THE FINE STRUCTURE OF THE DNP COMPONENT OF THE NUCLEUS

An Electron Microscopic Study Utilizing Autoradiography to Localize DNA Synthesis

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ABSTRACT

In the present investigation, the sites of deoxyribonucleic acid (DNA) synthesis and the fate of labeled deoxyribonucleoprotein (DNP) were studied in autoradiographs of ultrathin sections viewed with the electron microscope. Tritiated thymidine was employed as a label for DNA in the nuclei of proliferating cells of regenerating salamander limbs. In the autoradiographic method reported here, dilute NaOH was used to remove the gelatin of the emulsion after exposure and development. The exposed silver grains are not displaced by this treatment and the resolution of fine structure in the underlying section is greatly improved. Our observations suggest that the DNP component is a meshwork of interconnected filaments 50 to 75 A in diameter, which may be cross-linked to form what Frey-Wyssling would term a "reticular gel." The filamentous DNP meshwork is dispersed throughout the interphase nucleus during DNA synthesis, whereas in chromosomes, which are relatively inert metabolically, the meshwork is denser and is aggregated into compact masses. Dense chromatin centers in interphase nuclei are similar in fine structure to chromosomes and are also inert with respect to DNA synthesis. In the Discussion, the structure of the filamentous meshwork in chromatin is compared with that in chromosomes, and speculations are made as to the functional significance of the variations in DNP fine structure observed.

INTRODUCTION

Autoradiographic techniques used in conjunction with the electron microscope offer a new approach to the problem of relating the fine structure of cells to their metabolic activities, and they also provide a direct means of localizing chemical components within tissues fixed under optimal conditions for electron microscopy. Because thymidine is incorporated only into newly synthesized DNA and the energy of beta rays emitted by tritium is low, tritiated thymidine has been the compound of choice in preliminary analyses of the resolution of the technique (1) and in evaluating methods for removing the gelatin of the emulsion (see 2 for review). A technique utilizing alkaline solutions to dissolve the gelatin (3) has enabled us to study in detail fine structural features that would have been obscured by emulsion in conventional autoradiographs and to localize newly synthesized DNA within a specific nuclear component in growing cells of regenerating salamander limbs. The distribution of DNP (deoxyribonucleoprotein) observed agrees with

our own and previous studies of Feulgen-stained sections viewed with the light microscope (4–6). We have followed the fate of the labeled DNA in autoradiographs of limbs fixed at intervals after administration of tritiated thymidine and, here again, correlations with previous studies give us confidence in the technique.

In addition to providing evidence that attests to the reliability of the autoradiographic technique, the present paper presents several new observations on DNP fine structure and chromatin morphology during the interphase period of DNA synthesis. Current concepts of the structure of DNP are based primarily on studies of isolated nucleoproteins (6–9) and chromosomes (10–14). *Surprisingly little attention has been given to the morphology of the interphase nucleus.* Recent studies of nuclear morphology using histochemical approaches and special stains (6, 15, 16) have had to compromise quality of tissue preservation with necessities for special fixation. The meshwork arrangement of DNP filaments in the interphase nucleus described in the present report has received little emphasis in past studies, nor has much attention been given to the possibility that nucleoprotein macromolecules are cross-linked to form a proteinaceous gel in chromatin and chromosomes.

The present study has also demonstrated that the *synthetically active form* of the DNP component is a highly dispersed array of interconnected or overlapping filaments intimately intermingled with the nuclear sap. Light microscopists have long recognized that nuclei show definite variations in different functional states (17) and, indeed, much of the debate about the relation of heavily staining or dense chromatin ("heterochromatin," "basichromatin") to lightly staining or diffuse chromatin ("euchromatin") stems from such differences in nuclear structure. That these differences have functional significance is supported by our finding that dense chromatin shows little or no DNA synthesis. Other aggregated forms of DNP, such as chromosomes, have many structural features in common with dense chromatin and, likewise, are relatively inert metabolically. These compact nucleoprotein aggregates derive from the diffuse chromatin. We have restricted the use of the term chromosome to that distinct structural unit long recognized by morphologists as characteristic of the mitotic figure. We do this because broad "physiological" definitions of the word chromosome tend to obscure morphological differences in the organization of DNP in various phases of the cell cycle, differences which we hope to show could be of considerable importance in the metabolic functioning of the nucleoproteins involved.

MATERIALS AND METHODS

The salamander larvae (*Amblystoma opacum* and *Amblystoma maculatum*) used in the present experiments were 35 to 40 mm in length and weighed approximately 300 mg each. The forelimbs were amputated through the distal third of the humerus. In one experiment, the animals were injected intraperitoneally with 5 μc of tritiated thymidine (Schwarz Bioresearch, sp. act. 1.9 c/mM) on the 10th day after amputation of the limbs. Eight regenerating limbs were fixed 1 hour later. Half of these were fixed in Bouin's and prepared for routine light microscopic autoradiography by the techniques described in a previous report (18). Four of the limbs were fixed 1 hour in cold osmium tetroxide (1.2 per cent buffered in collidine, pH 7.4), embedded in prepolymerized methacrylate, and sectioned for electron microscopy (3). This experiment permitted us to compare autoradiographs of routine paraffin sections (5 to 10 μ in thickness) with autoradiographs of similar tissue fixed in osmium tetroxide and sectioned at 0.05 to 1.0 μ. The localization of the isotope over nuclei was similar in the two types of preparations, but the thin methacrylate sections coated with diluted emulsion showed better resolution and gave a lower grain count.

In the second experiment, the animals were injected with 1 μc of tritiated thymidine on the 8th day after amputation of the limbs, and the regenerating limbs were fixed at different intervals after thymidine administration to determine the fate of the DNA labeled at the time of the treatment. Twenty-four limbs were fixed in osmium tetroxide, two at each of the following intervals after injection: 10 minutes; 1, 2, 4, 6, 8, and 24 hours; 3, 4, 6, 8, and 10 days.

In preparing the osmium tetroxide–fixed tissues for autoradiography the same general procedures were used for light microscopy as for electron microscopy. Methacrylate sections were cut on a Porter-Blum microtome and the white to gold sections (0.05 to 0.1 μ in thickness) were mounted on carboned, celloidin-coated, Athene type grids. Blue-gold to colorless sections (0.2 to 1.0 μ) for light microscopy were floated onto clean glass slides and dried to the slide at 45°C (15 minutes). These slides were dipped directly into the emulsion (below), but it was necessary to mount the grids on some support, such as a glass slide, before dipping them. The most reliable method of mounting

the grids on the slides is to punch a hole slightly smaller than the grid in a piece of "Band aid" tape, mount the tape sticky side up on the slide and press the edges of the grid into the sticky edge of the punched hole. The slides with thick sections for light microscopy and the slides containing the grids were dipped in the melted (45°C) Ilford L4 emulsion recommended by Caro (19). The emulsion was diluted 7 to 8 times with distilled water. Some of the grids were coated with emulsion picked up with a platinum loop (3). After dipping, the slides were hung on clips to dry at room temperature and then placed in light-tight boxes which were kept in the refrigerator (4°C) 3 weeks to expose the emulsion. The emulsion was tested for background before each use; a blank slide was dipped, dried, developed, and examined in the light microscope before the experimental material was coated. The emulsion was discarded if the test slide showed more than 10 background grains per oil immersion field.

After appropriate exposure, the slides containing the coated sections and grids were developed in Dektol diluted with 2 parts water (grids for 2 minutes, light microscopy slides for 5 minutes), fixed in Hypo (5 minutes), and washed 10 minutes or longer in several changes of distilled water. The slides for light microscopy were treated with 0.05 N NaOH for 1 hour to remove most of the emulsion and then stained for 1 minute with hot (45°C) Toluidine Blue (1 per cent) in an aqueous solution of borax (1 per cent). They were cleared in xylol and mounted in balsam. Most of the grids were stained ½ hour with the Karnovsky lead stain (20) which is made up in 0.02 N NaOH. In all cases, a protective coat of carbon was deposited on the sections before they were examined in the electron microscope (Siemens Elmiskop I; RCA EMU 3E). Comparison of treated autoradiographs with those not subjected to alkaline treatment has convinced us that there is no significant loss or displacement of the exposed silver grains during removal of gelatin of the photographic emulsion (see also references 2, 3).

Some of the light microscopy sections were stained by the Feulgen technique (4). Uncoated sections were used because the acid hydrolysis in this procedure removes the grains of the autoradiographic emulsion. Leuchtenberger *et al.* (4) have shown that osmium tetroxide fixation gives essentially the same results with the Feulgen as formalin fixation. In order to compare the intensity of the Feulgen reaction with the fine structure of the nuclei studied, thin (0.2 to 1 μ) sections for the light microscope were cut adjacent to the thinner sections (0.1 μ) examined in the electron microscope. By this technique, clumps of chromatin can be identified in the same nucleus with the light and electron microscopes (5, 6).

RESULTS

The Structure of Interphase Nuclei and General Appearance of the Autoradiographs

The observations reported here were made on epidermal cells and blastema cells of regenerating *Amblystoma* limbs. The blastema cells that make up the bulk of the regenerate are rapidly growing, oval or stellate cells with a high nucleo-cytoplasmic ratio and relatively undifferentiated cytoplasm (18). The epidermis is also proliferating actively during the stages of regeneration studied here and the cells are characterized by tonofibrils, desmosomes, and other specializations that serve to distinguish them from blastema cells. The nuclei of blastema cells and epidermal cells are similar in fine structure, however, and no distinction will be made between them in the ensuing description. In autoradiographs of tissue fixed an hour after administration of tritiated thymidine, silver grains (*gr*, Figs. 1 to 5) can be seen overlying a large proportion of the nuclei. There is abundant evidence that tritiated thymidine is incorporated only into DNA in cells during the interphase synthetic period prior to division (21, 22) and it can be assumed that the labeled nuclei were actively synthesizing DNA at the time of exposure to the radioactive nucleoside. There is no significant labeling in the cytoplasm; soluble compounds have been removed in tissue preparation and the labeled DNA is bound to nuclear proteins. The number of silver grains varies from nucleus to nucleus, and this may be due to differences in the amount of thymidine incorporation in nuclei in different phases of the synthetic cycle (22, 23). Unlabeled nuclei (*N'*, Fig. 1) were probably in the interphase period prior to (G1) or following (G2) the synthetic period. In the illustrations to follow, the preferential distribution of the silver grains over one of the components of the nucleoplasm will be demonstrated. The fine structure of the nuclei themselves will be described first, and as a matter of convenience the structures referred to will be indicated in the same electron micrographs used to illustrate the autoradiographs.

