ULTRASTRUCTURE OF THE NUCLEOLUS DURING THE CHINESE HAMSTER CELL CYCLE

J. S. NOEL, W. C. DEWEY, J. H. ABEL, JR., and R. P. THOMPSON

From the Departments of Radiation Biology, and Physiology and Biophysics, Colorado State University, Fort Collins, Colorado 80521

ABSTRACT

Changes in the structure of the nucleolus during the cell cycle of the Chinese hamster cell in vitro were studied. Quantitative electron microscopic techniques were used to establish the size and volume changes in nucleolar structures. In mitosis, nucleolar remnants, "persistent nucleoli," consisting predominantly of ribosome-like granular material, and a granular coating on the chromosomes were observed. Persistent nucleoli were also observed in some daughter nuclei as they were leaving telophase and entering G_1. During very early G_1, a dense, fibrous material characteristic of interphase nucleoli was noted in the nucleoplasm of the cells. As the cells progressed through G_1, a granular component appeared which was intimately associated with the fibrous material. By the middle of G_1, complete, mature nucleoli were present. The nucleolar volume enlarged by a factor of two from the beginning of G_1 to the middle of S primarily due to the accumulation of the granular component. During the G_2 period, there was a dissolution or breakdown of the nucleolus prior to the entry of the cells into mitosis. Correlations between the quantitative aspects of this study and biochemical and cytochemical data available in the literature suggest the following: nucleolar reformation following division results from the activation of the nucleolar organizer regions which transcribe for RNA first appearing in association with protein as a fibrous component (45S RNA) and then later as a granular component (28S and 32S RNA).

INTRODUCTION

Recent studies of nucleolar fine structure have shown that the interphase nucleolus consists of: (*a*) fibrous regions that are networks of fibers (Brinkley, 1965; Bernhard, 1966; Bernhard and Granboulan, 1968) 50 A in diameter, up to 300–400 A in length (Marinozzi, 1964), are double-stranded (Terzakis, 1965) or tubular in substructure (Smetana and Busch, 1964), and are susceptible to ribonuclease digestion (Swift, 1963; Granboulan and Granboulan, 1964; Marinozzi, 1964); (*b*) granular regions composed of granules 150–200 A in diameter (Brinkley, 1965; Hyde et al., 1965; Marinozzi, 1964; Bernhard and Granboulan, 1968; Bernhard, 1966) with an attached filamentous substructure (Hay, 1968; Smetana and Busch, 1964) that is also ribonuclease-digestible (Swift, 1963; Granboulan and Granboulan, 1964; Marinozzi, 1964); (*c*) an amorphous matrix (Schoefl, 1964; Terzakis, 1965; Bernhard, 1966) which is pepsin-susceptible (Marinozzi, 1964; Lord and Lafontaine, 1969); and (*d*) the nucleolar-associated DNA containing chromatin (Bannasch and Thoenes, 1965; Harris, 1959) which incorporates ^{3}H-thymidine (Harris,

1959; Granboulan and Granboulan, 1964) and is composed of fibers 50–80 A in diameter (Hsu et al., 1967; Unuma, Arendell, et al., 1968; Unuma, Floyd, et al., 1968). Differences, however, are reported in interphase cell nucleoli concerning the various positions and associations of the fibrous and granular components. Marinozzi (1964) reports that the granules are intimately associated with the fibrous network in pancreas and nerve cells, while Schoefl (1964) and Granboulan and Granboulan (1965) report that the granular and fibrous moieties are well separated in monkey kidney cells. Bernhard (1966) describes the position of the granular component as distributed irregularly throughout or at the periphery of the nucleolus. In cell cultures, Phillips and Phillips (1969) have noted differences in nucleoli of adjacent cells but have found that in a single nucleus with more than one nucleolus, definite similarities exist between nucleoli.

Most authors agree that the nucleolus just before or during mitosis is represented by a general dispersion, dissolution, and fragmentation of the granular and fibrous components (Lafontaine and Chouinard, 1963; Jones, 1962; Brinkley, 1965; Hsu et al., 1965). The reconstruction of the nucleolus in the late mitotic or early G_1 periods is controversial, however. Yasuzumi and Sugihara (1965), studying ascites tumor cells, suggest that the nucleolus is reformed from accumulations of chromosomal-associated, ribosome-like granules carried through mitosis and then converted to fibers by polymerization changes. Others have observed aggregates of ribosomes during mitosis (Lafontaine and Chouinard, 1963; Jones, 1962) as well as nucleolar remnants which either are attached to the chromosomes or are free-floating in the cytoplasm, and which may be incorporated into daughter nuclei (Hsu et al., 1965; Brinkley, 1965; Heneen and Nichols, 1966). Several authors (Phillips and Phillips, 1969; Hay and Gurdon, 1967; Karasaki, 1965; Stevens, 1965) believe that the nucleolus is reformed by *de novo* synthesis characterized by the appearance of numerous small, fibrous bodies in very early G_1 which later become associated with ribosome-like granules. This mode of reformation does not involve the reutilization of any nucleolar remnants carried through mitosis.

To describe the events associated with nucleolar reformation following mitosis, the dynamic changes which occurred in the morphology of the nucleolus during the cell cycle were studied in synchronized Chinese hamster cells by using quantitative electron microscopic techniques.

MATERIALS AND METHODS

Growth Conditions, Labeling, and Synchrony

Male Chinese hamster lung cells of the Don strain (Hsu and Zenzes, 1964) growing in logarithmic phase were plated into Falcon Petri dishes (Falcon Plastics, Oxnard, Calif.) (2×10^6 cells/150 mm Petri dish) with 15 ml of McCoy's 5a medium + 20% fetal calf serum and incubated in an atmosphere of 6% CO_2. To label the DNA for radioautographic studies (to be reported later), ^{3}H-TdR (0.165 μCi/ml, 0.02 Ci/mmole and 0.66 μCi/ml, 0.08 Ci/mmole in experiments 22 and 25, respectively) was added for 24–36 hr before synchronizing the cells. (To insure continuous labeling, fresh medium containing ^{3}H-TdR was added at the 12th and 18th hour.) The cells were synchronized by adding Colcemid (0.06 μg/ml) for 2 hr before selectively removing the metaphase cells (Dewey and Miller, 1969). The synchronous metaphase cells were then plated into 60-mm Falcon plastic Petri dishes containing warm medium (1×10^6 cells/plate). The cells were pulse-labeled for 20 min with ^{14}C-TdR (0.5 μCi/ml, 0.041 Ci/mmole) at various times after plating to determine the position of the cells in the cycle (Dewey et al., 1966). In one experiment (130) the cells were trypsinized from the Petri dishes, suspended in medium, and the cell and nuclear volumes were measured by phase contrast microscopy (× 100) using an ocular micrometer.

An additional experiment using Chinese hamster ovary (CHO) cells (Tobey et al., 1967) grown on Falcon plastic Petri dishes in McCoy's 5a medium containing 5% fetal calf serum and 10% calf serum was conducted. The cells were synchronized without Colcemid treatment as described by Dewey and Miller (1969) to investigate possible Colcemid effects, as well as possible differences related to cell type.

Preparations for Electron Microscopy

The synchronized cells were removed from the Petri dishes after various incubation periods with medium containing 0.25% trypsin, and were centrifuged at 310 *g* for 5 min. The Don cells were suspended in 1% osmium tetroxide buffered with Veronal acetate (Palade, 1952) at pH 7.6. Then, the cells were pelleted and left in fresh osmium tetroxide for 30 min. In the confirming experiment, CHO cells were fixed in phosphate-buffered (Millonig, 1961) 2% osmium tetroxide, or were fixed in phosphate-buffered 3% glutaraldehyde (Sabatini

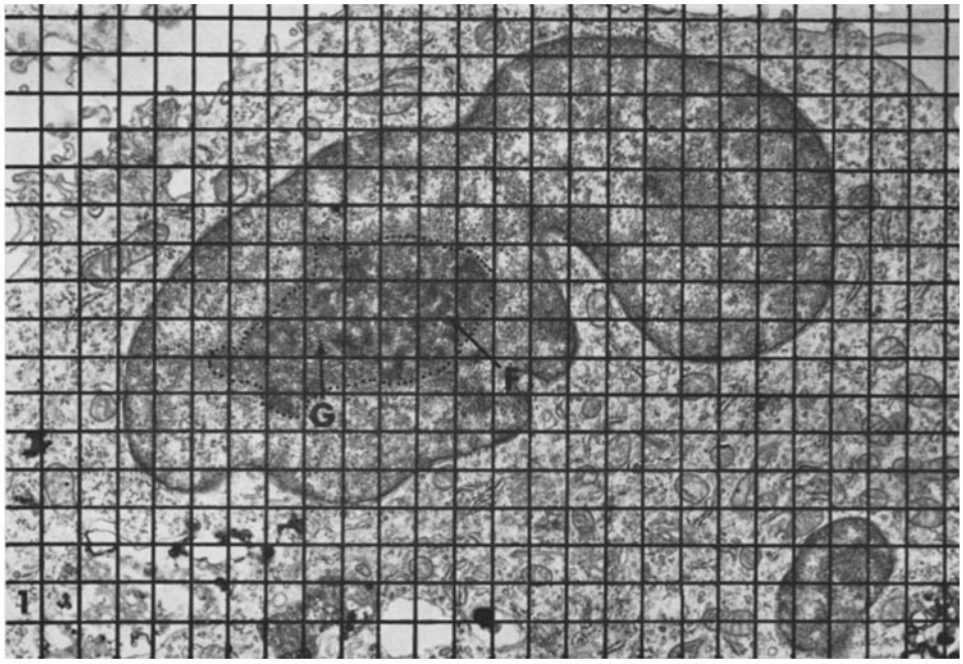


FIGURE 1 A ¼-inch grid was placed over a typical interphase cell, and a line was drawn around the periphery of the nucleolus. The horizontal and vertical line lengths over the nucleus, nucleolus, fibrous components, and unstained or vacuolar regions were measured and recorded. Arrows point to the fibrous (F) and granular (G) regions of the nucleolus. × 9900.

et al., 1962) and postfixed in phosphate-buffered 2% osmium tetroxide. When fixation was completed, the pellet was rinsed, dehydrated in a graded series of alcohols, and embedded in Epon 812 (Luft, 1961).

