

THE FINE STRUCTURE OF THE NUCLEOLUS DURING MITOSIS IN THE GRASSHOPPER NEUROBLAST CELL

BARBARA J. STEVENS, Ph.D.

From the Department of Anatomy, Cornell University Medical College, New York, and the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee. Dr. Stevens' present address is the Whitman Laboratory, University of Chicago, Chicago

ABSTRACT

The behavior of the nucleolus during mitosis was studied by electron microscopy in neuroblast cells of the grasshopper embryo, *Chortophaga viridifasciata*. Living neuroblast cells were observed in the light microscope, and their mitotic stages were identified and recorded. The cells were fixed and embedded; alternate thick and thin sections were made for light and electron microscopy. The interphase nucleolus consists of two fine structural components arranged in separate zones. Concentrations of 150 A granules form a dense peripheral zone, while the central regions are composed of a homogeneous background substance. Observations show that nucleolar dissolution in prophase occurs in two steps with a preliminary loss of the background substance followed by a dispersal of the granules. Nucleolar material reappears at anaphase as small clumps or layers at the chromosome surfaces. These later form into definite bodies, which disappear as the nucleolus grows in telophase. Evidence suggests both a collecting and a synthesizing role for the nucleolus-associated chromatin. The final, mature nucleolar form is produced by a rearrangement of the fine structural components and an increase in their mass.

INTRODUCTION

The nucleolus and its role in cellular functions have recently been the focus of widespread attention (see reviews in references 4, 36, and 39). Along with the problems posed concerning nucleolar participation in cellular RNA synthesis and metabolism, there exist several unanswered and relevant cytological questions, one of the more prominent of which is the cyclical behavior of the nucleolus during mitosis.

The disappearance of the nucleoli at the end of prophase and their re-formation in the daughter nuclei during telophase are nearly universal events in somatic cell division. However, the observations and accumulated records made of mitotic cells have not led to any generally accepted description

and interpretation of nucleolar dissolution and formation during mitosis. Moreover, specific studies on behavior of the nucleolus during mitosis have been carried out chiefly on plant cells (20, 21, 32, 39), and few descriptions are available for animal cells (15, 17).

The site of nucleolar formation in telophase at a specific chromosome position, the nucleolus organizing body, has been well established by earlier studies (14, 22, 25). The origin of the nucleolar material is less precisely fixed. Numerous authors (*e.g.*, 1, 8, 25, 42) state that at telophase, nucleolar material is accumulated at the nucleolar organizer from several sites on the chromosomal complex. Cytological information on the form, location, and

time of appearance of this material would aid in determining whether or not (a) the nucleolar substance is newly synthesized, (b) it represents a reassembly of previously existing substances, or (c) both processes are involved. The accumulation of material to form the nucleolar body occurs relatively rapidly in telophase. A more precise knowledge of the steps in this process should indicate the function of the nucleolar organizer in nucleolar formation. Morphological evidence of the fine structural changes during nucleolar growth and maturation, and of the process of disintegration at the end of prophase would also help to elucidate the role of the nucleolus in cellular syntheses.

The few electron microscope studies of the nucleolus during mitosis (20, 21, 39) have shown that it is possible to identify the nucleolar components by their fine structure and to observe their distribution and compartment with greater precision and in more detail than is permissible with the light microscope. Lafontaine and Chouinard's recent study (21) of nucleolar mitotic behavior in plant cells clarifies some of the points raised and offers a basis of comparison for the study reported here.

The present study attempts to relate the morphology and characteristics of the living cell at known mitotic stages with fine structural changes in the nucleolar cycle. The neuroblast cell offers many advantages for this analysis. Its large size (25 to 30 μ in diameter) and highly resolvable nuclear morphology allow detailed microscopic study under living conditions. Following fixation and embedding, it is possible to recognize the same cells in sections prepared for both light and electron microscopes. The neuroblast divides unequally in a unique and regular fashion, producing a column of small, daughter ganglion cells which projects dorsally from each neuroblast. In this way the neuroblasts maintained a fixed position in the embryo which permits precise identification and recording of their location and mitotic stages during extended periods. A large number of mitotic stages (up to 22) can be identified in the mitotic cycle. The median time for completion of a cycle at 38°C is 6 hours; an average time of 8 hours is recorded at 26°C (5, 12, 38).

MATERIALS AND METHODS

Embryos of the grasshopper, *Chortophaga viridifasciata* DeGeer (Acrididae: Orthoptera), at a stage equivalent to 14 days development at 26°C, were dissected

from the egg and freed from the surrounding chorion, yolk, and serosal membrane in Carlson's artificial culture medium (NaCl 0.68 gm, KCl 0.02 gm, CaCl₂ 0.02 gm, MgCl₂ 0.01 gm, NaH₂PO₄ 0.02 gm, NaHCO₃ 0.012 gm, glucose 0.77 gm, distilled water 100.0 ml). The amnion was ruptured along the mid-ventral line and drawn away from the embryo. The maxillary and thoracic appendages were cut off close to the body to expose the ventral surface. To reduce the tissue size the head and lower half of the abdomen were also removed. The remaining portion was transferred by pipette to a coverglass and mounted, ventral side down, in a small quantity of the culture medium. The coverglass was inverted over a depression slide and sealed with mineral oil. The hanging drop preparations were studied under the oil immersion lens of a standard light microscope.

The neuroblast cells lie in a single cell layer at the ventral surface and are covered by a thin layer of epidermal cells. They are arranged in nearly straight rows, interrupted only by the midventral line and the boundary limits between adjacent segments. This arrangement permits the mitotic stages of most of the neuroblasts in the large thoracic segments to be accurately identified and their positions to be recorded with the aid of a diagram of the ventral surface. Neuroblasts were identified in two or three segments in this manner, and the embryo was fixed within 5 to 10 minutes after the recording was begun.¹

The embryos were fixed in osmium tetroxide vapor by removing the coverglass, placing several drops of a 2 per cent osmium tetroxide solution in the depression and replacing the coverglass for 1 to 2 minutes. Postfixation was carried out for 2 hours at 0 to 4°C in a 3 per cent solution of potassium permanganate made with culture medium at pH 7.5 and containing no glucose. The tissue was rinsed in the same medium, dehydrated in a graded series of ethanol, and embedded in Araldite (10).

Alternate thick ($\frac{1}{2}$ to 1 μ) and thin sections through the neuroblast cell layer were made with a Porter-Blum microtome. The thick sections, used for light microscopy, were stained with azure-methylene blue (34); micrographs were taken for orientation and identification of the recorded neuroblasts. Thin sections having a known relationship to the thick ones were viewed without additional staining in an RCA EMU-2D electron microscope. The micrographs were segregated according to the recorded cell and section, and arranged in serial order for each cell.

¹ Additional details of the above techniques may be found in a recent review, CARLSON, J. G., and GAULDEN, M. E., Grasshopper neuroblast techniques, in *Methods in Cell Physiology*, (D. M. Prescott, editor), New York, Academic Press, Inc., 1964, 1, 229.

OBSERVATIONS

Nucleolar Cycle in the Light Microscope

The living interphase neuroblast cell contains two large, refractile and irregularly shaped nucleoli about 3 to 4 μ in diameter. They are formed at the subterminal secondary constriction on one of the longest chromosome pairs. The two units of this pair are generally found in opposite positions on the anaphase spindle and thus frequently occupy separate lobes of the telophase nucleus. By

of the middle telophase stage. Two tiny spheres first become visible in separate distal areas (*i.e.*, remote from centromere area) of the nuclear region, where the nuclear membrane is not yet apparent in the living cell. During this stage, which lasts about 26 minutes at 26°C, these refractile bodies increase rapidly in size until they reach a diameter of about 3 μ and contain several small refractile spots. The beginning of late telophase is defined as that moment when the nucleoli begin to lose their spherical outline and assume an irregular

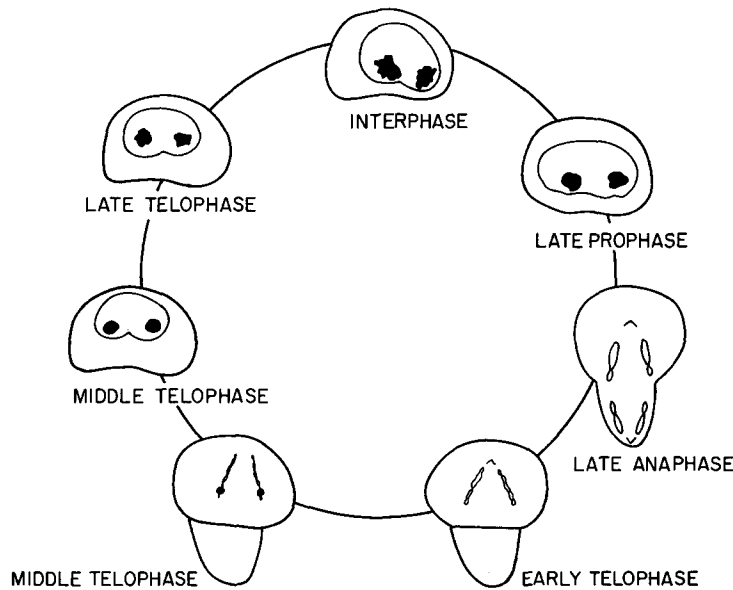


FIGURE 1 Diagram illustrating nucleolar morphology in the living neuroblast cell at various mitotic stages.

consequence, the nucleoli usually lie apart and have not been observed to fuse (13).

