

NUCLEOLI OF DIPLOID CELL STRAINS

Their Normal Ultrastructure and the Effects of Toyocamycin and Actinomycin D

STEPHANIE G. PHILLIPS and DAVID M. PHILLIPS

From the Department of Biology, Washington University, St. Louis, Missouri 63130

ABSTRACT

Nucleoli of cultured Chinese hamster or mouse cells in early passages had a loosely reticular substructure. Within the reticulum small, irregularly shaped, light fibrillar zones occurred which were contiguous with denser fibrillar zones. These denser zones appeared to be connected in some places to the particulate material which composed the mass of the nucleolus. Generally, electron-transparent spaces separated the particulate zones from the fibrillar areas. Treatment with toyocamycin, an agent which is reported to cause a blockage in the processing of ribosomal RNA, greatly inhibited the accumulation of newly synthesized RNA in the cytoplasm, as monitored by radioautography. Toyocamycin treatment caused the gradual disappearance of the granules from the particulate region of the nucleoli, and resulted ultimately in the nucleoli appearing homogeneously fibrillar. Actinomycin D treatment, which inhibited virtually all RNA synthesis, caused a segregation, and finally a disaggregation, of nucleolar components.

Nucleoli are currently thought to contain eight or more species of RNA (Weinberg et al., 1967; Weinberg and Penman, 1968; Muramatsu et al., 1966) as well as DNA (Miller, 1966; Barr and Plaut, 1966), 12–20 separable proteins (Vincent et al., 1966; Mundell, 1967), and other miscellaneous constituents such as lipid (Swift, 1959; Ghosh et al., 1969) and glycoprotein (Healy and Parker, 1967). Nucleolar structure has been analyzed extensively by means of electron microscopy, and the ultrastructural elements of which nucleoli are composed are morphologically similar in most cell types. In a statement summarizing a symposium on the nucleolus held in 1965, the Nucleolus Nomenclature Committee generalized that nucleoli possess *fibrillar components* about 50–80 Å in width, and *particulate components* about 150 Å in diameter, both of which contain ribonucleoproteins (*Nat. Cancer Inst. Monogr.* 1966. 23:573). The committee noted that particulate and fibrillar com-

ponents are frequently disposed in irregular and anastomosing threadlike elements about 0.1 μ in diameter termed *nucleolonemata*, but this is not a uniform feature of all nucleoli. The morphological relationships of the nucleolar components are subject to alteration if the cellular metabolism is changed, for instance, during differentiation (Hyde, 1966; Lane, 1967; Hillman and Tasca, 1969) or in response to drugs (Bernhard, 1966; Svoboda et al., 1967). Thus nucleolar structure is undoubtedly dependent upon some aspects of cellular metabolism, although the significance of the variable arrangements of the nucleolar elements is obscure at present, and the precise relationship between the biochemical constituents and the morphological components found in nucleoli is not understood.

The major function ascribed to nucleoli is the production of ribosomal RNA (Perry et al., 1961; Brown and Gurdon, 1964; Ritossa and Spiegel-

man, 1965). This study is particularly concerned with the cytological localization of ribosomal RNA precursors which are thought to occur in nucleoli. The study includes a description of the typical structural organization of nucleoli of diploid mammalian cells in short-term culture. The relationship of nucleolar structure to the production of ribosomal RNA was probed by means of two agents which affect nucleolar RNA synthesis in different specific ways. These agents are toyocamycin, which is reported to be incorporated into the 45S ribosomal RNA precursor molecule (the so-called *transcription unit*) (Perry et al., 1970) thereby preventing the normal cleavage of this molecule into 18S and 28S ribosomal RNA (Tavitian et al., 1968; 1969), and actinomycin D at a level which, in these cells, inhibits RNA synthesis entirely. Since the effects of toyocamycin have only recently been described, experiments were performed to corroborate the earlier reports on the action of this drug.

The experimental material consisted of short-term monolayer cell cultures originally derived from macerated whole fetuses of Chinese hamster or mouse. Only early passages were used as it was felt that cells in the early passages were more likely to be "normal." Both Chinese hamster and mouse cells were used because, for purposes of later comparison with two established cell lines (Phillips and Phillips, 1971), it was deemed necessary to delineate whether important species-specific differences in nucleolar structure existed.

MATERIALS AND METHODS

Cell Culture

Cell strains originally obtained by culturing dispersed Chinese hamster fetuses were used for experimentation in the 2nd through 10th passages. Cells derived from mouse fetuses were used in the 2nd or 3rd passage. Cells from cultures in these early passages appear to be morphologically uniform, spindle-shaped, fibroblastic-type cells and are predominantly euploid. Cells were grown on glass or plastic in Joklik's modified minimal essential medium (MEM) (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 10% fetal calf serum and were subcultured by trypsinization every 4-6 days. Cells to be used for labeling experiments were grown on glass coverslips in Leighton tubes. Those to be examined with the electron microscope were grown in Falcon plastic Petri dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.). Experiments were performed on cells on the 2nd day

after seeding when the cells were growing vigorously and had not yet reached confluency.

Radioautography

For studies on the incorporation of uridine-³H into RNA, cultures were exposed to uridine-³H, SA 0.2 Ci/mmole, at a concentration of 0.1 μ Ci/ml in "conditioned" medium for appropriate lengths of time. In experiments where cells were treated with toyocamycin, the inhibitor was used at a concentration of 0.3 μ g/ml and was added to cultures 20 min before the addition of uridine-³H. This concentration of toyocamycin was selected on the basis of results reported by Tavitian et al. (1968). Solutions of toyocamycin were always made up just before use. Toyocamycin (Vengicide) was originally kindly supplied by A. Tavitian and was later obtained from Myco Farm-Delft, Division of Royal Netherlands Fermentation Industries, Ltd., Delft, Holland. In cases where cells were treated with actinomycin D, the drug was used at a concentration of 5.0 μ g/ml and was added to cultures 20 min before the addition of uridine-³H. Actinomycin D (dactinomycin) was donated by Merck, Sharp & Dohme, Division of Merck & Co., Inc., West Point, Pa. After exposure to uridine-³H for appropriate lengths of time, cells were rinsed briefly with Hanks' salt solution, fixed with ethanol-acetic acid (glacial), 3:1, for $\frac{1}{2}$ hr, and stored for up to 24 hr in 70% ethanol at 4°C while awaiting further processing. RNase controls were digested for 1 $\frac{1}{2}$ hr at 37°C with 0.5 mg/ml RNase (Worthington Biochemical Corp., Freehold, N.J.) which had previously been briefly boiled to remove DNase activity. All cultures were extracted with 5% trichloroacetic acid (TCA) for 12 min at 4°C, rinsed for 1 hr in running water, and air dried. Coverslips were mounted on microscope slides, and the slides were dipped in Kodak NTB 2 emulsion, air dried, and stored in the dark at 4°C for 5 days. The radioautographs were developed in D-19 for 2 min at 20°C and stained with 0.025% azure B for 8 min at 4°C. Silver grains were counted over nucleoli, extranucleolar nucleoplasm, and cytoplasm of 50 random cells from each of two cultures for each experimental treatment. Thus each time point on the graphs shown represents grain counts averaged for 100 cells. An estimate of background grain density was obtained by counting grains over an adjacent empty region approximately equal in area to each cell scored. The 50 background grain counts were averaged for each slide scored, and these background estimates were subtracted from the average grain counts over the cellular compartments, dividing the background counts according to the average cellular area estimated to be occupied by cytoplasm, extranuclear nucleoplasm, and nucleolus. (The background is actually only of significance in grain counts over the cytoplasm after short labelling

periods, since the nuclei and nucleoli occupy relatively little area and incorporate relatively high amounts of precursor.) In the cultures treated with RNase, grains were scored only over cytoplasm or nucleus because nucleoli were difficult to identify after this treatment.

Electron Microscopy

Cultures for electron microscopy were grown on 35-mm in diameter Falcon plastic Petri dishes. Cultures were fixed in 0.5 × Hanks' balanced salt solution containing 2% glutaraldehyde (TAAB Laboratories, Reading, England) and a final collidine concentration of about 0.1 mole/liter at pH 7.2. The use of glutaraldehyde of high quality made further adjustments of pH unnecessary. Cultures were initially fixed at room temperature and subsequently placed at 5°C for 0.5–12 hr. After glutaraldehyde fixation, cultures were rinsed several times in 0.2 M collidine (total time 5–10 min) and postfixed in 1% OsO₄ in 0.1 M collidine (pH 7.2) for 1–4 hr. Cultures were subsequently dehydrated to 100% alcohol. The alcohol was then decanted and replaced with a few drops of Epon (Luft, 1961) which was spread over the surface by rocking the Petri dish. (Propylene oxide was omitted since it dissolves the plastic. Certain plastics, e.g., TAAB embedding medium, were also found to dissolve the Falcon plastic.) After polymerization, the Falcon plastic and Epon were separated, and pieces of Epon containing the cells were mounted with epoxy glue on blank Epon capsules. Sections were cut parallel to the culture surface, stained overnight in 3% aqueous uranyl acetate and subsequently in lead citrate (Venable and Coggeshall, 1965), and examined in a Siemens Elmiskop I or IA microscope.

Each experiment was repeated three times. Fresh cell lines were employed for each trial. Usually two different areas of a culture or two cultures treated in the same manner were examined for each treatment time. We have presented micrographs of cells with multiple nucleoli, and for each treatment we have presented one low magnification micrograph and enlargements of two of the nucleoli. The morphology of nucleoli does not appear to depend on the number of nucleoli per cell, and we felt that presenting cells with several nucleoli and accompanying them with enlargements would aid the reader in evaluating the extent of variability in nucleolar morphology.

