THE ORGANIZATION OF THE NUCLEOLUS IN MERISTEMATIC PLANT CELLS

A Cytochemical Study

A. LORD and J.-G. LAFONTAINE

From the Department of Biology, Faculty of Sciences, Laval University, Québec 10, Canada

ABSTRACT

The architecture of the nucleolus in *Allium porum* and *Triticum vulgare* meristematic cells has been investigated by means of digestions with various enzymes. After staining with azure B at pH4, plant nucleoli exhibit lighter regions which, under electron microscopy, correspond to the fibrillar zones characterizing these organelles. Evidence is presented indicating that these latter zones contain coarse convoluted filaments quite similar to the loops first demonstrated by La Cour (24) and which are assumed to originate from the nucleolar-organizing chromosomes. These coarse, 0.2μ wide filaments are remarkably resistant to the action of deoxyribonuclease, ribonuclease, pepsin, trypsin, or of various combinations of these enzymes and, moreover, they show insignificant incorporation of labeled thymidine even after long exposure to this DNA precursor. The clearing action of pepsin on different regions of the nucleolus lends support to the hypothesis that an amorphous material or matrix pervades the mass of this organelle. This effect is particularly striking within the particulate nucleolar zones themselves. Both ribonuclease and trypsin disorganize the RNP (ribonucleoprotein) nucleolar particles. The effect of the latter enzyme on the RNP particles is taken to indicate that they contain proteins particularly susceptible to trypsin which are essential for maintenance of their morphological integrity. Trypsin also interferes with azure B–staining of the nucleolar mass as a whole and, according to radioautographic data, extracts RNA throughout this organelle. Accordingly, the hypothesis is considered that RNA is complexed with proteins not only within the particulate nucleolar portions, as is already well known, but also in the fibrillar zones.

Recent electron microscopic studies have shown that the organizational pattern of the nucleolus varies to a certain extent with species. This diversity in nucleolar architecture is especially striking in animals (see references 3 and 19 for recent reviews). However, in plants, except for numerous vacuoles and lacunae, the nucleolar mass is generally composed of two main types of zones (22, 23, 25–28, 41, 47).[1] Some of these nucleolar portions consist predominantly of RNP (ribonucleoprotein) particles, which are believed to be embedded within an amorphous substance or matrix. Certain observations suggest that these particles are often organized in the form of threadlike structures, some 0.1 μ in diameter (26, 28). The remaining nucleolar regions in plants are usually referred to as fibrillar in texture. There is now good evidence that these latter zones are intimately associated with intranucleolar chromatin (22, 23, 25–28, 41).

As judged from available information on the biochemical composition of the nucleolus, there

[1] R. Vancoillie and J. G. Lafontaine. A correlated light and electron microscope study of the effects of actinomycin D on the architecture of the nucleolus in plant meristematic cells. In preparation.

presently exists a fair degree of agreement about the nature and importance of the main constituents of the nucleolus in both animal (9, 34, 51) and plant (4–8, 23) cells. According to data recently compiled by Vincent et al., (51), for instance, nucleoli from a wide variety of sources consist mostly of proteins (85–90%), RNA representing only 5–10% of the mass of these organelles. The situation is a great deal more uncertain as regards the actual concentration of DNA within nucleoli, and relevant data, therefore, differ markedly with authors and sources of material (5, 8, 9, 34, 37, 38, 51). These widely divergent reports undoubtedly result in part from the difficulty of preparing nucleoli free of contaminating chromatin.

Some progress has recently been made towards a better understanding of the organization of the nucleolus of both animal (2, 3, 16–18, 20, 30, 35, 36, 39, 46) and plant cells (25, 28, 48) by exploiting newly developed cytochemical techniques in the electron microscope. This report focuses on the application of such methods to the study of the architecture of plant nucleoli and, more particularly, presents observations concerning the localization and cytochemical characteristics of a coarse filamentous component which is believed to correspond to the nucleolar organizer.

MATERIAL AND METHODS

Fixation and Embedding

Roots of *Allium porum* and *Triticum vulgare* were used for the present investigation. Part of the material was fixed in 1% osmium tetroxide, pH 7.2, and embedded in Epon according to current procedures. Other roots to be utilized for cytochemical studies were fixed for 20–40 min in 6% ice-cold glutaraldehyde adjusted to pH 7.2 with 0.1 M cacodylate buffer and were embedded in glycol methacrylate (30).

Preparation of Isolated Nuclei

To demonstrate the presence within nucleoli of a coarse filamentous component of the type observed by La Cour (24) in a number of plant species, roots of *Allium porum* were fixed for a brief period in 5% formalin and then exposed to a 0.1% aqueous solution of "Tween 80" (Atlas Chemical Industries Inc., Wilmington, Del.) for a few minutes. Subsequent to gentle squashing of the preparation between slide and coverslip, favorable nucleoli were examined and photographed under phase-contrast microscopy.

Enzymatic Digestions

The hydrolyzing solutions were prepared as follows (30, 43):

1. Deoxyribonuclease[2,3] (2 × crystallized or electrophoretically purified), 0.1% in an aqueous solution containing 0.003 M $MgSO_4$ and adjusted to pH 6.5 with 0.01 N NaOH. Treatment was carried out at 37°C for 2–4 hr.
2. Ribonuclease[3] (5 × crystallized), 0.2% in distilled water adjusted to pH 6.8 with 0.01 N NaOH, was used for 2–3 hr at 37°C.
3. Pepsin[3] (3 × crystallized), 0.5% in 0.1 N HCl, pH 1.2, was employed for 2–3 hr at 37°C.
4. Trypsin[3] (2 × crystallized), 0.3% in distilled water adjusted to pH 8 with 0.01 N NaOH, was used for 2–3 hr at 37°C.

Staining and Microphotographic Procedures

Part of the 0.7-μ sections were stained with 0.25% azure B adjusted to pH 4.0 with McIlvaine's buffer (43). These stained preparations were photographed, submitted to enzymatic digestion, and then photographed a second time for comparison, after staining with azure B. Other preparations mounted in glycerine were photographed at 260 mμ with Leitz reflecting lenses before and after digestion with the foregoing enzymes. Ultrathin sections treated with enzymes were mounted on Formvar-coated copper grids and stained for 2–3 hr with a 1% aqueous solution of uranyl acetate. These sections were examined in a Philips EM-200 electron microscope equipped with an anticontamination device.

For light microscopic or UV preparations, the 0.7-μ sections, deposited, respectively, on glass and quartz slides, were immersed within the hydrolyzing solutions in coplin jars. As for the ultrathin sections, they were floated on the enzymatic solutions according to the technique described by Marinozzi (35). Control preparations in each case were subjected, for corresponding periods, to solutions of similar pH's and temperatures but containing no enzyme.

