FINE STRUCTURAL ALTERATIONS

OF INTERPHASE NUCLEI OF

LYMPHOCYTES STIMULATED TO

GROWTH ACTIVITY IN VITRO

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ABSTRACT

This report describes fine structural changes of interphase nuclei of human peripheral blood lymphocytes stimulated to growth by short-term culture with phytohemagglutinin. Chromatin is found highly labile, its changes accompanying the sequential increases of RNA and DNA synthesis which are known to occur in lymphocyte cultures. In "resting" lymphocytes, abundant condensed chromatin appears as a network of large and small aggregates. Early in the response to phytohemagglutinin, small aggregates disappear during increase of diffuse chromatin regions. Small aggregates soon reappear, probably resulting from disaggregation of large masses of condensed chromatin. Loosened and highly dispersed forms then appear prior to the formation of prophase chromosomes. The loosened state is found by radioautography to be most active in DNA synthesis. Small nucleoli of resting lymphocytes have concentric agranular, fibrillar, and granular zones with small amounts of intranucleolar chromatin. Enlarging interphase nucleoli change chiefly (1) by increase in amount of intranucleolar chromatin and alteration of its state of aggregation and (2) by increase in granular components in close association with fibrillar components.

INTRODUCTION

Nuclei of differentiated interphase cells generally contain some dispersed and some condensed chromatin. Electron microscopic radioautographic studies provide much support for the long-held view of light microscopists that dispersed portions of chromatin are metabolically active while highly condensed chromatin is inert. Thus, Hay and Revel (1) have identified nuclear regions containing a dispersed deoxynucleoprotein (DNP) meshwork, rather than condensed chromocenters, as zones of active DNA synthesis in proliferating cells of salamander larvae. Regions in which chromatin is diffusely arranged, but not those in which it is highly condensed, have similarly been shown by Littau and coworkers (2) to be active sites of RNA synthesis in isolated calf thymus nuclei in vitro.

These observations imply that labile fine structural organization of chromatin might be recognized in interphase nuclei during major alterations of their nucleic acid metabolism. Opportunity to seek evidence of such lability is presented by the increased synthesis of RNA and the replication of DNA which occur when highly differentiated cells enter premitotic interphase.

The remarkable transformation of peripheral

blood lymphocytes during their short-term culture in the presence of phytohemagglutinin (PHA) (3, 4) is advantageous for this purpose. An abundance of condensed chromatin is strikingly characteristic of the compact nuclei of small, "resting" lymphocytes. These cells demonstrate little or no mitotic activity under ordinary conditions of cell culture. In the presence of PHA, however, a large proportion of small lymphocytes grow large and divide within 48–72 hr (5–7). Rise in RNA synthesis begins within less than an hour (8) and continues exponentially for at least 24 hr (9) after treatment of lymphocyte cultures with PHA. Synthesis of DNA is appreciably increased only about 24 hr following treatment with PHA, then rises rapidly in the course of a subsequent 48–72 hr of culture (8).

The observations made herein verify changes in nuclear fine structure accompanying these metabolic changes. Radioautography has been of aid in relating DNA synthesis to the morphologic state of chromatin. Despite known asynchrony of growth of cultured lymphocytes (10, 11), a continuum of altered chromatin organization associated with the progress of interphase is suggested. Indeed, among such morphologic variables as cell or nuclear size and certain cytoplasmic features not considered here, the progress of late interphase appears to be most strikingly manifested by alterations in the organization of chromatin and related changes in nucleolar structure.

MATERIALS AND METHODS

Lymphocyte Culture

Suspensions of human lymphocytes were prepared from peripheral blood essentially by the procedure of Bach and Hirschhorn (12). Tissue culture medium NCTC-109 (13) was used, however, rather than Eagle's minimum essential medium. The procedure utilizes the adherence of granulocytes to flint glass surfaces to accomplish the separation of mononuclear from polymorphonuclear cells. Aliquots containing about 10^7 cells were cultured in 4 oz French square bottles in 8.0 ml of NCTC-109, (Hyland Laboratories, Los Angeles, Calif.) together with 2.0 ml of agamma newborn calf serum, 700 units of penicillin, and 0.7 mg of streptomycin. Phytohemagglutinin M (Difco Laboratories, Detroit, Mich.), 0.1 ml per culture, was added prior to incubation at 37°C in 5% CO_2–95% air for time periods ranging from 4 to 72 hr. Control specimens, designated 0 hr, were prepared directly for electron microscopy from non-incubated portions of concentrated cell suspensions.
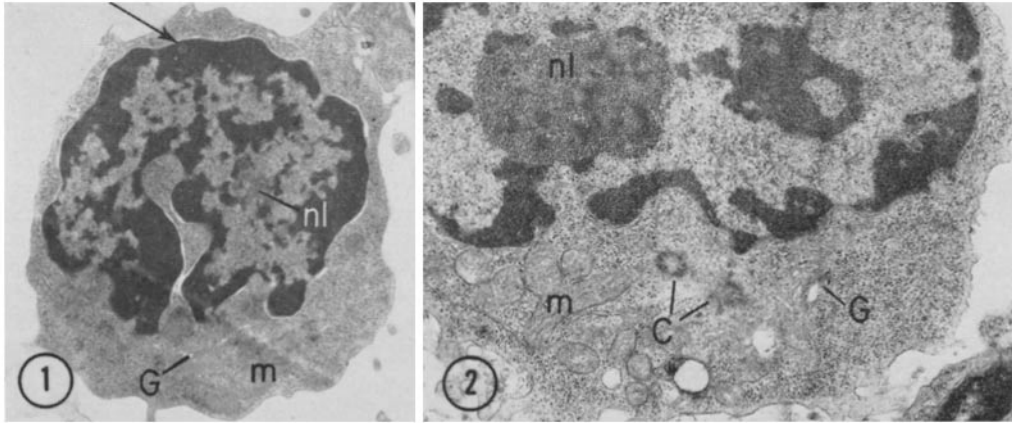
TABLE I

Enlargement and Division of Lymphocytes in PHA-Stimulated Cultures

The percentages of small lymphocytes and of large mononuclear cells are mean values based on measurement of 200 cells in Giemsa-stained smears from each of four cultures harvested at each time point. Mitotic indices are based on counts of 1000 cells of each of the same cultures. The cultures were treated with Colcemid for 2 hr before harvest.

Culture period	Small lymphocytes (<10 μ)	Large mononuclear cells (>10 μ)	Mitotic index
hr	%	%	%/hr
0	92	8	0
4	91	9	0
24	53	47	0.1
48	28	72	0.7
72	17	83	2.8

Giemsa-stained and acetic orcein-stained smears of original cell suspensions and of replicate cultures were prepared for differential cell counts, measurements of cell size, and evaluation of mitotic activity by light and phase microscopy. For the latter purpose the replicate cultures were treated with Colcemid (Ciba Pharmaceutical Co., Summit, N.J.), 0.04 μg/ml for 2 hr prior to harvest. Cells were measured by projecting their images to a ruled reticule through the prism of a drawing attachment mounted on a light microscope. The diameter of neighboring red blood cells in the smears, taken as 7 μ, was used as a standard of measurement (14). Leukocyte suspensions prepared as described from the blood of four normal adult donors whose cells were cultured for electron microscopy contained from 87 to 92% mononuclear cells and from 8 to 13% granulocytes. From 88 to 95% of mononuclear cells were small lymphocytes, the majority ranging, in Giemsa-stained smears, from 5 to 8 μ in diameter with nuclei from 4 to 6 μ. From 3 to 7% medium-sized lymphocytes (10–12 μ) and less than 1% large lymphocytes (13–15 μ) were present. Monocytes were 1–4% of mononuclear cells.

The presence of granulocytes and occasional monocytes was not a confusing factor in electron microscopy because granulocytes become necrotic within the initial 24 hr of culture (5), and monocytes do not increase in number (5) or transform to lymphocytes (15). At low magnification of the electron microscope the recognition of monocytes in culture is aided by their development of lysosomal and phagocytotic inclusions (15). Additionally, small lymphocytes stained by present methods demonstrate,

All figures are of thin sections of peripheral blood lymphocytes cultured for the time periods indicated in individual legends. Cells designated 0 hr are nonincubated, "mature", small lymphocytes. Directions of cellular and nuclear profiles are referred to the axes passing through nuclei and centrosphere regions.

Key to Symbols

C, centriole	*nl*, nucleolus
chi, intranucleolar chromatin	*np*, nuclear pore
chp, perinucleolar chromatin	*ns*, nucleolar nucleoplasmic space
G, Golgi apparatus	*pg*, perichromatin granule
gr, nuclear granular region	*sc*, nucleolar concentric structure
m, mitochondria	*si*, interstitial space of nucleolonema
mcy, cytoplasmic membrane	*za*, nucleolar agranular zone
min, inner nuclear membrane	*zf*, nucleolar fibrillar zone
mon, outer nuclear membrane	*zg*, nucleolar granular zone

FIGURE 1   0 hr cell. A deep invagination of the nuclear envelope is observed at the side facing the centro-sphere region. Numerous portions of the condensed chromatin network appear as minute aggregates interspersed among large chromatin masses. A small, round space (arrow) is recognized in the peripheral chromatin (see text). Ribosomes are densely packed in the cytoplasm. Step sections indicate that *nl* is the tangential profile of a nucleolus. × 10,000.

FIGURE 2   24 hr cell, a part in longitudinal section. The indentation of the nuclear envelope facing the centrosphere region is not so deep as in the 0 hr cell of Fig. 1. The cytoplasm is full of ribosomes, except where centrioles (*C*) and the Golgi apparatus (*G*) are seen. × 14,000.

because of abundant free ribosomes, appreciably darker cytoplasm than do monocytes, and this distinction remains evident in enlarged cells. Further, at all stages of culture, increase of the diameter of cells considered on the above grounds to be lymphoid is accompanied by roughly proportionate increase of nuclear size while, except in rare instances, cells with cytoplasmic characteristics of monocytes have smaller nucleocytoplasmic ratios.

Electron Microscopic Preparations

Cultures were harvested by transfer to 15-ml centrifuge tubes in which cells were sedimented for 10 min at 100 *g* (average). All but 1 ml of medium was aspirated, and the pellet of cells was resuspended in that volume. The cell suspension was mixed with 5 ml of 6% glutaraldehyde (16) in 0.1 M phosphate buffer, pH 7.4, at room temperature and quickly chilled in an ice bath. After 30 min, cells were again sedimented by centrifugation for 5 min at 100 *g*, then gently resuspended in 5.0 ml of phosphate buffer containing 0.2 M sucrose, and stored overnight at 5°C. Cells were lightly packed by centrifugation for 5 min at 70 *g* (average), and were postfixed by resuspension in 1% osmium tetroxide in 0.1 M phosphate–0.2 M sucrose, pH 7.4. Following this postfixation, no effort was made in succeeding steps to break up small cell clumps, which facilitated sedi-
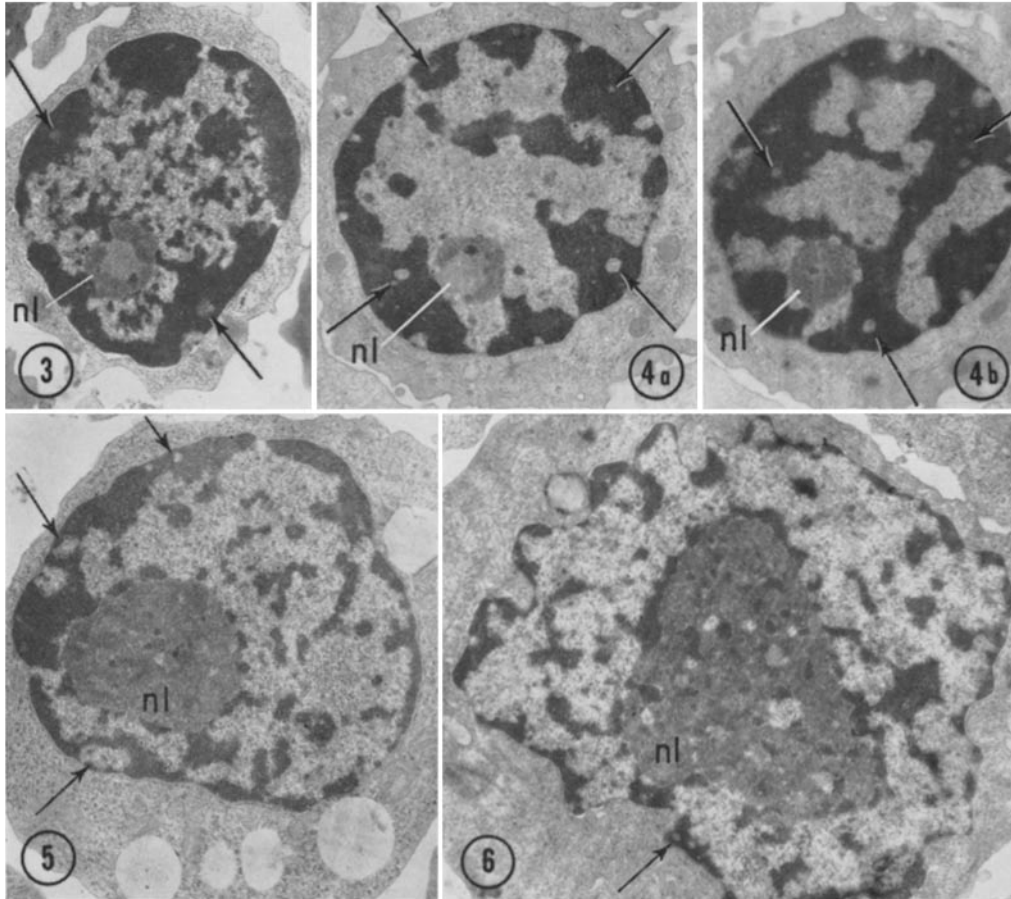
FIGURE 3 0 hr cell. A great number of minute chromatin aggregates are found in the nucleus. Inconspicuous, small round spaces are seen in the peripheral chromatin (arrows). Note that perinucleolar chromatin reaches the edge of the moderately dense core of the nucleolus. × 10,000.

FIGURES 4 *a* and *b* 4-hr cell cross-profiles at different levels. The profile in Fig. 4 *b* is closer to the nuclear periphery by about 0.7 μ. Comparison of these profiles indicates that the amount of condensed chromatin is lesser in the central nuclear region than in 0 hr cell, Fig. 3. Minute chromatin aggregates are greatly reduced in number, and coarse aggregates are more smoothly outlined. Numerous, small round spaces of low density (arrows) in large chromatin masses are conspicuous. × 10,000.

FIGURE 5 24 hr nucleus, oblique profile. Except for a few broad peripheral portions, chromatin aggregates show an approximately even distribution and roughly uniform thickness. Arrows indicate round spaces of low density in the thick regions of peripheral chromatin. × 10,000.

