and lead staining, which shows a mass of spherical and irregular granules and threadlike elements. The short threads are more prominent than in OsO_4-fixed sections, and they show an electron opacity similar to that of the granules and greater than that of the chromatin (*Ch*) and nucleolar components (*Nu*). Some threads appear to terminate in the dense granules (arrow). × 45,000.

FIGURE 8 Higher magnification of a Balbiani ring region similar to Fig. 7. The short, twisted threads have an uneven density and outline, suggesting a coiled structure. Spherical granules are closely associated with the threads (arrows). Acrolein/formalin-OsO_4 fixation; uranyl and lead stain. × 85,000.

FIGURE 9 A region of a Balbiani ring from a formalin-fixed gland. The uneven density of the short threads is more apparent than after double fixation, and filamentous forms can be observed within (arrows). Clumps of chromatin, *Ch*. Formalin fixation; uranyl and lead stain. × 85,000.


Nu
Ch
7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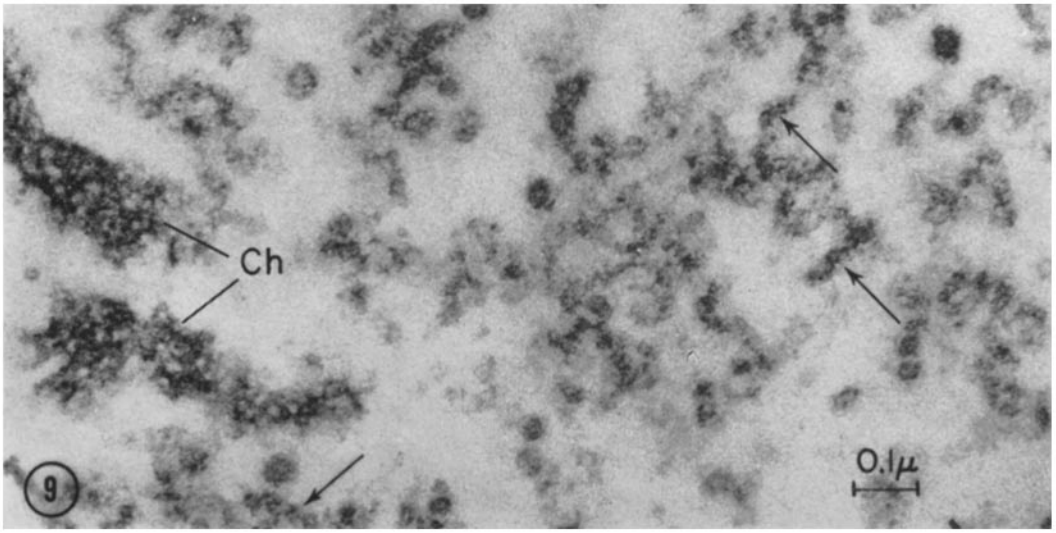
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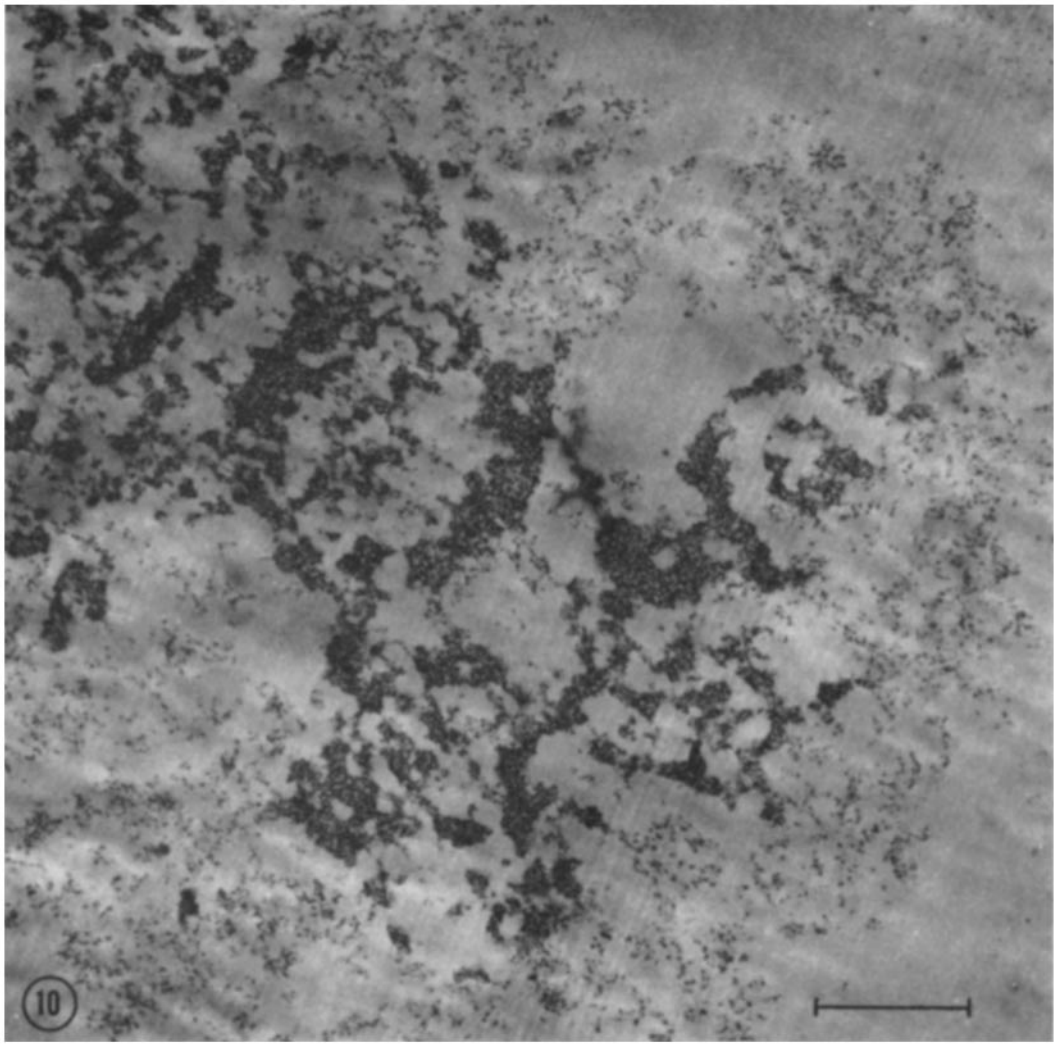

FIGURE 10 Micrograph illustrating the DNA distribution in the Balbiani ring. The section is from a formalin-fixed gland extracted with ribonuclease, and stained with sodium tungstate. The chromosome is sectioned obliquely and shows portions of the two Balbiani rings. One ring is at top and middle left, identified by the masses of small fibrils. The chromosome fans out into the other one, from the right side around to the bottom left. The compact bands of chromatin branch into the masses of fibrils, which represent the expanded portions of the Balbiani rings. × 20,000.

the fused membranes at the pore boundary. Such material has been previously described for many tissues (André and Rouiller, 1956; Baud, 1959; Watson, 1959; Gall, 1964). In the present study, it is worth noting that the diffuse material is more prominent after double fixation, and forms an ill defined sheath on both sides of the envelope (Fig. 24).