RESULTS

Light Microscopy and Radioautography

Cells in the early passages of Chinese hamster fetus-derived cultures had variable numbers of small, irregularly shaped nucleoli which stained strongly for RNA with azure B (Flax and Himes,

1952). Cells with six or more nucleoli were not uncommon in Chinese hamster cultures in early passages. As we have shown previously, variation in nucleolar number was a consequence of functioning of a high number of nucleolus-producing sites scattered through the Chinese hamster genome (Phillips and Phillips, 1969). In radioautographs of cells incubated with uridine-³H, it appeared that, regardless of the number of nucleoli per cell, all nucleoli were active in RNA synthesis.

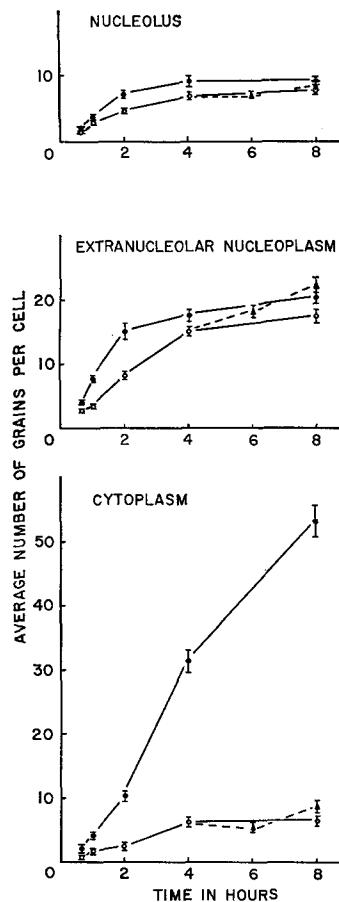


FIGURE 1 Average number of silver grains over nucleoli, nucleoplasm, and cytoplasm in radioautographs (5 day exposure) of cells incubated with uridine-³H, SA 0.2 Ci/mole, 0.1 μ Ci/ml, for various time intervals in the absence (filled circles) or presence (open circles) of 0.3 μ g/ml of toyocamycin. Toyocamycin was removed from some cultures after 4 hr, and the medium was replaced with conditioned medium containing the same amount of uridine-³H (triangles and dashed lines). Intervals represent standard errors of the mean.

The average amount of uridine-³H per cell incorporated into nucleolar or nucleoplasmic TCA-insoluble material rose rapidly for about the first 4 hr of incubation in uridine-³H and then leveled off, whereas the amount of cytoplasmic incorporation increased fairly linearly for 8 hr (Fig. 1). RNase removed more than 95% of the radioactivity.

The effect of actinomycin D, 5 µg/ml, on nucleoli of Chinese hamster cells was easily detectable with the light microscope. After 1–3 hr of actinomycin D treatment, nucleoli of most cells appeared smaller and rounder than control nucleoli, and often appeared segregated into two distinguishable components. After azure B staining these components often appeared as a refractile, central area which stained lightly, if at all, for RNA and a densely staining “cap” to one side of the refractile area. Other nucleoli appeared to be segregated into two roundish, refractile, unstained areas on opposite sides of a deeply stained central area. The deeply staining portion was generally much larger than the unstained area of the nucleolus. Nucleoli of cells treated for more than 3 hr with actinomycin D tended to disaggregate and were generally difficult to identify in the light microscope.

Actinomycin D at a level of 5.0 µg/ml abolished almost all RNA synthesis as determined radioautographically. For instance, in an experiment run in parallel with the one illustrated in Fig. 1, actinomycin D was added to two cultures before the addition of uridine-³H. After a 4 hr incubation, nucleoplasmic grain counts averaged 2.2% and cytoplasmic grain counts averaged 1.5% of those of the 4-hr control cultures.

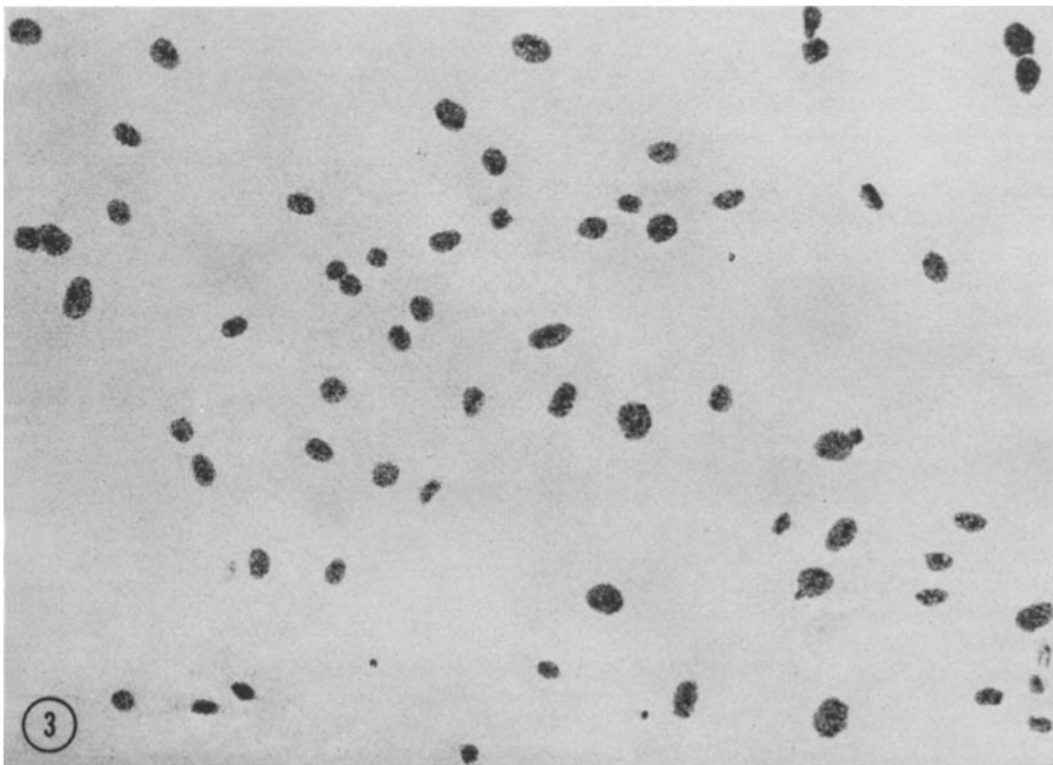
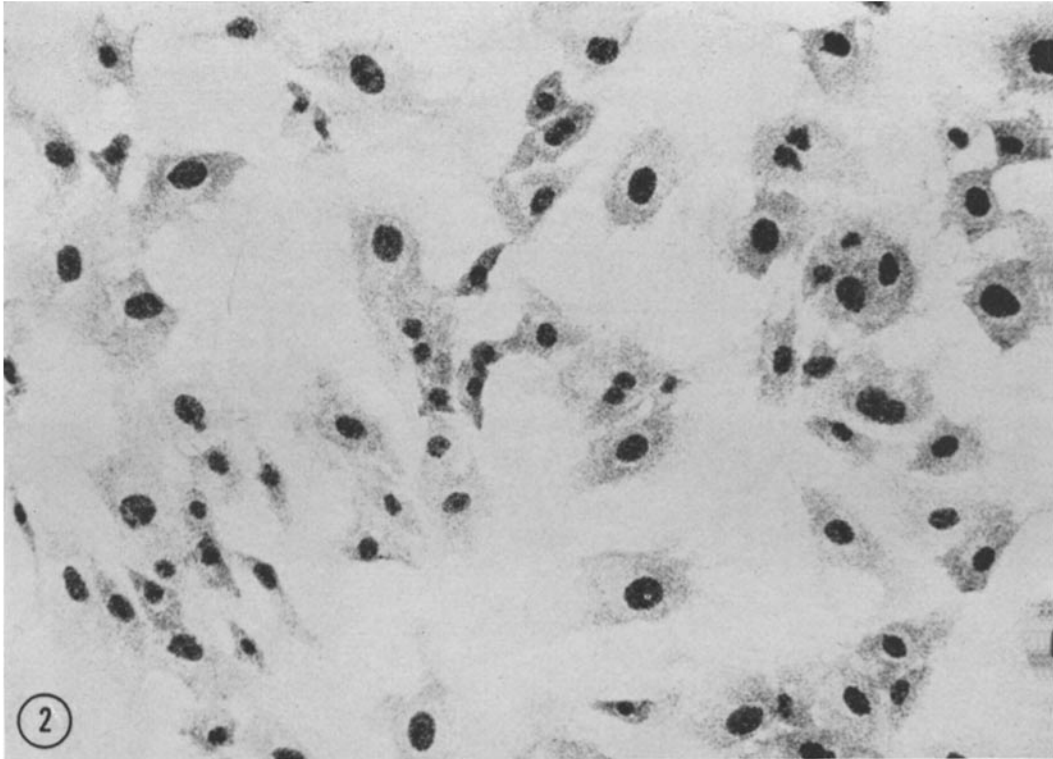
The effects caused by toyocamycin on nucleoli of Chinese hamster cells were much more subtle than those caused by actinomycin D. In cultures treated with toyocamycin, 0.3 µg/ml, for up to 12 hr, the nucleoli appeared to be of normal size, shape, stainability (with azure B), and number.

However, when cultures were treated for 4 hr with toyocamycin, rinsed repeatedly, and then incubated for 48 hr in medium lacking the analogue, most of the cells died. Thus, toyocamycin exerted an irreversible toxic effect on these cells.

