Effect of Thioacetamide on Rat Liver Regeneration

II. Nuclear RNA in Mitosis*

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ABSTRACT

The effect of thioacetamide on dividing cells of regenerating rat liver has been studied. Rats were given daily subcutaneous injections of thioacetamide as a 1 per cent solution at a dosage of 5 mg./100 gm. body weight for 7 to 10 days, subjected to partial hepatectomy, and sacrificed 28 to 31 hours later.

Thioacetamide treatment results in striking increases in the nuclear ribonucleoproteins of the liver cell without affecting the mitotic rate during regeneration (14). During mitosis, RNA-containing particles were seen within the spindle and coating the contracted chromosomes from prophase through metaphase or early anaphase. At telophase, prior to the reconstruction of the nuclear membrane, fine RNA-containing granules appeared within the compact chromosomal groups. These coalesced to form nucleoli corresponding in number to the number of nucleolar organizer regions. The nuclei and nucleoli showed a rapid increase in size during the reconstruction period when compared with corresponding figures of the control liver samples.

Electron micrographs of interphase nucleoli indicated a similar basic granular structure in both drug-treated and control animals.

The question is raised as to whether the increased nucleolar material merely made visible some of the nucleolar-chromosomal associations that normally occur in mitosis, or whether thioacetamide directly affects the synthetic activity of the contracted mitotic chromosomes.

INTRODUCTION

In the course of studying the effect of thioacetamide (CH_3CSNH_2) on the restoration of rat liver following partial hepatectomy, some interesting cytochemical events were observed (14). Thioacetamide induces striking increases in nuclear ribonucleoproteins (16). The nucleolus may enlarge 9- to 13-fold in volume within 10 days of treatment (12), facilitating the study of nucleolar-chromosomal associations during division. The present investigation is concerned with the effects of the increased nucleolar ribonucleoproteins on various phases of the mitotic cycle.

Materials and Methods

Male and female rats of the Wistar strain, weighing 150 to 200 gm., were given daily subcutaneous injections of thioacetamide (TA) as a 1 per cent solution at a dosage of 5 mg./100 gm. body weight for 7 to 10 days. Partial hepatectomy was then performed according to the procedure of Higgins and Anderson (8) whereby 70 per cent of the liver was removed. Animals were sacrificed 28 to 31 hours after partial hepatectomy, when the first burst of mitosis reaches a peak. The final TA injection was given 4 hours prior to sacrifice. Saline-injected, partially hepatectomized rats served as controls.

Liver samples were fixed in acetic acid-alcohol (1:3) and stained with azure B, 0.025 per cent, pH 4.0, for localization of nucleic acids (5). Under these conditions azure B stains deoxyribonucleic acid (DNA) relatively orthochromatically (blue-green), while ribonucleic acid (RNA) stains metachromatically (purple). Dye binding specificity was checked with deoxyribonuclease (0.15 mg./ml., pH 6.5, 1 hour, 30°C.) and ribonuclease (0.2 mg./ml., pH 6.5, 2 hours, 30°C.) digestion prior to staining. Small pieces of tissue were fixed in buffered OsO_4, embedded in methacrylate and prepared for examination with the electron microscope (2).

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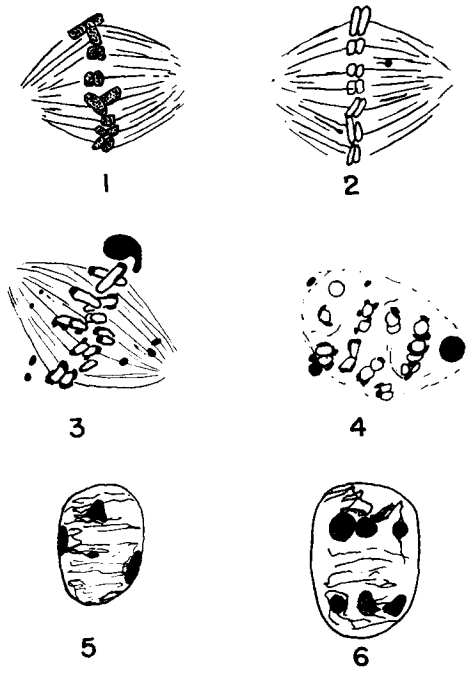
RESULTS

