A Reevaluation of Nuclear and Nucleolar Changes Induced in Vitro by Actinomycin D

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SUMMARY

Fine structural studies that included the use of various enzyme digestion procedures and autoradiography were carried out on ME-180 tissue culture cells treated with actinomycin D. Particular emphasis was placed on the evolution of the nucleolar changes that resulted from treatment with the antibiotic. Degradation of the nucleoloemal structure was the main effect of the drug. The fibrillar portion was involved first, indicating that it may contain an actively transcribing chromatin and may possibly be the site of synthesis of the 45 S precursor rRNA. The granular portion became involved later and gave rise to two fractions that, for convenience, were designated P1 and P2 fractions. All evidence indicates that P1 granules, which have been shown to reside on fibrils, are precursors to P2 granules, which lack such fibrillar support. It is suspected that P1 granules contain 32 S RNA and P2 granules contain 28 S RNA. Apart from granules, the P2 fraction was shown to contain microspherules that were found to be composed primarily of basic proteins. These microspherules appear to be identical with those observed in cells treated with adenosine. Fibrillar centers were found to persist longest under the action of actinomycin D. Their close association with chromatin suggests a chromosomal origin. Since chromatin is the target structure of actinomycin D, it is postulated that all nucleolar alterations are due to chromatin changes induced by the antibiotic. It was shown that actinomycin D has a condensing effect on chromatin. Therefore, if a dispersed form of chromatin forms the skeleton of the nucleolonema, it would condense and thus cause the disintegration of the nucleolonema.

INTRODUCTION

In view of the biochemical advances made in the clarification of ribosome biogenesis (3), we feel that there is a need for further exploration of the fine structure and macromolecular composition of nucleolar constituents. Correlation of fine structural data with recent biochemical results will further our understanding of the nature and function of the various structural entities composing mammalian cell nucleoli. Compounds such as adenosine (10, 15, 31), actinomycin D (2, 13, 29), and others (2, 8, 14, 27, 30, 34), known to induce profound structural alterations in mammalian cell nucleoli, should prove helpful in correlation of findings, especially if their biochemical effects are well known, as are those of actinomycin D (4, 11, 19, 25). For these reasons, we set out to study nucleoli of previously treated or untreated human tissue culture cells by cytochemical methods and electron autoradiography (21–24). This report summarizes results obtained with actinomycin D.

In vitro, actinomycin D-induced structural alterations of mammalian cell nucleoli have been described by several investigators (9, 13, 26, 29) and have been reviewed by Bernhard and Granboulan (2). Their findings are essentially identical with those presented here, but in this study an effort was made to give a more complete description of the evolution of the nucleolar changes and to characterize further the macromolecular composition of the various constituents. In addition, an attempt was made to correlate structural data with biochemical findings and to explain the structural changes on the basis of the known effects of actinomycin D. Whether the various hypotheses set forth are correct, future work will have to show, but it is hoped that they will stimulate interest in the structural aspects of ribosome biogenesis.

MATERIALS AND METHODS

Tissue Culture. For all experiments, the human cell line ME-180, derived from a metastatic carcinoma of the cervix, was used. The cell line is being maintained in our laboratory on Eagle's minimum essential medium supplemented with 30% fetal calf serum, streptomycin sulfate (25 mg/liter), and neomycin (100 mg/liter). Actinomycin D (Merck Sharp and Dohme, Westpoint, Pa.) was added to the medium in concentrations varying from 0.05 to 50 µg/ml. In all instances, the cells were grown in T-flasks. They were incubated in the medium containing actinomycin D while in the active growth phase. The average number of cells was 500,000 cells/ml medium. For autoradiography, cells were labeled with thymidine-3 H, 25 µCi/ml medium (specific activity, 10 Ci/mM) (Schwarz BioResearch, Inc., Orangeburg, N. Y.) for 18 hr and with uridine-3 H, 50 µCi/ml medium (specific activity, 28 Ci/mM) (Schwarz BioResearch, Inc.) for 30 min.