Sections of 500–800 A were cut on glass knives with a Porter Blum MT-2 ultramicrotome or LKB Ultratome III (LKB Instruments Inc., Rockville, Md.), mounted on Formvar-covered, carbon-coated, 100-mesh copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). Specimens were examined in a Philips 200 electron microscope (Philips Electronic Instruments, Mt. Vernon, N. Y.), at an accelerating voltage of 60 kv.

Quantitation and Analysis

Electron micrographs (× 10,000–20,000) of nuclei sectioned approximately in their central region and with a nucleolus or nucleoli containing clearly defined fibrous and granular regions were selected for quantitative study. These micrographs were randomized with respect to experiment numbers and sampling times and were examined under an illuminated magnifier. A line was drawn around the nucleolus to eliminate any peripheral, nucleolar-associated chromatin. A ¼-inch transparent grid (according to the procedure described by Loud et al., 1965) was placed over the print (Fig. 1), and its position was marked on the print for future reference. The following horizontal and vertical line lengths were measured and recorded: (*a*) nuclear line lengths, (*b*) nucleolar line lengths, (*c*) fibrous line lengths, and (*d*) line lengths of the unstained or vacuolar areas of the nucleolus. The nucleoli from at least 10 cells were measured for each sampling time in the two experiments.

Analysis of the data obtained from line length measurements was initiated by calculating ratios between the components as follows: (*a*) nucleolar line length:nuclear line length, (*b*) fibrous line length:nucleolar line length, (*c*) line length of unstained or vacuolar areas:nucleolar line length, and (*d*) the granular line length:nucleolar line length. Loud et al. (1965) established the relationship that the ratio of line length measurements over two components is equal to the ratio of the respective areas, as well as to the ratio of the respective volumes. Therefore, nucleolar line length:nuclear line length = nucleolar area:nuclear area = nucleolar volume:nuclear volume. By solving the above equa-

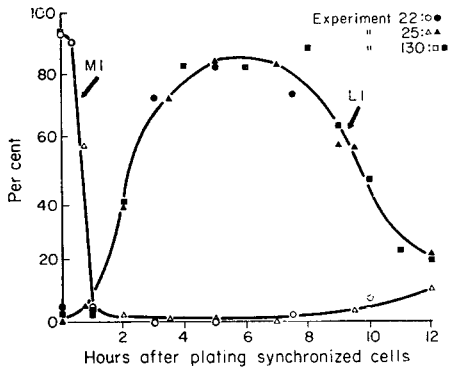


FIGURE 2 The per cent of cells in mitosis (MI) and the per cent of cells in S phase (LI as determined by pulse-labeling with ^{14}C-TdR) are indicated as a function of the incubation period. At 0 hr over 90% of the cells were in metaphase, and at 1 hr about 95% of the cells were in G_1. The labeling index (LI) indicates that some cells entered the S phase as early as 2 hr, and that by 3.0–3.5 hr the majority of the cells had entered this period. By 9.5 hr, the cells were entering G_2 and mitosis, although 50% still remained in the S phase.

tion for nucleolar volume, the relationship becomes: nucleolar volume = (nucleolar line length:nuclear line length) × (nuclear volume). The volumes of the fibrous and granular components were then calculated (Fig. 3) in a similar manner. Where applicable, standard errors of the mean were computed and indicated on the graphs.

RESULTS

The structure of the nucleolus was studied as the synchronous cells proceeded from metaphase through interphase into the next mitosis. The position of the cells in the cycle at the time they were fixed was determined from mitotic indices and the per cent of the cells incorporating ^{14}C-TdR during a 20 min pulse (Fig. 2). Morphological criteria such as the condensation of chromatin and dissolution or reformation of the nuclear membrane also assisted in identifying cells in or near mitosis (Robbins and Gonatas, 1964; Murray et al., 1965). The cells which were fixed at various times for analysis were distributed in the cycle as follows (Fig. 3): at 0 hr in metaphase; at 0.75 hr in anaphase, telophase, and early G_1; at 2–3.5 hr in G_1 and early S phase; at 5 and 7 hr primarily in S phase; at 9.5 hr in late S and G_2, and at 12 hr primarily in G_2, various stages of mitosis, and G_1. Metaphase cells were studied at both the beginning and end of the cycle due to the possibility that atypical, mitotic cells were produced from the 2-hr Colcemid treatment preceding collection.

The Nucleolus during Metaphase, Anaphase, and Telophase

The cells arrested in metaphase following the 2-hr treatment with Colcemid (Fig. 4) contained "persistent nucleoli" in 30% of the cells studied (13 out of 43). These nucleolar remnants existed

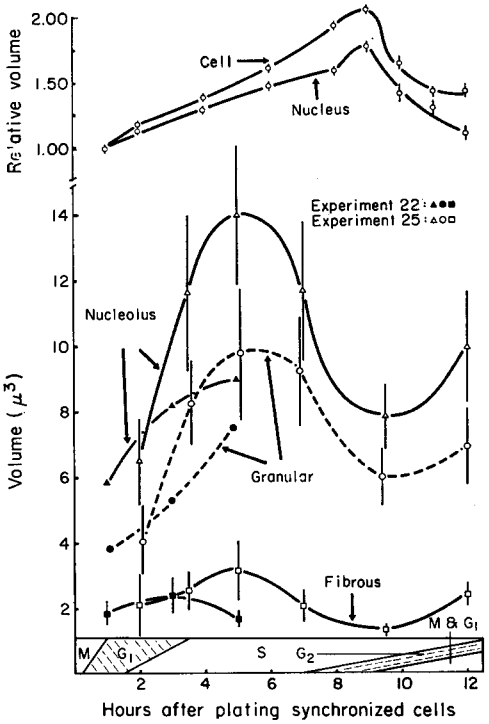


FIGURE 3 The volumes of the cell, nucleus, nucleolus, and fibrous and granular regions are illustrated. The position of the cells in the cycle was estimated from results in Fig. 2 and is illustrated at the bottom of the graph. The nucleolus more than doubled from the G_1 to the S phase, due to the accumulation of the granular component, as well as a possible slight increase in the fibrous component. The unstained or vacuolar areas of the nucleolus (not illustrated) remained relatively constant (3–9%) throughout the entire cycle. Nuclear and cell volumes are shown as relative volumes (nuclear volume, 1 = 84 μ^3; cell volume, 1 = 507 μ^3) with the peak at 9 hr indicating the completion of the cycle. To simplify the presentation of the data, the standard errors of the means, ±2.5–3.0 μ^3 for the nucleolus and ±1.8–3.0 μ^3 for the granular component, have not been plotted for experiment 22.

as well defined regions between the chromosomes and were characterized by the typical, 150–200 A granules interconnected by 50–75 A single nucleolar fibers. In addition, ribosome-like granules were dispersed along the surface of some of the chromosomes in anaphase and telophase cells as illustrated in Fig. 5. (Anaphase and telophase cells were identified by the formation of the nuclear membrane around the chromosomal elements.) Dense, fibrous regions characteristic of the nucleoli in interphase cells were not observed in any of the mitotic cells.

The Nucleolus during G_1 Phase

The inception of G_1 phase was marked by the complete reconstruction of the nuclear membrane and initiation of chromosomal decondensation. During early G_1, the granular coating along the surface of the chromosomes was still apparent (Figs. 6, 7, and 8 *b*) while dense granular patches resembling the persistent nucleoli were randomly distributed throughout the cytoplasm (Figs. 7 and 8 *a*). No structures characteristic of mature, interphase nucleoli were observed in the cells completing telophase. Numerous small, dense, fibrous patches, however, developed within and along regions of chromatin in virtually all of the cells observed in early G_1 (Fig. 7).[1] The fibrous material within these patches was similar to the fibrous material of mature nucleoli.

Further decondensation of the chromatin was evident as the cells progressed into the G_1 period (Fig. 9). The granular coating on the chromatin, as well as the persistent nucleoli, were no longer apparent. The small, fibrous patches coalesced, enlarged, and were gradually reduced in number (Fig. 9). By mid-G_1, they began to acquire the 150–200 A granular component found in mature, interphase nucleeli. At this point in the cycle all of the newly synthesized granular material was intimately associated with the fibrous components (Figs. 10 and 11). Shortly after the appearance of the first granular components, fully mature, intact, interphase nucleoli were observed. Quantitative measurements revealed that early G_1 cells had approximately twice as much granular material as fibrous material (Fig. 3).

[1] In the 22 late telophase or early G_1 nuclei observed at 0.75 hr before mature nucleoli were evident, fibrous areas which were separated from the ribosome-like granular material were present in 17 of these cells. However, in four of the five nuclei showing no fibrous areas, the initiation of chromatin decondensation had not occurred; therefore, these four nuclei were classified as late telophase.

Nucleolus during the S Phase

As the cells progressed into the S phase, the amount of nucleolar-associated granular material continued to increase dramatically. Many of these granules lost their association with the fibrous nucleolar component and collected into large, perinucleolar clumps (Figs. 12 and 13). Although there may have been a small increase in the amount of fibrous material during S, nucleolar growth was due primarily to a greater than twofold increase in the granular component (Fig. 3). The asynchrony which occurred near the end of the cycle (see Figs. 2 and 3) prevented distinguishing between cells in late S and G_2; however, in those cells near the end of the cycle, the amounts of both the fibrous and granular components began to decline.

