# REFORMATION OF NUCLEOLUS-LIKE BODIES IN THE ABSENCE OF POSTMITOTIC RNA SYNTHESIS

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## ABSTRACT

The dependence of nucleolar reformation on RNA synthesis that resumes in late anaphase or early telophase has been investigated in synchronously dividing *Amoeba proteus*. RNA synthesis was completely inhibited throughout all stages of mitosis and the early hours of interphase with high concentrations of actinomycin D. In such cells, nucleolus-like bodies that bind azure B and pyronin were apparent in the reformed nuclei. The bodies appear as dense, fibrous masses with loosely associated, finely fibrillar material. There are no characteristic granular regions in the reformed structures. It is suggested that the bodies probably represent mainly nucleolar protein and residual RNA which can bring about the reorganization of nucleoli in the absence of postmitotic RNA synthesis.

## INTRODUCTION

The synthesis of at least a major portion of the RNA in the nucleolus occurs at the nucleolus organizer of the chromosome (see, for example, Karasaki, 1965; Swift and Stevens, 1966). Much less is known about the site of synthesis of nucleolar proteins. Nucleolar proteins may be synthesized in the cytoplasm and may subsequently move to the nucleolus, but the possibility of protein synthesis within the nucleolus cannot be excluded. In any case, following mitosis the nucleolus is normally reformed by the accumulation of both RNA and protein. There is evidence, however, that the nucleolus may reorganize without the usual contribution of RNA synthesis in the nucleolus organizer. For example, nucleolus-like structures are present in nuclei lacking most of the cistrons for transcribing ribosomal (nucleolar) RNA (Jones, 1965; Hay and Gurdon, 1967).

To arrive at a better understainding of the involvement of RNA synthesis in nucleolar formation, we have examined by light microscope cytochemistry and electron microscopy the appearance of nucleoli following mitosis in *Amoeba proteus* in which RNA synthesis is completely inhibited by actinomycin D. In this ameba, all nuclear RNA synthesis ceases (Prescott, unpublished data), the nuclear envelope becomes fragmented, and the nucleoli have disappeared by the time metaphase is reached (Roth et al., 1960). In telophase, dense, granular nucleoli are found at the periphery of the mitotic chromosomes (Roth et al., 1960). The appearance of these structures is closely correlated with the resumption of transcription in the telophase stage.

## MATERIALS AND METHODS

### *Culture Methods*

*A. proteus* were cultured as described previously (Prescott and Carrier, 1964).

### *Assay for Inhibition of RNA Synthesis with Actinomycin D*

In a previous communication (Stevens, 1967), actinomycin D was used at a concentration of 100 μg/ml to inhibit RNA synthesis in *A. proteus*; this concentration was fully effective for RNA synthesis but did not have a discernible effect on DNA synthesis. In the present study, in order to bring about rapid and complete inhibiton of RNA synthesis, actinomycin D was used at a concentration of 1 mg/ml. Mitotic cells placed in this concentration of the antibiotic begin DNA synthesis on schedule at the end of telophase (there is normally no $G_1$ phase in the ameba cell cycle). The cells will survive up to 24 hr in this concentration of the antibiotic. To determine the efficacy of actinomycin, the following study was done.

*Tetrahymena pyriformis*, growing in synthetic medium (Elliott et al., 1952), were washed free of nutrient medium by means of centrifugation and were resuspended in pyrimidine-less medium containing 10 μCi/ml of 5-T-uridine (sp act 22 Ci/mmole). The cells were allowed to incorporate radioactivity over the next 30 hr, after which time 10 μCi/ml of both 5-T-uridine and 5-T-cytidine (sp act >20 Ci/mmole) were added. 4 hr later, the *Tetrahymena* were washed in organic ameba medium and fed to logarithmically growing amebae. Following 5 hr of feeding, the amebae were divided into two groups and actinomycin D was added to one of the groups to give a concentration of 1 mg/ml. At intervals, after addition of the antibiotic, nuclei were isolated by the Triton-spermidine method (Prescott et al., 1966), extracted with 5% trichloroacetic acid at 0–5°C, and assayed for the incorporated radioactivity in a windowless, low-background gas-flow counter. To reduce self-absorption, the nuclei were dispersed on planchets with 1 N formic acid. The results, which are given in Fig. 1, show complete inhibition of incorporation of the labeled precursors into RNA within at least 20 min after addition of actinomycin D, and probably well before 20 min.

### *Enzyme Digestion*

To determine the degree of specificity of labeling, amebae were fed on radioactive *Tetrahymena* for 16 hr. 25 nuclei were individually isolated by the Triton-spermidine method, placed singly on planchets, and assayed for incorporated radioactivity. The nuclei were digested for 6 hr at 37°C with 0.5% ribonuclease (Worthington Biochemical Corp., Freehold, N.J.) made up in phosphate buffer (pH 6.9). The ribonuclease solution was heated to 80°C for 10 min before use to remove any residual deoxyribonuclease activity. Following digestion, the nuclei were extracted with cold 5% trichloroacetic acid and assayed for any remaining radioactivity. Over 98% of the incorporated label was found to be removed by the ribonuclease digestion; control nuclei incubated in phosphate buffer alone at 37°C do not lose a significant amount of label.

### *Electron Microscope Preparation*

An hour or more following addition of actinomycin D (1 mg/ml) to a culture of amebae, 300–400 cells in division were collected by means of a braking pipette and kept in actinomycin D (experimental group). At 3-4 hours of interphase the cells were either: (*a*) fixed in 2% Veronal-acetate-buffered osmium tetroxide (pH 7.4) as described previously (Stevens and Prescott, 1965); or (*b*) prefixed in Karnovsky's glutaraldehyde-formaldehyde mixture (Karnovsky, 1965) at pH 7.3 and postfixed in 1% osmium tetroxide in cacodylate buffer after an overnight rinse in distilled water (Flickinger, 1968). Both samples were dehydrated in a graded series of ethanol, cleared in propylene oxide, and embedded in Araldite. Control groups of synchronized amebae were handled in a manner identical with that used for the experimentals, except for the absence of the antibiotic. Both control and experimental cells were examined by thick sectioning and staining with alkaline azure B (Stevens, 1966). Silver to pale-gold sections were stained with lead citrate (Venable and Coggeshall, 1965) and/or uranyl acetate (saturated aqueous solution). Microscopy was performed with a Hitachi HU-11 C electron microscope operated at 50 kv. It should be noted here that micrographs for clarification of results will be mainly those of the osmium tetroxide–fixed samples, since there is essentially little difference in the nuclear morphology between amebae fixed by the Karnovsky procedure and amebae fixed in osmium tetroxide.