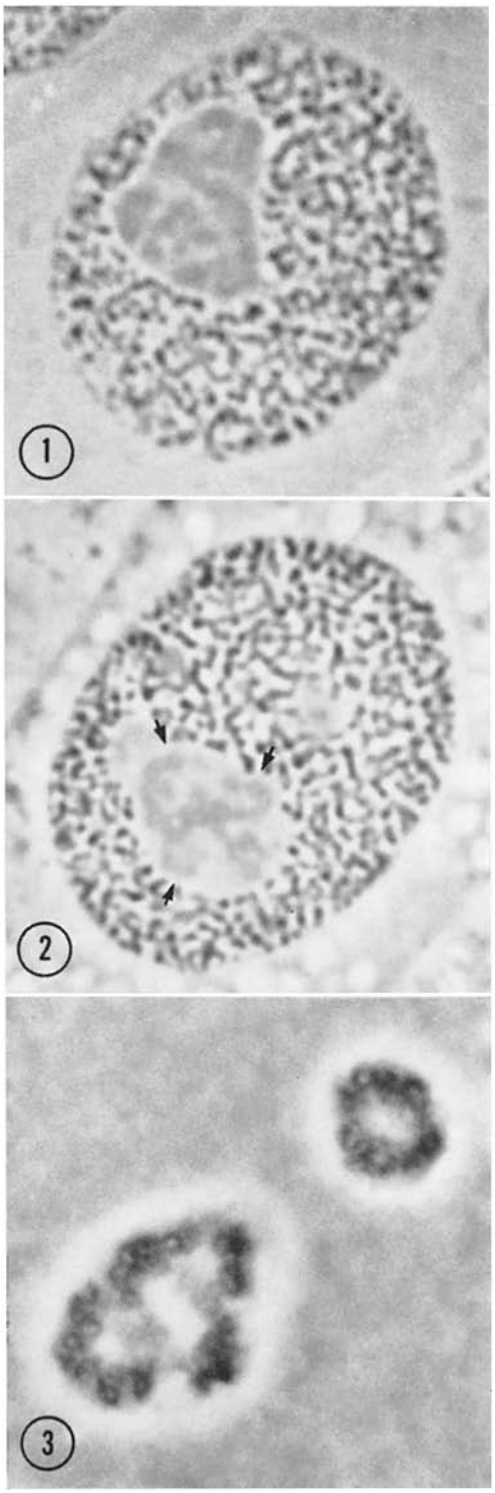
Radioautography

Roots of *Allium porum* and *Triticum vulgare* were immersed in distilled water containing tritium-labeled precursors[4] of DNA and RNA. Concentrations ranging from 5 to 125 μc/ml were used for cytidine-5-^{3}H (specific activity: 15 c/mmole) and of 2 μc/ml for thymidine-methyl-^{3}H (specific activity: 12 c/mmole). After incorporation periods varying from 5 to 60 min for cytidine and from 1 to 20 hr for

[2] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[3] Worthington Biochemical Corp., Freehold. N.J.

[4] Schwarz Bio Research, Inc., Orangeburg, N.Y.



thymidine, the root tips were fixed with either glutaraldehyde or osmium tetroxide and processed according to procedures described above.

Sections 0.5–0.7 μ in thickness were deposited on glass slides and covered by dipping in liquid Ilford L-4 emulsion. Before observation, the radioautographs were stained with 1% methylene blue in 1% borax.

OBSERVATIONS

The appearance of the nucleolus in *Allium porum* and *Triticum vulgare* under light microscopy is illustrated in Figs. 1 and 2. After methylene blue—staining or under phase-contrast microscopy, the presence of dense zones intermingled with lighter portions confers a rather heterogeneous appearance to this organelle. The examination of serial 0.5-μ-thick sections reveals, moreover, that these denser portions form complex three-dimensional patterns throughout the nucleolar mass as reported for other plant species (26–28). More suggestive images of these same nucleolar zones are obtained in preparations treated with "Tween 80": they are indeed then seen to contain coarse convoluted threads (Fig. 3), the nature of which is best studied under electron microscopy.

At the ultrastructural level, nucleoli of both *Allium porum* and *Tritucum vulgare* exhibit important particulate zones extending from their surface to more centrally located portions of their mass (Figs. 4 and 5). The RNP nucleolar granules, in

---

FIGURE 1 *Triticum vulgare* preprophase nucleus with nucleolus showing irregular dense regions which correspond to the fibrillar zones seen under electron microscopy (Fig. 4). Section stained by means of the Feulgen reaction and photographed under phase-contrast microscopy. × 3,000.

FIGURE 2 *Allium porum* preprophase nucleus from preparation processed as in Fig. 1. In this micrograph, the dense nucleolar regions take on a configuration which is highly suggestive of the presence of coarse twisted filaments. At certain places (arrows) at the periphery of the nucleolus, one gains the impression of a possible continuity between densely stained chromosome strands and parts of the intranucleolar skein. × 3,000.

FIGURE 3 Phase-contrast micrograph of nucleoli from isolated *Allium porum* nucleus treated with "Tween 80" for a few minutes. The coarse nucleolar filamentous components seem to have uncoiled to a limited extent only and exhibit an organization strikingly similar to that revealed under electron microscopy (Fig. 5). × 3,000.

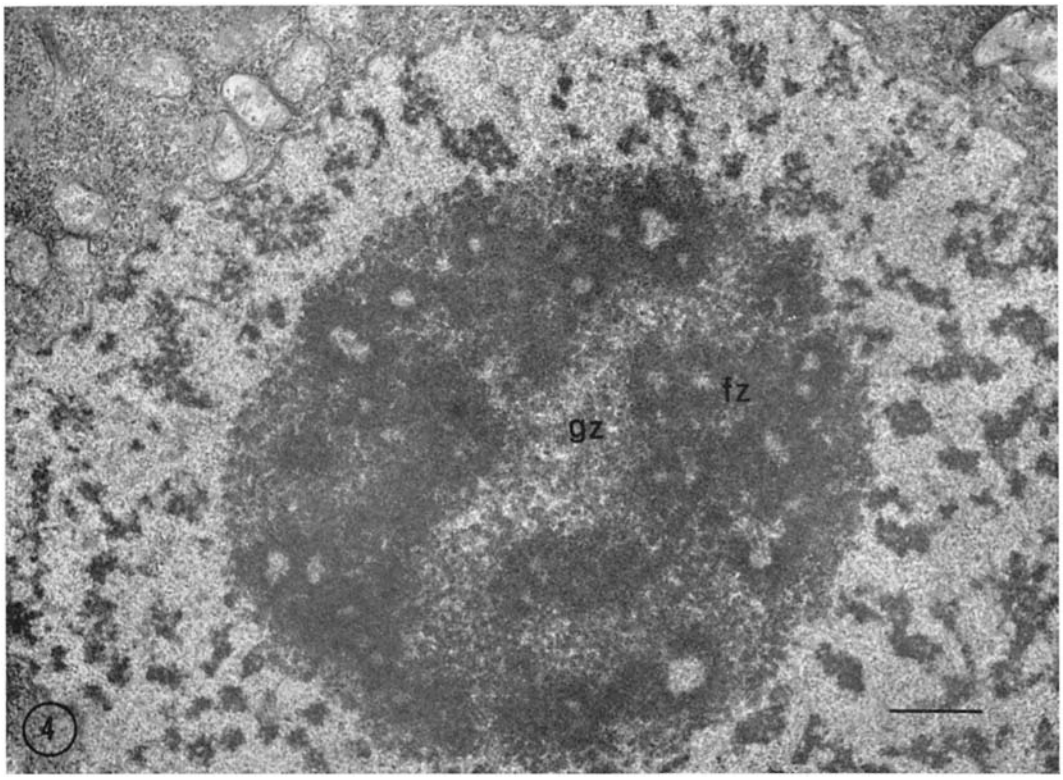


FIGURE 4 Portion of an interphase nucleus (*Triticum vulgare*) illustrating the dense irregular regions (*fz*) intermingled with granular zones (*gz*) which characterize the nucleolus. The former regions consist predominantly of closely packed fibrillar material, and their distribution and gross organization is seen to resemble that of the opaque zones detected in 0.5 μ preparations (Fig. 1). The light doughnut-like spaces distributed throughout the zones under discussion presumably correspond to those seen in detergent-treated nucleoli (Fig. 3). ×12,000.

favorable preparations, are seen to be arranged into coarse meandering threads, some 0.1 μ in diameter. Such an organization of the particles, it must be pointed out, is usually more conspicuous at the nucleolar periphery.

In addition to these particulate portions, the nucleolar bodies contain irregular areas consisting mostly of densely packed fibrillar material. In both species (Figs. 4 and 5), these zones are characterized by a rather complex architecture which is strikingly reminiscent of that observed in the dense portions of nucleoli treated with "Tween 80." On the basis of observations reported in later sections of this article, the structure of these nucleolar regions is best accounted for by assuming that they contain coarse convoluted filament some 0.2 μ in diameter.

The appearance of the different nucleolar zones after the action of a number of enzymes or combinations of these will now be described.

Ribonuclease

In azure B–stained preparations, both the nucleolus and cytoplasm appear purple, whereas chromatin takes on a blue-green color (Fig. 6). Digestion with ribonuclease completely removes this metachromatic staining throughout the nucleolar mass, but the chromosomes remain mostly unchanged (Fig. 7). Ribonuclease-treated nucleoli, as expected from their biochemical composition, still contain much material which is clearly visualized with either phase-contrast or UV optics.