The effects of toyocamycin on cellular RNA synthesis were investigated radioautographically in Chinese hamster cells. It appeared that toyocamycin at a level of 0.3 µg/ml greatly reduced incorporation of uridine-³H into cytoplasmic RNA without grossly affecting the amount of nuclear or nucleolar RNA synthesis. This is dramatically apparent in Figs. 2 and 3, which show overexposed radioautographs of Chinese hamster cells in the second passage incubated for 4 hr in uridine-³H in the presence and absence of toyocamycin. The results of grain counts in radioautographs of Chinese hamster cells cultured for various time intervals in the presence or absence of toyocamycin are given in Fig. 1. Grain counts over *nucleoli* of toyocamycin-treated cells ranged from 65% to 83% of the counts in control cells, averaging 76% of the level in untreated cells. The grain counts over *nucleoplasm* in toyocamycin-treated cells ranged from 44% to 85% of the control level at various time points, averaging 67% of counts in untreated cells. (It is to be remembered that much incorporation of uridine-³H into nucleoplasmic RNA is dependent upon normal nucleolar function; Perry et al., 1961.) *Cytoplasmic* incorporation of uridine-³H into RNA of toyocamycin-treated cultures was much more seriously affected. Cytoplasmic grain counts averaged roughly 30% of the control level at short (1 hr or less) time intervals and dropped to 12% of the control level by 8 hr. After 4 hr in toyocamycin, there appeared to be little further net cytoplasmic RNA synthesis. More than 85% of the radioactivity of toyocamycin-treated cells was RNase removable. Most of the residual radioactivity was in the nucleus and probably represented incorporation into DNA.

FIGURE 2 Unstained radioautograph of Chinese hamster cells in the fifth passage in culture grown for 4 hr in the presence of uridine-³H, 20 Ci/mole, 0.05 mCi/ml, 2 day exposure. The cells are rendered visible in this unstained preparation because of the very heavy label of nuclei and nucleoli and the moderately heavy cytoplasmic grain density. × 250.

FIGURE 3 Unstained radioautograph of culture which is sister to the one shown in Fig. 2. The conditions of incubation were the same except that toyocamycin, 0.3 µg/ml, was added to the culture 20 min before the addition of uridine-³H. In this case, only the nuclei and nucleoli are heavily labeled. Cytoplasmic grain density is very light. × 250.



Toyocamycin was removed from some cultures after 4 hr to test whether cells could recover normal RNA synthesis after toyocamycin treatment. These cultures were rinsed three times with Hanks' salt solution and incubated for an additional 2 or 4 hr in the absence of the drug. As can be seen in Fig. 1 little or no recovery of normal patterns of RNA synthesis was apparent in this time interval.

Results of light microscope observations on mouse cell strains were very similar to those on Chinese hamster cells (although no radioautography was done on mouse cells). Like Chinese hamster cells, normal mouse cells had variable numbers of nucleoli. Actinomycin D treatment caused the visible segregation of nucleolar components. Toyocamycin caused no noticeable change in nucleolar morphology; however, nucleoli of toyocamycin-treated cells contained many small, lightly staining (with azure B for RNA or fast green for total protein), roundish bodies in addition to the nucleoli. On the hypothesis that these bodies might be newly arising nucleoli, numbers of nucleoli were scored in 100 cells from each of three untreated (control) mouse cultures, three cultures treated for 4 hr with toyocamycin, and two cultures treated for 12 hr with toyocamycin. The average number of nucleoli per cell was found to be 3.4, 3.4, and 3.8, respectively. Since the average number of nucleoli per cell did not change significantly after 12 hr of toyocamycin treatment, it was considered unlikely that these cells contained either newly arising nucleoli or, for that matter, "dying" nucleoli. Thus, the origin of the lightly staining bodies remained unclear.

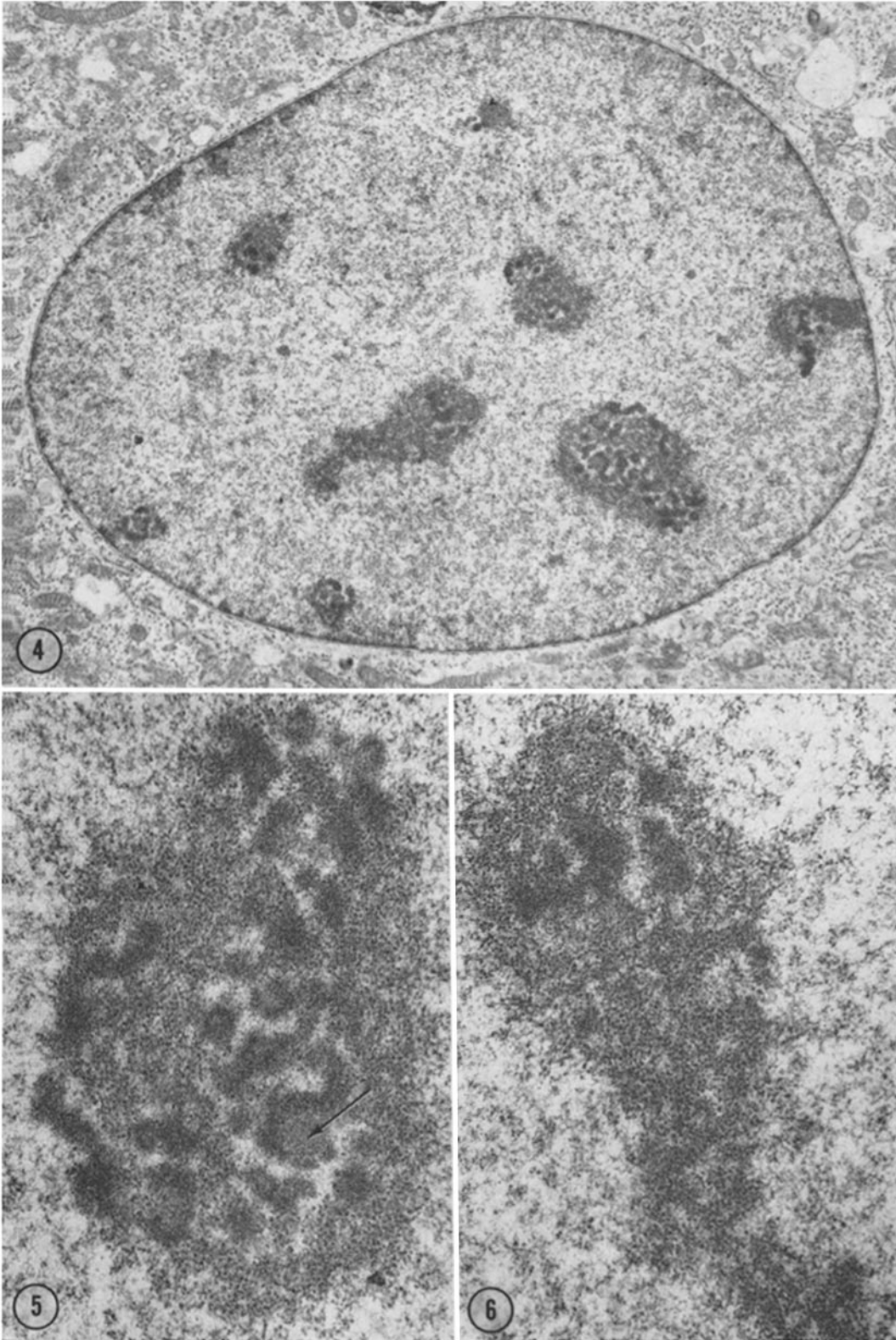
Nucleolar Fine Structure

UNTREATED CHINESE HAMSTER CELLS: The nucleoli of cells from normal, untreated Chinese hamster cultures were often quite irregular in outline (Figs. 4-6). They had a rather loose, reticular structure, but discrete strands of uniform diameter (nucleolonemata) were not well defined. More than half of the reticulum was composed of closely packed granules about 150 Å in diameter, which appeared to be embedded in a less dense, finely fibrous "matrix" material. This is the region commonly referred to as the *particulate* nucleolar component. The rest of the reticulum (the so-called *fibrillar* nucleolar component) was composed of two distinguishable types of fibrous material. The major component of the fibrillar regions was a very electron-opaque fibrous material. The minor