FIGURE 6 48 hr nucleus, oblique profile. The general configuration is similar to that of the 24 hr nucleus in Fig. 5 except for the roughly uniformly reduced thickness of peripheral chromatin and a large increase in the size and number of intranucleolar chromatin aggregates. Round spaces of low density, as found in thick portions of peripheral chromatin of 4- and 24-hr nuclei, are few in this enlarged nucleus (arrow). × 10,000.

mentation by a few minutes of centrifugation at 25 g. Cells were washed once with a fresh portion of phosphate buffer-sucrose and dehydrated through graded concentrations of alcohol and propylene oxide. Final pellets were embedded in Epon 812 (17), polymerization being carried out overnight at 40°C and for one day each at 50° and 60°C.

Sections 200–500 A thick were cut with the Porter-Blum MT-1 or MT-2 microtome and doubly stained with uranyl acetate (18) and lead hydroxied (19), or lead citrate (20). Sections were examined with the Hitachi HU-11B microscope.

Radioautography

Chromatin was labeled by adding thymidine-^{3}H (New England Nuclear Corp., Boston, Mass; NET-024, specific activity 6.7 c/mmole) directly to 46-hr-old cultures (100 μc/ml) and by harvesting 2 hr later. Cells were washed twice with fresh medium prior to being fixed, embedded, and sectioned as described above.

Radioautographs were prepared of sections mounted directly on Formvar-coated grids. After double staining, the sections were vacuum-coated with a thin layer of carbon. A drop of Kodak NTE emulsion, diluted and fractionated by the procedure of Salpeter and Bachmann (21), was applied, and the excess was removed by imbibition on filter paper as described by Granboulan and Granboulan (22). Before the sections were coated, control grids had been prepared to test for uniformity of the emulsion layer.

After exposure for 3 wk in helium-filled containers at 5°C, development including gold latensification was carried out according to Salpeter and Bachmann (21). Kodak Dektol was used as developer, and 20% sodium thiosulfate ("hypo") was used as fixer. Acid fixatives were found to affect the stability of lead staining, causing the deposition of particulate matter, especially over densely stained portions of chromatin or nucleoli. Although these deposits could be removed with dilute alkali, resultant radioautographs showed reduced stain intensity. The characteristic shape of grains developed in Dektol rather than in high resolution developers was found helpful in their identification. In order to minimize disturbance of the localization of grains, specimens were observed without removal of the gelatin layer.

OBSERVATIONS

Enlargement of Lymphocytes in Culture (Light Microscopy)

Increase of large mononuclear cells and simultaneous decrease of small lymphocytes during 72 hr of culture with PHA, as observed in Giemsa-stained smears, is indicated in Table I. The "blastlike" features in light microscopy of the enlarged cells have been well described (5, 6, 23, 24). Their abundance at 24 and 48 hr in the face of little mitotic activity in prior periods indicates the origin of the enlarged cells from small lymphocytes.

General Morphology of Cultured Lymphocytes (Electron Microscopy)

Descriptions and illustrations below are derived from observations of from 2000 to 10,000 cells harvested at each of the time periods 0, 4, 24, 48, and 72 hr following initiation of culture, a total of approximately 30,000 cells. For convenience, cells cultured for n hr will be referred to as n-hr cells and their nuclei or nucleoli as n-hr nuclei or nucleoli.

Several aspects of the general morphology of cultured lymphocytes deserve attention before entering into more detailed descriptions of nuclear changes. First, it has been noted in both light (25, 26) and electron microscopy (15, 23, 24, 27, 28) that the roughly spherical, small lymphocyte has a thickening of its otherwise thin cytoplasmic rim in the region where centrioles, Golgi apparatus, and small clusters of mitochondria are found (Fig. 1). Such polar arrangement may also be recognized in enlarged cells during culture. An axis passing through the nucleus and centrosphere region ("longitudinal axis") thus provides a convenient landmark for orientation of sections. One or more indentations of the nucleus in the centrosphere region, usually deep and narrow in 0 hr cells (Fig. 1) persist, but in quite variable form, in cultured cells. In 4- and 24-hr cultures they are generally broad and shallow (Fig. 2). They are most often again deep and narrow in enlarged cells of later cultures (Figs. 7 b and c).

Second, an eightfold increase in nuclear volume occurs during interphase in the 2–3 days of culture, as estimated from approximate doubling of nuclear diameters. Diameters are only rarely as great as 5 μ in 0 hr lymphocytes under present conditions of fixation. In 72-hr cultures more than half of nuclear profiles measure 6–9 μ and many as much as 10–12 μ. Major changes in nuclear constituents might be expected, and certain of the structural ones are to be described in this paper pending further study of their functional correlation.

Major Structural Changes in Growing Nuclei in Interphase

In most 0 hr nuclei (Figs. 1, 3) condensed chromatin consists of large peripheral and central aggregates and of minute aggregates, the latter forming an irregular trabecular pattern in the interspaces. The margins of the large aggregates are often irregular, apparently because of some continuity with the minute ones. Remaining space of low density consists of nucleoplasm which has granular regions (29) where interchromatin granules are found (Figs. 9 a and b) and diffuse chromatin regions (2) elsewhere.

In more than 80% of 4-hr nuclei, minute chromatin aggregates are greatly reduced in number, and large aggregates are more smoothly outlined but do not appear to be much different in distribution than those in 0 hr nuclei (compare Figs. 3 and 4 a). A concomitant, moderate increase in the amount of nucleoplasm occurs, more conspicuously at some sectional levels of a given nucleus than at others (compare Figs. 4 a and b). It is possible, therefore, that the minute aggregates of 0 hr nuclei transform from the condensed state to a diffuse state in 4-hr nuclei. Whether this accounts for the fate of all the minute aggregates or whether some are reorganized into the large aggregates of 4-hr nuclei remains for further study. Another feature of 4-hr nuclei is the appearance of numerous round spaces of low density within the large aggregates (Figs. 4 a and b). These are observed much less frequently in 0 hr nuclei (Figs. 1 and 3).

Some nuclei of 24-hr cells resemble those of 4 hr culture in that they contain relatively few chromatin aggregates in the central regions. In more than 60% of the whole population, however, large numbers of chromatin aggregates are roughly evenly distributed throughout central portions of nuclear profiles (Fig. 5). Some large aggregates remain in the peripheral portions. In such large portions of chromatin, round spaces of low density similar to those found in 4-hr nuclei are often recognized (arrows in Fig. 5).

Among large nuclei or 48- and 72-hr cultures, a variety of chromatin patterns such as shown in Figs. 6 and 7 a–c is observed. The 48 hr nucleus in Fig. 6 shows a structure which is qualitatively similar to the typical 24 hr nucleus seen in Fig. 5, except that in this larger nucleus the large peripheral chromatin aggregates have thinned at 48 hr

TABLE II

Nuclear Structure of Cultured Lymphocytes

Proportion of large nuclear profiles ($>6\ \mu$ in diameter) increases with culture time.

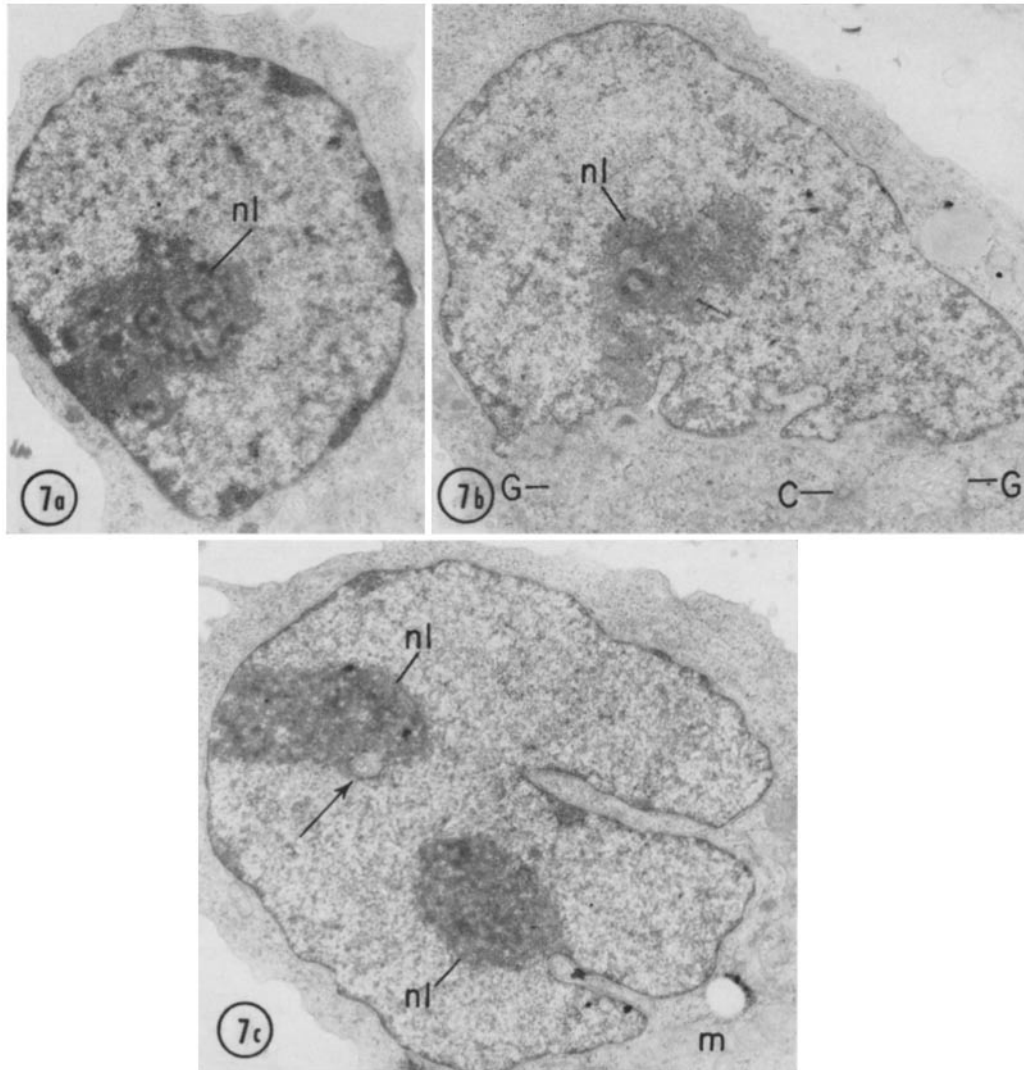| Culture period | Count under electron microscope | | Large nuclei |
	Large nuclei	Total nuclei	
hr	No.	No.	%
24	47	2,128	2
48	4,533	9,157	50
72	5,494	8,963	61

TABLE III

Nuclear Structure of Cultured Lymphocytes

Large nuclei with loosened or dispersed chromatin increase in number with culture time.

| Culture period | Large ($>6\ \mu$ diameter) nuclei | Chromatin state | | | |
		Condensed	Loosened	Dispersed	Mitotic
hr	No.	%	%	%	%
48	4,533	62	31	5	2
72	5,494	26	59	11	4

TABLE IV

Nuclear Structure of Cultured Lymphocytes

DNA synthesis was estimated by radioautography of 48 hr culture.

Batch	Nuclei examined	Thymidine-labeled nuclei (10–300 silver grains)
	No.	% examined
1	1,929	16.6
2	1,184	16.0
3	1,054	18.3
Total	4,167	Average 16.9

to approximately the width of central ones. In the 72 hr nucleus of Fig. 7 a, the peripheral chromatin is similar to that in the nucleus of Fig. 6, both in width and density, but the central aggregates are much reduced in width and are appreciably lower in density. Both peripheral and central aggregates in the nucleus of Fig. 7 b are greatly reduced in density as well as in width, and in Fig. 7 c chromatin is almost completely dispersed except at the periphery. Figs. 7 a and 7 b illustrate
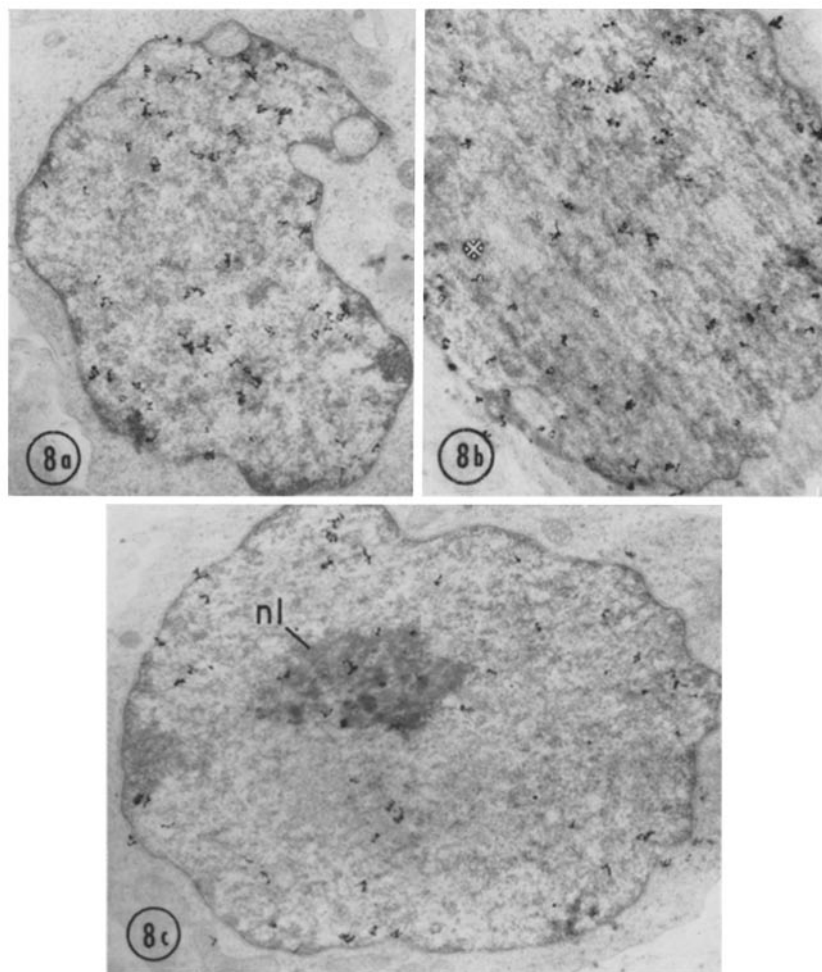
FIGURES 7 a–c Fig. 7 a, a 48 hr nucleus in oblique section, shows peripheral chromatin similar to that in Fig. 6 and central chromatin aggregates of reduced density and greater number and narrower width. Fig. 7 b, a 72 hr nucleus sectioned in the longitudinal axis, shows both peripheral and central chromatin aggregates reduced in density as well as in width. Fig. 7 c, a 72 hr nucleus sectioned longitudinally, shows few chromatin aggregates and peripheral chromatin of greatly reduced thickness. Nucleolonemal configurations are vaguely discerned in Figs. 7 a and b, and more distinctly in Fig. 7 c. In Figs. 7 b and c, more or less deep indentations of nuclear envelopes are seen at the sides facing the centrosphere regions. An arrow in Fig. 7 c indicates the cross profile of a probable very deep and somewhat tortuous indentation. Nucleoli appear closely associated with indentations. × 10,000.

what shall be termed herein loosened chromatin, and Fig. 7 c displays dispersed chromatin.