Our micrographs of the numerous granules found associated with nuclear pores have been arranged in a series, and are interpreted as representing the sequence of events in a nucleus-to-cytoplasm movement, during which the granules appear to undergo a process of elongation. The first step is shown in Figs. 18 and 19. The spherical granule, situated directly over the pore, becomes drawn out into a narrow point which is directed toward the center of the pore. A pro-

gressive elongation of the granule occurs so that the next step reveals a spherical portion remaining in the nucleus with a narrow process extending across the nuclear envelope and into the cytoplasm (Figs. 20 and 21). The granules may become completely transformed into a slender rodlike form, as shown in Figs. 22 to 24. Such a form may extend to a length of 1700 A and completely straddle the nuclear envelope. These rodlike forms are seldom regular in outline. Their diameter at the level of the nuclear envelope consistently measures about 150 to 200 A, but it may vary considerably along the rest of the length.

The slender width of the elongated granules as they lie within the pores is significant. As judged by the limits of the membrane edge, the measurable opening of the pore averages 600 A and would easily accommodate the 400 to 500 A granules. Particles of this size, however, were never observed within the pores. Rather, the elongated rod form seems to fill a central channel within the pore, about 200 A in diameter. Furthermore, there is some indication of a decrease in density of the rod at the nuclear envelope level in this channel (Figs. 22 and 24).

Tangential views of the nuclear envelope frequently demonstrate the same phenomenon. Regions in which the circular outline of the annulus is well delimited can be assumed to be positioned somewhere within the pore and/or its associated diffuse material. In such instances, a centrally placed dot or granule is often found (Fig. 29). The diameter of the dot ranges from 150 to 300 A. One further observation from tangential sections concerns the occurrence of dense, crooked strands immediately on the cytoplasmic surface of the nuclear envelope. A number of these strands are visible in Fig. 29. They have no obvious relationship to the annuli or pores and have a density similar to that of the cytoplasmic ribosomes.

No conclusive evidence is available to indicate the fate of the Balbiani ring granules. Whether the contents of the granules actually enter the cytoplasm is not certain, since most observations record a disappearance of the material on the cytoplasmic side of the nuclear envelope. Nevertheless, Figs. 25 to 28 offer some suggestions for this question. Sections transverse to the nuclear envelope often show ribosomes situated on the outer membrane (Figs. 22 and 24). Portions of the tubular rough endoplasmic reticulum which fills the cytoplasm of the cells are also found immediately adjacent to the outer membrane. Figs. 25 and 26 suggest a relation of the elongated granules with these ribosomal formations in the cytoplasm. Instances in which the major portion of a granule lies on the cytoplasmic side of the pore, as shown in Fig. 27, are comparatively rare. Finally, a few examples of apparently intact granules situated in the cytoplasm have been found (Fig. 28), as well as many others in which their presence is only suggested.

DISCUSSION

The puffing phenomenon observed in polytene chromosomes undoubtedly represents the actual functioning of genetic loci involved in the synthesis of specific RNA molecules (Beermann, 1956; 1963; Swift, 1962; Clever, 1964). The chromosome fibrils of a single band unfold to produce an expanded, diffuse region or puff, which exhibits a high rate of RNA synthesis. This morphological evidence of active synthetic regions along the chromosome thus provides support for concepts of differential gene activity in interphase chromosomes.

Some RNA puffs in *Drosophila* have already been shown to contain characteristic ribonucleoprotein particles of various sizes (Swift, 1962; 1963). Of special significance, however, is the particular example of the Balbiani ring in *Chironomus*. Not only is there a distinctive granular product attributable to these loci, but there is also evidence, in one case, that a specific message relating to the secretory product of the cell is controlled by a Balbiani ring (Beermann, 1961). The present study clearly shows that the 400 A granules originating at these loci are composed of ribonucleic acid and probably protein.

Structure of the Balbiani Ring

The observations reported here on the fine structure of the Balbiani ring give firm support to the chromosome model originally proposed by Beermann (1952) and shown in more detail in Beermann and Clever (1964). The region can be described as the unraveling of a thick, folded cable into individual filamentous elements, probably in the form of extended loops. Thin sections do not permit the actual observation of entire looped structures, but some further details of their fine structure have been noted. The lampbrush configuration suggested by Beermann and Bahr

(1954) in their earlier studies is supported, and three fine structural components are implicated in its formation: (1) Characteristic thick threads, 180 to 220 A in diameter, serve to form much of the lampbrush. (2) Dense granules, 400 to 500 A in diameter, appear associated with the thick threads in this region. (3) The presence of chromatin fibrils in the expanded portion of the Balbiani ring is demonstrated by ribonuclease digestion and nucleic acid staining.

The thick threads are characteristic only of the Balbiani ring, and do not appear free in the nucleoplasm. They occasionally seem to project from a loop axis, and may be closely associated with the granular component at their distal ends. In some cases they are convoluted into irregular coils. They stain densely with uranyl and lead stains, and this stainability is ribonuclease removable. The dense granules resemble the thick threads in their RNA content and staining affinities, and it seems likely that the spherical granules form by a configurational change from the threadlike component. The numerous examples of what appear to be incomplete granules support this suggestion. The granules obviously possess a complex structure, with a dense, beadlike cortex around a less dense core. The protein content of the threads and granules has not been accurately demonstrated in the electron microscope, but an extension of light microscope results strongly suggests the presence of a nonhistone protein associated with the RNA of these structures. Previous results of pepsin digestion on thin sections of glycolmethacrylate-embedded glands suggests that the protein is of a basic nature (Stevens, 1964). Radioautographic studies with labeled amino acids indicate that incorporation of protein precursors need not take place during puff formation (Clever, 1964). Thus, at least in some cases, the nonhistone puff protein seems to be preformed, and merely to accumulate at the puff during RNA synthesis. For this reason, it seems possible that one function of puff protein may be to package the newly formed RNA into a granule for transmission to the cytoplasm.