From the end of late telophase until soon after the start of late prophase, the nucleoli remain unchanged (Fig. 1). During the late prophase stage, when the chromosomes are visibly double, the nucleoli begin to become round, and by very late prophase these bodies are difficult to distinguish from the large chromosomes in the opaque karyolymph. Their disappearance is rapid and occurs 3 minutes before the dissolution of the nuclear membrane. No evidence of nucleoli or nucleolar material is found in the living cell between very late prophase and middle telophase.

The reappearance of the nucleoli marks the start

of the middle telophase stage. Two tiny spheres first become visible in separate distal areas (*i.e.*, remote from centromere area) of the nuclear region, where the nuclear membrane is not yet apparent in the living cell. During this stage, which lasts about 26 minutes at 26°C, these refractile bodies increase rapidly in size until they reach a diameter of about 3 μ and contain several small refractile spots. The beginning of late telophase is defined as that moment when the nucleoli begin to lose their spherical outline and assume an irregular

form. Growth continues during late telophase until the nucleoli attain their final mature size and form. The chromosomal association of the nucleoli at middle telophase is well demonstrated in histological sections (see Fig. 15 *b*). The spherical body appears to lie at the constriction of the nucleolar chromosome, symmetrically encircling the region. While not clearly demonstrable for the period from late telophase to early prophase, an association of the nucleolus with a chromosome segment can again be found in middle and late prophase.

Nucleolar Cycle in the Electron Microscope

FINE STRUCTURE OF THE INTERPHASE NUCLEOLUS: The mature interphase and very

early prophase neuroblast nucleolus appears in thin sections as dense, conspicuous body lying free in the karyolymph (Fig. 2 *a*). It is composed of several large central masses which project into narrow and twisting strands, 0.1 to 0.2 μ in diameter, at the nucleolar periphery, giving the general impression of a loosely encircling filament. The varying dimensions and the random arrangement of the component parts, however, suggest that the nucleolus is not a single, continuous filament but that it is made up of various anastomoses and fusions throughout the structure. The nucleolus may be in close proximity to chromatin material but one cannot always find a definite association or contact between these elements. Nucleoli are observed near the nuclear envelope but have never been found in actual contact.

Two fine structural components of the nucleolus can be described (Fig. 2 *b*). One of these, a homogeneous material, provides a background of medium density whose substructure is not well defined in micrographs, but which upon closer examination appears to contain single fibrils. The other component consists of dense granules, from 100 to 170 A in diameter, with an average of about 150 A. The granules are concentrated at the periphery of the large masses and form the predominant component in the surrounding narrow strands. The granules in these peripheral areas give the appearance of a dense rim encircling the large masses. The absence or diffuse scattering of granules in the central areas produces a more compact, homogeneous region.

In contrast with the nucleolar fine structure, the chromatin masses consist of aggregations of fine microfibrils, about 80 to 100 A in diameter. The

high electron opacity of the neuroblast chromatin usually observed after osmium tetroxide fixation is greatly reduced by the double fixation (osmium tetroxide vapor-potassium permanganate solution), thus allowing the more dense nucleolar components to be readily distinguished. It is likely that the oxidizing action of the permanganate ion has removed some of the chromatin substance, but the nucleolus appears to remain essentially intact and is comparable to the nucleolar image given after standard osmium tetroxide fixation. In addition to the nucleoli and chromatin, the nucleus contains small aggregates of interchromatin particles, from 150 to 300 A in diameter, located among the chromatin masses (Fig. 2 *a*).

NUCLEOLAR DISSOLUTION IN PROPHASE: The first changes in nucleolar structure to be observed with the electron microscope during prophase occur at the beginning of late prophase (for a more exact definition and description of stages in the neuroblast see references 13, 38). The nucleolus assumes a more compact form with fewer projections at the periphery. A greater portion of the structure is made up of the narrow strands containing dense granules.

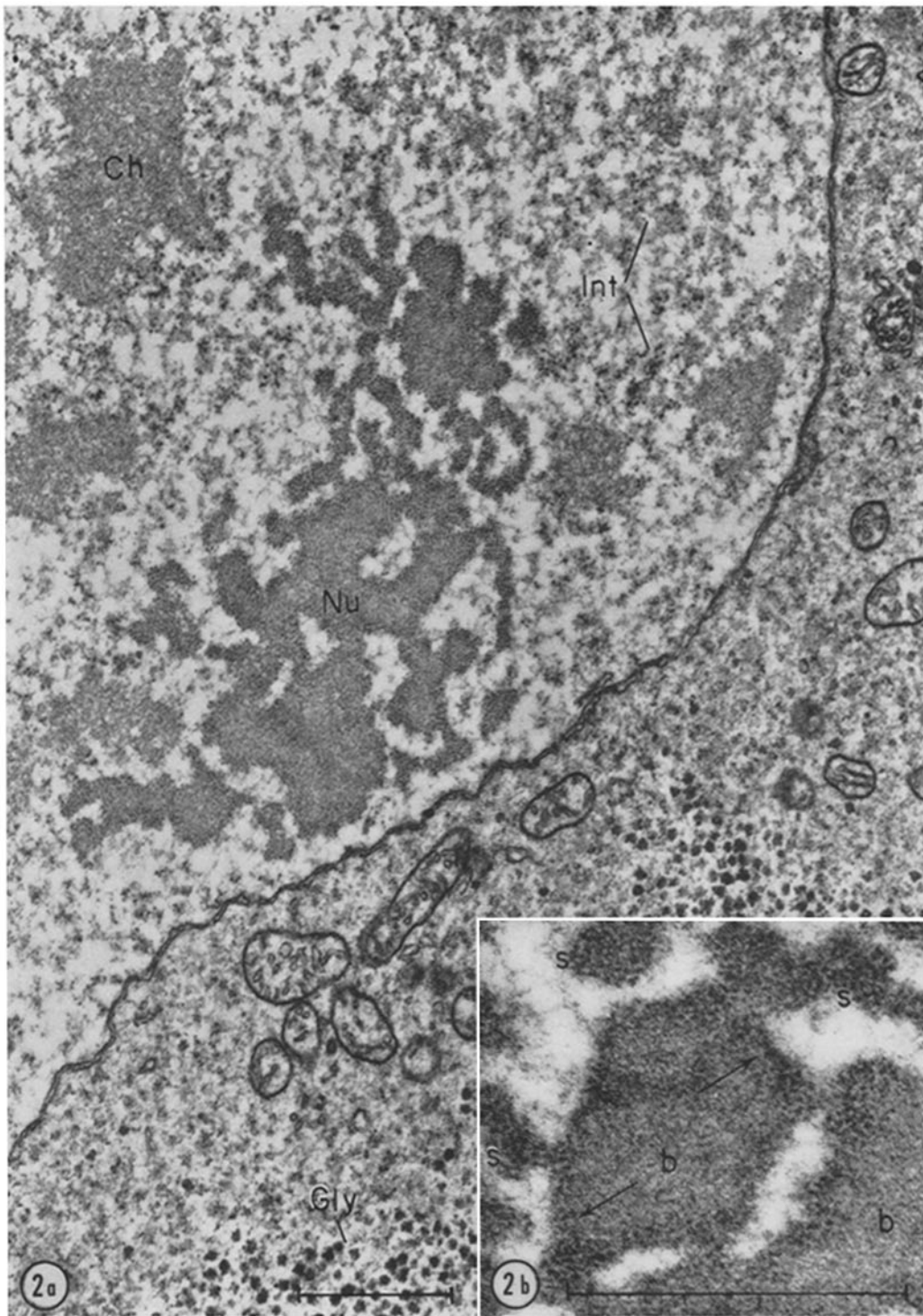
By the middle of late prophase, as the cell shape changes from a concavo-convex to a more rounded one, the nucleolar reorganization is more pronounced. The nucleolus becomes almost entirely composed of the dense granules organized into slender and twisting strands. These strands form a complex pattern of anastomoses. The change in form is accompanied by a gradual reduction in the homogeneous background substance (Fig. 3).

At very late prophase (Figs. 4 and 5), when prominent, uniform chromosomes fill the enlarged

The scale on all figures is equivalent to 1 μ .