The Nucleolus during the late G_2 Phase

At the end of the cycle (9.5 and 12 hr, during G_2) which was distinguishable by the gradual initiation of chromatin condensation and the wavy appearance of the outer nuclear membrane (Figs. 15 and 16), nucleolar volume decreased, principally due to a decrease in the granular material (Fig. 14). By late G_2 or early prophase, and definitely before many cells divided, the fibrous components of the nucleoli fragmented into small fibrous patches. The clumps of granules remained relatively unchanged during the remainder of G_2.

The Nucleolus during Prophase and Metaphase

Prophase cells, identified by condensed chromosomes enclosed in a relatively intact, nuclear envelope, contained persistent nucleoli (Fig. 17) which resembled those observed in the metaphase cells at the beginning of the cycle (Fig. 4). Dense, fibrous, nucleolar patches were no longer evident. Also, metaphase cells which had not been exposed to Colcemid since they had been synchronized contained typical persistent nucleoli which were comprised primarily of 150–200 A granules (Fig. 18). The granular component of the persistent nucleoli contained a substructure

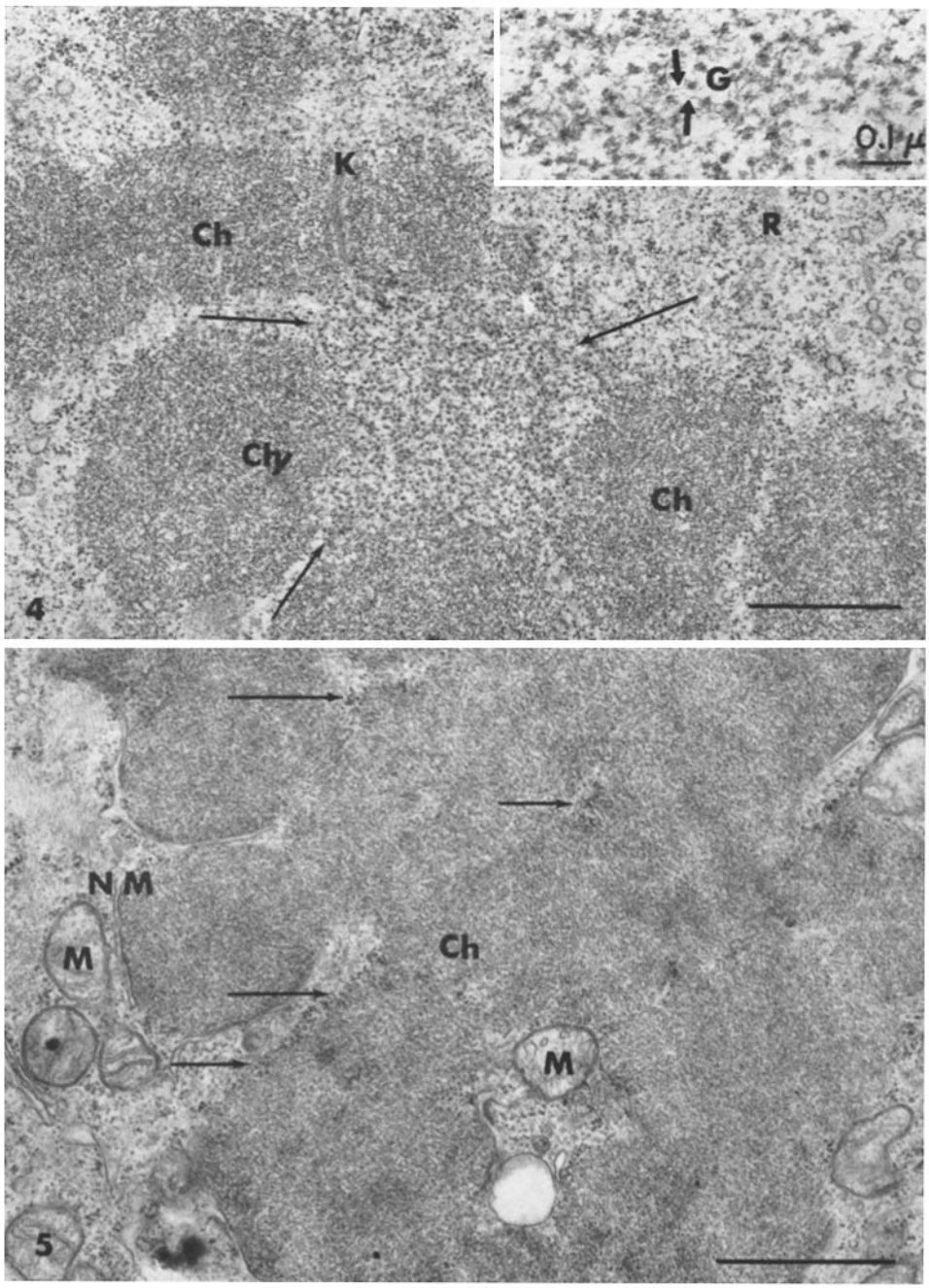
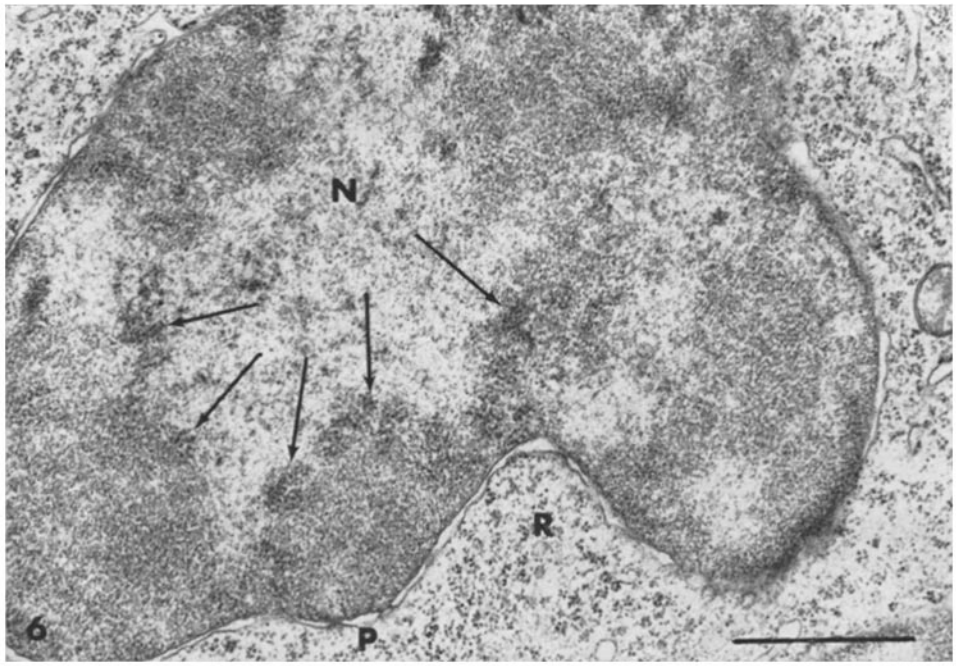


FIGURE 4 A high concentration of granular material (arrows) which is of nucleolar origin (persistent nucleolus) appears to be associated with the chromosomes (Ch) in a metaphase cell (0 hr). Ribosomes (R) are similar in size to the granular remnants. A kinetochore (K) is present on one chromosome. Magnification × 20,700. Insert: A region of the nucleolar remnant which is composed of granular material (G) with an interconnecting network of small, single, 50–75 A fibers (arrows). Magnification × 69,900.

FIGURE 5 An anaphase cell (0.75 hr) in which the nuclear membrane (NM) is reforming around the chromosomes (Ch). Concentrations of ribosome-like particles (arrows) appear to be associated with the surface of some of the chromosomes. Fibrous regions observed in interphase cells were never observed in mitotic cells. Mitochondria (M) are located near the chromosomal mass. Magnification × 24,570. (Distance markers in Figs. 5–19 are 1 μ unless noted differently.)



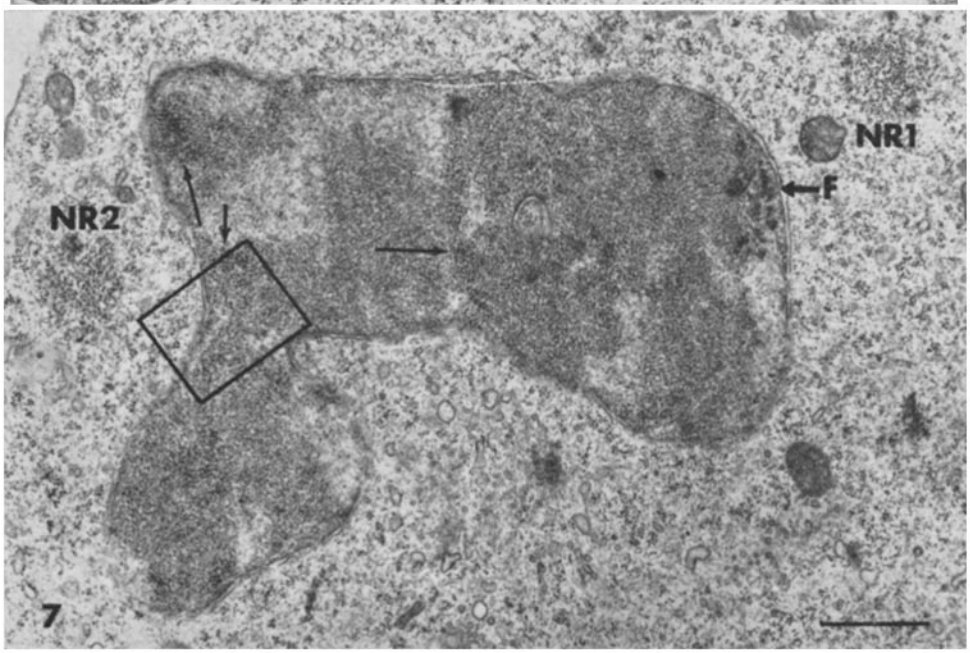


FIGURE 6 In an early G_1 cell (0.75 hr), the ribosome-like granules (arrows) associated with the chromosomes in Fig. 5 are still associated at the periphery of the condensed chromatin in the nucleus (N). Cytoplasmic ribosomes (R) are similar in size to the chromatin-associated granules (arrows). Nuclear pores (P) are evident in the nuclear membrane. Magnification × 24,570.