More study is needed on the structure of the fine chromatin fibrils within the Balbiani ring. They probably form the axes of looplike structures, and provide the continuity of the chromosome. The finest fibrils are about 50 A in diameter, but are often thicker, possibly because of secondary folding. In some places, they are continuous with the dense clumps of contracted chromatin.

Nucleocytoplasmic Transport

Although the electron microscope, of necessity, presents a static image of the cell, a dynamic picture can sometimes be discerned from the observations. Our results showing that thick threads and incomplete granules occur only in the Balbiani ring indicate that the 400 to 500 A granules in the nucleus are formed at this locus and subsequently move into all areas of the nucleo-

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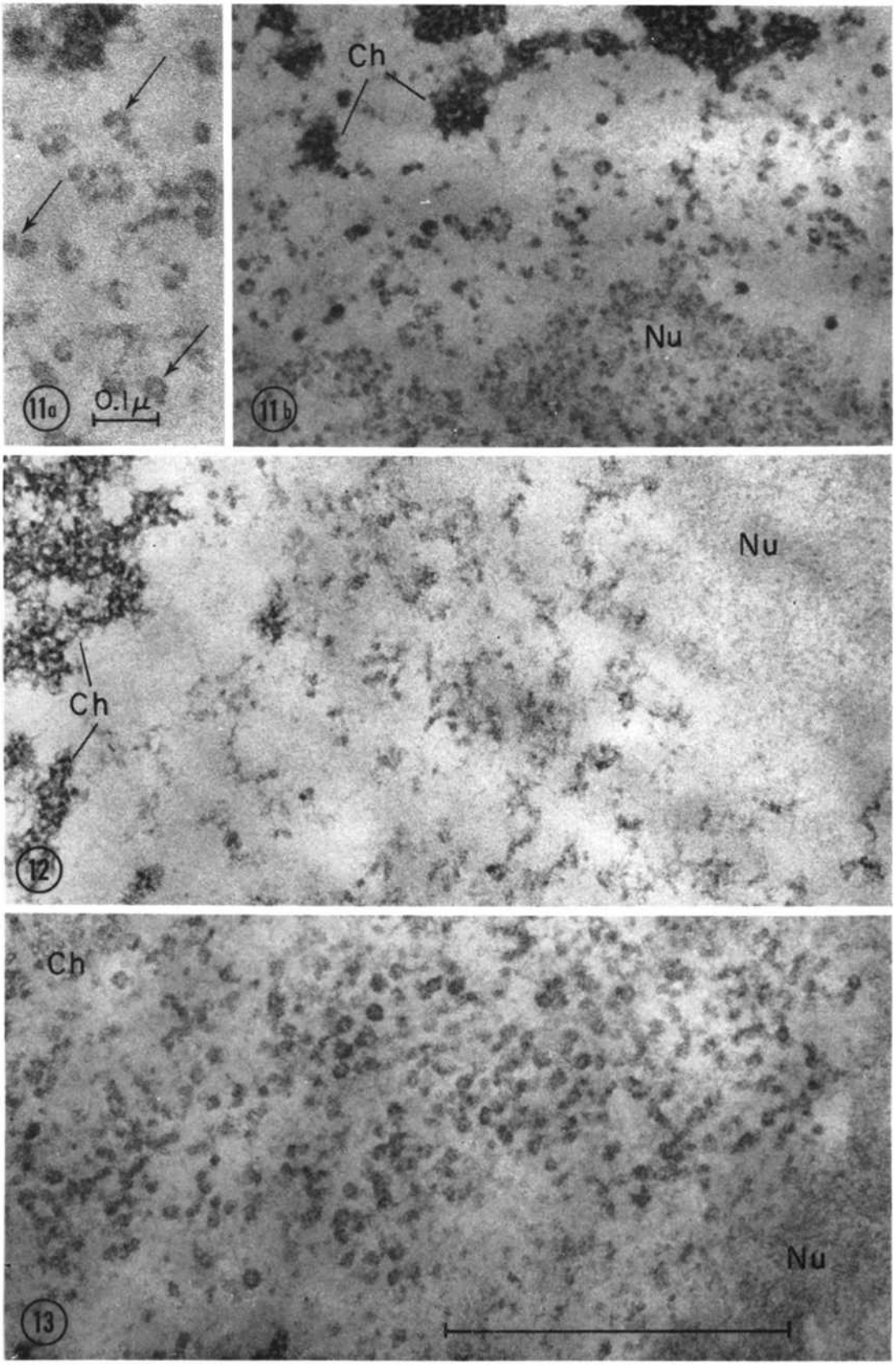
FIGURES 11 to 13 Cytochemistry of the Balbiani ring. Formalin fixation; sodium tungstate stain.

FIGURE 11 *a* and *b* Control. A region of a Balbiani ring is shown between areas of compact chromatin (*Ch*) and the nucleolus (*Nu*). Both the granular and threadlike components of the ring are intensely stained. In Fig. 11 *a*, at higher magnification, a region of lower density is seen in the center of some granules (arrows), giving the appearance of hollow beads. Fig. 11 *a*, × 100,000; Fig. 11 *b*, × 56,000.

FIGURE 12 Ribonuclease digestion. A portion of the Balbiani ring is situated between the densely stained chromatin (*Ch*) and the nucleolus (*Nu*). The only stainable remnants of the ring are a mass of fibrils which have a diameter of 50 to 100 A that is similar to to the dimensions of the chromatin fibrils. No granules or 180 to 220 A threads are visible. The nucleolus (*Nu*) has lost its stainability with sodium tungstate. × 56,000.