From the data of Table II it appears that the proportion of large nuclei in cell culture has increased strikingly at 48 hr and still further

(to at least 60%) at 72 hr. The fraction of smaller nuclear profiles at 48 and 72 hr has been found to consist of ones similar to those of 24 hr or earlier profiles or of tangentially sectioned ones.

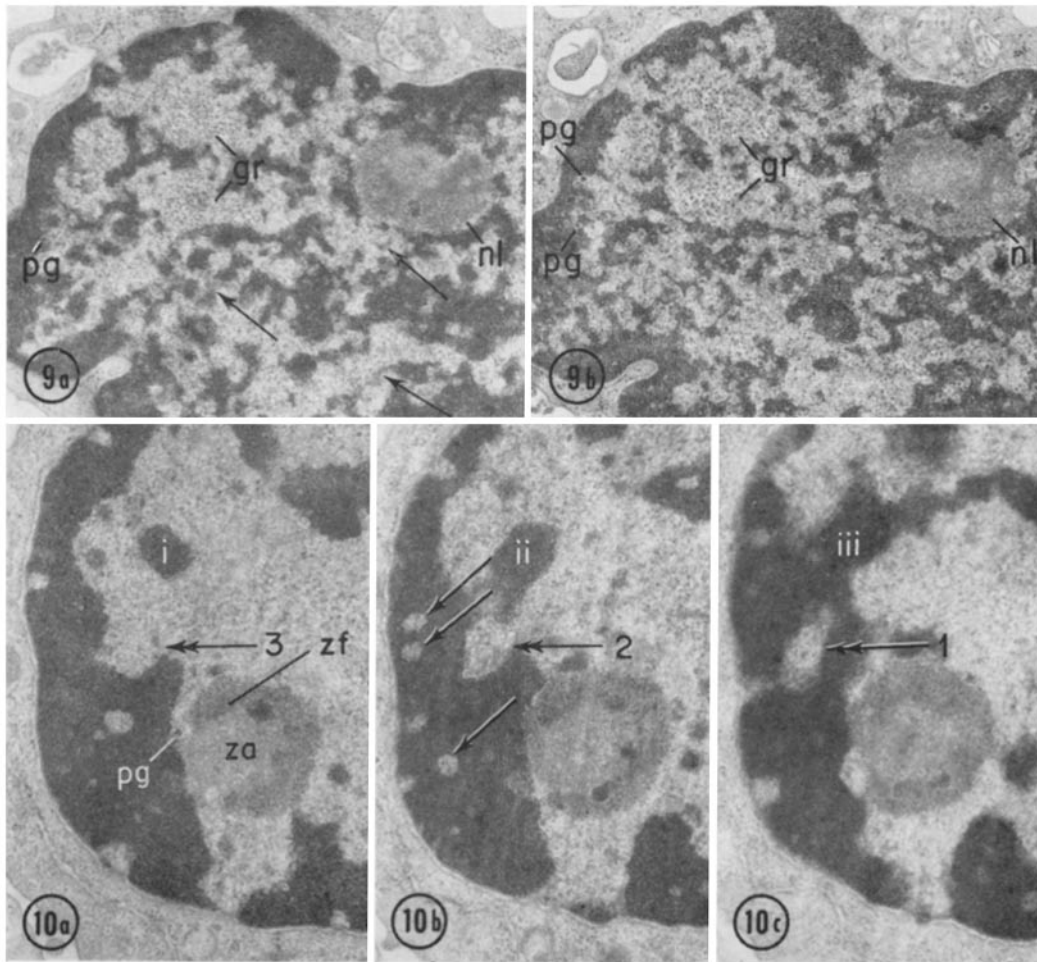In Table III the chromatin is shown to be

FIGURES 8 a–c 48-hr nuclear profiles, DNA radioautographs. Note the morphological similarity between Figs. 7 a and 8 a, Figs. 7 b and 8 b, and Figs. 7 c and 8 c. In Fig. 8 a, few silver rains, if any, are found on condensed peripheral chromatin. An asterisk in Fig. 8 b covers a dirt particle. × 10,000.

loosened or dispersed in a greater fraction of the large nuclei after longer culture, that is 72 hr compared to 48 hr. One finds upon examining the populations of cells of the separate culture periods evidence for a morphologic continuum from the appearance typified by Fig. 6 of condensed chromatin to that of Fig. 7 a for loosened chromatin and of Fig. 7 c for dispersed chromatin. It is of interest that although in 24 hr culture small numbers of nuclei are classified as "large" (2% in Table II) and display patterns comparable to those of Fig. 6 and 7 a; none of these has thus far been recognized to display chromatin

in the dispersed state of Fig. 7 c nor, indeed, of the advanced loosened state of Fig. 7 b.

In Table IV estimates of DNA synthetic activity in 48-hr nuclei are made using radioautography. About 17% are considered to have significant numbers of silver grains, above 10 and ranging up to 300 gr with low background densities, as exemplified in Figs. 8 a–c. All of such labeled nuclei show either loosened or dispersed chromatin patterns. The fraction of nuclei labeled agrees with data from light-microscope radioautography (5, 11).

In Table V further analysis of radioautographic

FIGURES 9 *a* and *b*   0 hr nucleus, serial sections. Granular regions are easily discerned because their over-all density is higher than that of the rest of the nucleoplasm. It can be surmised from serial sections that numerous minute chromatin aggregates together with coarser aggregates form a continuous network. In the thick (about 500 A) section of Fig. 9 *a*, certain portions of the network (arrows) appear lower in density than the remainder, but little variation in density is observed in the thin (roughly 250 A) section of Fig. 9 *b*. A chromatin aggregate which is continuous with the peripheral chromatin is in contact with the moderately dense core of the nucleolus, i.e. agranular zone. × 20,000.

FIGURES 10 *a–c*   4 hr nucleus, parts of the first, third, and fifth serial sections. An apparently isolated chromatin profile in Fig. 10 *a, i*, is seen to be in continuity with other chromatin profiles in Fig. 10 *b, ii*, and in Fig. 10 *c, iii*. Small, circular spaces, indicated by single-headed arrows in Fig. 10 *b*, show no corresponding structure in Figs. 10 *a* and *c* and are found to be isolated from the nucleoplasm. On the other hand, a large, oval-shaped space in Fig. 10 *c* (double-headed arrow *1*) anastomoses with the nucleoplasm, as shown in Fig. 10 *b* (double-headed arrow *2*) and in Fig. 10 *c* (double-headed arrow *3*). In the nucleolar profiles in Figs. 10 *a* and *b*, the nucleolar agranular zone (*za*) is found to be in contact with the peripheral chromatin. The fibrillar zone (*zf*) is significantly lower in density than is the condensed chromatin. Perinucleolar minute aggregates of chromatin as seen in Fig. 9 are virtually absent here in Fig. 10 and are found again in the 24 hr nucleolar profile of Fig. 11. × 20,000.
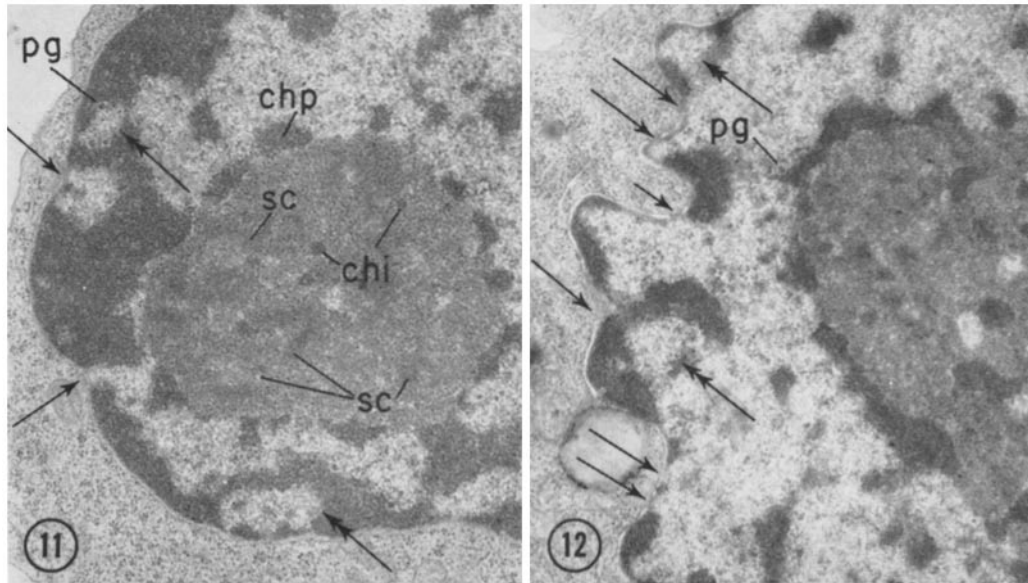
FIGURE 11 24 hr nucleus, a part of the profile of Fig. 5. Low density spaces of somewhat distorted oval configuration (double-headed arrows) in thick peripheral chromatin resemble the large space of low density in Fig. 10 c of a 4 hr nucleus (double-headed arrow 1 in Fig. 10 c). Only a small number of nuclear pores (single-headed arrows) disrupt the continuity of the thick peripheral chromatin. Perinucleolar chromatin aggregates (chp) are increased in number. In the nucleolus outer layers of circular structures (sc), i.e. fibrillar zones, are lower in density than chromatin aggregates. × 20,000.

FIGURE 12 48 hr nucleus, part of Fig. 6. Spaces indicated by double-headed arrows are partially encircled by chromatin aggregates. The reduced thickness of peripheral chromatin is accompanied by a striking increase in the number of nuclear pores (single-headed arrows). × 20,000.

TABLE V

Nuclear Structure of Cultured Lymphocytes

Labeled thymidine uptake is much greater in nuclei with loosened chromatin compared to dispersed chromatin.

| | Two adjacent large nuclei, labeled | | |
Consecutive section examined	(L) Nucleus with loosened chromatin	(D) Nucleus with dispersed chromatin	Ratio
	gr/μ^2	gr/μ^2	$(L)/(D)$
1	3.9	0.8	4.9
2	4.3	1.3	3.3
3	9.4	3.0	3.1
4	6.7	1.7	3.9

studies suggests that the loosened state of the chromatin is associated with the most DNA synthetic activity and supports the view that morphologically the developmental sequence of chromatin in interphase is from condensed to loosened to dispersed state. The table shows the silver grain counts on two adjacent nuclei (7 μ apart center-to-center), one of which nuclei presented the loosened chromatin state and one the dispersed. Four consecutive sections are counted, and in each the nucleus with loosened chromatin shows labeling three or more times greater than does the nucleus with dispersed chromatin. The different sections have different values of grain densities, possibly for technical reasons, but the relative distribution of label between the two nuclei is consistent, and the observation has been confirmed in several other pairs.

Chromatin Alterations Prior to S Phase; Possible Centers Related to Chromatin Disaggregation

From serial sections it would appear that most of the chromatin aggregates in a nucleus form a continuous network during all phases of culture.

Examples are shown in 0 and 4-hr nuclei in Figs. 9 and 10. In thick sections of 0 hr nuclei, a certain variation of density is observed among profiles of minute chromatin aggregates (Fig. 9 a of a 500 A section). When the section thickness is reduced, such variation becomes insignificant (Fig. 9 b of a consecutive 250 A section). Portions of chromatin aggregates which show lower densities in thick section may, therefore, be considered to represent tangentially sectioned areas. It would appear to follow that the density of all chromatin aggregates may be uniform.

Round spaces of low density are numerous in the large chromatin masses of 4-hr nuclei and appear to have the same texture and density as the nucleoplasm (Figs. 10 a–c). When studied in serial sections most of the small typically circular spaces appear isolated from the nucleoplasm (compare Fig. 10 b with Figs. 10 a and c); on the other hand relatively large spaces, often oval rather than circular (Fig. 10 c), frequently anastomose with the nucleoplasm (Figs. 10 a–c).