At the ultrastructural level (Fig. 9), ribonuclease disorganizes the cytoplasmic ribosomes and the

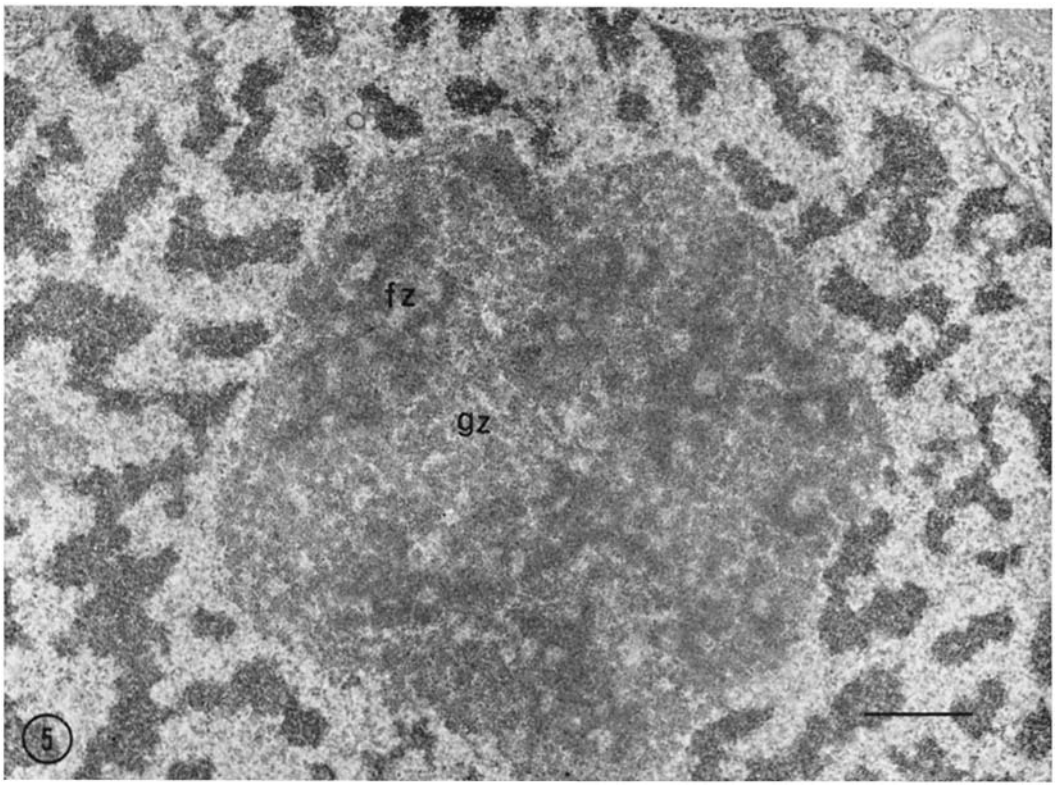


FIGURE 5 Electron micrograph of preprophase nucleus (*Allium porum*). The dense fibrillar nucleolar zones (*fz*) contain coarse filaments organized into an intricate pattern. The remaining portions (*gz*) of the nucleolus are granular in texture. The fact that chromosome strands appear to end abruptly on the nucleolar surface in the immediate vicinity of segments of loops is highly suggestive of a possible continuity between these structures. It is of interest to note that the diameter of the nucleolar loops is comparable to that of the chromosomes. × 14,000.

RNP nucleolar particles, which are no longer recognizable. Certain nucleolar zones show coarse, meandering threads some 0.1 μ in diameter consisting of fine fibrillar material. These latter structures undoubtedly correspond to similar threads, but containing RNP particles, which characterize the granular nucleolar zones in control preparations.

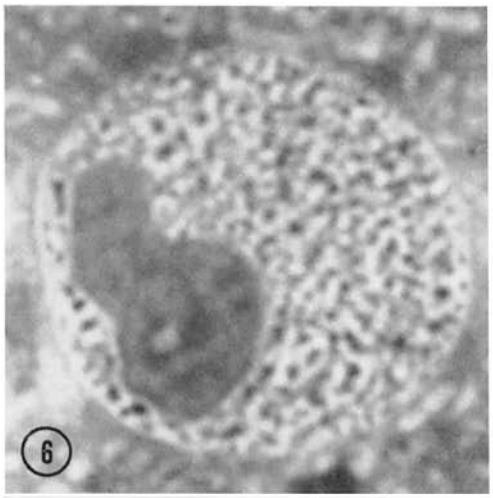
Digestion with ribonuclease is known (3, 52) to induce an increased stainability of chromatin with uranyl acetate. In our material, such procedures also emphasize, but to a rather limited extent, the presence within the nucleolus of a coarse skein already referred to above. This nucleolar component is best seen, however, when more extensive extractions of the nucleolar fibrillar zones are achieved by means of other enzymes.

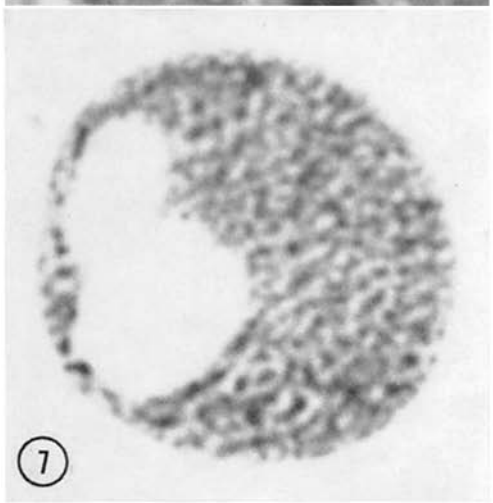
Pepsin

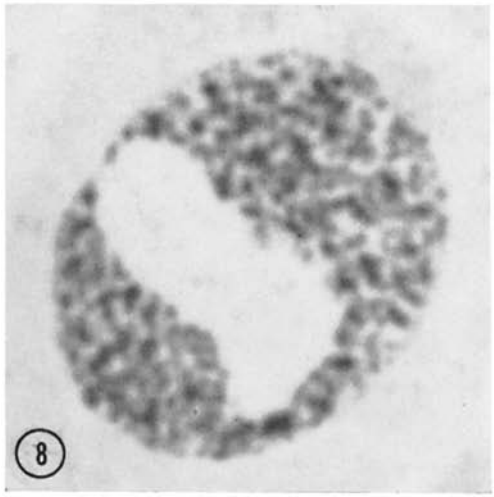
Under light microscopy, pepsin hydrolysis induces a very slight reduction in the azure B–staining intensity of the nucleolus and cytoplasm but the chromosomes appear unaffected. At the ultrastructural level (Fig. 10), the nucleolus is observed, indeed, to have lost part of its density. The fibrillar zones become quite conspicuous and more clearly exhibit the convoluted skein already observed in ribonuclease-treated preparations (Fig. 9). Since the RNP particles within the remaining nucleolar portions now also stand out more distinctly, it is most likely that pepsin extracts an amorphous material pervading the nucleolar mass as a whole.

Pepsin-Ribonuclease

When ribonuclease hydrolysis follows treatment with pepsin (Fig. 11), the RNP particles disappear







as such and the corresponding nucleolar areas become still more transparent. The skein characterizing the fibrillar portions does not seem to decrease in density more than in preparations subjected to the action of pepsin only. Likewise, staining of the chromosomes appears equivalent to that observed following pepsin digestion.

Trypsin

After 2–3 hr of exposure to trypsin (Fig. 8), the nucleolus no longer stains with azure B and the density of the cytoplasm is reduced to some extent, but less so than in ribonuclease-treated preparations. On the contrary, such extraction appears to induce a slight enhancement of the colorability of the chromosomes.

At higher magnification (Fig. 12), both the cytoplasmic ribosomes and nucleolar RNP particles are disorganized and no longer distinguishable as such as was also the case with ribonuclease-extracted preparations. The action of short extraction with trypsin on the fibrillar zones is difficult to evaluate, but more extended treatments remove much material and the nucleolar mass eventually takes on a uniformly amorphous appearance. In order to verify that nucleolar RNA was really extracted by trypsin, 0.7-μ sections labeled with uridine-^{3}H or cytosine-^{3}H were subjected to the

---

FIGURES 6, 7, and 8 This series of micrographs (*Triticum vulgare*) depicts the action of ribonuclease and trypsin on nuclear structures. × 3,000.

