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STUDIES ON THE ULTRASTRUCTURE OF THE PEA NUCLEOLUS

AND RELATED NUCLEAR STRUCTURES

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INTRODUCTION

Biochemical studies in these laboratories have shown that pea nucleoli are completely equipped for protein synthesis and do, indeed, synthesize proteins in vivo (Birnstiel and co-workers, 1961-1963). Chemically, they not only have RNA and protein but also DNA as an integral part of their make-up (Birnstiel et al., 1961; 1963). They have ribosomes that compare very well to those of cytoplasmic origin in regard to both structure and composition (Birnstiel et al., 1962). Such findings prompted us to look into the ultrastructure of these nucleoli with a two-fold purpose, first, to localize the reported main chemical components and second, to correlate, if possible, structure with reported function.

A third purpose arose after a brief survey of published studies on nucleolar ultrastructure. Such reports often conflict as the following paragraphs will attempt to show. Almost all such studies have been made on thin sections of plant and animal material.

LaFontaine (1958a) and LaFontaine and Chouinard (1963), working with Vicia and Allium reported that the nucleolus is differentiated into a granular region and an amorphous region. In his later paper, this author finds the amorphous region loosely packed with 60-100 A fibrils. That this component is fibrillar in nature is confirmed by other reports dealing with nucleoli from animal cells (Miller, 1962; Jacob and Sirlin, 1963). However, these other authors do not agree in other respects with LaFontaine. For example, Jacob and Sirlin (1963) believe that there is no real differentiation of the nucleolus. They find that sometimes the nucleolus consists of only the fibrillar component (as does Miller, 1962) and assume that the apparent differentiation arises due to regions of the same component being either more or less dispersed. The granular component is reported to consist of particles that average about 150 A in diameter (Porter, 1954; Bernhard et al., 1955; Swift, 1958; LaFontaine and Chouinard, 1963; Jacob and Sirlin, 1963; etc.) in a wide variety of plant and animal cells. Such particles are susceptible to ribonuclease and resemble cytoplasmic ribosomes according to Marinozzi (1962). The same author reports (with rat pancreas material) an inner core in the nucleolus that consists of 50-100 A wide network of proteinaceous strands. Whether this inner core is synonymous with the "amorphous" or fibrillar component (which also is generally reported to be internal to the granular region) reported by other authors is not known.

Again, while many authors report (LaFontaine, 1958b; Brown and Ris, 1959; Swift, 1959a; Jacob and Sirlin, 1963; etc.) a similarity between structural elements in nucleoli and chromosomes, only a few (for example, Davis, 1960) contend that DNA forms an integral part of the nucleolus proper. Indeed, many doubt the existence of DNA in nucleoli. Studying rat liver cells, Watson and Aldridge (1961) observed that "clumps of dense and possibly stranded material ... lie on the surface of the nucleolus and also congregate at the surface of the nucleus, in general following the pattern of feulgen positive material." Such a criticism could be offered for other efforts to localize DNA in nucleoli in situ like, for example, the studies of autoradiography at the electron microscope level by Hay and Revel (1962, 1963). Thus, proof for the existence of DNA in nucleoli at the ultrastructural level is yet to be found.

According to LaFontaine (1958a), Porter (1958) and Sun (1961), extremely dense particles characterize nucleoli of many plant species. However in a later paper, LaFontaine (LaFontaine and Chouinard, 1963) did not find such dense particles in his studies of the same material. The nature of these particles is not well understood (Sun, 1961). Tandler (1960) feels that such regions may be composed of inorganic phosphates.

Thus the ultrastructure of nucleoli remains far from clear. While we have also included studies of pea nucleoli in situ, our

primary effort was to study isolated nucleoli as they offer the possibility of observing structure in a new material that was relatively well characterized biochemically.

MATERIALS AND METHODS

Materials

The choice of material for this study was Pisum sativum var. Alaska. This is the same material as that used for biochemical investigations in these laboratories. All chemicals that were used in this investigation are of chemical reagent grade. Sugar, C & H "Bakers Special," was used in the initial process of separating the embryonic axes from the cotyledons. Deoxyribonuclease (crystalline) and ribonuclease (5x recrystallized) were obtained from Sigma, St. Louis, Mo.

Methods

Isolation of nucleoli. The following is a brief account of the process as developed by Birnstiel et al. (1961) and Rho et al. (1961) in these laboratories.

Approximately 100 to 150 lbs. of pea seeds (var. Alaska) were washed with detergent, soaked in running water at room temperature for 12-18 hours and sprayed continuously for another 20-24 hours. The young seedlings were harvested by a semi-automatic three step process

involving the liberation of the axes by crushing the pea seeds between grooved rollers, a floatation of the axes from the cotyledons in an approximately 0.3 M sucrose solution and a sieving step in a mechanical shaker.

All the subsequent operations were carried out at 0-4 C unless otherwise specified. The embryonic axes were then sterilized with a 0.05% solution of Purex for three minutes. After thorough rinsing with distilled water the seedlings were soaked for 20 minutes in a 0.05 M $CaCl_2$ solution and once again rinsed thoroughly. The nuclei were then liberated by passing the axes through the "pea-popper" (Rho and Chipchase, 1962) in a sucrose grinding medium (0.6 M sucrose, 6 mM $CaCl_2$, and 1 mM $MgCl_2$). After filtration through miracloth, the homogenate was then spun at 350 x g for 10 minutes in an International Centrifuge to sediment most of the nuclei and starch. The nuclei were further purified by pelleting them through a 2 M sucrose density gradient in the Spinco SW 25 rotor at 8000 rpm for 20 minutes. The purified nuclei were resuspended in 25-30 ml of high density (1.315) sucrose and ground in a Servall Omnimix at 40 volts with 0.25-0.30 ml of 0.1 M sodium citrate for periods of 30 seconds at a time till there were more than ten isolated nucleoli per every whole nucleus. If the initial preparation of whole nuclei was clumped, it was dispersed by a preliminary grinding step at 100 volts for not more than three seconds. Subsequently, this medium containing the broken-up nuclei was diluted

17% by volume with distilled water and spun at 4000 rpm in the Spinco SW 25 rotor for 20 minutes to sediment out most of the nuclei. The nucleoli were then pelleted at 14000 rpm from this supernatant. Such a preparation contains mostly nucleoli, a little of other nuclear material and rarely, whole nuclei.

The nucleolar pellet was then resuspended in a minimal amount of 2 M sucrose solution and dialyzed against a 1 M sucrose solution containing $CaCl_2$ (0.5 mM) and $MgCl_2$ (0.1 mM) for 12 hours (approximately) and then for 4-6 hours against a 0.4 M sucrose solution containing the same amounts of Ca^{++} and Mg^{++} ions. This was the basic isolated material used in these studies and also the material subjected to enzymatic extraction experiments. These will from now on be referred to as sucrose-citrate (SC) nucleoli.

Incubation and chemical determinations. Sucrose-citrate nucleoli were incubated as specified in Table 1. Controls were kept at 0 C also. After incubation the nucleoli were chilled to 0 C and aliquots were set aside for chemical determinations of RNA, DNA and protein. The rest of the chilled nucleolar suspension was used to prepare the material for electron microscopy. These experiments have been repeated three times.

RNA was extracted for 48 hours in 1 N perchloric acid at 0 C. The DNA was subsequently extracted with 0.5 N perchloric acid for 20 minutes at 70 C (Ogur and Rosen, 1950). Nucleotides were measured

Table 1. Incubation Conditions of Different Experiments.

Treatment	Concentration in μg/ml of nucleolar suspension			pH at start			Duration in minutes	Temperature
	1	2	3	1	2	3		
Nucleoli alone	-	-	-	-	-	-	-	0 C
Nucleoli alone	-	-	-	6.7	7.1	6.9	30	30-34 C
Nucleoli alone -ribonuclease	5	50	50	6.5	6.6	6.9	30	30-34 C
Nucleoli alone -deoxyribonuclease	5	5	5	6.5	6.6	6.9	30	30-34 C

Incubation mixture: 8-10 ml of nucleolar suspension in 0.4 M sucrose with 0.5 mM $CaCl_2$ and 0.1 mM $MgCl_2$; 1/2 ml per 10 ml of initial suspension of tris buffer at pH 7; final pH adjusted after addition of enzymes where applicable.

The figures 1, 2 and 3 refer to, in order, experimental details for experiments denoted as 2 SC, 3 SC and 4 SC, respectively. Conditions for experiment 5 SC were the same as for 3 SC in (2) above.

by determining their absorbencies at 258 mμ and 268 mμ on a complete UV spectrum as obtained with the Cary spectrophotometer. Protein determinations were carried out on the residual samples by the biuret reaction. According to Birnstiel et al. (1962), the treatment with perchloric acid does not cause detectable hydrolysis of biuret positive materials.

Electron microscopy. Isolated subnuclear fractions and sections of fresh seedlings formed the main materials for this study. Also, samples taken directly from various stages of the isolation procedure were studied. Osmium tetroxide, either buffered with s-collidine (pH 7.2-7.4) in equal proportions, or mixed with an equal volume of 0.4M sucrose (with Ca^{++} and Mg^{++} ions, pH 5.8-6.6) was used as the fixative for periods of 30 minutes to 12 hours. DeRobertis (1956) found that Ca^{++} ions may help in the preservation of nuclear structures. In addition, Caulfield (1957) considers a non-electrolyte like sucrose preferable to NaCl (as is the case with the fixative formulated by Kellenberger et al. (1958)) as an osmoregulator for natural preservation of structures. Therefore, our use of a Ca - Mg - sucrose-osmium fixative seems logical.

The material was then dehydrated through graded series of alcohol and two changes of propylene oxide. The dehydrated material was then infiltrated with Luft's (1961) epoxy resin for two to six hours. We used the following formulation:

Epon 812 (Shell Chemical Company)	5.92 g
Dodecenyl succinic anhydride (DDSA)	1.79 g
Nadic methyl anhydride (NMA)	4.04 g
2,4,6-tri(dimethylaminomethyl) phenol	0.2 ml

The material was then transferred into freshly prepared plastic in gelatin capsules and polymerized at 60 C overnight. Ultrathin sections (350-700 A) of the material were then obtained with the aid of the LKB 4800 Ultratome. Sections were supported on 200 mesh copper grids previously covered with a parlodion film coated by evaporation of carbon.

The following compounds were employed as post-stains in an effort to improve the contrast of the cellular organelles being studied:

0.01 M aqueous solution of uranyl acetate (UAc), pH 4.4, 20-45 minutes.

1% $Pb(ClO_4)_2$, pH 4.4, for one to twelve hours.

$Pb(OH)_2$ solution (Karnowski, 1961) for 30-75 minutes, pH over 10.

The lead compounds produce sufficient contrast if used by themselves. However, sometimes, staining with UAc was combined with $Pb(ClO_4)_2$ in an effort to understand whether there is any differential staining of the nucleolar components by these two stains. Except for lead hydroxide, the same batch of staining solutions was used throughout these experiments.

A rather extensive literature can be found on the subject of staining for electron microscopy (Gersch et al., 1957; Huxley and co-workers, 1957, 1960 and 1961; Watson, 1958a, 1958b, 1962; Zobel and Beer, 1961; Swift and Adams, 1962; etc.). However, very few reports claim any degree of specific staining of any tissue component by any of these stains. According to Beer's classification (Beer, 1962), the above listed stains are members of a group of heavy metal ions forming mostly coordination complexes with tissue components. Control of physical and chemical conditions under which a stain is used, particularly the concentration and pH, are considered to be critical in staining (Wolfe et al., 1962). They found that UAc in concentrations of 10^{-5} M and 10^{-4} M stained DNA preferentially, while at 10^{-2} M and 10^{-1} M, both DNA and protein films were stained. Watson (1958b) considers that lead hydroxide is the most generally useful of the heavy metal stains. However, there is some difficulty in choosing the correct time of staining; if the time is too short, only the surface of the section is stained and a misleading impression of the fine structure of the specimen is obtained. If the time of staining is too long, extraction of tissue component occurs. Also, it is difficult to prevent a dense carbonate settling on the surface of the section while it is being stained. We have tried to overcome this precipitation by staining in a CO_2 free atmosphere and washing the grids exhaustively in previously boiled and cooled distilled water. We reject any grids in which we suspect any extraction had occurred. Osmium

tetroxide itself provides additional contrast to the material, particularly for the lipoprotein membranes of the cell. Barr's (1954) work on numerous biological compounds suggests that staining properties of the fixative on proteins would be dependent on their amino acid constitution. From empirical considerations it is suggested that protein reactions with OsO_4 are roughly dependent on tryptophan, cysteine and histidine/2 content in the protein.

The stained sections were examined in a Phillips EM 200 electron microscope, using 80 kv, and 20μ objective apertures. Photographs were taken on 35 mm Kodak non-perforated fine grain positive film and enlarged 6.5 times on suitable printing papers.

PART A. OBSERVATIONS ON NUCLEOLI

SECTION 1. Ultrastructure of Nucleoli in situ

These observations were made on cells of the hypocotyl regions of 48-72 hour old pea seedlings. They are presented here because we need a reference for our observations on isolated nucleoli. Further, they will help to provide the link between the published work and our results on isolated nucleoli.

Observations to date suggest that pea nucleolar ultrastructure varies according to the type of tissue in which it is located, that is, nucleolar structure changes as the cell differentiates. The term "differentiation" is used to describe here the stage of vacuolization of a cell. Thus, a meristematic cell has no vacuoles and the total cell volume is occupied by a large nucleus and the cytoplasm. Semi-differentiated cells have a few vacuoles. The cells appear larger, but as yet the cytoplasm and the nucleus occupy 50% or more of cellular volume. Cells in a fully differentiated state have a major portion of their volume devoted to a single vacuole leaving a very small area in the proximity of the cell wall to the cytoplasm and the nucleus.

In general, nucleoli in meristematic cells are most electron dense after post staining with heavy metals. As the cells become more differentiated, the density of nucleoli approach more closely that of the rest of the nucleus (Figs. 1-3).

Nucleoli in meristematic pea cells in interphase. These commonly appear like that represented in figure 1. Their structural details compare well with most published descriptions of nucleolar ultrastructure from both plant and animal tissues.

Such nucleoli are characterized by a distinct granularity (Fig. 1) because they contain many 120-160 A (a few also of 80 and 180 A) diameter particles. Though the particles are not restricted to a narrow peripheral ring (as reported by some workers), such granules appear always to be more concentrated in the peripheral areas of the nucleolus. Often, they form wedge shaped or finger like projections to the center of the nucleolus (Fig. 1). In appearance, these granules compare well with the 150 A particles reported by LaFontaine (1958a; Vicia; Allium), LaFontaine and Chouinard (1963; Vicia), and by Jacob and Sirlin (1963; salivary gland cells in sciarids). Marinozzi (1962) reports that such particles compare well to cytoplasmic ribosomes. Further, such granules appear often to be arranged as a string of beads (Fig. 1). Sometimes, however, they do not appear as distinct particles or granules but rather as denser portions of 50-80 A strands.

The second structural feature to be observed in these pea nucleoli is the presence of 60-100 A fibrils or strands (Figs. 1 and 26). Such fibrils occupy small patches that are usually located in the central region of the nucleolus (Fig. 1). Such strands appear to be of the same density as the chromatin strands outside. Neither the chromatin strands nor the above mentioned nucleolar strands appear to be double. The thinnest strands that can be identified in these nucleoli average 20-30 A in width. Because the areas where such strands are usually present are devoid of the 120-160 A particles, we will call such areas the non-particulate areas of the nucleolus.

The non-particulate areas of meristematic pea nucleoli are also characterized by some large, densely staining granules. Such granules appear similar to the ones that have been reported in many plant nucleoli by Sun (1961), LaFontaine (1958b), and others. In size they range from 160-320 A in diameter. A majority of such granules average 210-230 A in diameter. Often, 20-30 A wide strands are associated with these granules (arrows, Fig. 1; also in Fig. 2).

Nucleoli in semi-differentiated cells. As cells differentiate more and more, the granularity of the nucleolus becomes less and less evident (Figs. 2 and 3). Figure 2 describes the type of nucleolus seen most commonly in semi-differentiated cells. Such a nucleolus appears somewhat intermediate in appearance between the predominantly granular

nucleoli such as that of figure 1 and the predominantly fibrillar nucleoli (Fig. 3) commonly found in well-differentiated cells.

The nucleolus of a non-meristematic pea cell has very few, if any, recognizable 120-160 A particles. More often, the absence of such 120-160 A particles is decisive. Loosely distributed 60-100 A wide strands (thick arrows, Fig. 2b), a few thin strands of about 20-25 A, and large dense granules are the only distinct structural components of such nucleoli.

A further point of interest in semi-differentiated nucleoli is the appearance of the dense granules (see Fig. 2b). These granules now appear to be less compact (than the ones seen in Fig. 1) and sometimes appear to fray out into strands (arrows, Fig. 2b). Sometimes regions of such granules are less dense and give the appearance of having either convoluted or coiled strands (some arrows, Fig. 2b). These observations thus suggest that these granules may in reality be coils of strands.

Nucleoli in well-differentiated cells (Fig. 3). These have been the least well studied nucleoli in this investigation. However, the nucleoli that we have observed so far in such cells lack both the large dense granules and the 120-160 A particles. Such a nucleolus has for the most part coarse strands of approximately 80-100 A diameter. Furthermore, it is difficult to recognize any differences in structure between many regions in the nucleolus and the denser areas of chromatin (see Fig. 3b).

In summary, these studies on in situ pea nucleoli indicate clearly the variability of nucleolar ultrastructure. Preliminary evidence indicates that this may be related to the stage of differentiation of the cells in which the nucleoli are located. Also, these studies suggest that the most often found pea nucleolus is essentially fibrillar in nature, since meristematic cells form only a minor fraction of the cell population of a pea seedling.

SECTION II A. Chemical Analyses of Isolated Nucleolar Fractions

The isolated nucleolar fraction used in this study is a crude preparation consisting mostly of nucleoli, some chromatin, and occasional whole nuclei. The chemical composition of such a fraction is represented in Table IIa and is compared with data obtained with purer fractions obtained from the same material by other workers in Table IIb. On the average, a purified nucleolar preparation is composed of approximately 12% RNA, 6% DNA, and 80-85% protein (Birnstiel et al., 1962).

The chemical composition of isolated pea nucleoli is dependent on many factors. The age of seedlings from which the nucleoli are isolated is one factor (Birnstiel, unpublished data). Further, the fractionation pattern and efficiency of recovery depend largely on the previous experience of the nuclei and are very sensitive to $CaCl_2$ concentration (Birnstiel et al., 1962). Also, prolonged grinding beyond an optimum time results in a partial elution, especially of RNA, from nuclear components (Birnstiel, personal communication). Some or all of the above factors, combined with the fact that the isolated nucleolar fraction for these structural studies was not carefully purified, allow a rational-

ization of the data represented in Tables II a and b.

Since the present investigation dealt mainly with dialyzed nucleolar material, an analysis of the supernatant of dialyzed nucleoli was done in an effort to ascertain whether dialysis itself eluted a significant portion of the nucleolar components. Such an analysis (from the supernatant of about 20 ml nucleoli) yielded approximately 34 μg of RNA (less than 15%), 180 μg of protein (less than 15%), and did not contain more than 5 μg of DNA (less than 5%), indicating that there was negligible loss, if any, of these components from the nucleolar fraction during dialysis. Also, there was no significant difference in quantity of RNA and DNA between incubated (32 C, 30 minutes) and non-incubated (stored at 0 C) nucleolar fractions. There was, per ml of incubated fraction 2.2 μg less of RNA (ca. 10%) and 2.6 μg more of DNA (ca. 12%).