FIGURE 2 *a* An interphase neuroblast cell. A portion of the nucleus appears at the left, containing clumps of chromatin (*Ch*), dispersed groups of dense 150 to 300 A interchromatin particles (*Int*), and a large irregular-shaped nucleolus (*Nu*). The nucleolus is represented here in its mature, interphase form. The organelle consists of several main masses surrounded and joined by narrow, twisting strands. No definite association of the chromatin is observed. The cytoplasm contains numerous mitochondria and dense masses of glycogen granules (*Gly*). $\times 23,000$.

FIGURE 2 *b* A higher magnification of a portion of an interphase nucleolus. Two fine structural components are arranged into separate zones. The twisted strands encircling the nucleolus (*s*) are principally composed of dense 150 A granules. The dense granules also form a narrow rim at the periphery of the main masses (arrows). The central regions of these masses consist of a compact material forming a homogeneous background (*b*). $\times 50,000$.



nucleus, the nucleolus is recognizable as a loose aggregation of the 150 Å granular component. Little of the background material remains, and the density of the nucleolus approaches that of the chromosomes (Fig. 5). Nucleolar size is likewise reduced. An examination of the karyolymph at this stage reveals a definite increase in electron opacity which appears to result from a greater concentration of dense 150 Å particles in the areas between chromosomes. Whether this effect is due to an actual addition of material, or merely to a crowding of previously existing particles caused by the enlarging chromosomes is difficult to determine. The final steps in the dissolution of the nucleolus at the end of very late prophase have not been actually observed in the electron microscope.

NUCLEOLAR ORIGIN IN ANAPHASE AND TELOPHASE: The components of the nucleolus are not seen during prometaphase, metaphase, and early and middle anaphase. At late anaphase, as cytokinesis occurs and the chromosomes move closer together at the poles, a double membrane (identical in structure with the nuclear envelope), can be observed at the chromosome surfaces. Consecutive sections show that each chromosome tends to form a separate membrane, except near the proximal (centromere) ends where the chromosomes are fused into a tight ring at the neuroblast pole and a membrane forms at their common periphery. Distally, the chromosomes are separately enclosed to form partial karyomeres (38).

Once the nuclear contents are thus isolated from the cytoplasm, small, dense areas appear embedded in the chromosomes at their periphery (Fig. 6). Higher magnification shows that the increased density of the areas is due to accumulations of 150 Å granules, similar to those of the mature nucleolus (Figs. 7 and 8). The density also results from the presence of a finely divided background material which is not apparent throughout the rest of the chromosome. The dense areas are numerous and randomly placed along the edges of the chromosomes.

As the chromosomes move closer together and begin to lose their smooth outline, the double membrane surrounding adjacent chromosomes becomes continuous to form a single, complete nuclear membrane enclosing the very early telophase nucleus (Fig. 9). The small, granular regions in the surface regions of the separate anaphase chromosomes are now recognized both as thin layers between adjoining chromosomes and as small pockets or layers of dense granular material lying in, or along, the free chromosomal surfaces (Figs. 9 to 11).

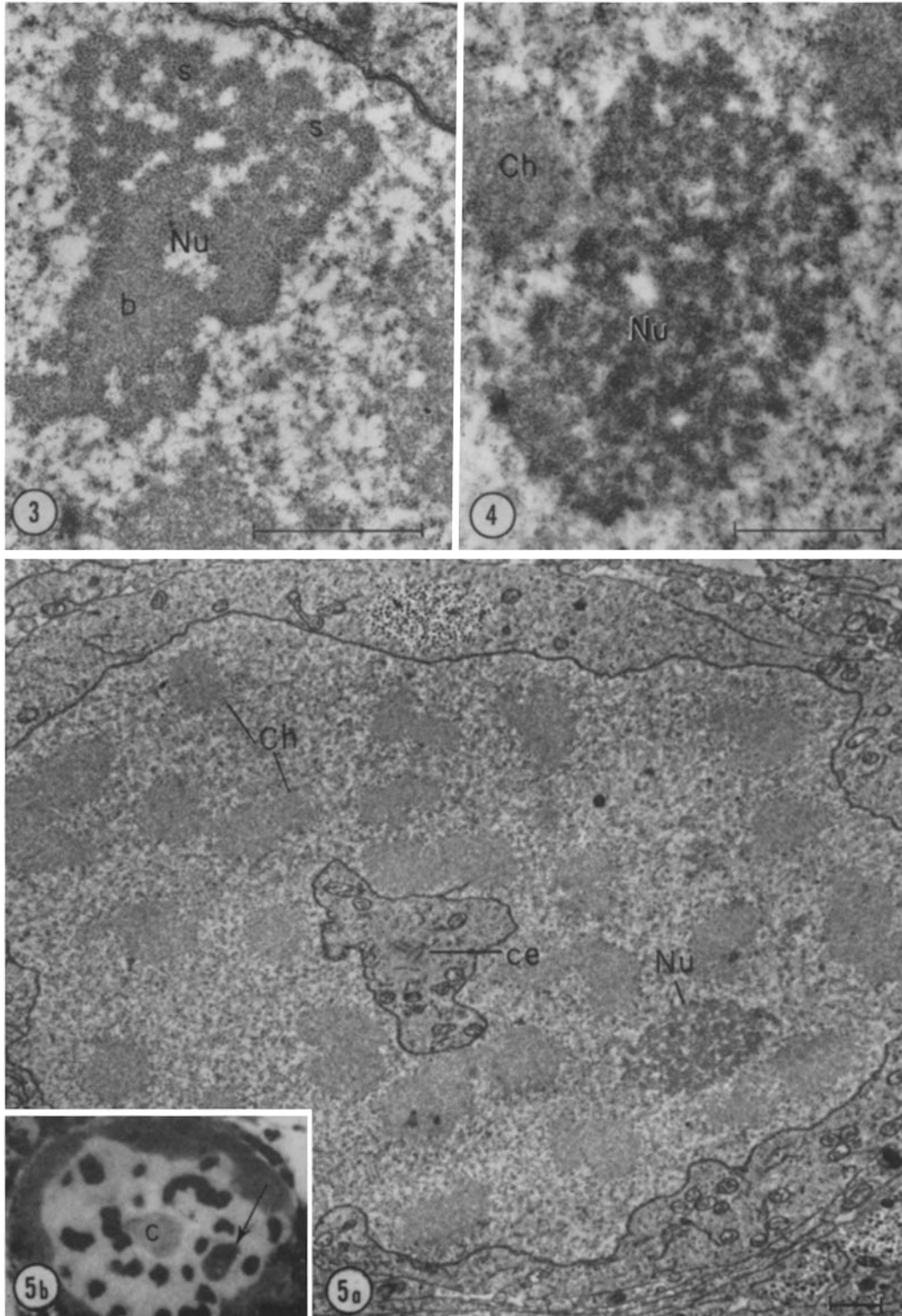
In the following stage, early telophase, the visible portions of the chromosomes are smaller and ragged in outline, and the volume of the surrounding karyolymph increases. The accumulations of nucleolar-like material occur as larger, defined bodies (0.2 to 0.3 μ in diameter), adhering to the chromosome surfaces (Fig. 12). High mag-

FIGURE 3 Neuroblast nucleolus (*Nu*) at late prophase. The form is more round and more compact than in interphase (Fig. 2 *a*). The peripheral strands (*s*) show frequent interconnections and project less freely from the surface. Regions containing the homogeneous background component (*b*) appear reduced in size. $\times 26,000$.

FIGURE 4 A nucleolus at very late prophase. None of the homogeneous background component remains. The nucleolus consists of a loose cluster of dense granules which are arranged into a mass of narrow and twisted strands. A direct association of chromatin (*Ch*) with the nucleolar body is indicated. $\times 23,000$.

FIGURE 5 a Another section from the very late prophase cell shown in Fig. 4. Numerous chromosomes (*Ch*) with a large and uniform diameter fill the enlarged and lobated nucleus. Note the increased electron opacity of the karyolymph. The rounded nucleolus (*Nu*) shows a density only slightly higher than that of the chromosomes. The central cytoplasmic core contains numerous small mitochondria and a pair of centrioles (*ce*). $\times 8,000$.

FIGURE 5 b A light micrograph of an adjacent thick section from the very late prophase cell. The round, vacuolated nucleolus (arrow) can be distinguished from the more intensely staining chromosomes. The plane of sectioning illustrates the doughnut-like shape of the neuroblast nucleus and shows the core of cytoplasm (*c*) which completely traverses the center of the nucleus.



nification distinctly shows the granular component of these bodies (Fig. 14). Micrographs indicate that, coincident with an increase in size, there is a decrease in number of these bodies. The same bodies are visible in adjacent $\frac{1}{2} \mu$ sections in the light microscope as intensely stained, deep-purple dots lying between the more lightly stained, blue chromosomes.