The nuclei of blastema cells and epidermal cells are similar in structure to the interphase nuclei of other growing cells. They are large and "vesicular" or lightly staining as viewed in the light microscope, and they contain one or two prominent nucleoli. In very low power electron micrographs, these nuclei seem to be relatively homogeneous

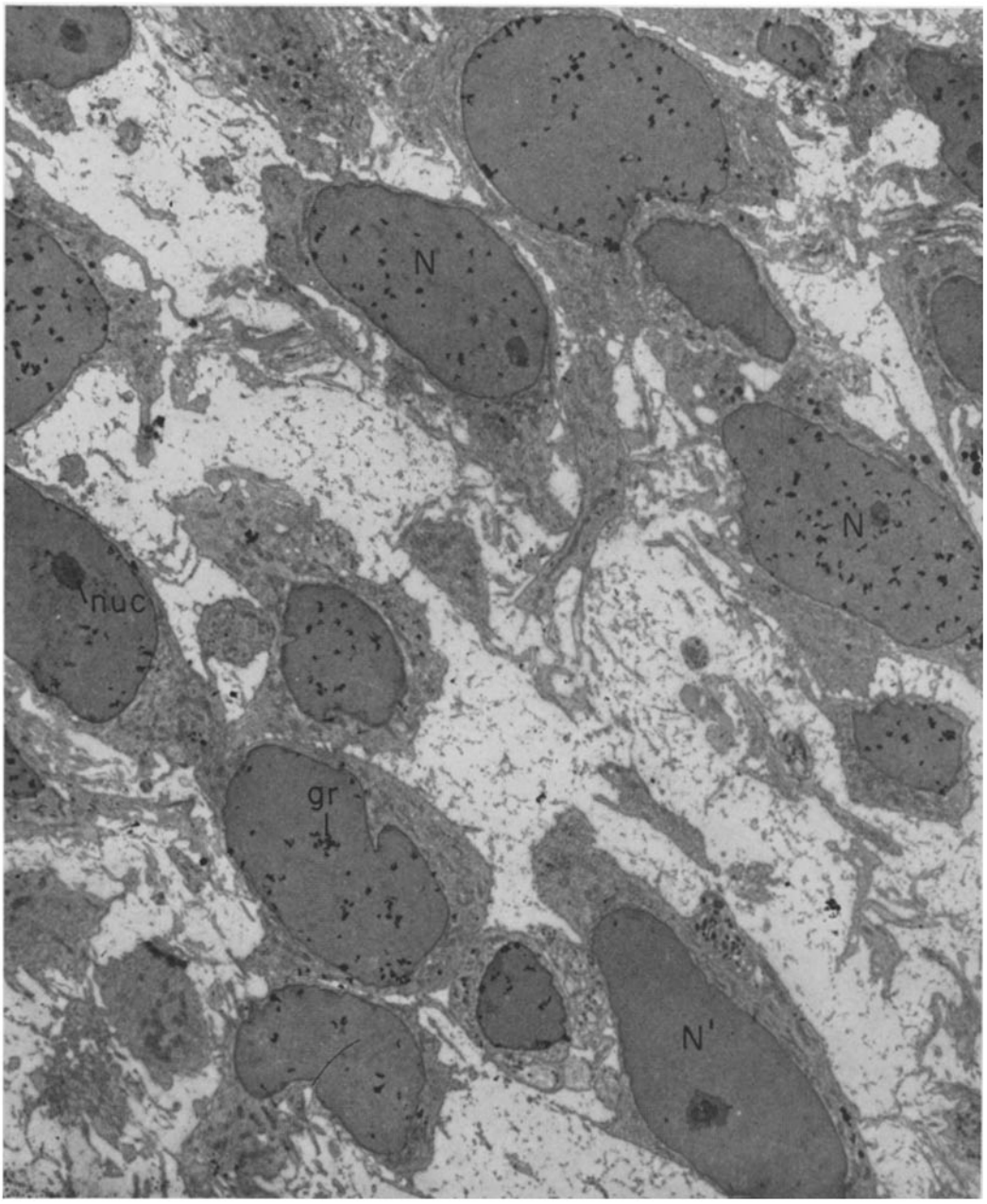


FIGURE 1

A very low power electron micrograph of an autoradiograph showing a group of blastema cells in a regenerating limb fixed 1 hour after injection of 5 μc tritiated thymidine. The nuclei (*N*) appear relatively homogeneous at this magnification. The sites of DNA synthesis in these nuclei are indicated by the developed silver grains (*gr*) which appear as dense, irregularly shaped particles. One of the nuclei is not labeled (*N'*). This cell presumably was not synthesizing DNA when the isotope was administered to the animal. A few background grains are found, but these are usually smaller than the grains exposed by the isotope. The good localization of the grains over the nuclei is typical of the autoradiographs we have obtained by the methods described in the text. The gelatin base of the emulsion was removed by staining according to Karnovsky's method. (*nuc*), nucleolus. × 2,000.

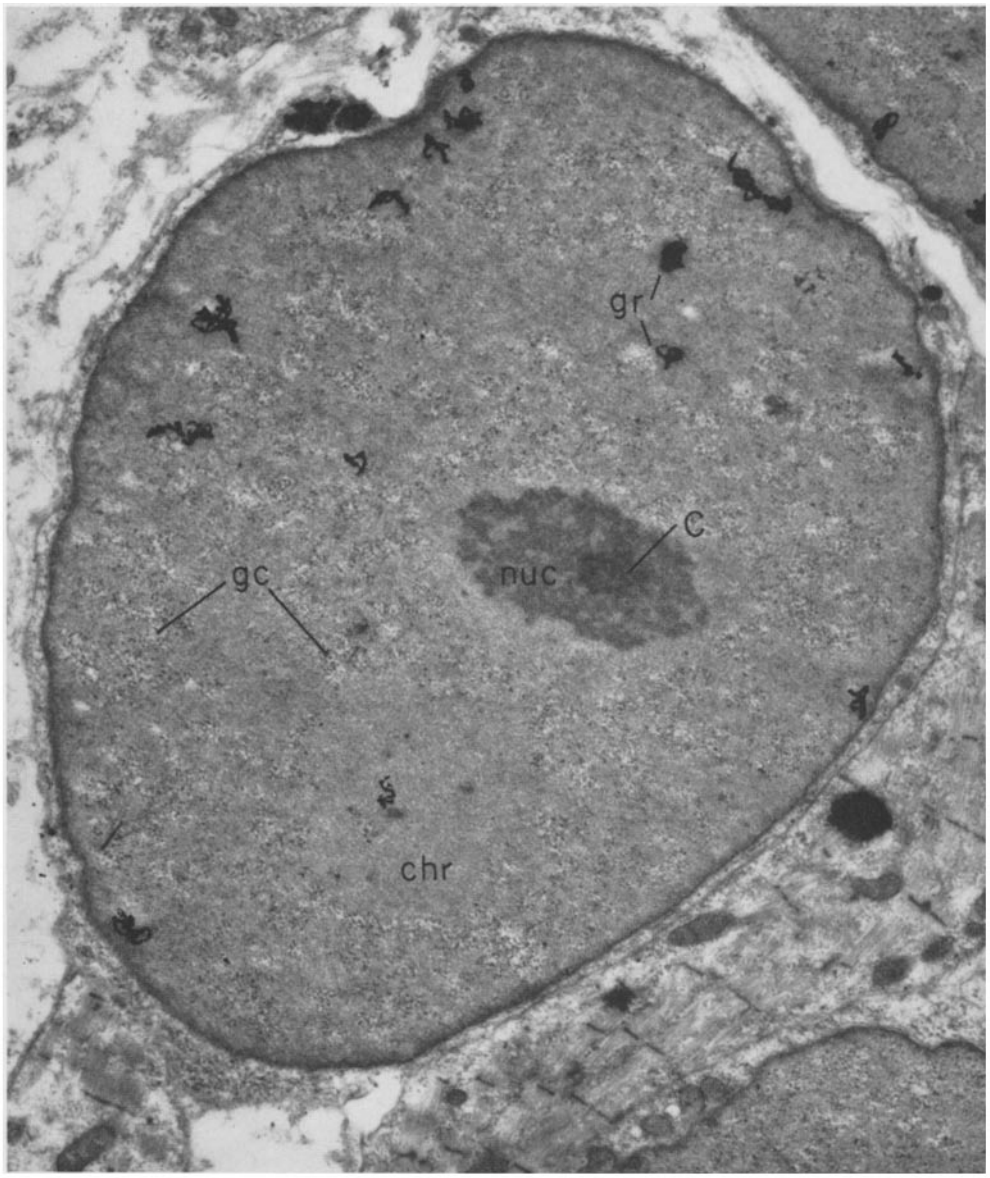


FIGURE 2

When they are viewed at a slightly higher magnification the nuclei of blastema cells reveal a finely textured component (*chr*) intermingled with small granules (*gc*). The nucleolus (*nuc*) shows an electron-opaque central part (*C*) surrounded by a less dense peripheral part. The developed silver grains (*gr*) are seen to be preferentially located over the finely textured component of the nucleus. (Further examples of the distribution and labeling of the DNP component of the nucleus are presented in Figs. 3 to 6.) Two developing muscle fibers appear in the lower part of the figure. The regenerate was fixed 1 hour after administration of tritiated thymidine to the animal. × 9,000.