Data in Table II c indicate the effectiveness of the various extraction treatments employed in this study. The conditions of incubation, dosage, etc., have already been specified in Table I under materials and methods. The protein values in the table have not been corrected for the contribution made by the enzymes themselves. Further, the data are subject to the following limitations. During the various extraction treatments, the treated materials clump, making resuspension difficult. Thus, aliquots separated for chemical analyses do not necessarily have strictly the same amount of material, making

comparison difficult. Also, the experiments are not comparable with each other on an absolute basis since the amount of material per ml of suspension was not standardized.

Data represented in Table II c indicate then that about 60% of the RNA is removable by ribonuclease treatment and that increasing the dosage of enzyme by tenfold did not result in its further loss (compare 2 SC to 5 SC). The data for 4 SC ribonuclease treatment is considered an aberration that might be due to an abnormally large amount of material (as a result of clumping) in the portion allotted for analysis as compared to the control. Deoxyribonuclease was used at a constant dosage of 5 μg per ml of nucleolar suspension so as to keep its ribonuclease activity to a minimum. At such dosages, deoxyribonuclease was able to effect at least a 60% reduction in DNA content of the nucleolar fraction.

In summary, the chemical composition of our nucleolar fraction is different from those obtained for purified nucleolar preparations. Our chemical data thus reflect the mean composition of a nucleoli-rich fraction containing some chromatin as contaminant. Dialysis and incubation did not alter the gross chemical composition of this fraction significantly. Finally, our data on the effectiveness of nuclease extraction do show that a major proportion of nucleic acids was extracted by the respective enzymes.

TABLE II. Data of Chemical Analyses on Isolated Nucleolar Fractions.#

a. Composition of isolated nucleolar fractions.

Expt.	%RNA	%DNA	%Protein	Remarks
4 SC	7.7	6.9	85.4	material dialysed to 0.4 M sucrose with Mg and Ca ions
5 SC*	5.8	7.2	87.0	material diluted to above concentration

The sum of RNA, DNA and protein is taken as 100.

b. Relative composition of RNA, DNA and protein in isolated subnuclear fractions.

Source	Fraction	RNA :	DNA :	Protein
2 SC	crude nucleoli	1.2	1	-
4 SC	crude nucleoli	1.1	1	12.4
5 SC*	crude nucleoli	0.75	1	11.2
Birnstiel et al.(1962)	nucleolar apparatus	2.6	1	12
Birnstiel et al.(1962)	chromatin	0.45	1	2.5
Birnstiel et al.(1962)	whole nucleus	0.75	1	6

TABLE II (Cont'd)

c. Composition of isolated nucleolar fractions after various extraction treatments.[a]

Component	Control	Ribonuclease treated	Deoxyribonuclease treated	Expt.[#]
RNA	22.7	8.7, 38	15.2, 64	2 SC
	7.7	5.8, 75	10 , 130	4 SC
	19.7	8.4, 43	26.7, 136	5 SC*
DNA	19.4	13.9, 74	5.8, 30	2 SC
	6.9	13.8, 210	2.8, 41	4 SC
	26.7	22.3, 87	4.7, 18	5 SC*
Protein**		VALUES NOT DETERMINED		2 SC
	85.8	123.8, 156	83.3, 92	4 SC
	298	276 , 93	250 , 84	5 SC*

[a] All values in micrograms per ml of nucleolar fraction. Underlined numbers represent per cent remaining.

[#] Material alloted for chemical analysis in expt. 3 SC was lost before actual determinations could be made.

* Control was incubated for one hour at 32 C whereas the treated materials were incubated for half an hour only.

** Protein values indicated in ribonuclease and deoxyribonuclease treated columns include the contribution made by the enzymes themselves.

SECTION II B. Ultrastructure of Isolated Nucleoli

a. Characteristics of Control Nucleoli

Most of our studies on the ultrastructure of isolated nucleoli have been done on crude nucleolar fractions dialyzed to 0.4 M sucrose (with Ca^{++} and Mg^{++} ions) and incubated in the same solution for half an hour at 30-33 C. First, however, a preliminary experiment was done to be sure that dialysis does not extract drastic amounts of the nucleolar components from the nucleoli. Chemical determinations (previous section) showed that there was no major loss of either RNA, DNA, or protein from the nucleolar fraction during dialysis or incubation. We will now establish that such preparative procedures do not destroy the ultrastructure of nucleoli but, in fact, alter it in such a way as to favor a more critical analysis.

Comparison of structure in non-dialyzed and dialyzed control nucleoli. In a preliminary experiment, non-dialyzed control nucleoli, fixed in equal amounts of 2% osmium tetroxide and 2 M sucrose (we will recall that the nucleolar pellets are taken up in 2 M

sucrose) were compared with dialyzed control nucleoli fixed in a 1% solution of OsO_4 in 0.4 M sucrose (with Ca^{++} and Mg^{++} ions).

It is necessary for us to digress at this point to report the existence of a new nuclear body in pea cells (Fig. 4). In figure 4 an ovoid body is seen attached to the larger oval structure, the nucleolus. At least at the ultrastructural level, such a body has not been described so far in published literature. Our studies to date indicate that they are different from nucleoli, chromatin or chromosomes, the "elementary nucleoli" of Jacob and Sirlin (1963), or the paranucleolar bodies reported by LaFontaine (1958b). We have named these bodies "karyosomes." Karyosomes have been seen not only in isolated nucleolar fractions (prepared at different times and in different ways), but also within isolated whole nuclei (Fig. 14) and in in situ pea nuclei (Fig. 26). Karyosomes need not be attached to nucleoli always and, in fact, are found more often free amongst chromatin masses (Figs. 8, 9 and 22). Because we think these bodies have not been described before, we have allotted Part B of this dissertation to the detailing of our observations on their structural characteristics.

Figures 4-7 show nucleolar ultrastructure before and after dialysis. Both non-dialyzed (Figs. 4 and 5) and dialyzed (Figs. 6 and 7) nucleoli show the same basic pattern of nuclolar ultrastructure. At least two distinct components can be observed in these nucleoli. One component is a lightly staining material that forms a network in the

nucleolus (Figs. 4 and 6). This background array of strands is referred to as nucleolonemata by Busch et al. (1963) in their descriptions of similar structures in isolated nucleoli of Walker tumor cells. However, because the term "nucleolonemata" was originally given by Estable and Sotelo (1951, 1955) for a network of strands seen in the light microscope, we will refer to our background strands as "strands of the nucleolonemata." The second component we refer to is clusters of densely staining material which seem attached to the strands of the nucleolonemata. (We will refer to these dense structures from now on as the clusters of dense granules.)

Figures 4, 5, 6 and 7 show nucleoli stained with $Pb(OH)_2$ or $Pb(ClO_4)$ separately (Figs. 4 and 7) or with UAc and $Pb(ClO_4)_2$ (Figs. 5 and 6). When a nucleolus is stained with $Pb(ClO_4)_2$ alone (Fig. 7), the nucleolonemata are very much less evident than when stained in combination with UAc (Figs. 5 and 6) or with $Pb(OH)_2$ alone (Fig. 4). The only structures that stand out clearly now are the clusters of dense granules (Fig. 7). When UAc is used in combination with this stain (Fig. 5), the visualized nucleolar ultrastructure appears very similar to that seen after staining with $Pb(OH)_2$ alone (Fig. 4). However, the UAc and $Pb(ClO_4)_2$ combination appears to obliterate the substructure of the dense granules that is evident when $Pb(OH)_2$ alone is used (compare Figs. 4 and 5). According to Wolfe et al. (1962), both DNA and protein would be stained (rather than DNA alone) by UAc at the

concentrations we have used (0.01 M). Therefore, we have argued that $Pb(OH)_2$ alone would permit a more complete and detailed visualization of nucleolar ultrastructure.

In a non-dialyzed nucleolus the strands of the nucleolonemata measure 75-120 A in diameter and occasionally show further substructure (Figs. 4 and 5). Such 75-120 A strands do not seem to form a true network in the nucleolus; rather, they seem to run alongside or across each other without branching. The interstrand region appears structureless and of the same density as the supporting film of parlodion. Relatively large areas, empty or nearly so, appear circular in many isolated nucleoli. We will refer to such regions as vacuoles.

The clusters of granules enmeshed in the nucleolonemata are interpreted not to be portions of the strands of the nucleolonemata. Often they appear to be dense regions of strands or as short dense fibrils (Figs. 4-7). When there is no close packing of such dense structures, they appear as short dense fibrils. They appear as strands when many such clusters of "granules" are close together and seem to be interconnected with each other. Where the interconnected granules form a chain, such a chain or strand measures 60-120 A in diameter (mode, 80 A).

A third component can be occasionally seen in these nucleoli. These are single strands that appear somewhat more compact than the

strands of the nucleolonemata. These single strands are usually seen in close association with the dense fibrils. They are approximately 20 A wide (arrows, Fig. 4).

Dialysis produces nucleoli with a less compact structure (Figs. 6 and 7). The strands of the nucleolonemata now average mostly 200 A wide (range 100-200 A). The interconnected dense fibrils seen earlier also in non-dialyzed control nucleoli now average 80-120 A in width. Sometimes, they now appear to be divisible into two 40-60 A wide strands (Figs. 6 and 7).

The strands of the nucleolonemata appear to be coalesced into dense amorphous patches in certain regions of dialyzed control nucleoli. In such regions, the single 20 A wide strands that were reported to be occasionally seen in non-dialyzed control nucleoli are more often seen (Fig. 6). Also, the dense fibrils appear to be much more concentrated in such areas as well as around the "vacuoles." Such dense amorphous patches have not been seen in non-dialyzed control nucleoli.

The following observations on dialyzed and incubated nucleoli belong to a different experiment from the one described above. Hence we cannot make direct comparisons of nucleoli in Figures 4-7 with nucleoli in Figures 8-20 (these belong to the same (3 SC) experiment), for we have not standardized the age at which the seedlings are harvested. Further, there is a large variation in the rate of growth of these seedlings from time to time. Thus, while we might isolate nudleoli in one experiment largely from meristematic or near

meristematic cells, in another experiment we might isolate the bulk of nucleoli from more differentiated cells. We have already shown (section I) that nucleolar ultrastructure varies with cellular "differentiation."

Nucleolar ultrastructure as seen in dialyzed and incubated control nucleoli. A survey of dialyzed and incubated nucleoli (incubated for 30 min. at pH 7 in 0.4 M sucrose containing Ca^{++} and Mg^{++} ions, tris, at 30-33 C) indicates a further loosening of nucleolar ultrastructure (Fig. 8). A small percentage of nucleoli are found to be partially disrupted. This class of nucleoli has been the most intensively investigated. They show, apparently, a wide variation in structure (Figs. 8-13). Of these, nucleoli such as those in Figures 11 and 12 are found much less often.

The strands of the nucleolonemata in dialyzed and incubated nucleoli appear ill defined and much more diffuse (Figs. 8, 9 and 10). At times they lose their stranded appearance altogether and appear as an amorphous mass of lightly staining material (Figs. 11 and 13). In any case, they form the lightest structures or material in the nucleolus. Also, they seem to form the "background" of the nucleolus (especially clear in Fig. 12).

Many incubated nucleoli show large vacuoles, for example Figure 8. Their shape and consistent appearance suggest that they are not artifacts of isolation.

A new component that we could not see in other types of control nucleoli is noticeable in these dialyzed and incubated nucleoli. These are particles that average mostly a diameter of 120 A and sometimes 150-180 A. They appear often oval in shape and seem to have a hollow core when seen against a light background (Figs. 9-13). Such granules strongly resemble the 120 A particles we saw earlier in in situ meristematic pea nucleoli not only in size but also in their appearance and arrangement. As before, they still appear to be arranged as a string of beads or as portions of 60-100 A wide strands (Figs. 10, 12 and 13). It is difficult to decide whether such 60-100 A strands (if they are real and not a result of linear arrangement of many such granules) are part of the nucleolonemata or not.

Though such 120 A particles are present all over the nucleolus, they tend, however (as in in situ nucleoli), to be present in greater quantities in the peripheral areas of the nucleolus (Figs. 10 and 12). The paucity of such particles in nucleoli of Figures 9 and 11 may be attributable to a loss during isolation and incubation.

Patches of dense material that appear amorphous for the most part, except for some dense particulate structures similar to the ones that occurred sporadically in dialyzed nucleoli, are often seen in dialyzed and incubated nucleoli. In dialyzed and incubated nucleoli the amount of nucleolor area such dense patches may occupy varies widely, as does the size, shape, and amount of particulate structures

that seem to be present in these areas.

Such dense patches tend to occur especially around vacuoles in these nucleoli (for example, Fig. 8). As before, in the case of dialyzed nucleoli, we once again interpret such patches to be the result of a coalescing of the strands of the nucleolonemata.

Also occurring in these areas are single strands similar in appearance and dimensions (20 A wide) to the single strands that were observed in non-dialyzed and dialyzed nucleoli (arrows, Fig. 11, for example).

It is difficult to analyze the structure of the dense "granules" because of their presence in the densest areas of the nucleolus. They present a varied appearance in these nucleoli. It is possible that this component represents a loosened form of the dense clusters of granules we observed in non-dialyzed and dialyzed control nucleoli. In Figure 12 this dense material is clearly seen to exist as loosely coiled, short double strands that sometimes interloop. We apply the same interpretation to the densest material in Figure 10. These double strands are 80-120 A wide and resolve sometimes to two 40 A (approximately) wide strands. Similar dimensions, it may be recalled, were reported for the strands of the dense clusters of granules in non-dialyzed and dialyzed nucleoli. In Figures 10 and 12, regions of such strands appear to be coiled in tight loops or coils, usually towards one end, to form dense granules of varying size. In other nucleoli (Figs. 9 and 13), structures

with a similar density occur as relatively long, 80-120 A wide, strands. We interpret the associated dense granules to be due to regions where such strands are coiled tightly. The nucleolus in Figure 11 shows only vestiges of such structures and we assume that this is due to a partial disruption.

Such 80-120 A strands appear to be different from all other strands in the nucleolus. Strands or granules of like density can be seen also amongst chromatin strands (Figs. 8, 9 and 13). Further, they compare in density and dimensions to some strands in karyosomes. Since the structure of karyosomes will be discussed in part B of this dissertation, we can discuss this aspect further only after that section.

Strands of chromatin material are often found wrapped around isolated nucleoli (Fig. 8, for example). Sometimes they appear to intrude into the nucleolus (Figs. 8, 9 and 10; arrows). However, we interpret such an association of chromatin as an artifact of the isolation process.

Nucleoli fixed at various stages of isolation. In a further effort to understand structural organization in the nucleolus, whole nuclei that were (a) freshly released from young pea seedlings, (b) purified in 2 M sucrose (Fig. 14), and (c) present in the dialyzed and incubated fraction (Fig. 15) were studied. Also observed were nucleoli freshly released from sucrose-citrate nuclei, but not spun to obtain a nucleoli rich fraction (Fig. 16).

Whole nuclei in (a) and (b) above show nucleoli consisting essentially of amorphous masses of uniform density (Fig. 14). The only particulate structures that can sometimes be discerned are a few 25-40 A wide strands, 120 A particles similar in appearance and distribution to those seen in situ (Fig. 1) and in isolated nucleoli (Fig. 12), and a few dense granules that appear similar to those in the attached karyosome (Fig. 14), and also appear analogous to the dense clusters of granules seen in isolated nucleoli (for example, Fig. 4).

Nucleoli contained in nuclei which have remained intact through grinding, fractionation, dialysis, and incubation show relatively much more compact structure than isolated nucleoli in the same pellet (Fig. 15). Nonetheless, they reveal apparently double, 75-100 A wide strands, 120 A particles, and also some single strands that are 25-40 A wide. Dense amorphous patches, reminiscent of those seen in isolated, dialyzed, and incubated nucleoli (Figs. 6-13), can be seen in the nucleolus and also elsewhere in the nucleus (Fig. 15).

A study of freshly broken sucrose-citrate nuclei fixed prior to centrifugation (Fig. 16) reveals a nucleolar structure essentially like that of dialyzed and incubated nucleoli (compare Figs. 16 and 13). The following observations are worth pointing out, however. Dense amorphous patches similar to those seen in dialyzed nucleolar fractions are not seen in these nucleoli. Therefore, we assume that such patches arise as a result of incubation and dialysis.

These freshly isolated nucleoli do not show a peripheral band of chromatin wrapped around them as did centrifuged nucleoli. Therefore, such an association of chromatin may be concluded to represent an artifact of centrifugation.

Besides the 120-160 A particles, two components are clearly visible in the nucleolus in Figure 16. The denser material appears stranded and seems embedded in the lightly staining material. We interpret the lightly staining material as belonging to the nucleolonemata. The dense stranded material, we think, is what coils up in non-dialyzed and dialyzed control nucleoli to form the clusters of dense granules (compare with Figs. 4-7). Their resemblance to equally dense material in dialyzed and incubated control nucleoli is much more easily evident.

These studies then confirm that nucleolar structure in dialyzed and incubated nucleoli presents a reliable source of information for an understanding of the ultrastructural organization of the nucleolus. Comparison of nucleoli in whole nuclei or in situ nuclei with isolated nucleoli indicates that the clarity of structures seen in the latter could be due to a soluble component in pea nuclei. This component is what obscures structural details in situ or in whole nuclei but which is lost to a varied extent during isolation of nucleoli and the subsequent preparative processes of dialysis and incubation.

b. Effect of Enzymatic Extraction Treatments on Isolated Nucleoli.

Alteration of nucleolar structure by ribonuclease. When dialyzed nucleoli are incubated with ribonuclease for one half hour, most of the particulate structures in the nucleolus are destroyed. Only a few dense particles or strands belonging to the densest areas of control nucleoli are left (Figs. 17 and 18). Except for these structures which seem either analogous to, or part of, the dense clusters of strands and granules, the nucleolus has no discernible structure and consists of a uniform density material.

According to our chemical analyses (Table II c), most of the protein in nucleoli remains unaffected after ribonuclease digestion. Therefore, we assume that the amorphous residue that now pervades these nucleoli is chiefly protein. Since such a result is produced only after ribonuclease treatment, we conclude that the now absent structures in nucleoli, the ribosome-like 120 A particles and the strands of the nucleolonemata (we include here both the double strands and single strands, using the term nuclolonemata in its most general sense), are essentially ribonucleoprotein in chemical composition. However, we cannot say that all stranded structures that are now absent are composed of ribonucleic acid for the following reasons. Firstly, we cannot preclude a partial removal of DNA by ribonuclease in these experiments since commercial preparations of ribonuclease are not proven to be

specific for removing only RNA from a material that contains both the nucleic acids. Secondly, a collapsing of structure (due to the presence of mainly a proteinaceous mass now in nucleoli) could obliterate any fine stranded structures that may be sandwiched in them. We have consistently observed that many nucleoli treated with ribonuclease appear smaller than control nucleoli.

The susceptibility of the very dense structures (related to the dense clusters of granules) varies widely (Figs. 17 and 18). Nucleoli in which there is a drastic reduction of such granules to small dense specks do occur, but relatively rarely, after ribonuclease treatment (Fig. 18a). Nucleoli in which these structures appear only slightly affected (Fig. 18b) and in which they appear to remain as vestigial structures (Fig. 17) are much more common.

Such a variable susceptibility might perhaps be caused either by a fluctuating RNA content in these dense structures or by steric hindrance. The latter implication that these granules or strands are in different organizational states in different nucleoli is supported by our earlier observations (in dialyzed and incubated nucleoli) that incubation uncoils these "granules" to varying degrees (Figs. 9-13).