Normal Regenerating Liver.—Interphase rat liver cells stained with azure B are shown in Fig. 1. The DNA of the chromosomes stains blue-green, while the RNA of the nucleoli and cytoplasmic components stains deep purple. Chromosomal RNA of rat liver nuclei does not stain with basic dyes under routine staining procedures. From the work of several investigators it appears that RNA is present in chromosomes but in a form often not available for basic dye binding (11, 6, 4, 25). Thus the metachromatically stained nuclear RNA in the rat liver cell, *i.e.* the nucleolus, represents a fraction not intimately associated with the chromosomal structure itself. In regenerating liver the nucleoli are typically enlarged, irregular in shape, and contain increased amounts of RNA (24, 20).

The nucleus increases in volume at prophase. The chromosomes contract into visible threads staining blue-green with azure B. As the nuclear membrane is dissolved the nucleolus disappears. At metaphase the metachromatic spindle contrasts with the blue-green staining of the condensed chromosomes aligned to form the metaphase plate (Fig. 3). When such sections are treated with deoxyribonuclease prior to staining, only the spindle fibers and surrounding cytoplasm are stained (Fig. 4). At telophase the chromosomes form optically homogeneous vesicles which subsequently fuse. As soon as the nuclear membrane becomes visible, distinct RNA bodies appear within the chromosome mass. The number of these granules corresponds to the number of nucleolar organizer regions of the rat, 4 per diploid nucleus (18). The nucleoli enlarge and often fuse as the daughter cells separate and the reconstruction period commences (Fig. 7).

Thioacetamide-Treated Regenerating Rat Liver.—Interphase liver cells of thioacetamide-treated, partially hepatectomized rats, stained with azure B are shown in Fig. 2. The nuclear volume is greatly increased and the DNA of the chromosomes diluted such that the blue-green staining is barely perceptible. The nucleoli are disproportionately enlarged and dense. They stain metachromatically with increased intensity. As in the control liver cells, the nucleoli are characteristically irregular in shape and tend to contact the nuclear membrane during this period of regeneration.

At prophase the chromosomes contract into visible threads in typical fashion. When the nuclear membrane breaks down, the nucleolus



TEXT-FIGS. 1 to 6. Outline drawings of the micrographs shown in Figs. 3 to 8 respectively. The solid black structures ranging in size from small granules to large bodies represent ribonucleoprotein staining after DNA extraction.

fragments but does not completely disappear. RNA-containing particles of varying sizes may be seen scattered among the contracted chromosomes. At prometaphase the mitotic spindle takes form. The fibers are comparable in diameter to the control spindle. The larger fragments of nucleolar material are often pushed to the edge of the spindle and finally into the cytoplasm. Such fragments frequently persist throughout metaphase (Figs. 5 and 6). When sections treated with deoxyribonuclease are stained with azure B, the chromosomes no longer stain. However, a metachromatically stained skeleton-like outline of the metaphase chromosome plate is often clearly visible (Figs. 5 and 6). This is interpreted as representing RNA-containing material at the surface of the chromosomes and not chromosomal RNA believed to be present within the fabric or matrix of the chromonemata. Small RNA granules are also seen lying between the spindle fibers. At anaphase, when the daughter chromatids separate, the RNA-containing granules are rarely seen. Occasionally very fine particles at the edges of the

separating chromosome groups are discernible. At telophase, when the chromatids form typical vesicles, increased numbers of fine RNA granules are seen at the cytoplasmic interface and within the chromosomal masses. Toward the end of telophase, RNA bodies appear, corresponding in number to the number of nucleolar organizer regions. The nuclear membrane becomes visible at this time. A striking feature of late telophase in the thioacetamide-treated liver cell is the rapid increase in the size of the nucleoli (Fig. 8). Both the nuclei and nucleoli are much larger at the time of cytokinesis than are corresponding figures in the control liver cells (compare Figs. 7 and 8). Text-figs. 1 to 6 show diagrammatically some of the nucleolar-chromosomal associations that occur during mitosis.