Electron Microscopy. The cells were fixed in suspension with cacodylate-buffered 2% glutaraldehyde for 20 to 30 min, washed with Millonig's buffer, dehydrated with ethanol, and embedded in Epon. In a few instances, postfixation with 1% osmium tetroxide in Millonig's buffer was carried out. The enzyme digestion and autoradiographic techniques used in

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these experiments have previously been described (23, 24). The following enzymes were purchased: RNase, crystallized 5 times, Calbiochem, Los Angeles, Calif.; pepsin, crystallized 2 times, Worthington Biochemical Corp., Freehold, N. J.; and trypsin, 1-300, Nutritional Biochemicals Corp., Cleveland, Ohio. Sections were cut with a diamond knife on an LKB Ultratome and stained with uranyl acetate and lead citrate. The staining procedure recently described by Bernhard (1) was used for selected sections. These sections were stained with uranyl acetate in 50% ethanol for 5 min, floated on a 0.2 M EDTA solution in distilled water for 30 or 120 min, and stained with lead citrate for 3 min. The sections were examined in a Siemens Elmiskop IA.

RESULTS

Introductory Remarks

In order to clarify the terminology used in this text, a brief description of the principal nucleolar structures follows. Essentially, 5 components can be differentiated: the fibrillar and granular substances, the fibrillar centers, the nucleolar chromatin, and the amorphous matrix. Nucleoli of ME-180 cells show, in most instances, a well-formed nucleolus that contains the fibrillar and granular elements (Fig. 1). In the meshes of the nucleolomema, one frequently finds a loose fibrillar substance of low electron density that is invariably associated with the fibrillar component of the nucleolomema. We have referred to this substance as “fibrillar centers” (23). The nucleolar chromatin appears in 2 forms, the intranucleolar chromatin found in the meshes of the nucleolomema and the nucleolus-associated chromatin located at the nucleolar surface. An amorphous proteinaceous matrix is associated with the nucleolomema and the fibrillar centers.

Structural Changes Inducible with Actinomycin D

The structural alterations observed in ME-180 cells following actinomycin D treatment do not essentially differ from those described by others (9, 13, 26, 29), but, despite this, a brief description is considered necessary to understand the digestion results. The description applies to changes induced with actinomycin D in a concentration of 2.5 μg/ml medium.

Following a 30-min treatment, electron-opaque nodules of 70 to 150 μm in size are observed in the nucleus that appear to be associated with the dispersed chromatin (Fig. 2). For convenience, we will refer to these nodules as “microspherules.” The nucleoli show minor changes. The fibrillar substance, instead of being distributed along nucleolomematic strands, forms round globules that vary greatly in size. In most instances, these globules are associated with fibrillar centers. After a 1-hr treatment (Fig. 3), the various nucleolar components are in the process of sorting out. The fibrillar centers retain their association with the fibrillar substance. A granular electron-opaque substance has emerged that is not present in nucleoli of untreated cells. We will refer to this substance as P2 fraction (particulate Fraction 2), in contrast to the P1 fraction (particulate Fraction 1), which will designate the regular granular component of nucleoli. The P2 fraction is found throughout the nucleolus after a 1-hr treatment. It contains microspherules that appear to be identical with those seen in extranucleolar areas after a 30-min treatment. After a 2-hr treatment, the segregation of the nucleolar components is evident (Fig. 4). The P2 fraction is now assembled on the nucleolar surface, often forming crescent-shaped caps over the P1 fraction, which forms the central mass. The fibrillar substance is usually part of this central mass, but the fibrillar centers are extruded onto the surface. Actinomycin D treatment longer than 2 hr produces further changes, the progress of which varies greatly in individual cells. The P1 fraction shrinks gradually and ultimately seems to disappear, while the P2 fraction transforms from crescent-shaped caps to rounded masses (Figs. 5 and 6). One gets the impression that the P1 fraction actually converts into the P2 fraction. Later the P2 fraction will disappear also. Its fate is not clear at the present time. A close structural relationship of the P2 fraction with groups of interchromatin granules can, in some instances, be demonstrated, but this may merely be incidental (Fig. 7). Along with these developments, the fibrillar centers become detached from the main nucleolar mass and outlast the P1 and P2 fractions. In this process, the fibrillar substance is disrupted. Part of it remains attached to the main nucleolar mass (P1 fraction), while the remainder migrates along with the fibrillar centers. The fibrillar substance associated with the fibrillar centers usually forms globules that frequently show a bi- or tripartite distribution (Figs. 8 and 9).