The spaces of low density found in the thick portions of peripheral chromatin aggregates in 24-hr nuclei often resemble the relatively large spaces of 4-hr nuclei (compare Figs. 10 and 11). In enlarged nuclei of later cultures, the peripheral chromatin is reduced in thickness, and such spaces are seldom seen. Nonetheless, portions of nucleoplasm which are partially encircled by chromatin aggregates can be found in some of the enlarged nuclei (Fig. 12).
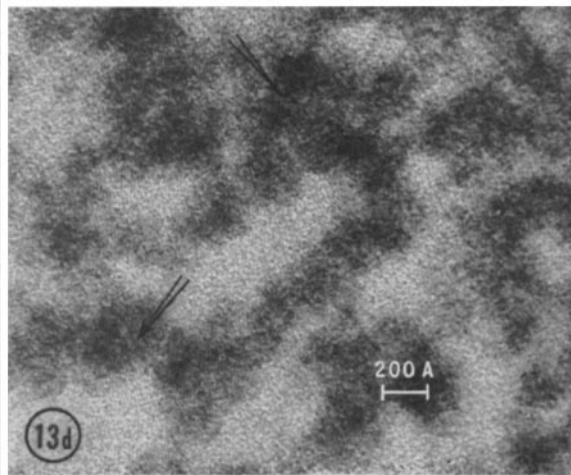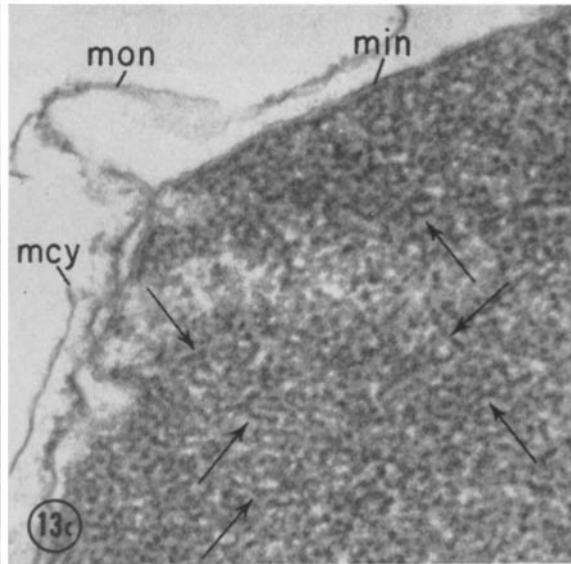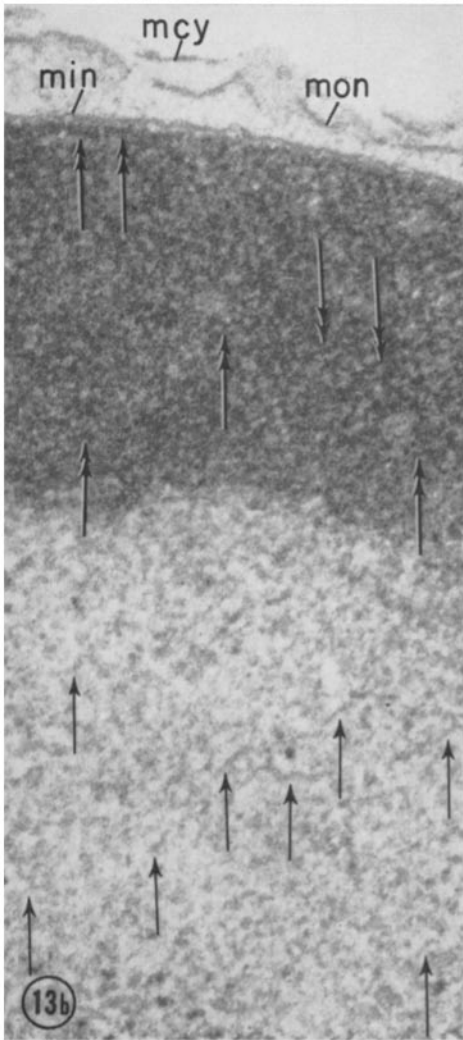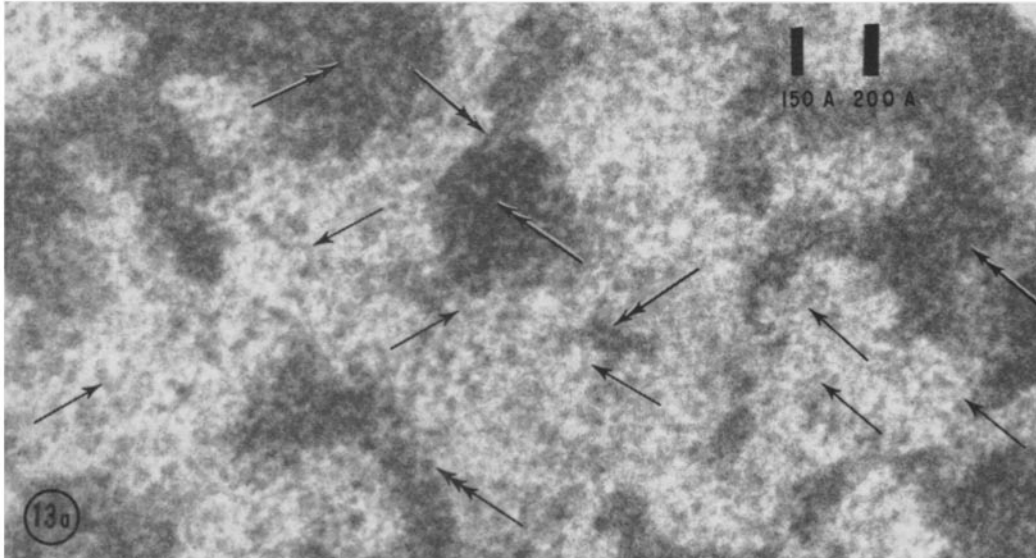
These observations raise the possibility that small isolated spaces in condensed chromatin of 4-hr nuclei may be centers for the transformation of portions of condensed chromatin into the diffuse state. The enlargement of such spaces and their anastomosis with the nucleoplasm appear to be parts of the disaggregation process of large chromatin masses as well as of the expansion of the nucleoplasm. Together with the disappearance of minute chromatin aggregates during the first 4 hr of culture, these events provide considerable character to the initial period of the growth process. Pogo and coworkers have reported (8) that early increases of RNA and protein synthesis in lymphocyte cultures may involve the activation of genetic loci which in resting lymphocytes are repressed. The possible functional role of the small spaces is an intriguing one awaiting more definitive studies.

Nuclear pores are rather few in 24-hr and earlier cultures (Fig. 11). When the large peripheral chromatin aggregates undergo subdivision in later cultures, a striking increase in number of pores is recognized (Fig. 12), suggesting an enhanced nucleocytoplasmic interaction.

Structural Organization of Condensed Chromatin

It is now known from studies of isolated chromatin or chromosomes (30–35) that fibers of roughly 200–250 A in diameter are the main constituents

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FIGURES 13 a–d   Parts of 0 hr nuclei. Fig. 13 a is a part of Fig. 9 a, an intact nucleus in a 500 A section. Vaguely defined fibrous structures are found both in condensed chromatin (double-headed arrows) and diffuse chromatin regions (single-headed arrows). Structures of granular appearance are occasionally seen (triple-headed arrows). In a mildly lysed nucleus in Fig. 13 b, the fibrous structures are more commonly and easily observed (double- and single-headed arrows in condensed chromatin and diffuse chromatin and diffuse chromatin regions). They are appreciably higher in density in the condensed than in the diffuse chromatin regions in both the intact and the mildly lysed nuclei. The matrix in which they are embedded also appears to be higher in density in condensed chromatin. The fibrous nature of chromatin becomes more conspicuous in a drastically lysed nucleus, Fig. 13 c. The chromatin is still attached to the inner nuclear membrane. Fibrous structures of roughly 200–250 A diameter are shown at high magnification in Fig. 13 d. They are neither smoothly outlined nor clearly defined. Minute fibrils, about 20 A in width, are found (double-stemmed arrows). The fibrous structures in the intact nucleus in Fig. 13 a, on the other hand, are not so thick as 200 A but close to 150 A. Note that the width of the fibrous structures in Fig. 13 b of a mildly lysed nucleus appears approximately the same as that of the structures in Fig. 13 c of a drastically lysed nucleus, despite the much higher magnification of Fig. 13 b. Fig. 13 a, × 100,000; Fig. 13 b, × 80,000; Fig. 13 c, × 50,000; Fig. 13 d, × 300,000.

150 A 200 A

13a

mcy

min

mon

13b

mon

min

mcy

13c

13d

200 A

of chromatin. Short structures which appear to be chromatin fiber segments are observed in condensed chromatin, as well as in diffuse chromatin regions in Fig. 13 a (double- and single-headed arrows). These segments are found with considerable frequency in thick sections, as is that of Fig. 13 a, but their image quality is inevitably degraded.

The chances of including long segments of these fibers in thin sections might increase if the fibers were released from tight and tortuous binding. Study of this point has been attempted by observing nuclei of 0 hr cells which have been lysed to various degrees with distilled water. Fig. 13 b shows a portion of a cell lysed to a moderate degree in which the cytoplasmic system is partially destroyed but the over-all organization of the swollen nucleus is still preserved. In this preparation fibrous structures are more easily recognized than in intact nuclei. Those in diffuse chromatin regions are appreciably lower in density than those in condensed chromatin regions, both in intact (Fig. 13 a) and in mildly lysed nuclei (Fig. 13 b).

When cells are lysed more extensively the nuclear organization is destroyed but the fibrous nature of chromatin becomes more conspicuous (Fig. 13 c). The widths of extended fibrous structures in these preparations are roughly in agreement with those of isolated chromatin fibers under direct observation, and minute filaments approximating the diameter of the DNA molecule may be found within them (arrows in Fig. 13 d). It is to be noted that even at the high resolution presented the structure of the chromatin fiber is still unresolved. Whether the occasionally observed granular structures (Fig. 13 a) indicate the existence of a granular component in chromatin or merely represent cross-profiles of chromatin fibers is not clear.
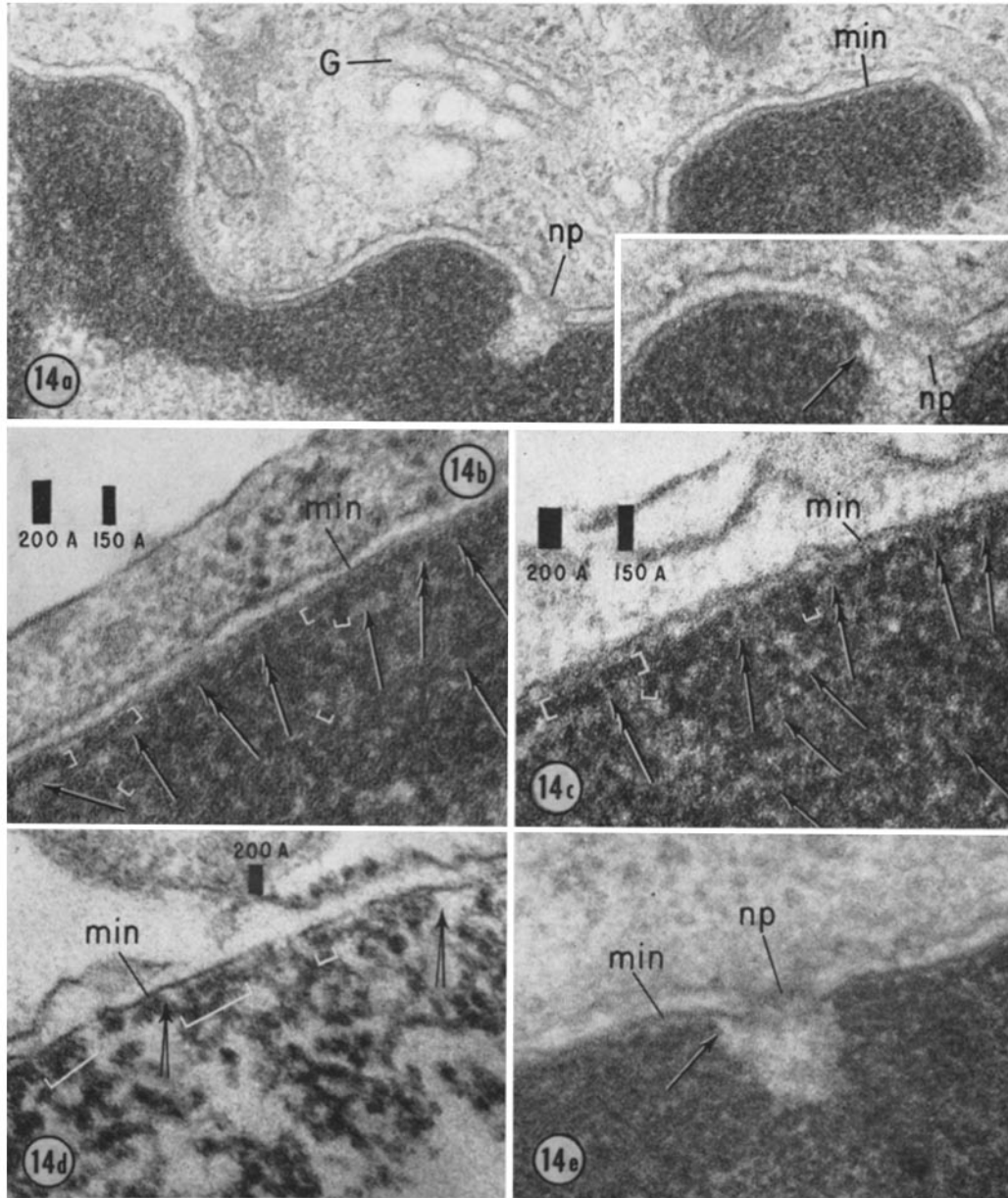
In locations where well-defined cross-profiles of the inner nuclear membrane are seen, an approximately uniform space of moderate density, about 50 A in width, is generally distinguished between the membrane and the large peripheral chromatin aggregates (Fig. 14 a). Among those dense components of the aggregates which are considered to be various profiles of chromatin fibers, lie interspaces of quite similar density to the space of moderate density (single-headed arrows in Fig. 14 b). Structural continuity of these interspaces with the space of moderate density is sometimes recognizable (double-headed arrows in Fig. 14 b). Such continuity is observed more clearly in the mildly lysed nucleus of Fig. 14 c.
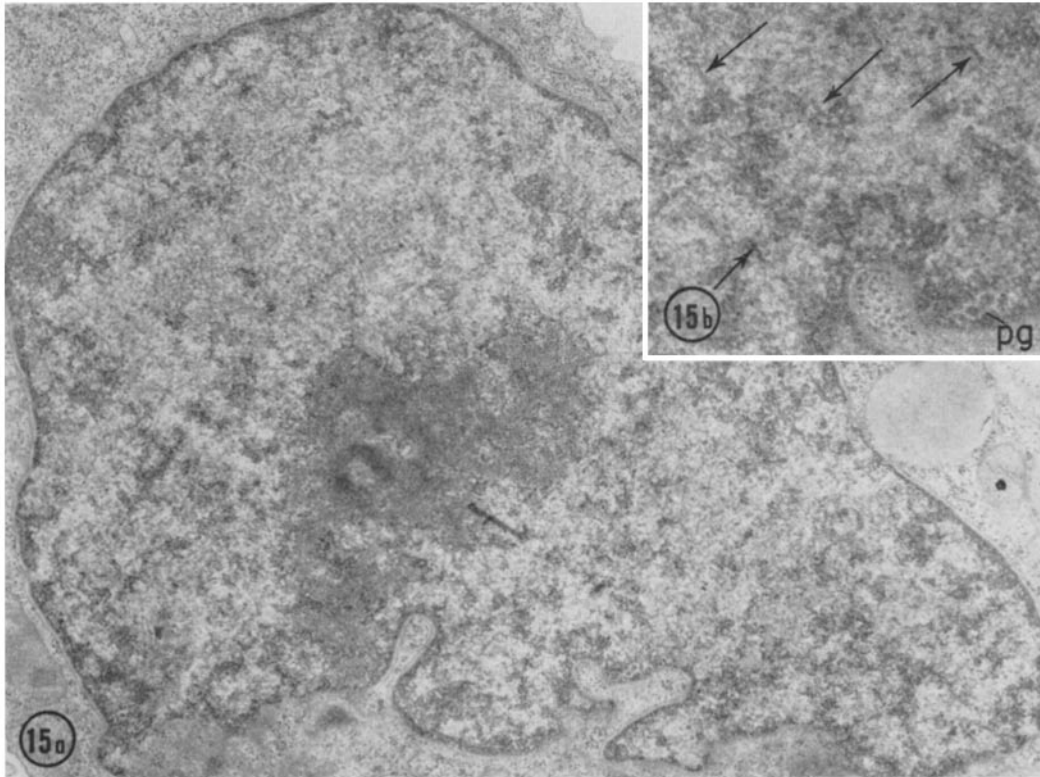
Of interest, it has been found that condensed chromatin is not easily dispersed by osmotic shock nor easily detached from the inner nuclear membrane (Fig. 13 c of nucleus after lysis with water). A few sites of such detachment may be found after drastic cell lysis (Fig. 14 d). These findings suggest that the substance which fills these less dense spaces may, among other possible functions, serve to bind chromatin fibers together and to anchor chromatin to the inner nuclear membrane (33).