Nucleolar structure after deoxyribonuclease treatment. Isolated nucleoli when incubated with deoxyribonuclease show few, if any, structural alterations (Figs. 19-21). This consistent similarity of deoxyribonuclease treated nucleoli to control nucleoli suggests that

DNA plays a negligible role in nucleolar ultrastructure. It also confirms the ribonucleoprotein nature of most of the nucleolar structures as suggested by a study of their ribonuclease susceptibility. That deoxyribonuclease has been active is evident from its effect on chromatin, whose structure now appears relatively destroyed (Figs. 19 and 20).

The only structures that seem susceptible to the action of deoxyribonuclease are the densely staining clusters of particulate structures (Figs. 19-21). The common deoxyribonuclease effect on such "granules" is seen in the dense structures in Figures 19 and 20. That these structures do contain DNA is suggested by the resemblance to deoxyribonuclease treated chromatin (Fig. 19). The chromatin appears collapsed and does not seem to have clearly defined double strands as were seen in control nucleoli (Fig. 19). That a major proportion of such "granules" can still be seen as integral structures is clear evidence for a major non-DNA containing component in them (Figs. 19 and 20).

A marked degradation of the nucleolar dense "granules" like that of Figure 21 is rare. Such an occurrence may be due either to a presence of DNA in nucleoli in reality, or it may be due to a non-specific degradation of these structures during incubation with deoxyribonuclease.

Observations and conclusions regarding the composition of the nucleolar clusters of dense "granules." The following considerations

enter in any rationalization of the observed variability of ribonuclease and deoxyribonuclease on these dense strands or "granules."

(i) Deoxyribonuclease modifies these structures in a drastic way only rarely. However, there always seems to be some undefinable change in the appearance of these granules when compared with controls.

(ii) While ribonuclease affects these structures most of the time to a recognizable extent, it does not destroy them completely. Sometimes it has only a relatively mild effect on these structures.

These two observations have to contend with the following experimental limitations. (a) The ribonuclease preparations we used in these experiments are not necessarily pure. They may have some deoxyribonuclease activity. (b) The RNA in our nucleolar fraction is almost certainly complexed with protein. Therefore, the effectiveness of ribonuclease will be less than if we were dealing with pure preparations of RNA only. Even if the latter were the case, it is well known that there always seems to be a resistant "core" of RNA left after ribonuclease treatments under drastic conditions.

With regard to deoxyribonuclease, we feel confident that (a) above does not apply to any recognizable extent in our experiments. We know that (b) above does apply (chemical analyses, section II a). Further, experiments in this laboratory (Bonner, personal communication) have shown that deoxyribonuclease is roughly 20 times less

effective in degrading DNA from preparations of deoxyribonucleoprotein than from pure "solutions" of DNA.

However, we rely on some built-in controls in our sections to judge the effectiveness of these enzymes. For example, we look at chromatin in deoxyribonuclease treated sections and for the absence of the ribonucleoprotein particles in ribonuclease treated sections.

Nonetheless, even here we are limited by a possible ineffectiveness of these enzymes being related, in part, to steric hindrance. That is, the dense strands could be so knotted up that the enzymes cannot get to all the sites of breakage that they will normally affect.

We conclude that RNA is present always to a major extent and DNA to a (relatively) minor extent in these dense structures in nucleoli, taking a majority of our observations to represent the reality. We then postulate that the observed variability is due to one or more of the following causes:

(i) The RNA and DNA contained in these structures exist as nucleoproteins.

(ii) They are so knotted up around each other that they offer steric hindrance to enzyme action.

That they are sometimes loose enough to offer more sites on which the enzymes can act is clear from our observations in previous sections on isolated nucleoli. That such a loosening of coils may occur in nucleoli <u>in situ</u> is also worthwhile noting in this connection. We will

recall that we observed meristematic nucleoli with large, compact "granules" (Fig. 1). In semi-differentiated cells these same structures appeared to be composed of coils of strands (Fig. 2). Finally, in differentiated cells we observed only dense strands (Fig. 3). Such changes of these structures, we believe, are indicative of the metabolic activity of these structures in nucleoli. Further discussion of the significance, nature and origin of these dense strands in nucleoli will, however, have to be deferred to the end of section I of part B.

(iii) Finally, a complete disappearance of these structures after enzyme action may not be possible because of the following reason. The nucleases produce nucleotides (from the respective nucleic acids) which are phosphate esters of the various constituent bases. The phosphate that is now open could complex (we are assuming here that the nucleotides so produced are not washed out) with the heavy metals of our post-staining solutions to produce relict specks of the structures previously seen in control nucleoli. Such "specks" with the mass of protein remaining (that was previously complexed with the nucleic acids in these structures) could give rise to the vestigial structures we see after treatment with ribonuclease (especially) of our nucleolar fraction.

Summarizing, we conclude that the nucleolar dense clusters of "granules" are composed of ribonucleoprotein strands to a major extent and, to a relatively small extent of DNA or deoxyribonucleoprotein.

DISCUSSION AND CONCLUSIONS

In summary, the foregoing observations on sucrose-citrate isolated nucleoli show that they have four major classes of structures. These are:

(i) A material that appears amorphous and stains lightly with heavy metals after fixation with OsO_4. This, we infer, accounts for a major portion of the proteinaceous mass in pea nucleoli. We will call this amorphous mass the "nucleolar sap"; (ii) Single (20 A) and apparently double (60-80 A) ribonucleoprotein strands; (iii) 120-160 A oval ribonucleoprotein particles; and (iv) often, but not always present, densely staining coiled strands that contain RNA and DNA, probably as ribonucleoprotein and deoxyribonucleoprotein.

Organizationally speaking, the "amorphous" protein material pervades the nucleolus. Interspersed in this "nucleolar sap" of protein is a mesh of ribonucleoprotein strands. Extreme dehydration, coupled with a partial loss of protein and RNA (as, for example, during isolation, fixation of isolated nuclei and dehydration of nucleoli in 2 M sucrose), may produce a network of "nucleolonemata." Thus, we visualize the presence of nucleolonemata as an artifact caused by an

agglutination or rolling up of protein to the mesh of ribonucleoprotein strands referred to in (ii) above.

We infer this because "nucleolonemata" vary in dimensions, appearance, and are not seen in nucleoli in situ. Estable and Sotelo (1951, 1955) described and first gave the name nucleolonemata for a network of strands they saw under the light microscope. Therefore, these structures were 0.2 micron or over in thickness. The "nucleolonemata" described in nucleoli from Walker tumor tissue by Busch et al. (1963) appear as non-uniform "strands" that can be more accurately described as irregular amorphous masses.

LaFontaine and Chouinard (1963) describe 150 A granules assembled into 0.1 micron wide thread-like structures in in situ Vicia faba nucleoli but conclude that they cannot establish a direct correspondence of such structures with the nucleolonemata of Estable and Sotelo (1951, 1955). Further, our studies on in situ pea nucleoli did not reveal any threadlike structures that could correspond to "nucleolonemata."

We differ from the concept of Marinozzi (1962) that the nucleolus consists basically of a proteinaceous network of 50-100 A wide strands. Our observations show that at least in the case of peas, nucleoli consist of a basic structure of ribonucleoprotein strands. We infer this because a compacting of nucleolar size occurs following ribonuclease treatment. Following extraction with 1 M NaCl or 10 mM sodium citrate, nucleoli appear essentially to consist of strands less than 40 A

in width. There is no visible compacting following such treatments which effect a 50% or more extraction of nucleolar proteins (Hyde et al., personal communication).

We conclude that the 120-160 A particles seen both in isolated nucleoli and in situ nucleoli represent nucleolar ribosomes. Their ribonuclease sensitivity, their resemblance to cytoplasmic ribosomes, and their similarity in appearance and dimensions to ribonucleoprotein particles seen in many other nucleoli of both plants and animal cells (Miller, 1962; Jacob and Sirlin, 1963; LaFontaine and Chouinard, 1963; etc.) allow such a conclusion.

We observed both in isolated and in situ nucleoli that the nucleolar ribosomes appeared to be either opposed to, or appear as portions of 50-80 A strands. Such an arrangement was seen also in Vicia faba nucleoli by LaFontaine and Chouinard (1963). Such an appearance could arise as a result of these particles being attached to the ribonucleoprotein strands that form the basic structure of the nucleolus.

Isolated calf thymus nuclei have been shown to have 150 A ribonucleoprotein particles attached to 80 A wide ribonucleoprotein strands (Georgiev and Chentsov, 1962). These authors identify such structures as "nucleonemata." The nucleonema is one of the structural elements of chromosomes, the "residual chromosome," according to these authors, and they infer that nucleonemes form the structural elements of nucleoli. While our studies do not pertain to chromatin (we have

noted often the presence of 120-160 A particles in chromatin also), these studies of Georgiev and Chentsov (1962) offer supporting evidence for the existence in nucleoli of 60-80 A strands with attached 150 A particles.

We speculate that one of the functions of nucleoli is to make ribosomes (or their subunits) that will eventually be incorporated into the cytoplasm. Besides the above inference of nucleoli having 60-80 A ribonucleoprotein strands with attached ribosomes, we also offer the following observations suggestive of such a role for the nucleolus.

Studies on nucleoli in situ in peas show that nucleolar ribosomes are present less and less as cells differentiate and eventually disappear altogether in differentiated cells. Studies by Birnstiel and Hyde (1963) suggest that ribosomes are not the main functional units of nucleolar protein synthesis in pea nucleoli. These data, as well as other studies (Flamm and Birnstiel, 1963), suggest that nuclear ribosomes take a minor part in protein synthesis.

Further, as have been observed in many published studies on nucleolar ultrastructure, we also observe a peripheral concentration of these ribosomes in pea nucleoli. We suggest that this location may be preparatory to their transfer to the cytoplasm. Thus, according to the concept we have proposed above, the nucleolar ribosomes are a product of the synthetic activity of the nucleolus and do not form a structural

unit of the nucleolus. For this reason, we do not consider ribosomes as functional units of nucleoli.

The fourth structural component of pea nucleoli is the dense, predominantly RNA containing coiled strands. We will recall (last subsection in section II B.b) that we related these structures to the large dense "granules" in nucleoli seen in situ in meristematic and semi-differentiated pea cells.

We believe the RNA contained in these dense structures is a different kind of ribonucleoprotein--a ribonucleoprotein characterized by a high turnover rate--from that of the nucleolar ribosomes and ribonucleoprotein strands of the "nucleolonemata." These above-mentioned ribonucleoprotein structures have already been discussed in the foregoing paragraphs.

Our concept of two types of RNA is in agreement with the results of several studies (Love and Baradwaj, 1959; Stolk, 1959, 1961; Vincent and Baltus, 1960). Such studies indicate that the two types of RNA are metabolically different.

Lettre and Siebs (1954a, 1954b, 1961) also found that there are two types of RNA in the nucleolus. They characterized these as an RNA of the nucleolonema and an RNA of the "pars amorpha." The nucleolonema consists of parts of the nucleolar chromosomes. The "pars amorpha" represents the RNA or ribonucleoprotein which is being synthesized or accmulated by the nucleolar chromosomes. That the

nucleolus "contains a ribonucleoprotein which turns over rapidly," was also concluded by Busch et al. (1963).

Biochemical studies of protein synthesis in nucleoli in vivo (tobacco) and in vitro (peas) also locate the most active fractions not in the desoxycholate extractable fraction (ribosomes) but in a different ribonucleoprotein fraction (Flamm and Birnstiel, 1963). This "other ribonucleoprotein" fraction that is most active in protein synthesis in nucleoli, we think, is contained in the dense clusters of granules (the "pars amorpha" RNA) in our micrographs. We have already observed that the nucleolar dense "granules" or coils of strands vary in their shape, size and number (and are mainly located in the "pars amorpha"). We suggest that this indicates a high turnover of active products of nucleolar synthesis in these regions.

We have already concluded that these dense regions consist predominantly of RNA or ribonucleoprotein and DNA or deoxyribonucleoprotein to a much less extent. Such small amounts of DNA, we think, cannot account for all the 6-8% of DNA shown by chemical analyses (Table II a; Birnstiel et al., 1961; Birnstiel and Hyde, 1963). In agreement with the opinion of Birnstiel and Hyde (1963), we also believe that a major part of the 6-8% DNA shown in "nucleoli" by chemical analyses is attributable to DNA contained in karyosomes (see Part B).

PART B

SECTION I. Observations on a New Nuclear Body--"Karyosomes"

Introduction

During our observations on isolated nucleoli, we noticed nuclear bodies that resembled in shape small nucleoli but staining much more intensely (Figs. 4, 8 and 9). We have called these bodies karyosomes.

Karyosomes are often found amongst chromatin masses (Fig. 22). Sometimes they are found attached to nucleoli. Frequently, if not always, chromatin material appears intimately attached to karyosomes (Fig. 22).

Karyosomes have also been seen in isolated whole nuclei (for example, Fig. 14) and *in situ* in pea meristematic cells in interphase (Fig. 26). They show a characteristic structure in isolated material prepared in different ways and at different times (Figs. 22-25). Finally, we have so far observed only one organized karyosome per nucleus.

Fine Structure of Isolated Karyosomes

The detailed descriptions of the fine structure of karyosomes

which follow are deduced from our studies on sucrose-citrate isolated subnuclear fractions in which they occur frequently and are therefore easily discernible for investigation. Investigations of structure of karyosomes in situ have yet to be made. Karyosomes measure about 1/3 to 1/5 the size of nucleoli (see inserts to Figs. 8, 9 and Fig. 14). They are devoid of vacuoles like those in nucleoli proper. Further, the dense granules are larger and more closely packed than in nucleoli (Figs. 8, 9, 22 and 25). Ribosomes are not present in karyosomes. We will recall that ribosomes, the 120-160 A oval particles, were present in nucleoli. These are some of the characteristics of karyosomes which help us differentiate them from nucleoli.

Our observations of karyosomal fine structure show the following structural components to be present in a karyosome: (i) 60-80 A wide double strands that resemble chromatin strands in density; (ii) densely stained double strands, 80-120 A in diameter; (iii) a few strands approximately 20 A in diameter; and (iv) a nucleolar-like background matrix.

The 60-80 A wide double strands are recognizable, in favorable sections, in the lighter regions of the karyosome (Figs. 22 and 23). They are composed of two 25-30 A wide strands.

Often, these 60-80 A wide strands appear to weave through the karyosome as long, continuous strands. In the process they may form free loops or intertwine with another such strand (Fig. 22). Also, such

strands interconnect the very dense regions of the karyosome (Figs. 22-25).

At low magnifications, the densest regions in the karyosomes appear as compact, large "granules" (Figs. 8, 9 and 14), and measure, when well compacted, 350-600 A in diameter.

In favorable cases, the dense granule-like regions in karyosomes are seen to represent coiled strands (Fig. 22). Thus, we interpret the karyosomal dense "granules" to consist of dense double strands wound into large coils. Such a dense double strand appears to be itself helically arranged and 80-120 A in width. The individual strands average 25-30 A in diameter.

Therefore, the karyosomes appear to have two kinds of double strands in them in terms of dimensions and density. While this is possible, it may be that the dense double strand of the "granules" (80-120 A wide) and the 60-80 A wide strands of the lighter regions of the karyosome represent one and the same strand. Thus, the increased dimensions of the strands in the "granules" could be a result of their being helically arranged. An alternative possibility is that the increased dimensions and density are due to our inability to resolve an absorbed component (which is densely staining) from the 60-80 A strands.

Often these dense coiled regions appear to have in association single strands, 18-23 A in diameter (arrows, Figs. 22, 24 and 25). In addition, a few 40 A wide strands are also present in such dense

areas of the karyosome. However, while the near 20 A wide strands do not appear to form part of the double strands, the latter do appear to be an integral part of such double strands.

These dense "granules" in karyosomes appear to be different from the nucleolar clusters of dense granules which also have a similar density. While the latter were observed to vary in their distribution, size and shape (part A, Section II B.b), the dense granules of the karyosome are uniformly distributed, never absent, and present a reproducible structure in material isolated and prepared by the same procedures. Further, we will show that these karyosomal dense regions react differently to nucleases and thus have a different chemical composition from those in nucleoli.

An amorphous background structure is sometimes visible in karyosomes (Fig. 23). In density, such a background matrix is also seen in nucleoli (compare Figs. 23 and 24 with Figs. 8-12).

When karyosomes are found attached to nucleoli (Figs. 23 and 24), the connecting link appears to be this amorphous background structure. Karyosomes may appear either as if they are part of the integral structure of the nucleolus (Fig. 24) or as being connected to the nucleolus by bands of such amorphous material, or just lie in close proximity.

Chromatin strands are almost always found in intimate association with the structures in a karyosome (Figs. 22 and 23). Association with chromatin of karyosomes, we believe, is not an isolation artifact

(as we believed to be the case for nucleolar association of chromatin) because of its regularity and, in addition, for the following reasons. Chromatin strands do not form a peripheral band around karyosomes as they do around isolated nucleoli. On the contrary, they appear to form actual connections with the structure of karyosomes at several points (Figs. 22 and 23). Also, we will show that such connections are real in a subsequent section dealing with deoxyribonuclease treated karyosomes.

Occasionally, certain regions of chromatin can be seen to compare favorably with the dense regions of karyosomes both in isolated nucleolar fractions (Fig. 22) and in in situ pea nuclei (Fig. 26). We interpret these regions to consist of not only chromatin strands (similar to those present in many areas containing chromatin) but also a component or components that stain densely. Our experience with lead hydroxide suggests that protein alone will not "stain" so heavily. Also, because we are dealing with subnuclear structures, we have argued that the dense material or component is a nucleoprotein complex other than of DNA. We will present the possibility that such a component could be of a ribonucleoprotein nature in a later section.

Because karyosomes formed part of the isolated nucleolar fraction, it has been possible to study the effects of ribonuclease and deoxyribonuclease on karyosome structures also.

Effects of ribonuclease on karyosome structure. Ribonuclease treated karyosomes show a background structure that appears essentially amorphous. The appearance of this background matrix is the same as in ribonuclease treated nucleoli (Fig. 27). Therefore, we conclude, as we did with nucleoli, that the background matrix of karyosomes is of a ribonucleoprotein nature. We will recall that single strands of about 20 A diameter were seen in control karyosomes. Since these single strands may no longer be seen in ribonuclease treated karyosomes, we attribute to them also a ribonucleoprotein chemical composition.

The dense coiled regions appear to be the least affected in ribonuclease treated karyosomes. This is in sharp contrast to the nucleolar clusters of dense granules which were more often affected by ribonuclease than deoxyribonuclease, and more drastically.

However, these dense coiled regions do not appear to be as intact as in untreated karyosomes (Figs. 27 and 28). They appear to be partially eluted or as collapsed structures (Fig. 28). Such an eluted appearance could be due either to the removal of an RNA component that was originally present within these dense regions or merely a non-specific action of ribonuclease. Nonetheless, if such an effect is due to a removal of RNA from these dense regions, we do not know how this component was organized into the original dense "granules."

Double strands are still present in the dense regions of the

karyosomes or interconnecting such dense areas. Therefore, we may infer that such strands are not of a ribonucleoprotein composition.

Effects of deoxyribonuclease on the structure of karyosomes. Deoxyribonuclease treated karyosomes appear distinct from control karyosomes. They lack their main distinguishing component--namely, the large, closely packed densely staining "granules." Most of such regions now appear to be vestigial. Such strands that now remain appear to be single and measure, on an average, about 20 A in diameter (Figs. 29 and 30; arrows).

The effectiveness of deoxyribonuclease treatment in removing DNA from chromatin is clearly seen by a greatly reduced capacity of chromatin to bind with lead ions (Fig. 29). However, they do retain their stranded appearance in some instances (Fig. 29). Such a stranded appearance of chromatin after deoxyribonuclease treatment was also noticed by Ris (1962) with calf thymus chromatin fibrils. He interprets such a retention of stranded structure to be due to protein.