One example of an early telophase stage (Fig. 13) shows an extra large accumulation of the nucleolar-like material apparently confluent with a chromosome along its width. The fine structure, showing 150 A granules, dispersed on a homogeneous background, and the higher density of this mass distinguishes it from the adjoining chromosome. Its large size and distal position in one of the nuclear lobes suggest that this mass represents the initial formation of the nucleolus.

Micrographs of two neuroblasts recorded in middle telophase demonstrate the next step in nucleolar development (Figs. 15 to 19). Nuclear size is greater, and the chromosomes are recognizable as rod-like structures that are smaller and more irregular than in the previous stage. Small, dense bodies, 0.3 to 0.7 μ in diameter, are evident in the increased expanse of the nucleus. The bodies have lost their chromosomal attachments, but have a composition similar to that of the small masses of the previous stage (Fig. 16). In these bodies, the 150 A granules are evenly arranged against the background substance. The spacing of the granules can be described as less compact than that found in the peripheral regions of the mature nucleolus.

In addition to the freely situated bodies, consecutive thick and thin sections of both middle telophase cells exhibit a pair of larger round structures, 1.2 to 1.7 μ in diameter, in each nucleus. These structures have a composition similar to the small bodies, but a slightly increased concentration of the granules at the surface and a more extensive homogeneous region at the center are distinguish-

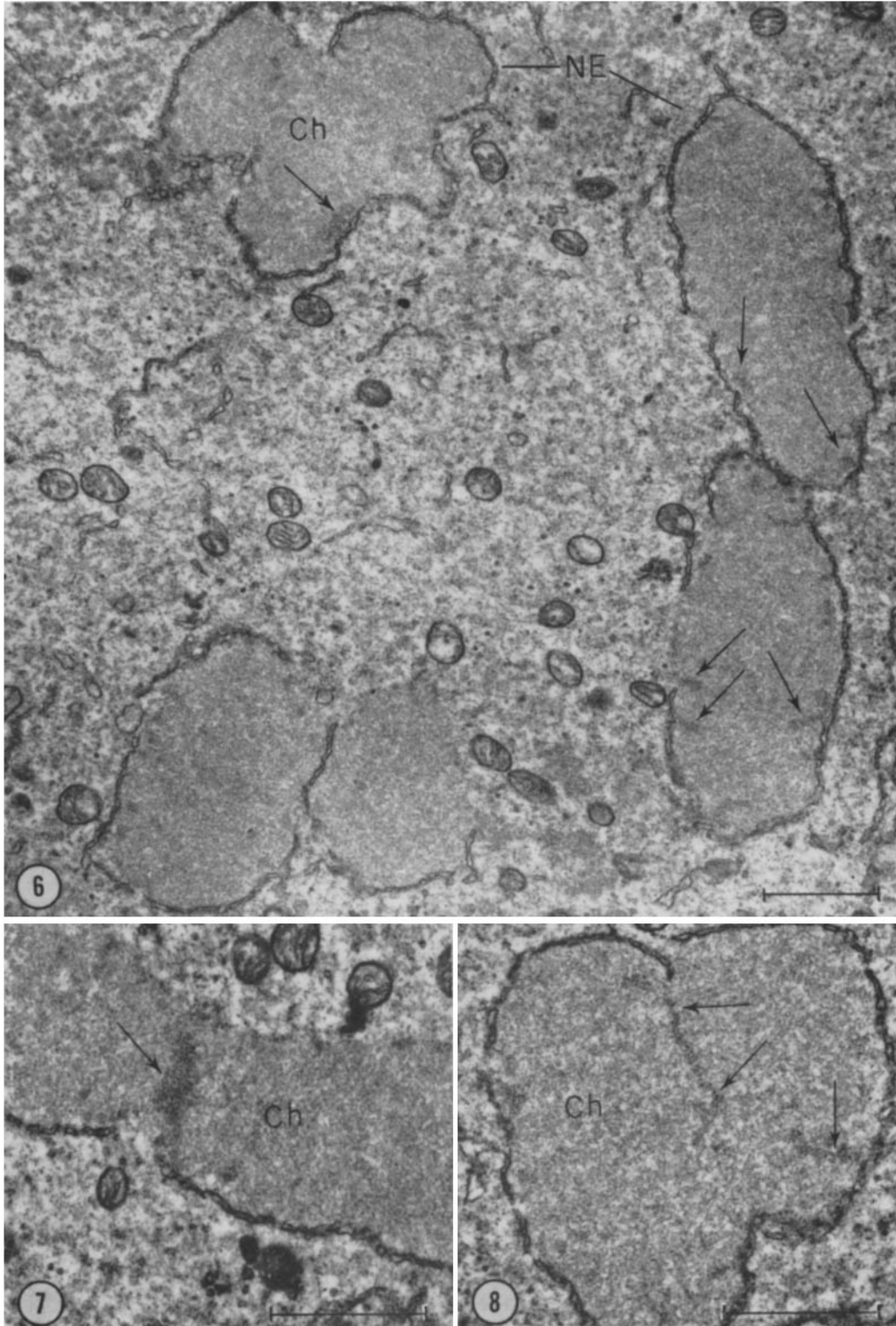
ing features (Fig. 19). The direct attachment of one such large body to a chromosome segment is shown in Fig. 15 *a*, where the body is intimately attached to the chromosome substance. These large, paired structures can be identified as young middle telophase nucleoli on the basis of the mitotic stage, appearance and staining qualities in the $\frac{1}{2} \mu$ sections, their characteristic fine structural composition, and the extensive area of chromosomal contact.

The simultaneous presence of the small 0.3 to 0.7 μ bodies with the young middle telophase nucleoli is clearly demonstrated in both the light and electron microscopes. The small bodies stain as intensely as the nucleoli in $\frac{1}{2} \mu$ sections (Fig. 15 *b*). Their fine structural contents, and their appearance and growth prior to the formation of the true nucleoli permit these bodies to be termed "prenucleolar bodies."

A series of consecutive thin sections (Figs. 17 *a* to *d*) suggests a possible fate of the prenucleolar bodies in middle telophase. A lobe-like protrusion is noted at the round, even surface of one of the young nucleoli. The contents of the small lobe appear to be confluent with the nucleolar substance and to exhibit a density and fine structure resembling more closely that of the nucleolus than the chromatin (Fig. 18). These micrographs are offered as suggestive evidence for fusion of a prenucleolar body with a nucleolus and thus indicate a possible means of nucleolar growth.

FINAL GROWTH AND MATURATION: The rapid increase in size of the nucleoli during middle telophase results in a body 2.5 to 3.0 μ in diameter by the end of the stage. At the start of late telophase the nucleolus is a solid mass characterized by a slightly uneven outline, a dense rim at the surface caused by a concentration of the dense granules, and the maintenance of a chromosomal association in a small central cavity (Fig. 20). The small, free bodies, or prenucleolar bodies, of middle telophase have disappeared by the end of the stage.

FIGURES 6, 7, and 8 Adjacent thin sections from a neuroblast cell in late anaphase. The chromosomes (*Ch*) appear as homogeneous masses of uniform density. Each is incompletely enclosed by a nuclear envelope (*NE*), forming at the chromosome surface. Small areas or pockets of a slightly denser material appear randomly scattered along the chromosome surface or embedded in the chromosome itself (arrows). Higher magnification in Figs. 7 and 8 illustrates the presence of dense granules, having a diameter of about 150 A, in the small areas. Fig. 7 suggests the presence of a background material adds to the density of these areas. Fig. 6 $\times 18,000$; Figs. 7 and 8, $\times 24,000$.



During late telophase, additional nucleolar growth is accomplished by an increase of both fine structural components. The profile becomes more irregular, and several encircling narrow strands begin to form (Fig. 21). A chromatin connection is still apparent as a strand traversing a cavity in the nucleolus.

Midway through late telophase the solid nucleolar mass separates into a number of smaller ones (Fig. 22). The development of the peripheral strands, composed principally of the dense granules, continues to increase the complexity and the surface area of the nucleolus. Toward the end of late telophase a chromatin association is no longer obvious, and the final, mature nucleolar form is achieved.

Contemporary with the changes in nucleolar shape and size during the late telophase period, a fine structural reorganization is indicated. The dense particles contained in the small bodies originating at the chromosomal surfaces, as well as those contained in the young middle telophase nucleolus, are less concentrated and less confined to the peripheral regions than in the mature interphase nucleolus. The concentration and localization of the granules at the periphery in the mature form suggest a directed displacement of this component during the final period of nucleolar maturation. A comparison of Figs. 19 and 2 *a* and *b* demonstrates this point and shows a more homogeneous arrangement of granules in the middle telophase form as compared with the interphase form.