FIGURE 13 Deoxyribonuclease digestion. The granules and thick threads of the Balbiani ring appear unchanged by the enzymatic extraction. The nucleolus (*Nu*) also is unchanged and continues to stain. A portion of extracted chromatin (*Ch*) is shown at left; thick sections of the same block were Feulgen-negative. × 56,000.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11b
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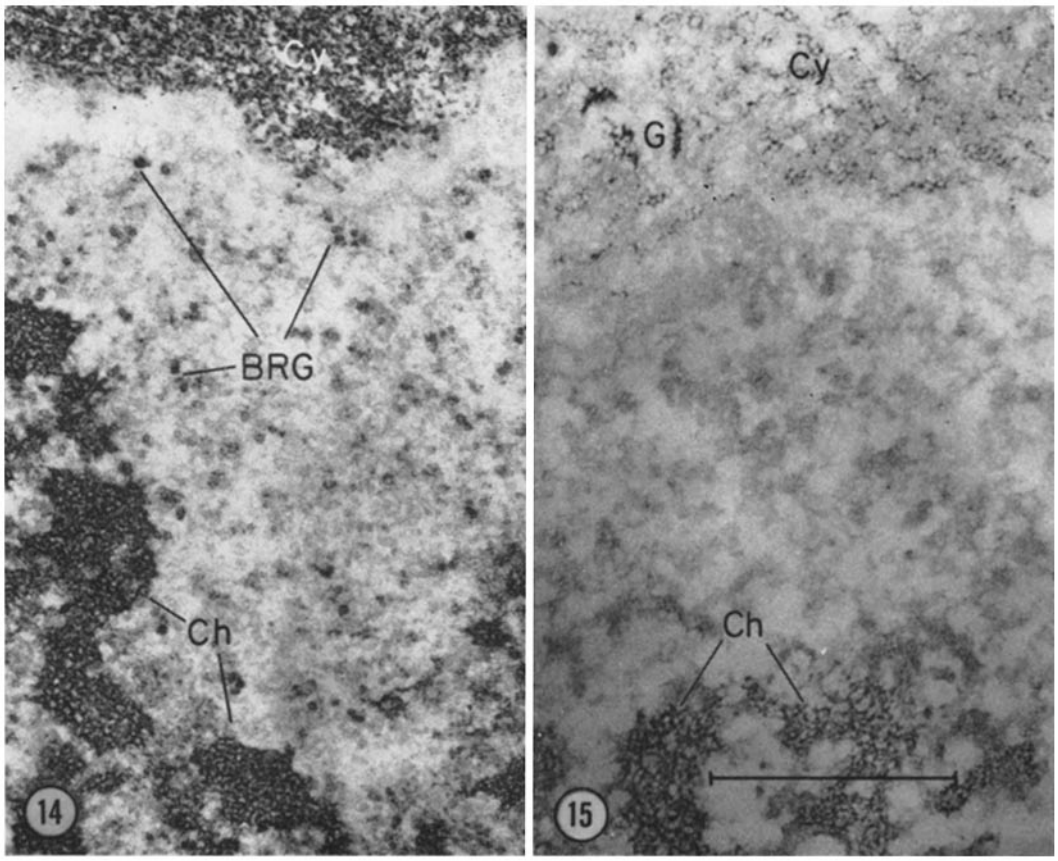
Nu
12
Ch
Nu
13
Ch
Nu



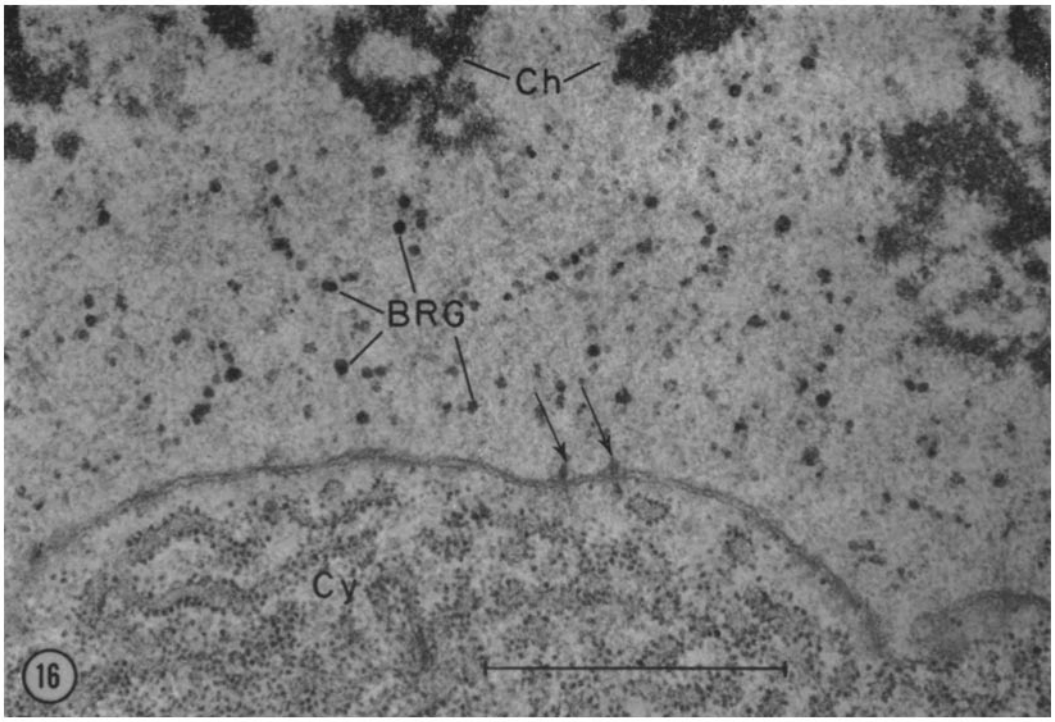
FIGURES 14 and 15 Cytochemistry of Balbiani ring granules. Formalin fixation; uranyl stain. × 33,000.

FIGURE 14 An area of nucleoplasm which contains numerous, single Balbiani ring granules (*BRG*). The cytoplasm (*Cy*) and chromatin (*Ch*) likewise stain intensely with uranyl.

FIGURE 15 A comparable area of nucleoplasm from a ribonuclease-extracted gland. No Balbiani ring granules can be identified in the nucleus between the chromatin (*Ch*) and the cytoplasm (*Cy*). Only an amorphous substance remains in this region, presumably proteinaceous in nature. In the cytoplasm, the densely stained material represents the secretory product contained in the cisternae of the endoplasmic reticulum. Concentrations of the same material are apparent in saccules of a Golgi body (*G*).

plasm. As these specific puffs are generally active throughout most of the larval life (Beermann, 1956), a constant production of granules is implied. An accumulation of granules at the nuclear margin is sometimes observed. Such is the case, in particular, near the end of the prepupal period when deep invaginations of the nuclear envelope are produced and large numbers of granules are seen in these invaginations. These indirect indications and the numerous observations of granules partially engaged in the nuclear pores support the existence of a transport mechanism from nucleus to cytoplasm. It appears unlikely that the migration of the granules is in the reverse direction, particularly in view of the many radioautographic studies supporting RNA movement from nucleus to cytoplasm (see Zalokar, 1961).