### *Light Microscope Cytochemistry*

Nuclei were isolated at 3–4 hr of interphase from the above control and experimental samples by the Triton-spermidine method. After fixation in acetic acid:alcohol (3:1), the nuclei were stained with acidic azure B bromide according to Flax and Himes (1952), or 0.5% aqueous pyronin.

## RESULTS

The data in Fig. 1 show that 1 mg/ml of actinomycin D completely inhibits RNA synthesis in 20 min or less. Since amebae were exposed to actinomycin D for at least an hour before mitosis (exposure to the antibiotic for more than 2 hr prevents entry into mitosis), we are confident that

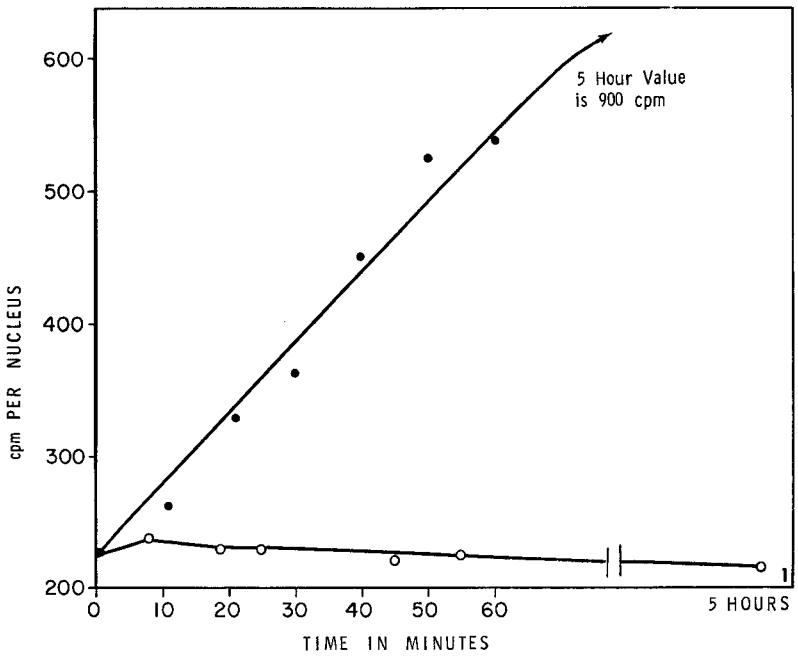



FIGURE 1 Effect of actinomycin D on incorporation of 5-T-uridine and 5-T-cytidine into RNA of *A. proteus*. Logarithmically growing amebae were fed for 5 hr on *T. pyriformis* that had been heavily labeled with 5-T-uridine and 5-T-cytidine. Subsequently, actinomycin D was added to give a final concentration of 1 mg/ml. Nuclei were isolated at indicated times in Triton-X-100, extracted for acid-soluble material, and counted in a low-background gas-flow counter. Each point represents the corrected average counts per minute per nucleus of a total of 12 or more nuclei. *Solid circles*—control (no actinomycin D); *open circles*—experimental (1 mg/ml actinomycin D added).

no RNA synthesis took place between mitosis and the time of fixation at 3–4 hr of interphase.

Mitosis in *A. proteus* takes about 30 min; during the last quarter of this time, nucleolar reformation begins (Roth et al., 1960). Light microscope preparations of untreated, synchronized amebae fixed at 3–3.5 hr. of interphase display irregularly shaped nuclei that contain richly basophilic nucleoli (Fig. 2). Isolated nuclei from control amebae that have been stained with either azure B bromide or pyronin verify the presence of large amounts of RNA within the organelles.

The nucleoli, which are usually located immediately beneath the nuclear membrane, often appear in a sheet configuration at this stage of interphase. This arrangement of the structures probably is related to the location of the nucleoli by 4–4.5 hr of postmitosis (Stevens, unpublished observations). That is, by 4–4.5 hr of interphase, the nucleoli have pulled away from the nuclear envelope and are observed in the nucleus as a sheet of nucleolar material immediately surrounding the centrally located chromatin. Subsequently, the nucleolar material dissociates into normal-sized nucleoli that are found in their usual, peripheral location at 5–5.5 hr of interphase.

Normal amebae nucleoli have been described as homogeneously granular after osmium tetroxide fixation (Roth et al., 1960; Stevens, 1967); a typical fibrillar region is not readily apparent. However, favorable micrographs of osmium tetroxide–fixed material give one the impression that areas of increased density occupy the predominantly granular nucleoli (Fig. 6); these areas become even more pronounced after fixation of amebae in Karnovsky's fixative (Fig. 7). Resolution of the substructure in these regions is extremely difficult, but it appears that the areas are a tightly interwoven network of fibrils and granules (Fig. 7, insert; see also Flickinger, 1968).