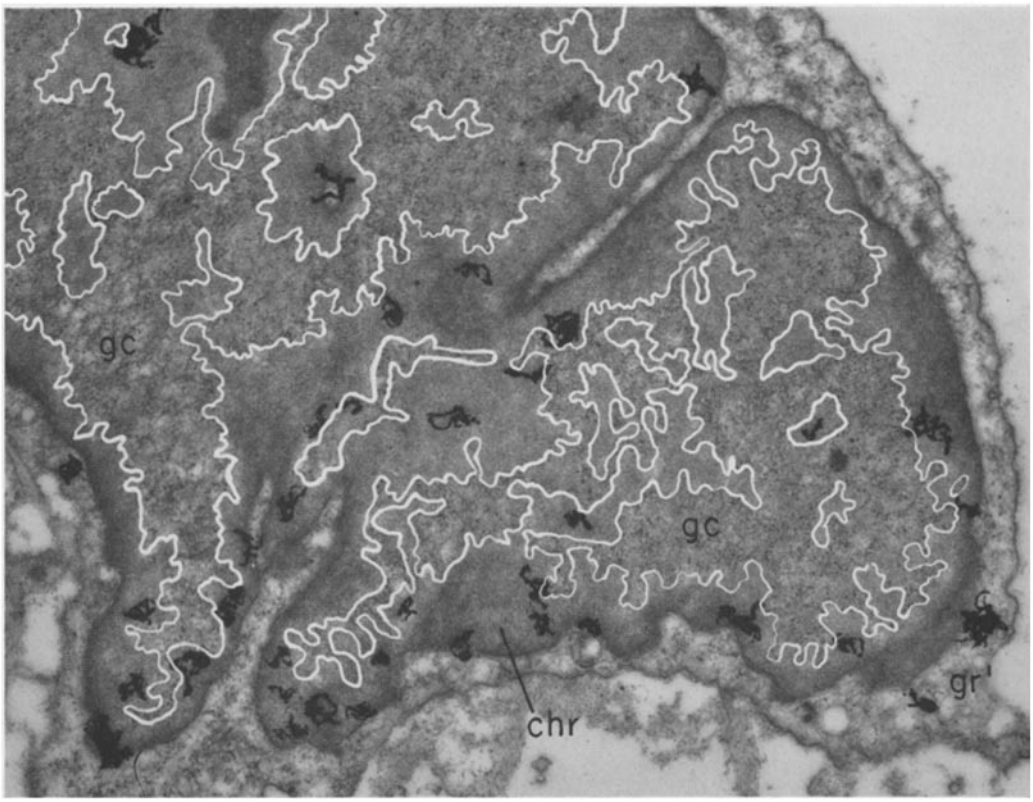


FIGURE 3

In this electron micrograph, those regions of the nucleus composed exclusively of the finely textured material (*chr*) have been outlined with white ink. Practically all of the silver grains are found in the areas thus defined and it seems reasonable to conclude that this is the DNP component of the nucleus. A few grains (*gr'*) lie outside of the nuclear envelope. They were probably exposed by β-rays of higher than average energy emitted by radioactive DNP within the nucleus. This cell is from a limb fixed 1 hour after administration of tritiated thymidine to the salamander. *gc*, granular component. × 11,500.

and the prominent nucleoli appear as dense bodies (Fig. 1). At higher magnifications, it can be seen that the nucleoplasm actually consists of two distinct components (Figs. 2 to 7). One of the components is a finely textured material which is dispersed throughout the nucleus (*chr*, Fig. 2) and along the nuclear envelope (*chr*, Fig. 3). This component may form small aggregates (*chr*, Fig. 4) or it may be diffusely distributed in narrow strands that are difficult to distinguish from the second, more coarsely textured component (Fig. 5). The coarse-textured component (*gc*, Figs. 2 to 6) is composed of dense "granules" embedded in a material of low electron opacity (Fig. 6), which might be termed the nuclear sap. At higher magnifications, the so called granules seem to have a core of low electron opacity (*gc*, Fig. 7). They are in intimate contact with each other and with the fine-textured component (*chr*, Fig. 7). They are larger (300 to 500 A) than the ribonucleoprotein granules of the cytoplasm (*rnp*, Fig. 6) and they are often irregular in shape. Although the term "granule" falls short of accurately defining their heteromorphic appearance, these structures will be referred to as the "granular component" of the nucleus for purposes of the present description.

The large nucleoli assume various shapes and show a great diversity in their organization. Usually they have a dense central portion and a less dense peripheral portion (Figs. 2 and 6), but sometimes the periphery is denser than the central part. Either the central or the peripheral

part may be arranged in strands, the nucleolonema of light microscopy. The fine-textured component of the nucleoplasm fills the interstices of the nucleolonema. At higher magnifications, the nucleolonema seems to be composed of dense, punctate subunits (*sub*, Fig. 7), which may show considerable regularity in their array. The nucleolus is surrounded by the finely textured component and does not come into contact with the granular component of the nucleus. The nucleus itself is contained within a nuclear envelope which has the usual inner and outer membranes and occasional pores.

Sites of Incorporation of Thymidine in Interphase Nuclei

Although background grains are minimal in these autoradiographs (Fig. 1), there is occasional scatter outside the nucleus, probably due to β-rays of higher than average energy emitted by the tritium within DNA (*gr'*, Fig. 3). It is difficult to estimate the exact resolution of our autoradiographs (3). It is possible, however, to localize the radioactivity to structures at least as small as 0.2 μ in diameter, the size of the smallest silver grains here. We have analyzed a large sample of micrographs of labeled nuclei (over 500) and it is quite clear that radioactive DNA is localized within the finely textured component described above. Figures 2 to 7 are typical examples of autoradiographs of a regenerate fixed an hour after administration of tritiated thymidine. The fine-textured component has been outlined in white in Fig. 3 to make the association of silver grains with this component more apparent. In many nuclei, the boundaries of the fine-textured component are sufficiently sharply defined that its distribution is clear without such aids (*chr*, Figs. 2 and 4). In other nuclei, particularly those with the most active DNA synthesis and the greatest incorporation of thymidine, the fine-textured component is so intermingled with the granular or coarse-textured component that it is difficult to define the boundaries between them (Figs. 5 and 6). In autoradiographs of interphase nuclei which have passed through a mitotic cycle subsequent to the initial incorporation of thymidine (24 hours to 10 days postinjection), the silver grains are similarly localized over the fine-textured component. It will be referred to hereafter as chromatin, even though the smaller clumps identifiable in electron micrographs (Figs. 5 and 6) are barely detectable as a stained or colored material in histological sections examined with the light microscope.

Significant numbers of silver grains are never found in regions of the nucleus composed almost exclusively of the granular material (*gc*, Fig. 3) and this coarse-textured component does not become labeled subsequent to mitosis. Silver grains are occasionally seen overlying nucleoli (Fig. 6). Since the chromatin component extends into the nucleolus, filling the interstices of the nucleolonema (Fig. 7), it seems quite likely that the radioactivity associated with the nucleolus is due to the presence of DNP filaments in these regions (3, 24).

The Structure of the DNP Component Viewed at High Magnification

Since DNP appears to be localized within the finely textured component of the nucleus, it seemed of some interest to study this component at higher magnification The textured appearance of the DNP-containing regions of the nucleus is due to an interlacing pattern of what appear to be short filaments of moderate electron opacity. The filaments can barely be detected in routine sections 500 to 1000 A in thickness (arrows, Fig. 7), but they can be seen clearly in electron micrographs of very thin (<500 A) sections (arrows, Fig. 8). They appear to be connected to form a fine meshwork (Fig. 8; see also Fig. 9 inset, lower right corner). The interstices of the mesh are less than 200 A wide and the filaments are 50 to 75 A in diameter. Because of these small dimensions, there is considerable superimposition of filaments in sections of routine thickness, and, therefore, their arrangement is usually difficult to make out. The net-like appearance visible in thin sections seems to result from branching or anastomosis of individual filaments (Figs. 8 and 9). If the filaments intersect the plane of section, they appear punctate or indistinct. It is difficult to say whether or not a single filament continues from section to section, making numerous contacts with other filaments, or whether each filament is actually very short, ending at the junction with the next filament. Since it seems likely that the filaments in the chromatin meshwork are composed of DNP macromolecules (see Discussion), they will be called "DNP filaments" to distinguish them from other types of filamentous structures.