DISCUSSION

Nucleolar Fine Structural Features

In its general form and fine structure, the nucleolus of the interphase neuroblast cell closely

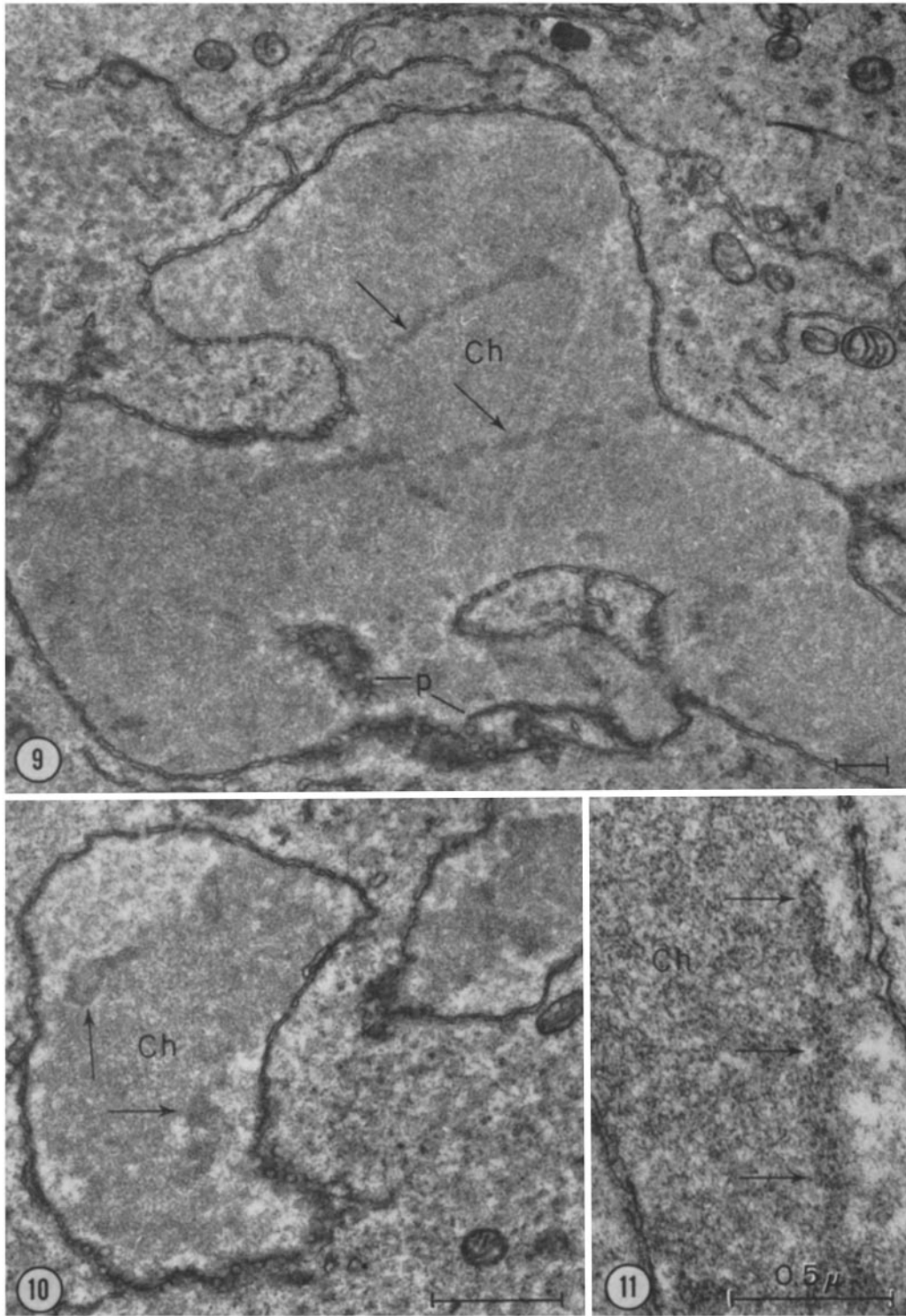
resembles the typical invertebrate or plant cell nucleolus. The separation of the fine structural components into two distinct zones and the solid nature of the nucleolus are consistent with descriptions of nucleoli from dipteran salivary glands (15, 27, 40) and several plant cells (20, 21).

The dense, granular component of the neuroblast nucleolus corresponds closely to the 150 to 200 Å granules described in nucleoli from a wide variety of plants, invertebrates, and vertebrates (3, 21, 26, 30, 40). The non-granular, homogeneous regions in the neuroblast nucleolus can be equated with a second component, a compact matrix substance, common to nucleoli of many organisms. This second component is found by many studies to contain closely packed, dense fibrils, having a diameter of 50 to 100 Å and a length of up to 300 or 400 Å (3, 15, 21, 26–28). In vertebrate nucleoli, the fibrils are compactly arranged in the matrix substance to form the internal dense network of the nucleolus. In plant and invertebrate cells, the fibrils and matrix substance are placed into a distinct and homogeneous zone that frequently occupies the central area of the nucleolus (27, 40).

The matrix substance, which often conceals the presence of the fibrils, is probably protein (it is easily extractable by pepsin, see reference 26). Both the fibrils and granules have been demonstrated by cytochemical methods to contain ribonucleic acid (26, 27). Results indicate that the fibrils are more labile than the 150 Å granules and are removed by lead staining after pepsin treatment.

It is interesting to note the effects of the combined osmium tetroxide and permanganate fixation on the fine structure of the neuroblast. Double fixation was chosen in an attempt to combine the qualities of good membrane preservation given by permanganate, and the generally good

FIGURES 9, 10, and 11 Adjacent thin sections from a neuroblast cell in very early telophase. The nucleus is completely enclosed by the nuclear envelope containing uniformly spaced pores (*p*). Chromosome forms (*Ch*) can still be recognized within the nucleus. Between adjacent chromosomes in Fig. 9, narrow layers of the dense granular material are present (arrows). Similar layers (Fig. 11), or clumps (Fig. 10) of dense granules embedded in a slightly dense material are found at the free chromosome surfaces (arrows). The granular material appears to have separated from the chromosome substance and to lie in irregularities at the surface (Fig. 10). An increasing volume of karyolymph is observed between the chromosomes and the nuclear membrane. Fig. 9, $\times 8,000$; Fig. 10, $\times 21,000$; Fig. 11, $\times 50,000$.



cytoplasmic and nuclear preservation obtained with osmium. Standard osmium fixation revealed many clusters of dense 150 to 200 Å particles in the cytoplasm; following the double fixation, however, there is no indication of these cytoplasmic particles. By contrast, the dense 150 Å granules of the nucleolus are well preserved, suggesting a definite chemical difference between the two kinds of presumably RNA-containing granules. The inability to demonstrate distinctly the fibrillar component in the central, homogeneous areas of the neuroblast nucleolus may be due to its lability during the fixation procedure or to its concealment by the background substance.

Nucleolar Dissolution

The electron microscope reveals that the disappearance of the neuroblast nucleolus in late prophase occurs in two distinct steps. The preliminary loss of the homogeneous background component and the consequent reorganization of the remaining nucleolar substance in the first step coincide with the loss in refractility and rounding of nucleoli seen in living cells. While the final dissolution was not observed in thin sections, it is likely that this step involves a fragmentation of the remaining nucleolar material and a scattering of the granules in the nucleus. The timing of the disappearance of the nucleolus, just prior to nuclear membrane breakdown, favors the hypothesis that the granular component remains free and is released into the cytoplasm at prometaphase. Similarly, the over-all increase observed in electron opacity of the late prophase karyolymph suggests that the granules become distributed in the karyolymph and are not closely associated with the chromosomes. Various light microscopic studies have indicated the presence of RNA in prophase and metaphase chromosomes (16, 19,

24), but its origin is not known. It may lie on the surface of but not be an integral part of the chromosome (18).

Several definite changes in nucleolar function can be related to fine structural changes in the nucleolus at late prophase prior to its disappearance at very late prophase. The cessation of RNA synthesis is reported to occur in late prophase (9, 31). The loss of the background component and the accompanying change in organization of the nucleolus may be related to the discontinuation of nucleolar RNA synthesis.