Frequently observed in close association with the nucleoli are (*a*) granular masses that contain particles of varying size and density (Figs. 4 and 5), the largest of which has a characteristic mul-

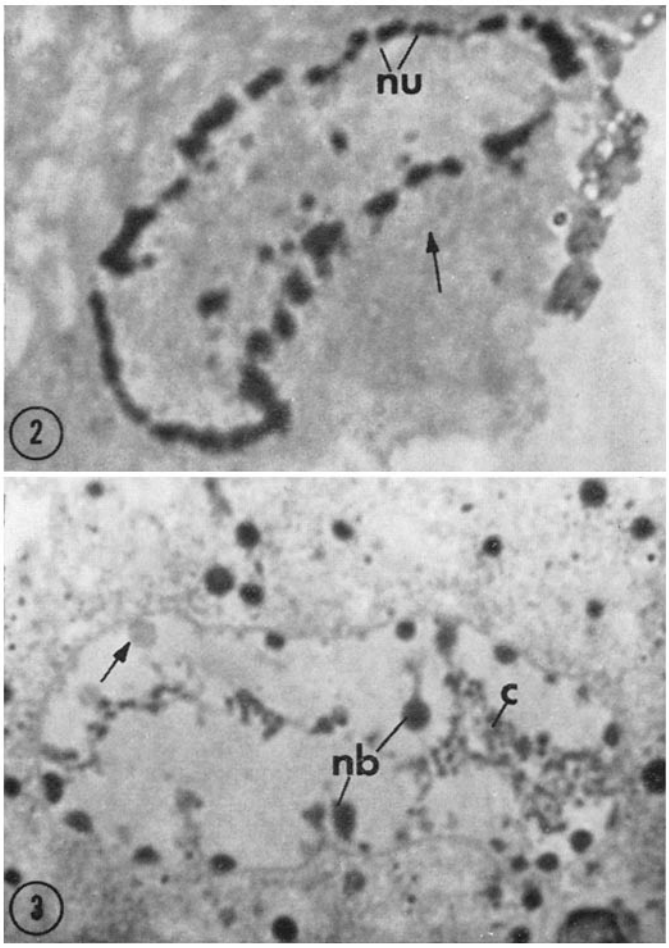



FIGURE 2 Light micrograph of a thick section (ca. 0.3 $\mu$) of *A. proteus* (control sample). Amebae in early cytokinesis were selected manually, allowed to complete division, and processed for electron microscopy at 3.5 hr of interphase (see Materials and Methods). Stain, azure B, pH 9.5–10.0. *Arrow*, nuclear membrane; *nu*, nucleoli. ca. × 2000.

FIGURE 3 Light micrograph of a thick section (ca. 0.5 $\mu$) of *A. proteus* (experimental sample). Amebae were exposed to actinomycin D (1 mg/ml) for 1 hr. Subsequently, cells in early cytokinesis were selected manually, allowed to complete divsion, and prepared for electron microscopy at 3–4 hr of interphase (see Materials and Methods). Stain, azure B, pH 9.5–10.0. *nb*, nucleolus-like bodies; *arrow*, granular mass; *c*, chromatin. ca. × 2000.

berry appearance (Stevens, 1967) and can attain a diameter of up to 1200 A) (Fig. 5); the mulberries are also found separated from other granular material in regions surrounding the nucleoli (Fig. 8); and (*b*) the striking clusters of helices (Figs. 5 and 8) that have been reported in detail in earlier communications (Stevens and Prescott, 1965; Stevens, 1967). In the more central regions of normal nuclei, networks of condensed material are seen (Fig. 4); at higher magnifications, this network appears as strands of finely fibrillar, interconnected material (Fig. 9). Preliminary cytochemical studies indicate that the fibrillar substance is probably DNA (chromatin) (Stevens, 1967). The remaining nucleoplasm is filled with loosely distributed granules and fibrils.