The same sequence of nucleolar changes is observed if actinomycin D is applied in concentrations lower than 2.5 μg/ml, but the changes seem to develop at a slower pace. The reverse is true for concentrations higher than 2.5 μg/ml. The changes develop and progress more rapidly. With extremely high concentrations, such as 50 μg/ml, the evolution of the changes is curtailed. After 2 hr, the P1 fraction has almost disappeared (Fig. 10). The P2 fraction is scattered throughout the nucleus. Fibrillar centers may be observed, but they show no relationship to the P2 fraction.

The nucleolar changes are accompanied by nuclear changes. Most ME-180 cells of a culture in the active growth phase show a primarily dispersed chromatin (Fig. 11), while only a few cells contain a significant amount of condensed chromatin. Under the action of actinomycin D, the dispersed chromatin appears to condense to irregular aggregates, which are scattered throughout the nucleus. The extent of condensation seems to depend on the exposure time and the concentration. The longer the exposure time and the higher the concentration, the more pronounced is the effect, but it takes approximately 1 hr for the effect to manifest itself, regardless of concentration (Fig. 12). Interchromatin granules aggregate in discrete groups with increasing condensation of the chromatin and thus become quite prominent. After prolonged actinomycin D treatment, one frequently observes irregular condensations within groups of interchromatin granules, which appear to be made up of granules and an amorphous substance (Fig. 13) and resemble somewhat the P2 fraction.
Staining Property of Microspherules and $P_2$ Fraction

Previously, Recher (22) described electron-dense nodules in nuclei of cells treated with adenosine. He was able to show that these adenosine-induced nodules appear unstained or bleached in sections that are stained with uranyl acetate and then treated with the chelating agent EDTA before they are poststained with lead citrate (1). Because of the structural resemblance of these nodules to microspherules, we stained sections of actinomycin D-treated cells by the same method. As illustrated in Fig. 14, the microspherules appear bleached following a 30-min exposure of the sections to EDTA. An exposure time of 2 hr bleaches the entire $P_2$ fraction and also the chromatin (Fig. 15). The microspherules and the $P_2$ fraction are not extracted by this staining procedure. This can be shown by restaining the sections with uranyl acetate and lead citrate. They appear again as electron opaque, as if they had never been exposed to the chelating agent.

Enzyme Digestion Studies

All enzyme digestion procedures were carried out on cells that were treated with actinomycin D (2.5 $\mu$g/ml) for 2 hr.

RNase. RNase digestion does not appear to change the electron opacity of microspherules (Fig. 16). The electron density of the $P_1$ and $P_2$ fractions, however, appears reduced.

Pepsin. Brief 20-min pepsin digestion removes the amorphous matrix of the $P_2$ fraction. The granules present in this fraction become very distinct and are in size and appearance identical with those of the $P_1$ fraction (Figs. 17 and 18). The microspherules are also sensitive to pepsin. They appear to consist of an amorphous matrix, highly sensitive to pepsin, and fine fibrils, less sensitive to pepsin (Fig. 17). Pepsin digestion for 40 min removes the entire $P_2$ fraction, including the microspherules (Fig. 19). The matrix of the $P_1$ fraction is also removed, but the granules remain. They resist even prolonged pepsin digestion and can be removed only if RNase digestion precedes that of pepsin. The fibrillar substance frequently associated with the $P_1$ fraction is most resistant to pepsin. It can, however, be partially removed if the cells are subjected to vigorous pepsin digestion. The proteinaceous matrix of the fibrillar centers is also sensitive to pepsin (Fig. 20). Fine fibrils approximately 70 A thick, which show an intricate relationship with chromatin, are exposed. These fibrils retain the relationship with chromatin after the centers have separated from other nucleolar components.

RNase and Pepsin. Double digestion with RNase and pepsin removes the $P_2$ fraction completely (Fig. 21). The $P_2$ granules, normally resistant to brief pepsin digestion, cannot be demonstrated if RNase digestion precedes that of pepsin, even if pepsin digestion is carried out for only 2 to 3 min, indicating that $P_2$ granules contain RNA. Double digestion removes the amorphous matrix of the $P_2$ fraction and the $P_1$ granules. At their place, a fine filamentous network is observed, which is anchored on chromatin. This network cannot be demonstrated unless the granules are removed first. It therefore appears that the granules actually reside on these filaments.

Trypsin. Trypsin digests the microspherules and the proteinaceous matrix of the $P_2$ fraction (Fig. 22). Trypsin-resistant granules that resemble those of the $P_1$ fraction remain in the $P_2$ fraction. Trypsin seems to have no effect on other nucleolar structures.