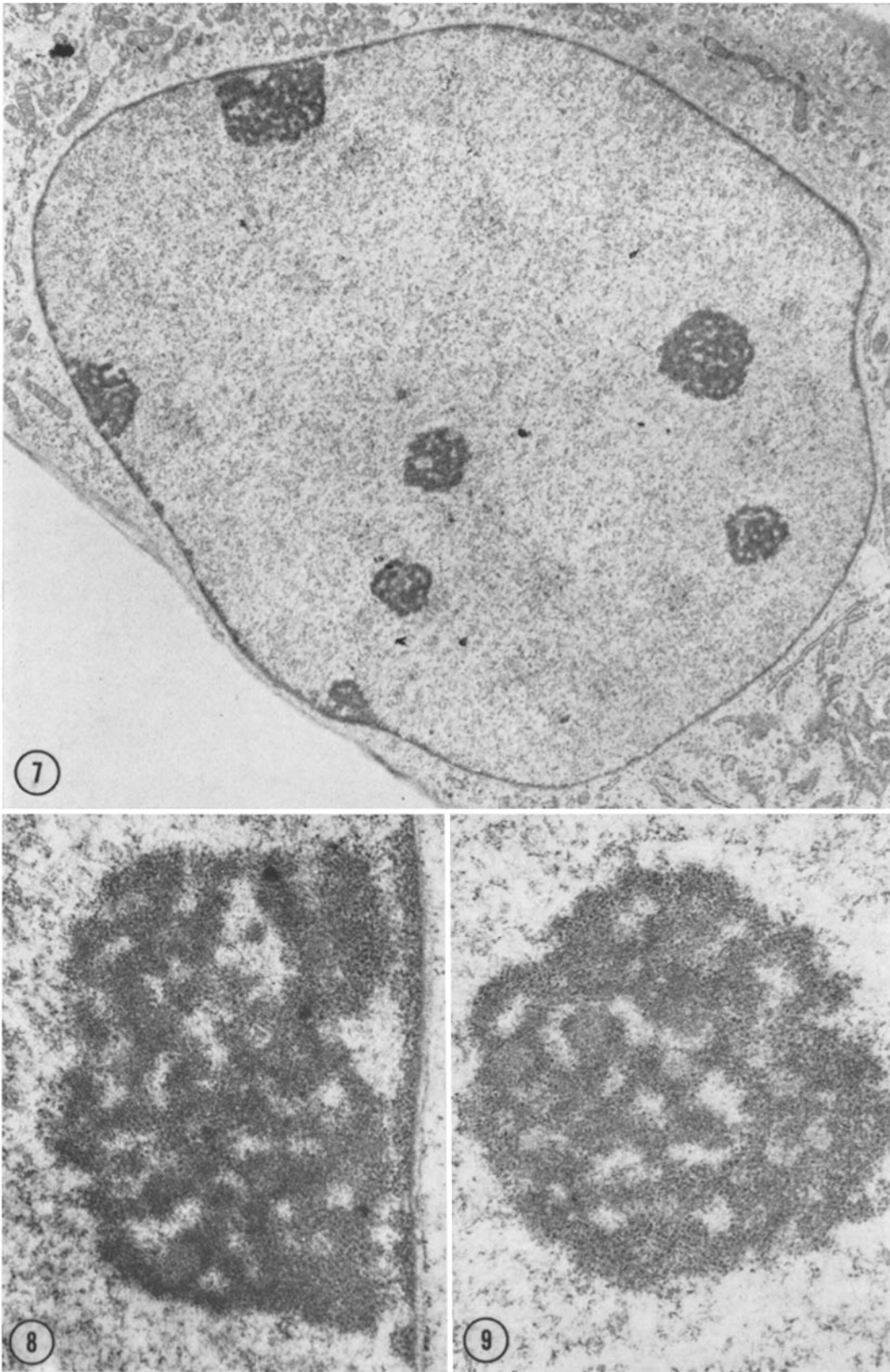
component was a lighter fibrous material which often appeared as a small sphere partially surrounded by and intimately associated with the dense fibrous material. The lighter fibrous material rarely, if ever, appeared to be in contact with particulate zones. The dense fibrous material appeared to merge at some points with particulate portions of the reticulum, but generally the particulate and fibrous strands of the reticulum were clearly separated by electron-transparent spaces which were similar in appearance to the surrounding nucleoplasm (Figs. 5 and 6). The relationship of nucleolar components to one another thus appeared to be quite specific. The light fibrous component merged into a denser fibrous component which, in turn, merged with the particulate component.

TOYOCAMYCIN EFFECTS ON CHINESE HAMSTER CELLS: Treatment of Chinese hamster cells with toyocamycin caused the gradual disappearance of the light fibrous component and the granules of the nucleolus. A change in nucleolar morphology was quite noticeable after a 2 hr treatment with the analogue. Nucleoli of cells treated for 2 hr with toyocamycin were somewhat rounder than those of control cells, and the three types of nucleolar regions, particulate, dense fibrous, and light fibrous, were much more difficult to identify as morphological entities. Dense fibrous and particulate regions were contiguous rather than separated as in controls, and the concentration of granules in the particulate zone appeared reduced (Figs. 7-9). After 4 hr of treatment, separate nucleolar regions could generally not be distinguished. Nucleoli appeared to be composed entirely of one dense fibrous zone which included occasional poorly defined granules, and the nucleolar material was disposed in anastomosing strands separated by electron-transparent spaces, giving an over-all appearance reminiscent of nucleolonemata. In cells from cultures treated 8 or 12 hr with toyocamycin, nucleoli were exclusively composed of dense fibrous material disposed, as described, in nucleolonemata-like strands (Figs. 10-12).

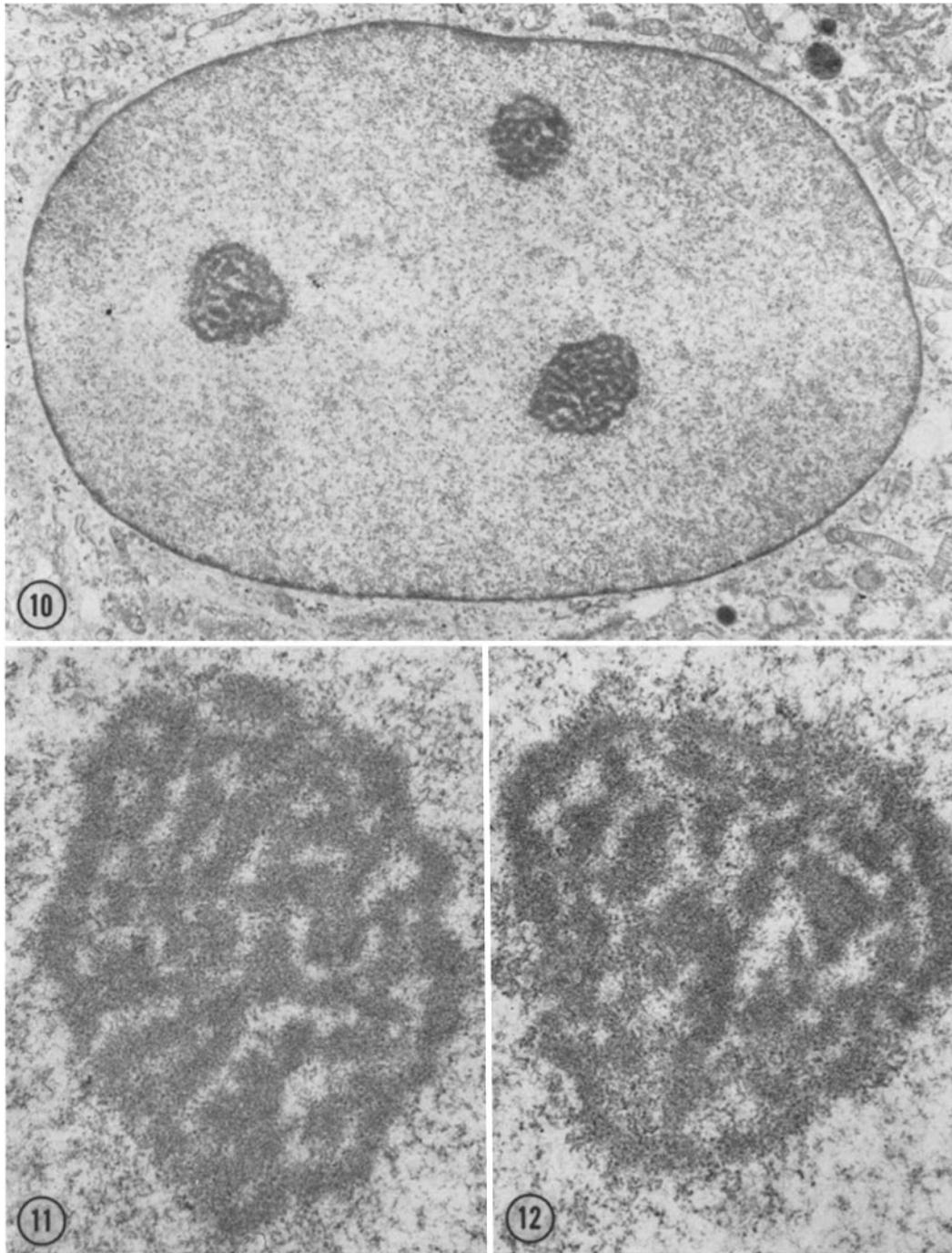
ACTINOMYCIN D EFFECTS ON CHINESE HAMSTER CELLS: As we have previously reported (Phillips and Phillips, 1969), actinomycin D treatment caused rapid and obvious segregation of nucleolar components in Chinese hamster cells. In cells exposed to actinomycin D for 45 min-2 hr, most nucleoli were segregated into three compo-



FIGURES 4-6 Untreated Chinese hamster cell, and enlargements of two of the nucleoli. Nucleoli are composed of a granular region which is often separated by electron-transparent areas from dense fibrous region. Small, light, fibrous regions (arrow) can sometimes be discerned juxtaposed to dense fibrous regions. Fig. 4, $\times 8500$; Figs. 5 and 6, $\times 35,000$.



FIGURES 7-9 Chinese hamster cells treated for 2 hr with toyocamycin, and enlargements of two of the nucleoli. Nucleoli appear more rounded than nucleoli of control cells, and fibrous and granular zones are more interspersed and contiguous and less distinguishable as separate regions. Fig. 7, $\times 6500$; Figs. 8 and 9, $\times 29,000$.



FIGURES 10-12 Chinese hamster cell incubated for 8 hr with toyocamycin. Nucleoli appear similar to those of 4-hr treated Chinese hamster cells except that there are even fewer granules. Fig. 10, $\times 5500$; Figs. 11 and 12, $\times 26,000$.

nents. A *granular* component often made up most of the nucleolar mass. Adjacent to the *granular* component was an *electron-opaque fibrous* component, and adjoining that was a *lighter fibrous* zone. As in untreated control nucleoli, the lighter fibrous zone comprised a relatively small amount of the mass of the whole nucleolus. Some segregated nucleoli of actinomycin D-treated cells possessed a fourth component which appeared as an electron-opaque rim or tab of granular material peripheral to the lighter granular component. Nucleoli of cells treated for 3 hr with actinomycin D often appeared to be disaggregated, and nucleolar material of varying morphology was seen in the nucleoplasm (Figs. 13-15). Cells treated for 4 hr with actinomycin D contained very little nucleolar material which could be easily recognized.

NUCLEOLAR FINE STRUCTURE IN MOUSE CELLS: Nucleoli of untreated mouse cells were very similar in appearance to nucleoli of Chinese hamster cells. The three nucleolar components bore similar morphological relationships to one another (Figs. 16-18). In mouse cells, as in Chinese hamster cells, toyocamycin treatment caused the progressive disappearance of the light fibrous material and the nucleolar granules (Figs. 19-24). Nucleoli of toyocamycin-treated mouse cells, however, generally appeared more spherical, compact, and homogeneously fibrillar than correspondingly treated Chinese hamster cells (Figs. 22-24). Actinomycin D effects in mouse cells also were similar to those described in Chinese hamster cells (Figs. 25-27).