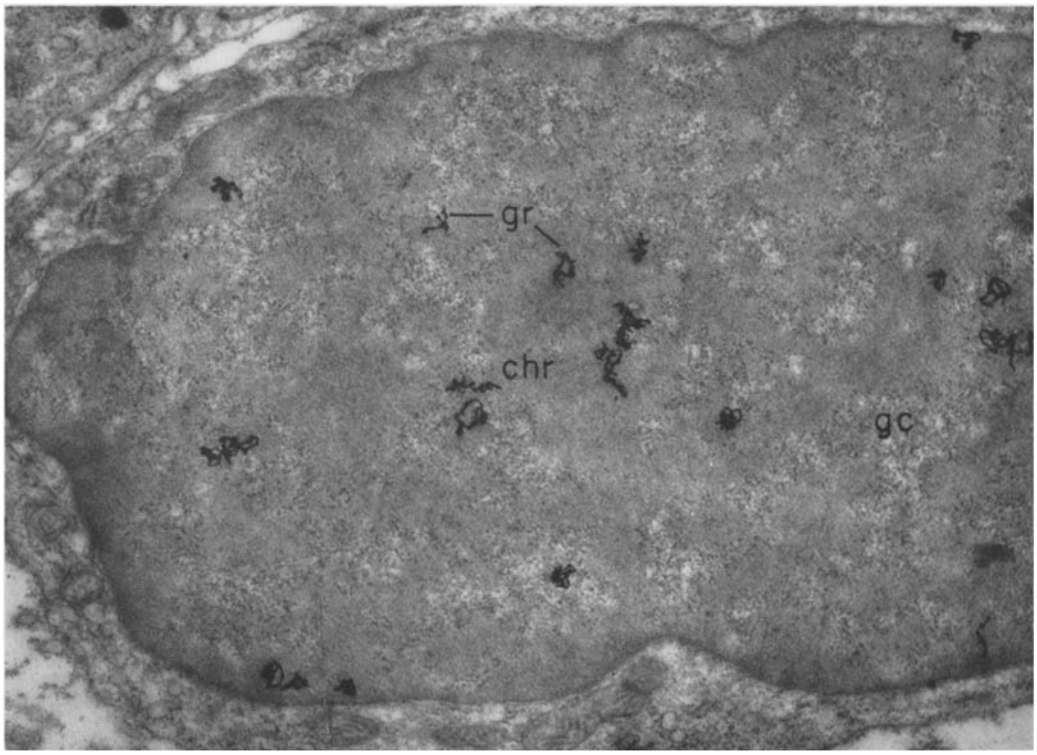


FIGURE 4

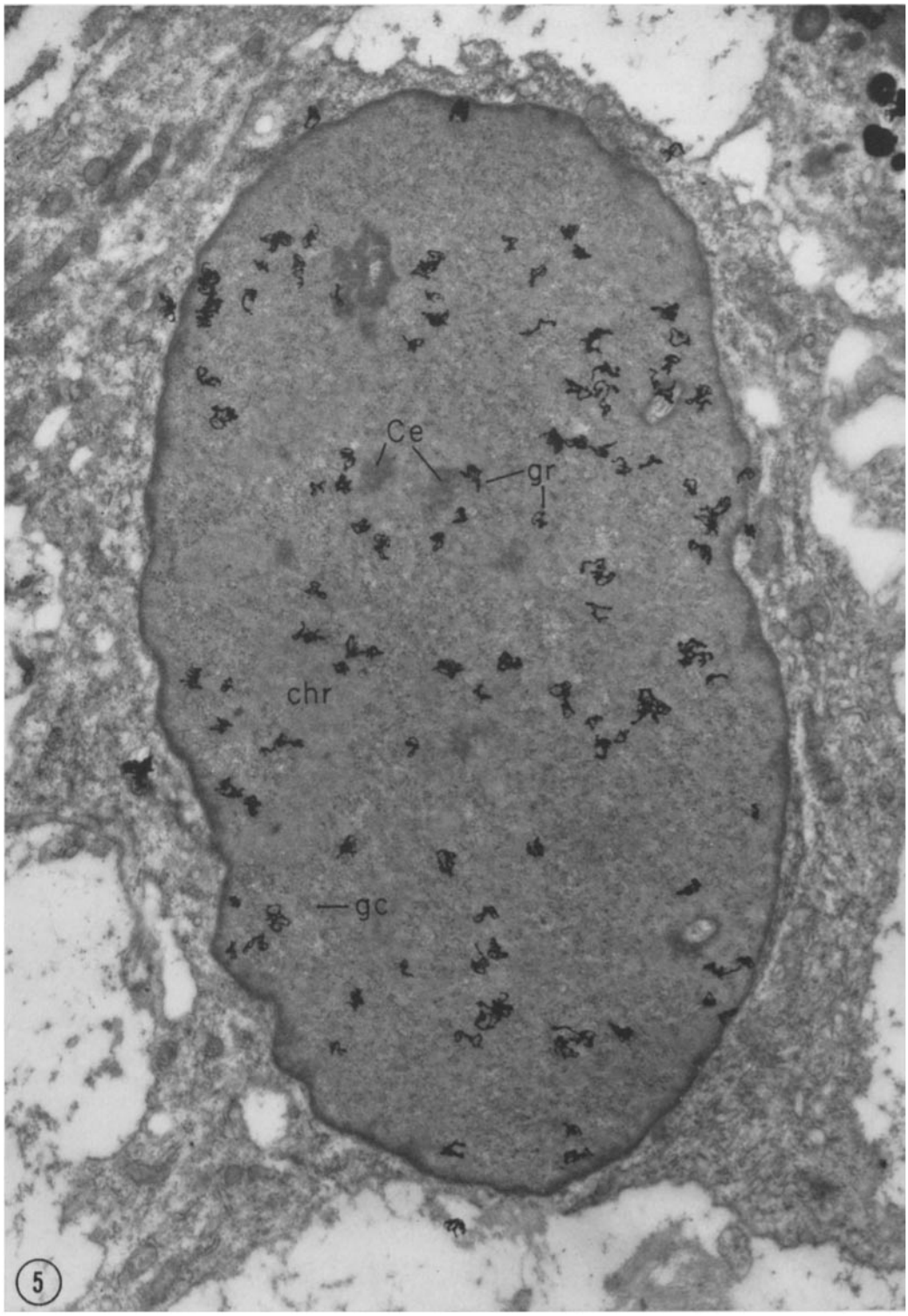
A blastema cell fixed 1 hour after tritiated thymidine injection. In the nucleus illustrated here, the finely textured material (*chr*) is disposed in strands and clumps which have relatively sharply defined boundaries. Almost all of the silver grains (*gr*) lie within these boundaries. The granular component (*gc*) of the nucleus is not labeled. The smaller strands of the finely textured material would not be resolved clearly in light microscope preparations but the DNP component illustrated here corresponds to what has been called "euchromatin" by early morphologists. × 11,500.

Variation in Electron Opacity, Staining Properties and Synthetic Activity of Chromatin

In light microscopy studies in the past, it has been customary to distinguish the more lightly staining chromatin dispersed throughout the nucleus, the true chromatin (euchromatin), from more densely staining clumps sometimes called heterochromatin, basichromatin, chromocenters,

FIGURE 5

Electron micrograph of a very heavily labeled nucleus. The chromatin is very diffuse and is not as sharply segregated from the granular component as in the nuclei shown in the previous illustrations. Under these circumstances, it is much more difficult to decide which component of the nucleus is labeled. In areas where the chromatin (*chr*) forms small clumps, however, it is clear that the silver grains are associated with the finely textured component. In some regions, the central portion of a chromatin clump (*Ce*) may be denser than the periphery. Similar areas of dense chromatin ("heterochromatin") occur commonly along the nuclear envelope. Although grains (*gr*) are found on the less dense chromatin surrounding the centers, the dense chromatin itself is unlabeled. As in Fig. 3, a few grains are found over the cytoplasm close to the nuclear envelope. The specimen was fixed 1 hour after treatment with tritiated thymidine. *gc*, granular component. ×9,000.


Ce
gr
chr
gc
5

or karyosomes. In the present electron microscopic study, we noted electron-opaque "centers" in larger chromatin aggregations along the nuclear envelope and throughout the nucleus (*Ce*, Figs. 5 and 6). Silver grains may be located near these electron-scattering regions in specimens fixed an hour after treatment, but they are more likely to be located over the dense chromatin at later time intervals. That is to say, we have the impression that the dense chromatin is less active in DNA synthesis and forms from the less dense, loosely organized chromatin. To test the possibility that the electron-opaque chromatin might correspond to the strongly staining regions of the interphase nucleus viewed in the light microscope, we compared electron micrographs of thin (500 A) sections (Fig. 9) with photomicrographs of adjacent thin sections (<1 μ) stained with the Feulgen reaction (inset, upper left corner, Fig. 9). Even in very thin sections (6, 14), it is difficult to match perfectly the small accumulations of dense chromatin with their counterparts in adjacent sections studied with the light and electron microscopes (arrows, Fig. 9). Larger regions of dense chromatin (*Ce*, Fig. 9) can be compared in such sections. The more closely compacted, electron-opaque chromatin does show a more intense staining reaction and thus, by some definitions of the term, is "heterochromatin." The dense chromatin has the same filamentous structure as the diffuse chromatin, but the DNP filaments seem denser and more closely knit, and the interstices of the mesh also seem to have greater electron opacity (inset, lower part of Fig. 9). In some respects the dense chromatin resembles the chromosomal DNP meshwork to be described in the next section and it is not impossible that in the nuclei depicted here they represent centers of formation of chromosomes. In non-dividing cells, such as the nucleated erythrocyte, the similarity must be explained on some other basis. We will return to this point after describing the fine structure of chromosomes in more detail.

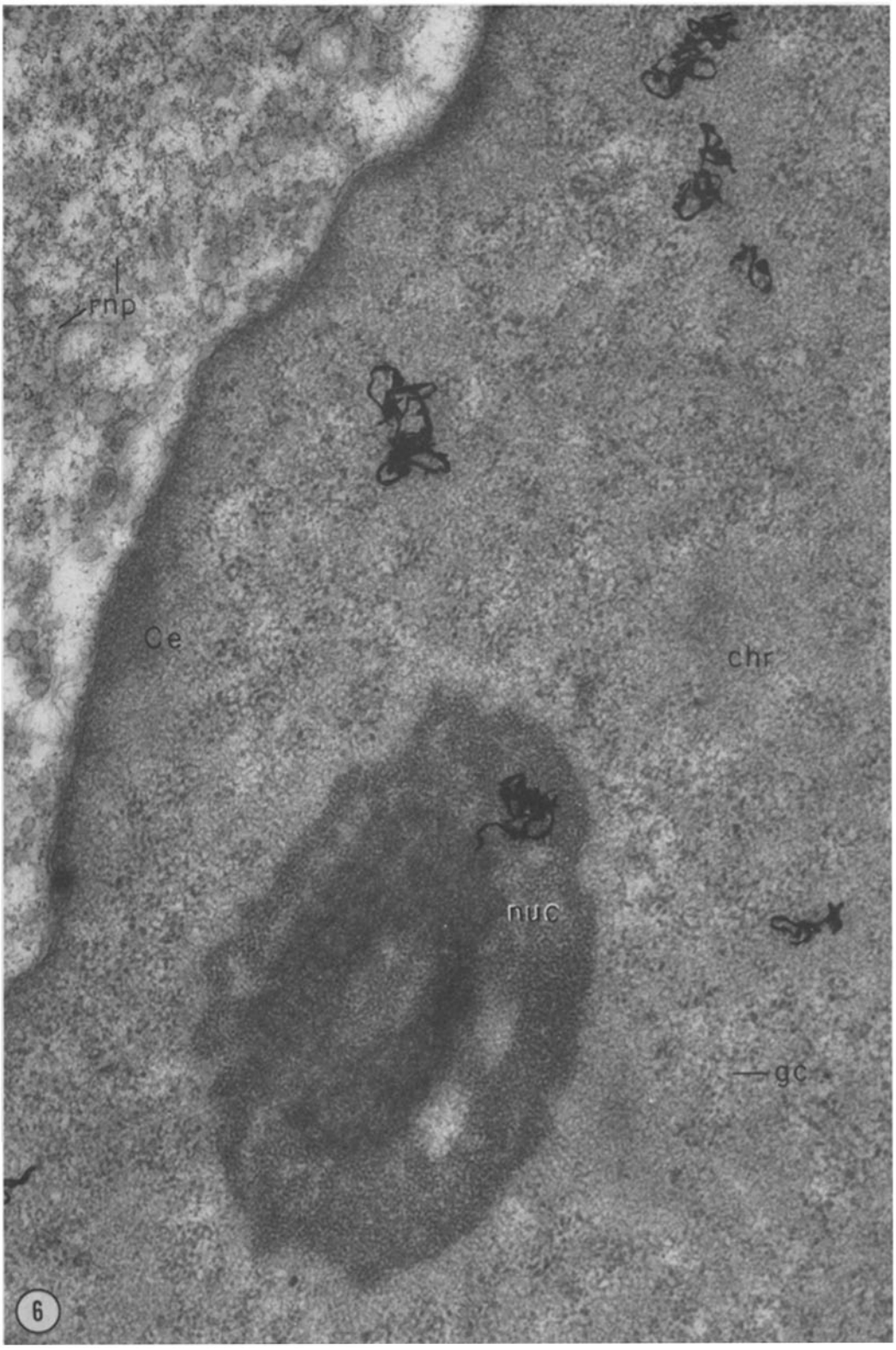
The Fine Structure of the Chromosome

Autoradiographs of sections of regenerating limbs fixed at hourly intervals after treatment with tritiated thymidine were studied with the light microscope to detect the first appearance of labeled mitoses. The first labeled chromosomes were found 6 hours after injection of the isotope, and it can be assumed that they formed from the chromatin in the interphase nuclei that incorporated thymidine 6 hours previously. Thus, the period of interphase between the end of DNA synthesis and the beginning of cell division (G2) in the salamander regenerate is of the same order of magnitude as that encountered in other vertebrates (21, 22). By 4 to 6 days the grain count over labeled nuclei has decreased to about a quarter of that present in autoradiographs of limbs fixed on the day of injection. This decrease in grain count is due to dilution of the isotope by repeated cell division and, after 10 days, the only labeled cells that can be detected are in cartilage and muscle where cell division has ceased. The grains are always associated with the filamentous component described here as chromatin, and the granular component of the nucleus does not become radioactive at any of the time intervals studied.