Fig. 6 shows an untreated preparation. The chromosomes stain blue-green whereas the nucleolus takes on a purple color. In such preparations the nucleolus exhibits a rather heterogeneous appearance and is seen to contain more intensely stained regions scattered throughout its mass.

The same nucleus is illustrated in Fig. 7 after treatment with ribonuclease. The cytoplasm and the various nucleolar regions then no longer stain with azure B. The chromosomes, however, appear unchanged.

FIG. 8. Extraction with trypsin removes azure B staining from both cytoplasm and nucleolus, as was also the case with ribonuclease (cf. Fig. 7). It may be noted that RNA has been removed to a comparable extent from the different nucleolar zones and that only a few scattered small areas of intranucleolar chromatin still show a faint staining. The chromosomes throughout the non-nucleolar portions of the nucleus, on the other hand, retain their staining capacity with azure B and even appear slightly denser than in control preparations.

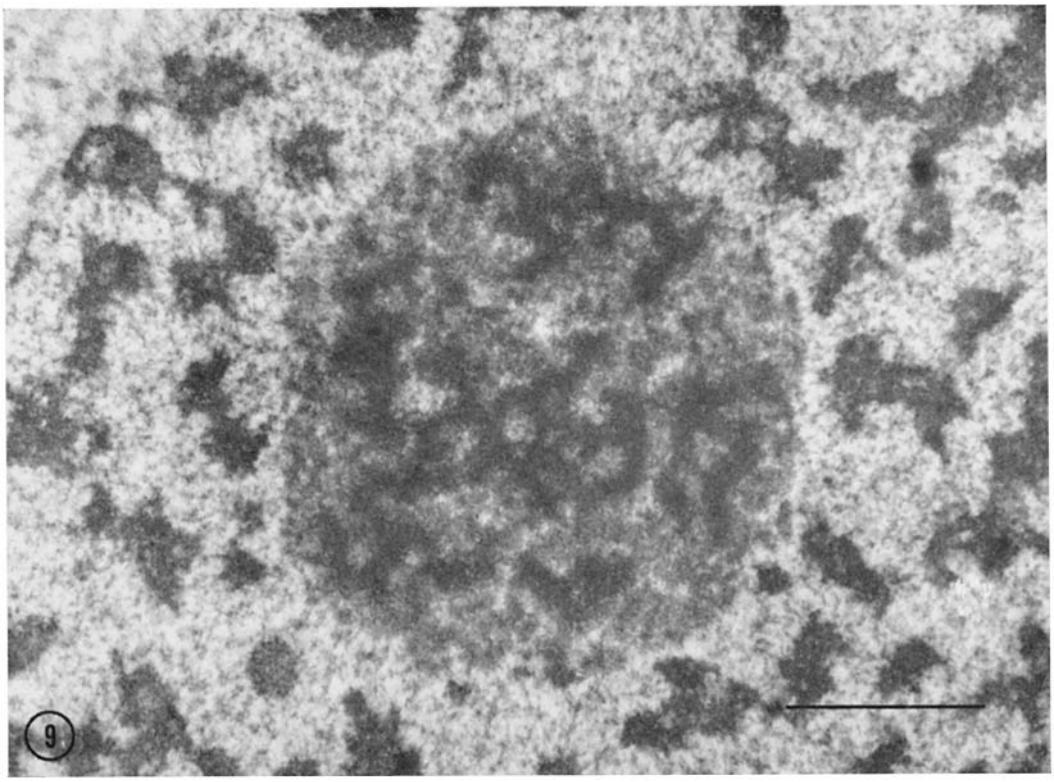


FIGURE 9 Micrograph of a preprophase nucleus (*Allium porum*) digested with ribonuclease. The cytoplasmic ribosomes as well as the nucleolar RNP particles are no longer observable. The nucleolus is now clearly seen to contain a coarse skein which, after staining with uranyl acetate, exhibits a density closely matching that of the chromosomes. ×26,000.

action of this enzyme and then radioautographed. After identical exposure, nucleoli in control preparations are heavily labeled, whereas those treated with trypsin hardly show any radioautographic grains, as expected.

Preparations hydrolyzed with both trypsin and ribonuclease, in that order, do not differ to any noticeable extent from those extracted with trypsin only.

Deoxyribonuclease

This enzyme is found to reduce the coloration of chromosomes in preparations stained with azure B (Fig. 14) but, even after prolonged digestion, certain segments of chromosomes still exhibit a blue-green color characteristic of these structures in control sections (Fig. 13).

In the course of the present study, deoxyribonuclease has been consistently observed to accentuate the heterogeneous appearance of the nucleolus following azure B staining. Regions of this organelle which, in control preparations (Fig. 13) are less densely stained with azure B, now appear still more transparent (Fig. 14). Except for some reduction in the density of the chromosomes and in the fibrillar areas of the nucleolus, electron microscopy shows no easily noticeable alteration of the organization of these nuclear structures.

When used subsequent to digestion with pepsin or trypsin, deoxyribonuclease greatly alters the architecture of both the chromosomes and nucleolus. The latter structure, for instance, becomes a great deal more transparent except for the coarse skein which remains unaffected and, as a result, stands out with considerably more contrast (Fig. 15) than in preparations treated with other enzymes. The observed disorganization of the particulate zones, even with electrophoretically purified deoxyribonuclease, is rather puzzling and warrants further investigation.

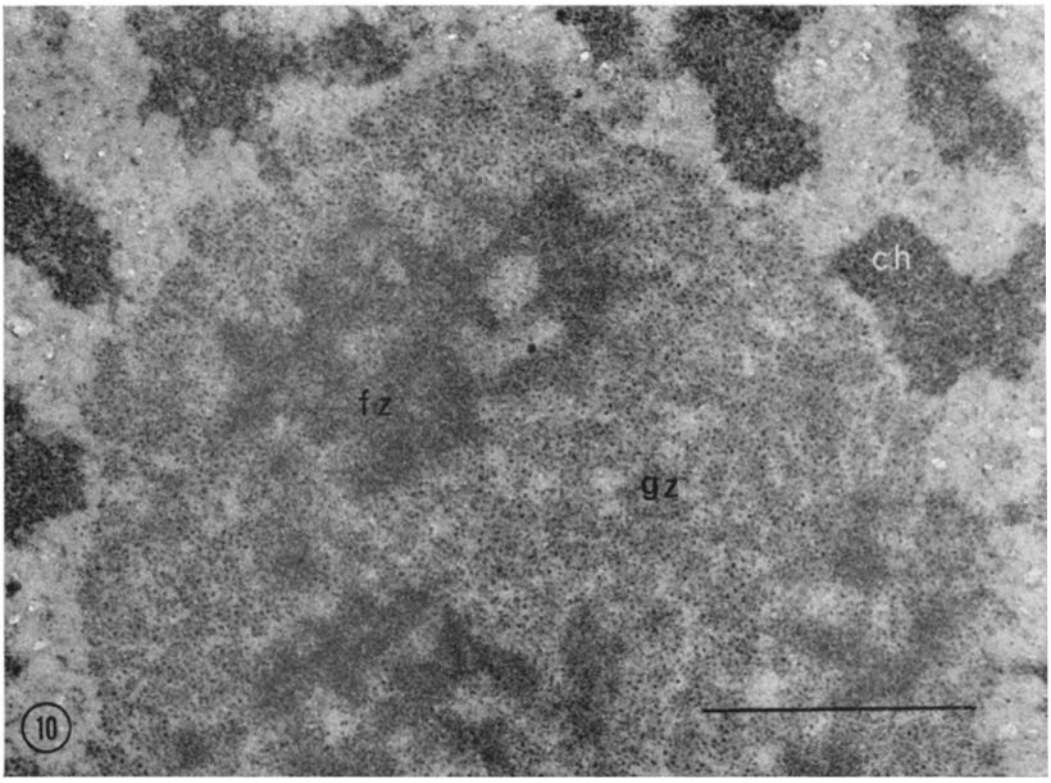


FIGURE 10 This figure represents a portion of a preprophase nucleus (*Allium porum*) after extraction with pepsin. The granular nucleolar zones (*gz*) are now more transparent and the RNP particles are more conspicuous than in control preparations. It is also more easily recognized that the fibrillar zones (*fz*) consist predominantly of convoluted coarse threadlike structures or loops. The chromosomes (*ch*), unlike the nucleolus, have lost little of their density as a result of pepsin extraction. ×36,000.