The nuclear envelope and its pores or discontinuities described here are entirely similar to those described in studies of other cell types (Gall, 1964; Watson, 1959; André and Rouiller, 1956; Baud, 1959). The finding of a diffuse material within the opening and extending a short distance into the nucleus and cytoplasm is also well corroborated by



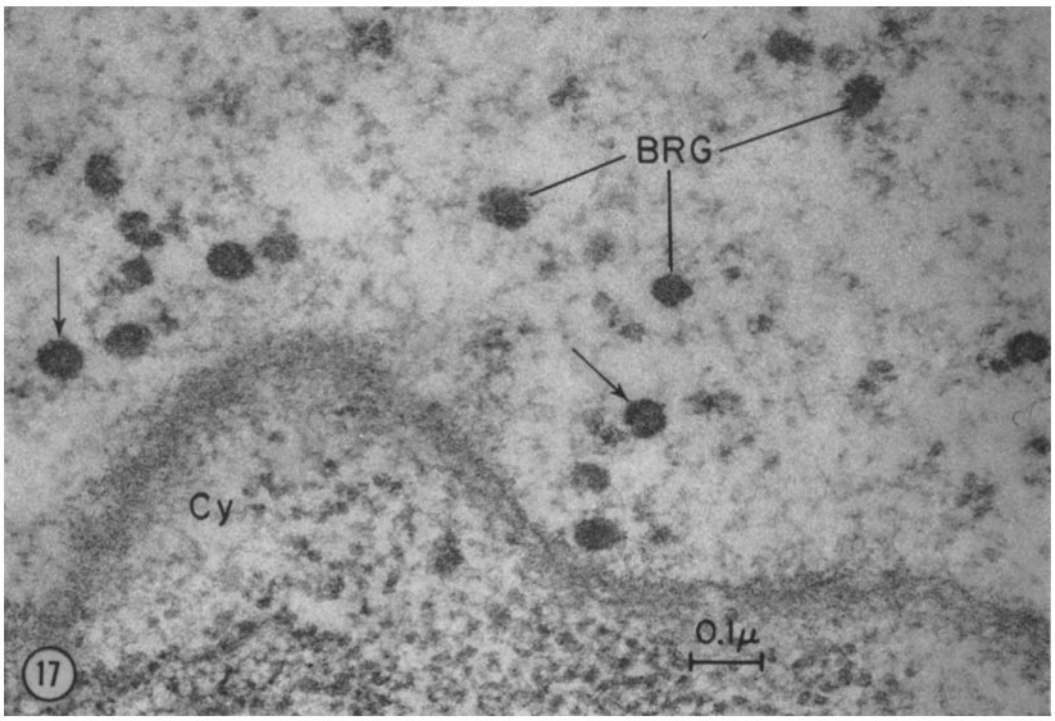


FIGURE 16 Typical view of nucleus near the nuclear envelope. Numerous round and dense Balbiani ring granules (*BRG*) are dispersed in the nucleoplasm around the chromosomes (*Ch*). At the nuclear surface, two of these granules can be seen in intimate association with the nuclear envelope (arrows). The cytoplasm (*Cy*) is filled with tubules of the endoplasmic reticulum. Acrolein/formalin-OsO_4 fixation; uranyl and lead stain. × 40,000.

FIGURE 17 A region of the nucleus near the nuclear envelope, showing dense and spherical Balbiani ring granules (*BRG*). The granules have a diameter of about 500 A, and some of them exhibit a central region of lower density (arrows). Cytoplasm, *Cy*. OsO_4 fixation; uranyl and lead stain. × 100,000.

other reports. There are several lines of evidence which imply that this material has an active role in effecting nucleocytoplasmic interchanges.

In studies on the nuclear uptake of colloidal gold particles injected into amoebae, Feldherr (1965) has found that particles of a maximum size of 125 to 145 A diameter are able to enter the nucleus. He noted also an accumulation of the particles within and adjacent to the nuclear pores of isolated oocyte nuclei after 5 min exposure to gold particles (Feldherr and Harding, 1964). He concluded that the electron-opaque pore material is able to regulate nucleocytoplasmic transport. A system of active transport of RNA from nucleus to cytoplasm in *Musca* nurse cells was likewise postulated by Bier (1965) from his studies on the effects of oxygen deficiency and low temperature on RNA movements.

Ito and Loewenstein (1965), measuring the electrical resistance of the nuclear envelope in *C. thummi*, have found high values which deny the possibility of a freely porous structure. The resistance observed, of the order of 1 ohm-cm^2, should represent a strong barrier to ion diffusion, and these investigators speculate that the electron-opaque material of the pores is responsible for the diffusion barrier. Although the ultrastructure and dimensions of amphibian oocyte nuclear envelopes are quite similar to those of salivary gland cells, oocyte nuclei exhibit almost negligible electrical resistance and are completely permeable (Wiener et al., 1965). Differences in the ill defined material of the pore complex, as yet unrecognizable in the electron microscope, could well account for this divergence.