The chromosomes appear to form by aggregation of the chromatin component of the nucleus, which at the same time increases in density. In

FIGURE 6

A higher magnification electron micrograph of an interphase nucleus fixed during the period of DNA synthesis. In some areas, the chromatin is found as distinct clumps or strands (*chr*). In others, the chromatin is extremely dispersed and comes into intimate association with the granular component (*gc*) of the nucleus. Even though there is close intermingling of the granular component with the chromatin, it can be clearly seen that the silver grains are associated with the finely textured component. One silver grain is found over the nucleolus. This is probably due to the presence of chromatin in the strands of the nucleolonema. The granular component of the nucleus is composed of electron-opaque particles 300 to 500 A in diameter. These particles are larger than cytoplasmic ribonucleoprotein granules (*rnp*) and they are also different in appearance from the small punctate structures (*nuc*) that compose the nucleolonema. From a regenerate fixed 1 hour after administration of thymidine. *ce*, dense chromatin. × 28,500.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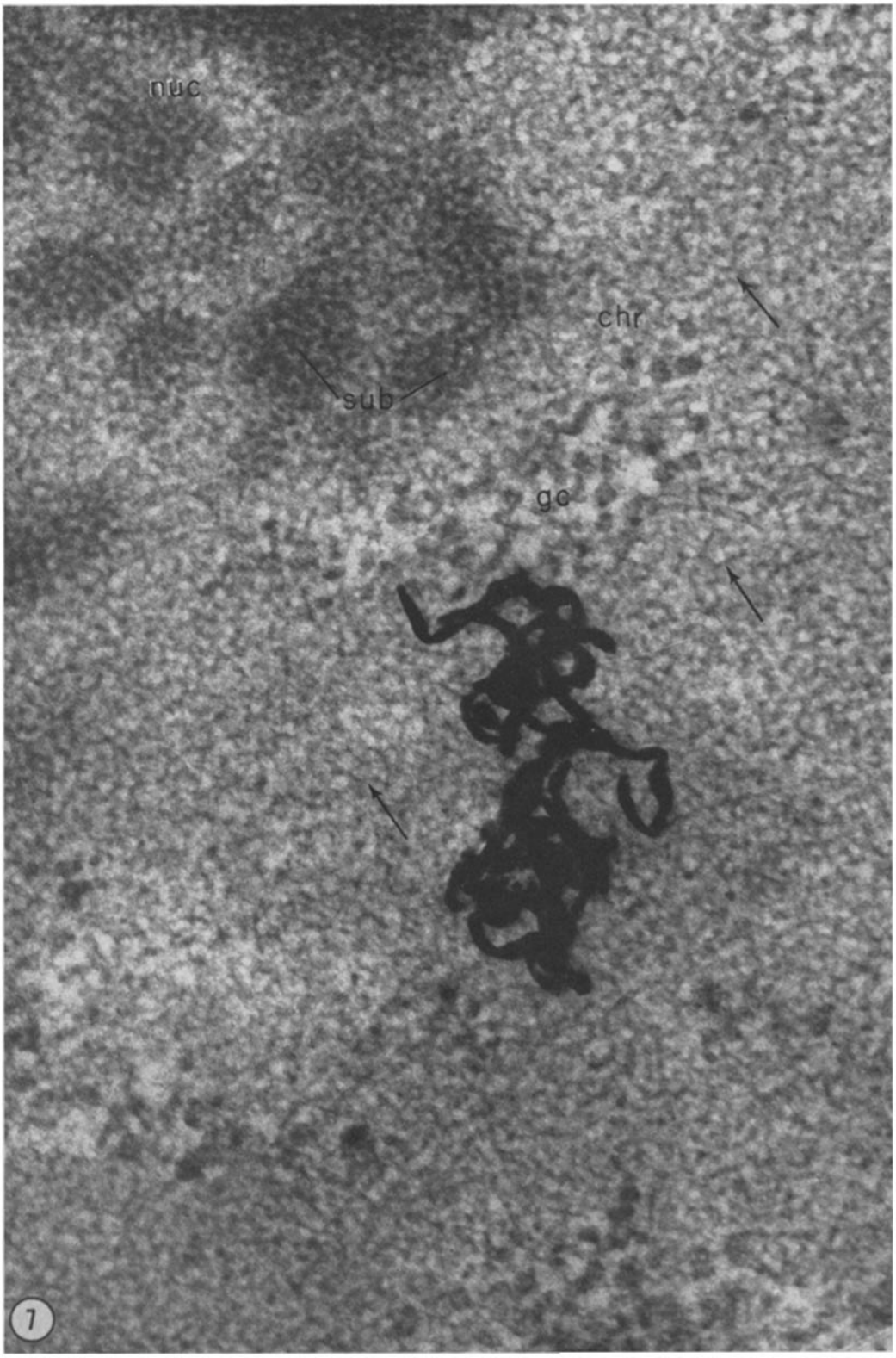
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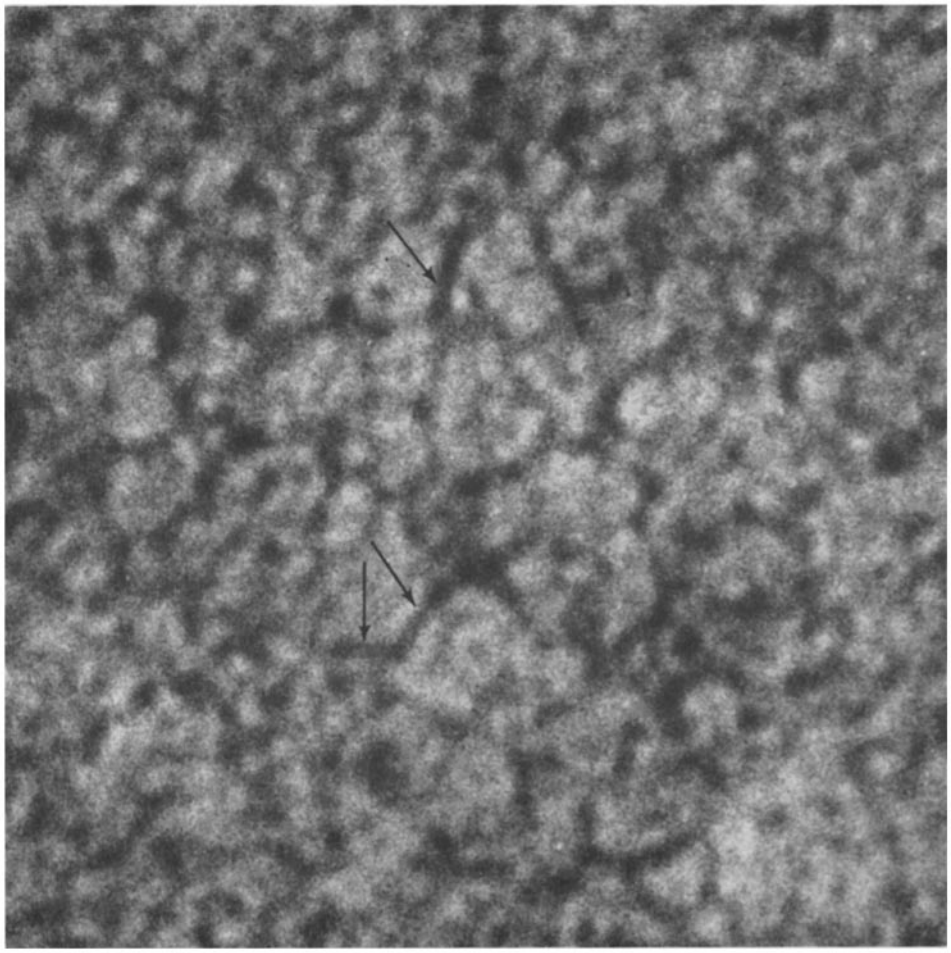
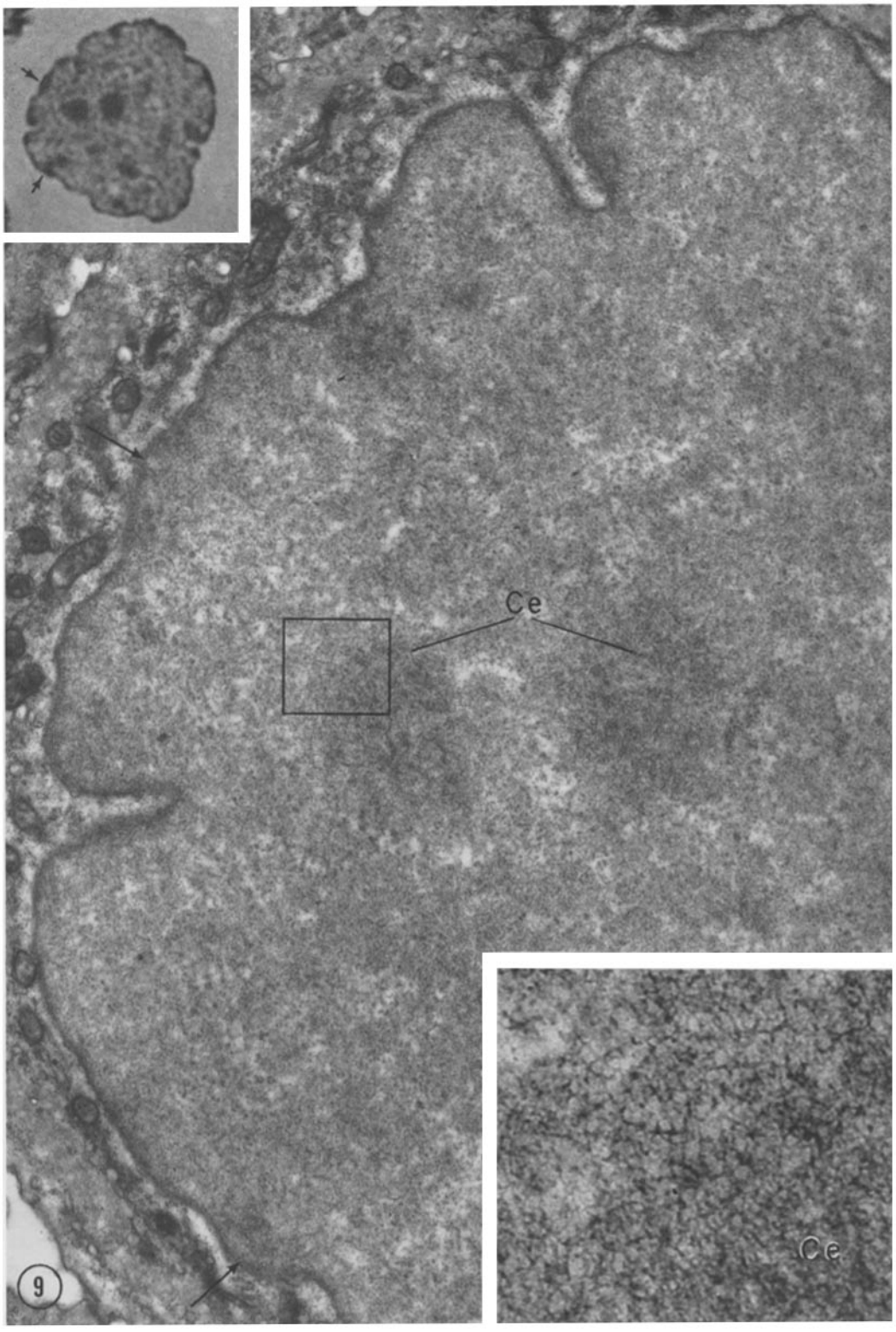


FIGURE 8

Higher magnification electron micrograph of a small portion of a very thin section through the chromatin of an epidermal cell nucleus. The filamentous nature of the meshwork comprising the chromatin is seen to better advantage in thin sections because there is less superimposition of the individual elements. The filaments (*arrows*) appear to branch and may interconnect with adjacent filaments. The interstices of the mesh so formed are less than 200 A wide. In cross-section the filaments appear punctate. Where the plane of sectioning is oblique the filaments are indistinct. × 350,000.