Additional information on the space of moderate density may be supplied by two further observations. In early prophase the inner nuclear membrane, bare of chromatin aggregates, has the thickness of a unit membrane and shows no recognizable underlying space of moderate density separating it from the nucleoplasm. Second, in intact interphase nuclei, short segments of the inner membrane next to nuclear pores may be occasionally found to be free from attachment of peripheral chromatin (insert in Figs. 14 a and e). It appears that these places are also free of the substance of the space of moderate density. Such observations appear to relate this material to the presence of nondispersed chromatin.

The profiles of chromatin fibers in intact or mildly lysed nuclei are significantly narrower, frequently about 150 A or less (Figs. 13 a, 14 b and c), than those of artificially separated chromatin fibers which are often 200 A or more in width (Fig. 13 d). This can be seen more clearly in Fig. 13 b and c. Despite the fact that the magnification of Fig. 13 b is much higher than that of Fig. 13 c, the profiles of chromatin fibers in Fig. 13 b of a mildly lysed nucleus appear to be of approximately the same width as those in Fig. 13 c of a drastically lysed nucleus. Such relationship is also seen in Figs. 14 b and d of intact and drastically lysed nuclei. Possibly chromatin fibers when released and separated so forcibly may shrink and broaden. An additional possibility is that the vague outlines and broadening of fibers as in Fig. 13 d may be a result of a continued adherence of fiber-binding substance following disruption of chromatin aggregates.

FIGURES 14 a–e   0 hr and 4-hr nuclei, parts including the nuclear envelope. In Fig. 14 a of a 4 hr nucleus, the space of moderate density, about 50 A thick, is observed between the inner nuclear membrane and the peripheral chromatin. In condensed chromatin of a 0 hr nucleus in Fig. 14 b, interspaces of quite similar density (single-headed arrows) are found among dense components considered to be various profiles of chromatin fibers. Continuity between the interspaces and the space underlying the inner nuclear membrane is also observed (double-headed arrows). This is more definitely seen in the mildly lysed 0 hr nucleus in Fig. 14 c, an enlarged portion of Fig. 13 b. A few portions of the inner nuclear membrane where chromatin fibers are detached are seen in Fig. 14 d of a drastically lysed 0 hr nucleus (double-stemmed arrows). In Fig. 14 e of a 4 hr nucleus and also in the insert in Fig. 14 a, peripheral chromatin does not quite reach the edges of nuclear pores (np) at the positions indicated by arrows. In such cases, the nucleoplasm appears to be in direct contact with the inner nuclear membrane, which shows the thickness of a unit membrane, about 60 A. Note, in Figs. 14 b and c, that the widths of the chromatin fibers marked by small brackets are not so wide as 200 A but are close to 150 A. On the contrary, in Fig. 14 d, the widths of many fibers are close to 200 A. Fig. 14 a, × 70,000 (insert, × 120,000); Fig. 14 b, × 120,000; Fig. 14 c, × 150,000; Fig. 14 d, × 90,000; Fig. 14 e, × 120,000.

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Figures 15 a and b 72 hr nucleus in Fig. 7 b and its part at higher magnification. The width of chromatin aggregates in Fig. 15 a of a 250 A section is not much different from that of the minute chromatin aggregates of the 0 hr nucleus of Fig. 9 b in a section of comparable thickness, but the density is significantly lower. It can be seen in Fig. 15 b that chromatin fibers (arrows) are more loosely arranged and more easily recognized in this loosened state of chromatin than in condensed chromatin. A certain variation of density is observed among such fibers. Perichromatin granules (pg) are still found in association with this loosened state of chromatin. Fig. 15 a, × 18,000; Fig. 15 b, × 40,000.
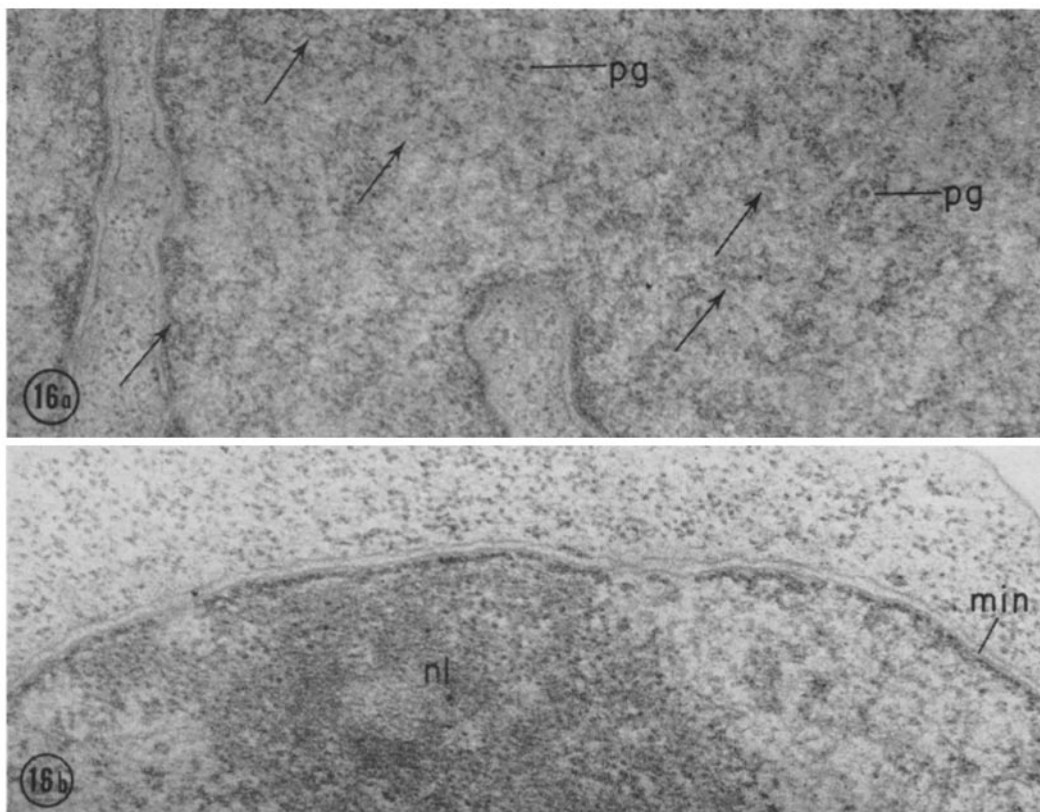
Structural Alterations of Chromatin during S Phase and the Localization of DNA Synthesis

As described above, nuclei in S phase are characterized by the presence of the loosened or the dispersed state of chromatin. Chromatin fibers in the loosened state are more separately arranged than in the condensed state (compare Figs. 15 a and 9 b which are of sections of similar thickness), making it easier to observe the fibrous nature of chromatin (arrows in Fig. 15 b). The width of such fibers, most commonly about 150 A (Fig. 26), is the same as that of the fibers found in condensed chromatin of intact nuclei. When chromatin is highly dispersed, the fibrous nature becomes even more conspicuous (arrows in Fig. 16 a). A certain variation in density is recognized among

chromatin fibers, in both the loosened and dispersed states (Figs. 15 b and 16 a). Whether this reflects a real difference is not clear at present.

Perichromatin granules (36–38) consistently maintain a close association with chromatin aggregates throughout the transformation process (Fig. 9–12 and 15 b). In nuclei with highly dispersed chromatin, the halos of low density still provide a positive means of identification (Fig. 16 a).

As in the nuclei of prior stages, the space of moderate density continues to be observed between the peripheral chromatin and the inner nuclear membrane wherever cross-profiles of the membrane are clearly seen, even when peripheral chromatin is greatly reduced to a thickness con-
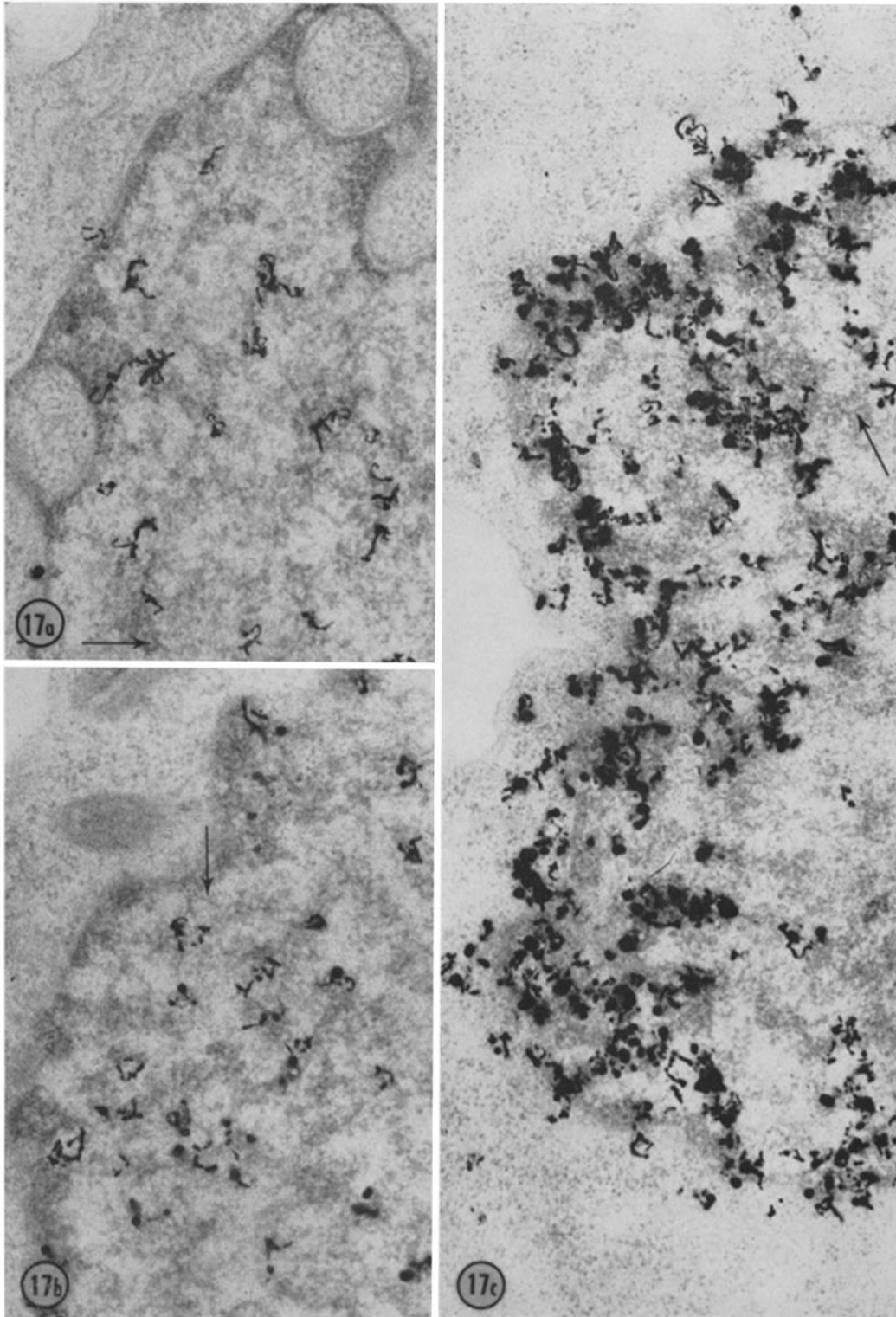
Figures 16 *a* and *b* 72-hr nuclei, portions with dispersed chromatin. In Fig. 16 *a*, chromatin fibers of variable density are readily found (arrows). Halos of low density reveal the existence of perichromatin granules (*pg*) even in this highly dispersed state of chromatin. In Fig. 16 *b*, the space of moderate density, about 50 A in width, is observed between the inner nuclear membrane and the peripheral chromatin of such reduced width as to be consistent with a monolayer of chromatin fibers. Fig. 16 *a* × 40,000; Fig. 16 *b*, × 48,000.

sistent with a monolayer of chromatin fibers (Fig. 16 *b*).

In the nucleus of Fig. 8 *a* which is considered to be in an early period of S phase, silver grains are almost exclusively found in association with loosened portions of chromatin and rarely on condensed peripheral chromatin. Parts of nuclei at higher magnification in Figs. 17 *a* and *b* show this association with loosened chromatin even more clearly, and it is further emphasized in Fig. 17 *c*. In the nucleus of Fig. 18, a significant number of grains is found in association with portions of peripheral chromatin so thin as to be considered monolayers of chromatin fibers.

Although DNA synthesis appears most active

in loosened chromatin, as described above, whether it occurs in diffuse chromatin remains unsettled. In an attempt to determine whether it occurs in diffuse chromatin regions of nuclei containing loosened chromatin, silver grain counts were made over a total of 30 such nuclear profiles with 4000 total counts. 5% of the grains were found unassociated with loosened chromatin aggregates and 95% associated with aggregates, after correction had been made for a background count found in perinuclear zones to be equivalent to 7% of the total count. This relatively low level of grain count in diffuse chromatin regions in nuclei with loosened chromatin does not permit safe conclusion, considering the possible artifacts involved (39, 40).

FIGURES 17 a–c 48-hr nuclei, DNA radioautographs. In Fig. 17 a, a part of the nucleus of Fig. 8 a which is considered to be in an early stage of S phase, most of the peripheral chromatin still appears to be condensed, and the majority of silver grains are associated with the inner, loosened portions of chromatin. The association of silver grains with the loosened chromatin network is more clearly seen in Fig. 17 b. In Fig. 17 c, numerous silver grains are distributed roughly on the network of loosened chromatin. Profiles of chromatin fibers are easily seen (arrows). × 35,000.

Nucleolar Changes

Nucleoli are observed in thin sections of 0 hr lymphocytes with a frequency consistent with estimates of the relative sizes of nucleolus and nucleus. Although light microscopists have not uniformly found nucleoli in resting small lymphocytes, Maximow in 1928 (25) and later Bloom (26) described them; others confirmed their presence with use of electron microscopy (41). The typical appearance when the plane of section is appropriate is shown in Figs. 19 *a* and *b*. The nucleolus, roughly circular and 1–1.5 μ in diameter, appears composed of three concentric layers. The central region (*za*) of moderate density, about 0.5 μ in diameter, and the midzone (*zf*) of higher density, about 0.1 μ in width, appear to be fibrillar. The outermost layer (*zg*), on the other hand, is composed of a matrix of moderate density in which granules about 150 A in diameter are recognized. These layers from center to periphery will be referred to as agranular, fibrillar, and granular zones, respectively. Small clumps of intranucleolar chromatin, quite frequently found within the fibrillar zone (*chi*, Fig. 19 *b*), have not been seen to penetrate the agranular zone. Fibrous components may be recognized within the matrix of the granular zone in closely focused micrographs (arrows in Fig. 19 *b*).

Nucleoli of cells harvested at 4 hr are seldom appreciably enlarged. In many, however, there is indication of an increase of the number and concentration of granules in the granular zone (Fig. 20).