DISCUSSION

Distribution of RNA

A sizeable body of information is presently available concerning the concentration and metabolic activity of RNA within the nucleolus. It is also known that RNA is distributed throughout the mass of this organelle (2, 3, 16, 18–20, 23, 26–30, 33, 35, 36, 45, 47, 48).[1] In plant cells, staining of 0.7-μ sections with azure B before and after ribonuclease confirms this finding (Figs. 6 and 7). A comparison of electron micrographs with 0.5-μ thick preparations reveals, moreover, that the particulate nucleolar zones stain more densely with azure B than the remaining fibrillar portions.

After hydrolysis with ribonuclease, the RNP particles (Fig. 9) have disappeared as such, thus increasing the transparency of the corresponding zones which now show coarse, threadlike structures approximately 0.1 μ in diameter consisting exclusively of closely packed, fine fibrillar material. These formations are assumed to correspond to similar threads, but containing RNP particles, seen in control preparations (26, 28). It is not clear from our observations whether the fine fibrillar material now composing these threads originates mostly from the disorganized RNP particles or represents a material within which granules are immersed in intact nucleoli. However, the fact that this substance is resistant to extraction with ribonuclease indicates that it is predominantly proteinaceous in nature.

The observations that the fibrillar zones of the nucleolus stain less intensely with azure B than do the adjoining particulate areas and, moreover, that their texture and density are not affected notice-

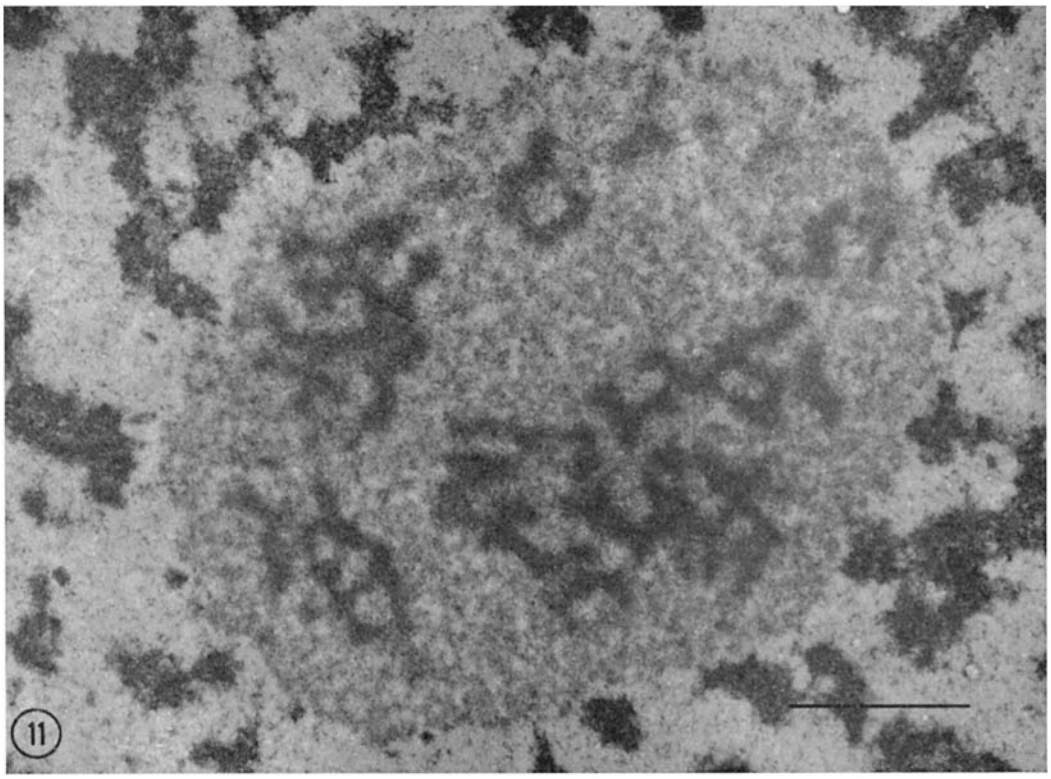


FIGURE 11 Preparation (*Allium porum*) digested with both pepsin and ribonuclease, in that order. The nucleolar RNP particles have disappeared as such (compare with Fig. 10) and the corresponding zones now consist of fine fibrillar material which is difficult to analyze. Such digestion brings out most clearly the fibrillar nucleolar zones, the bulk of which is formed by the coarse loops referred to in earlier figures. ×24,000.

ably by ribonuclease digestion strongly suggest that the former zones contain a smaller proportion of the total nucleolar RNA. Biochemical evidence supporting such a conclusion comes from the finding that isolated nucleoli have a much lower RNA: protein ratio (1:7) after removal of their RNP particles (23). This ratio is estimated as 1:2 for the nucleolar RNP particles themselves (7).

Nucleolar Proteins

From biochemical studies, nucleoli of both animal and plant cells have been known for some time to consist mostly of proteins (5, 9, 10, 34, 51). Although these proteins are apparently quite heterogeneous and their exact nature is still partly unresolved, they are thought to belong to three or four broad classes (6, 9, 10).

In the present study, digestion of the nucleolus with pepsin was observed to affect both the fibrillar and particulate areas of this organelle (Fig. 10). These results closely match those reported by Marinozzi (35) and are best explained, in accord with this author, by assuming that an amorphous matrix pervades the nucleolar mass and is susceptible to extraction with pepsin.

On the basis of the specificity of action of pepsin, it may be concluded that the matrix pervading the nucleolar mass consists of proteins. Unfortunately, whereas this specificity of action of proteinases on undenatured proteins is well documented (12), it is generally recognized that many uncertainties still remain (discussed in reference 29) about their mode of action on fixed tissues. In the case at hand, the problem of identifying the types or proteins extracted by pepsin is further complicated by the demonstration that the various nucleolar protein

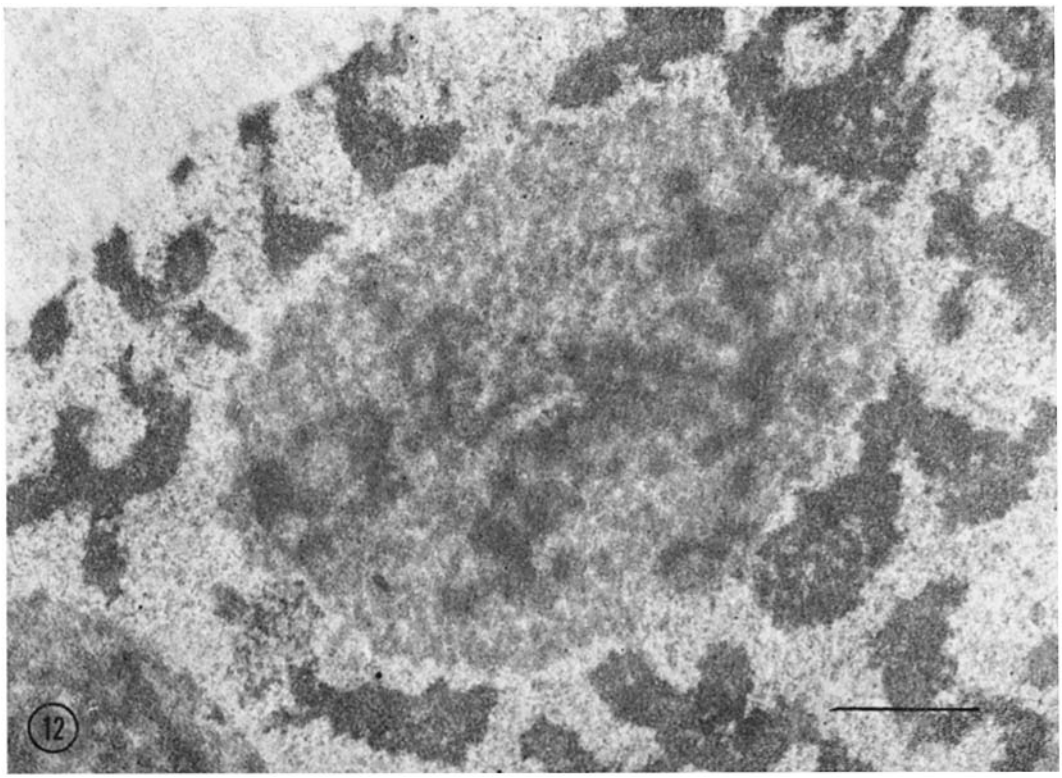
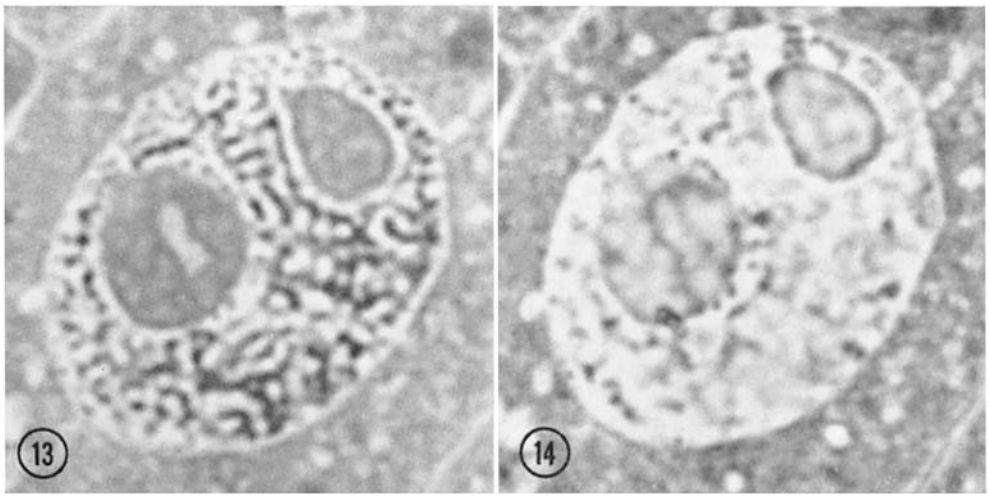