FIGURE 7

At a high magnification the chromatin is seen as a meshwork of short filaments of moderate electron opacity (*arrow*). The individual filaments are 50 to 75 A in diameter and are best seen in very thin sections (Fig. 8). Part of a nucleolus is found in the upper left corner of the figure and it can be seen that the chromatin (*chr*) component extends between the strands of the nucleolonema (*nuc*). At this magnification the interchromatin granules (*gc*) appear to have a central core of lower electron opacity. They are more irregular in shape and larger than the small structures (*sub*) which form the nucleolonema. A silver grain appears over the chromatin. From a nucleus in a limb fixed 1 hour after administration of tritiated thymidine. × 83,500.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sectioned, osmium tetroxide–fixed material of the somatic chromosomes depicted here (Figs. 10 to 12), chromosomal coils and other structural subunits described in classical acetic acid squashes are not seen. Even in thicker sections and whole mounts of salamander cells viewed in the light microscope, formation of chromosomes seems to involve a coming together of the diffuse, interconnected strands of chromatin (see the excellent colored illustrations of Bloom and Leider, 25) rather than by a "coiling" up of chromatin threads. The chromosomes are strongly Feulgen positive as seen in the light microscope (inset, Fig. 10) and in the electron microscope at low magnification they appear very dense and homogeneous. Even at metaphase, they are so closely associated that it is difficult to tell in sections where one ends and the next begins (*chm*, Fig. 10). The granular component is excluded from the confines of the chromosomes and now surrounds the DNP component. Nuclear and cytoplasmic granules are intermingled with membrane-bounded vesicles derived from the nuclear envelope and endoplasmic reticulum, and these various organelles and cell components become dispersed along the mitotic spindle during cell division.

At higher magnifications, a fine filamentous meshwork similar to that observed in chromatin can be detected within the chromosome (Fig. 11, inset). The seemingly short, interconnected filaments (~50 to 75 A in diameter) have the same general appearance as the filaments comprising the diffuse chromatin (Fig. 8) and, here again, they will be referred to as DNP filaments. The major fine structural differences between the filamentous meshworks in the two cases are that in the interphase nucleus the DNP meshwork is more dispersed and is in close relationship with the granular component; and that the DNP meshwork has a greater electron opacity in chromosomes than in chromatin. The increased density seems to be due to increased electron scattering by both the filaments and the material of the interstices of the meshwork. The differences in density are quite apparent when interphase nuclei and mitotic figures are compared in the same field (Fig. 12). The electron opacity of the chromosomes is not due merely to osmium or to the lead stain employed to increase the contrast of the section; unstained chromosomes and chromosomes fixed in formalin are also very dense (25).

Certain types of chromatin share some of the morphological and physiological properties of the chromosomal DNP. The electron opacity of the chromatin centers (*Ce*, Fig. 12) and of erythrocyte nuclei (*rbc*, Fig. 13) closely approximates that of the chromosomes. The similarity between these structures extends to their appearance after Feulgen staining. The chromosomes (*chm*, Fig. 14), the dense chromatin centers (*Ce*, Figs. 9 and 14), and the red blood cell nuclei (*rbc*, Fig. 14) are intensely Feulgen positive, while the diffuse chromatin of the interphase blastema or epidermal cell nucleus is only lightly stained. Differences in staining reaction and electron opacity are also correlated with an inherent density difference that can be observed in living preparations, as well as in unstained fixed preparations of cells studied with the light microscope. This correlation may even extend to the physiological properties of the several types of chromatin, for the chromosomes, red blood cell nuclei, and dense

FIGURE 9

This figure compares the appearances of the dense chromatin "centers" in serial sections of the same nucleus viewed in the light and electron microscopes. The main portion of the figure is an electron micrograph of a thin (< 500 A) section. The inset in the upper left corner is an adjacent section (< 1 μ thick) of the same nucleus stained with the Feulgen reaction and photographed in the light microscope. (Neither of the preparations is an autoradiograph). It is difficult to match small accumulations of dense chromatin in the two sections (*arrows*). There are, however, two large regions of dense chromatin which can readily be compared in the two sections illustrated (*Ce*). The dense chromatin shows a more intense staining reaction than the surrounding diffuse chromatin. The inset in the lower portion of the figure is an enlargement of the area indicated by a square in the main figure. It can be seen that the dense chromatin has the same filamentous structure as the diffuse chromatin. There is, however, an over-all increase in the density of the filaments as well as interstices of the mesh. × 17,000. Lower inset, × 67,000. Upper inset, × 2,000.

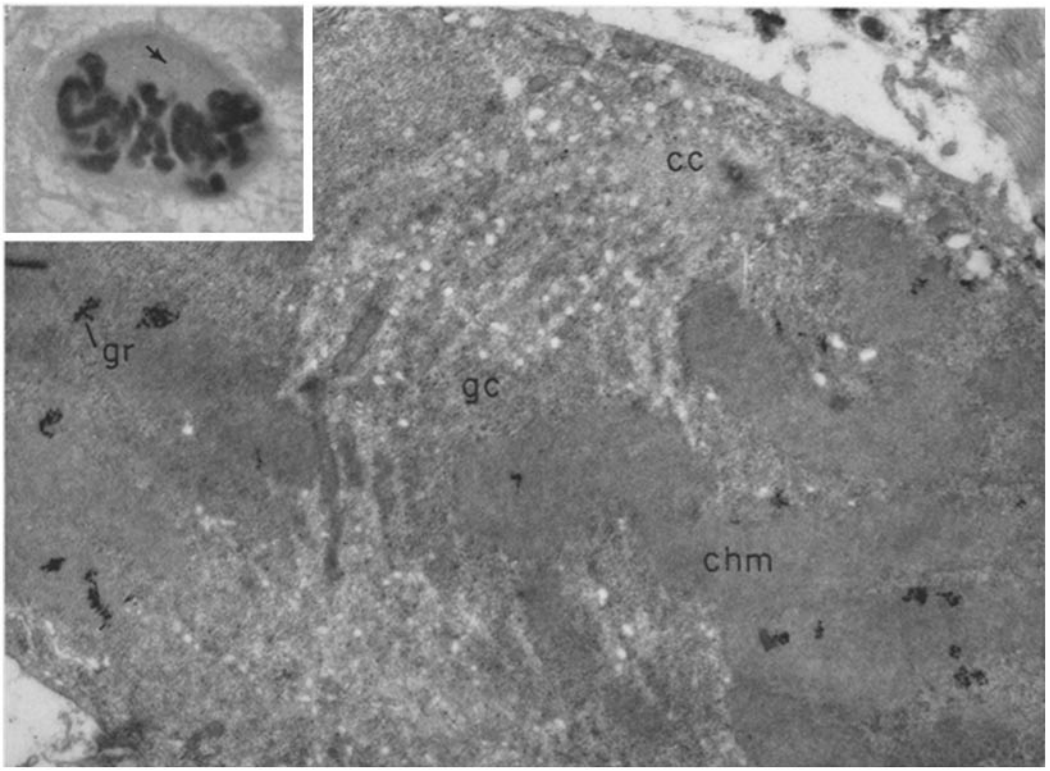
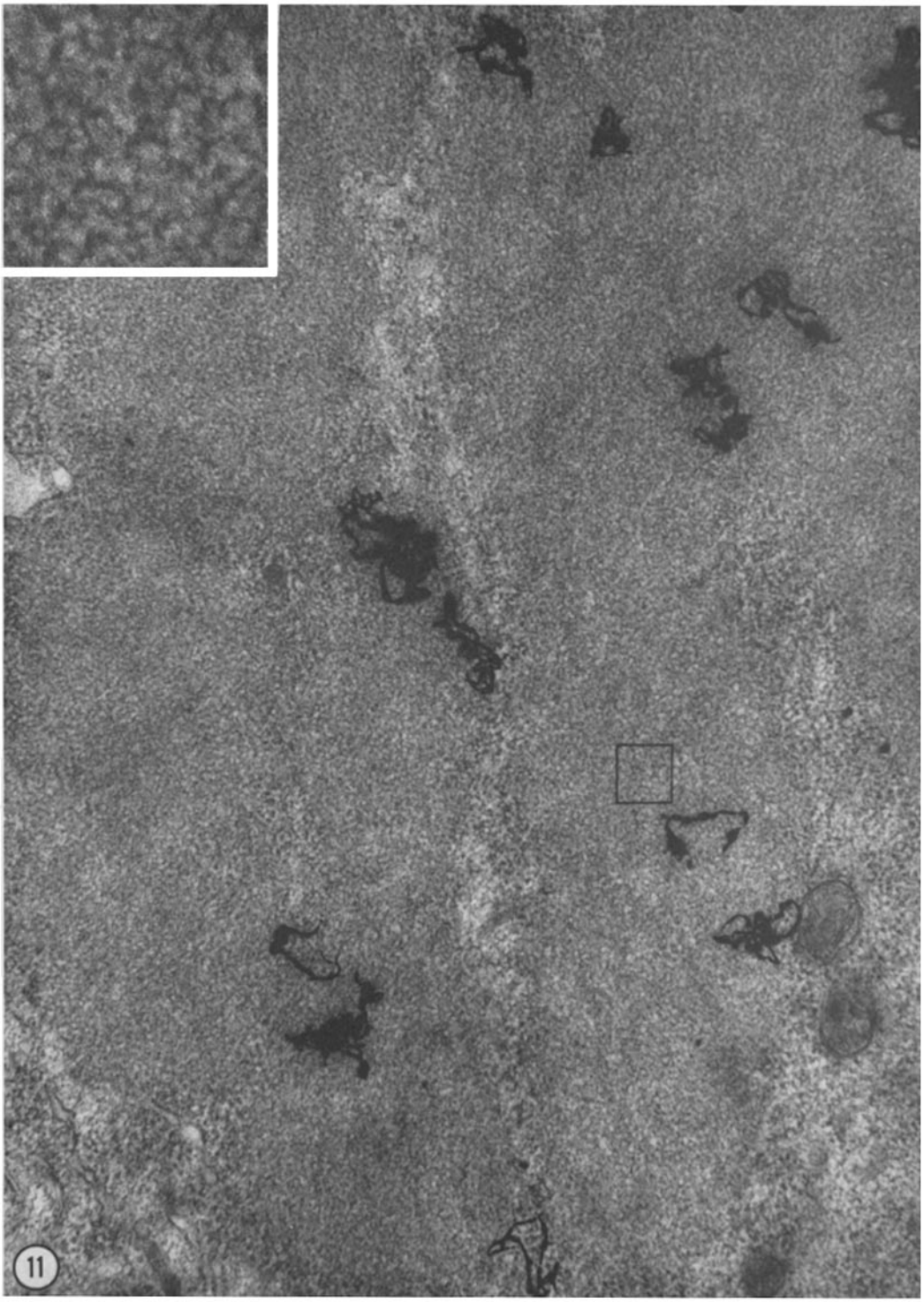