FIGURE 7 An early G_1 cell (0.75 hr) is illustrated with the nucleolar remnants (NR1 and NR2) in the cytoplasm, chromatin-associated ribosome-like granules (arrows) and a newly formed fibrous nucleolar component (F) in the nucleus. In 95% of the early G_1 cells observed, the fibrous component was present and always separated from the granular material which was associated with the decondensing chromatin. No fibrous material was noted, however, in those late telophase or early G_1 cells in which chromatin decondensation had not been initiated. Magnification × 14,352.

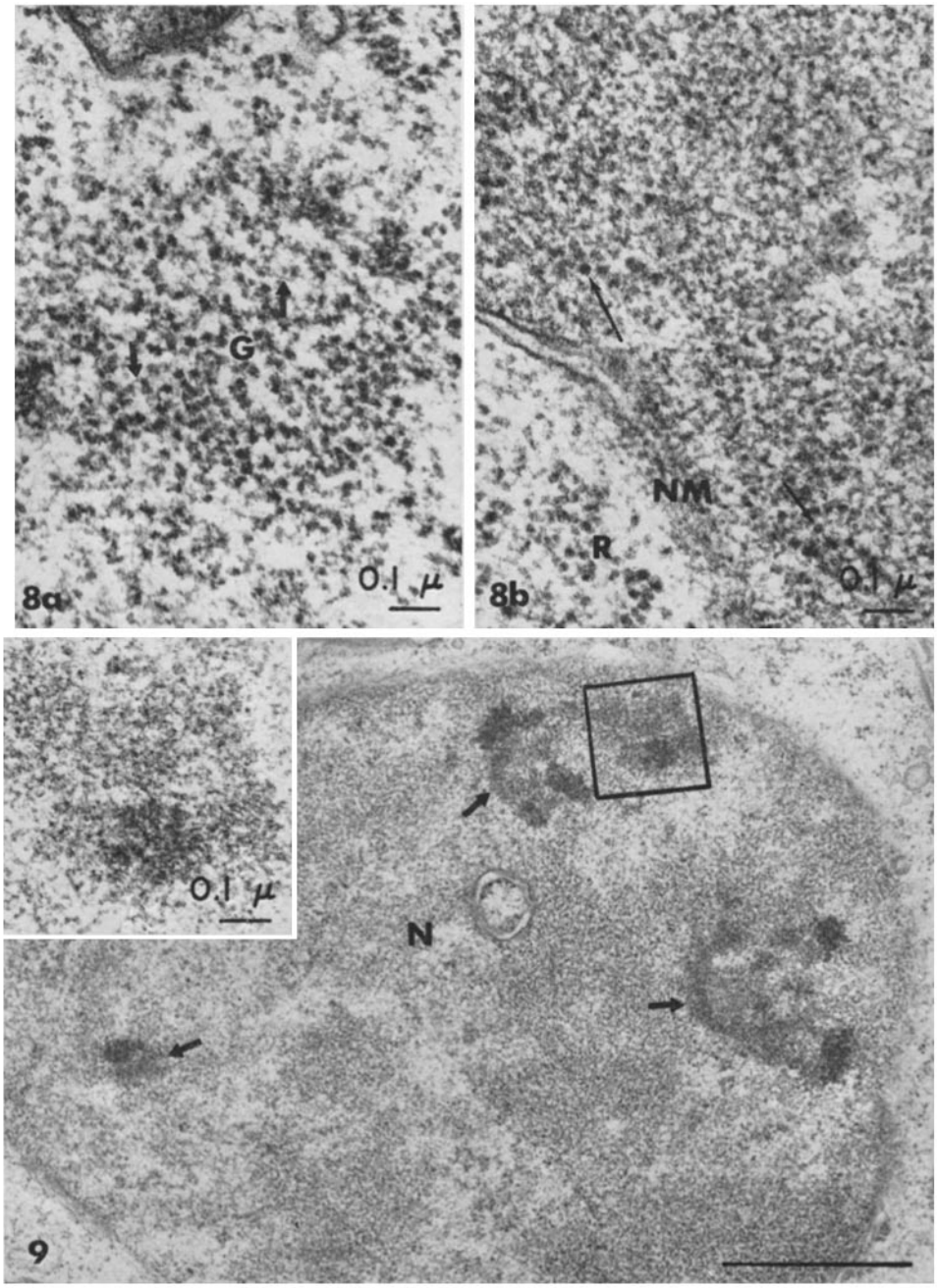


FIGURE 8 (*a*) A higher magnification of the nucleolar remnant NR1 (in Fig. 7), which is still predominately granular (G) (compare with Fig. 4), with some fine interconnecting 50–75 A fibers (arrows). Magnification × 69,900. (*b*) Granular material (arrows) and cytoplasmic ribosomes (R) at higher magnifications of the region outlined in Fig. 7. The nuclear membrane (NM) is present but lacks distinction due to the plane of sectioning. In Figs. 8 *a* and *b*, the cytoplasmic ribosomes, the granular component of the nucleolar remnant, and the chromatin-associated, ribosome-like granules are all similar in size and appearance. Magnification × 69,900.

FIGURE 9 A G_1 cell (0.75 hr) nucleus (N) with three fibrous regions (arrows). Decondensation of the chromatin has progressed further than in Fig. 7, and the chromatin-associated granules illustrated in Figs. 5 and 6 are not evident. Magnification × 25,480. Insert: A higher magnification of one of the developing nucleoli, showing its predominately fibrous nature. Magnification × 69,900.

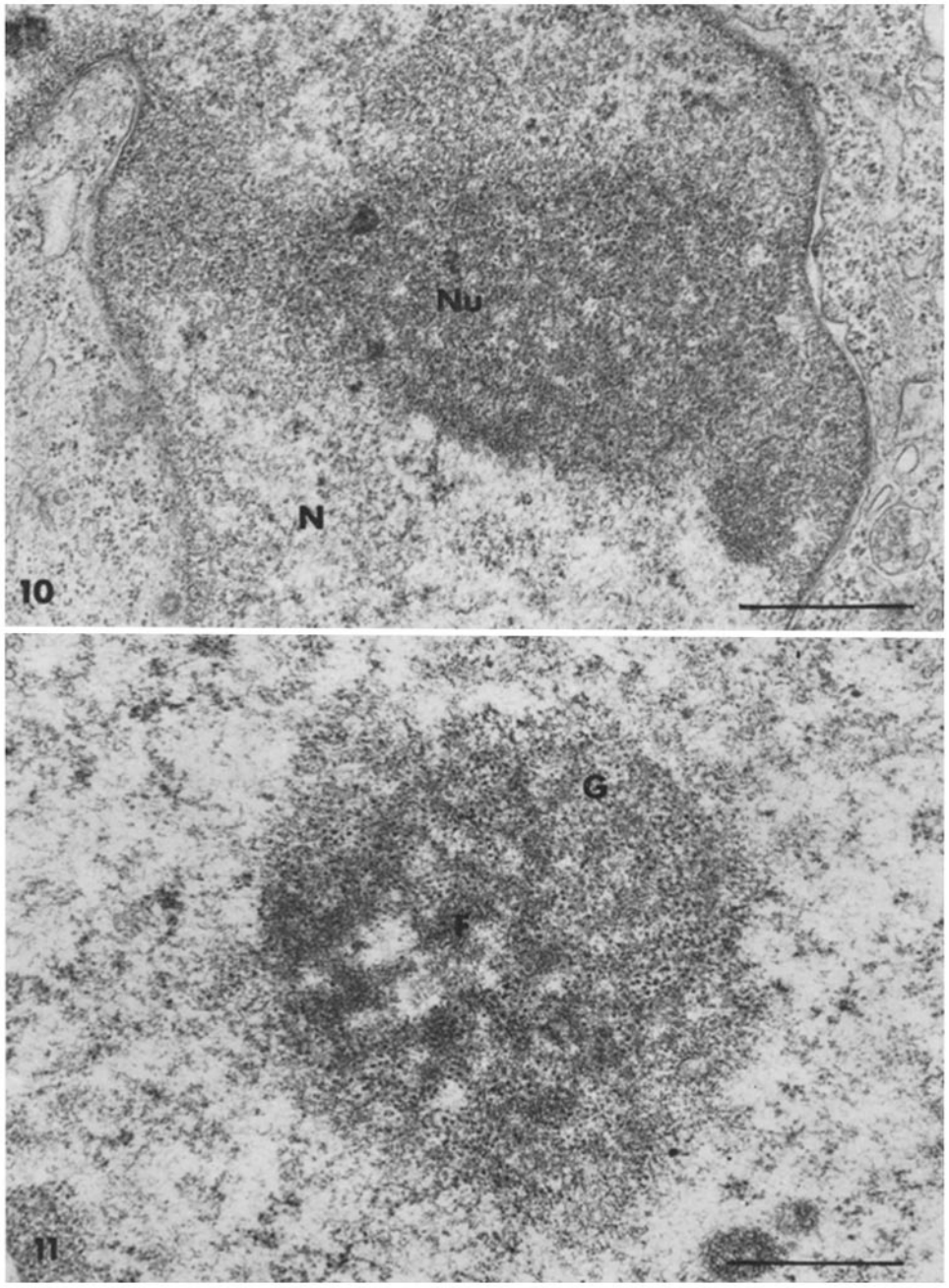


FIGURE 10 In the G_1 (2 hr) cell nucleolus (Nu), the appearance of the granular component appears intimately associated with the fibrous regions. The chromatin has dispersed into small, scattered areas throughout the interphase nucleus (N). Magnification × 23,800.