Similar (to deoxyribonuclease treated chromatin strands) stranded structure can also be seen inside the karyosome (Figs. 29 and 30). Because of this consistent resemblance of karyosomal strands to chromatin strands both before and after deoxyribonuclease digestion, we conclude that such strands are made up of deoxyribonucleoproteins.

Because deoxyribonuclease changes the appearance of chromatin strands, we can clearly see that chromatin strands are attached

intimately with karyosomal structures (Fig. 29). Thus, it is possible that the deoxyribonucleoprotein strands outside and inside the karyosome are part of the same strands of chromatin.

In summary, we have presented suggestive evidence for the chemical composition of karyosomes. These are (i) deoxyribonucleoprotein--composing the double strands seen in the karyosome; (ii) a ribonucleoprotein component composing the matrix of the karyosome (along with a large amount of "amorphous" protein) and the single strands that were seen to be associated with the dense "granules" in the karyosome. This ribonucleoprotein (and probably the protein also) is very similar to that present in the nucleolus; (iii) another ribonucleoprotein component in the dense "granules" comparable in density to the nucleolar clusters of dense granules and also to a ribonucleoprotein component is present in much less amounts in karyosomes than in nucleoli.

SECTION II. Observations on an Ubiquitous Ribonucleoprotein Component in Pea Nuclei

We called attention earlier to the similarity in density and composition between the dense structures in the karyosome and certain areas of chromatin nearby (Fig. 22). We suggested that such dense regions in chromatin are not pure nucleohistone but contain, additionally, another component. That such may be the case is further supported by Figures 29 and 30. If the increased density in the controls was due to purely nucleohistone masses, then deoxyribonuclease treated material in Figures 29 and 30 should not contain areas of such high density.

On the contrary, a chromatin-like stranded material that stains densely is evident outside the deoxyribonuclease treated karyosome in Figure 29. Such dense strands (they measure on an average 160 A in diameter) were seen in control fractions also (Figs. 9, 11, 13, 15 and 22). We have not definitely seen such strands in ribonuclease treated material. However, we are not certain whether they are ribonuclease susceptible, because until now we have not learned to recognize them by any other criteria than their density and, sometimes, their coarse appearance (Figs. 27 and 29).

In situ, such a dense material appears to be recognizable in differentiated cells as a coarse, stranded, dense material (see Fig. 3b). Other observations in regard to these dense, chromatin-like strands are still in progress. One of our observations is that such a material resists 1 M NaCl extraction also (Hyde, personal communication).

Chromatin-like strands that are similarly dense and that are resistant to deoxyribonuclease and 2 M NaCl treatment have been reported also by Ris (1962) in isolated and in situ calf thymus nuclei. He describes them as an interchromosomal, coarse fibrillar material of about 200-250 A thickness. He assigns a non-histone-RNA chemical composition to this material. Georgiev and Chentsov (1960), who have made similar studies on isolated thymus nuclei, have also described a fibrous material (which they call "nucleonemes") that resists 2 M NaCl and deoxyribonuclease extraction.

Thus, there is a clear similarly between the coarse fibrillar material in calf thymus nuclei and the chromatin-like dense strands in pea nuclei. Lacking clear evidence that our material is ribonuclease susceptible, we assume that the dense chromatin-like strands in pea nuclei do represent a ribonucleoprotein component different from ribosomes.

Such a densely staining material presumably composed of ribonucleoproteins seems to be ubiquitous in pea nuclei. Using density

alone as a criterion, we find that there is a common structural element in chromatin, nucleoli and karyosomes. These are the dense chromatin-like strands amongst chromatin, dense strands or "granules" (clusters of dense granules) in nucleoli that resist deoxyribonuclease but not ribonuclease extraction and a dense material in karyosomal granules that also seems to resist deoxyribonuclease extraction. However, in karyosomes this component is less important in granule structure than in nucleoli (compare Figs. 29 and 19, deoxyribonuclease treated karyosome and nucleolus, respectively).

That such a ribonucleoprotein component can be present as "particles" instead of as strands is supported by some studies by Swift (1959, 1962a, 1962b). Studying rat liver and salamander pancreas nuclei, this author noted dense particles averaging 15 mμ and 40 mμ diameters in the interchromosomal areas of such nuclei. Similar size particles were also seen in nucleoli. Swift also points out that while the nucleolar particles were "readily extracted by ribonuclease or perchloric acid," the interchromatin particles appeared to contain both nucleic acids (as judged by their susceptibility to ribonuclease and deoxyribonuclease). These latter particles were "prominent in nuclei treated with deoxyribonuclease, but much less so in ribonuclease or perchloric acid extracted material." (Swift, 1962b). Thus, we infer that the dense material in pea nuclei (in nucleoli, karyosomes and chromatin) is composed of a ribonucleoprotein component in association with varying amounts of deoxyribonucleoprotein.

DISCUSSION

The term "karyosome" was introduced by Ogata (1883). The same type of structure was called a "chromatin nucleolus" by Montgomery (1898). Both authors were describing animal nuclei and the nucleolus was called by these authors a "plasmosome." Gates (1942) quotes these authors as observing that the plasmosome is "paler, staining with basic dyes."

In the protozoan Ceratomyxa (Noble, 1941), the karyosome stains with feulgen, like "the peripheral chromatin of the nucleus. It is formed, at least in part, from the chromosomes, but divided to form two centrioles, which soon disintegrate. It may contain both nucleolar and chromatin material" (quoted from Gates, 1942).

Thus, the karyosomes described in published literature signify a body containing both nucleolar and chromatin material. However, they have been described in animal cells only. We are justified in choosing the term "karyosome," therefore, only in so far as the nuclear body we describe in peas also contains both nucleolar and chromatin material.

Our inquiries into the nature of karyosomes take two approaches.

Firstly, we can ask whether karyosomes can be identified with any previously described organelle--ephemeral or permanent--of nuclei. Secondly, we can seek to deduce their function, from what is known of their chemical composition, and cytological structure and behavior.

In our studies, karyosomes were found to be associated either with the nucleolus or chromatin. In the latter case, the association with the chromatin strands was seen to be a true connection. Thus, karyosomes are not structures that are always associated with either the nucleolus or chromatin. This suggests that karyosomes may serve a function interrelating the nucleolus and chromatin.

Karyosomes do not represent nucleolar elements being assembled by chromatin. Elementary nucleoli (Jacob and Sirlin, 1963), paranucleolar bodies (LaFontaine, 1958b), or the unknown nucleolar substance revealed by the silver staining method (Das and Alfert, 1959) exist only during anaphase and telophase. We have so far found karyosomes only in interphase cells.

We can alternatively assume karyosomes are "young" nucleoli. Or, simply that the karyosome is a second nucleolus in the cell. Carrying this hypothesis further, we might speculate that they are attached to nucleoli because they will ultimately be incorporated into the bigger nucleolus. We consider these possibilities as unlikely since nucleoli in our studies have not shown decisively the large amounts of DNA shown by karyosomes.

The remaining possibilities are that karyosomes represent condensed regions of chromosomes ("chromocentres," or heterochromatin) or the nucleolar organizers described in many plants (review by Gates, 1942). Karyosomes are not identifiable as either of these structures for the following reasons. Our studies of karyosomes to date have shown only one karyosome per nucleus. Further, our efforts so far to locate karyosomes in situ have shown that these bodies do not appear in many sections of nuclei.

However, further studies are necessary to conclusively decide that karyosomes are not either nucleolar organizers or heterochromatic regions of chromosomes. For this purpose, it would be desirable to study a plant material like maize or tobacco, where chromocenters and nucleolar organizers have been well chracterized at the light microscopic level.

Our knowledge of karyosomes then depends on further studies of their origin, development and fate in the cell. Lacking such studies as yet, we seek speculative correlations with existing biochemical data derived from studies on pea subnuclear components.

In this regard, some studies of RNA and protein synthesis in pea nuclei by Birnstiel et al. (1961; in press) and Chipchase and Birnstiel (personal communication) are worthy of note. Protein synthesis was studied by incubating isolated whole nuclei with radioactive leucine in a suitable incubation medium. Results from such studies showed that

the nucleolar fraction was the principal site of incorporation in terms of total activity. However, in terms of specific activity, the mode was found neither in the nucleolar fraction nor the chromatin fraction, but in a fraction sedimenting intermediary between the nucleoli and the bulk of the chromatin. These authors examined and observed that these interfractions contain small, deeply stained (with methylene blue) bodies which they describe as "chromatin particles" (Birnstiel, personal communication).

The specific activity associated with these interfractions was not dissociable by desoxycholate or sodium citrate treatment. Treatment with 2 M NaCl (which dissociates nucleohistone and some of the residual ribonucleoprotein), however, resulted in a loss of the radioactivity. Thus, they have biochemical evidence that the most active sites of protein synthesis in pea nuclei occur in closest association with deoxyribonucleoproteins. Recent studies of Chipchase and Birnstiel (personal communication) also show such an interfraction to contain active sites of RNA synthesis.

The "chromatin particles" sediment at centrifugal forces somewhat higher than nucleoli and occur in highest concentration in chromatin I, a fraction characterized by its high DNA content (Birnstiel et al., 1962). Since our observations have shown that karyosomes are structures containing large amounts of DNA, we consider it very liekly that karyosomes and "chromatin particles" are identical. Under such an

assumption, karyosomes would become the most active sites of protein and RNA synthesis in pea nuclei.

The ephemeral nature of karyosomes (they have so far been found in situ only occasionally), their presence in nuclei of very young pea cells, and their association with chromatin suggest that karyosomes could be considered as regions of intense genetic activity of chromatin or chromosomes.

In this regard, we may usefully bring up the phenomenon of puffing of the giant salivary gland chromosomes of Diptera. There can be little doubt by now (Beerman, 1952, 1957; Breuer and Pavan, 1955; Becker, 1959; etc.) that such puffs are an expression of the action of specific chromosomal loci, functioning in different tissues in relation to particular stages of development or states of physiological activity. Reviewing such information, Swift (1962a) points out that "puff formation in Drosophila involves the accumulation of an RNA-nonhistone component protein complex at certain loci." We suggest that karyosomes could similarly represent an organized or activated state of interphase pea chromatin.

Since there is a similarity (especially when karyosomes are attached to nucleoli) in density between the "granules" in karyosomes and the dense clusters of granules in nucleoli, we suggest that the karyosomes may be involved in the transfer of an RNA or ribonucleoprotein component from the chromatin to the nucleolus.

Such a presumption finds supporting evidence from some studies of Rho and Bonner (1961). These authors conclude that the primary site of RNA synthesis in pea nuclei is in the chromatin. Further, they state that "the RNA, assembled in close association with the DNA of chromatin, would appear according to the present view to be transferred to the nucleolus, where, over a substantial period of time, it is modified or processed for its ultimate cellular function."

This "ultimate cellular function" of the nucleolus could be protein synthesis occurring, we suggest, at the sites of the clusters of dense granules--the "pars amorpha" RNA, which Lettre and Siebs (1954a, 1954b, 1961) suggest represent the RNA or ribonucleoprotein that is being synthesized or accumulated by the nucleolar chromosomes.

SUMMARY

An electron microscope study of isolated and *in situ* pea nucleoli was made in an effort to rationalize their ultrastructure.

Nucleoli were isolated for this purpose by a standard procedure involving complexing the purified nuclei with 0.1 M sodium citrate and breaking them in a 1.315 density sucrose in a Servall Omnimix. The so isolated nucleoli were enriched into a crude nucleolar fraction by differential centrifugation. This formed the basic material for this study. Besides, nucleoli contained in whole nuclei and those *in situ* were also studied.

Some of the isolated material was digested with ribonuclease and some with deoxyribonuclease before fixation. Chemical analyses of the isolated nucleolar fractions show that the enzymatic treatments were effective to the extent of removing at least half of the respective nucleic acids.

The materials to be studied electron microscopically were fixed with 1% osmium tetroxide, embedded in Epon, thin-sectioned and post-stained with various heavy metal stains that are commonly used in electron microscopy.

Studies of pea nucleoli in situ indicate that basically the pea nucleolus is composed of 60-100 A wide fibrils. 120-160 A diameter particles that resemble cytoplasmic ribosomes and some dense granules that are thought to represent coiled strands were also observed, but only in young pea nucleoli.

The structural features of isolated nucleoli are very similar to those observed in in situ nucleoli. These studies show that the basic structure of the nucleolus consists of a mesh of ribonucleoprotein strands, 80-120 A in diameter, and composed of two substrands, interspersed in an amorphous proteinaceous mass. Ribosomes of 120-160 A diameter and single strands of 20-25 A width are also noticeable in these nucleoli. Ribonuclease treatment consistently shows these structures to be composed essentially of ribonucleoproteins.

Further, isolated pea nucleoli show some very dense structures generally in the inner regions of the nucleolus. They present a varied structure and appear to contain 80-120 A wide, coiled double strands. They are partially susceptible to ribonuclease and to deoxyribonuclease also, albeit to a small extent. Thus, both DNA and RNA are thought to be present in these dense coiled strands of pea nucleoli.

The ribonucleoprotein contained in these structures is thought to be of a different type from that present otherwise in these nucleoli. These observations are discussed along with some published biochemical evidence and the possibility is raised that these dense regions

represent the active sites of protein synthesis in pea nucleoli. Ribosomes are thought to be products of nucleolar synthesis.

In addition, the studies on isolated nucleolar fractions have disclosed the presence of ovoid bodies amongst chromatin and sometimes attached to nucleoli. We call these structures karyosomes. Karyosomes have so far been seen in situ only in meristematic cells in interphase.

Karyosomes contain structures common to both nucleoli and chromatin. Their basic structure is similar to that of the nucleolus but they further contain closely packed, coiled strands that are essentially composed of deoxyribonucleoproteins and a small amount of the same type of ribonucleoprotein which has been observed in the inner regions of pea nucleoli.

Karyosomes are thought to represent the most active centers of RNA and protein synthesis in interphase pea nuclei and have been likened to the puffs of salivary gland chromosomes in Diptera representing the action of specific chromosomal loci.

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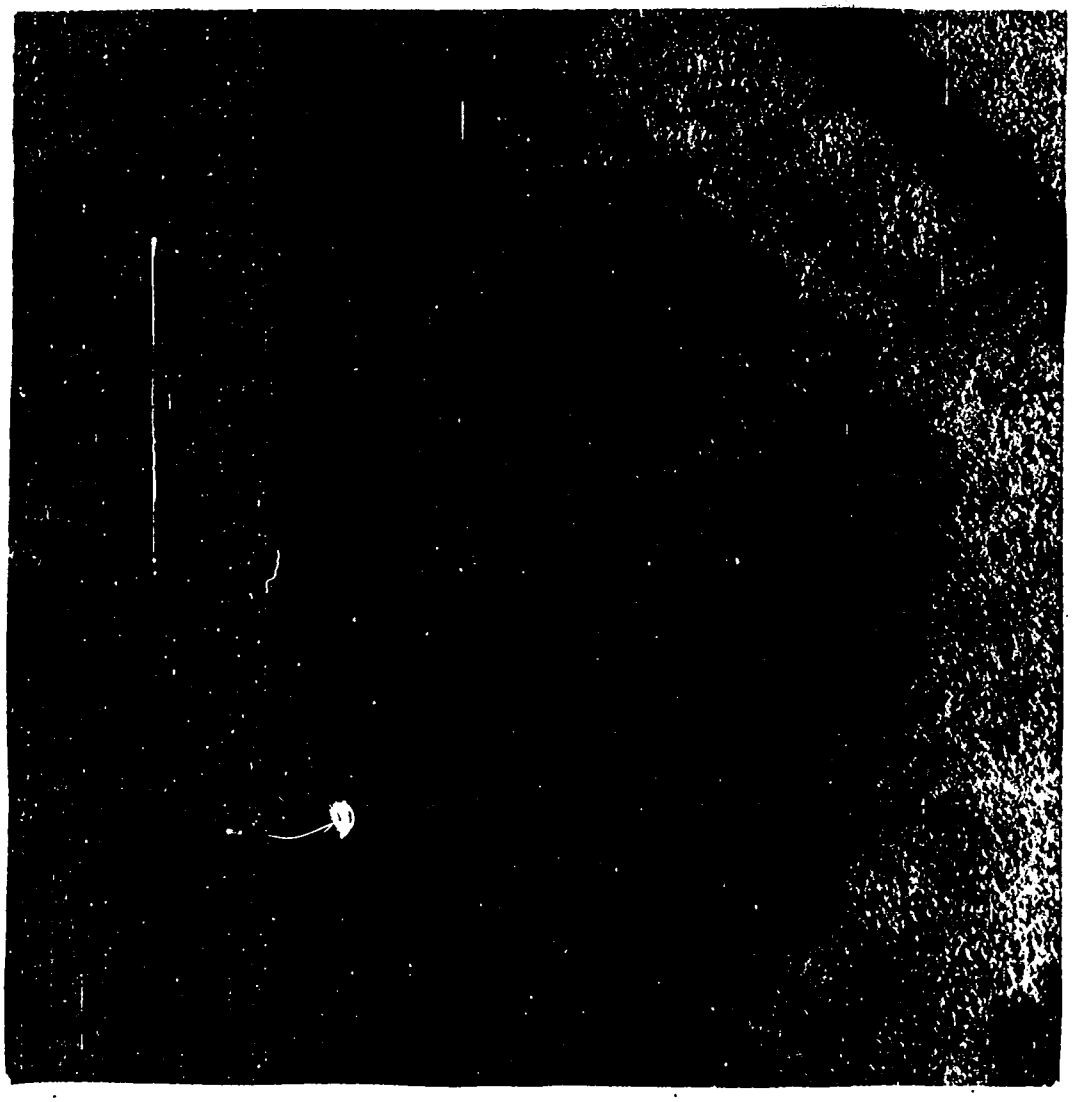
Fig. 1. Electron micrograph of a pea nucleolus in a meristematic cell in interphase. Two components, one particulate and the other fibrillar, are visible in the nucleolus. The particulate component is largely made up of 120-160 A diameter particles. Such particles can often be seen (especially in the top half of the micrograph) to be arranged as a string of beads. The fibrillar component of the nucleolus is present in small patches as 60-100 A wide fibrils associated with some large, densely staining granules. Arrows point to a few thin strands of 20-25 A diameter present in association with such granules. Post-stained with lead hydroxide. x 48,500.