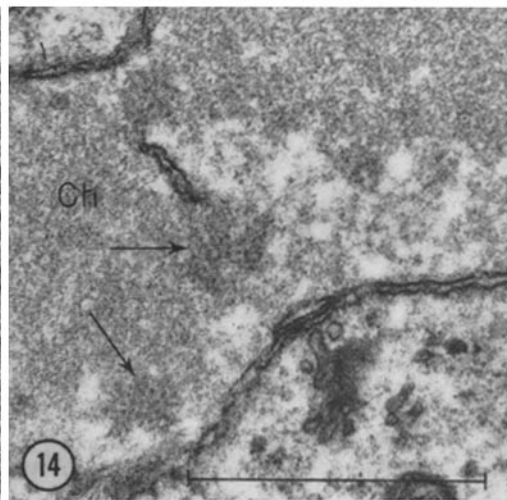
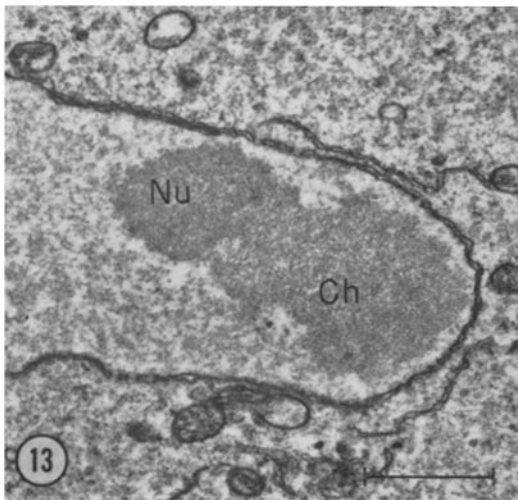
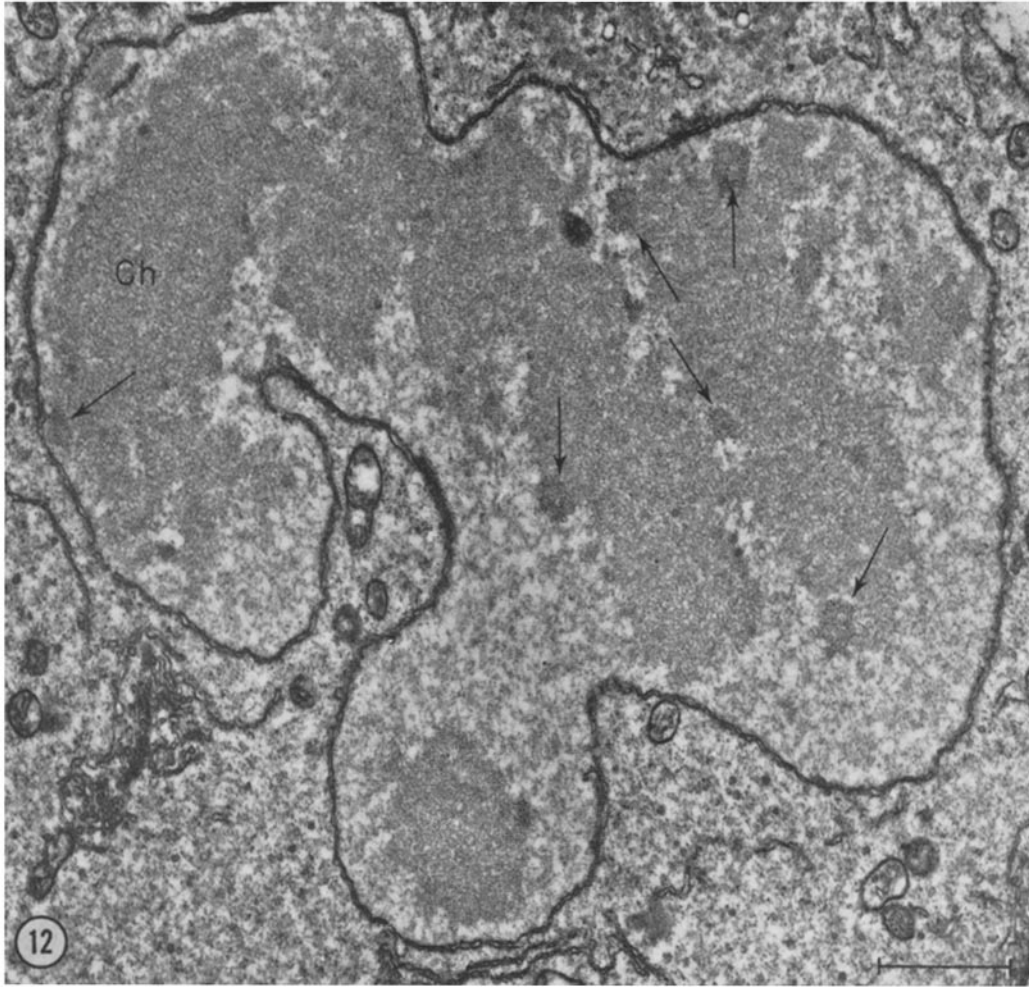
Among the earliest fine structural changes observed in nucleoli of cultured cells treated with actinomycin D is the segregation of the nucleolar components into separate zones (35). The nucleolar disorganization and the persistence of nucleolar granules² in these treated cells, whose RNA synthesis is completely arrested (33), provide an interesting correlation with the similar situation shown in late prophase neuroblast cells. Finally, a loss of sensitivity of the neuroblast to ultraviolet microbeam irradiation of the nucleolus has been shown to occur in late prophase (12, 13).

Nucleolar Formation

Although cytologists have long stated that nucleolar substances are collected from various sites on the chromosomal complex at telophase and are accumulated at the nucleolar organizer locus to form the nucleolus (1, 25, 42), the light microscope has not been able to localize and demonstrate the phenomenon accurately. Electron microscopy, however, has clearly shown nucleolar

² *Footnote added in proof:* Recent observations, however, indicate that the behavior of the nucleolar granules in response to actinomycin D treatment is dependent upon the cell type and its normal nucleolar organization.

FIGURES 12, 13, and 14 Three thin sections from a neuroblast cell in early telophase. The chromosomes (*Ch*) are smaller and less regular in outline than in the previous stage (Figs. 9, 10, and 11). Small rounded bodies are situated in depressions of the chromosome surfaces (arrows). Dense granules are apparent in these bodies at higher magnification (Fig. 14). One thin section of a distal nuclear lobe (Fig. 13) shows a large mass (*Nu*) of dense material lying in close contact with a chromosome segment (*Ch*). A slightly greater density and a granular nature distinguish this body from the fibrillar chromosome substance. Its location and structure suggest that the large body represents the elementary nucleolus (It is not possible to distinguish this body in preparations for the light microscope). Figs. 12 and 13, $\times 17,500$; Fig. 14, $\times 40,000$.



material at the surface of the early telophase chromosomes in *Vicia* (21) and at various sites on salivary gland chromosomes in *Bradysia* (15). A similar demonstration is made in the late anaphase and very early telophase stages of the neuroblast, prior to the appearance of the true nucleolus.

The micrographs of *Vicia* show a definite layering effect of the fibrillogranular nucleolar material along the early telophase chromosome surfaces. The impression given by observations on late anaphase neuroblasts (Figs. 6, 8) is that the granular material at this stage is frequently situated within the chromosome at certain sites, as well as at the surface. By very early telophase the material has often been organized into small, round bodies (Figs. 9, 10), in contrast with the sheath-like layers observed in *Vicia*. The significance of these differences in the formation of prenucleolar material is not evident.

Whether the prenucleolar material was incorporated into the chromosomes at an earlier time and is separated out during this period, or if it is synthesized *in situ* cannot be answered from the present micrographs. If some or all of the prenucleolar material is preformed, the chromosomes in the neuroblast provide the only means of transport and storage during mitosis. As the nuclear membrane is constructed after mitosis, it is tightly applied to the chromosome surfaces (Fig. 6) and no spindle or cytoplasmic material becomes included in the daughter nuclei.

The series of thin sections of a middle telophase nucleolus suggesting that at least some of the prenucleolar bodies may become incorporated into the nucleolus (Figs. 17 *a* to *d*, 18) points to a definite function for the nucleolar organizer in the

neuroblast. The lobe appearing on the usually perfectly round surface of the immature nucleolus suggests a simple fusion process. Furthermore, the extremely rapid growth of the nucleolus in the early period of middle telophase implies that extranucleolar material is added at this period, which coincides with the disappearance of the prenucleolar bodies. Owing to the brief duration of middle telophase, few examples of the stage were available, and other fusion phenomena were not recorded. Other possibilities, that the lobe represents a fragment of associated chromatin or that prenucleolar bodies are lost by disintegration, as Swift (39) has proposed, cannot be excluded.