In 24-hr cultures nucleoli are frequently enlarged to a diameter of 2 μ or more as illustrated by comparison of Figs. 20 and 22 *a* (compare also Figs. 10 and 11). The complex structure now present may be seen to be composed of the basic components of the 0 hr nucleolus. Agranular zones are quite regularly accompanied by peripheral fibrillar components, the two often assuming a somewhat modified concentric arrangement. These more or less well-defined concentric structures (*sc* in Fig. 22 *a*) measure 0.3–0.4 μ in diameter, one-half or less the size of those in 0 hr nucleoli. Some fibrillar zones (*zf*) are found in the absence of agranular zones in Figs. 21 and 22 *a*. Whether these exist independently of agranular zones or appear to do so because of a sectional plane in the periphery of a concentric structure is not certain.

In addition to a great increase of granules in 24-hr nucleoli, the arrangement of granular zones as distinct outermost layers of concentric structures is obscured or totally lost. Fibrous components (arrows in Fig. 21) are identified only on careful inspection. As in 0 hr nucleoli, their being recognized also remains greatly dependent on the focus of micrographs. When only moderately underfocused, the density of granules is exaggerated, obscuring fibrous components, as in the circled area of Fig. 22 *a*. Within the smae area sharply focused in the insert, fibrous components are more readily seen. They are also well demonstrated within the large and small broken circles of Fig. 22 *b*, a portion of the same nucleolus at higher magnification. Superposition of granules on fibrous components (double-headed arrows) lends difficulty to the estimation of sizes of both components.

Fibrils of lesser width (about 60 A) than the fibers of granular zones are observed in agranular and fibrillar zones (Fig. 22 *b*, single-headed arrows). Similar fibrils have been identified in the nucleoli of ascites tumor cells by Yasuzumi and Sugihara (42).

Fibrillar zones in 24-hr nucleoli, although reduced in size, are increased in number (Fig. 21). Commonly, unlike in 0 hr nucleoli (Figs. 19 *a* and *b*), these zones are sufficiently dense so that they are not readily distinguished from intranucleolar condensed chromatin aggregates. In some instances, distinction is possible only because condensed chromatin aggregates are of coarser texture than fibrillar zones (Figs. 21 and 22).

The nucleus rarely displays more than one nucleolar profile during the initial 24 hr of culture. Two to four profiles are not exceptional at 48 and 72 hr.

In the enlarged nucleolus of a 48 hr cell in Fig. 6, perinucleolar chromatin surrounds almost the entire nucleolar circumference, and intranucleolar aggregates are increased in size and number. In the same nucleolus at higher magnification in Fig. 23, the granular component is conspicuous, and agranular zones still appear as the central components of concentric structures. Several round spaces of density lower than that of agranular zones are present. They resemble the nucleoplasm in density and texture and, in some instances, direct continuity has been observed between such spaces and the nucleoplasm. For these reasons they will be designated nucleoplasmic

spaces (ns in Fig. 23). They are occasionally recognized in a 24 hr nucleolus (arrows in Fig. 22 a).

Unlike agranular zones, which are typically accompanied by fibrillar components, nucleoplasmic spaces are often closely bordered by chromatin aggregates. Frequently, however, these relationships are obscure. In Fig. 23, for example, both fibrillar zones and chromatin aggregates appear to border on agranular zones labeled za_1 and za_2. In such loci, moreover, distinction between fibrillar zones and chromatin aggregates on one hand, and between agranular zones and nucleoplasmic spaces on the other, are equivocal. There is some indication of continuity between chromatin aggregates and fibrillar zones in both density and texture, but this is by no means clearly demonstrated.

Well-defined nucleolonemata, which have been shown to be composed of fibrillar and granular components (43–46), are only rarely observed in nuclei of early interphase. So long as appreciable amounts of condensed chromatin are present, the nucleolus more often shows the complex arrangement of concentric structures, variably dense agranular zones, nucleoplasmic spaces, and chromatin aggregates illustrated in Fig. 23. Emergence of a nucleolonema appears in large measure related to the loosening and dispersion of chromatin. In the 72 hr nucleus of Fig. 24, for example, chromatin is loosened rather than condensed, and a nucleolonema is vaguely discerned. Perinucleolar (chp) and intranucleolar chromatin (chi) are scarcely more dense than other components of the nucleolus and are distinguished principally by their coarse texture.

A more distinct nucleolonema is recognized in Fig. 25, a portion of a 48 hr nucleolus with highly dispersed chromatin. While some fibrillar components still appear as parts of concentric structures, other portions form parts of a nucleolonemal thread about 0.1 μ in width. Fibrous components of the granular zones are rather readily distinguished in the circled areas of the nucleolonema, possibly because of some loss of granular density.

Agranular zones, occasionally still recognized as the central components of concentric structures, are frequently in the presence of a nucleolonema seen as parts of its interstitial spaces. This is illustrated in zones labeled za and za_1, respectively, in Fig. 26, a part of the 72 hr nucleus of Fig. 15 a at higher magnification. Segments of loosened chromatin fibers (arrows) are also well demonstrated in the interstitial spaces and along the periphery of this nucleolus.
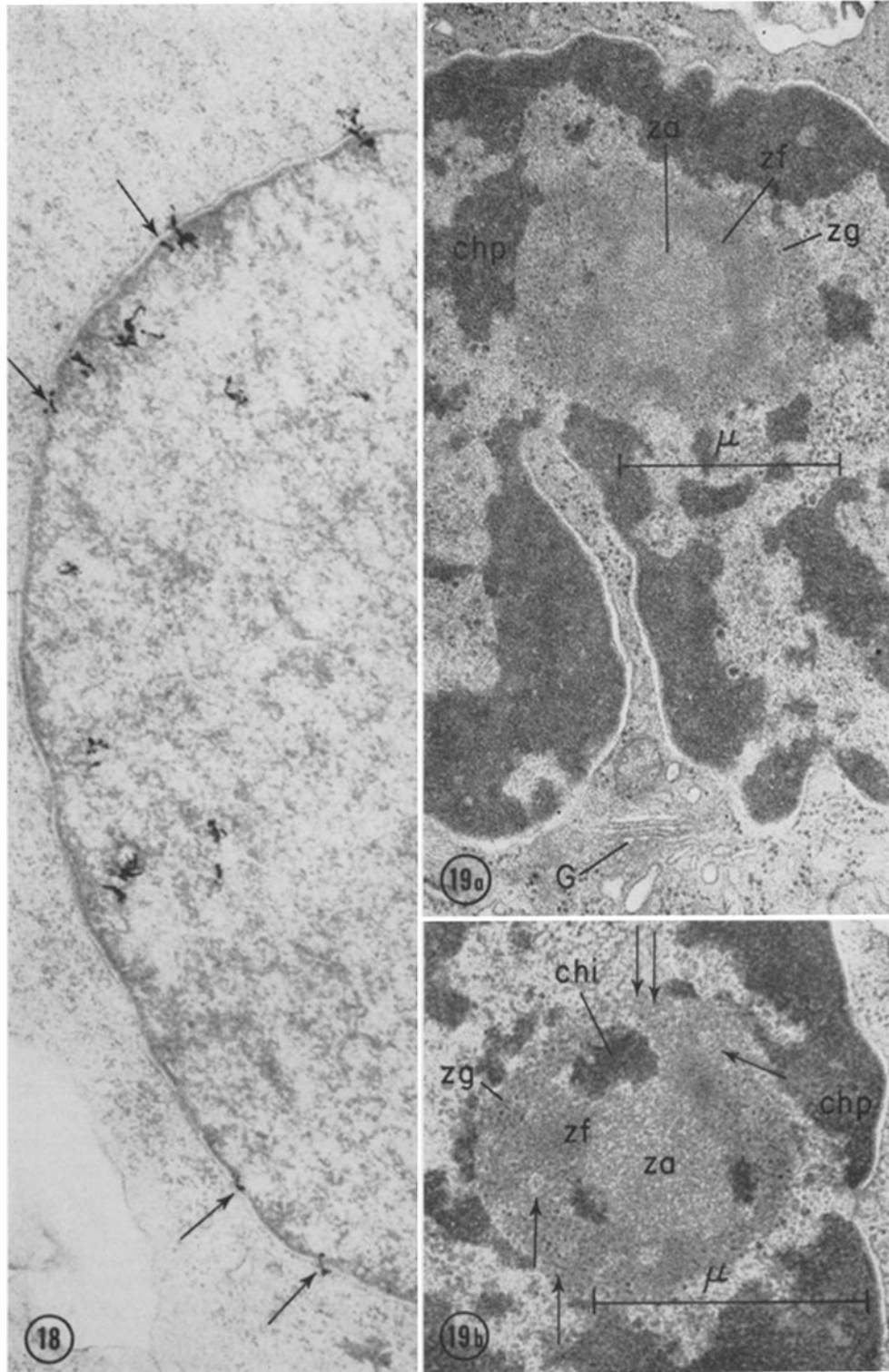
Radioautographic Localization of Sites of DNA Synthesis in the Nucleolus

Labeling of the nucleolus by radioactive thymidine is regularly observed in nuclei with loosened and dispersed chromatin. The histogram of Fig. 29 summarizes some observations of the number of silver grains found per unit area over nucleolar as compared to nuclear (excluding nucleoli) profiles.

Assigning to the nucleolus grains closely associated with its periphery the mean value of ratios of nucleolar to nuclear labeling in 36 nuclei is close to unity, which suggests that the concentration of nucleolus-associated DNA is of similar order to that in the nucleus generally. The high standard deviation, 0.62, among individual nuclei might be explained by asynchrony of DNA synthesis in the nucleolus and in the nucleus proper.

FIGURE 18   48-hr nuclei, with dispersed chromatin, DNA radioautograph. A number of silver grains (arrows) are found in association with portions of peripheral chromatin which are comparable in thickness to the width of a monolayer of chromatin fibers. $\times$ 30,000.

FIGURES 19 a and b   0 hr nucleoli. A central agranular zone (za) is bounded by a roughly concentric fibrillar zone (zf) and this, in turn, by a granular zone (zg). The fibrillar zone is somewhat denser than the agranular zone or the matrix of the granular zone but is lighter than condensed chromatin. In Fig. 19 b, three intranucleolar chromatin aggregates (chi) are recognized in the vicinity of the fibrillar zone. In this figure, of finer focus than 19 a, linear components (arrows) are evident in the granular region. The nucleolus in Fig. 19 a is found near the tip of the deep invagination of the nuclear envelope facing the centrosphere region. Fog. 19 a, $\times$ 32,000; Fig. 19 b, $\times$ 40,000.
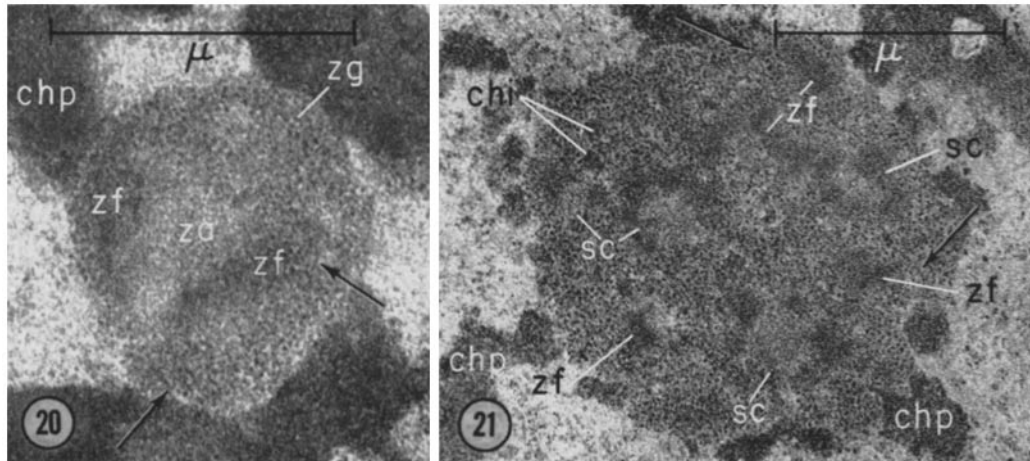
FIGURE 20 4 hr nucleolus. In size and morphology the nucleolus is similar to the 0 hr nucleolus, but in this section fibrillar zones (zf) do not form a complete concentric layer about the agranular zone (za). The granular zone (zg) appears to contain more abundant granules than in the 0 hr nucleolus. A careful inspection reveals coarse fibrous components in the granular zone (arrows). × 40,000.

FIGURE 21 24 hr nucleolus. The size is now roughly twice that of 0- or 4-hr nucleoli. Some of the agranular and fibrillar zones are concentrically arranged in structures (sc) which are smaller but otherwise similar to the concentric structures of 0- or 4-hr nucleoli. The granules are more abundant than in prior periods. Careful inspection still reveals fibrous components in the zone (arrows). Note that the over-all amount of fibrillar component relative to that of granular component is not much different than in 0 or 4-hr nucleoli. Small perinucleolar chromatin aggregates are again numerous. × 30,000.
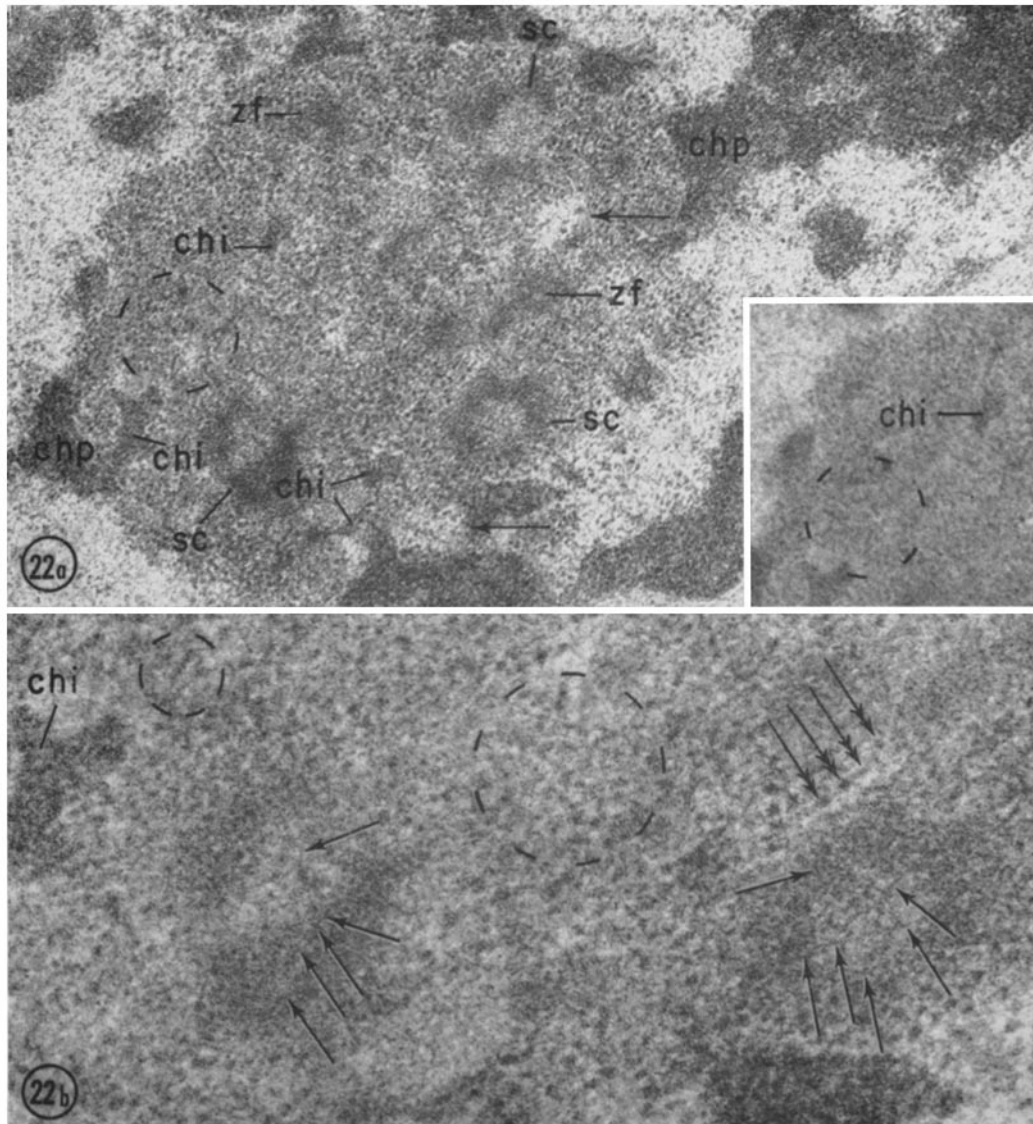
The identity of labeled nucleolar components is especially difficult to determine prior to formation of a nucleolonema. This is, in part, because the similar density of intranucleolar chromatin in an early stage of loosening to the density of adjoining nucleolar regions frequently does not permit clear distinctions among the several components (Fig. 24). With the formation of a nucleolonema, portions of intranucleolar chromatin occupy interstitial spaces. These spaces may be recognized in radioautographs despite inferior resolution due to superimposed emulsion or obscurement of underlying structures by silver grains (Fig. 27 a). Thus, in nucleoli with nucleolonemata the location of grains can be made out somewhat more satisfactorily.