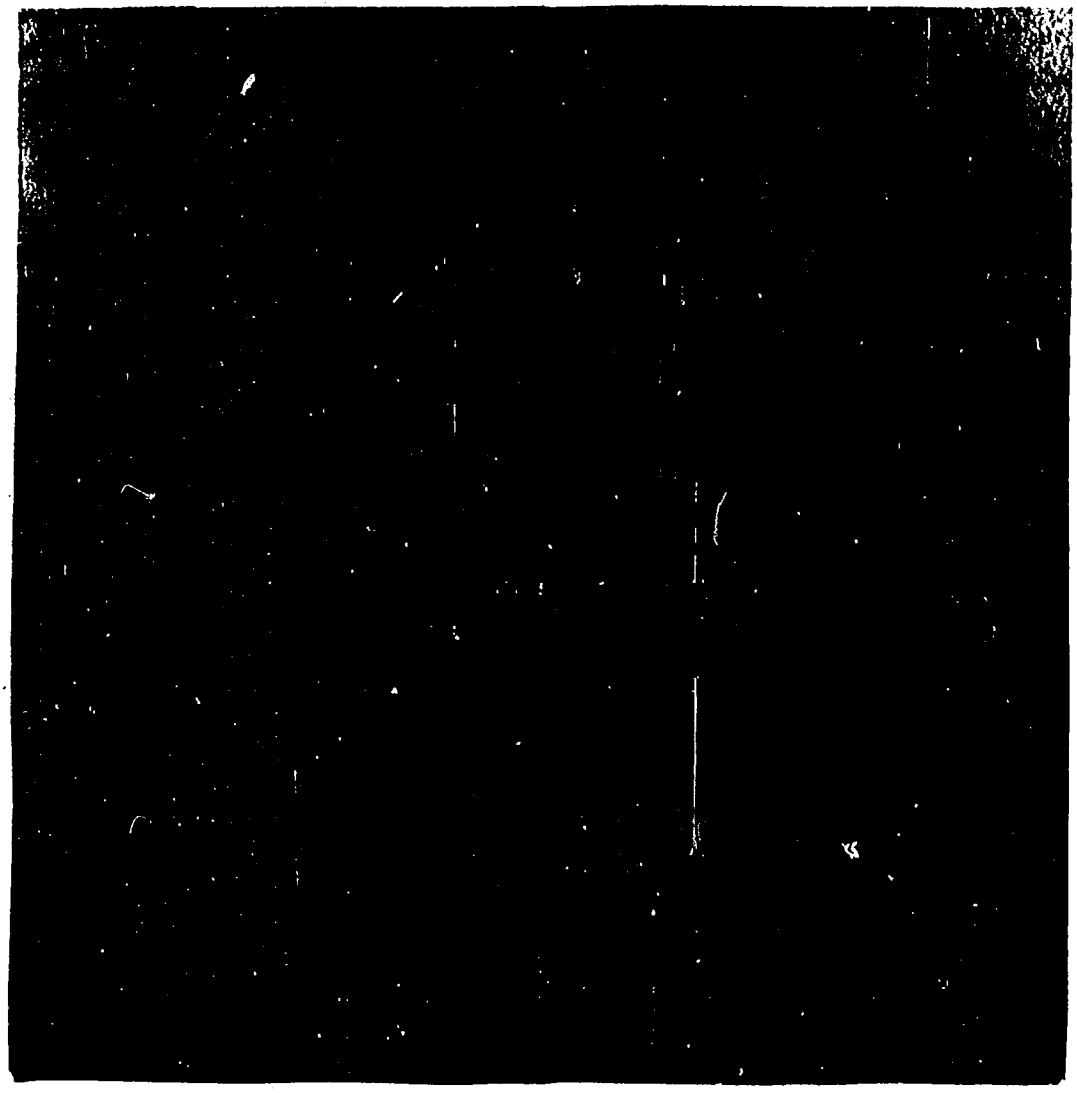


Fig. 2. Electron micrograph of a nucleus in a semi-differentiated cell showing the nucleolus to be distinctly fibrillar. Very few of the 120-160 A particles can be seen. Most of the strands present in the nucleolus average 60-100 A in diameter. Thinner strands of 20-25 A width are also present in such nucleoli. Lead hydroxide stained. x 60,000.

Fig. 2b. Higher magnification of a nucleolus in a semi-differentiated cell. At many places the dense granules appear as if they are in reality coiled strands (short thick arrows). The long arrow points to some granules again showing associated thin strands. Post-stained with lead hydroxide. x 120,000.

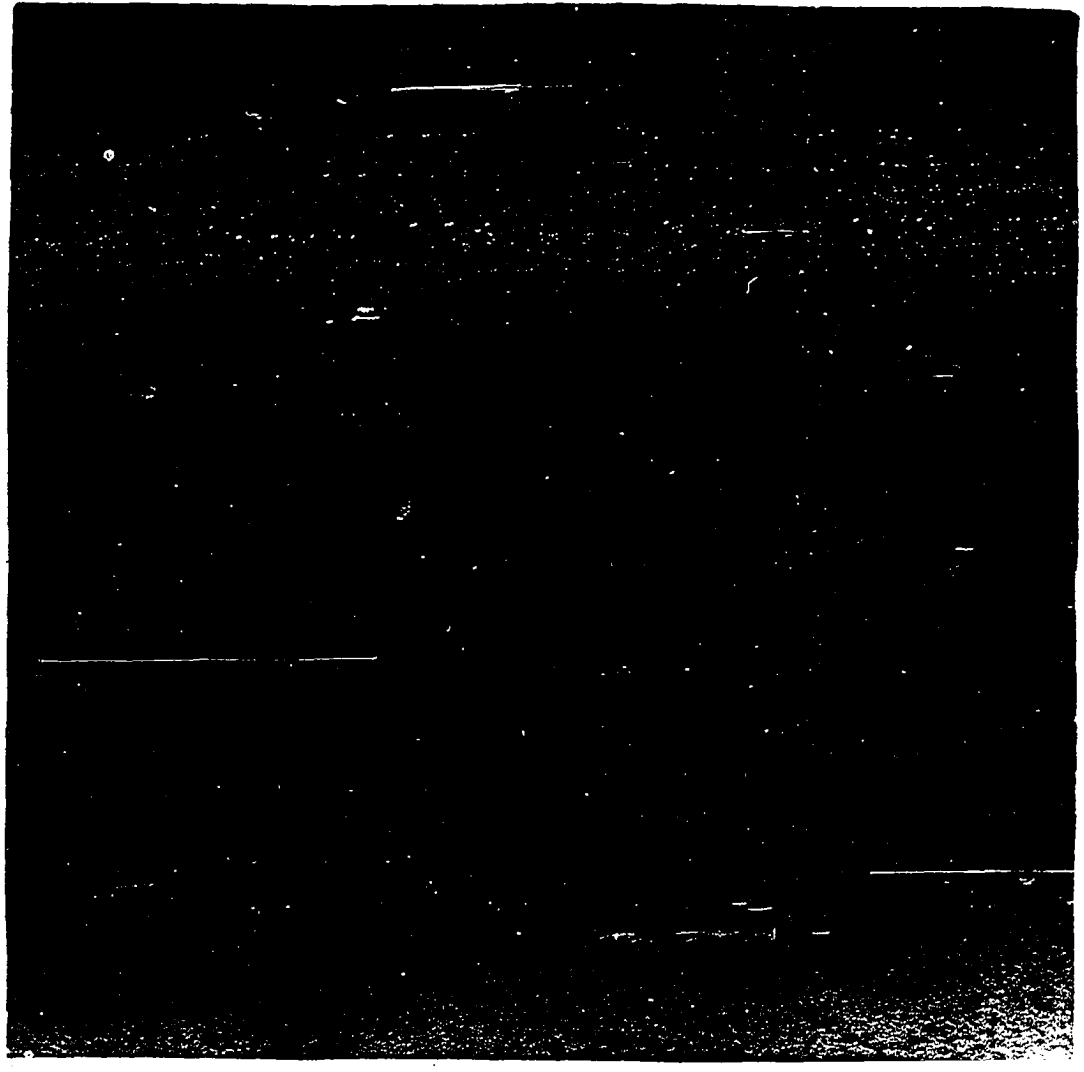


Fig. 3. Electron micrograph of a fully differentiated pea cell showing the nucleus in close proximity to the cell wall. The nucleolus lacks not only the 120-160 A particles of meristematic cell nucleoli but also the large, dense granules seen in the previous two types of nucleoli. See also figure 3b. Lead hydroxide stained. x 13,000.

Fig. 3b. Higher magnification of the nucleolus in figure 3. The nucleolus shows for the most part ill-defined, coarse strands. Its structure is almost indistinguishable from that of the chromatin outside. Arrow points to a region of chromatin having an increased density. Explanation in text, p. 47. Post-stained with lead hydroxide. x 120,000.

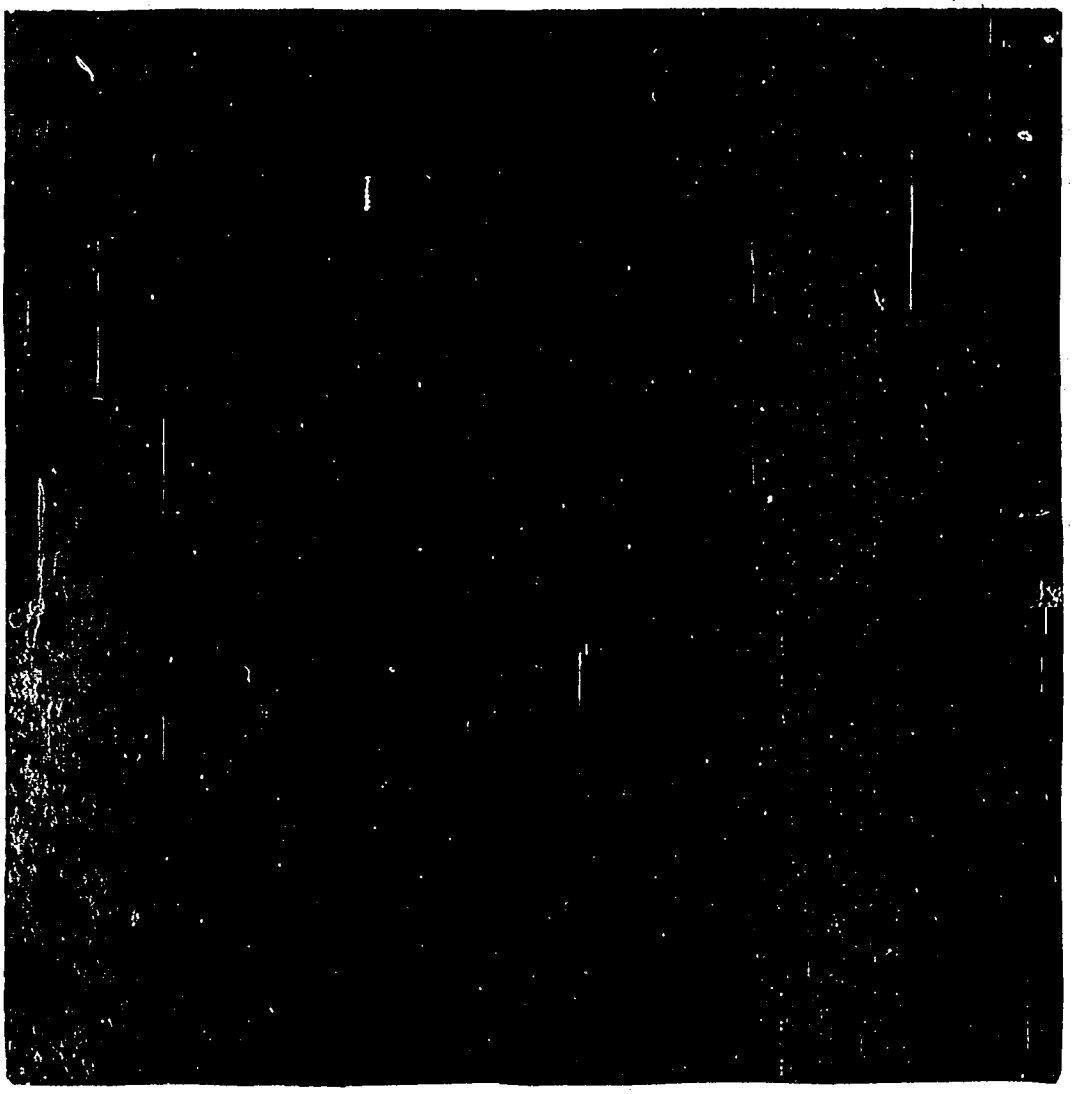


Fig. 4. Electron micrograph of an isolated nucleolus (N) fixed before dialysis. The attached nodule-like structure is a new nuclear body, the karyosome (K). See text for explanation. Two components can be clearly seen in the nucleolus. One is the lightly stained array of strands in the background. The densely stained clusters of granules form the second component. In certain regions (for example, region of long arrow) these granules tend to appear as strands. The short arrows point to regions where some thin strands are seen in association with these granules. One large vacuole (V) can also be seen in the nucleolus. Lead hydroxide stained. x 60,000.

Fig. 5. Electron micrograph of a non-dialyzed nucleolus post-stained with uranyl acetate and lead perchlorate. Comparison with figure 4 shows that the two procedures of post-staining enable the visualization of nucleolar ultrastructure to the same extent. However, the dense clusters of granules do not reveal as much a detailed structure as in figure 4. x 48,500.


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Fig. 6. Electron micrograph of a dialyzed nucleolus stained with both uranyl acetate and lead perchlorate. Comparison with fig. 5 shows that dialysis does not destroy nucleolar ultrastructure but does produce a general loosening up of its structure. In the process, amorphous dense patches (arrows) are often produced in these nucleoli. Thin strands, averaging 20 A in diameter, are seen more frequently in such dense patches. x 48,500.


6

Fig. 7. Electron micrograph of a dialyzed nucleolus showing structures visualizable with lead perchlorate alone. Though the dense clusters of granules are the only structures clearly visible in the nucleolus, their substructure is much more clear than that in fig. 6, where uranyl acetate was also used. x 74,000.


7

Fig. 8. Survey micrograph of a control nucleolar fraction fixed after dialysis and incubation. Besides nucleoli, such a fraction also shows a small amount of other sub-nuclear structures. Arrows point to strands of chromatin that have wrapped themselves around the nucleoli during centrifugation. The insert shows an isolated karyosome at the same magnification to compare size and general appearance with the nucleoli. Post-stained with lead hydroxide. x 21,600.

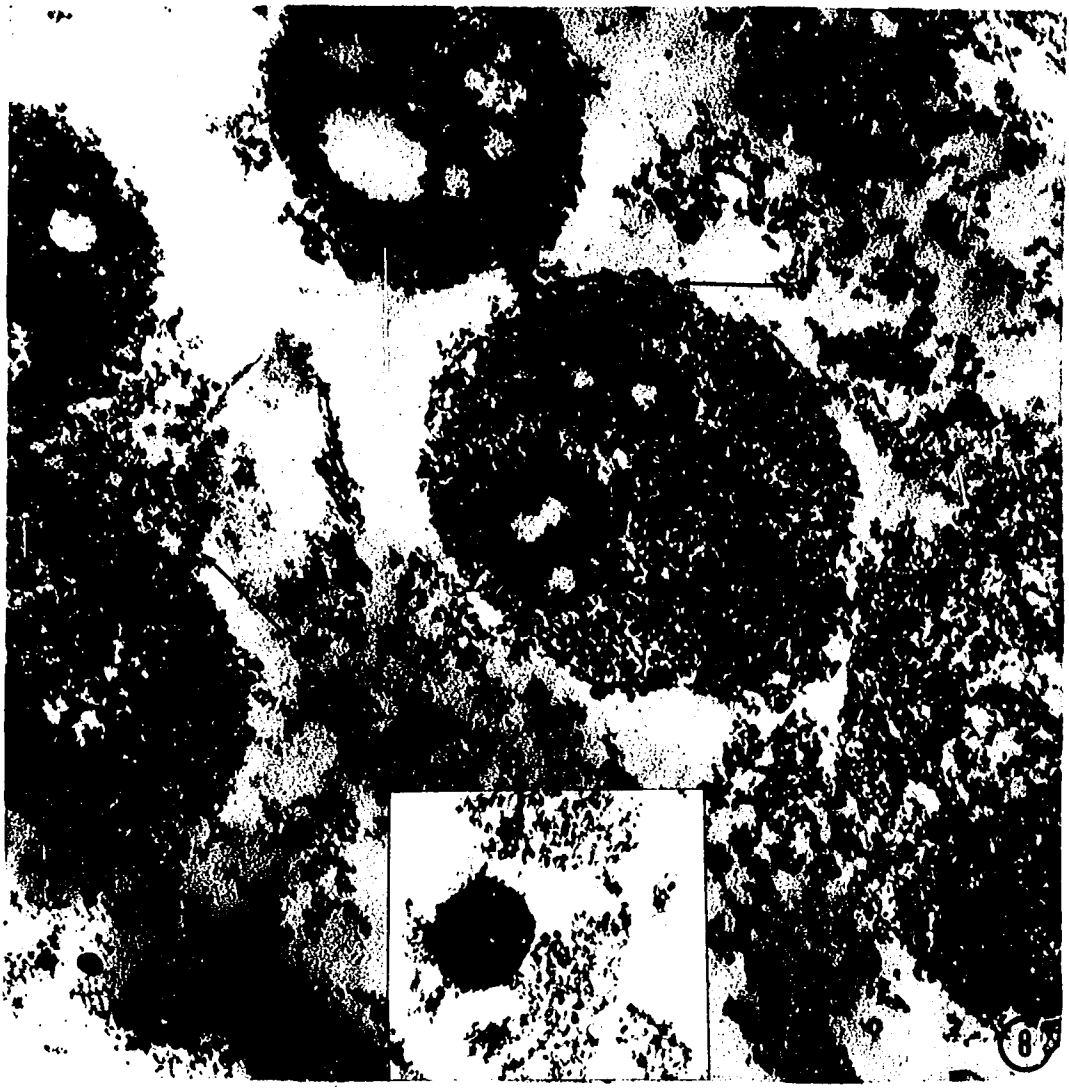


Fig. 9. Higher magnification of one of the nucleoli in figure 8. The background array of strands in such nucleoli appear as ill-defined structures. Areas of the nucleolus have (particularly around vacuoles (V))dense patches containing some dense strands that seem to be coiled in places into small knots. The thin arrows point to some oval particles averaging 120-160 A in diameter. These are thought to represent nucleolar ribosomes. The thick arrows point to chromatin strands that sometimes seem to intrude into the nucleolus. Two types of chromatin strands, one less dense than the other, can also be noticed outside the nucleolus, for example, in the upper right hand corner of the micrograph. The insert (same magnification) shows a karyosome that was present in an adjacent section. Lead hydroxide stained. x 74,000.