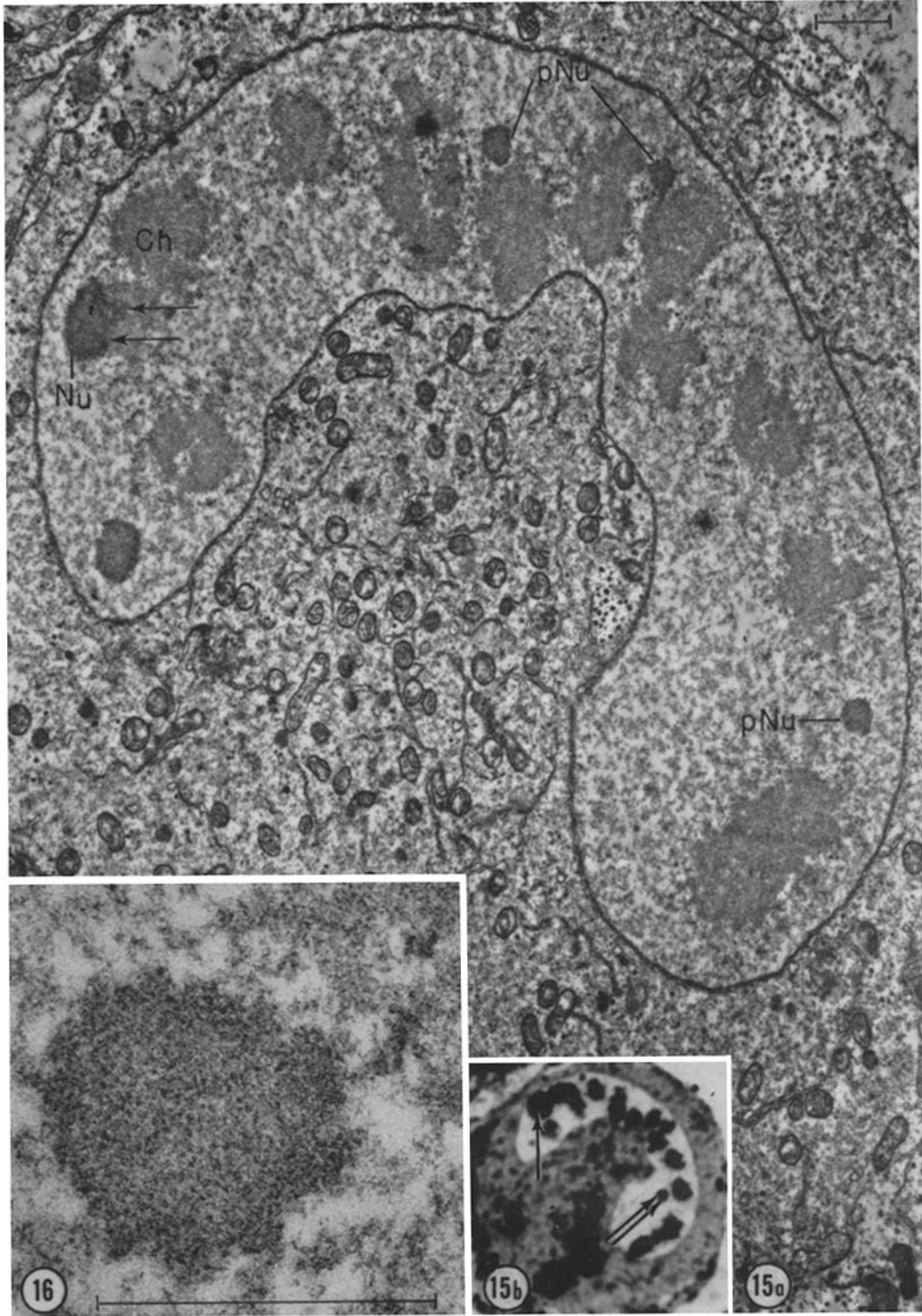
Light microscopy reports, however, have noted the fusion of prenucleolar bodies or layers into larger masses and finally into the mature nucleolus (*e.g.*, references 7, 8, 25, 32, 41). Further evidence for an organizer is supplied by several classic demonstrations of the control of nucleolar number, mass, and RNA content by the nucleolar organizer locus (22, 23, 25). Likewise, the inability of cells from mutant strains presumably lacking the locus to form a normal nucleolus is reported (2, 11). In such cells, numerous small nucleolar-like bodies are produced which contain RNA, but do not fuse with each other. Similar bodies have been noted in micronuclei produced by x-irradiation (7), and in neuroblasts treated in midmitosis with ultraviolet irradiation (6).

A variation in fine structure between the early and middle telophase nucleolus and the prenucleolar bodies indicates a possible role of synthesis for the organizer. The prenucleolar bodies contain a quantity of evenly distributed, dense granules, while the elementary nucleolus has a

FIGURE 15 *a* A neuroblast in middle telophase. Rounded or slightly elongated segments of the chromosomes (*Ch*) can be distinguished in the nucleus. In addition, several small bodies of higher density lie free in the karyolymph and are identified as prenucleolar bodies (*pNu*). A portion of a middle telophase nucleolus appears at *Nu* and demonstrates an extensive area of contact with the adjoining chromosome segment (arrows). Other thin sections of this same nucleolus are shown in Fig. 17. $\times 11,000$.

FIGURE 15 *b* A light micrograph of an adjacent thick section of the same cell. The nucleolus (arrow) is shown as a small and regular sphere, in contrast to the irregular chromosome shapes. A spherical prenucleolar body appears at the double arrow.

FIGURE 16 A higher magnification image of one of the prenucleolar bodies. The body is formed by dense granules uniformly arranged in a homogeneous background material. $\times 51,000$.



greater content of the homogeneous component and fewer granules, which tend to be placed peripherally. Synthesis of a specific substance at the organizer locus is suggested by two observations on early telophase cells: the elementary nucleolus shows intimate, extensive attachment to the chromosome and is of considerable size in spite of the fact that the prenucleolar bodies at other sites on the chromosomes are not yet free to migrate to the organizer site (Fig. 13). A function of protein synthesis has been previously proposed for the organizer (37, 43), fitting well with cytochemical studies which demonstrate protein in the homogeneous component. Other studies (29) have shown that RNA synthesis is initiated at the organizer. If there is a specific product of the organizer, it may participate in the aggregation of nucleolar material at the locus and explain the lack of a true nucleolus in mutant strains (2, 11), or in irradiated cells in which the organizer has been inactivated (6, 7).

The mode of assembly of the prenucleolar material at the nucleolar site may differ among cell types. The material is progressively accumulated by an apparent "flow" from the interchromosomal layers to the nucleolus in *Vicia*, while in the neuroblast the material is formed into defined bodies which apparently migrate through the nucleus to the nucleolus. Such observations suggest that the manner of nucleolar assembly may depend on nuclear size, volume, and chromatin content during the telophase stages.

Nucleolar Growth and Maturation

The stages from early middle telophase to the end of late telophase show a growth and a gradual

reorganization of the nucleolus, both in form and in distribution of the components. The continuing increase in size indicates a continued synthesis and/or collection of materials. The increasing complexity of form serves to increase the surface area exposed to the nuclear contents and suggests an augmented capacity for synthesis and release of nucleolar products.

Numerous reports have indicated morphological alterations in the nucleolus in relation to cyclic or functional changes. Porter (30) states that the outer cortex of the nucleolus, composed of 150 to 200 A granules, is narrow in telophase and becomes wider by prophase. Cells actively synthesizing protein, *e.g.* embryonic cells and certain types of cancer cells (4), commonly exhibit large and prominent nucleoli with a well developed internal network. The present study shows that the nucleolus is composed of two distinct fine structural components whose quantity and distribution vary considerably and in a definite pattern during the mitotic cycle, changes that are undoubtedly associated with functional contributions of the nucleolus to the cyclic changes in the cell.