Mouse cells were utilized to extend the investigation into the nature of the homogeneous fibrillar material of which the nucleoli of toyocamycin-treated cells were composed. Mouse cells were treated for 4-12 hr with toyocamycin and then for 2 hr more with both toyocamycin and actinomycin D. Nucleoli of these cells assumed peculiar contorted configurations and appeared segregated into three distinguishable zones: a light fibrous zone, a dense fibrous zone, and a zone reminiscent of the dense granular zone which sometimes appeared in cells treated with actinomycin D alone (Figs. 28-30).

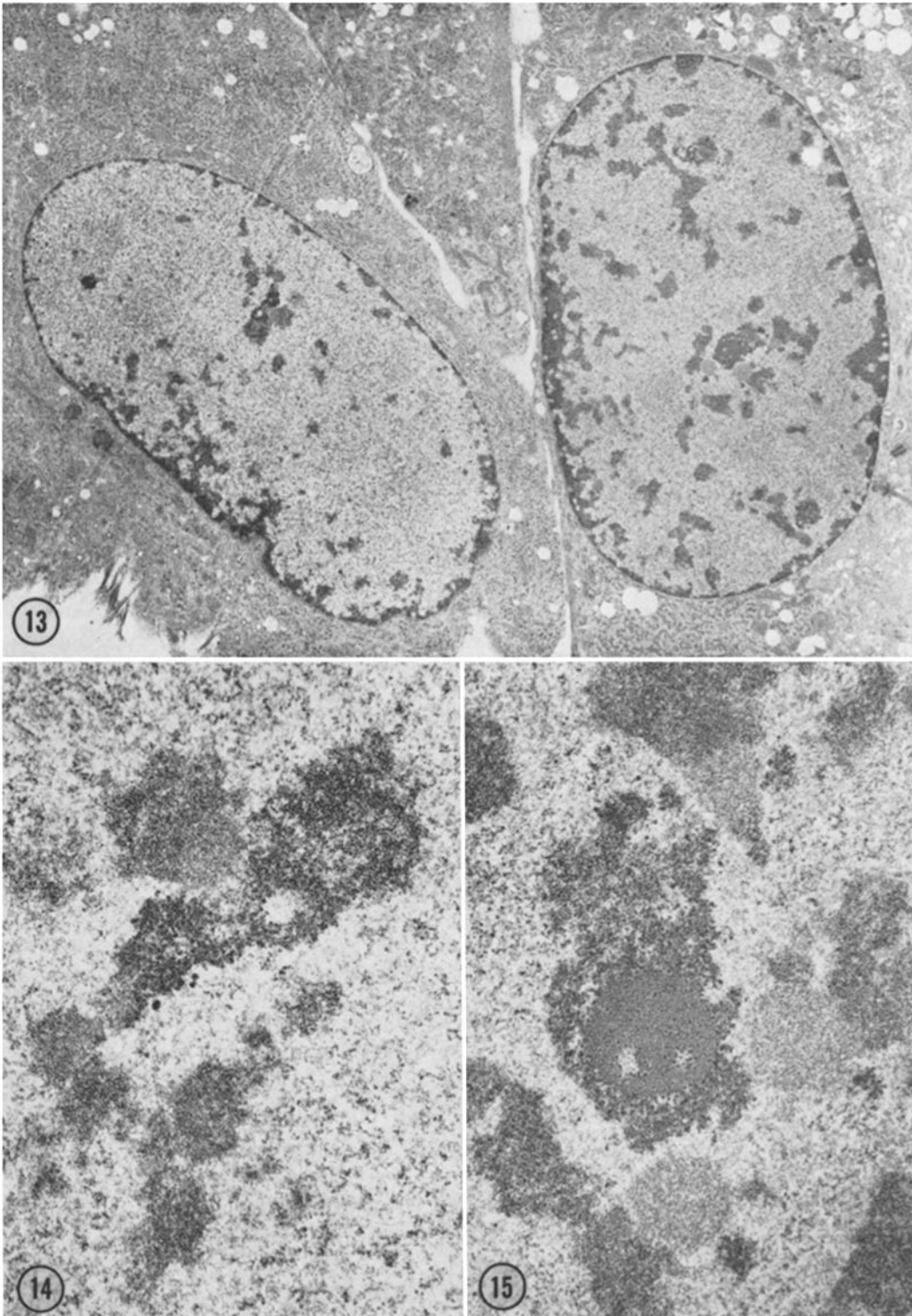
DISCUSSION

Toyocamycin Effects

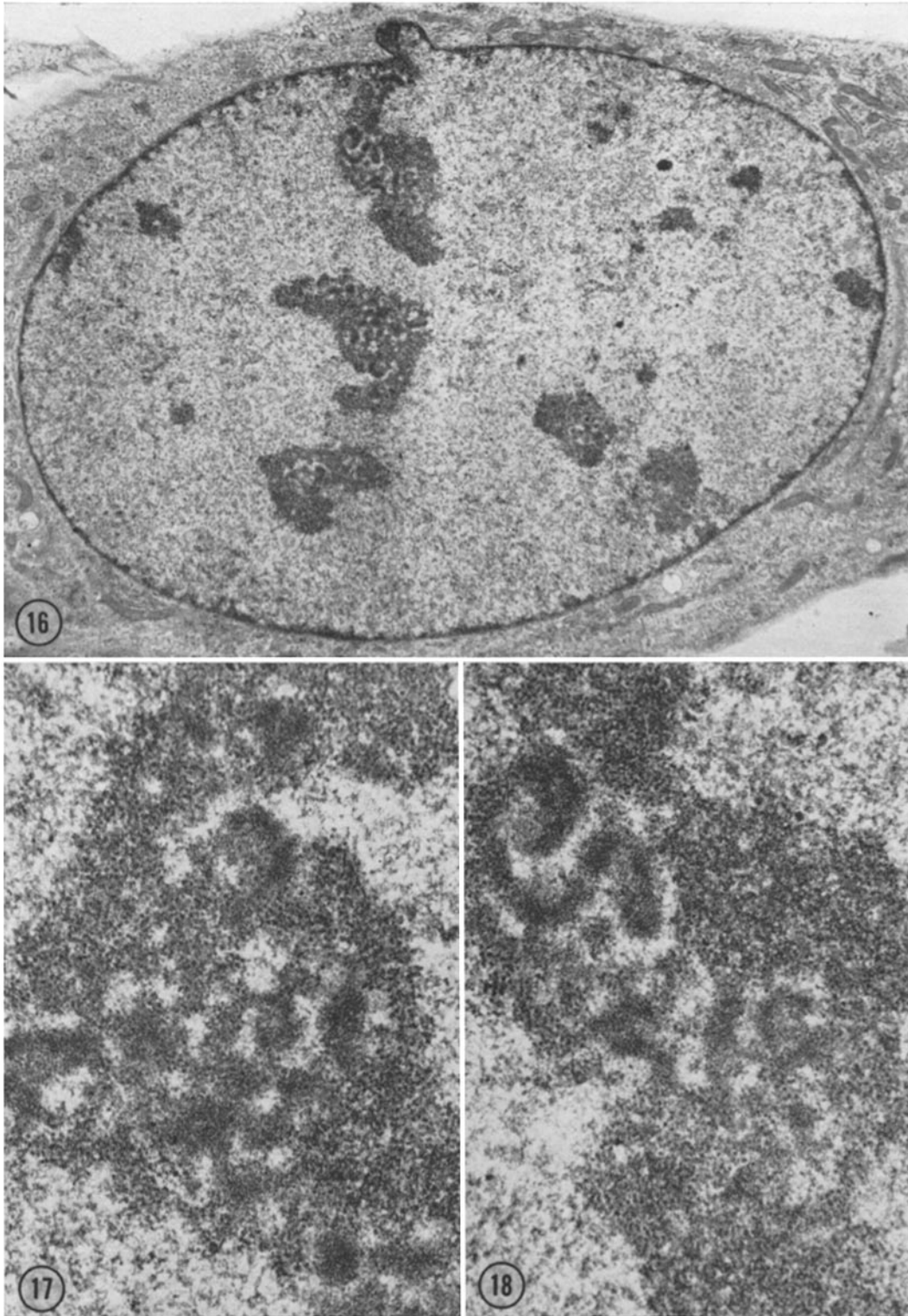
The radioautographic results presented in this paper support the idea that, as reported by

Tavitian et al. (1968; 1969), the adenosine analogue toyocamycin does cause a blockage in the processing of ribosomal RNA. In Chinese hamster cells this blockage apparently did not result in a build-up in the amount of newly formed RNA in the nucleolus, but our data do not distinguish whether newly formed nucleolar RNA was degraded and new RNA synthesized (i.e., there was turnover) or whether nucleolar RNA synthesis simply came to a halt after prolonged incubation with toyocamycin. In any case, the appearance of newly formed RNA in the cytoplasm was profoundly inhibited by this drug. The work of Tavitian et al. suggests that, at least in L cells, toyocamycin is incorporated into the 45S ribosomal RNA precursor, but that the molecule containing toyocamycin is not normally processed and that no ribosomal 18 or 28S RNAs appear in the cytoplasm. Our results suggest that in mouse and Chinese hamster cells, toyocamycin may have a similar effect. Thus the major RNA component in nucleoli of treated cells is probably an early ribosomal RNA precursor.