Autoradiographic Studies

ME-180 cells exposed to thymidine-$^3$H for 18 hr and treated with actinomycin D for 2 hr show no labeling of the $P_1$ and $P_2$ fractions (Fig. 23). If exposed to uridine-$^3$H for 30 min and treated with actinomycin D for 2 hr, labeling is observed in both fractions (Fig. 24).

DISCUSSION

It is now well established that the formation of ribosomes is not a single event but a rather complex process involving several steps. The process begins in the nucleolus by the transcription of a high-molecular-weight RNA with a sedimentation coefficient of 45 S RNA (18). This 45 S ribosomal precursor RNA is cleaved, yielding 18 S and 32 S RNA. The 32 S RNA is further converted to 28 S RNA. The 18 S RNA ultimately becomes part of the smaller ribosomal subunit, and the 28 S RNA becomes part of the larger subunit. Actinomycin D is known to block the transcription of 45 S RNA (19), but it does not interfere with the further processing of 45 S RNA synthesized prior to the block (5, 17, 20, 33). The result is a gradual depletion of the 45 S RNA in the nucleolus. This loss of 45 S RNA does not manifest itself clearly in the fine structural changes induced by actinomycin D.

The earliest fine structural nucleolar changes induced by actinomycin D are observed in the fibrillar substance of the nucleolonema after approximately 20 to 30 min. In untreated cells, the fibrillar substance is distributed along nucleolonemal strands located near fibrillar centers. Under the action of actinomycin D, the strands disintegrate, and the fibrillar substance collects in droplets that are usually associated with fibrillar centers. It appears as if there is a degradation of the supportive structure of these strands. Since actinomycin D seems to be the cause of this degradation, it is possible that the supportive structure is an activated form of chromatin. Actinomycin D is known to exert its effect by binding to DNA, thus preventing RNA transcription by RNA polymerase (11, 25). In the early phase of treatment, it seems to bind preferentially to the chromatin available for transcription (28); that is, in structural terms, the dispersed or decondensed chromatin (7, 16). The findings presented here have shown that the antibiotic effects condensation of the dispersed chromatin. Therefore, if a dispersed chromatin forms the supportive structure of these strands, it would condense and possibly retract, thus causing disintegration.

Bernhard and Granboulan (2) suggested that fibrillar areas in nucleoli might be the morphological substrate of the 45 S RNA, but they had reservations, stating: "If the morphological substrate of the 45 S fraction is the fibrillar part in the nucleolus, one would expect this compound to react first..."
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to actinomycin D. This could not be demonstrated, however.” It is evident that our findings do not support his latter statement. The very first manifestation of the actinomycin D effect that we observe is a structural reorganization of the fibrillar substance. Since others (26, 29) have described identical changes in other cell lines, it appears to be a manifestation typical for actinomycin D.

After approximately 40 to 60 min of actinomycin D treatment, changes can be observed in the granular nucleolonema. The nucleolonemal structure degrades. The granules, instead of being distributed along nucleolonemal strands, form compact, rounded masses that we have referred to as the P1 fraction. Concomitant with this degradation, a new granular fraction, the P2 fraction, which is invariably associated with the P1 fraction, appears. Both the P1 and the P2 fractions harbor granules that are of identical size and appearance and contain RNA. The P2 fraction emerges at a time when RNA synthesis has ceased. The RNA of the P2 granules therefore must derive from an existing source of RNA. Because of the close association of the P1 and P2 fractions, it is likely that the P1 granules are precursor granules to the P2 granules. The fact that the P1 fraction is depleted while the P2 fraction evolves also supports this view. Schoefl (29), who studied the effects of actinomycin D in baboon kidney cells, came to essentially the same conclusion.

The P1 granules are quite resistant to pepsin, while P2 granules are easily destroyed by pepsin digestion. The P1 granules are supported by fine fibrils (Fig. 21), which appear to be remnants of the fibrillar reticulum described elsewhere (24). The P2 granules lack such a support by fibrils. The P1 granules apparently convert into P2 granules by being released from these fibrils. The nature of the fibrils has not been established. Their association with chromatin suggests a chromosomal origin, but, thus far, neither digestion nor autoradiographic studies have revealed their macromolecular composition.