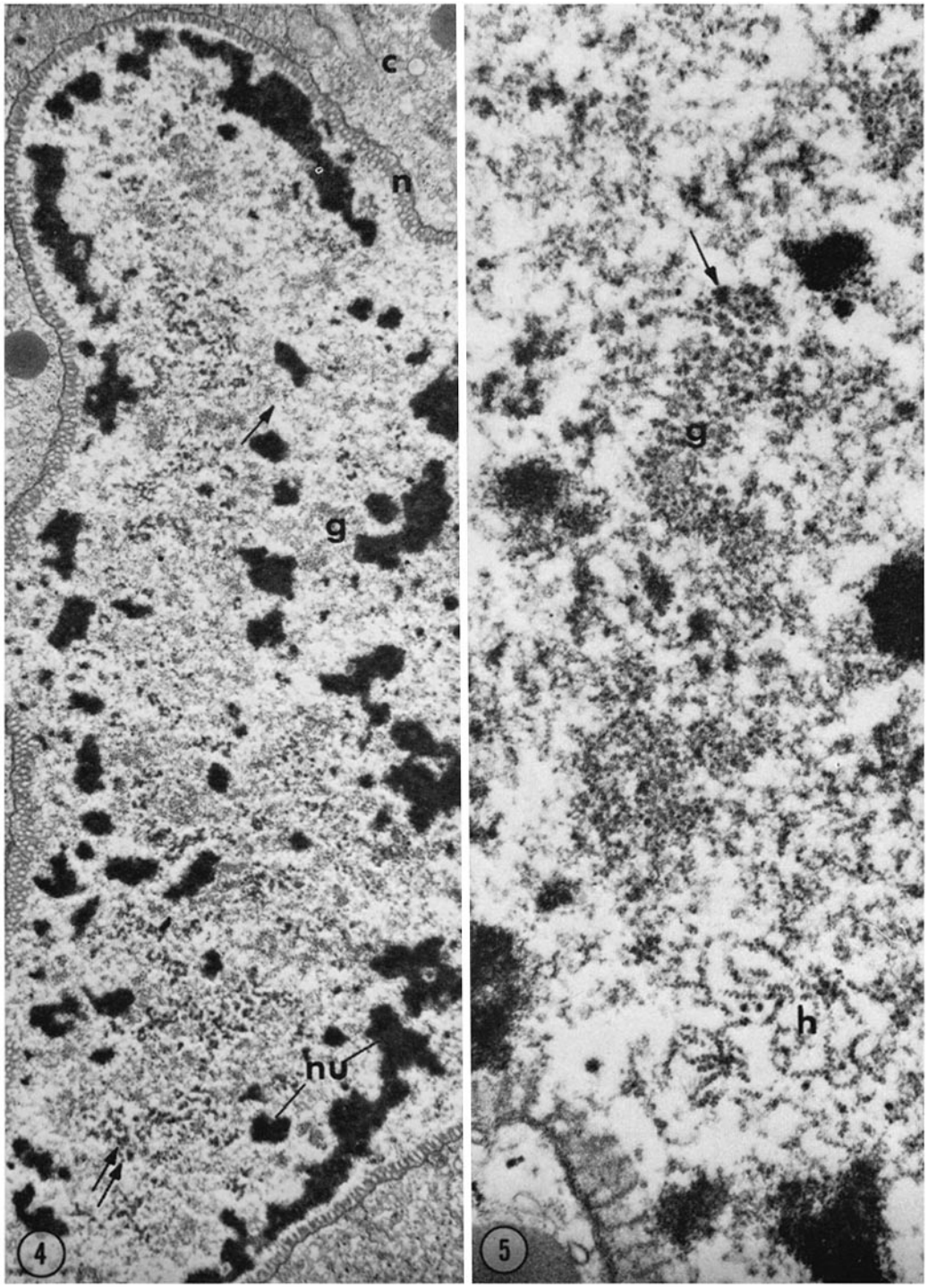



FIGURE 4 Electron micrograph of a section of control amebae (see Fig. 2 for details). The nucleoli (*nu*) of amebae are usually peripherally located in the nucleus, but at 3.5 hr of interphase some nucleoli have begun to penetrate the interior regions of the nucleus (see Results). The granular masses (*g*) and helices (single *arrow*) are frequently found in close proximity to nucleoli. The dense networks (double *arrows*) spanning the more central regions of the nucleus represent chromatin. *n*, nuclear membrane with associated honeycomb regions; *c*, cytoplasm. ca. × 5500.

FIGURE 5 High-magnification electron micrograph of a different area of the same nucleus as was seen in Fig. 4. The helices (*h*) and granular masses (*g*) with associated mulberries (*arrow*) are present in regions surrounding the nucleoli. ca. × 25,000.

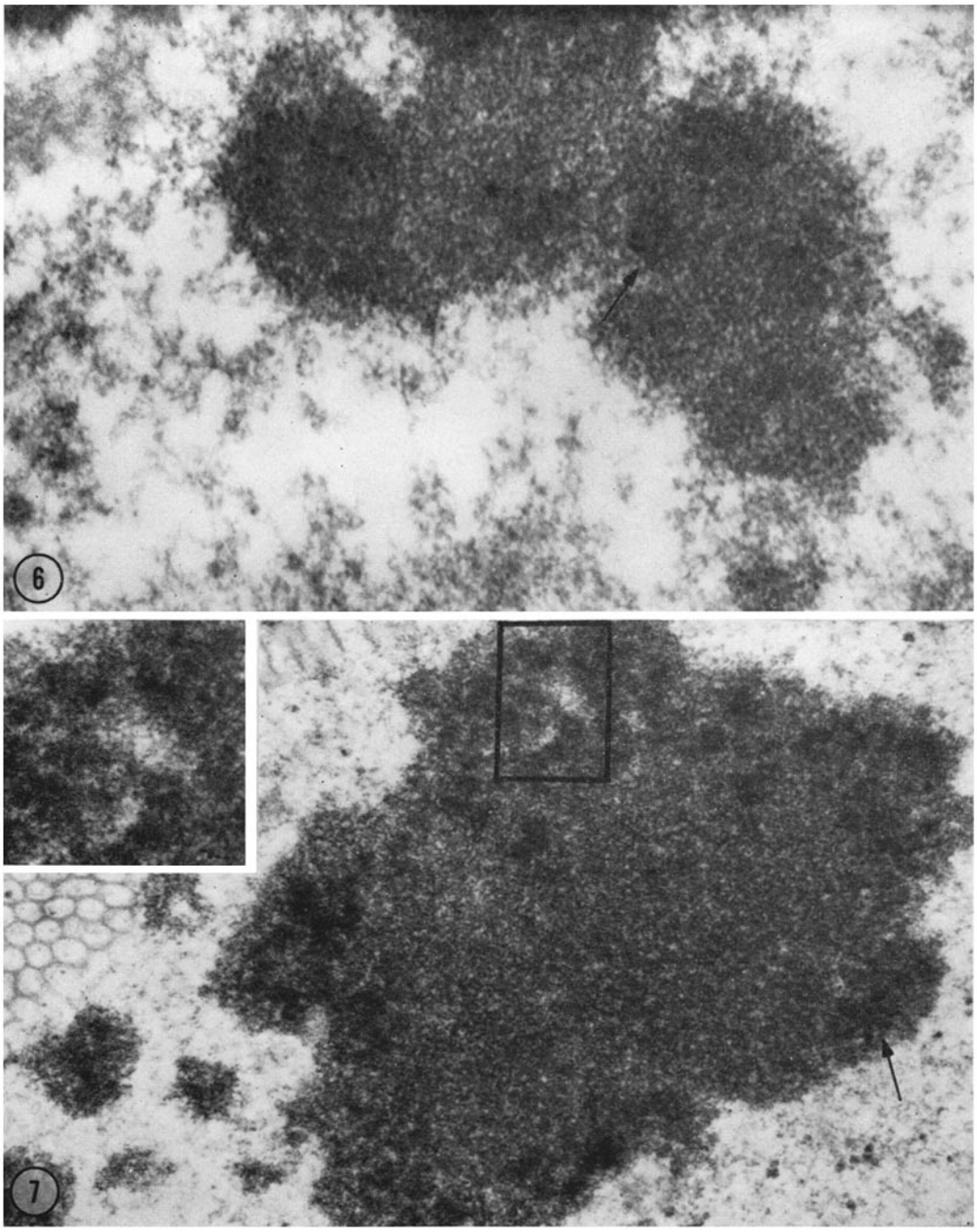
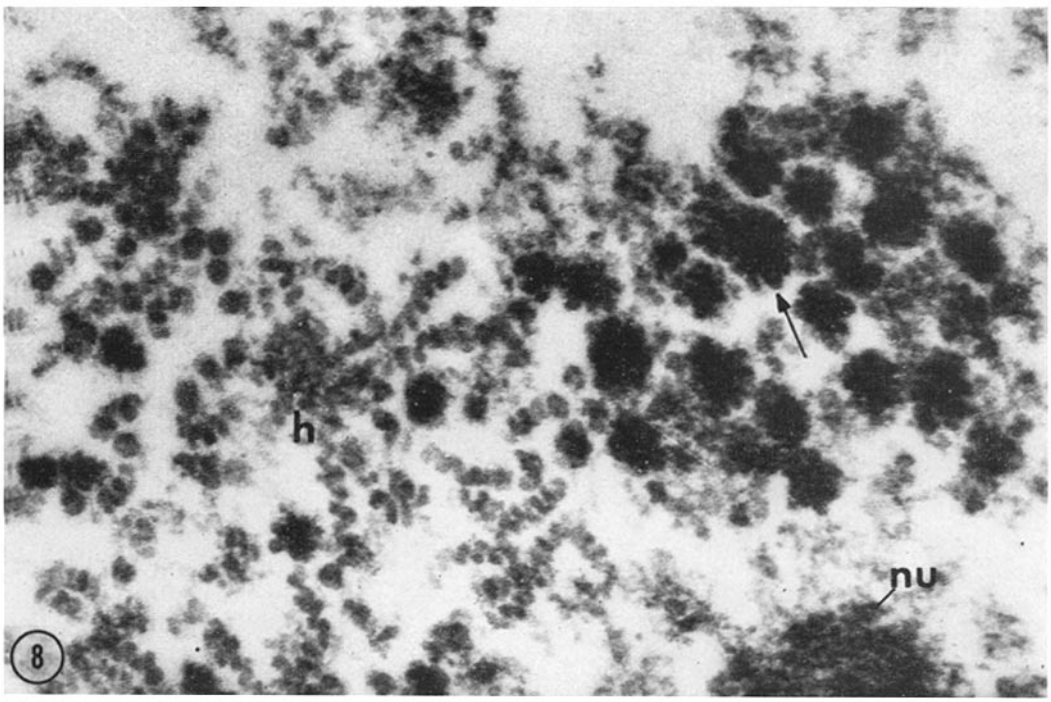