We found that treatment of Chinese hamster and mouse cells caused a gradual disappearance of the 150-A granules which normally characterize the particulate zone of the nucleolus, so that, after 4 hr or more of exposure to toyocamycin, cells contained nucleoli which were homogeneously fibrous. These findings suggest that in mouse and Chinese hamster cells the region of the nucleolus which contains the early ribosomal RNA precursor molecule is fibrillar in appearance. Heine (1969) found that toyocamycin treatment of HeLa cells caused a loss of nucleolar granules similar to what we have reported here. There are several other lines of evidence which suggest that the fibrillar region of the nucleolus contains the earliest ribosomal RNA precursor molecules and that later, during processing, these molecules appear in the particulate zone. There is evidence that DNA penetrates into the fibrillar compartment of the nucleolus in a variety of organisms (Miller, 1966; Jacob, 1966; Lane, 1967; LaCour and Wells, 1967; Ebstein, 1969). Nascent RNA formed in association with this DNA might thus be expected to be found in the fibrillar zone. Indeed, in mammalian cells in culture (Geuskens and Bernhard, 1966), dipteran salivary gland cells (Pelling, 1964; von Gaudecker, 1967), and amphibian oocytes (Karasaki, 1965; Macgregor, 1967; Lane, 1967), very short pulses (5 min-2 hr,



FIGURES 13-15 Chinese hamster cell treated for 3 hr with actinomycin D, and enlargements. Although the process of nucleolar disintegration is more advanced in some cells than others, by 3 hr only what appear to be nucleolar remnants are observed in most cells. It is difficult to equate these structures with normal nucleolar components. Fig. 13, $\times 5500$; Figs. 14 and 15, $\times 33,000$.

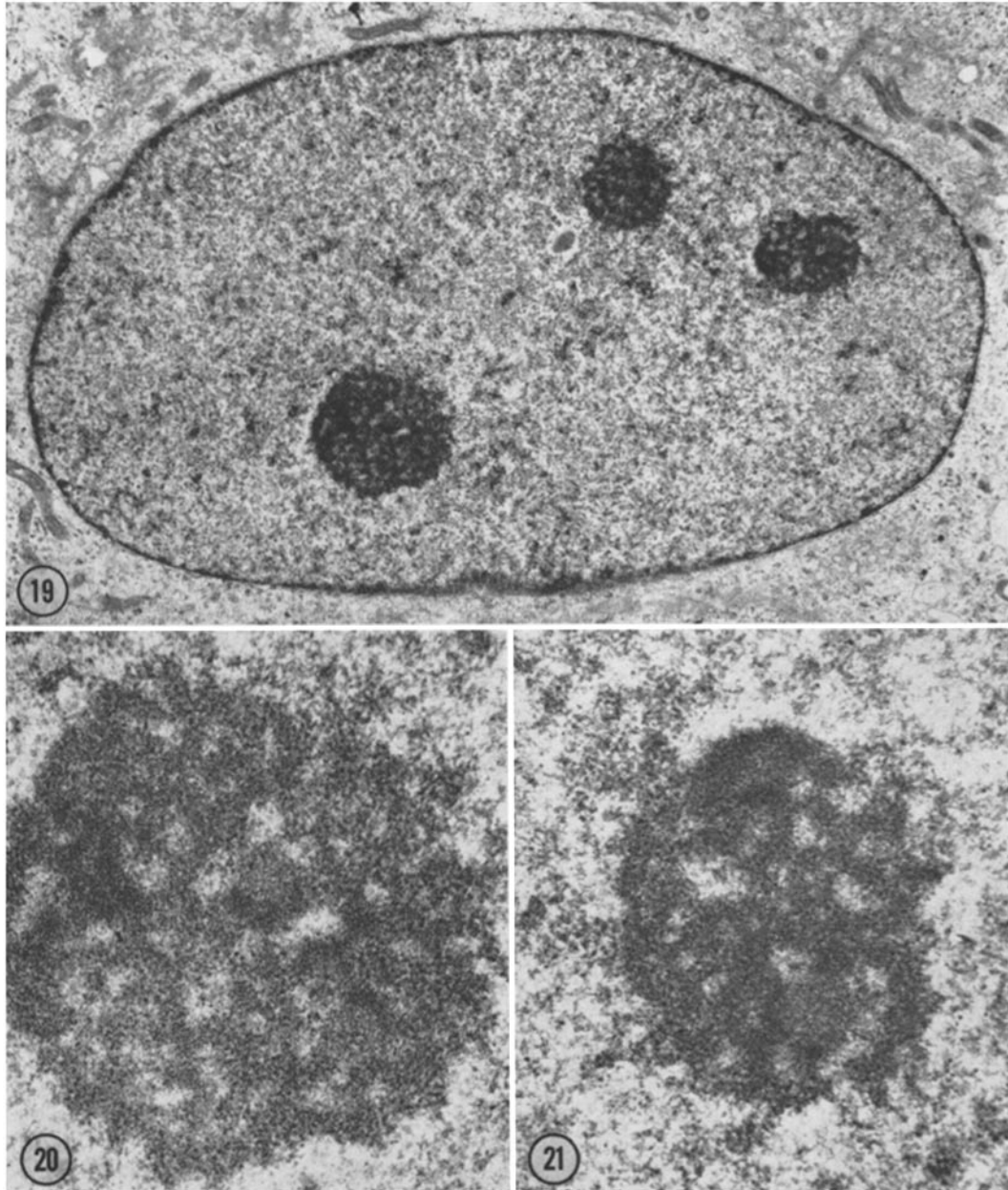


FIGURES 16-18 Untreated mouse cell (third passage), and enlargements of two of the nucleoli. The morphology of these nucleoli is indistinguishable from that of control Chinese hamster nucleoli. Fig. 16, $\times 5500$; Figs. 17 and 18, $\times 28,000$.

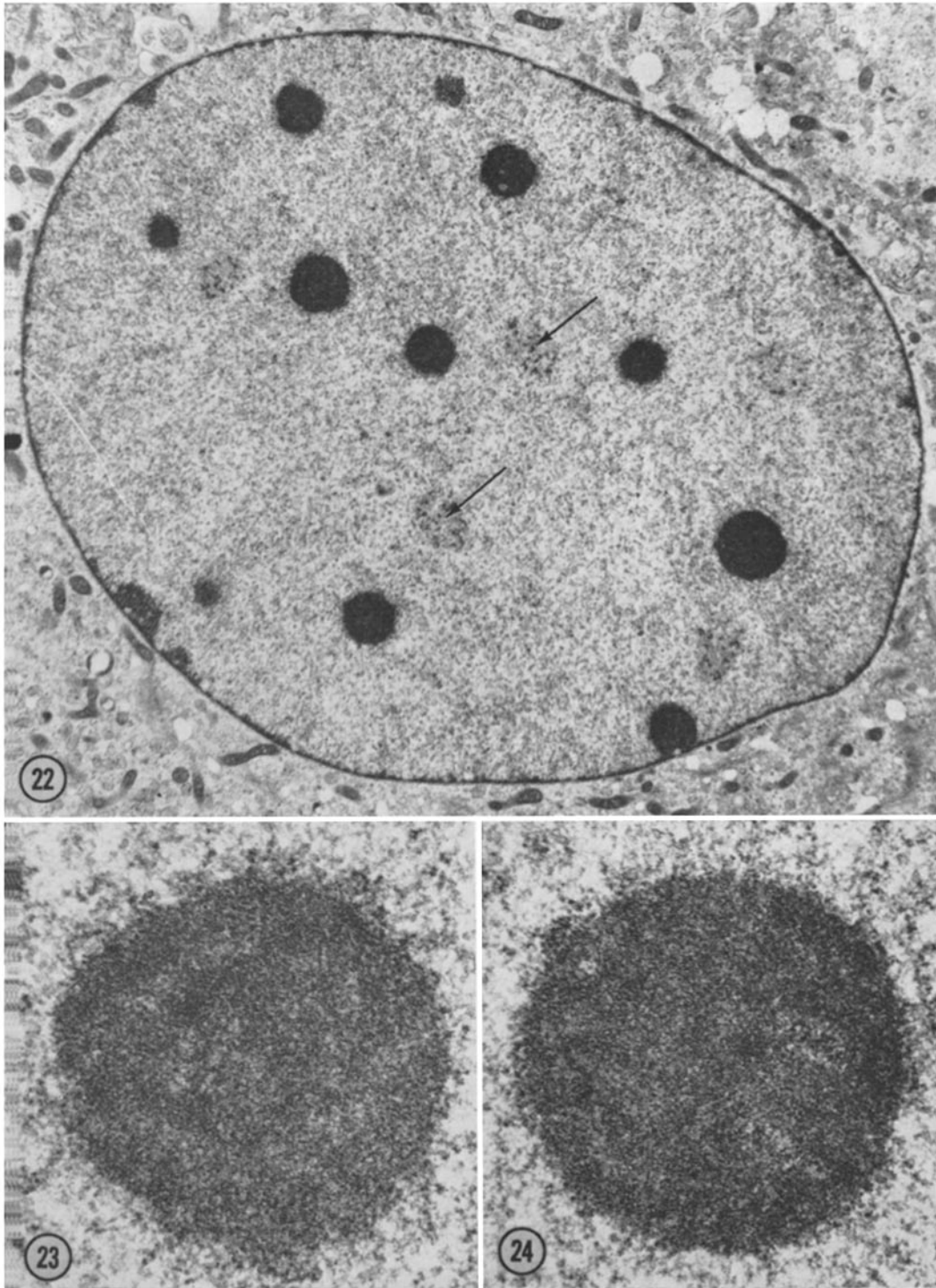
depending on the organism) with radioactive RNA precursors are sufficient to label the fibrillar regions of the nucleolus. Radioactivity is found in association with the particulate region of the

nucleolus only after longer incubation in the presence of the RNA precursor.