Electron Microscopy.—Electron micrographs of interphase nucleoli are shown in Figs. 9 to 14. The nucleoli of control liver cells have a coiled thread-like structure which gradually becomes less dense and granular at the peripheral edges where the nucleolar and chromosomal material intermingle (Figs. 9 and 12). The nucleoli of the TA-treated cells are characteristically larger and denser (Figs. 10 and 11). There is a fairly sharp line of demarcation between the nucleolar and chromosomal material in contrast to the gradual transition between the two substances seen in the controls. The entire nucleolar outline stands out as a tightly coiled mass of anastomosing threads interspersed with several large vacuoles (Figs. 10 and 11). These vacuoles, which resist all staining procedures (13), do appear to have some structure (Fig. 13). In thin sections one can resolve the granular nature of the nucleolar material. The granules of both control and TA-treated nucleoli are similar in size and appear to be arranged along fine threadlike filaments (Figs. 12 and 14). They correspond to the 100 to 150 A granules previously resolved in nucleoli of various cell types (19, 1, 9). Recently Yasuzumi *et al.* (29) have interpreted the granular elements as representing gyres of the helicoidal filaments of the nucleolonema.

DISCUSSION

The presence of RNA-containing material at the surface of the contracted chromosomes described above does not occur in all dividing cells. There is a wide range of heterogeneity in the response of the liver cells to thioacetamide, such that not all cells show the enlarged nucleoli to the same degree (12).

Several questions may be raised in analyzing some of the above observations. How can we explain the persistence of large nucleolar fragments within both the spindle and cytoplasm? Does thioacetamide induce the synthesis of a particular species of RNA that is more resistant to dissolution? Or is the dense and massive nature of the nucleolus more difficult to degrade? Is the presence of RNA-containing granules at the surface of the metaphase chromosomes (Figs. 5 and 6) due solely to the fragmentation of the nucleolus followed by an aggregation of the particles onto the chromosomal surfaces? Or does the drug stimulate RNA synthesis in the dividing cell despite the contracted state of the chromosomes?

Electron microscope studies indicate that the basic granular structure of the interphase nucleoli is similar in both control and thioacetamide-affected cells. The increased density and large vacuoles of the nucleoli and the decreased electron density of the chromosomal material in the drug-treated cells no doubt reflect biological differences. Analysis of the purine and pyrimidine bases of nuclear RNA is in progress in order to determine whether a specific kind of RNA is synthesized in response to the drug.

If we assume that the enlarged nucleolus of the thioacetamide-treated liver cell merely makes visible some of the nucleolar-chromosomal associations that normally occur in mitosis, the following scheme may be postulated: A fraction of nucleolar ribonucleoproteins resists dissolution during prophase and becomes associated with the contracted chromosomes through metaphase. At anaphase this material is eliminated or degraded. At early telophase fine RNA-containing granules are prematurely visible within the chromosomal mass as a result of the increased stimulation of nuclear RNA synthesis induced by the drug. This "pre-nucleolar" material aggregates at specific nucleolar organizer regions in mid-telophase, giving rise to true nucleoli. This interpretation seems probable in view of previous reports on the persistence of nucleolar material through anaphase (30, 3, 22) and the elimination or decrease of chromosomal RNA at anaphase during mitosis (10) and meiosis (23, 26). Support for an association of nucleolar material with the chromosomes during mitosis comes from the recent work of Woods and Taylor (28). After short exposure of *Vicia faba* meristems to tritium-labelled cytidine only the nucleoli were