Extracts of the HeLa cell nucleoli contain primarily 45 S and 32 S RNA (18), no measurable amounts of 18 S RNA, and only small amounts of 28 S RNA (33). If these findings apply to nucleoli of other human tissue culture cells, then one may ask what RNA species is present in the P1 granules. The P1 fraction is derived from the granular substance of the nucleolonema; therefore, P1 granules are identical with the granules observed in nucleoli of untreated cells. Since the granular fraction of nucleoli of untreated cells usually forms the bulk of the nucleolar mass, it is unlikely that the granules contain 28 S RNA because of the small amounts of 28 S RNA present (33). Kinetic studies with radioactively labeled RNA precursors indicate that 45 S RNA disappears rapidly (5, 17, 33) when cells are exposed to actinomycin D. P1 granules survive, however, for many hours if the dose of the antibiotic is not excessive but sufficient to suppress 45 S RNA synthesis. This makes it also unlikely that the P1 granules contain 45 S RNA. Exclusion of 45 S and 28 S leaves the 32 S RNA as the species likely to be present in P1 granules. Should P1 granules contain 32 S RNA, the conversion of the P1 to P2 granules or, in other words, the release of P1 granules from the fibrils on which they reside may well mean transition of their 32 S RNA to 28 S RNA. Girard et al. (5) have shown that, in HeLa cells after long periods of actinomycin D treatment, the radioactive RNA in the 32 to 28 S region shifts to predominantly 28 S. This shifting to 28 S may result from the conversion of the P1 to P2 granules.

The P2 fraction, apart from the granules and the proteinaceous matrix, contains electron-dense nodules, which we referred to as microspherules. Digestion studies have shown that these microspherules are composed primarily of basic proteins. Unuma and Busch (32) observed similar structures in Ehrlich ascites and Novikoff hepatoma cells after high doses of actinomycin D. These authors were first to use the term “microspherules,” a term we adopted because of the structural similarity and identical location.

Earlier, Recher (22) described similar structures in nucleoli of ME-180 cells treated with adenine. These adenine-induced nodules have many characteristics in common with microspherules (21, 22) and should therefore be designated by the same term.

The structures most susceptible to the bleaching action of EDTA (microspherules, P2 matrix) are also most susceptible to trypsin digestion. It therefore appears that the bleaching effect is due to the presence of basic proteins. Bernhard (1) found that deoxyribonucleoprotein-containing structures are bleached by the staining method. From the above findings, one would have to conclude that the basic protein component complexed to DNA, rather than the DNA itself, must be responsible for the effect.

Most puzzling are the structures that we referred to as fibrillar centers. Their intrinsic relationship with chromatin becomes very evident under the action of the antibiotic. The fibrils are continuous with those of the chromatin, and in some instances it is even difficult to draw a sharp line between the 2 structures (Fig. 20). This close relationship suggests that they are of chromosomal origin.

Actinomycin D has a condensing effect on chromatin, an effect that has been little emphasized in the past. Schoefl (29) has stated that “the overall amount of chromatin tended to increase during the experimental period.” Goldstein et al. (6) observed an increase of Feulgen-positive material in HeLa cells treated with actinomycin D. It seems to us that the findings by these authors actually refer to an increase of the condensed chromatin, rather than an overall increase of chromatin. The magnitude of the effect seems to depend on the saturation level of the chromatin with actinomycin D. The effect is most evident in cells treated with high doses sufficient to suppress even DNA synthesis (Fig. 10). Actinomycin D, known to block DNA-dependent RNA synthesis, brings about a structural change in chromatin that, on the basis of autoradiographic results (7, 16), must be interpreted as inactivation of chromatin.

The effect that actinomycin D exerts on chromatin could easily explain the nucleolar events if one assumes that a dispersed chromatin forms the backbone of the nucleolonema. In a recent report (24), we described a RNase- and pepsin-resistant fibrillar reticulum, which we considered the skeleton of the nucleolonema. The reticulum is closely associated with the condensed nucleolar chromatin and, thus, might be a dispersed form of chromatin.