Agranular nucleolus-like bodies rather similar in appearance to the nucleoli of cells treated with



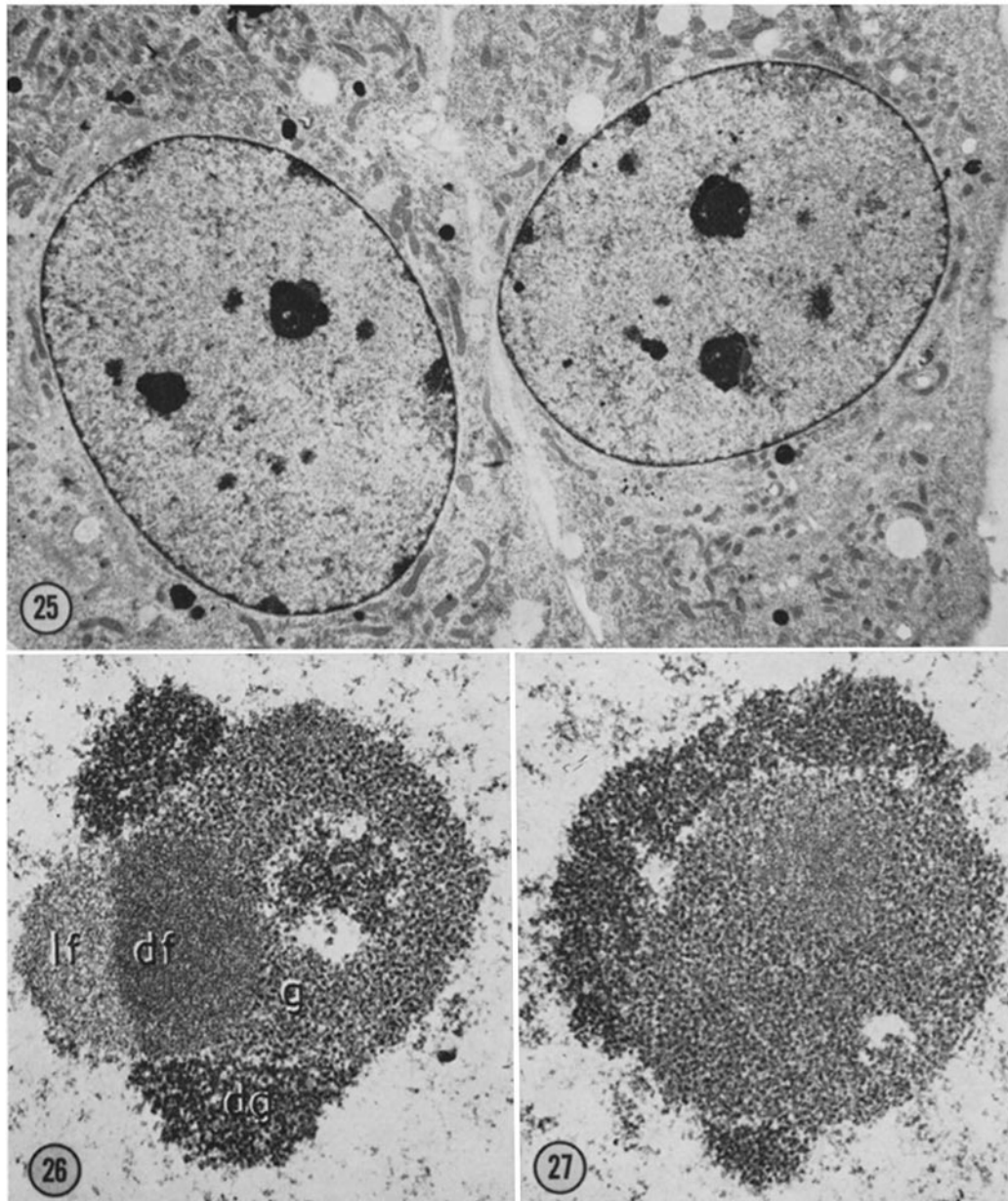
FIGURES 19-21 Mouse cell (third passage) treated for 2 hr with toyocamycin, and enlargements of two of the nucleoli. Morphological effects of the drug are similar to those seen in Chinese hamster nucleoli. Fig. 19, $\times 9000$; Figs. 20 and 21, $\times 31,000$.



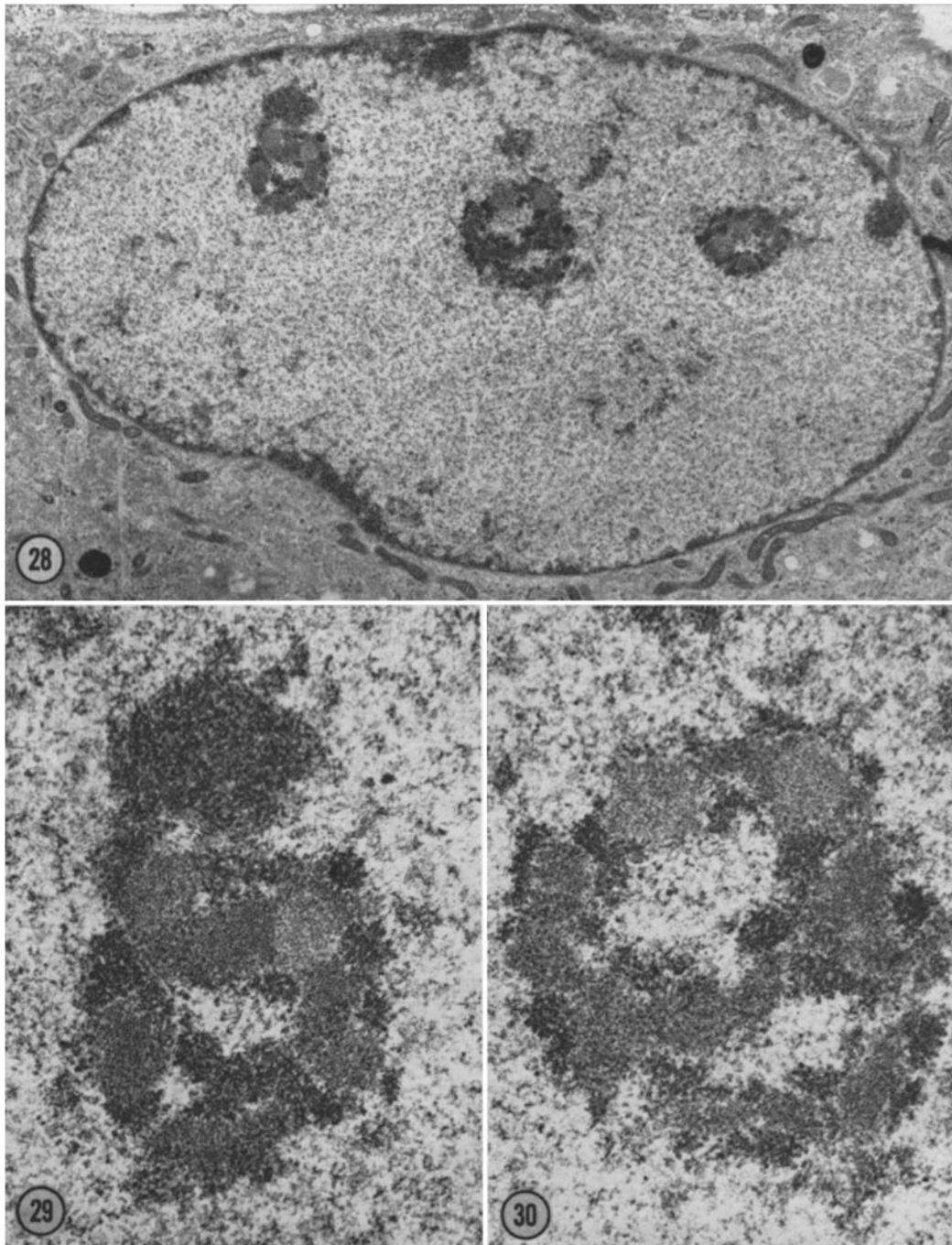
FIGURES 22-24 Nucleoli of this mouse cell (third passage) which was incubated for 12 hr in toyocamycin are spherical and composed entirely of fibrous material. Other faint accumulations of material (arrows) may correspond to faintly staining regions observed in the light microscope. Fig. 22, $\times 6500$; Figs. 23 and 24, $\times 33,000$.

toyocamycin occur in special cases where no ribosomal RNA is being synthesized. For instance, in 0-nu ("anucleolate") mutant larvae of *Xenopus* (Hay and Gurdon, 1967) and in corn microspores lacking the nucleolar organizer region (Swift and

Stevens, 1966), multiple nucleolus-like bodies occur which are principally fibrillar in appearance but may also possess a few peripherally located granules. Completely fibrillar pronucleoli occur in early embryogenesis in amphibia (Karasaki,



FIGURES 25-27 Mouse cells (third passage) treated with actinomycin D for 1½ hr. In addition to the usual granular (*g*), dense fibrous (*df*), and light fibrous (*lf*) zones, a fourth dense granular (*dg*) zone is distinguishable in many actinomycin D-treated mouse cells. Fig. 25, $\times 4500$; Figs. 26 and 27, $\times 33,000$.



FIGURES 28-30 Mouse cells treated with toyocamycin for 12 hr and subsequently treated with toyocamycin plus actinomycin D for 2 hr are segregated into three distinguishable zones: a light fibrous zone, a dense fibrous zone, and a zone reminiscent of the dense granular zone which sometimes appeared in cells treated with actinomycin D alone. Fig. 28, $\times 7500$; Figs. 29 and 30, $\times 33,000$.

1965), sea urchins (Karasaki, 1968), and mice (Hillman and Tasca, 1969). Though ribosomal RNA synthesis is lacking or negligible in these cases, the nucleolus-like bodies do contain RNA. Presumably, this is not the same type of RNA that occurs in the fibrillar nucleoli of toyocamycin-treated cells. It is doubtful whether the fibrillar material of the nucleolus-like bodies can be equated with the fibrillar zones of nucleoli which are active in ribosomal RNA synthesis. Hillman and Tasca showed that at the embryonic stage when nucleolar RNA synthesis commences, the fibrillar pronucleoli obtain peripheral granules, and only this new particulate region appears to incorporate uridine-³H (see Fig. 12, Hillman and Tasca, 1969).