labelled, including prophase nucleoli. In prometaphase nuclei, when the nucleolus disappeared, labelled nucleolar material was found associated with the chromosomes. Metaphase and anaphase chromosomes, however, were not labelled, indicating that the nucleolar material normally did not remain associated with the chromosomes for long. In telophase they found the nucleoli were again radioactive. It has generally been believed that nucleolar material starts to reform in mid-telophase when the chromosomes are almost fully unravelled. Prenucleolar material has been described as first appearing along the chromosomal surfaces (27, 21), then to aggregating at late telophase under the control of the nucleolar organizer regions of specific chromosomes (7, 17). Recently Lafontaine (15), working with meristem cells of *Allium cepa* and *Vicia faba*, has provided the most convincing evidence of this sequence. Using electron microscopy he described prenucleolar bodies at late anaphase scattered between and within the chromosome masses. In the course of telophase, concomitant with the unravelling of the chromatids, a coalescence of the scattered prenucleolar substance occurred resulting in fewer and larger "nucleolar-like" structures. In late telophase nuclei he found only large nucleoli. It is interesting to note that the unravelling or swelling of the contracted chromosomes occurred before the condensation of the prenucleolar substance was seen. On the other hand the synthesis of the prenucleolar material appeared to be initiated in late anaphase, when the chromosomes were still highly contracted. This observation places doubt on the supposition that all synthetic activity of the chromosomes is necessarily blocked when the chromosomes are tightly coiled during mitosis. Studies of ribonucleic acid metabolism utilizing autoradiography with tritium-labelled cytidine are in progress. This approach should contribute to the understanding of nucleolar-chromosomal associations during division beyond the morphological level.

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Addendum. Since this manuscript was submitted, analyses of nuclear RNA base ratios (ion exchange column) have been completed (T. R. Breitman, Graduate Department of Biochemistry, Brandeis University). The results show essentially no difference between control and TA-treated animals.

Bibliography

1. Bernhard, W., Bauer, A., Gropp, A., Haguenau, F., and Oberling, C., *Exp. Cell Research*, 1955, **9,** 88.
2. Caulfield, J. B., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 827.
3. Estable, C., and Sotelo, J. R., *in* Fine Structure of Cells, Symposia of 8th Congress of Biology, Leiden, New York, Interscience Publishers, Inc., 1954, 89.
4. Flax, M. H., and Himes, M. H., *Anat. Rec.*, 1951, **111,** 465.
5. Flax, M. H., and Himes, M. H., *Physiol. Zoöl.*, 1952, **25,** 297.
6. Gay, H., *Am. J. Bot.*, 1949, **36,** 796.
7. Heitz, E., *Planta*, 1931, **12,** 775.
8. Higgins, G. M., and Anderson, R. M., *Arch. Path.*, 1931, **12,** 186.
9. Horstmann, E., and Knoop, A., *Z. Zellforsch. u. mikr. Anat.*, 1957, **46,** 100.
10. Jacobsen, W., and Webb, M., *Exp. Cell Research*, 1952, **3,** 163.
11. Kaufmann, B. P., McDonald, M. R., and Gay, H., *Nature*, 1948, **162,** 814.
12. Kleinfeld, R. G., *Cancer Research*, 1957, **17,** 954.
13. Kleinfeld, R. G., and Koulish, S., *Anat. Rec.*, 1957, **128,** 433.
14. Kleinfeld, R. G., and von Haam, E., *Cancer Research*, 1959, **19,** 769.
15. Lafontaine, J. G., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 777.
16. Laird, A. K., *Arch. Biochem. and Biophysics*, 1953, **46,** 119.
17. McClintock, B., *Z. Zellforsch. u. mikr. Anat.*, 1934, **21,** 294.
18. Ohno, S., Kaplan, M. D., and Kinosita, R., *Exp. Cell Research*, 1959, **16,** 348.
19. Porter, K. R., *J. Histochem. and Cytochem.*, 1954, **2,** 346.
20. Price, J. M., and Laird, A. K., *Cancer Research*, 1950, **10,** 650.
21. Rattenbury, J. A., and Serra, J. A., *Portugaliae Acta Biol., Series A*, Lisbon, Universade Faculdade de Ciências Instituto Botânico, 1952, **3,** 239.
22. Ris, H., *in* Analysis of Development, Philadelphia, W. B. Saunders Co., 1955, 91.
23. Ris, H., and Kleinfeld, R. G., *Chromosoma*, 1952, **5,** 363.
24. Stowell, R. E., *Arch. Path.*, 1948, **46,** 164.