FIGURE 12 Portion of a preprophase nucleus (*Allium porum*) in a preparation digested with trypsin. The texture and density of the chromosomes appear to be hardly affected by this treatment. The nucleolar mass, however, is more transparent than in control preparations, and the RNP particles are no longer recognizable. A similar disorganization of the cytoplasmic ribosomes is observed. Note the persisting nucleolar loops. ×28,000.

fractions contain quite similar concentrations (6, 9, 10) of those amino acid residues which normally react with this enzyme.

When pepsin hydrolysis is followed by ribonuclease (Fig. 11), the RNP nucleolar particles and the cytoplasmic ribosomes are completely disorganized (3, 28, 35, 36). The nucleolar particulate areas lose much of their density but some amorphous material still persists. This substance most likely consists of residual proteins from the disorganized RNP particles and possibly also of part of the matrix material not extracted by pepsin. Such sequential extraction with both ribonuclease and pepsin accentuates the conspicuousness within the fibrillar zone of the nucleolus of a coarse skein (Fig. 11), the nature of which will be discussed further on in relation to intranucleolar chromatin loops.

In spite of the reservations just made concerning the specificity of proteinases under the present experimental conditions, indications exist that certain of the proteins extracted by trypsin are different from those hydrolyzed by pepsin. For instance, it is clear from previous cytochemical observations (28, 30) and from those presented here (Fig. 12) that the nucleolar RNP particles, as well as the cytoplasmic ribosomes, are particularly sensitive to the action of trypsin. Whether this disorganization of the cytoplasmic ribosomes arises uniquely as a result of the hydrolysis of their basic proteins (13, 32) by trypsin is difficult to assess at present. The same uncertainties hold in the case of the nucleolar RNP particles which similarly react to trypsin. Indeed, one cannot exclude the possibility that trypsin also affects the neutral and acidic protein fractions which, according to recent bio-



FIGURES 13 and 14 These two micrographs illustrate a preprophase nucleus before (Fig. 13) and after digestion with deoxyribonuclease (Fig. 14). In the control, except for a large central vacuole, the nucleolus is seen to exhibit numerous lightly stained zones or lacunae presumably corresponding to the fibrillar portions of this organelle (refer to Fig. 4). The nucleolar surface, the periphery of the large vacuole, and the areas in between the lacunae consist predominantly of RNP particles and, therefore, stain more intensely with azure B. Following digestion with deoxyribonuclease, the various lacunar zones become still lighter compared to the remaining nucleolar portions, thus suggesting that material is extracted by this enzyme. The chromosomes generally stain only faintly as a result of this treatment, but certain segments, nevertheless, appear rather unaffected. ×3,000.

chemical analysis (6, 9), contain combined lysine arginine concentrations comparable to that of the basic nucleolar protein moiety.

The biochemical make-up of the fibrillar zones of the nucleolus is still poorly documented, but certain observations indicate that these zones contain a higher protein: RNA ratio (23, 44) than the particulate zones. This conclusion supports our finding that the latter nucleolar zones stain more intensely with azure B than the fibrillar portions.

The fact that trypsin, besides disorganizing the RNP particles, also destroys the ability of the nucleolar mass as a whole to react with azure B at pH4 (Fig. 8) may be taken as evidence that it has removed a great deal of the nucleolar RNA. Such removal of RNA is confirmed by the additional finding that preparations labeled with uridine-^{3}H, when radioautographed, no longer show photographic grains over the nucleolus after treatment with this same enzyme. (See Note Added in Proof.)

These cytochemical data raise the possibility that a large proportion of the RNA throughout the nucleolus is complexed with proteins susceptible to the action of trypsin. The particulate nucleolar constituents are naturally well known, as a result of both biochemical (6, 7) and cytochemical (2, 3, 28–30, 35, 36) studies, to consist of ribonucleoprotein complexes. The presence of such complexes within the nucleolar fibrillar zones, however, has so far been inferred only from cytochemical observations (28, 35, 36). Considering the fact that these fibrillar zones are intimately associated with intranucleolar chromatin in the form of coarse loops (Figs. 10, 11, 15), it is plausible that the RNA rapidly synthesized in these areas in turn associates with proteins which, according to our results, would be of a type highly susceptible to the hydrolyzing action of trypsin. Such an interaction between newly synthesized RNA and proteins might stabilize the RNA moiety during its subsequent transport to the particulate zones of the nucleolus where, conceivably, it is processed further and forms the RNP (40) particles. Similar assumptions about the possible role of such ribonucleoprotein complexes in the economy of the nucleolus have already been presented by Vincent (50).