Actinomycin D Effects

Our findings, and those of other workers, on the effects of actinomycin D on nucleolar structure seem to complicate analysis of the composition of the various nucleolar compartments. We found that treatment of cells with actinomycin D at a level sufficient to block almost all RNA synthesis caused nucleoli to segregate into four distinguishable components. Such a segregation cannot be explained on the basis of known effects of actinomycin D on RNA metabolism. In the presence of actinomycin D, ribosomal RNA precursor molecules are reported to disappear rapidly from the nucleolus (Weinberg et al., 1967). But no morphological component is found to disappear in the presence of actinomycin D. We found that, of the remaining components, at least one component (possibly a particulate zone) stained intensely for RNA even after 3 hr of exposure to actinomycin D. Stevens (1964) found that extended treatment of dipteran salivary glands with actinomycin D caused disappearance of RNA from the particulate but not from the fibrillar nucleolar component. However, our data on Chinese hamster and mouse cells suggest that the particulate zone of the segregated nucleoli contains most or all of the RNA and that the fibrillar zones are principally proteinaceous.

The situation is more puzzling when one considers the results of actinomycin D treatment of cells which were previously exposed to toyocamycin for extended periods. In these cells, in which processing of ribosomal RNA is inhibited at an early stage and in which the nucleoli have a uniformly fibrillar appearance, actinomycin D nev-

ertheless causes segregation into at least three distinguishable compartments. This supports the idea that actinomycin D has an effect on the arrangement of nonRNA (principally proteinaceous) nucleolar constituents.

Nucleolar Morphology

This study has presented a description of the appearance of typical nucleoli of mouse and Chinese hamster cells in short-term culture. We would like to stress that the description is a summary of the results of many separate experiments in which large numbers of cells were examined. Extensive examination was necessary because changes in nucleolar morphology were sometimes subtle and, more important, there was much variability in the appearance of nucleoli between cultures treated in the same way and even among cells in the same culture. It is not known whether the variability in nucleolar morphology was related to the stage of the cell cycle, the state of health of the individual cell, or other factors, but it became evident during this study that the appearance of nucleoli is quite labile and may be affected by a variety of factors.

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REFERENCES

- BARR, H. J., and W. PLAUT. 1966. Comparative morphology of nucleolar DNA in *Drosophila*. *J. Cell Biol.* 31:C17.
- BERNHARD, W. 1966. Ultrastructural aspects of the normal and pathological nucleolus in mammalian cells. *Nat. Cancer Inst. Monogr.* 23:13.
- BROWN, D. D., and J. B. GURDON. 1964. Absence of ribosomal RNA synthesis in the anucleolate mutant of *Xenopus laevis*. *Proc. Nat. Acad. Sci. U.S.A.* 51:139.
- EBSTEIN, B. 1969. The distribution of DNA within the nucleoli of the amphibian oocyte as demonstrated by tritiated actinomycin D radioautography. *J. Cell Sci.* 5:27.
- FLAX, M. H., and M. H. HIMES. 1952. Microspectrophotometric analysis of metachromatic staining of nucleic acids. *Physiol. Zool.* 25:297.
- GEUSKENS, M., and W. BERNHARD. 1966. Cytochimie ultrastructurale du nucléole. III. Action de l'ac-

- tinomycine D sur le metabolisme de RNA nucléolaire. *Exp. Cell Res.* **44**:579.
- GHOSH, S., R. LETTRÉ, and I. GHOSH. 1969. On the composition of the nucleolus with special reference to its filamentous structure. *Z. Zellforsch. Mikrosk. Anat.* **101**:254.
- HAY, E. D., and J. B. GURDON. 1967. Fine structure of the nucleolus in normal and mutant *Xenopus* embryos. *J. Cell Sci.* **2**:151.
- HEALY, G. M., and R. C. PARKER. 1967. Selective nucleolar uptake of α -acid glycoprotein by mammalian cells in tissue culture. *Biochim. Biophys. Acta.* **148**:556.
- HEINE, U. 1969. Electron microscopic studies on HeLa cells exposed to the antibiotic toyocamycin. *Cancer Res.* **29**:1875.
- HILLMAN, N., and R. J. TASCA. 1969. Ultrastructural and autoradiographic studies of mouse cleavage stages. *Amer. J. Anat.* **126**:151.
- HYDE, B. B. 1966. Changes in nucleolar ultrastructure associated with differentiation in the root apex. *Nat. Cancer Inst. Monogr.* **23**:39.
- JACOB, J. 1966. Intranucleolar deoxyribonucleic acid components in insect cells as revealed by electron microscopy. *Nature (London)*. **211**:36.
- KARASAKI, S. 1965. Electron microscopic examination of the sites of nuclear RNA synthesis during amphibian embryogenesis. *J. Cell Biol.* **26**:937.
- KARASAKI, S. 1968. The ultrastructure and RNA metabolism of nucleoli in early sea urchin embryos. *Exp. Cell Res.* **52**:13.
- LACOUR, L. F., and B. WELLS. 1967. The loops and ultrastructure of the nucleolus of *Iphæion uniflorum*. *Z. Zellforsch. Mikrosk. Anat.* **82**:25.
- LANE, N. J. 1967. Spheroidal and ring nucleoli in amphibian oocytes. Patterns of uridine incorporation and fine structural features. *J. Cell Biol.* **35**:421.
- LUFT, J. H. 1961. Improvements in epoxy resin embedding methods. *J. Biophys. Biochem. Cytol.* **9**:409.
- MACGREGOR, H. C. 1967. Pattern of incorporation of [3 H] uridine into RNA of amphibian oocyte nucleoli. *J. Cell Sci.* **2**:145.
- MILLER, O. L. 1966. Structure and composition of peripheral nucleoli of salamander oocytes. *Nat. Cancer Inst. Monogr.* **23**:53.
- MUNDELL, R. D. 1967. The occurrence of ribosomal proteins in nucleoli of starfish oocytes. *Biochem. Biophys. Res. Comm.* **28**:117.
- MURAMATSU, M., J. L. HODNETT, W. J. STEELE, and H. BUSCH. 1966. Synthesis of 28-s RNA in the nucleolus. *Biochim. Biophys. Acta.* **123**:116.
- PELLING, C. 1964. Ribonukleinsäure-Synthese der Riesenchromosomen. Autoradiographische Untersuchungen an *Chironomus tentans*. *Chromosoma.* **15**:71.
- PERRY, R. P., T.-Y. CHENG, J. J. FREED, J. R. GREENBERG, D. E. KELLEY, and K. D. TARTOF. 1970. Evolution of the transcription unit of ribosomal RNA. *Proc. Nat. Acad. Sci. U.S.A.* **65**:609.
- PERRY, R. P., A. HELL, and M. ERRERA. 1961. The role of the nucleolus in ribonucleic acid and protein synthesis. I. Incorporation of cytidine into normal and nucleolar inactivated HeLa cells. *Biochim. Biophys. Acta.* **49**:47.
- PHILLIPS, D. M., and S. G. PHILLIPS. 1971. Distinctive characteristics of nucleoli of two established cell lines. *J. Cell Biol.* **49**:803.
- PHILLIPS, S. G., and D. M. PHILLIPS. 1969. Sites of nucleolus production in cultured Chinese hamster cells. *J. Cell Biol.* **40**:248.
- RITOSSA, F. M., and S. SPIEGELMAN. 1965. Localization of DNA complementary to ribosomal RNA in the nucleolus organizer region of *Drosophila melanogaster*. *Proc. Nat. Acad. Sci. U.S.A.* **53**:737.
- STEVENS, B. J. 1964. The effect of actinomycin D on nucleolar and fine structure in the salivary gland of *Chironomus thummi*. *J. Ultrastruct. Res.* **11**:329.
- SVOBODA, D., A. RACELA, and J. HIGGINSON. 1967. Variations in ultrastructural nuclear changes in hepatocarcinogenesis. *Biochem. Pharmacol.* **16**:651.
- SWIFT, H. 1959. Studies on nucleolar function. In *A Symposium on Molecular Biology*. R. E. Zirkle, editor. University of Chicago Press, Chicago. 266.
- SWIFT, H., and B. J. STEVENS. 1966. Nucleolar-chromosomal interaction in microspores of maize. *Nat. Cancer Inst. Monogr.* **23**:145.
- TAVITIAN, A., S. C. URETSKY, and G. ACS. 1968. Selective inhibition of ribosomal RNA synthesis in mammalian cells. *Biochim. Biophys. Acta.* **157**:33.
- TAVITIAN, A., S. C. URETSKY, and G. ACS. 1969. The effect of toyocamycin on cellular RNA synthesis. *Biochim. Biophys. Acta.* **179**:50.
- VENABLE, J. H., and R. COGGESHALL. 1965. A simplified lead citrate stain for use in electron microscopy. *J. Cell Biol.* **25**:407.
- VINCENT, W. S., E. BALTUS, A. LOVLIE, and R. E. MUNDELL. 1966. Proteins and nucleic acids of starfish oocyte nucleoli and ribosomes. *Nat. Cancer Inst. Monogr.* **23**:235.
- VON GAUDECKER, B. 1967. RNA synthesis in the nucleolus of *Chironomus thummi*, as studied by high resolution autoradiography. *Z. Zellforsch. Mikrosk. Anat.* **82**:536.
- WEINBERG, R. A., U. LOENING, M. WILLEMS, and S. PENMAN. 1967. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1088.
- WEINBERG, R. A., and S. PENMAN. 1968. Small molecular weight monodisperse nuclear RNA. *J. Mol. Biol.* **38**:289.