FIGURE 10

Electron micrograph of a metaphase figure in a regenerate fixed 24 hours after the animal had been injected with tritiated thymidine. The chromosomes (*chm*) are labeled (*gr*). The inset in the upper corner shows a similar metaphase figure in a thick section stained by the Feulgen reaction (not subjected to autoradiography). The chromosomes are homogeneous in appearance and are much denser than the diffuse chromatin of interphase nuclei synthesizing DNA. They are closely associated with each other, and it is sometimes difficult to distinguish them as separate structures. The granular component (*gc*) seen in interphase nuclei becomes excluded from the DNP regions during prophase. In this figure, the granular component completely surrounds the chromosomes and is also found in linear arrays extending toward the cell center (*cc*). In the light micrograph (*inset*), the cell center (arrow) appears as a lightly staining area in the same region of the cell. The spindle is presumably located between the chromosomal and the polar regions of the cell, but it is not visible as a distinct entity in osmium-fixed preparations. × 8,000. Inset, × 1,500.

FIGURE 11

This higher magnification electron micrograph shows a small portion of two chromosomes in an anaphase figure. The animal was injected with tritated thymidine 24 hours prior to fixation, and the chromosomes are labeled. Like the more diffuse chromatin of interphase nuclei, the chromosomes are seen to consist of a filamentous meshwork. The inset is an enlargement of the area marked by a square in the middle of the figure. The short filaments composing the chromatin have the same dimensions (50 to 75 A in diameter) and the same general appearance as the filamentous component of chromatin. The chromosomal DNP meshwork resembles the chromatin centers of interphase nuclei in electron opacity (Figs. 9 and 12). × 28,500. Inset, × 120,000.


11

chromatin centers do not incorporate tritiated thymidine. The possible functional significance of the diffuse arrangement of synthetically active chromatin will be considered in the discussion.

DISCUSSION

By the use of autoradiographic techniques in conjunction with electron microscopy, it has been possible to visualize with high resolution the structure of nuclei in active phases of DNA synthesis and to localize directly the sites of thymidine incorporation in such nuclei. Some aspects of the interpretation of the autoradiographs and the significance of the observations have already been considered, but further discussion of the fine structure of the DNP-containing component of the nucleus seems warranted. The morphology of the chromosomes that formed subsequently from the labeled chromatin has also been studied, and the fine structure of chromosomes will be compared to that of chromatin. In the final section of the discussion, the possible significance of the morphological variations observed in the DNP component of the nucleus in different functional states will be considered.

Fine Structure of the DNP Component of the Interphase Nucleus

The component of the interphase nucleus which incorporates tritiated thymidine appears in electron micrographs of osmium tetroxide–fixed material as a finely textured material of low density, arranged in an interlacing network throughout the nucleus. At high magnification, the fine-textured nature of the DNP component is seen to be due to minute filaments 50 to 75 A in diameter, which branch or connect with one another to form a tight meshwork. These filaments have a punctate appearance in cross section. The interstices of the mesh are not much larger than the filaments themselves, and, therefore, the filaments are often difficult to resolve except in very thin sections parallel to their long axis. It is tempting to think that the filaments are macromolecules of DNP. Isolated DNA molecules viewed in the electron microscope are ~20 A in

FIGURE 12

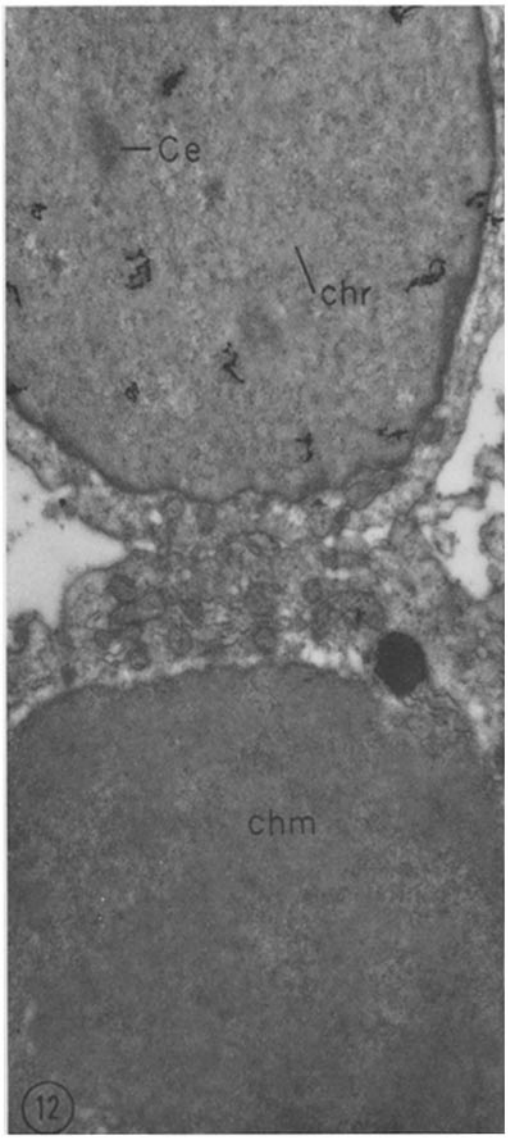
Electron micrograph showing, in the same field, an unlabeled prophase (lower half of the figure) and a labeled interphase nucleus. The regenerating limb was fixed 1 hour after injection of tritiated thymidine. The electron opacity of the chromosomes (*chm*) is comparable to that of the dense centers (*Ce*) found in interphase chromatin. The synthetically active chromatin (*chr*) of the interphase nucleus is more diffuse and much lower in electron opacity. × 8,500.

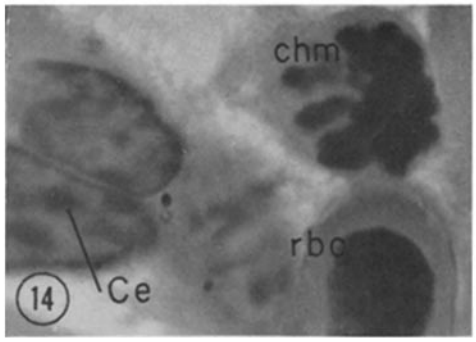
FIGURE 13

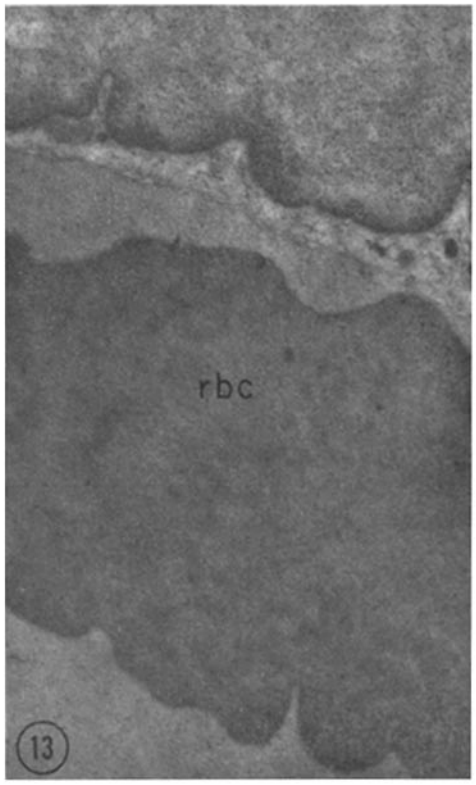
Electron micrograph, showing in the same field, a diffuse interphase nucleus of an endothelial cell and a dense nucleus of a red blood cell. The chromatin of the erythrocyte nucleus (*rbc*) is much more electron opaque than the diffuse chromatin of the interphase nucleus and in this respect it resembles the chromosomes depicted in Fig. 12. Dense chromatin and chromosomes also resemble each other in their staining reactions for light microscopy (Fig. 14). × 9,500.

FIGURE 14

Two interphase nuclei with diffuse chromatin, a dividing cell, and part of an erythrocyte nucleus are shown here in a Feulgen-stained section photographed with the light microscope. The chromatin of the erythrocyte nucleus (*rbc*) and the chromosomes (*chm*) of the dividing cell show an intense Feulgen reaction, whereas the diffuse chromatin of the interphase nuclei depicted at the left of the figure is more lightly stained. The chromatin centers of the interphase nuclei (*Ce*) are more intensely stained than the diffuse chromatin (see Fig. 9). Thus, the electron opacity of chromatin and the intensity of the Feulgen reaction appear to be correlated. The possible relation of these morphological characteristics to physiological activity is considered in the text. × 1,800.


Ce
chr
chm
12


chm
rbc
Ce
14


rbc
13

diameter, and individual nucleoprotein macromolecules have a diameter of 30 to 50 A (7–9). Stoeckenius (7) and others have speculated that the DNA molecule forms the core of the nucleoprotein filaments and protein, the shell; the basic amino groups of the protein encircling the DNA helix may form bonds with the phosphate radicals of the nucleic acid (26). Small filaments (25 to 200 A in diameter) have been described in chromatin and chromosomes by a number of investigators (10–14), and also in various DNP-containing organelles, such as the chloroplasts of *Chlamydomonas* (27), the kinetoplasts of trypanosomes (28), and the nucleoids of bacteria (29) and certain algae (30). It thus seems possible that DNP in the cell has a filamentous structure wherever it occurs. Many of the reported differences in size of the filaments may be due to methods of specimen preparation (13). Some of the variation in plants and lower animals, however, is probably due to differences in the types of protein combined with the DNA (30). We have seen no evidence of pairing of filaments in somatic cells, but Ris (31) has described 40 A filaments arranged in 100 A units in sperm heads. Here again, the differences in size and arrangement may reflect differences in protein content, for the sperm nucleus has unique nuclear proteins and is highly differentiated in structure and very specialized in function.