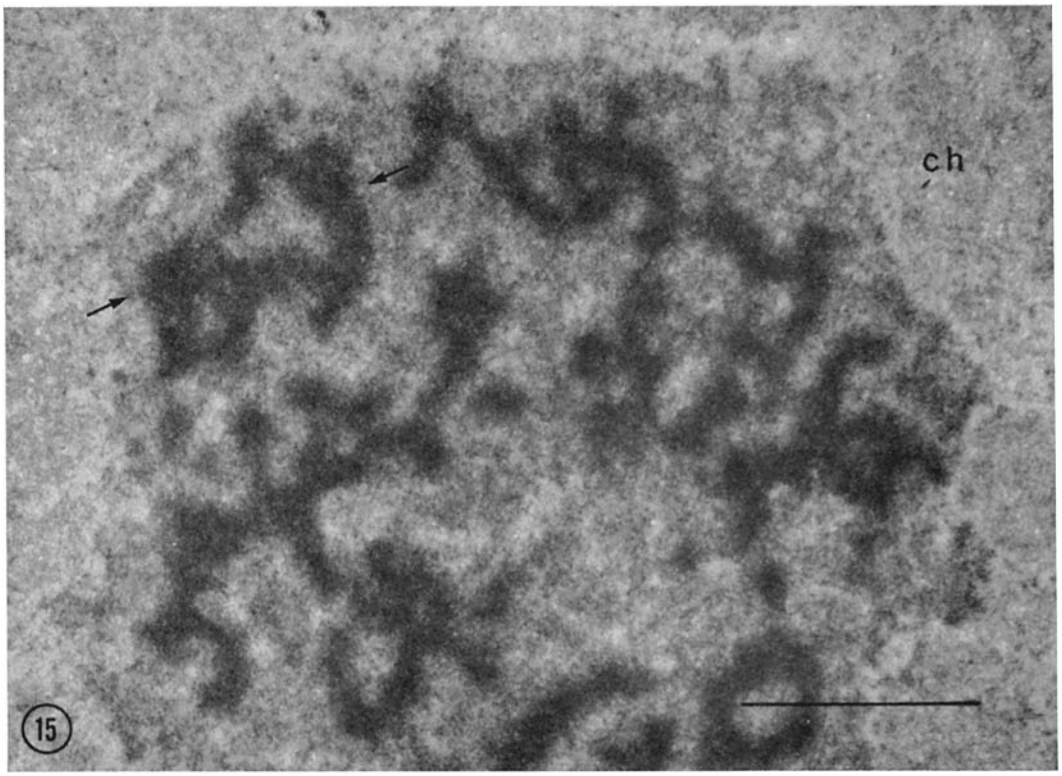


FIGURE 15 Although extraction with deoxyribonuclease gives rise to little noticeable change in the ultrastructural nucleolar architecture, important modifications are observed when this enzyme is preceded by pepsin or trypsin. The present micrograph (*Allium porum*) illustrates the effect on nucleolar structures of such a double pepsin-deoxyribonuclease extraction. The chromosomes (*ch*) show little contrast but remain fibrillar in texture. The nucleolar loops persist as such and, to all appearances, are unaffected. The fact that the granular zones are somewhat disorganized is unexpected and not easily explained at the present time. A number of the complex filamentous patterns (arrows) observed, judging from their dimensions, are thought to represent cross- or oblique sections of a coiled structure formed of the 0.2 μ coarse nucleolar filaments. This coiled nucleolar component would account for the doughnut-like patterns observed in isolated nucleoli (Fig. 3). ×31,000.

Intranucleolar Chromatin

Biochemical analysis of isolated nucleoli has furnished a large body of evidence to the effect that DNA is a constant and relatively important constituent of this organelle. Although in certain instances chromatin inclusions are readily observed after the Feulgen reaction (see reference 31 for further discussion), this is generally not the case. In recent years, therefore, demonstration of intranucleolar DNA has rather resulted from the exploitation of other experimental approaches such as radioautography (3, 11, 15, 17, 21, 38), enzymatic digestion (2, 3, 17, 28, 46), or electron microscopy (2, 3, 16–18, 22, 23, 25–28, 41, 49). Unfortunately, in most cases it is still uncertain to what exact extent this chromatin is related to the nucleolar organizer proper. A solution to this problem appears, hopefully, to be emerging from recent data which indicate that these nucleolar-organizing chromosome segments form loops throughout the mass of the nucleolus (24, 25, 28).

These chromosome loops, according to our observations, are part of the irregular dense zones forming complex three-dimensional patterns throughout the mass of the nucleolus (26, 28). It is easily verified, moreover, that this skein in turn corresponds to the coarse, meandering threads detected, under electron microscopy, within the

fibrillar zones of this organelle (Figs. 4 and 5). Such an intimate association between the loops under discussion and the latter nucleolar areas becomes still more evident after extraction with various enzymes (Figs. 9–12, 15).

The findings that this coarse nucleolar filamentous component is Feulgen-negative and, after long exposure to thymidine-^{3}H, shows very low incorporation activity in radioautographs would seem to suggest that it contains little DNA. If this interpretation is correct and, if the fact that the loops in question are some 0.2 μ in diameter is considered, one must further assume that such loops consist mostly of non-DNA substances which are remarkably resistant to the various enzymes utilized here. Recent observations by La Cour[5] point to lipids as important constituents of these filamentous structures. Such substances have indeed been reported in nucleoli on several occasions (1, 39, 42, 53), and the possibility must therefore be envisaged that they are complexed with the nucleo-protein moiety of the loops, thus protecting the latter from extraction by both nucleases and proteinases.

Apart from the cytochemical characteristics of the nucleolar loops which remain partly unsettled, much still has to be learned also about their ultrastructure. Our observations show that the nucleolar loops consist of microfibrils indistinguishable from those commonly observed in chromosomes or within the fibrillar zones of the nucleolus. For lack of further definitive information we do not feel justified at present to attempt to resolve these microfibrils into finer components and are not in a position either to verify the rather complex organization recently reported for such nucleolar loops in other plant species (25). A higher-resolution investigation of these structures is now being undertaken.

Finally, one should point out that the coarse nucleolar filaments depicted here are highly reminiscent of structures observed in a variety of animal material following quite different preparative procedures (14, 19, 31, 39). It is possible, therefore, that further work will shed light on the relationship of these latter filamentous nucleolar components and those described previously (24, 25) in plants as well as in the present report.

This investigation was supported by research grant No. A-1018 from the National Research Council of Canada.

Received for publication 10 June 1968, and in revised form 28 October 1968.

Note Added in Proof: M. Tunis (1968. *Science* **162**:912) has recently shown that commercial preparations of trypsin generally contained sufficient amounts of ribonuclease to degrade RNA. In view of these findings, the possibility must be envisaged that part of the observed action of trypsin on nuclear structures is due to the presence of ribonuclease as a contaminant. To minimize the effect of this last enzyme, observations were also made on specimens treated with increasing dilutions of trypsin solutions. It was found that 0.7 μ sections hydrolyzed during 3 hr with trypsin solutions diluted 40-fold (50 μgr/ml) still did not show any azure B staining over the nucleolus whereas the cytoplasm remained pale pink. Similar results were obtained with trypsin solutions diluted 100-fold relative to the concentration normally used in this study. Finally, when solutions of trypsin diluted 200-fold (10 μgr/ml) were used, only certain zones located within the central portion of the nucleolus lost their ability to stain with azure B. According to Tunis, this last concentration of trypsin still contains sufficient ribonuclease to hydrolyze RNA in vitro. Work has now been undertaken to purify our trypsin preparations by gel filtration and thus verify the action of this enzyme on fixed material.

BIBLIOGRAPHY

1. Albuquerque, R. M., and J. A. Serra. 1951. Nucleolar composition and the nucleolar zones of the chromosomes. *Port. Acta Biol. Ser. A.* **3**:187.
2. Bernhard, W. 1966. Ultrastructural aspects of the normal and pathological nucleolus in mammalian cells. *Nat. Cancer Inst. Monogr.* **23**:13.
3. Bernhard, W., and N. Granboulan. 1968. Electron microscopy of the nucleolus in vertebrate cells. *In* Ultrastructure in Biological Systems: The Nucleus. A. J. Dalton and F. Haguenau, editors. Academic Press Inc., New York. **3**:81.
4. Birnstiel, M. L., and W. G. Flamm. 1964. Intranuclear site of histone systhesis. *Science.* **145**:1435.

[5] L. F. LaCour. Personal communication.