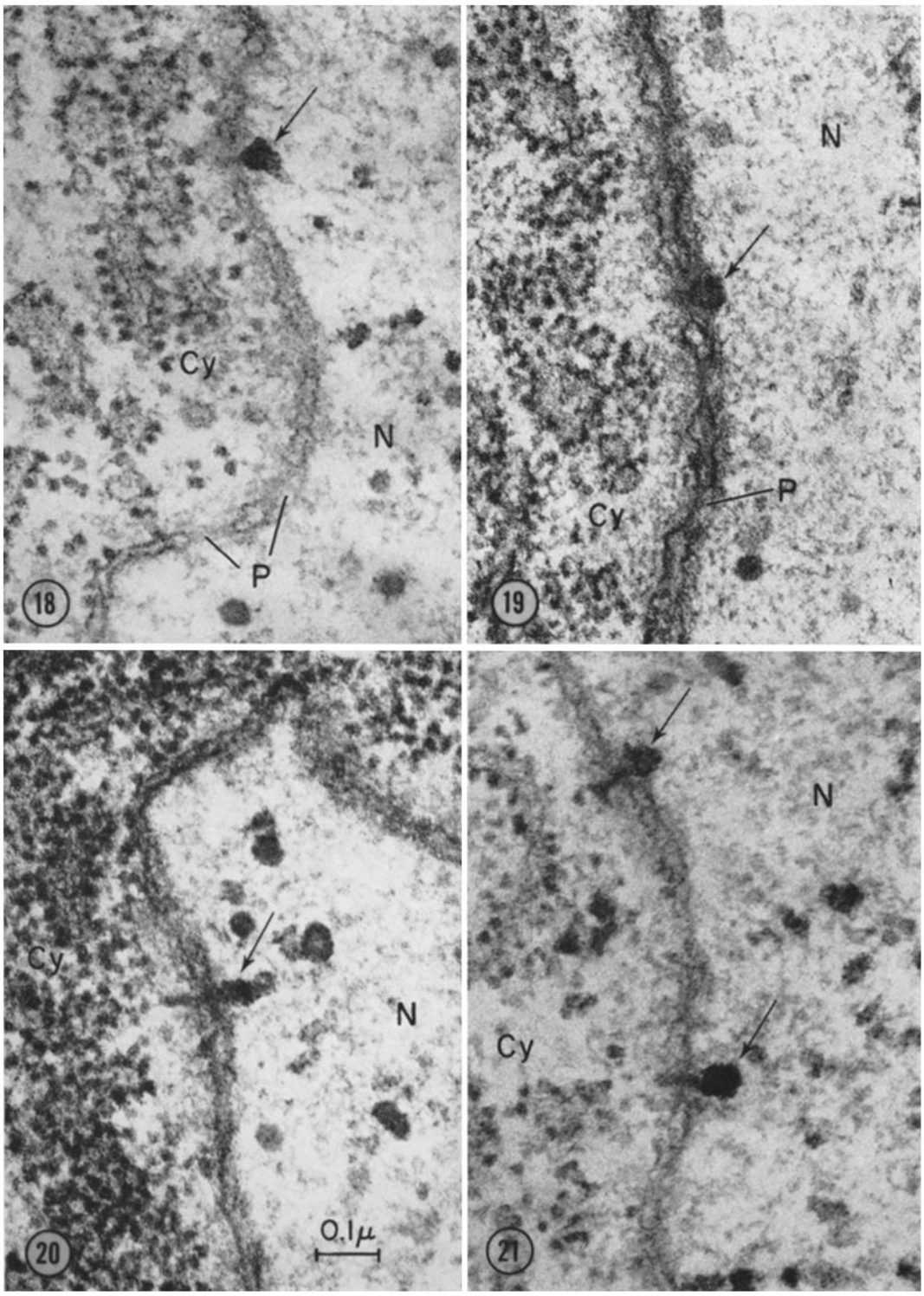
If this diffuse material does indeed act as a barrier to free movement across the envelope, our observations indicate that an apparent central channel, about 200 A in diameter, may at least temporarily exist within it. The central distribution within the pores of colloidal gold particles in Feldherr's studies gives further support for the existence of a central channel.

The drawing out of the granules in the direction of the pore, seen as the first step in the process, may involve a binding to the annular material, such as Feldherr and Harding (1964) postulated. The further step of elongation may be due, at least partially, to factors residing in this material and/or the cytoplasm. The transformation of the granules into rodlike forms and their ultimate disappearance in the cytoplasm speak for some form of configurational change at the nucleocytoplasmic boundary, perhaps through the influence of the differing ionic environment known to exist in the cytoplasm. It may also involve the separation of the RNA from the protein components, and the possible reutilization of the protein moiety, as indicated in the cytonucleoprotein described by Goldstein (1958). There is general agreement that newly formed messenger RNA in the cell is most likely protected by a protein coat and does not exist as a naked molecule (Latham and Darnell, 1965; McConkey and Hopkins, 1965).

There are many references to the presence of a small, dense "central globule" in nuclear annuli viewed tangentially (Afzelius, 1955; Gall, 1964; Watson, 1959). Such granules have been interpreted as helping to effect the diffusion barrier (Wiener et al., 1965), as organized material within a channel at the level of the pore (Watson, 1959), and as actually representing material passing through the pore (Gall, 1964). The present ob-

FIGURES 18 and 19 First step in the proposed process of nucleocytoplasmic transport of Balbiani ring granules. The spherical granule (arrows) has been slightly drawn out toward the center of a nuclear pore. Nucleus, *N*; cytoplasm, *Cy*; nuclear pores, *P*. Fig. 18, Acrolein/formalin-OsO_4 fixation; lead stain. Fig. 19, OsO_4 fixation; lead stain. × 100,000.

FIGURES 20 and 21 Succeeding step in the nucleocytoplasmic transport of Balbiani ring granules. The granule (arrows) has elongated to a rodlike form which penetrates the center of the nuclear pore and extends a short distance into the cytoplasm (*Cy*). The portion of the granule remaining in the nucleus (*N*) retains its spherical shape. Fig. 20, Acrolein/formalin-OsO_4 fixation; lead stain. Fig. 21, Same fixation; uranyl and lead stain. × 100,000.


Cy
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18
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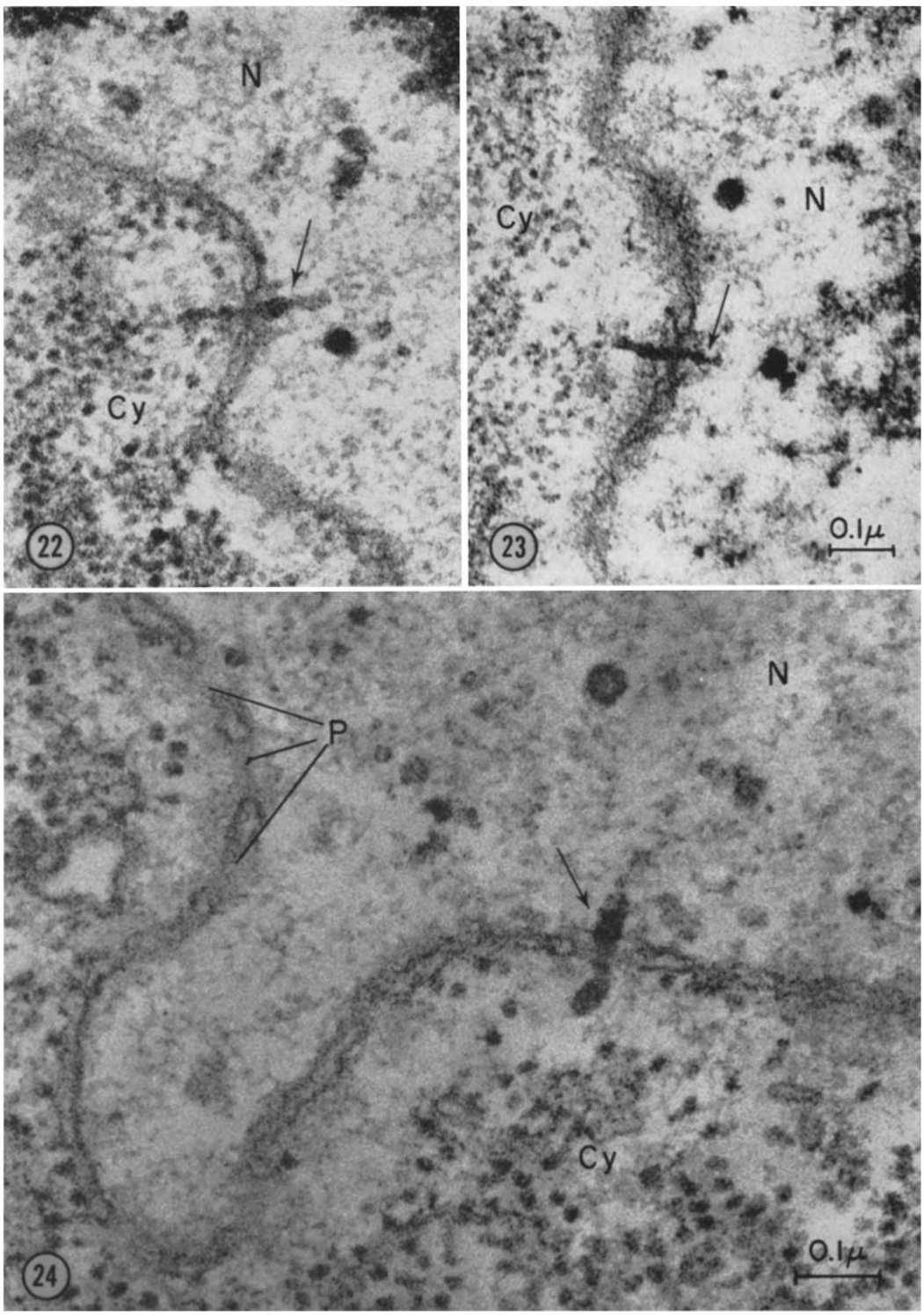