FIGURE 11 In late G_1 or early S (3.5 hr) the amount of granular material is in excess of the amount noted in Fig. 10; however, some of the granular material (G) is still closely associated with the periphery of the fibrous regions (F). Magnification × 23,800.

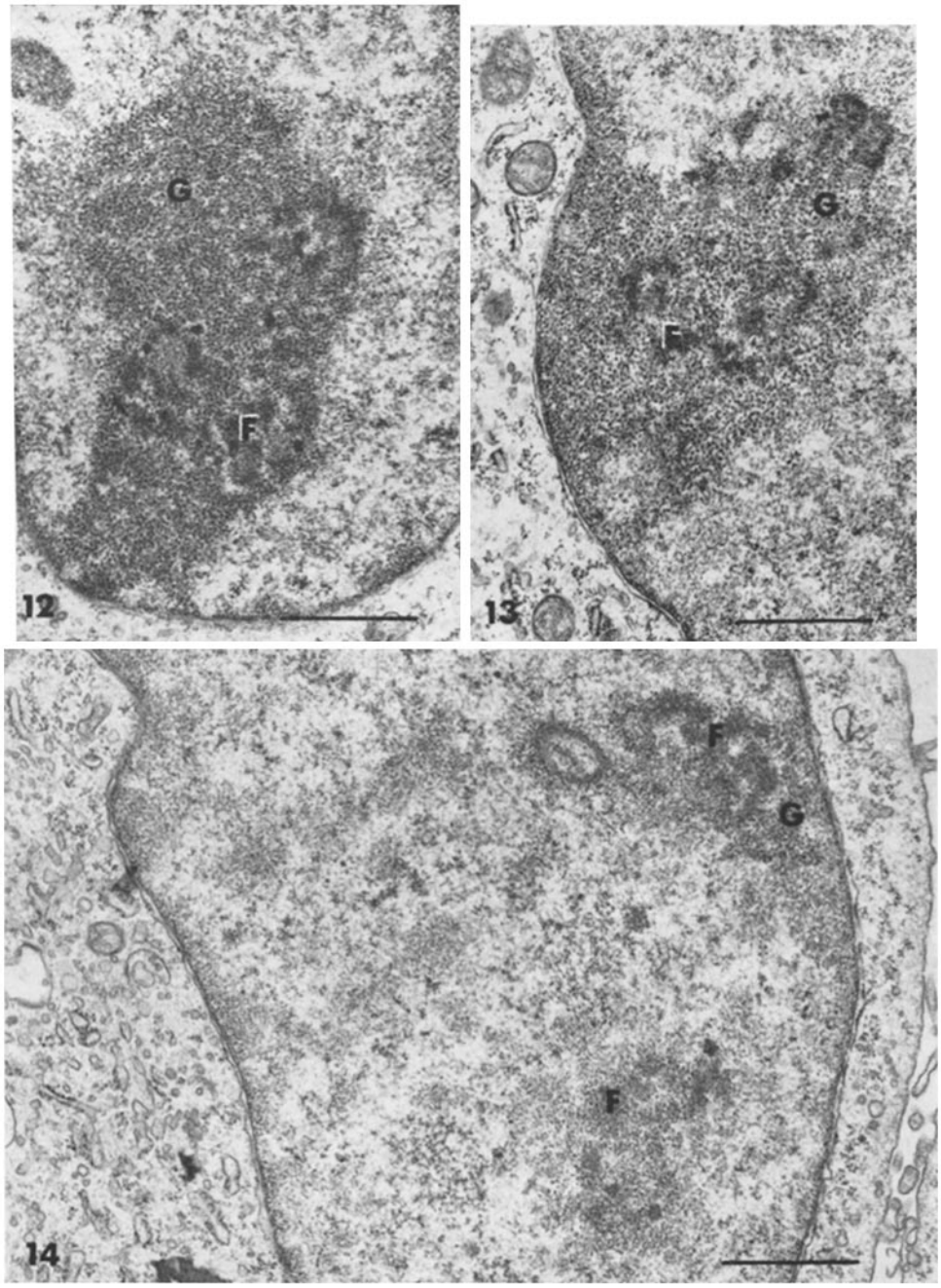


FIGURE 12 The S phase nucleolus (5 hr) is characterized by more than a twofold increase in the granular component (G) and a possible small increase in the fibrous component (F), which results in an over-all increase in nucleolar mass (see Fig. 3) Magnification × 18,768.

FIGURE 13 The large nucleolar mass remains evident in the middle S phase (7.0 hr) primarily due to the abundance of the granular regions (G), while the fibrous regions (F) remain relatively constant. Magnification × 18,768.

FIGURE 14 During late S or early G_2 phase (9.5 hr), the size of the nucleolus has diminished due to a decrease in the amount of the granular component (G) and possibly some of the fibrous component (F). Magnification × 18,768.

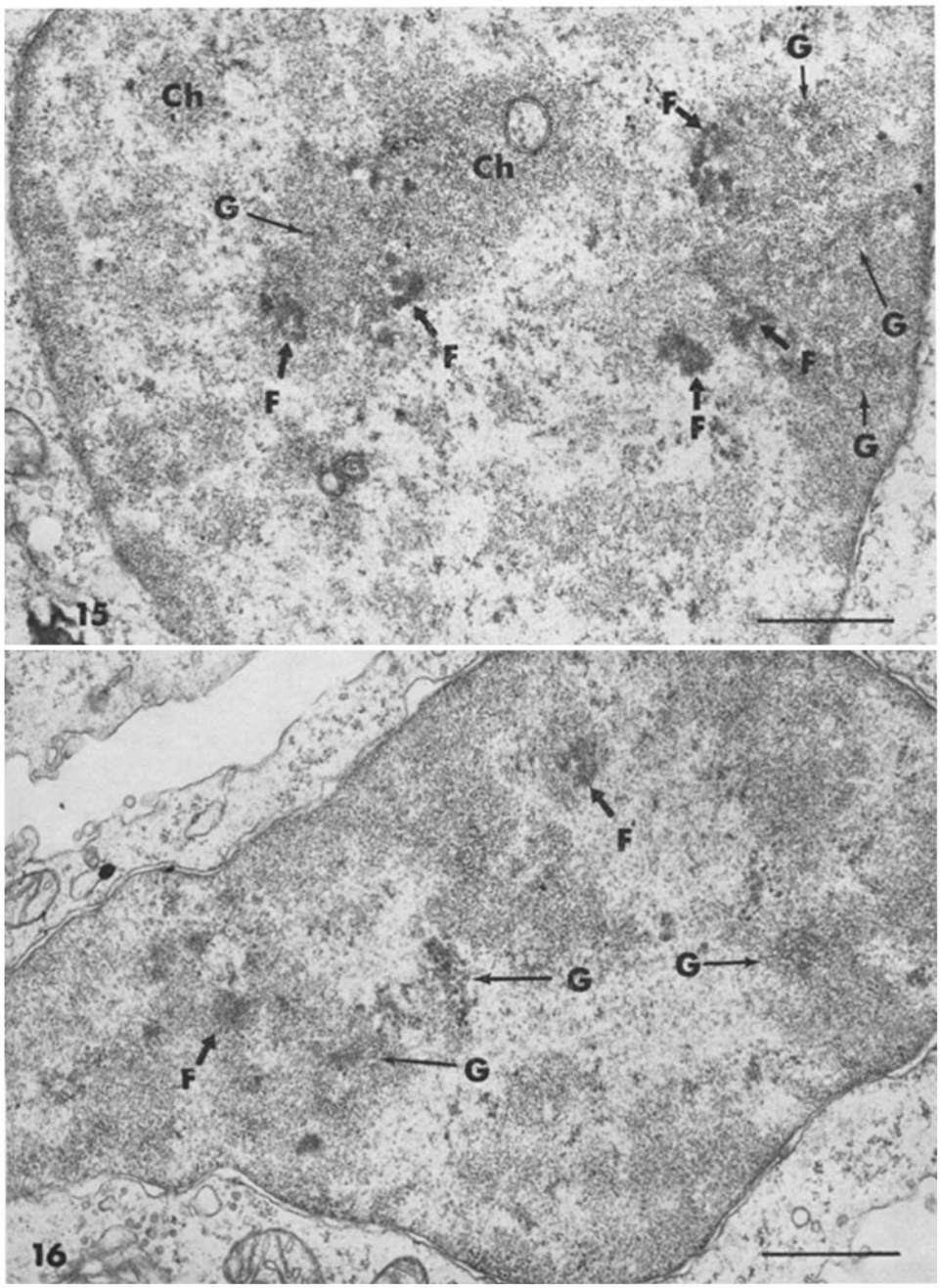


FIGURE 15 A G_2 or preprophase cell nucleus (12.0 hr) evidenced by the initiation of chromatin condensation (Ch). The nucleolus shows dispersion and dissolution into small granular (G) and fibrous (F) regions, although some fibrous and granular associated regions are still evident. Magnification × 18,768.

FIGURE 16 An early prophase cell (12.0 hr) characterized by the condensation of chromatin and the loosening of the nuclear membrane. The nucleolus has further dispersed into numerous small regions of dissociated fibrous (F) and granular regions (G). Magnification × 18,768.