25. Swift, H., *Texas Rep. Biol. and Med.*, 1953, **11,** 755.
26. Swift, H., Rebhun, L., Rasch, E., and Woodard, J., 14th Symposium of the Society for the Study of Development and Growth, Princeton, Princeton University Press, 1955, 45.
27. Van Camp, G. M., *Cellule*, 1924, **34,** 5.
28. Woods, P. S., and Taylor, J. H., *Lab. Inv.*, 1959, **8,** 309.
29. Yasuzumi, G., Sawada, T., Sugihara, R., Kiryama, M., and Sugioka, M., *Z. Zellforsch. u. mikr. Anat.*, 1958, **48,** 10.
30. Zirkle, C., *Bot. Gaz.*, 1928, **86,** 402.

Explanation of Plates

Plate 186

Fig. 1. Regenerating rat liver taken 28 hours after partial hepatectomy stained with azure B. The nucleoli and cytoplasm (RNA) stain deep purple. The chromatin (DNA) stains pale blue-green. × 1250.

Fig. 2. Thioacetamide-treated regenerating rat liver taken 28 hours after partial hepatectomy. Note the enlarged, dense nucleoli. Azure B. × 1250.

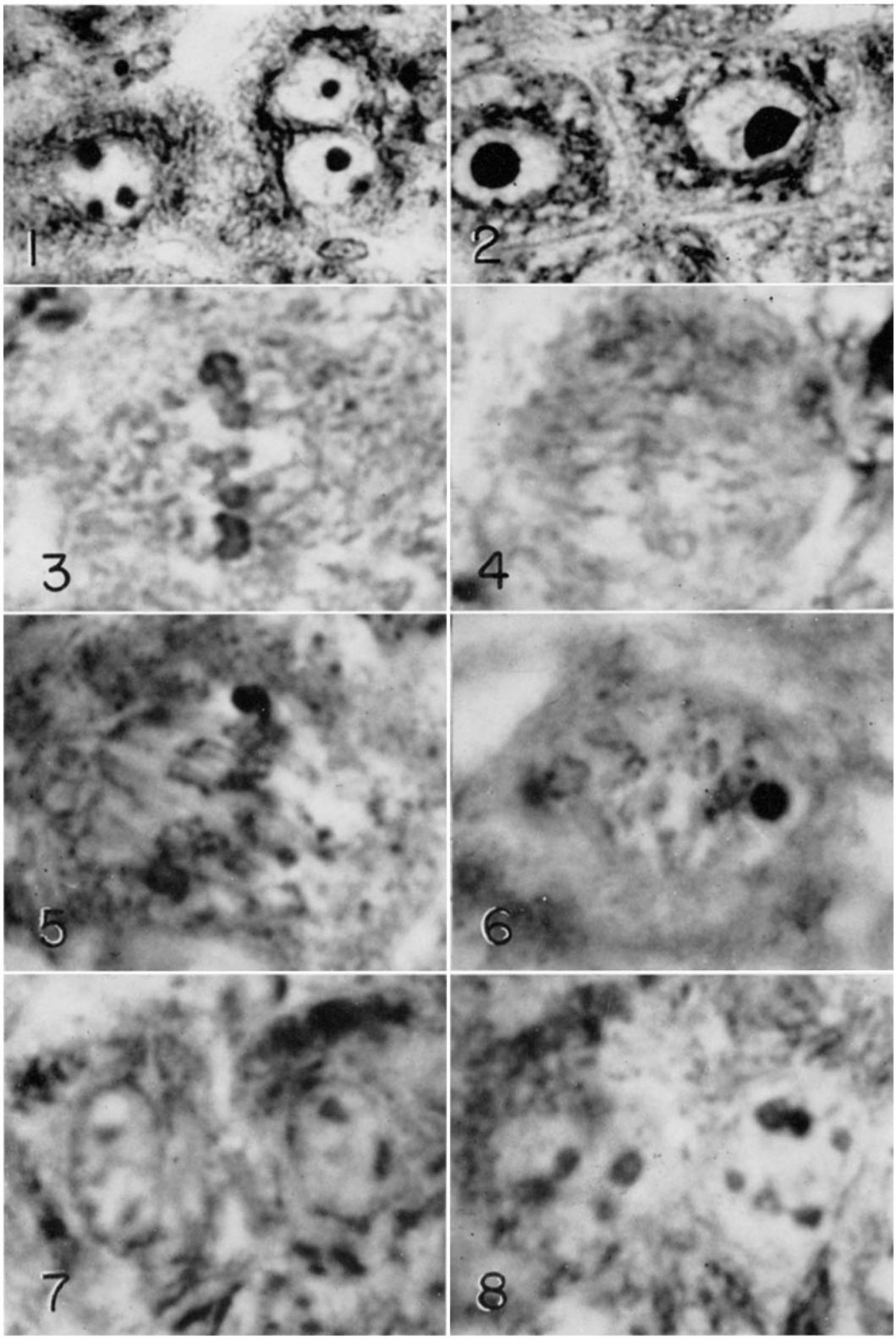
Fig. 3. Metaphase of control regenerating rat liver stained with azure B. The chromosomes stain blue-green. The spindle and cytoplasm stain purple. × 2300.