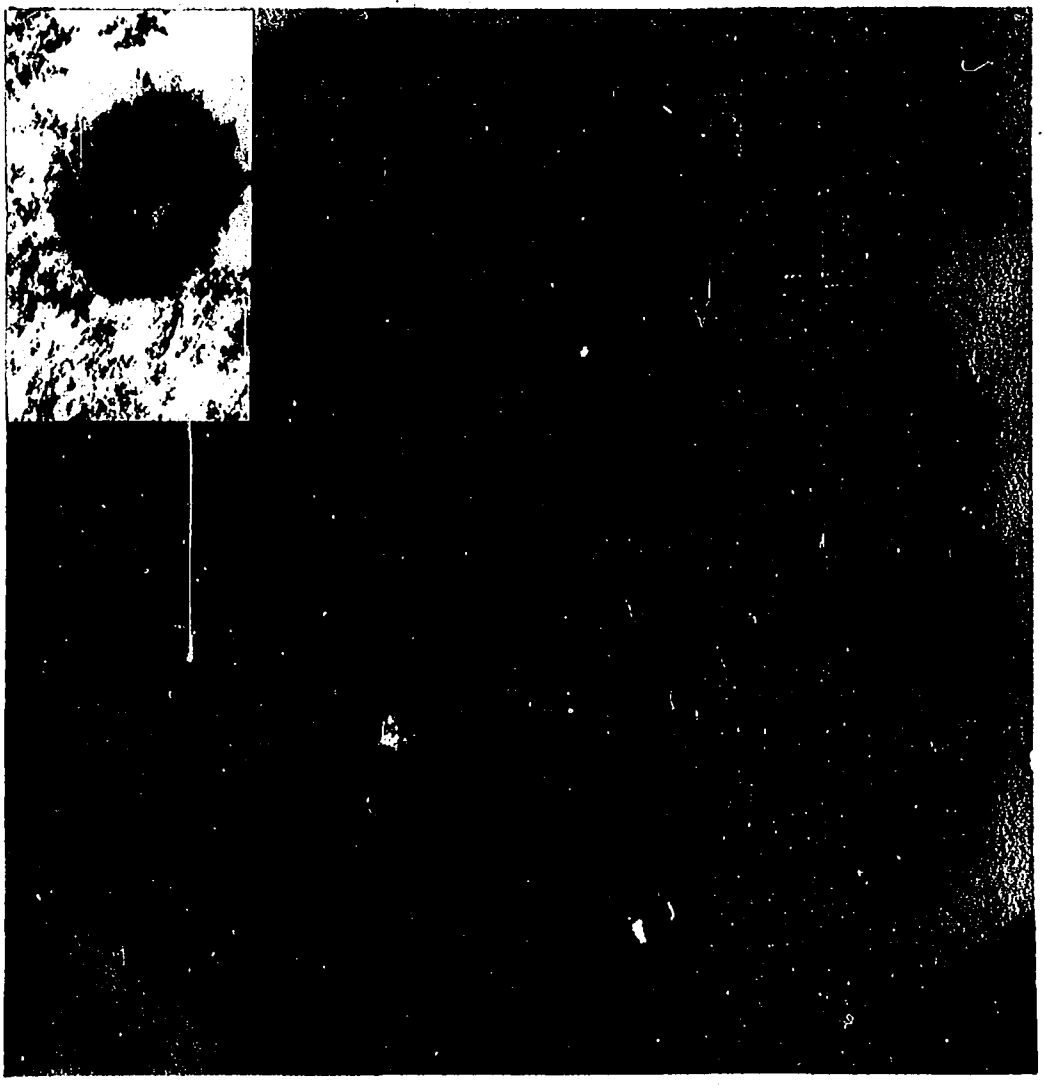


Fig. 10. Another dialyzed and incubated control nucleolus. The thin arrows point again to the 120-160 A particles. The thick arrow shows one of the dense patches where strands of about 20 A width can be clearly seen. Lead hydroxide stained. x 48,500.

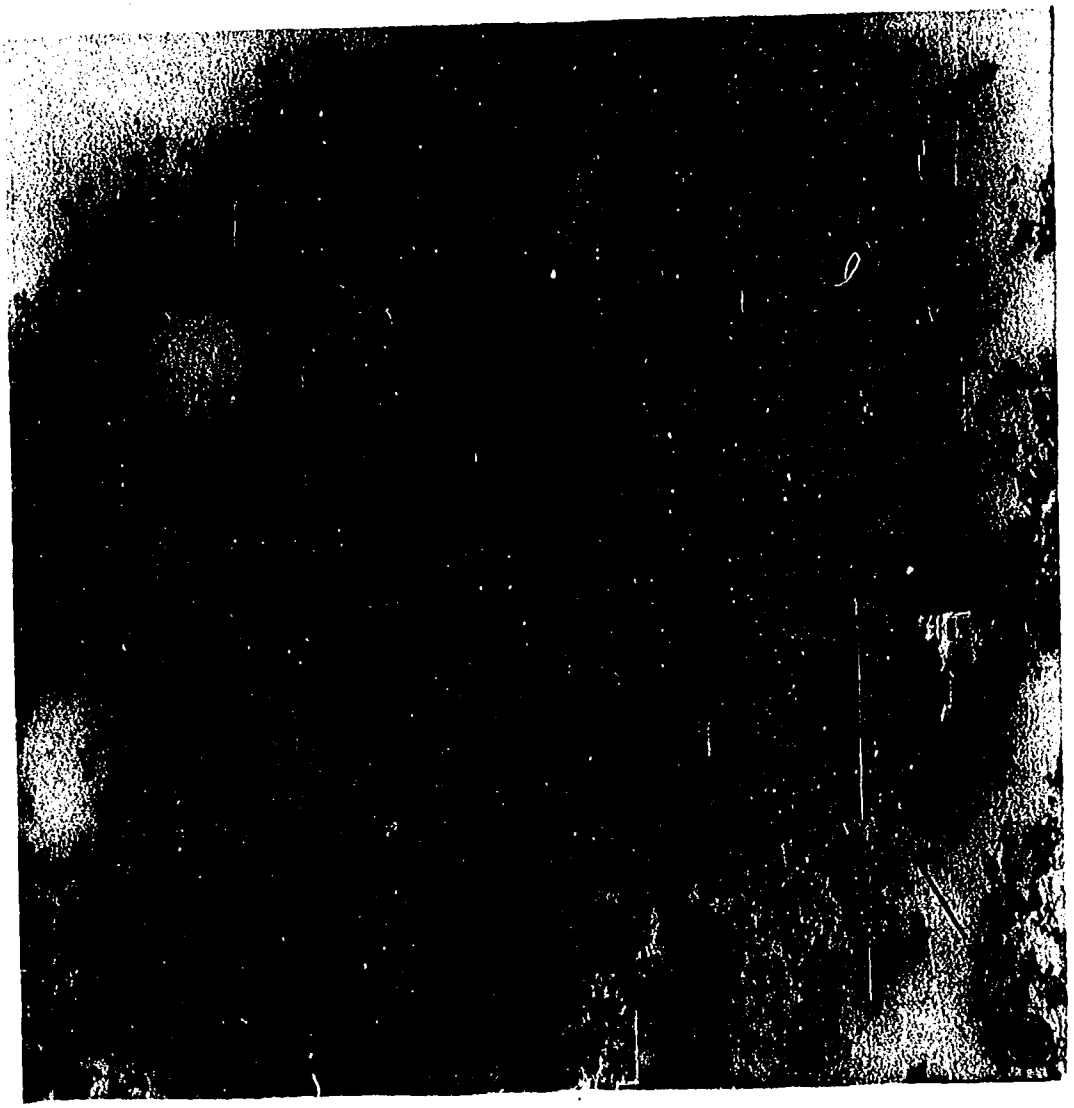


Fig. 11. A partially disrupted nucleolus found in the dialyzed and incubated nucleolar fraction. It is presented here mainly to show the single strands in the dense patches (thin arrows). The thick arrows point to a dense strandy material amongst chromatin. Explanation in text, p. 47. Lead hydroxide stained. x 74,000.

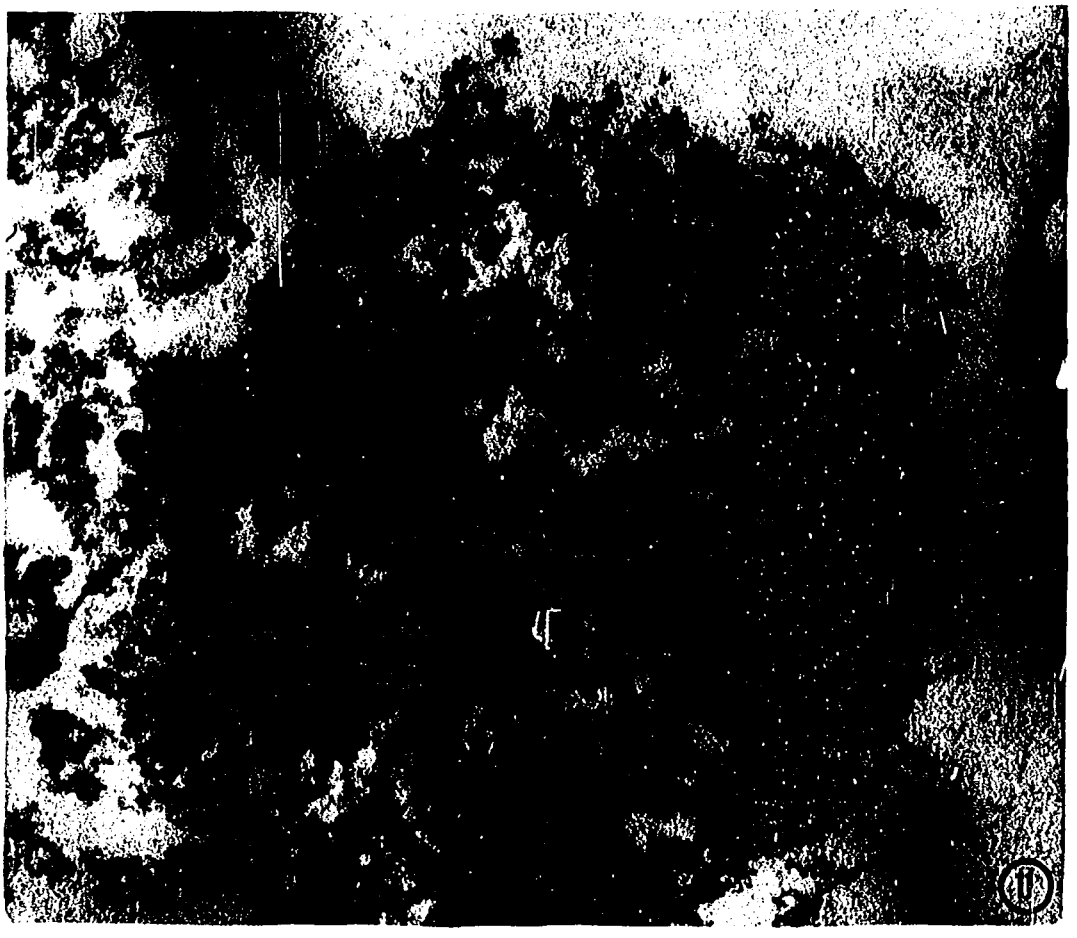


Fig. 12. This micrograph of a dialyzed and incubated nucleolus shows clearly two aspects of nucleolar ultrastructure. The thin arrows point to regions where the 120-160 A particles appear as if they are portions of strands or arranged as a string of beads. The densest material in the nucleolus is uncoiled enough at places to appear as 80-120 A dense strands (for example, region of thick arrow) which sometimes form tight loops. Lead hydroxide stained. x 74,000.

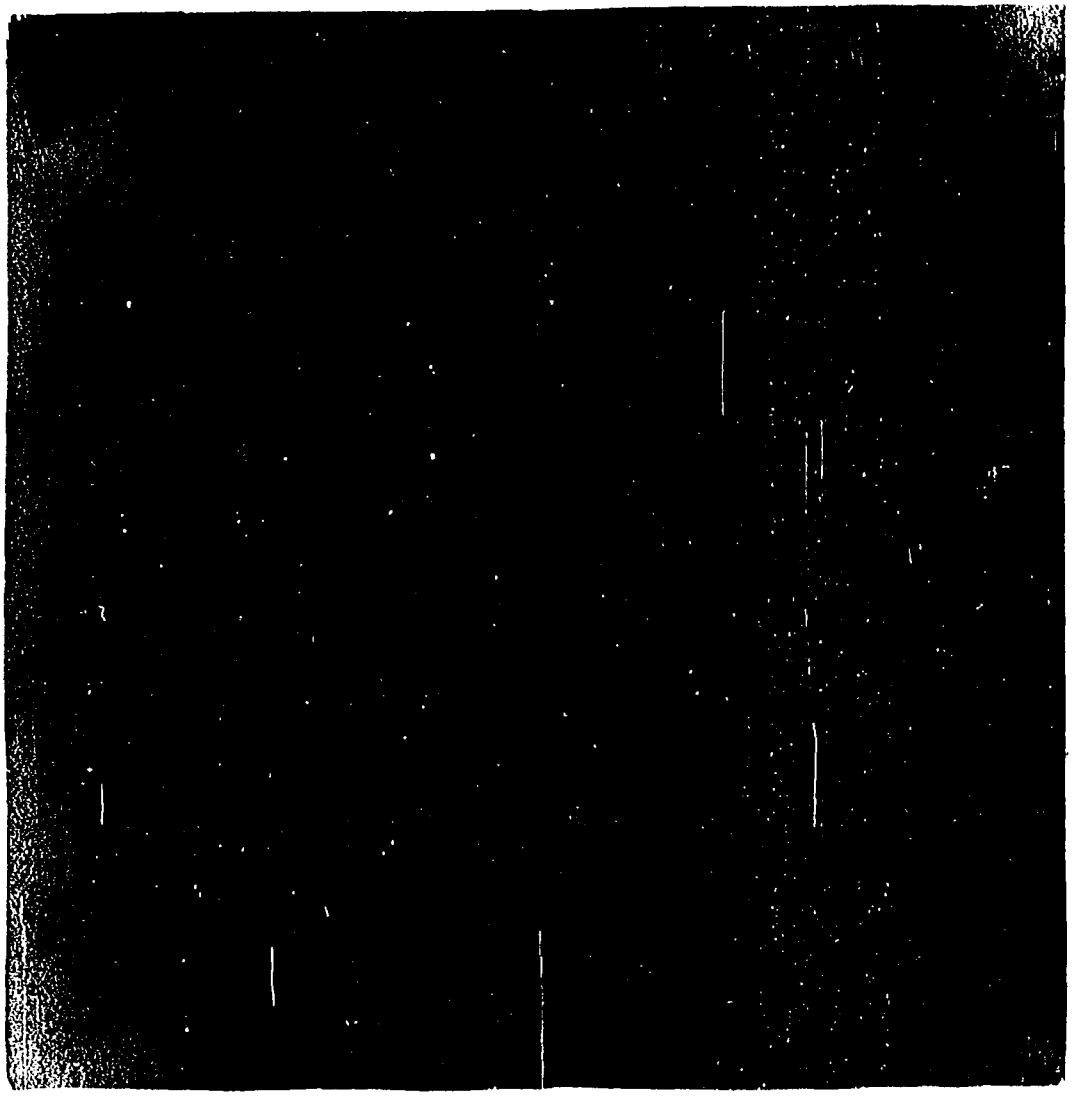


Fig. 13. This micrograph of a control nucleolus (dialyzed and incubated nucleolar fraction) shows again the same type of arrangement of the 120-160 A particles as was seen in figure 12 (thin arrow). However, the nucleolus shows relatively long, dense strands in the densest regions. The thick arrow points to chromatin strands that have become associated with the nucleolus during the isolation process. Lead hydroxide stained. x 74,000.


13

Fig. 14. Electron micrograph of a whole nucleus fixed immediately after the nuclei were purified in 2 M sucrose. The nucleolus does not show a definite structure except for a few vestigial dense granules. The karyosome (K), however, has still its characteristic appearance. Like the nucleolus, chromatin (C) is also present in amorphous masses. Lead hydroxide stained. x 48,500.

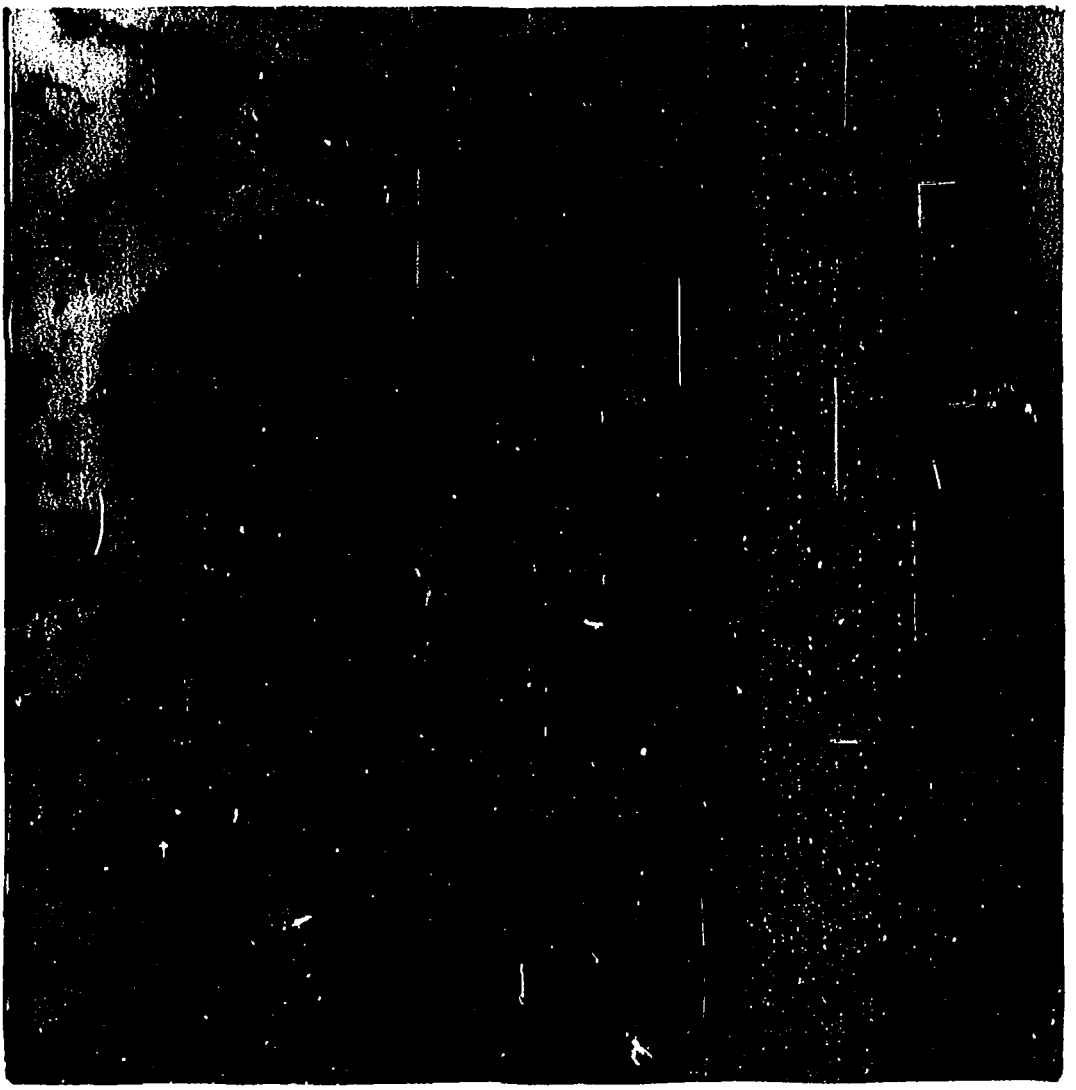


Fig. 15. Electron micrograph of a whole nucleus found in a dialyzed and incubated nucleolar fraction. The nucleolus appears as a compact structure. Nevertheless, it does reveal, on close examination, most of the features seen in isolated nucleoli. Notice the presence of densely stained amorphous patches and strands (arrow) amongst chromatin. Lead hydroxide post-stained. x 48,500.

Fig. 16. This micrograph of a nucleolus, fixed immediately after the breaking of nuclei, serves to confirm the reality of most of the structures seen in isolated and incubated nucleoli. The nucleolus is composed of the 120-160 A particles (thin arrow region, for example), some densely staining strands and the lightly staining background strands. The beaded arrangement of the 120-160 A particles is visible at many places. The densely staining strands (for example, thick arrow) are similar to the dense structures seen in other control nucleoli. Lead hydroxide stained. x 28,500.