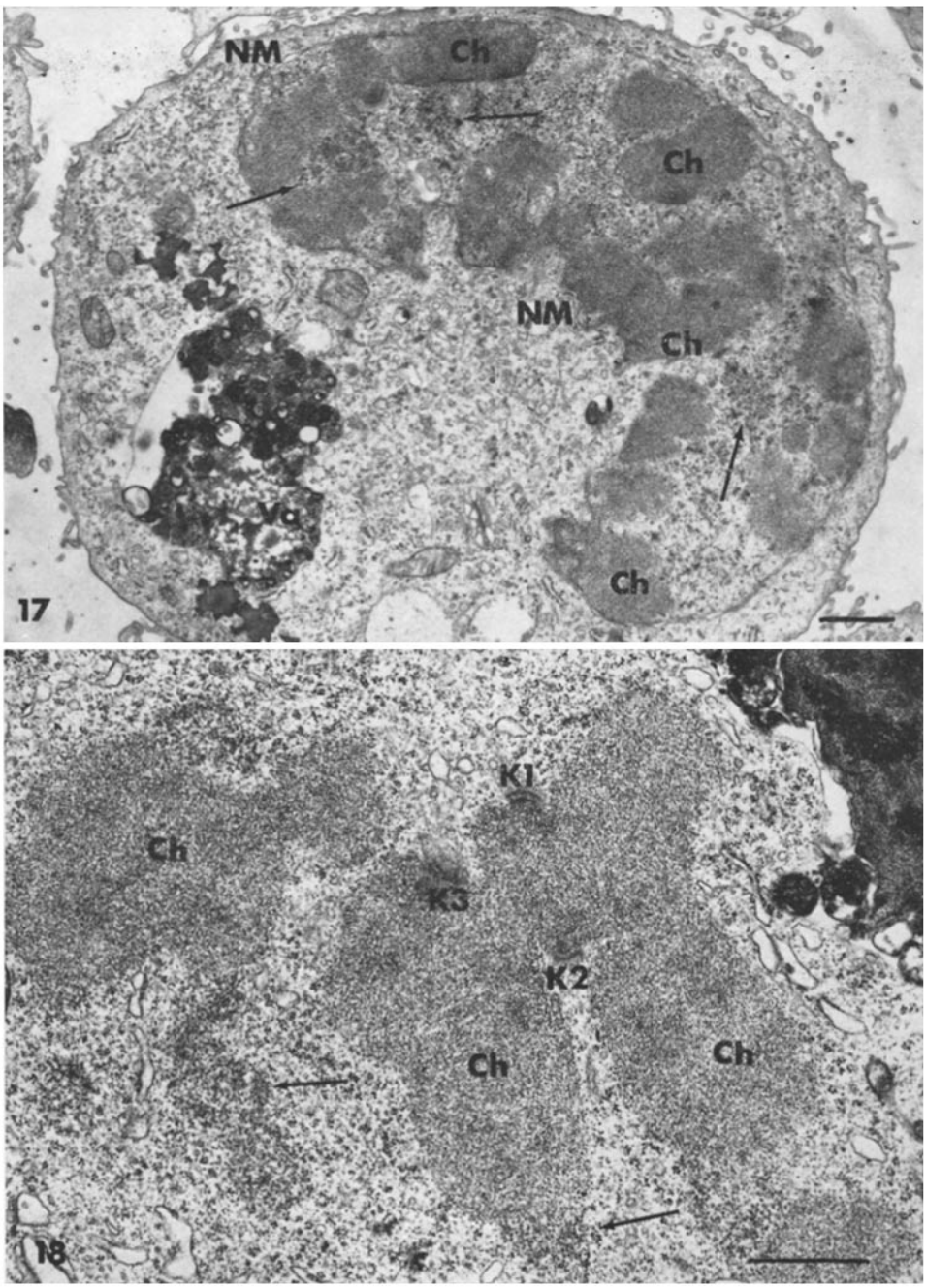


FIGURE 17 A prophase cell (12.0 hr) in which chromatin condensation is relatively complete with some remnants of the nuclear envelope (NM) still surrounding the chromosomal mass (Ch). Persistent nucleoli (arrows) are associated with the chromosomes. A large autophagic vacuole (Va) is present in the cytoplasm. Magnification × 9936.

FIGURE 18 A metaphase cell (12.0 hr) with evidence of persistent nucleoli (arrows) that are associated with the chromosomes (Ch). Kinetochores (K1, K2, and K3) are present on two chromosomes, with an indication of spindle fibers at K3. Magnification × 16,560.

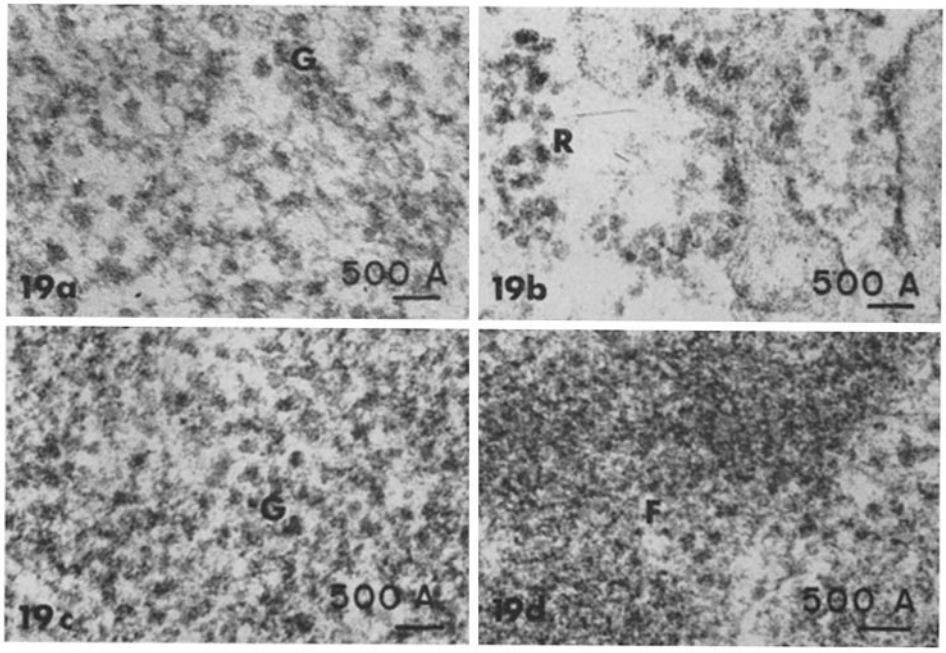


FIGURE 19 High magnifications of (*a*) the granular portion of the persistent nucleolus (G) in Fig. 4, (*b*) cytoplasmic ribosomes (R), (*c*) the granular region (G) of a mature nucleolus, and (*d*) the fibrous region (F) of a mature nucleolus. A fundamental substructure composed of two 20–25 A filaments which constitute a 50–75 A fiber is evident in each of the above RNA-containing units. Note the similarity between the 150–200 A granules in (*a*), (*b*), and (*c*). Magnification × 121,050.

of 20–25 A filaments. These filaments were similar to the filaments in the 50–75 A fibers that constituted the 150–200 A granules observed in the granular portions of mature nucleoli and cytoplasmic ribosomes (Fig. 19).

Nucleolar Changes in CHO Cells Synchronized without Colcemid and Fixed with Glutaraldehyde

The nucleolar changes noted in the Don cells synchronized with Colcemid were confirmed in the glutaraldehyde-fixed CHO cells synchronized without Colcemid. The granular nucleolar remnants were noted in both mitotic and early G_1 cells. The small, dense, fibrous patches were not observed in mitosis and only appeared as the cell entered early G_1. Then, later in G_1, the fibrous-associated granular component appeared (Fig. 20 *a*), and the mature nucleolus enlarged as the cell progressed through G_1 into the S phase. The relative diameters estimated for 20 nucleolar transections randomly selected at each of three sampling times were: 1.00 ± 0.05 (standard error of mean) for G_1 cells fixed at 1.0 hr, 1.41 ± 0.13 for S cells fixed at 8.0 hr, and 1.06 ± 0.06 for cells (a mixture of late S, G_2, and G_1) fixed at 14.0 hr. The position of the CHO cells in the cycle as a function of time after plating is plotted elsewhere (Dewey et al., 1971). This increase in nucleolar size observed in S phase cells resulted from the accumulation of the granular component (Fig. 20 *b*).

General Observations on Interphase Nucleoli

In addition to the line length measurements that were taken and recorded, other general observations on the interphase nucleoli were made. The average number of nucleolar transections per cell (the number of nucleoli observed per cell selected at random in a tissue section) was 1.5 (70% with at least one nucleolus) and remained constant throughout the interphase cycle (1–12 hr). The fraction of the nucleoli with a predominance of fibrous material at the periphery of

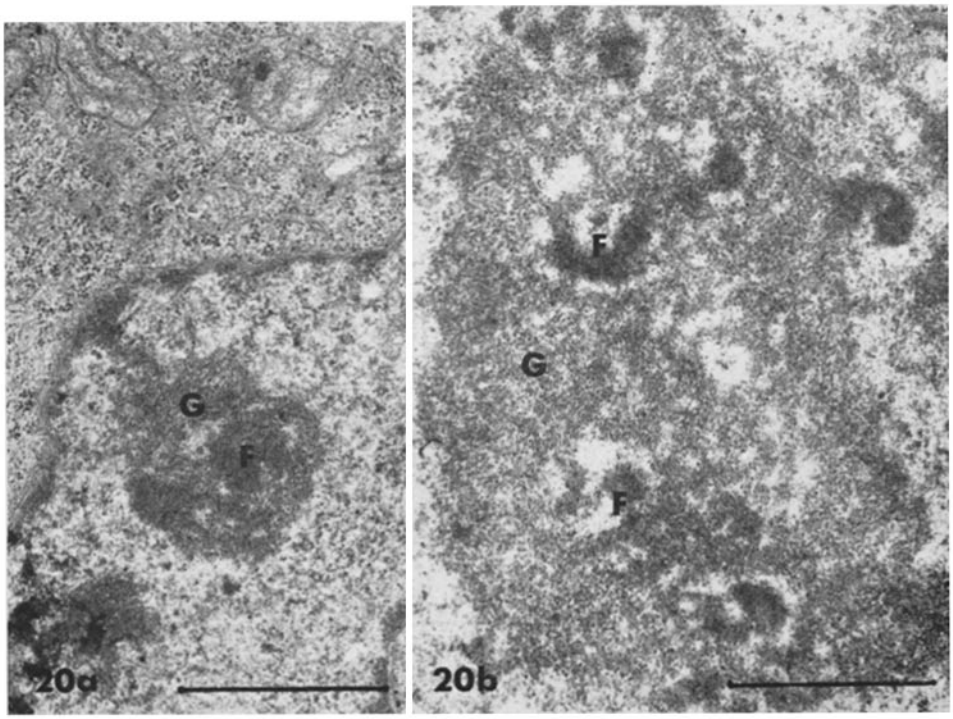


FIGURE 20 Glutaraldehyde-fixed CHO cell nucleoli. (*a*) In G_1 (1.0 hr), the newly formed nucleolus is small and composed primarily of fibrous (F) material. (*b*) In S (8.0 hr), the nucleolus is larger and has a predominately granular (G) appearance with scattered regions of fibrous (F) material. Magnification × 26,900.

the nucleolus appeared to increase from 0.3 at 2–3.5 hr to 0.45–0.65 at 5–12 hr. However, there was no apparent change, throughout the cycle, either in the fraction of the nucleoli associated with the nuclear envelope (0.5–0.8) or in the fraction of the nucleoli connected by chromatin with the nuclear membrane (0.25–0.65). Thus, no striking changes in the number, location, or over-all structure of the nucleolus during the interphase cycle were observed. Additional studies of more sections would be needed to establish any small change which may occur.