FIGURE 6 High-magnification electron micrograph of a nucleolus of osmium-fixed, control amebae. Note regions of increased density (*arrow*) within the predominantly granular nucleolus. × 44,000.

FIGURE 7 High-magnification electron micrograph of a nucleolus of Karnovsky's-fixed, control amebae. Densely staining areas (*arrow*) are obvious in the granular matrix of the nucleolus. *Insert* shows a magnified view of the densely staining region indicated in the Karnovsky's fixed nuceolus. ca. × 27,000; *Insert*, × 41,000.



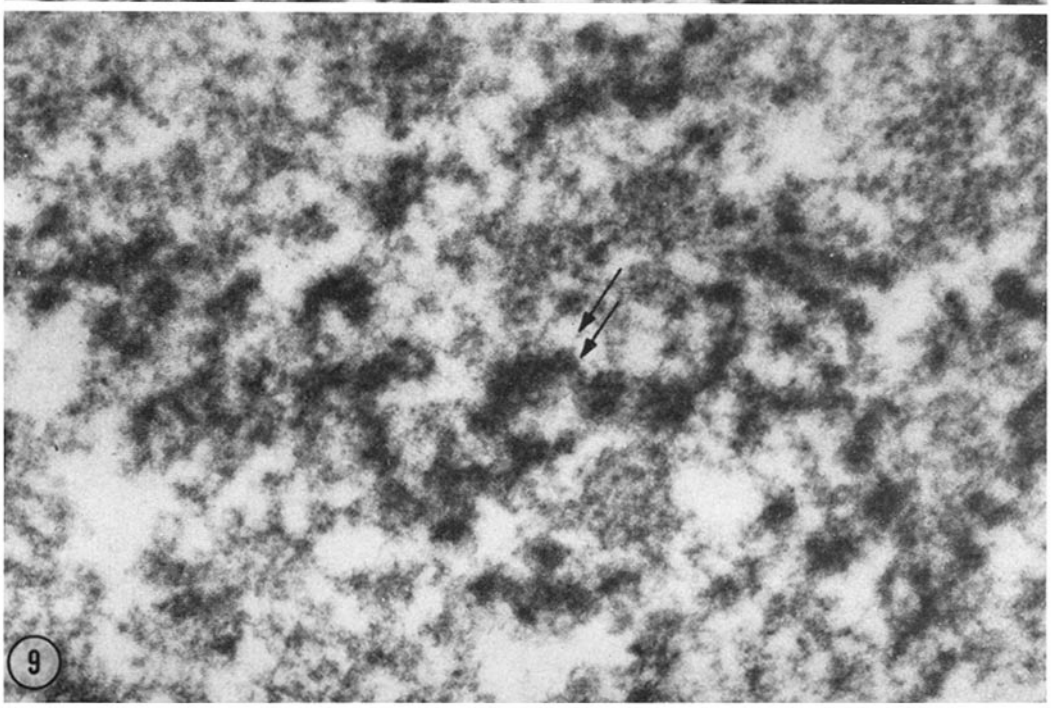




FIGURE 8 High-magnification electron micrograph of a section of control sample (see Fig. 2 for details) showing mulberries (*arrow*) (independent of granular mass) in close association with helices (*h*); *nu*, portion of a nucleolus. ca. × 76,000.

FIGURE 9 High-magnification electron micrograph of a section of control sample (see Fig. 2 for details) showing condensed chromatin networks (double arrows). The chromatin appears as electron-opaque masses of fibrillar material. ca. × 60,000.