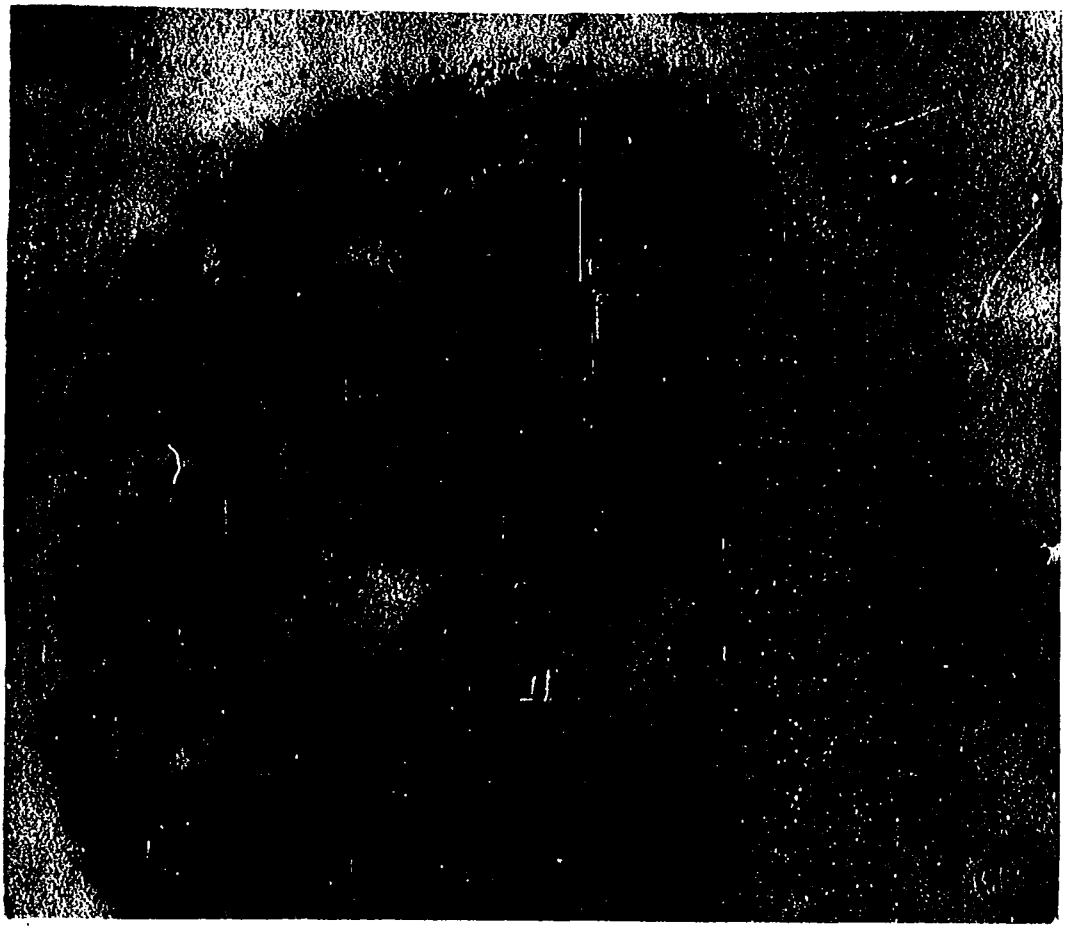
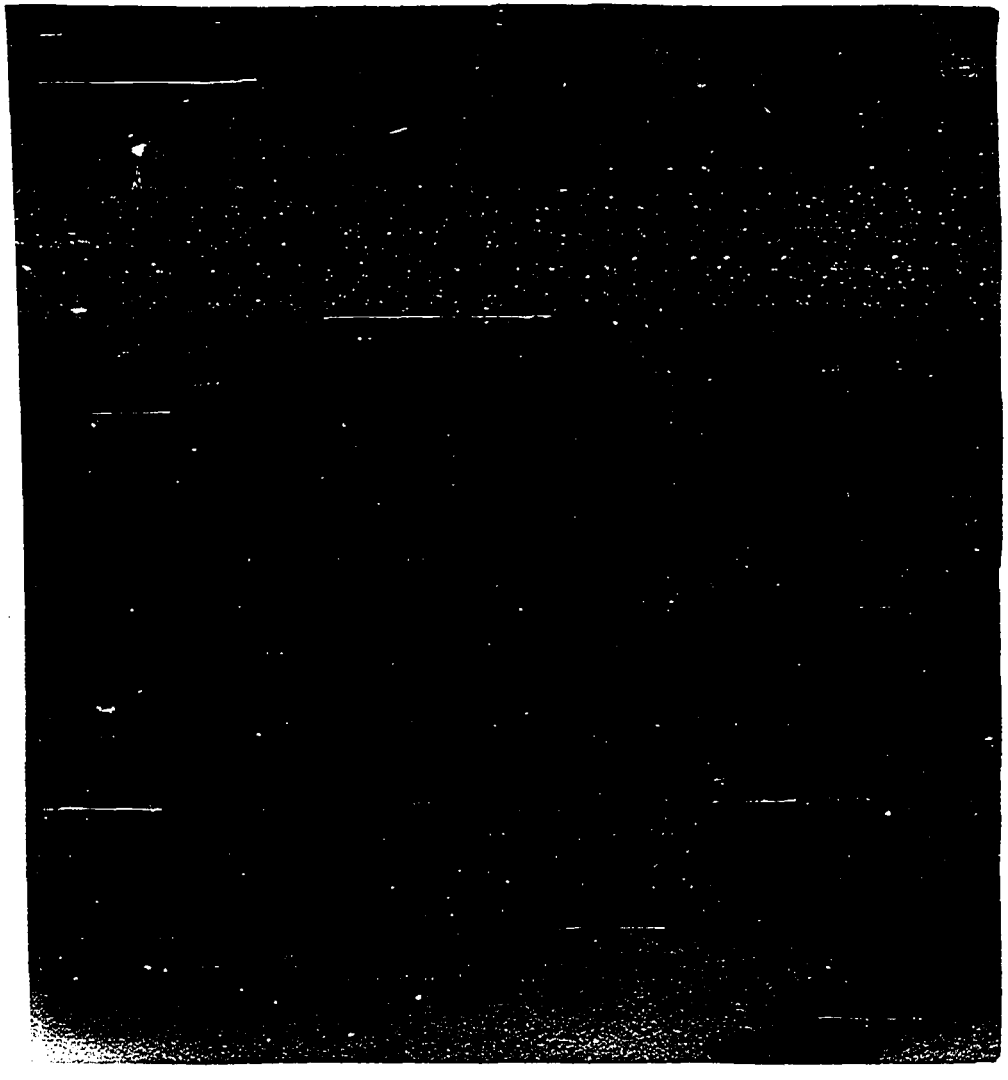


Fig. 17. Electron micrograph of a nucleolus fixed after ribonuclease treatment. In place of most particulate structures seen in the nucleolus before, an amorphous background now prevails. The densest material in the nucleolus is seen to be only partially susceptible to the action of ribonuclease. Lead hydroxide stained. x 74,000.



Figs. 18a and 18b. Nucleoli showing two extreme cases of susceptibility to ribonuclease treatment. Fig. 18a shows a nucleolus in which only remnants of the dense strands remain. Fig. 18b shows the other extreme case that is also relatively rare, where the dense coiled strands remain almost unaffected by ribonuclease treatment. Arrows in fig. 18b show the edge of the nucleolus. Lead hydroxide stained. x 74,000.

18

Fig. 19. A deoxyribonuclease treated nucleolus. Most nucleolar structures are still well preserved. In contrast, the structure of chromatin appears destroyed (arrow). Lead hydroxide stained. x 74,000.

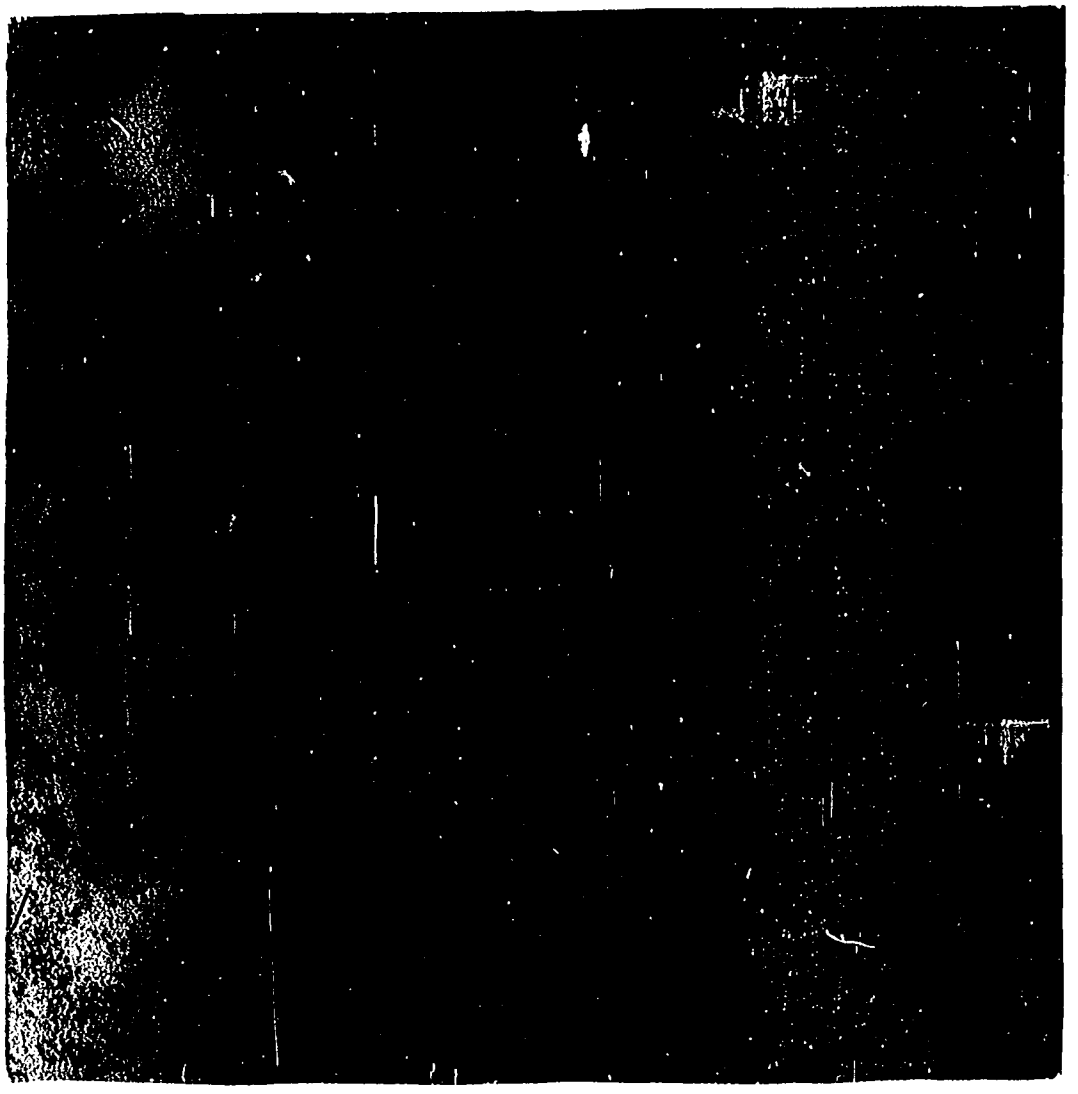


Fig. 20. Higher magnification of a deoxyribonuclease treated nucleolus showing limited susceptibility of the dense coiled strands to enzyme treatment. The nucleolus appears to be otherwise well preserved. Lead hydroxide stained. x 191,000.

Fig. 21. A relatively rare case of a deoxyribonuclease nucleolus showing drastic effect on the dense structures in the nucleolus. The common appearance of chromatin (C) after deoxyribonuclease treatment is as pictured in this micrograph. The dense strands that are seen amongst chromatin are thought to be of a ribonucleoprotein composition (text, p. 47). Lead hydroxide stained. x 60,000.

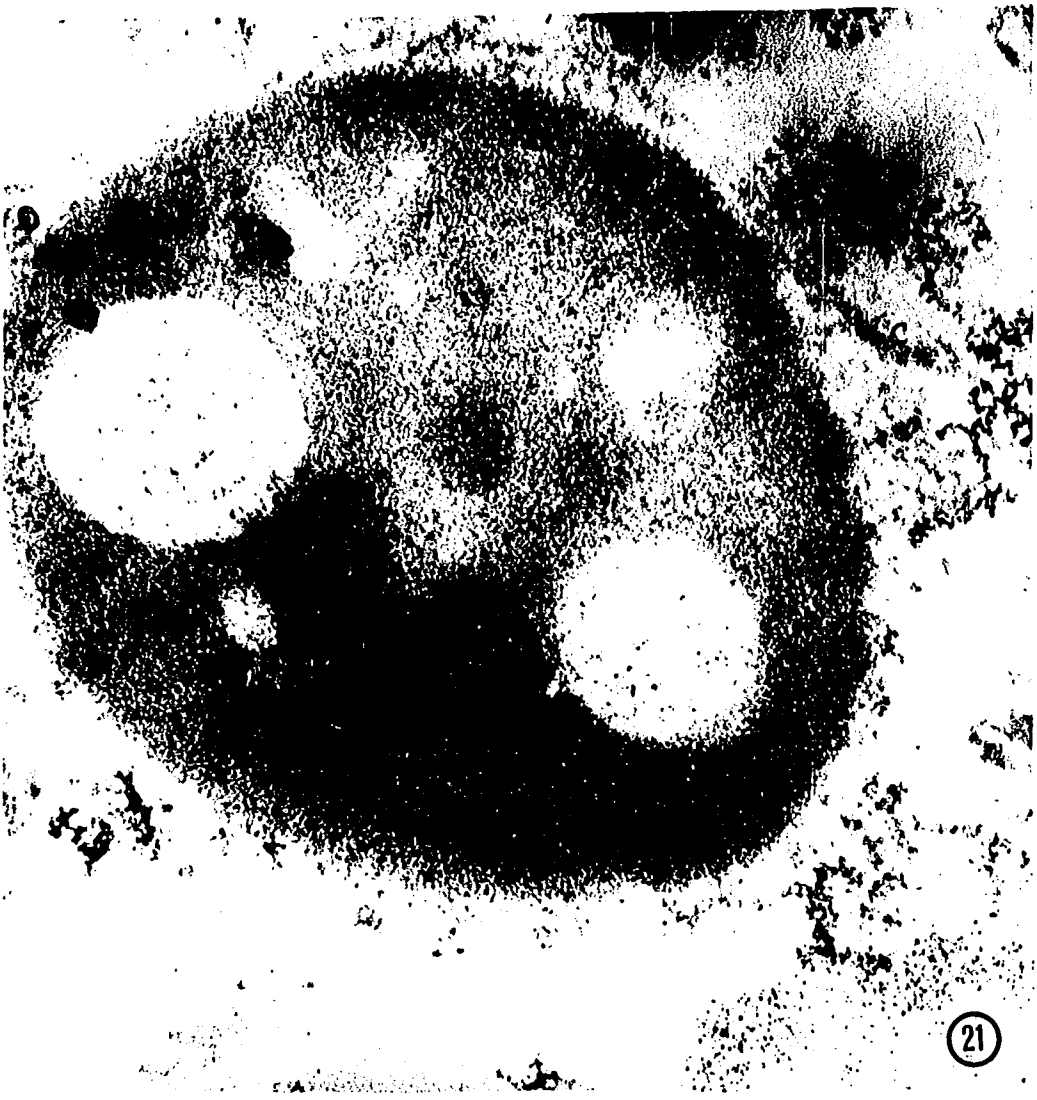

21

Fig. 22. Electron micrograph of a karyosome found in the dialyzed and incubated nucleolar fraction (Experiment 3 SC). The karyosome is composed of (i) lightly staining double strands (long thick arrows) that weave through the karyosome, (ii) closely packed dense coiled strands (short thick arrows) and (iii) an amorphous matrix that can be seen in the intergranular regions. The long thin arrows point to regions where approximately 20 A wide strands are present in close association with the dense granule-like regions.

The similarity of structural components of the very dense regions of chromatin (lower center region of the micrograph, for example) to the dense areas inside the karyosome is striking. Note the intimate connection of the chromatin strands with the structure of the karyosome.

Lead hydroxide post-stained. x 120,000.

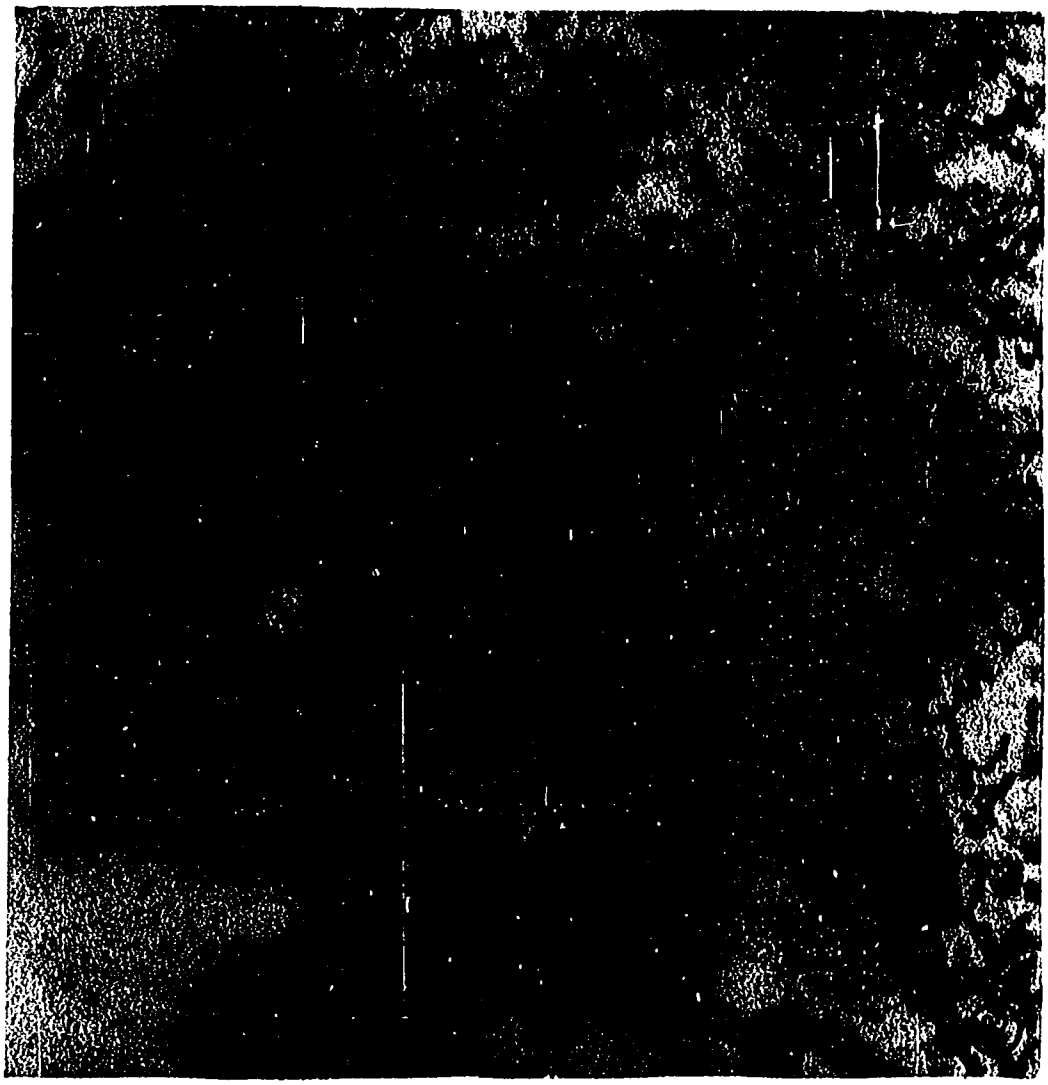


Fig. 23. Thin section of a karyosome found attached to a nucleolus (N) in the same isolated nucleolar fraction as that of the karyosome in figure 22. The differences in appearance are attributed to this being a somewhat thinner and grazing section. The amorphous background of the karyosome is well evident as are the single strands present in association with the dense, granule-like regions (thin arrows). The thick arrows again point to regions showing the inter-connecting strands. Lead hydroxide stained. x 191,000.