DISCUSSION

This study of nucleolar ultrastructure during the Chinese hamster cell cycle has suggested dynamic relationships between the fibrous and granular portions of the nucleolus. A cell completing telophase contains remnants of the granular portions of the nucleoli from the previous cycle (Figs. 4–6). Immediately as the cell enters G_1, new nucleoli containing only fibrous regions (Figs. 7–9) appear. As the cell proceeds through G_1, the granular portions of the nucleoli appear in intimate association with the fibrous regions (Fig. 10), which strongly suggests that the fibrous material is a precursor for the granular material. By the time the cell reaches the middle of the S phase, the granular material increases by a factor of two (Figs. 3, 12, and 13). Then, as the cell leaves the S phase, enters G_2, and prepares for division, the granular material again decreases in amount (Fig. 14). This decrease in the granular portion is possibly preceded by a decrease in the fibrous portion. When the cell enters division, only remnants of the nucleoli resembling the granular portion of intact nucleoli are observed (Figs. 15–18). Thus, the nucleoli appear to be synthesized at the beginning of the cycle, starting first with the fibrous regions which serve as precursors for the granular regions.

At least two theories exist concerning the mode of nucleolar formation: (*a*) that nucleoli arise from material carried through mitosis (Moses,

1964; Yasuzumi and Sugihara, 1965) providing material for nucleolar reformation (Yasuzumi and Sugihara, 1965), and (*b*) that reformation occurs as DNA cistrons in the nucleolar organizer are activated for transcription. Our present study, as well as the studies of others (Karasaki, 1965; Stevens, 1965; Hay and Gurdon, 1967; Phillips and Phillips, 1969) which suggest that the fibrous region is the precursor for the granular region, are consistent with the second hypothesis. Other supporting evidence is provided from several labeling experiments which have shown that the synthesis of RNA stops during mitosis (Taylor, 1960; Prescott and Bender, 1962; Das, 1963; Arrighi, 1967), and that the fibrous component of the nucleolus is the first to label and that about 30 min later the granular component becomes labeled (Granboulan and Granboulan, 1964, 1965; Harris, 1959; Unuma, Arendell, et al., 1968; Unuma, Floyd, et al., 1968; Marinozzi, 1964; Karasaki, 1965; Gaudecker, 1967; Jacob, 1967; Geuskens and Bernhard, 1966). This fibrous component probably contains the protein-associated, RNase-sensitive matrix fibers attached to a DNase-sensitive axis fiber isolated by Miller and Beatty (1969) from amphibian oocyte nucleoli. Also, a study of amphibian embryogenesis showed that the lack of nucleoli during the blastula stage was followed, during the gastrula stage, by immature nucleoli which contained only fibrous components; then, as embryological development continued, the nucleoli enlarged primarily due to the appearance and subsequent enlargement of the granular regions (Karasaki, 1965). Furthermore, Shinozuka et al. (1970) reported that, after the administration of ethionine, nucleolar reformation consisted of the appearance of a rod-shaped, predominately fibrous material followed by the presence of both the fibrous and granular components in the maturing nucleolus. Thus, the hypothesis is well supported that nucleolar reformation after division results from the activation of DNA in the nucleolar organizer regions which transcribe for RNA, first appearing as a fibrous component, containing both DNA and RNA, and then later as a granular component.

Biochemical studies are also relevant to the morphological changes observed. The general scheme for ribosomal formation which has been shown to be associated with transcription from DNA cistrons associated with the nucleoli is as follows: the first ribosomal RNA precursor to be produced is the 45S RNA (110S with associated proteins)[2] which has a mean life of about 16 min before it cleaves into 18S and 32S–35S precursors (78S with associated proteins) (Liau and Perry, 1969; Willems et al., 1969; Penman et al., 1966; Perry, 1966; Perry, 1965; Perry and Kelley, 1966). The 18S RNA component migrates immediately to the cytoplasm where it is found as a 40S ribonucleoprotein (RNP) subunit of a ribosome. The 32S–35S precursors which remain in the nucleolus and have a mean life of about 40 min split to form a 28S RNA unit (62S with associated protein), which in turn remains in the nucleolus for another 20 min before it migrates to the cytoplasm and is found as the 60S RNP subunit of the ribosomes (Willems et al. 1969; Perry, 1964, 1965; Girard et al., 1964; Girard, Latham, et al., 1965). This rapid turnover (16-min mean life) of the 110S RNP subunit and the slow turnover (60-min mean life) of the 62S–78S RNP subunits implies that the 110S RNP pool would be much smaller than the 62S–78S pool. Furthermore, the decrease in the protein-binding capacity of the 62S–78S RNP particles (44% protein) compared with the 110S RNP particles (57% protein) should cause the 62S–78S RNP particles to have a more compact conformation than the 110S RNP particles (Liau and Perry, 1969). These relationships, when related to the sequence of labeling in the nucleolus and the changes in nucleolar structure during the cell cycle, strongly suggest that the fibrous component in mammalian cells is composed of the 110S RNP subunits and is a precursor for the granular component which is composed of the 62S–78S RNP subunits (Perry, 1964; Hay, 1968).

The appearance in mitotic cells of ribosome-

[2] In the mutant, *Xenopus laevis*, pseudonucleoli containing no 45S RNA (Brown and Gurdon, 1966) are described as small fibrous bodies (Hay 1968). These bodies probably correspond to the protein moiety normally associated with the 45S RNA (110S RNP) subunits. Also, when actinomycin D (2μg, ml) was added to synchronous metaphase cells and the cells were examined in the G_1 and early S periods, a fibrous core surrounded by some granular material was observed (unpublished data). Since the stains (uranyl acetate and lead citrate) normally used in electron microscopy are relatively nonspecific (Zobel and Beer, 1965), the DNA, and chromosomal and nucleolar proteins, should be stained even in the absence of RNA.

like particles appearing both as loose aggregates, designated as persistent nucleoli (Hsu et al., 1965; Brinkley, 1965; Heneen and Nichols, 1966), and as a coating of particles intimately associated with the chromosomes (Yasuzumi and Sugihara, 1965), is consistent with the hypothesis presented. As the cell reaches prophase, RNA synthesis ceases (Taylor, 1960; Prescott and Bender, 1962; Das, 1963; Arrighi, 1967); therefore, the fibrous component with a mean life of about 16 min should become virtually depleted during the 45-min period required to complete division, while the longer-lived granular component should persist. These persistent RNP granules which are carried through division into the next cycle as they remain intimately associated with the chromosomes or are lost as aggregates to the cytoplasm (Figs. 5–7, 17, and 18) are probably not involved in nuclear reconstruction. Thus, according to the hypothesis, the reformation of nucleoli in mammalian cells after division results from the transcription of nucleolar organizer DNA to produce new 110S RNP (45S RNA). This new RNA, associated with the DNA which coded for its production, appears morphologically in association with protein as fibrous material. Then, after 16 min, the fibrous material is converted to 62S–78S RNP (28–32S RNA) which appears morphologically as granular material.

Appreciation is expressed to Dr. Glenwood P. Epling for his advice and encouragement.

This work was supported in part by the National Institutes of Health grant no. CA08618-03.

Received for publication 6 July 1970, and in revised form 25 January 1971.

REFERENCES

ARRIGHI, F. E. 1967. Nucleolar RNA synthetic activity in Chinese hamster cells *in vitro* and the effects of actinomycin D and nogalamycin. *J. Cell Physiol.* **69**:45.

BANNASCH, P., and W. THOENES. 1965. Zum Problem der nucleolaren Stoffabgabe. Elektronen mikroskopische Untersuchungen am Pankreas der weissen Maus. *Z. Zellforsch. Mikrosk. Anat.* **67**: 674.

BERNHARD, W. 1966. Ultrastructural aspects of the normal and pathological nucleolus in mammalian cells. *Nat. Cancer Inst. Monogr.* **23**:13.

BERNHARD, W., and N. GRANBOULAN. 1968. Electron microscopy of the nucleolus in vertebrate cells. *In* The Nucleus. A. Dalton and F. Haguenau, editors. Academic Press Inc., New York. 81–149.

BROWN, D. D., and J. B. GURDON. 1966. Size distribution and stability of DNA-like RNA synthesized during development of anucleolate embryos of *Xenopus laevis*. *J. Mol. Biol.* **19**:399.

BRINKLEY, B. R. 1965. The fine structure of the nucleolus in mitotic division of Chinese hamster cell *in vitro*. *J. Cell Biol.* **27**:411.

DAS, N. K. 1963. Chromosomal and nucleolar RNA synthesis in root tips during mitosis. *Science (Washington).* **140**:1231.

DEWEY, W. C., and H. H. MILLER. 1969. X-ray induction of chromatid exchanges in mitotic and G_1 Chinese hamster cells pretreated with Colcemid. *Exp. Cell Res.* **57**:63.