FIGURES 22, 23, and 24 Three examples of Balbiani ring granules completely transformed into rodlike shapes (arrows) and extending across the nuclear envelope from nucleus (*N*) to cytoplasm (*Cy*). The long rods lie in the center of the nuclear pores and assume a diameter of about 200 A at this level. These structures in Figs. 22 and 24 exhibit a slightly lower density immediately within the pore. Other pores, *P*. Fig. 22, OsO_4 fixation; uranyl and lead stain. Fig. 23, Acrolein/formalin-OsO_4 fixation; same stain. × 100,000. Fig. 24, Acrolein/formalin-OsO_4 fixation; lead stain. × 128,000.

servations lend further support to the latter interpretation. The smallest diameter detected, about 150 A, probably represents material at the level of the envelope, while the larger diameters of 300 A could be portions of Balbiani ring granules on the nuclear side of the envelope.

The salivary glands of *Chironomus* afford exceptionally good material for study of the formation and transfer of ribonucleoprotein particles, as the Balbiani ring produces a large number of granules of characteristic shape and size. It seems likely that a similar particle transfer occurs in other synthesizing cells. Some morphological candidates for these ribonucleoprotein particles in other cells include the variety of RNA puff granules in *Drosophila* (Swift, 1962) and the heterogeneous granules associated with lampbrush loops in amphibian oocytes (Gall, 1956). It is of interest that particles of a given size are characteristic of a particular chromosomal region. This is what one would expect if the size of the messenger RNA determined the size of the ribonucleoprotein particle, with the polydisperse character of messenger RNA being reflected in the heterogeneity of particle size.

In mouse and rat tissue nuclei, granules about 300 A in diameter have been observed. Their characteristic enclosure in chromatin material has produced the name "perichromatin granules" (Watson, 1962). In a study by Jézéquel and Marinozzi (1963), perichromatin granules are reported to be sensitive to ribonuclease digestion and to be located in relation to the interchromatin spaces of the nuclei that lie in the axes of the nuclear pores. These observations, and the finding of an increased frequency of perichromatin granules during liver regeneration, suggest that these granules may be a vertebrate counterpart of the Balbiani ring granules in *Chironomus*.

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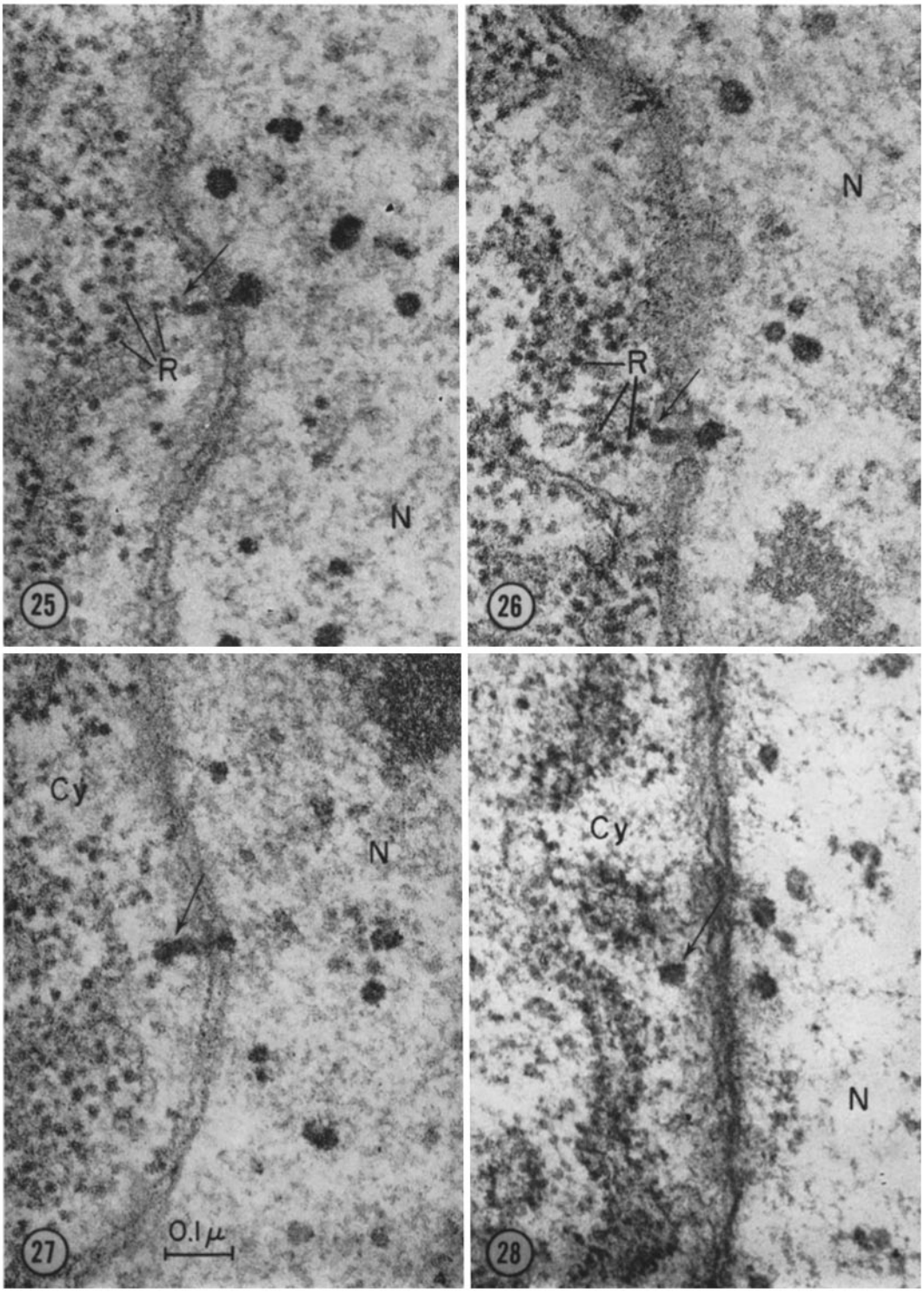
FIGURES 25 and 26 Illustrations of a possible fate of the Balbiani ring granules. The rodlike portion of the granule as it apparently penetrates a nuclear pore is shown in a close association with ribosomes (*R*) in the cytoplasm (arrows). Fig. 25, Acrolein/formalin-OsO_4 fixation; lead stain. Fig. 26, Same fixation; uranyl and lead stain. × 100,000.