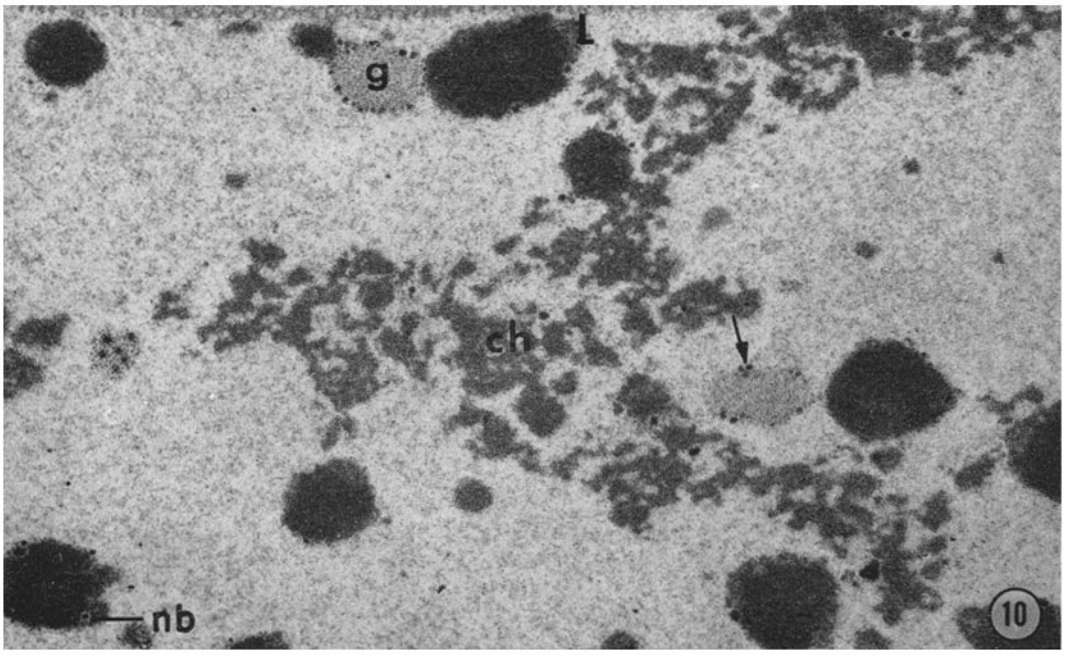



FIGURE 10 Electron micrograph of a section of experimental sample (see Fig. 3 for details). Nucleolus-like bodies (*nb*) are present in the reformed nucleus and appear as dense, fibrous structures that have loosely associated material (*L*). The granular masses (*g*) are obvious in the reformed nucleus and often are found closely applied to the nucleolus-like bodies. Mulberry-like structures (*arrow*) are found associated with the granular masses, the nucleolus-like bodies, and material that is tentatively defined as chromatin (*ch*). The latter is usually displaced toward one side of the nucleus in the experimental sample. ca. × 9500.

Light microscope examination of sections of amebae treated with actinomycin D prior to and during mitosis and during early interphase shows the effects of the antibiotic on nucleolar reformation (Fig. 3). The nucleolar material is reduced in amount and does not occur in the sheetlike configuration seen in control nuclei. Instead, structures that stain intensely with alkaline azure B in sectioned material, or with acidic azure B bromide or pyronin in isolated nuclei, are observed. Because of their staining properties these structures will hereafter be referred to as nucleolus-like bodies. The more lightly staining areas (Fig. 3) in sections of the actinomycin D–treated amebae are not found in the control nuclei but, as pointed out below, they probably represent altered granular masses. Material that is tentatively identified as chromatin (Fig. 3) appears highly condensed and displaced toward the side of the nucleus.

The nucleolus-like bodies in amebae, prepared in either Karnovsky's or osmium tetroxide fixative, are composed primarily of dense, fibrous material; there is no discernible granularity in these structures (Figs. 10, 11, and 13). In favorable micrographs of the osmium tetroxide–fixed material, however, very slender fibrils ($< 100$ A in diameter) can occasionally be resolved (Fig. 11). The outer limits of the nucleolus-like bodies exhibit caps of loosely organized material of somewhat lower electron opacity (Figs. 10–12). This material bears a striking similarity to the exaggerated, displaced masses that were identified at the light microscope level as chromatin (Figs. 10 and 12). At higher magnifications, both the loosely associated nucleolar material and the chromatin appear to have a finely fibrillar substructure (Fig. 12).

The very dense, finely particulate substance overlying the nucleolar and the chromatin material in the control (Fig. 6) and the experimental (osmium tetroxide–fixed) cells (Figs. 11–14) is observed consistently in treated and untreated material. This substance (which is noted in both stained and unstained preparations) was initially thought to correspond to the "foamy particles" of