The breakdown of the nucleolonema, particularly of its
fibrillar portions, appears to be analogous to the disappearance of the loops of lampbrush chromosomes, which was observed with actinomycin D (12). The granular nucleolenema responds to actinomycin D later than the fibrillar nucleolenema and is labeled later, following short pulses of tritiated RNA precursors (7). This raises the question of whether its supportive structure can be an actively transcribing chromatin. It is possible that no transcription takes place in this portion of the nucleolenema but that the granules pass through a maturation phase before they are released into the nucleoplasm. The fibrillar nucleolenema then would be the transcription site of the 45 S RNA, and the granular nucleolenema would be a site of maturation of the 32 S RNA-containing particles. The RNA would either migrate from the fibrillar to the granular nucleolenema, or strands of the fibrillar nucleolenema would simply convert to strands of the granular nucleolenema. In this conversion, the 18 S RNA-containing particles would most likely be released, and the 32 S RNA-containing particles would be retained for further maturation. Under normal conditions, the 32 S RNA-containing particles would mature to particles with 28 S RNA and be released immediately into the nucleoplasm. Under the action of actinomycin D, however, these particles would not be released immediately but would pass first into the P2 fraction and be released later.

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Fig. 1. Nucleolus of ME-180 cell. The nucleolonema is well formed and is composed of fibrillar (FS) and granular (GS) substance. The fibrillar substance is located next to fibrillar centers (FC). The chromatin in this instance is inconspicuous. Fixation, glutaraldehyde and osmium tetroxide; stain, uranyl acetate and lead citrate. × 40,000.

Fig. 2. Nucleus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 0.5 hr. One microspherule is seen (arrow). The fibrillar substance (FS) of the nucleolus forms droplets that vary in size and are associated with fibrillar centers (FC) in most instances. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 20,000.

Fig. 3. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 1 hr. A particulate substance (P2) that contains microspheres is observed. It differs from the granular substance (P1) by its higher electron density and coarser texture. The various constituents are in the process of sorting out. Fibrillar centers (FC) remain associated with the fibrillar substance (FS). Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 4. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr. The various constituents are segregated. The P1 fraction forms caps over the P2 fraction. The fibrillar center (FC) is extruded onto the surface but is still associated with the fibrillar substance (FS). Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 5. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 6 hr. The P1 fraction roughly equals the amount of the P2 fraction. The fibrillar center (FC) has separated from the main nucleolar mass. Notice the close association of the interchromatin granules (IG) with the P2 fraction. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 6. Nucleus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 8 hr. The nucleolus shows a large P2 fraction but no P1 fraction. The fibrillar center (FC) and the associated fibrillar substance (FS) are separated from the main mass. Notice the close relationship of the interchromatin granules (IG) with the P2 fraction. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 7. Nucleus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 8 hr. The P1 fraction shows a close association with groups of interchromatin granules (IG). Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 8. Nucleolar area of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 4 hr. The fibrillar center (FC) is associated with fibrillar substance, which shows a bipolar distribution. Left, P2 fraction. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 9. Nucleolar area of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 8 hr. The fibrillar center (FC) is associated with fibrillar substance, which shows a tripolar distribution. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 10. ME-180 cell that has been treated with actinomycin D (50 μg/ml) for 2 hr. The cytoplasm appears well preserved. The nucleolus shows marked condensation of the chromatin. The nucleolus is disintegrated. The P2 fraction is scattered through the nucleoplasm. There is no evidence of any P1 fraction. A fibrillar center (FC) is separated from other nucleolar structures. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 18,000.

Fig. 11. ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 30 min. The nuclear chromatin is largely dispersed. The nucleolus (Nu) shows droplet formation of the fibrillar substance. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 18,000.

Fig. 12. ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr. The nucleoli (Nu) show segregation of their constituents. The chromatin is largely condensed. Compare with chromatin in Fig. 11. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 18,000.

Fig. 13. Nucleolar area of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 8 hr. It shows a group of interchromatin granules (IG) that includes 2 irregular condensations (arrows). These condensations are composed of granules and an amorphous matrix. Are they remnants of the P2 fraction? Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 14. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr. The P2 fraction contains round, electron-lucent areas that are sites of microspheres. Fixation, glutaraldehyde; stain, uranyl acetate, EDTA for 30 min, and lead citrate. × 30,000.

Fig. 15. Nuclear area of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr. The P2 fractions appear bleached, but the sites of microspheres can still be recognized by their higher electron lucency (arrows). The marginal chromatin (double arrows) appears bleached also. The P1 fraction is unaffected. Fixation, glutaraldehyde; stain, uranyl acetate, EDTA for 2 hr, and lead citrate. × 30,000.