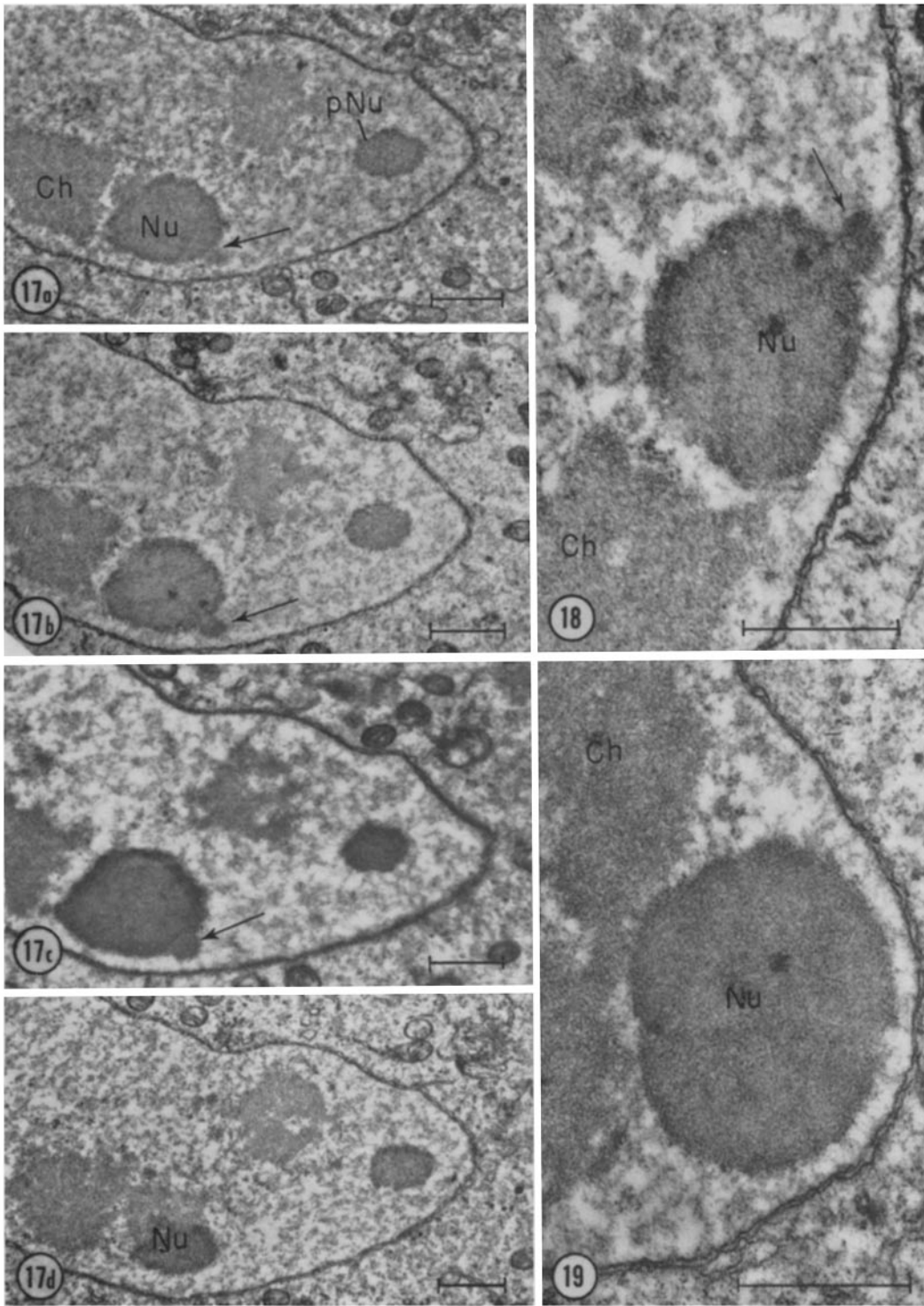
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FIGURE 17 Consecutive thin sections of the nucleolus (*Nu*) from the middle telophase cell shown in Fig. 15. Arrows indicate a lobe-like protrusion at the surface of the round nucleolus, suggesting a possible fusion of one of the prenucleolar bodies with it (see text). A prenucleolar body is shown at *pNu*. *a*, *b*, and *d*, $\times 11,500$; *c*, $\times 12,500$.

FIGURE 18 Higher magnification of Fig. 17 *b*, showing the fine structure of the nucleolus (*Nu*) and its lobe-like protrusion (arrow). $\times 26,000$.

FIGURE 19 The other nucleolus from the middle telophase cell shown in Figs. 15 to 18. The round and dense nucleolus (*Nu*) is easily distinguished from the chromatin substance (*Ch*). At the surface of the nucleolus, a slightly higher concentration of granules produces a narrow rim. $\times 26,000$.



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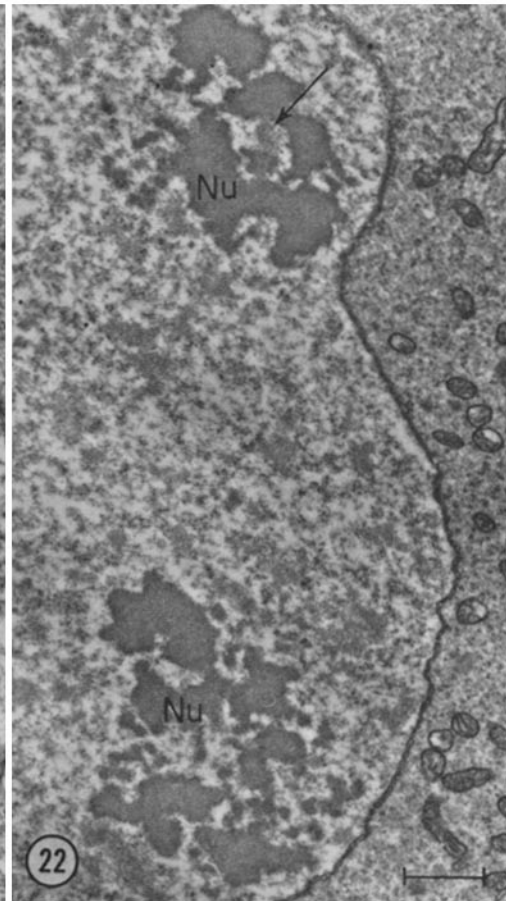
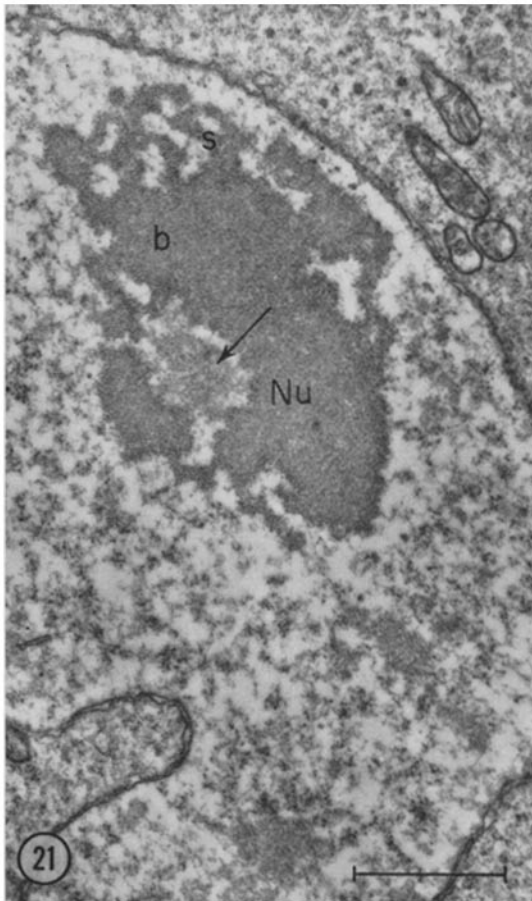
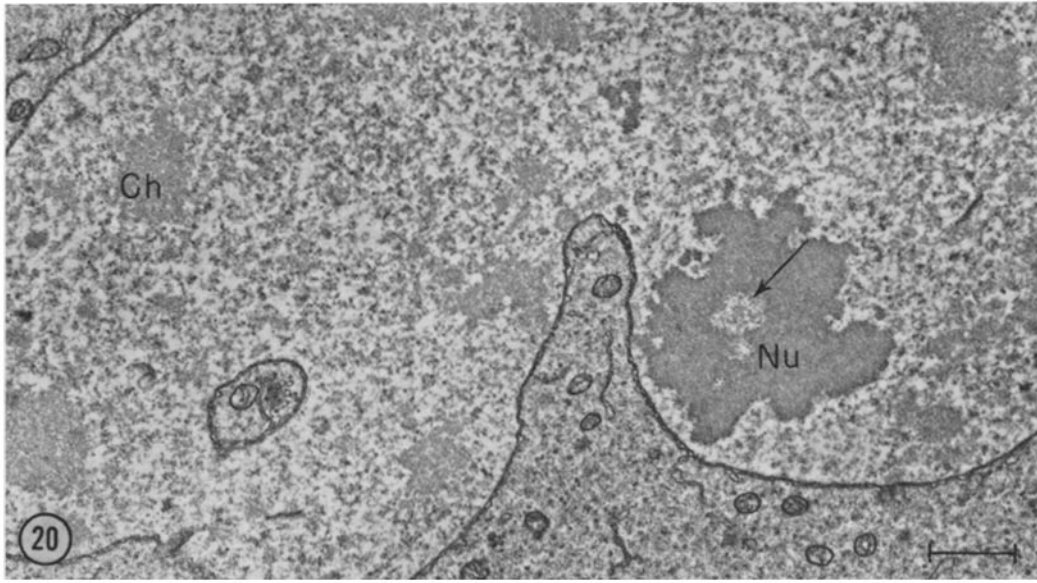
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FIGURE 20 A neuroblast at the start of late telophase. A portion of the nucleus is shown containing several chromatin masses (*Ch*) and a nucleolus (*Nu*). The nucleolar outline is irregular and suggests the formation of projecting strands. A slightly increased density is apparent at the nucleolar surface. Arrow indicates chromatin material in a central cavity of the nucleolus. $\times 11,500$.

FIGURE 21 The nucleolus (*Nu*) from a neuroblast in the early period of late telophase. The granular and homogeneous components are beginning to separate into two zones. Granules are chiefly concentrated at the periphery and in the twisted strands (*s*) forming at the surface. The central region (*b*) contains fewer granules and has a homogeneous composition. A chromatin association is observed in a nucleolar cavity (arrow). $\times 20,000$.

FIGURE 22 A neuroblast in the middle period of late telophase. The pair of nucleoli (*Nu*) demonstrate a further degree of complexity. The main body has formed into several irregular masses and the number of peripheral strands has increased. A continued chromatin association is suggested (arrow). $\times 11,500$.



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