DEWEY, W. C., R. M. HUMPHREY, and B. A. SEDITA. 1966. Cell cycle kinetics and radiation-induced chromosomal aberrations studied with ^{14}C and ^{3}H labels. *Biophys. J.* **6**:247.

DEWEY, W. C., H. H. MILLER, and D. B. LEEPER. 1971. Chromosomal Aberrations and Mortality of X-Irradiated Mammalian Cell: Emphasis on Repair. *Proc. Nat. Acad. Sci. U. S. A.* **68**: In press.

GAUDECKER, B. VON. 1967. RNA synthesis in the nucleolus of *Chironomus Thummi* as studied by high resolution autoradiography. *Z. Zellforsch. Mikrosk. Anat.* **82**:536.

GEUSKENS, M., and W. BERNHARD. 1966. Cytochimie ultrastructurale du nucléole. III. Action de l'actinomycine D sur le métabolisme du RNA nucléolaire. *Exp. Cell Res.* **44**:579.

GIRARD, M., H. LATHAM, S. PENMAN, and J. E. DARNELL. 1965. Entrance of newly formed messenger RNA and ribosomes into the HeLa cell cytoplasm. *J. Mol. Biol.* **11**:187.

GIRARD, M., S. PENMAN, and J. E. DARNELL. 1964. The effect of actinomycin on ribosome formation in HeLa cells. *Proc. Nat. Acad. Sci. U. S. A.* **51**:205.

GRANBOULAN, N., and P. GRANBOULAN. 1964. Cytochimie ultrastructurale du nucléole. I. Mise en evidence de chromatine á l'intérieur du nucléole. *Exp. Cell Res.* **34**:71.

GRANBOULAN, N., and P. GRANBOULAN. 1965. Cytochimie ultrastructurale du nucléole. II. Étude des sites de synthèse du RNA dans le nucléole et le noyau. *Exp. Cell Res.* **38**:604.

HARRIS, H. 1959. The initiation of deoxyribonucleic acid synthesis in connective tissue cells, with some observations on the function of the nucleolus. *Biochem. J.* **72**:54.

HAY, E. D. 1968. Structure and function of the nucleolus in developing cells. *In* The Nucleus. A. Dalton and F. Haguenau, editors. Academic Press Inc., New York. 2–79.

HAY, E. D., and J. B. GURDON. 1967. Fine structure of the nucleolus in normal and mutant *Xenopus* embryos. *J. Cell Sci.* **2**:151.

HENEEN, W. K., and W. W. NICHOLS. 1966. Persistence of nucleoli in short term and long term

cultures and in direct bone marrow preparations in mammalian materials. *J. Cell Biol.* **31**:543.

HSU, T. C., F. E. ARRIGHI, R. R. KLEVECZ, and B. R. BRINKLEY. 1965. The nucleoli in mitotic divisions of mammalian cells *in vitro*. *J. Cell Biol.* **26**:539.

HSU, T. C., B. R. BRINKLEY, and F. E. ARRIGHI. 1967. The structure and behavior of the nucleolar organizers in mammalian cells. *Chromosoma.* **23**: 137.

HSU, T. C., and M. T. ZENZES. 1964. Mammalian chromosomes *in vitro*. XVII. Idiogram of the Chinese hamster. *J. Nat. Cancer Inst.* **32**:857.

HYDE, B. B., K. SANKARANARAYANAN, and M. L. BIRNSTIEL. 1965. Observations on fine structure in pea nucleoli *in situ* and isolated. *J. Ultrastruct. Res.* **12**:652.

JACOB, J. 1967. An electron microscope autoradiographic study of the site of initial synthesis of RNA in the nucleolus of *Smittia*. *Exp. Cell Res.* **48**:276.

JONES, P. 1962. Paramitotic granulation and ribosome bodies in erythroblasts. *J. Ultrastruct. Res.* **7**:308.

KARASAKI, S. 1965. Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J. Cell Biol.* **26**:937.

LAFONTAINE, J. G., and L. A. CHOUINARD. 1963. A correlated light and electron microscope study of the nucleolar material during mitosis in *Vicia faba*. *J. Cell Biol.* **17**:167.

LIAU, M. C., and R. P. PERRY. 1969. Ribosome precursor particles in nucleoli. *J. Cell Biol.* **42**:272.

LORD, A., and J. G. LAFONTAINE. 1969. The organization of the nucleolus in meristematic plant cells. A cytochemical study. *J. Cell Biol.* **40**:633.

LOUD, A. V., W. C. BARANY, and B. A. PACK. 1965. Quantitative evaluation of cytoplasmic structures in electron microscopy. *Lab. Invest.* **14**:996.

LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.

MARINOZZI, V. 1964. Cytochimie ultrastructurale du nucléole—RNA et protéines intranucléolaires. *J. Ultrastruct. Res.* **10**:433.

MILLER, O. L., JR., and B. R. BEATTY. 1969. Visualization of nucleolar genes. *Science* (*Washington*). **164**:955.

MILLONIG, G. 1961. Advantages of a phosphate buffer with OsO_4 in fixatives. *J. Appl. Phys.* **32**:1637.

MOSES, M. J. 1964. The nucleus and chromosomes: A cytological perspective. *In* Cytology and Cell Physiology. G. H. Bourne, editor. Academic Press Inc., New York. 423–558.

MURRAY, R. G., A. S. MURRAY, and A. PIZZO. 1965. The fine structure of mitosis in rat thymic lymphocytes. *J. Cell Biol.* **26**:601.

PALADE, G. E. 1952. A study of fixations for electron microscopy. *J. Exp. Med.* **95**:285.

PENMAN, S., I. SMITH, E. HOLTZMAN, and H. GREENBURG. 1966. RNA metabolism in the HeLa cell nucleus and nucleolus. *Nat. Cancer Inst. Monogr.* **23**:489.

PERRY, R. P. 1964. Role of the nucleolus in ribonucleic acid metabolism and other cellular processes. *Nat. Cancer Inst. Monogr.* **14**:73.

PERRY, R. P. 1965. The nucleolus and the synthesis of ribosomes. *Nat. Cancer Inst. Monogr.* **18**:325.

PERRY, R. P. 1966. On ribosome biogenesis. *Nat. Cancer Inst. Monogr.* **23**:527.

PERRY, R. P., and D. E. KELLEY. 1966. Buoyant densities of cytoplasmic ribonucleoprotein particles of mammalian cells: distinctive character of the ribosome subunits and the rapidly labeled components. *J. Mol. Biol.* **16**:255.

PHILLIPS, S. G., and D. M. PHILLIPS. 1969. Sites of nucleolus production in cultured Chinese hamster cells. *J. Cell Biol.* **40**:248.

PRESCOTT, D. M., and M. A. BENDER. 1962. Synthesis of RNA and protein during mitosis in mammalian tissue culture cells. *Exp. Cell Res.* **26**:260.

REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**:208.

ROBBINS, E., and N. K. GONATAS. 1964. The ultrastructure of a mammalian cell during the mitotic cycle. *J. Cell Biol.* **21**:429.

SABATINI, D., K. BENSCH, and R. J. BARRNETT. 1962. New fixatives for cytological and cytochemical studies. *In* Electron Microscopy. S. S. Breese, Jr., editor. Academic Press Inc., New York. **2**:L3.

SCHOEFL, G. I. 1964. The effect of actinomycin D on the fine structure of the nucleolus. *J. Ultrastruct. Res.* **10**:224.

SHINOZUKA, H., I. M. REID, K. H. SHULL, H. LIANG, and E. FARBER. 1970. Dynamics of liver cell injury and repair. I. Spontaneous reformation of the nucleolus and polyribosomes in the presence of extensive cytoplasmic damage induced by ethionine. *Lab. Invest.* **23**:253.

SMETANA, K. and H. BUSCH. 1964. Studies on the ultrastructure of the nucleoli of the Walker tumor on rat liver. *Cancer Res.* **24**:537.

STEVENS, B. J. 1965. The fine structure of the nucleolus during mitosis in the grasshopper neuroblast cell. *J. Cell Biol.* **24**:349.

SWIFT, H. 1963. Cytochemical studies on nuclear fine structure. *Exp. Cell Res. Suppl.* **9**:54.

TAYLOR, J. H. 1960. Nucleic acid synthesis in relation to the cell division cycle. *Ann. N. Y. Acad. Sci.* **90**:409.

TERZAKIS, J. A. 1965. The nuclear channel system of human endometrium. *J. Cell Biol.* **27**:293.

TOBEY, R. A., E. C. ANDERSON, and D. F. PETERSEN. 1967. Properties of mitotic cells prepared by

mechanically shaking monolayer cultures of Chinese hamster cells. *J. Cell Physiol.* **70**:63.

UNUMA, T., J. P. ARENDELL, and H. BUSCH. 1968. High resolution autoradiographic studies of the uptake of ^{3}H-5-uridine into condensed and dispersed chromatin of nuclei and granular and fibrillar components of nucleoli of Novikoff hepatoma ascites cells. *Exp. Cell Res.* **52**:429.

UNUMA, T., L. R. FLOYD, and H. BUSCH. 1968. Selective removal of the perinucleolar nucleolus-associated chromatin from the isolated nucleoli of livers of thioacetamide-treated rats. *Exp. Cell Res.* **52**:101.

WILLEMS, M., D. PENMAN, and S. PENMAN. 1969. The regulation of RNA synthesis and processing in the nucleolus during inhibition of protein synthesis. *J. Cell Biol.* **41**:177.

YASUZUMI, G., and R. SUGIHARA. 1965. The fine structure of nuclei as revealed by electron microscopy. II. The process of nucleolus reconstitution in Ehrlich ascites tumor cell nuclei. *Exp. Cell Res.* **40**:45.

ZOBEL, C. R., and M. BEER. 1965. The use of heavy metals as electron stains. *In* International Review of Cytology. G. H. Bourne and J. F. Danielli, editors. **18**:363.