FIGURE 27 An example in which the major portion of a Balbiani ring granule (arrow) lies in the cytoplasm (*Cy*). Acrolein/formalin-OsO_4 fixation; uranyl and lead stain. × 100,000.

FIGURE 28 An example of an apparently intact Balbiani ring granule (arrow) lying in the cytoplasm (*Cy*). OsO_4 fixation; lead stain. × 100,000.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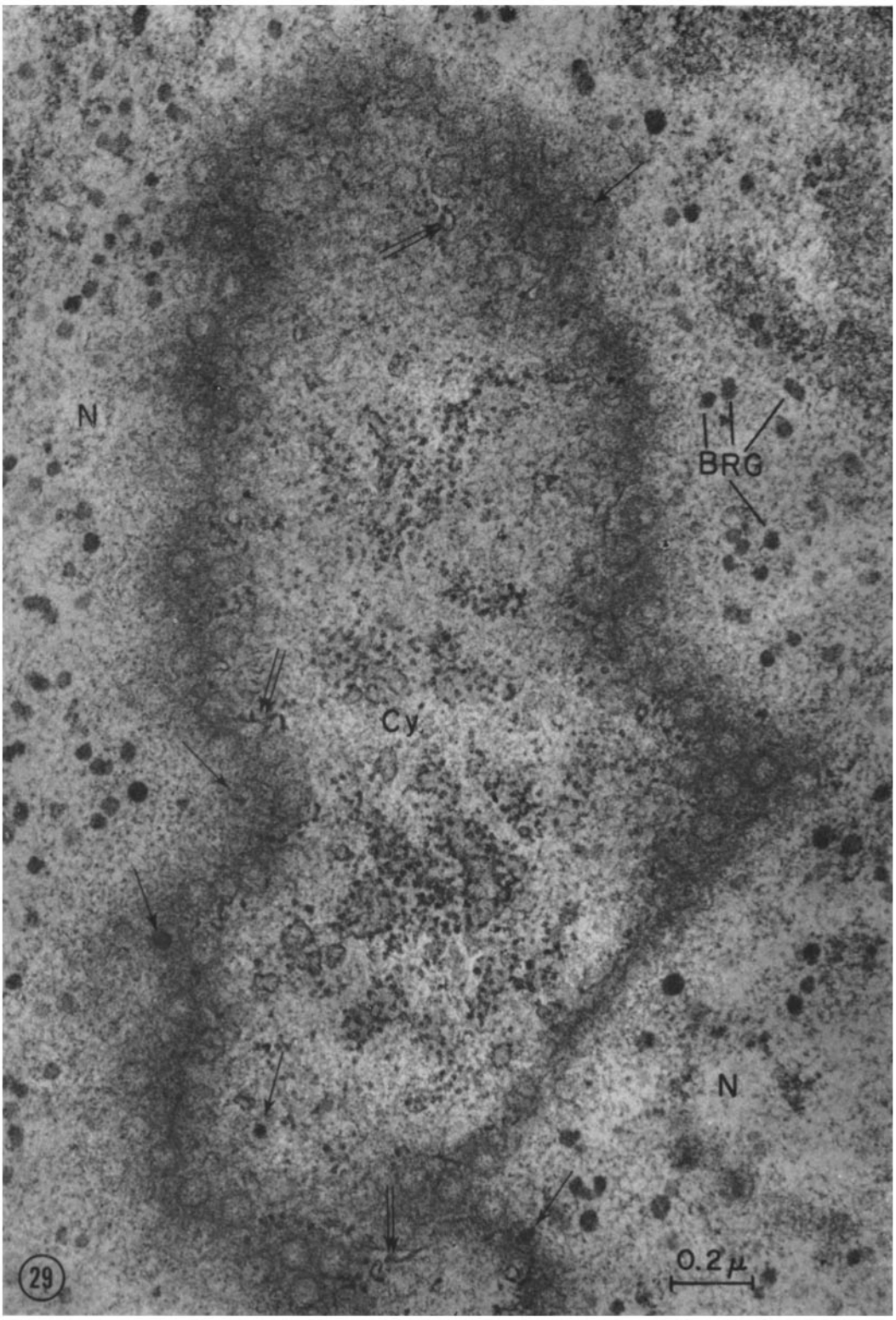

FIGURE 29 A tangential section of the nuclear envelope showing the numerous annuli arranged in a hexagonally spaced array. Several instances of Balbiani ring granules lying within annuli are visible (arrows). Immediately on the cytoplasmic side of the envelope are seen many dense, curled threads, whose contents and function are unknown (double arrows). These curled threads have no obvious relation to the annuli. Many Balbiani ring granules (*BRG*) appear in the nucleus (*N*). OsO_4 fixation; lead stain. × 63,000.

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