5. BIRNSTIEL, M. L., and B. B. HYDE. 1963. Protein synthesis by isolated pea nucleoli. *J. Cell Biol.* **18**:41.
6. BIRNSTIEL, M. L., M. I. H. CHIPCHASE, and W. G. FLAMM. 1964. On the chemistry and organisation of nucleolar proteins. *Biochim. Biophys. Acta.* **87**:111.
7. BIRNSTIEL, M. L., M. I. H. CHIPCHASE, and B. B. HYDE. 1963. The nucleolus, a source of ribosomes. *Biochim. Biophys. Acta.* **76**:454.
8. BIRNSTIEL, M. L., J. H. RHO, and M. I. H. CHIPCHASE. 1962. Fractionation of isolated pea nuclei. *Biochim. Biophys. Acta.* **55**:734.
9. BUSCH, H., R. DESJARDINS, D. GROGAN, K. HIGASHI, S. T. JACOP, J. MURAMATSU, T. S. RO, and W. J. STEELE. 1966. Composition of nucleoli isolated from mammalian cells. *Nat. Cancer Inst. Monog.* **23**:193.
10. BUSCH, H., and C. M. MAURITZEN. 1967. Nuclear proteins. *In* Methods in Cancer Research. H. Busch, editor. Academic Press Inc., New York. **3**:391.
11. CAMARGO, E. P., and W. PLAUT. 1967. The radioautographic detection of DNA with tritiated actinomycin D. *J. Cell Biol.* **35**:713.
12. DIXON, M., and E. C. WEBB. 1964. Enzymes. Longmans, Green and Co. Ltd., London. 240.
13. ELSON, D. 1967. Ribosomal enzymes. *In* Enzyme Cytology. D. B. Roodyn, editor. Academic Press Inc., New York. 407.
14. ESTABLE, C. 1966. Morphology, structure, and dynamics of the nucleolonema. *Nat. Cancer Inst. Monogr.* **23**:91.
15. FRAYSSINET, C., C. LAFARGE, and R. SIMARD. 1968. Incorporation de thymidine tritiée dans le DNA du foie de rat. Différence d'activité spécifique entre le DNA nucléolaire et nucléaire total. *Expt. Cell Res.* **49**:40.
16. GEUSKENS, M., and W. BERNHARD. 1966. Cytochimie ultrastructurale du nucléole. *Exptl. Cell Res.* **44**:579.
17. GRANBOULAN, N., and P. GRANBOULAN. 1964. Cytochimie ultrastructurale du nucléole. I. Mise en évidence de chromatine à l'intérieur du nucléole. *Exp. Cell Res.* **34**:71.
18. GRANBOULAN, N., and P. GRANBOULAN. 1965. Cytochimie ultrastructurale du nucléole. II. Etude des sites de synthèse du RNA dans le nucléole et le noyau. *Exp. Cell Res.* **38**:604.
19. HAY, E. D. 1968. Structure and function of the nucleolus in developing cells. *In* Ultrastructure in Biological Systems: The Nucleus. A. J. Dalton and F. Haguenay, editors. Academic Press Inc., New York **3**:1.
20. HAY, E. D., and J. B. GURDON. 1967. Fine structure of the nucleolus in normal and mutant *Xenopus* embryos. *J. Cell Sci.* **2**:151.
21. HAY, E. D., and J. P. REVEL. 1963. The fine structure of the DNP component of the nucleus. *J. Cell Biol.* **16**:29.
22. HYDE, B. B. 1967. Changes in nucleolar ultrastructure associated with differentiation in the root tip. *J. Ultrastruct. Res.* **18**:25.
23. 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.
24. LACOUR, L. F. 1966. The internal structure of nucleoli. *In* Chromosomes Today. C. D. Darlington and K. P. Lewis, editors. Oliver & Boyd, Edinburgh and London. **1**:150.
25. LACOUR, L. F., and B. WELLS. 1967. The loops and ultrastructure of the nucleolus of *Ipheion uniflorum*. *Z. Zellforsch. Mikrosk. Anat.* **82**:25.
26. LAFONTAINE, J. G. 1968. Structural components of the nucleus in mitotic plant cells. *In* Ultrastructure in Biological Systems: The Nucleus. A. J. Dalton and F. Haguenau, editors. Academic Press Inc., New York. **3**:151.
27. LAFONTAINE, J. G., and A. LORD. 1966. Ultrastructure and mode of formation of the nucleolus in plant cells. *Nat. Cancer Inst. Monogr.* **23**:67.
28. LAFONTAINE, J. G., and A. LORD. 1969. Organisation of nuclear structures in mitotic cells. *In* Handbook of Molecular Cytology. A. Lima-de-Faria, editor. North-Holland Publ. Co., Amsterdam. In press.
29. LEDUC, H. L., and W. BERNHARD. 1961. Ultrastructural cytochemistry. Enzyme and acid hydrolysis of nucleic acids and protein. *J. Biophys. Biochem. Cytol.* **10**:437.
30. LEDUC, E. H., and W. BERNHARD. 1962. Water-soluble embedding media for ultrastructure cytochemistry. Digestion with nucleases and proteinases. *In* The Interpretation of Ultrastructure. *Symp. Intern. Soc. Cell Biol.* R. J. C. Harris, editor. Academic Press Inc., New York. **1**:21.
31. LETTRÉ, R., W. SIEBS, and N. PAWELETZ. 1966. Morphological observations on the nucleolus of cells in tissue culture, with special regard to its composition. *Nat. Cancer Inst. Monogr.* **23**:107.
32. LOW, R. B., and I. G. WOOL. 1966. Mammalian ribosomal protein: Analysis by electrophoresis on polyacrylamide gel. *Science.* **155**:330.
33. MACGREGOR, H. C. 1967. Pattern of incorporation of (^{3}H)-uridine into RNA of amphibian oocyte nucleoli. *J. Cell Sci.* **2**:145.
34. MAGGIO, R., P. SIEKEVITZ, and G. E. PALADE. 1963. Studies on isolated nuclei. II. Isolation and chemical characterization of nucleolar and nucleoplasmic subfractions. *J. Cell Biol.* **18**:293.
35. MARINOZZI, V. 1964. Cytochimie ultrastructurale

du nucléole. RNA et protéines intranucléolaires. *J. Ultrastruct. Res.* **10**:433.

36. MARINOZZI, V., and W. BERNHARD. 1963. Présence dans le nucléole de deux types de ribonucléoprotéines morphologiquement distinctes. *Exp. Cell Res.* **32**:595.
37. MC LEISH, J. 1964. Deoxyribonucleic acid in plant nucleoli. *Nature.* **204**:36.
38. MC LEISH, J, 1968, Chemical and autoradiographic studies of intranucleolar DNA in *Vicia faba*. *Exp. Cell Res.* **51**:157.
39. PAWELETZ, N., W. SIEBS, and R. LETTRÉ. 1967. Untersuchungen zur argentaffinreaktion des nukleolus. *Z. Zellforsch. Mikrosk. Anat.* **76**:577.
40. PERRY, R. P. 1967. The nucleolus and the synthesis of ribosomes. *Progr. Nucl. Acid Res. Mol. Biol.* **6**:219.
41. PORTER, K. R. 1960. Problems in the study of nuclear fine structure. *In* Elektronenmikroskopie. *Intern. Kongr. Elektronenmikrosk.* 4. *Berlin, 1958.* W. Bargmann et al., editors. Springer Verlag, Berlin. **2**:186.
42. SERRA, J. A., and M. M. P. SEIXAS, 1962. Demonstration of lipids in nucleoli and nucleolar regions of the chromosomes by a specific test. *Rev. Port. Zool. Biol. Gen.* **3**:255.
43. SWIFT, H. 1955. Cytochemical techniques for nucleic acids. *In* The Nucleic Acids. E. Chargaff and J. N. Davidson, editors. Academic Press Inc., New York. **2**:51.
44. SWIFT, H. 1959. Studies on nucleolar function. *In* A Symposium on Molecular Biology. R. E. Zirkle, editor. The University of Chicago Press, Chicago. 266.
45. SWIFT, H. 1962. Nucleic acids and cell morphology in dipteran salivary glands. *In* The molecular control of cellular activity. J. M. Allen, editor. McGraw-Hill Book Company, Inc., New York. 73.
46. SWIFT, H. 1963. Cytochemical studies on nuclear fine structure. *Exp. Cell Res.* **9** (Suppl.): 54.
47. SWIFT, H., and B. J. STEVENS. 1966. Nucleolar-chromosomal interaction in microspores of maize. *Nat. Cancer Inst. Monogr.* **23**:145.
48. THOMAS, P. 1966. Etude, en microscopie électronique, de l'action de la pepsine et de la ribonucléase sur des cellules méristématiques de radis et de courge. *Compt. Rend.* **262**:745.
49. UNUMA, T., K. SMETANA, and H. BUSCH. 1967. A morphologic study on the chromatin areas associated with the nucleolus. *Exp. Cell Res.* **48**:665.
50. VINCENT, W. S. 1963. The nucleolus. *In* Genetics Today. *Proc. Intern. Congr. Genet. 11th, The Hague, 1963.* S. J. Geerts, editor. Pergamon Press, London. **2**:343.
51. 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.
52. YOTSUYANAGI, Y. 1960. Mise en évidence au microscope électronique des chromosomes de la levure par une coloration spécifique. *Compt. Rend.* **250**:1522.
53. ZAGURY, D. 1957. Existence d'un complexe liporibonucleoprotidique à groupements sulfhydrilés au sein du nucléole. *Compt. Rend.* **244**: 1825.