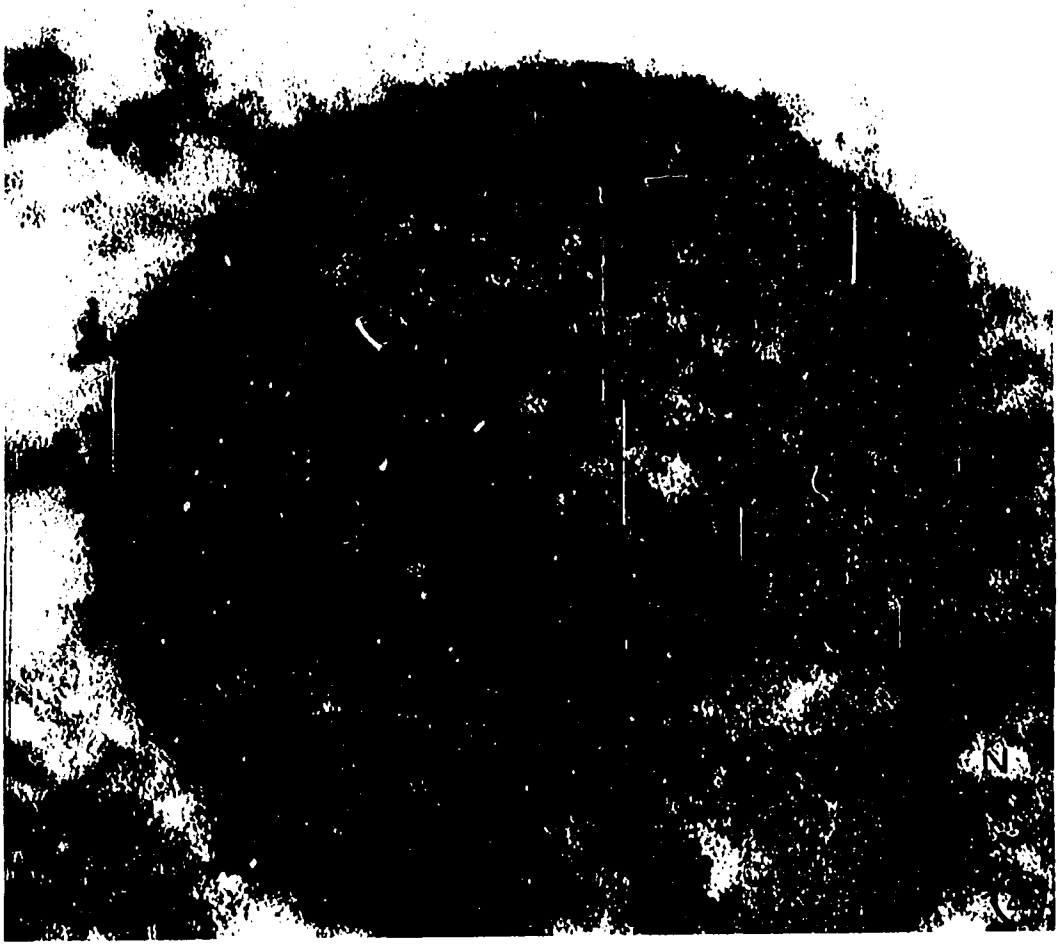

N

Fig. 24. Isolated karyosome found attached to a nucleolus in a non-dialyzed nucleolar fraction (Experiment 1 SC). The similarity of the background structure in the nucleolus and karyosome is again evident. The karyosome appears here attached to the nucleolus (N) as though a part of its integral structure. The arrows point to the same structures as in figures 22 and 23. Lead hydroxide stained. x 120,000.

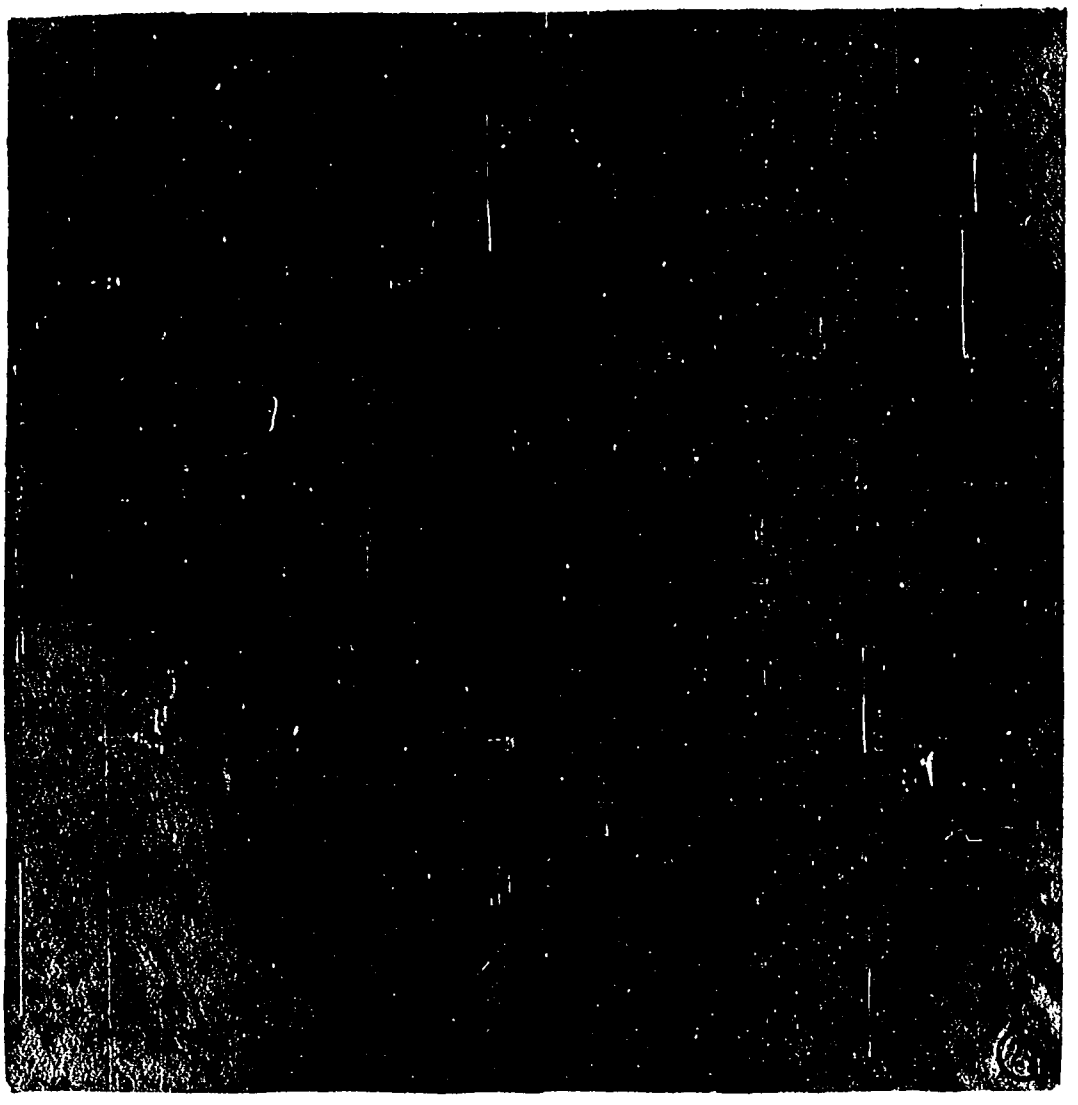
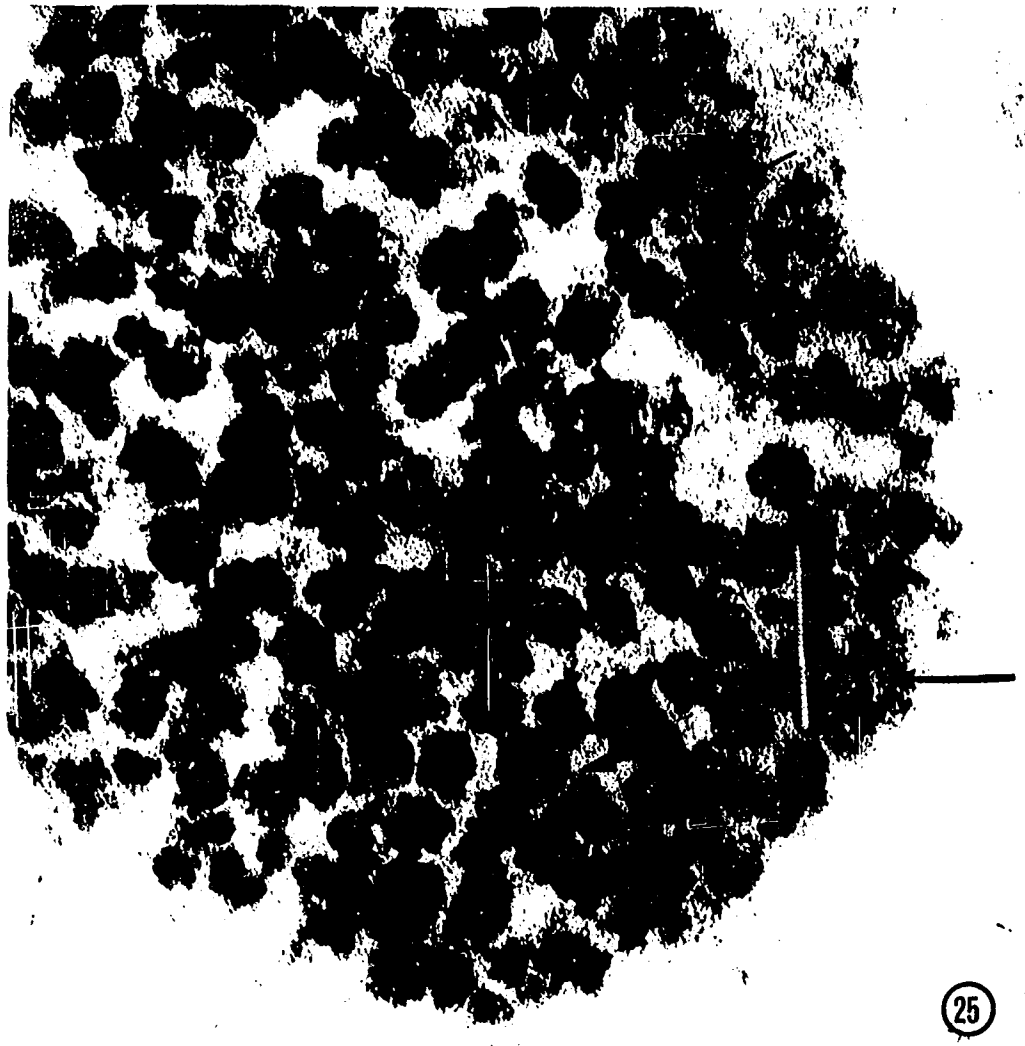


Fig. 25. An isolated karyosome observed in a "diluted control" fraction (Experiment 5 SC). The same structural details again characterize the karyosome. Arrows point to same structures as before. Lead hydroxide staining. x 154,000.



25

Fig. 26. Electron micrograph of an *in situ* karyosome in a meristematic cell in interphase. Arrow points to a region in chromatin that compares favorably with the general structure in the karyosome. K--karyosome. N--nucleolus. C--chromatin. Lead hydroxide staining. x 48,500.

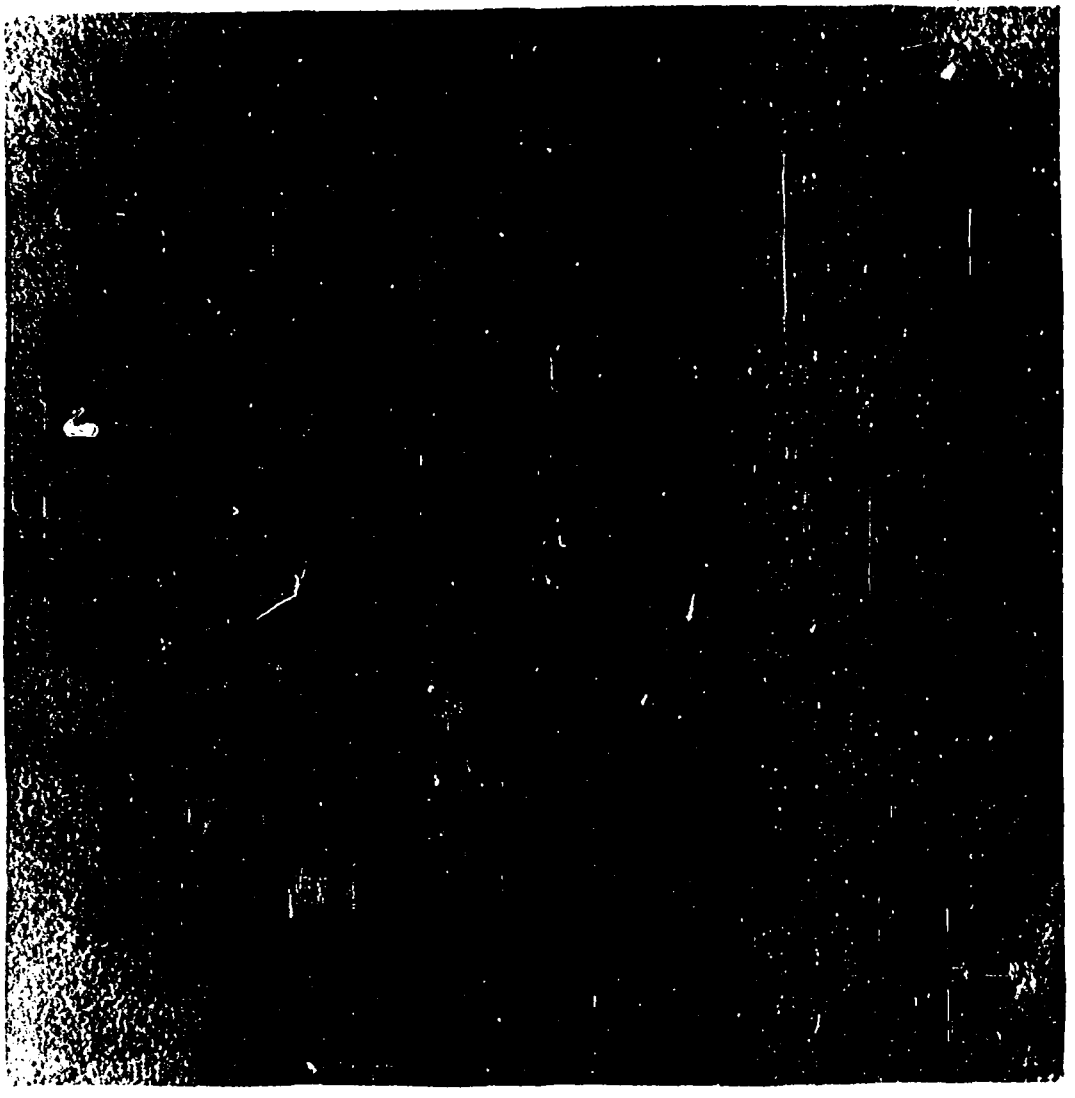


Fig. 27. The structure of an isolated karyosome after ribonuclease treatment (Experiment 3 SC). The similarity in background structure of the nucleolus and the karyosome is now confirmed by their similar appearance after ribonuclease treatment. The double strands, however, are still evident. Lead hydroxide stained. x 191,000.

Fig. 28. An isolated karyosome affected by ribonuclease treatment (Experiment 3 SC). The dense, granule-like regions appear eluted. Lead hydroxide stained. x 191,000.

Fig. 29. A decxyribonuclease treated karyosome from experiment 3 SC. Except for the marked lack of the dense regions and interconnecting double strands, the karyosome shows the same structures as before. The densely stained strands (arrow) amongst chromatin are thought to be composed of a non-histone-protein and RNA complex. See text, p. 47. K--karyosome. C--chromatin. Lead hydroxide staining. x 120,000.

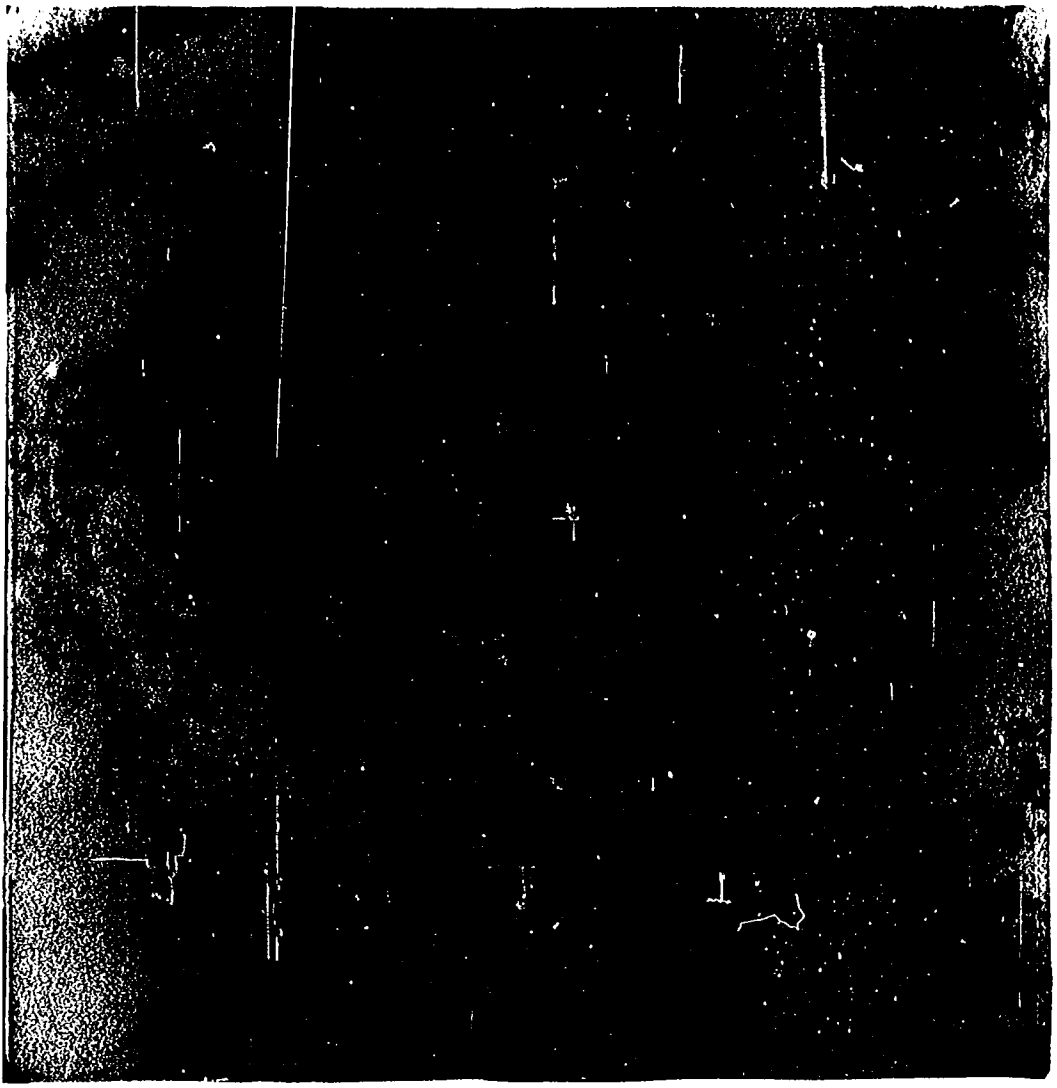


Fig. 30. Another karyosome from experiment 3 SC showing the effect of deoxyribonuclease treatment. Vestiges of the granule-like regions and the interconnecting double strands are apparent as before. Lead hydroxide stained. x 191,000.