Fig. 4. Control metaphase figure similar to the one shown in Fig. 3, stained with azure B after extraction of DNA with deoxyribonuclease. Only the spindle and cytoplasm stain. × 2300.

Figs. 5 and 6. Metaphase figures of thioacetamide-treated regenerating rat liver stained with azure B after extraction of DNA with deoxyribonuclease. Note the large nucleolar fragments pushed to the outer edge of the spindle. RNA-containing material can be seen coating the surfaces of the chromosomes in both the longitudinal section of Fig. 5 and the oblique view of Fig. 6. × 2300.

Fig. 7. Reconstruction nuclei of control regenerating rat liver stained with azure B. × 2300.

Fig. 8. Reconstruction nuclei of thioacetamide-treated regenerating rat liver stained with azure B. Note the increased size of the nuclei and nucleoli when contrasted with the corresponding control of Fig. 7. × 2300.



(Kleinfeld and von Haam: Effect of thioacetamide on rat liver regeneration)

PLATE 187

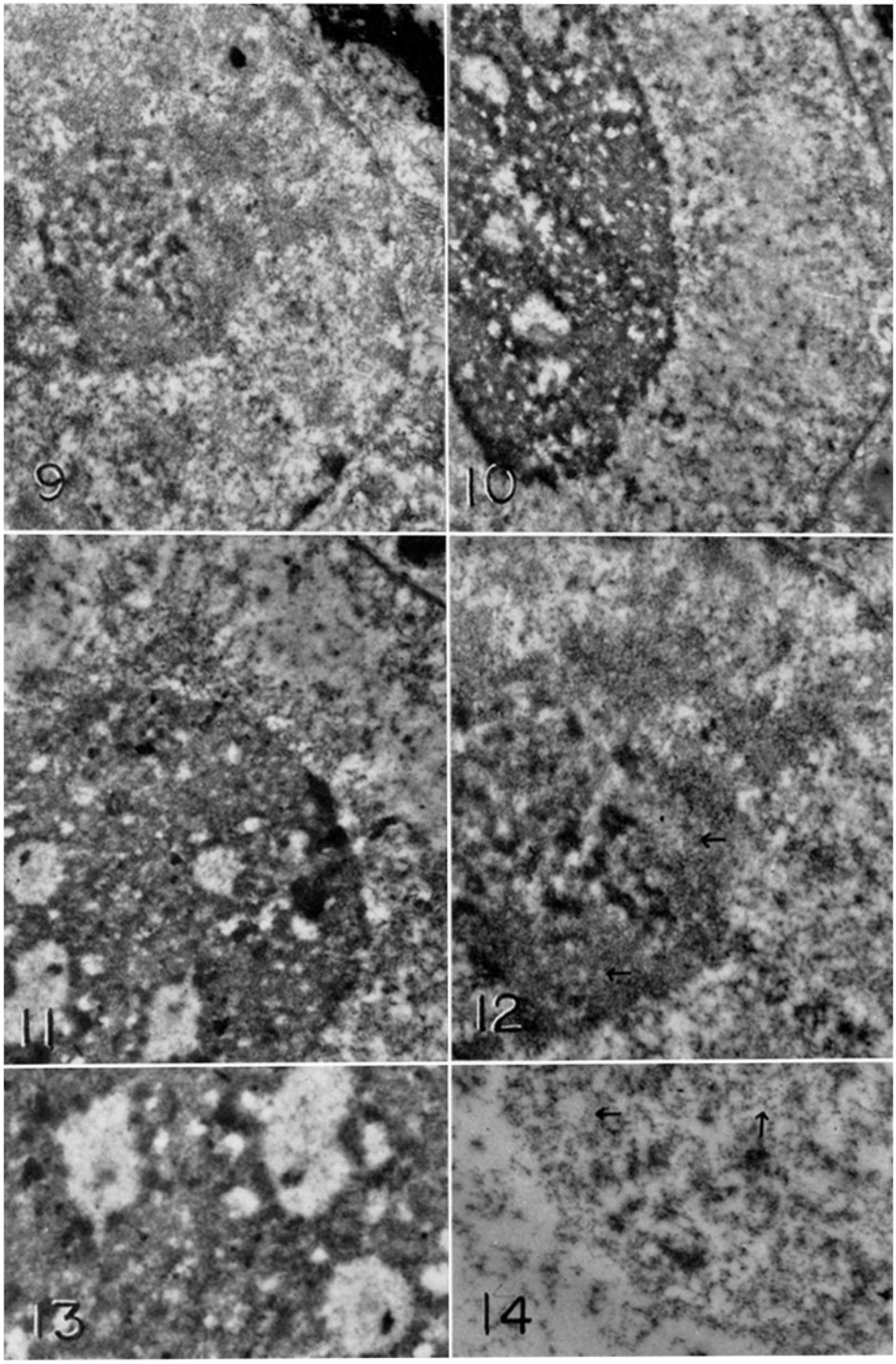
FIG. 9. Electron micrograph of a portion of a regenerating rat liver cell. The nucleus contains a nucleolus composed of a central thread-like structure which becomes granular and less dense peripherally where nucleolar and chromosomal material intertwine. × 16,500.

FIGS. 10 and 11. Electron micrographs of portions of thioacetamide-treated regenerating rat liver cell nuclei. The enlarged, dense nucleoli contain numerous vacuoles. Note the sharp line of demarcation between nucleolar and chromosomal material in contrast to the control shown in Fig. 9. × 16,500.

FIG. 12. Higher magnification of the control nucleolus shown in Fig. 9. The electron-dense granules of the nucleolus appear to be arranged along a fine filament (arrows). × 26,500.

FIG. 13. Higher magnification of the nucleolar vacuoles in the thioacetamide-treated nucleolus of Fig. 11. The vacuoles are composed of relatively electron-transparent material. × 26,500.

FIG. 14. Higher magnification of a very thin area of a thioacetamide-treated nucleolus. The electron-dense granules of the nucleolus are similar in size and orientation to those seen in the control nucleolus of Fig. 12 (arrows). × 26,500.



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