In Table VI are recorded counts of grains associated with the nucleoli in 30 nuclear profiles which displayed a total of about 4000 grains, 30 or more in each nuclear profile. The background of grains, counted in extranuclear regions of radioautographs, is calculated to account for about 7% of the 449 grains associated with nucleoli. As

in loosened chromatin of the nucleus (Fig. 27 a), the loosened chromatin in interstitial spaces of the nucleolonema and that closely associated with the nucleolar periphery are seen in the table to be extensively labeled, accounting for more than three-quarters of grains associated with nucleoli. Somewhat unexpectedly, about 20% of grains appear to be more intimately related to the nucleolonemal thread, predominantly its fibrillar zone, than to its interstitial spaces or perinucleolar chromatin (Fig. 27 b). Although this suggests the presence of DNA in the nucleolonema, a positive conclusion is precluded by the uncertainty of localizing grains relatively large in comparison to the narrow regions concerned.

Chromosome Formation

The 48 hr nucleus of Fig. 28 a demonstrates a greatly reduced layer of peripheral chromatin, but in other nuclear regions large aggregates of chromatin are present. The thin peripheral layer of chromatin is very similar to that observed in the nucleus of Fig. 7 c, in which other chromatin

FIGURES 22 a and b   24 hr nucleolus and its part at higher magnification. The nucleolus is similar to that in Fig. 21 in over-all configuration and size. In Fig. 22 a, which is at moderate underfocus, coarse fibrous components of the granular zone are not clearly seen. The circled area, shown at sharp focus in the insert, demonstrates fibrous components. Arrows indicate spaces of low density which show a texture similar to that of the nucleoplasm. In Fig. 22 b of higher magnification, the single-headed arrows indicate fibrillar structures of about 60 A width in the agranular and fibrillar zones. Fibrous components are seen in the small and large broken circles of the granular zone. Many granular components appear to lie on the profiles of these fibrous components. This is particularly evident in the region indicated by double-headed arrows. Fig. 22 a, × 40,000; Fig. 22 b, × 100,000.

is highly dispersed and which has been considered to be a late interphase nucleus. The configuration of Fig. 28 a appears to represent the initiation of chromosome formation at an early stage of prophase. It closely resembles the pattern described by Davies and Tooze (47) as an early prophase nucleus of a newt erythroblast. The nucleus in Fig. 28 b is quite similar to that in
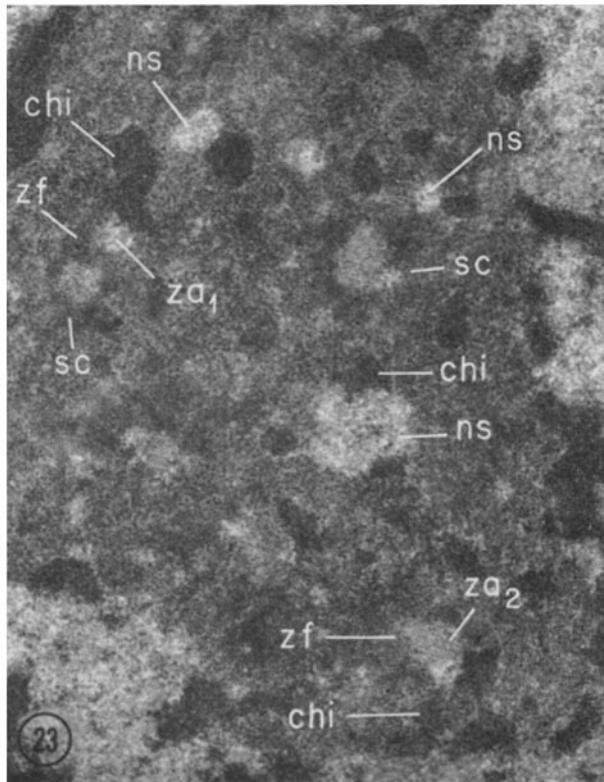
FIGURE 23 48 hr nucleolus, an enlargement on Fig. 6. Intranucleolar chromatin aggregates (chi) are much more numerous than in the 24-hr nucleoli of Figs. 21 and 22 a. Some are closely associated with nucleoplasmic spaces (ns). Agranular zones of concentric structures (sc) are denser than nucleo plasmic spaces. In areas marked za_1 and za_2, however, sharp distinction between nucleoplasmic spaces and agranular zones and between fibrillar zones and chromatin aggregates may not be possible. $\times$ 30,000.

Fig. 28 a with respect to the persistence of peripheral chromatin, but the other chromatin aggregates display dendrite-like processes about 500–700 A in width.

In the 48 hr nucleus of Fig. 28 c the thin peripheral layer of chromatin is no longer observed, apparently because of its incorporation into the typical prophase chromosomes now present. These display more distinct dendrite-like configurations. Chromatin at the inner nuclear membrane is limited to those multiple points at which the chromosomes contact the membrane (insert of Fig. 28 c).

A fairly distinct nucleolonema is observed in the nucleus of Fig. 28 a. In the more typically early prophase nucleus of Fig. 28 c, in which it may be assumed that chromatin is more completely assembled into chromosomes, a nucleolonema is not clearly defined.

DISCUSSION

Differences of fine structural appearance of nuclei resulting from various methods of fixation, embedding, and staining, as well as problems of inferring three-dimensional configurations from thin sections, have been extensively studied and reviewed (1, 16–20, 48–50). The present observations call attention to less well-recognized effects of altered metabolic activity on the fine structure of nuclei. The small resting lymphocyte with its abundant condensed chromatin presents, in the course of its transformation by PHA, a somewhat exceptional opportunity to observe, in particular, the lability of chromatin organization. The dynamic behavior observed here is relevant to interpretations of "normal" structure of interphase nuclei as well as to its alterations in pathologic states.

The major observation of Hay and Revel (1) that DNA synthesis in proliferating cells occurs chiefly in regions of "dispersed DNP meshwork" appears to be confirmed and further extended in the present studies. With the aid of the different techniques used here, the major locus of synthesis has been found to be in loosened chromatin (Figs. 8 a, 17 a and b), a state regarded as having
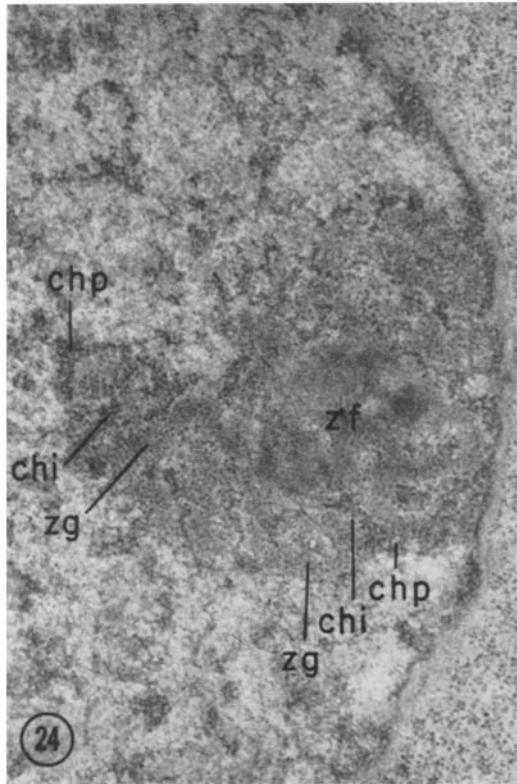
FIGURE 24 72 hr nucleolus of nucleus with loosened chromatin. In portions of the nucleolus, fibrillar (zf) and granular (zg) components form a vaguely discerned nucleolonemal configuration. Small aggregates of the loosened chromatin (chi), distinguishable from fibrillar and granular components by their coarse texture, are seen within the nucleolonemal meshwork. × 30,000.

derived from a disaggregation of masses of condensed chromatin during premitotic growth of the interphase nucleus. Thymidine incorporation was not found in condensed chromatin aggregates (Fig. 17 a) in consonance with earlier observations (1), nor was it found in clearly significant amount in diffuse chromatin regions of nuclei with loosened chromatin (Table V).

The activity of diffuse chromatin regions in RNA synthesis has been shown in isolated calf thymus nuclei by Littau and associates (2) and was noted again in unpublished work on our cultured lymphocytes. This activity is found in cells containing, along with diffuse chromatin regions, large aggregates of chromatin. In the

proliferating cells studied here, we note in addition the presence of minute aggregates of chromatin, part of the chromatin network in 0 hr nuclei (Figs. 3 and 9), their disappearance in the 4 hr cultured cell including those in the perinucleolar region (Figs. 4 and 10), followed by the reappearance of minute aggregates and of perinucleolar chromatin in 24-hr cells (Figs. 5 and 11). All this sequence remains to be correlated with the progress of nuclear RNA and protein synthesis in these premitotic cells.

The subsequent loosening of the condensed aggregates of the early premitotic period to form the patterns of the 48- and 72-hr nuclei, as seen in Figs. 7 a and b, is accompanied by DNA replicating activity. As yet it is not clear whether the low density spaces among the loosened chromatin are of the same composite as the low density areas of the 0, 4-, and 24-hr nuclei which are termed diffuse chromatin regions. In both, however, RNA synthesis must be proceeding, but only in the former, the 48–72 hr nucleus with loosened chromatin, is significant DNA replication occurring.

The relationship of the space of moderate density described above to the fibrous lamina of Fawcett (46) in invertebrate cells or the nuclear limiting zone recently shown by Patrizi and Poger (51) in various mammalian cells is not clear. The space of moderate density appears narrower than the other structures reported and, as suggested under Observations, may contain material concerned in the organization of chromatin aggregates. The existence of such material may reasonably be postulated. In whole mount preparations of nuclei of honeybee embryonic cells, DuPraw has demonstrated attachment of chromatin fibers to the nuclear envelope at the edges of annuli (33).