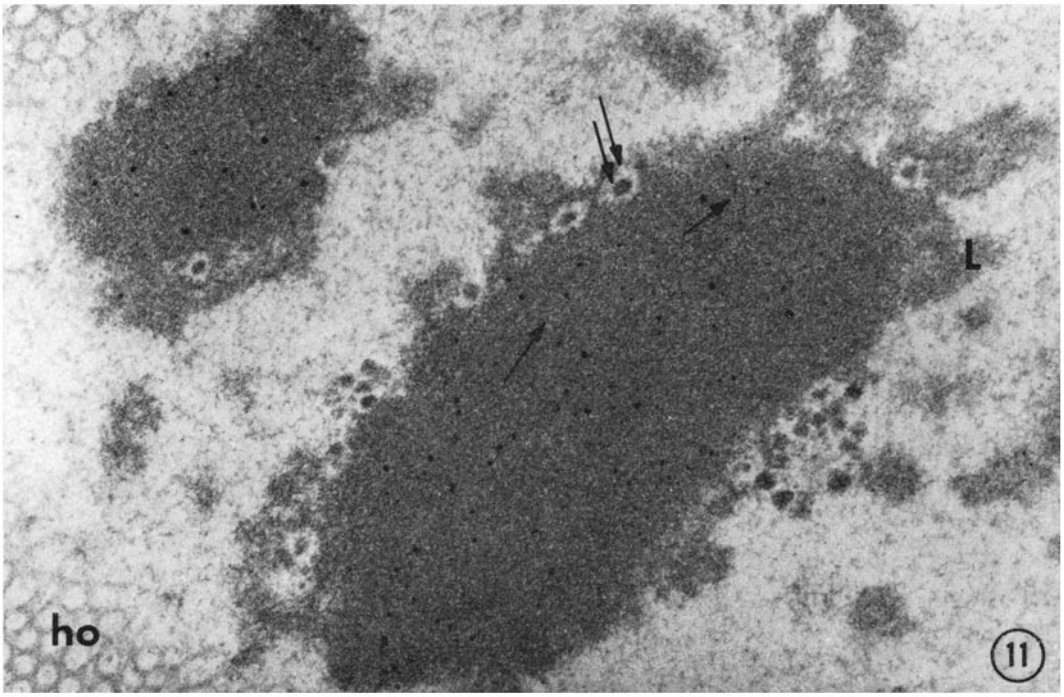



FIGURE 11 High-magnification electron micrograph of a section of experimental sample (see Fig. 3 for details) showing the nucleolus-like bodies in the reformed nucleus. The bodies are primarily composed of dense, fibrous material in which very slender fibrils (<100 A in diameter) can occasionally be resolved (single *arrows*). The mulberry-like structures (double *arrows*) appear to be segregating out of the nucleolus-like bodies. The loosely associated material (*L*) seen at the periphery of the nucleolar structures appears at this magnification to be finely fibrillar. *ho*, honeycomb region of nuclear membrane. ca. × 25,000.

Cohen (1957). However, the location of the substance over the chromatin and the absence of the material when amebae are preserved with Karnovsky's fixative (Fig. 7) may indicate that it represents an artifact of fixation.

The lightly staining areas resolved in thick sections of the actinomycin D–treated amebae appear, at the level of the electron microscope, to be granular masses that have been altered as a result of the antibiotic (Figs. 10, 12, and 13). The granular masses have a lowered electron opacity, and the particles composing the areas are more uniform in size. Moreover, the masses are more clearly delineated in nuclei of the treated amebae than in the control nuclei, and frequently are contiguous with the nucleolus-like bodies (Figs. 10 and 13). In addition to the small, lightly staining particles composing the major portion of the granular areas, the most prominent components of the granular masses are large, dense, spherical structures that range from 300 to 1200 A in diameter (Figs. 10–14). These structures are most often arranged as a peripheral ring outlining the granular areas (Figs. 10 and 13), but are also present within and at the periphery of the nucleolus-like bodies (Figs. 10, 11, and 13), and dispersed throughout the chromatin material (Figs. 10 and 14). Although the spherical structures are somewhat abnormal in appearance, we must tentatively conclude that they represent the mulberries observed in nuclei of untreated amebae.

As reported previously (Stevens, 1967) helices, are not observed in actinomycin D–treated amebae. The remaining nucleoplasm is sparsely filled by homogeneous material of low electron opacity (Fig. 10). The nuclear envelope with its associated honeycomb region is present in the treated cells; however, it may be abnormal in composition, since the morphology of this organelle is not as sharply defined in the experimental cells as it is in the control nuclei.

## DISCUSSION

The nucleoli of *A. proteus* are considered to be functionally analogous to the nucleoli of higher cell types (Stevens, 1967; Flickinger, 1968), although morphological dissimilarities may exist. The ameba nucleoli are predominantly granular; the fibrillar and granular regions typical of most animal cell nucleoli are not easily identified in the ameba nucleoli. However, the morphology of the nucleolus in amebae fixed in Karnovsky's fixative indicates that the fibrillar areas may be tightly packaged within the granular material and therefore not easily resolved (Fig. 7; see also Flickinger, 1968). The morphological alterations of the nucleoli after treatment of interphase amebae with high concentrations of actinomycin D support this view. After exposure to the drug for long periods of time, ameba nucleoli segregate into fibrillar, granular, and amorphous regions (Flickinger, 1968). Similar responses have been noted in nucleoli after treatment of higher cell types with actinomycin D (Jézéquel and Bernhard, 1964; Schoefl, 1964; Stenram, 1965; Bernhard, 1966; Heine et al., 1966).