Fig. 16. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr and digested with RNase (1 mg/ml) for 1.5 hr at 37°. The fibrillar substance (FS) and the P2 and P1 fractions show a decreased electron density. FC, fibrillar centers. The microspheres seem to have their usual density. Fixation, glutaraldehyde prior to digestion; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 17. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr and digested with pepsin (0.1 mg/ml) for 20 min at 37°. The amorphous matrix of the P2 fraction is removed. The P1 granules are discrete. The sites of microspheres are still recognizable (arrows). They seem to contain tiny fibrils. The P1 fraction and the fibrillar substance (FS) are little affected. The fibrillar substance contains some positive material. Fixation, glutaraldehyde prior to digestion, osmium tetroxide following digestion; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 18. Nucleolus of ME-180 cell treated identically as cell in Fig. 17. The P2 granules show a loose arrangement. Notice the fibril (arrow) that bridges chromatin and the P2 fraction. This is observed quite frequently. The fibrillar substance (FS) contains a moderate amount of lead-positive material. FC, fibrillar centers. Fixation, glutaraldehyde prior to digestion, osmium tetroxide following digestion; stain, uranyl acetate and lead citrate. × 30,000.

Fig. 19. Nucleolus of ME-180 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr and digested with pepsin (0.1 mg/ml) for 40 min at 37°. The P2 fraction is totally removed. It is assumed that it was located in areas wherever the P1 fraction shows straight borders (P1?). The amorphous matrix of the P2 fraction is also removed. The P1 granules are densely packed and appear slightly swollen. They are larger than those in Figs. 17, 18, and 20 because of the longer digestion time. FS, fibrillar substance. Fixation, glutaraldehyde prior to digestion, osmium tetroxide following digestion; stain, uranyl acetate and lead citrate. × 30,000.
Fig. 20. Nucleolus of ME-i80 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr and digested with pepsin (0.1 mg/ml) for 20 min at 37°. The amorphous matrix of the fibrillar centers (FC) is removed. Fine fibrils of approximately 70 A, which show continuity with chromatin fibrils, are exposed (arrow). The fibrillar substance (FS) and the P1 fraction have been little-affected by pepsin. The P2 fraction is missing in this section. Fixation, glutaraldehyde prior to digestion, osmium tetroxide following digestion; stain, uranyl acetate and lead citrate. X 30,000.

Fig. 21. Nucleolus of cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr and digested with RNase (1 mg/ml) for 1 hr and pepsin (0.1 mg/ml) for 40 min at 37°. The P1 granules are removed. A fine fibrillar reticulum is exposed that is anchored on chromatin. The P2 fraction is totally removed; possible sites are indicated (P2 †). The fibrillar substance (FS) shows a slightly decreased electron density. Individual fibrils of the fibrillar centers (FC) resisted double digestion. Fixation, glutaraldehyde prior to digestions, osmium tetroxide following digestions; stain, uranyl acetate and lead citrate. X 30,000.

Fig. 22. Nucleolus of ME-i80 cell that has been treated with actinomycin D (2.5 μg/ml) for 2 hr and digested with trypsin for 1.5 hr at 37°. The amorphous matrix of the P2 fraction is removed. The P2 granules can still be recognized but are not very discrete. They resemble those of the P1 fraction. Rounded defects (arrows) are likely to be sites of microspherules. Notice the lead-positive material in the fibrillar substance (FS) and the fibrillar center (FC). The latter is detached from the main nucleolar mass. Fixation, glutaraldehyde prior to digestion, osmium tetroxide following digestion; stain, uranyl acetate and lead citrate. X 25,000.

Fig. 23. ME-i80 cell that has been labeled with thymidine-3H (25 μCi/ml) for 18 hr and then treated with actinomycin D (2.5 μg/ml) for 2 hr. Note the massive labeling of the chromatin, while there is no labeling of the P1 and P2 fractions. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate; exposure, 4 weeks. X 30,000.

Fig. 24. Nucleolus of ME-i80 cell that has been labeled with uridine-3H (50 μCi/ml) for 30 min and then treated with actinomycin D (2.5 μg/ml) for 2 hr. Both the P1 and P2 fractions are labeled. There is also some extra nucleolar labeling. FS, fibrillar substance. Fixation, glutaraldehyde; stain, uranyl acetate and lead citrate; exposure, 3 months. X 30,000.
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A Reevaluation of Nuclear and Nucleolar Changes Induced in Vitro by Actinomycin D

Louis Recher, Letty G. Briggs and Nikko T. Parry

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