The nucleolus of the small, resting lymphocyte, except for its central round zone of moderate density, is quite similar to the primary nucleolus observed by Karasaki (29) in cultured explants of amphibian tissues. The central zone, because of the contrast of its texture with the coarsely granular zone and for convenience of description, we have called "agranular." It closely resembles the "nucleolar vacuole" which Swift (52) described in ascites tumor cells and showed to consist largely of protein. The "light zone" surrounded by a "dense zone," observed by Yasuzumi and Sugihara (42) also in ascites tumor cells, appears similar. Fawcett (46) has pointed
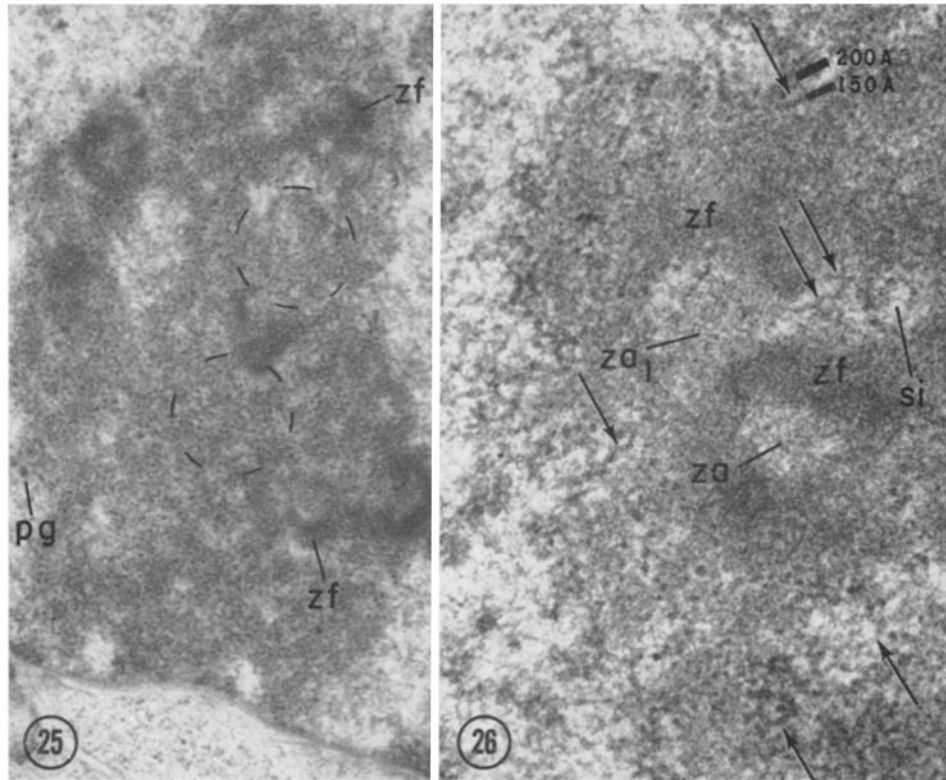
FIGURE 25   48 hr nucleolus of nucleus with highly dispersed chromatin. A nucleolonema is more clearly recognized. Fibrillar zones (zf) appear as portions of the nucleolonema. Within the circled areas, fibrous components of granular zones are somewhat readily distinguished, apparently because of a decrease in the number of granules. × 30,000.
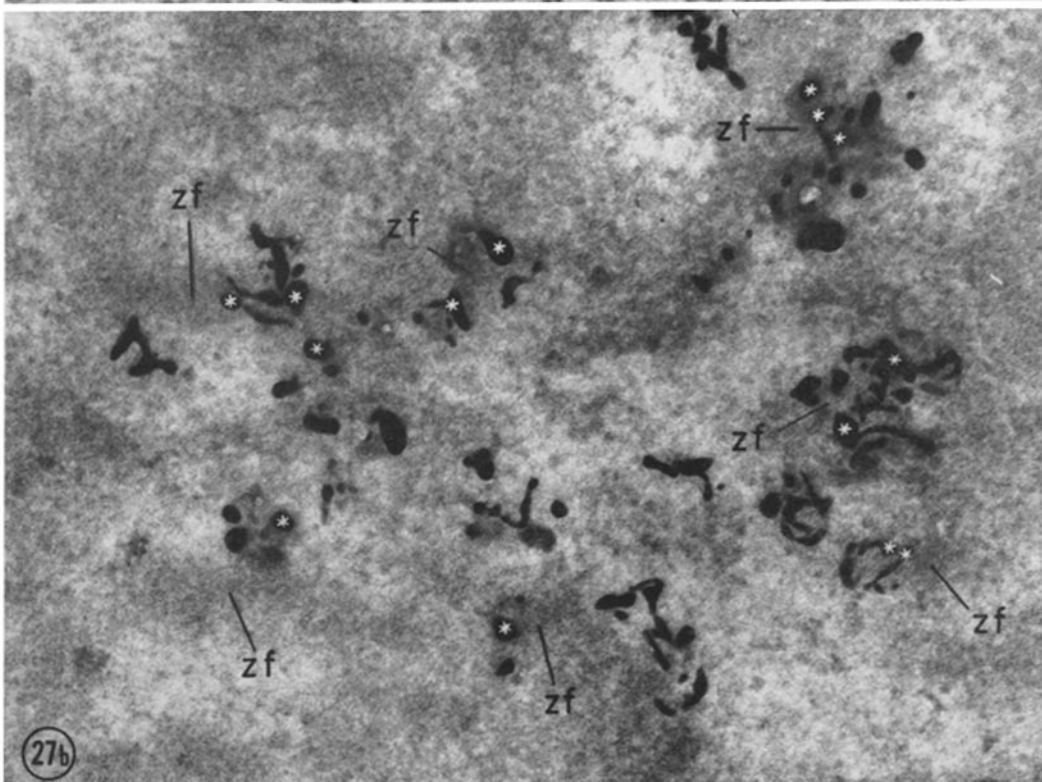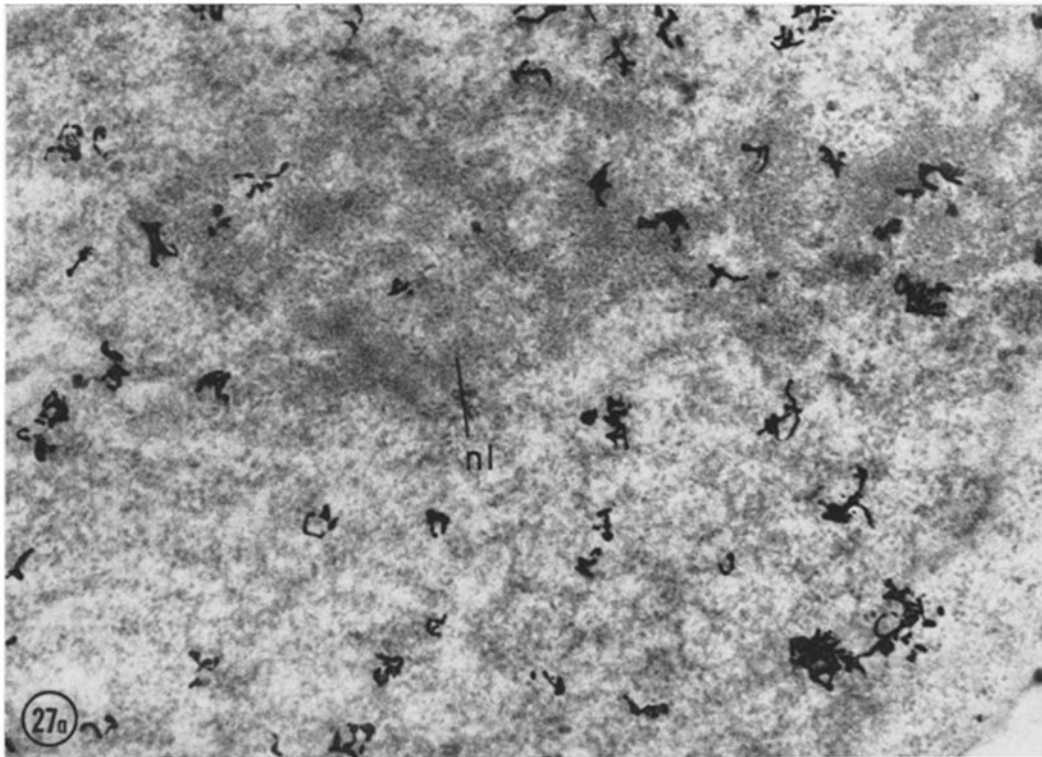
FIGURE 26   72 hr nucleus, a part of Fig. 15 a. Due to loosening of chromatin, profiles of chromatin fibers (arrows) are readily found along the nucleolar periphery as well as in interstitial spaces. The width of loosened chromatin fibers is not so large as 200 A but is close to 150 A. The agranular zone, za_1, with fine fibrillar components is now in continuity with the interstitial space (si) where coarse profiles of chromatin fibers are found. × 60,000.

out that similar bodies of moderate density in nucleoli of spermatogonia and Sertoli cells probably correspond to the *pars Amorpha* seen with the light microscope. A preprophase nucleolus of a plant cell, illustrated by Lafontaine (53), displays, in addition to a peripheral granular zone and a fibrillar zone, a lighter central area which "shows chromatin material corresponding to the nucleolar organizer."

Whether all of these observations deal with the same entity and whether this may be a ubiquitous component of the nucleolus need clarification. It is for this reason worthy of note that the con-

spicuous agranular component of the nucleolus of the small lymphocyte is less readily distinguished in the more complex nucleoli of PHA-stimulated cells. It appears first to become intimately associated with nucleoplasmic spaces and, with the appearance of the nucleolonema, to be included together with chromatin in interstitial spaces. Indeed, without the clear observation of the agranular zone in the 0 hr nucleolus, the presence of this component in the large, complex nucleoli observed at 24 hr or later might be overlooked.

It is perhaps not surprising that nucleolar chromatin is found in these growing lymphocytes

FIGURES 27 a and b   48-hr nuclei, parts in DNA radioautographs. In Fig. 27 a, the loosening of chromatin is well advanced and the nucleolus now shows a clear nucleolonema. Silver grains in the nucleolus are found in association with interstitial spaces or along the nucleolar periphery. In other nuclear regions grains appear to be associated with the network of loosened chromatin. In Fig. 27 b, a considerable number of silver grains (marked by asterisks) is more clearly present over the nucleolonema in addition to those associated with interstitial spaces. Silver grains over the nucleolenoma appear to be located over fibrillar zones. Fig. 27 a, × 40,000; Fig. 27 b, × 90,000.

Nuclear Structure of Cultured Lymphocytes

DNA synthesis in nucleoli examined by radioautography.

Nucleolar region	Grain count	
	No.	*% of total*
Interstitial space	143	31.8
Nucleolar periphery	206	45.9
Fibrillar zone	84	18.7
Granular zone	16	3.6
Total	449	100.0

to increase in amount and to be altered in morphologic state. There is much evidence that this portion of chromatin functions in the synthesis of ribosomes as reviewed by Perry (54) and by Georgiev (55), and accelerated synthesis of ribosomal RNA has been shown by Rubin and Cooper (56) to occur early in the response of lymphocytes to PHA. With the increase of intranucleolar chromatin found here at early stages of culture, a continued intimate relationship of chromatin to fibrillar zones is noted and is accompanied by enlargement of the granular component of nucleoli. In the phase of active DNA synthesis, the emergence of a nucleolonema appears to be related to the transformation of aggregates of condensed intranucleolar chromatin to a loosened, and further, to a dispersed state.

These structural changes of the nucleolus, which accompany the metabolic alterations of cell growth, would appear to present interesting opportunities for further study of nucleolar function.

Finally, a word about terminology may be in order. There is no intent in this paper to introduce new terms or overlook previous ones. Any such occurrence noted should be ascribed to complexity of the subject or ignorance of the authors or both. Biochemical and functional studies of the sort being widely pursued may be expected to clarify morphological terminology.
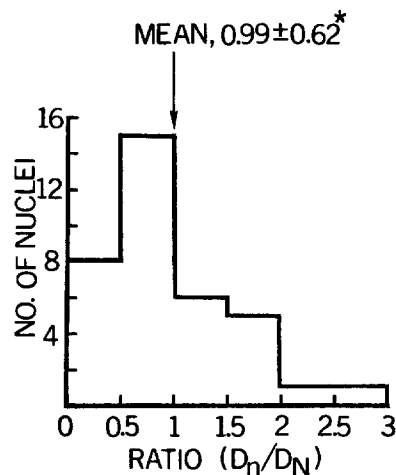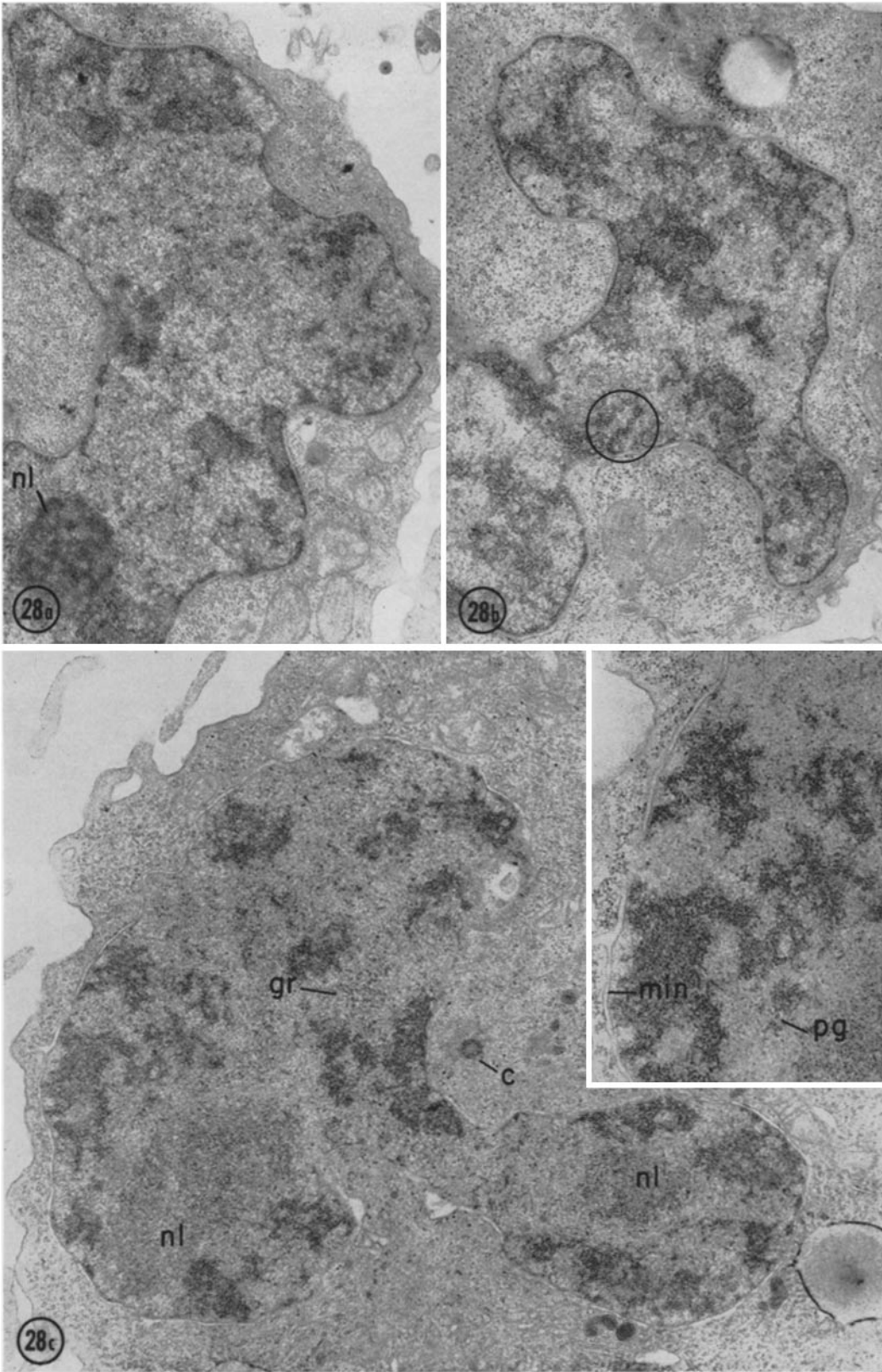

FIGURE 29 The variation in the ratio of the grain number per μ^2 over the nucleolar profile (D_n) to the number per μ^2 over the remainder of the nuclear profile (D_N) is shown for 36 nuclei. The asterisk indicates the standard deviation of the ratios. Counts over individual nuclei ranged from 40 to 250, with a total count of about 5000 grains and mean background of about 7% (less than 10% in each nucleus). The grain number per μ^2 ranged from 1 to 14 with an average value of 5.8 per whole nucleus.

FIGURES 28 a–c   48-hr nuclear profiles. In Fig. 28 a a thin layer of peripheral chromatin persists augmented here and there by thick aggregates. A nucleolonemal configuration is found in the nucleolus. The over-all configuration of the nucleus in Fig. 28 b is similar to that in Fig. 28 a, but chromatin aggregates show a more complex, dendrite-like structure, with branches roughly 500–700 A in width (circle). These configurations are believed to represent very early stages of prophase. In a typical early prophase nucleus (Fig. 28 c), dendrite-like chromatin aggregates are more characteristic of chromosomes. The nuclear membrane is here devoid of peripheral chromatin except where contacted at a few points by chromosomes. This is particularly well observed in the insert, an enlarged portion of the nucleus. Nucleolonemata cannot be distinctly discerned. Nuclear granular regions (gr) are still seen. Nuclei at this stage are frequently variable in outline; this nucleus shows an oval profile at a level about 0.5 μ apart from the present section. Note the persistence of perichromatin granules (pg, insert of Fig. 28 c). Figs. 28 a and b, × 20,000; Fig. 28 c, × 15,000 (insert, × 27,000).

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