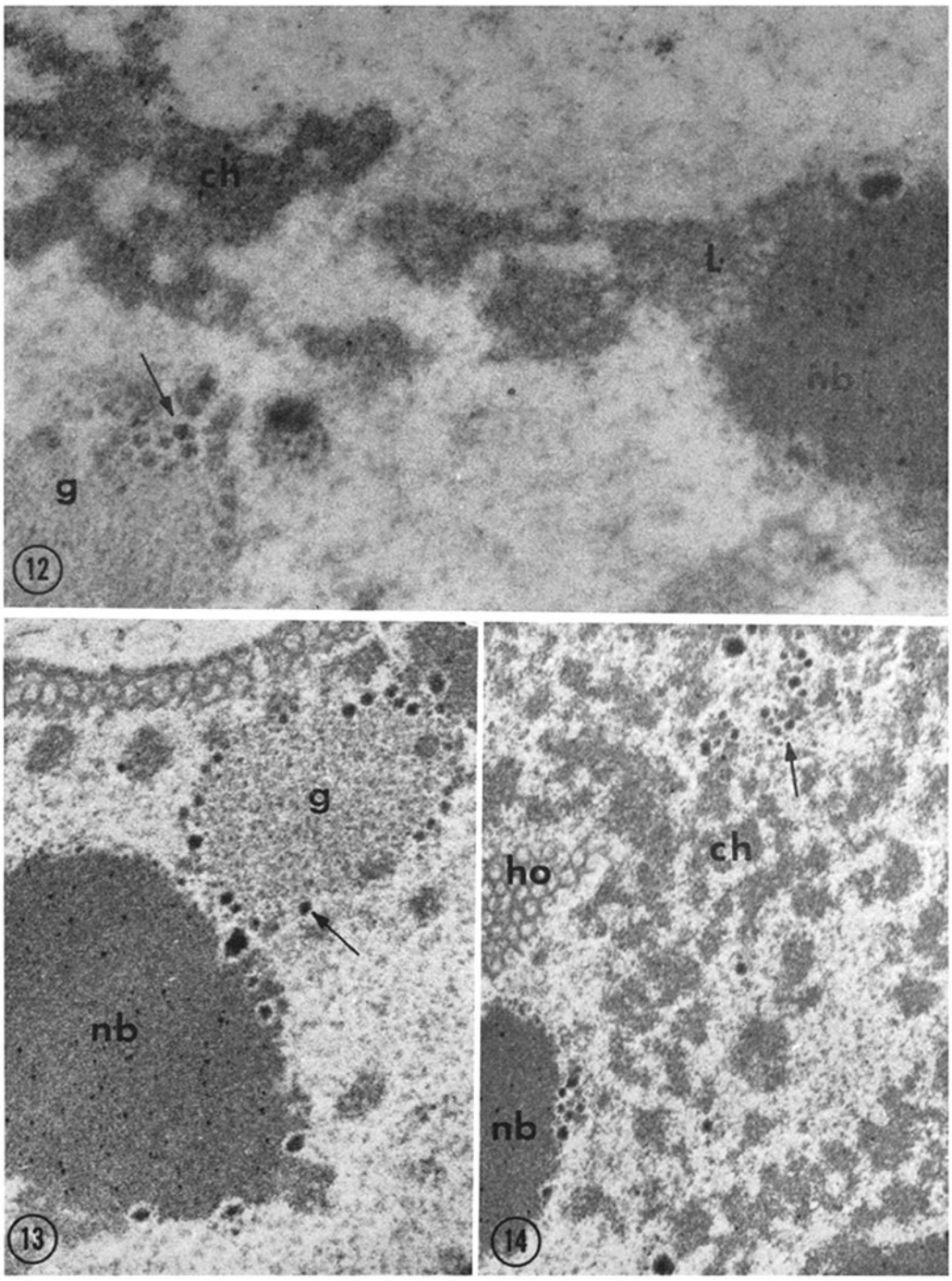
Treatment of dividing amebae with actinomycin D as described in this paper does not totally inhibit formation of structures that can be identified as nucleoli. These nucleolus-like bodies consist mainly of highly condensed, fibrous material in which a granular region is not clearly resolvable; in its texture the substance resembles the fibrous component that becomes apparent in nucleoli after treatment of logarithmically growing amebae with lower concentrations of actinomycin D (Flickinger, 1968). In the present study, the fibrous matrix of the nucleolus-like structures may reflect the fibrillar material of the dense regions observed in Karnovsky's-fixed, untreated amebae (Fig. 7); however, due to the difficulty in resolving these regions, even in very thin sections, this cannot be stated with certainty. It is interesting to note, though, that nucleolus-like bodies similar to those described in this report have been observed in nuclei that lack the nucleolus organizer regions of the chromosome (Jones, 1965; Swift and Stevens, 1966), and also in nuclei of developing *Xenopus* embryos treated with actinomycin D (Sameshima et al., 1970).

The inhibition of RNA synthesis by actinomycin D during mitosis in HeLa cells has led Hodge et al. (1969) to conclude recently that post-mitotic protein synthesis is not dependent on newly synthesized RNA. In that same study the authors also mention that micronucleoli are present in the reformed nuclei of the treated cells. Since post-mitotic protein synthesis may not require resumption of transcription at the end of mitosis, much of the material composing the reformed nucleolus-like structures seen in this study almost certainly reflects reorganized nucleolar protein synthesized pre- and/or postmitotically. This theory receives support from the observation that the structures stain intensely in sectioned material with basic solutions of azure B bromide. Azure B is a specific stain for nucleic acids at acidic pH's, but proteins can also bind the dye at basic pH's (Flax and Himes, 1952). That the reformed structures in the

FIGURE 12 High-magnification electron micrograph of a section of experimental sample (see Fig. 3 for details). Note the similarity between the loosely associated nucleolar material (*L*) and that which has been tentatively defined as chromatin (*ch*). Both appear finely fibrillar in texture. The granular mass (*g*) and the nucleolus-like body (*nb*) have associated mulberry-like structures (*arrow*). The dense, finely particulate substance overlying the chromatin and nucleolus-like body may be an artifact of fixation (see Results). ca. × 30,000.

FIGURE 13 High-magnification electron micrograph of a section of experimental sample (see Fig. 3 for details). This micrograph demonstrates the close association of the granular mass (*g*) with the nucleolus-like bodies (*nb*). The mulberry-like structures (*arrow*) often appear radially arranged in a granular mass and are frequently observed at the periphery of the nucleolus-like bodies. ca. × 23,000.

FIGURE 14 Electron micrograph of a section of experimental sample (see Fig. 3 for details). This micrograph shows the displacement of the fibrillar material tentatively defined as chromatin (*ch*) and the presence of mulberry-like structures (*arrow*) in the chromatin masses. *nb*, nucleolus-like body; *ho*, honeycomb region of nuclear membrane. ca. × 11,000.


ch
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actinomycin D–treated amebae must also contain RNA is evidenced by their affinity for stains specific for RNA (i.e., acidic azure B bromide and pyronin). This RNA may reflect residual predivision synthesized RNA that has returned to the nucleus (Rao and Prescott, 1967).

The type of RNA contained within these reformed nucleolus-like bodies is not known, but it is interesting to note here that the alteration of nucleoli to completely fibrillar structures can be achieved in cells grown at elevated temperatures (Simard and Bernhard, 1967; Simard et al., 1969). Biochemical analyses of cells cultured at the higher temperature indicate a partial inhibition of synthesis of ribosomal precursor RNA(45S), and complete inhibition of maturation of ribosomal RNA (Warocquier and Scherrer, 1969).

It may be argued that the fibrillar material, which is loosely associated with the nucleolus-like bodies, actually represents a nucleolar component; however, we feel that this fibrillar material actually reflects nucleolus-associated chromatin, since it seems to be originating from the finely fibrillar, chromatin material in adjacent areas (see Figs. 10, 12, and 14).

In conclusion, the evidence obtained in the present study indicates that reformation of nucleolus-like structures is not totally dependent upon postmitotic RNA synthesis. The structures formed most probably represent accumulations of nucleolar protein and predivision synthesized RNA. The results also reaffirm the previous conclusion that the helices of *A. proteus* represent RNA-containing structures (Stevens, 1967).

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