The filaments observed in the chromatin of interphase nuclei are so short, or, rather, so tightly cross-linked with other filaments, that they do not completely meet our expectations of DNP structure based on previous studies of chromatin in somatic cells. Most workers (see 12, 13), who have recognized that chromatin is filamentous rather than granular in nature, have described individual "microfibrils," not an interconnected meshwork of filaments. Yet, Yasuzumi (32) has clearly illustrated a net-like pattern of interlacing filaments in the chromatin of nucleated red blood cells. Moses (14) has suggested that the microfibrils in meiotic chromosomes may branch and anastomose, and Claude (33) has illustrated branching filaments in interphase nuclei. Huxley and Zubay (6) have reported a fine meshwork in isolated nucleohistone and have speculated that here the DNA chains might be arranged in a well ordered, cross-linked fashion. Using formalin fixation, Bloom and Leider (25) have demonstrated a coarse reticular framework in salamander chromosomes, although they believe the DNA component corresponds to a material which has an amorphous appearance in their electron micrographs. Differences in fixation undoubtedly contribute to the variations in these descriptions of the arrangement of the DNP filaments (33). This is a problem that is difficult to resolve, but we have confidence in the quality of the osmium fixation achieved in the present study because of the excellent preservation of cytoplasmic structure and because of the close resemblance of the fixed nuclei to living nuclei in size, shape, and density. Huxley and Zubay (6) have found reassurance along these lines from the fact that their osmium tetroxide–fixed nucleoprotein preparations have the same x-ray diffraction pattern as unfixed material.

The possibility that DNP macromolecules in the nucleus are cross-linked to form a reticular type of framework is not incompatible with biochemical data. The insoluble precipitates formed *in vitro* by DNA and basic protein at low salt concentrations have been thought to be "three dimensional, cross-linked networks" held together by salt bridges (34). DNP is probably insoluble in its natural form in the living cell and it would not be unreasonable to expect it to occur as a cross-linked meshwork rather than as long fibrils. Stoeckenius (7) noted "frequent contact points or crossings" of isolated DNP molecules spread from a solution of 1 M NaCl, and at lower ionic strength the DNP formed more extensive aggregates. Perhaps the greatest appeal of the meshwork model proposed here comes from a consideration of the predicted structure of insoluble gels. Frey-Wyssling (35) has suggested that, "Gels with reticular structure are characterized by the existence of a framework whose constituent parts occupy definite mutual positions. The reticular state is . . . distinguished . . . by the fact that the strands or strings of the framework cannot be solvated completely and maintain certain *junctions*." The speculation that the chromatin of the interphase nucleus is an insoluble "reticular" gel (permeated by the nuclear sap) is not unreasonable. The structure of such a gel might be a meshwork, or framework if you like, of DNP macromolecules cross-linked to form numerous "junctions" and arranged in "definite mutual positions."

The Structure of the Chromosome

The chromatin labeled by incorporation of tritiated thymidine does become completely

confined to chromosomes during mitosis. Although some investigators have interpreted the chromosomal unit of structure as granular in nature or consisting of fibrils with smaller subunits, high resolution studies of very thin sections of chromosomes reveal a filamentous meshwork of the same dimensions as that in chromatin, but of greater density. It is not completely clear why the chromosomal DNP meshwork is so much more dense than the chromatin from which it originated. The increased Feulgen reaction is probably due to an increase in the concentration of DNA (36). It is certainly true that the DNP filaments are more closely knit and extraneous materials such as the granular component are excluded from the chromosome. In examining the electron micrographs, however, we have the impression that the DNP filaments, and perhaps the interstices of the mesh, are actually darker, that is, that they have increased their electron-scattering power. It is tempting to postulate the presence of, or increase in the concentration of, a third substance such as calcium or magnesium in the chromosomal DNP gel, since we know that removal of cations by chelating agents disrupts the chromosome (37). This point will require further study, however (38).

There has been a tendency among cytologists to look with dismay upon the somewhat amorphous image of the chromosome obtained by electron microscopists. The concept of the chromosome as a highly ordered structure with precisely oriented genes located along its coiled chromatid cores has become well accepted as a result of the classical cytogenetic studies of the earlier part of this century. It is difficult to accept the fact that osmium fixation does not reveal coils or cores in well preserved somatic chromosomes. Intrachromosomal structures of the type described by Moses (39) and Fawcett (40), and other differentiated components such as loops and threads (11, 40) have been seen only in highly specialized "chromosomes." The coils in ameba nuclei described by Pappas (42) have not been seen in ameba chromosomes (43). Although there is abundant evidence that osmium preserves remarkably well the cytoplasmic and extracellular proteins of tissues, many cytologists have ignored electron microscopic studies of osmium-fixed chromosomes, believing that the true picture is seen in acetic acid squashes. Indeed, most chromosome models continue to be based on light microscopy studies (see review by Swift, 41). There is, however, nothing basically incompatible with genetic theory in the view of the chromosome obtained by electron microscopic studies. A gel consisting of a homogeneous appearing meshwork of DNP macromolecules could be well ordered. Mazia (37) has speculated that divalent bonds play a role in binding together chromosomal proteins and he suggests that the chromosome could be a "biochemical continuum" in the sense that the same class of bonds existing within the units also characterizes the boundaries between units. If the chemical units composing the "continuum" are similar (and we know they are) and if they are linked by the same bonds that exist within the units (as Mazia suggests), then we might not expect to detect visible structural differences between one part of a chromosome and the next. The structural order for which we search may exist at a dimensional level that will remain a part of the chemical domain until new techniques are developed for its delineation.

Significance of the Dispersed Chromatin of Synthetically Active Interphase Nuclei

It has been recognized for some time that chromosomes are relatively inert metabolically (44, 45). It seems worthwhile, then, to study the morphology of the dispersed interphase DNP engaged in replication if we are to understand better the structural arrangement of metabolically active nucleoproteins. It is quite possible that the intermingling of the DNP meshwork and other structural components which occurs in nuclei synthesizing DNA places the DNP in a better position for interactions with the substrates and other molecular components required for nucleic acid synthesis. If the DNA is in the center of the protein filament described in the previous section, then this theory does not explain any better than others (41, 44) how the helices of the DNA could become uncoiled as demanded by current biochemical speculations concerning the morphology of DNA during nucleic acid and protein synthesis (46). What our observations do suggest is that the DNP is in a very different morphological and chemical environment during DNA synthesis than at other times. The chromosome seems to be a firm nucleoprotein gel, probably relatively impervious to the substrates mentioned above. Indeed, it withstands harsh manipulation and can be fixed for microscopy in a much more consistent manner than the dispersed chromatin. From a

teleological point of view, one might reason that the chromosome is an admirably stable carrier for inactive genes during cell division, whereas the dispersed chromatin within the controlled chemical environment of the nucleus is adapted for more extensive metabolic interaction (see reviews by Gall, 11, and Mazia, 45).

If we accept the theory that "condensed" chromatin such as that composing chromosomes is a metabolically inert nucleoprotein gel as compared with the dispersed chromatin of nuclei during DNA synthesis, then we are easily led to further speculation about the significance of nuclear structure in general. Bearing in mind the difficulties sometimes encountered in fixing the relatively unstable chromatin of the interphase nucleus, we find it still possible to distinguish clearly two extremes in the morphology of interphase nuclei. On the one hand, we have the large, so called "vesicular" or lightly staining nucleus, with prominent nucleoli, which the light microscopist has long associated with cells that have a highly basophilic cytoplasm. This is the type of nucleus found in the growing epithelial and blastema cells described in the present report, and in gland cells, neurons, and many embryonic cell types. What these cells seem to have in common is active nucleic acid and protein synthesis, either nuclear or cytoplasmic in nature. Viewed in the electron microscope, this type of nucleus is seen to contain highly dispersed strands of the filamentous meshwork. At the other extreme in nuclear morphology is the nucleus whose chromatin always appears heavily clumped and darkly stained in the light microscope. The nucleus of the red blood cell in the amphibian is an example of this extreme, and viewed in the electron microscope is seen to consist almost entirely of chromatin composed of a very dense filamentous meshwork (32; Fig. 13, this paper). Like the chromosomes, this chromatin is intensely Feulgen positive in the light microscope and it is tempting to speculate that it is also relatively inert metabolically. Circulating nucleated red blood cells show no DNA synthesis and preliminary studies with labeled amino acid precursors suggest that protein turnover in salamander erythrocytes is not very great. Interphase nuclei engaged in DNA synthesis do contain clumps of dense chromatin which give a strong Feulgen reaction in the light microscope and these chromatin "centers" resemble chromosomes in their fine structure. It is possible that the dense chromatin, or what might be called "heterochromatin" by some definitions (47), and the dense DNP meshwork of the chromosome are both manifestations of a relatively stable form of the DNP gel. Regions of chromatin in synthetically active interphase nuclei that are heterochromatic probably duplicated their DNA at a different interval in the synthetic period than the diffuse chromatin labeled immediately by the administration of thymidine. Indeed, Taylor (22) has suggested that homologous regions can be either heterochromatic or euchromatic at different times. It is interesting to note also that the chromatin of the synthetic zone of the macronucleus of *Euplotes* is of low electron opacity and highly diffuse in appearance, whereas the intensely Feulgen-positive, electron-opaque, chromatin bodies of the remainder of the macronucleus do not synthesize DNA (48, 49). Moreover, synthetically active DNP regions (puffs) of certain polytene interphase "chromosomes" are less dense than the heterochromatic bands (see 41).

There are, of course, interphase nuclei with morphological characteristics intermediate between the extremes discussed here, and there are nuclei which appear to be exceptions to the rule. The mature plasma cell, for example, has a clumped nucleus but presumably is synthesizing protein. It should be possible by means of autoradiographic techniques, such as those described here, to determine the exact extent of the correlation of nuclear morphology with metabolic activities of the cells involved. As a working hypothesis for the present, we would like to suggest that the dispersed DNP gel, or what has sometimes been called "euchromatin" (47) or "diluted chromatin" (48), is the metabolically active form of the DNP meshwork and that the denser, more heavily staining forms of chromatin are relatively inert, at least with respect to nucleic acid and protein synthesis.

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