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ULTRASTRUCTURAL AND RADIOAUTOGRAPHIC INVESTIGATION OF THE NUCLEOLAR CYCLE IN *PHYSARUM POLYCEPHALUM*. CHARACTERIZATION OF DNA-CONTAINING SUBUNITS

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SUMMARY

The present study has been mainly focused on the nucleolar cycle in the slime mould *Physarum polycephalum*. The ultrastructural characteristics of the interphase nucleolus, in this species, are quite similar to those of nucleoli in other organisms: it is essentially constituted of large particulate zones surrounding denser regions which are predominantly fibrillar in texture. The latter nucleolar zones, following fixation with osmium tetroxide, are characterized by the presence of opaque granules approximately 25 nm in diameter. Contrary to the situation which generally prevails in other eukaryotes, the late prophase nucleolus fragments into numerous globular bodies which are recognizable by the presence of opaque particles. These fibrillo-granular nucleolar fragments persist during mitosis and are observed to become incorporated in the newly formed nucleolus. High-resolution radioautographic observations reveal that these nucleolar remnants contain DNA. The present observations together with recent biochemical data from other authors on the characteristics and mode of duplication of nucleolar DNA in *P. polycephalum* have led us to the hypothesis that the nucleolus, in this organism, contains several distinct globular subunits each containing ribosomal DNA as a key component. The existence of such morphological subunits appears to account for the unusual behaviour of the nucleolus during the cell cycle.

INTRODUCTION

In most higher organisms, the nucleolus undergoes complete disorganization at the end of prophase and reforms during the ensuing interphase. Although cytochemical (Jacobson & Webb, 1952; LaCour & Chayen, 1958; Kaufmann, McDonald & Gay, 1948), radioautographic (Feinendegen & Bond, 1963) and ultrastructural observations (Papsidero & Braselton, 1973) have indicated that part of the nucleolar material may persist in the form of a coating on the mitotic chromosomes, no definite evidence has appeared, so far, to the effect that this substance is subsequently integrated within the reforming nucleolar body. In cases where the persisting nucleolar material appears as more conspicuous remnant bodies which accompany the chromosomes to the cell poles (Brinkley, 1965; Hsu, Arrighi & Klevecz, 1965), electron-microscopic observations have revealed that portions of this substance may be incorporated within the telophase nucleus. According to more recent ultrastructural investigations on

growing oocytes (Chouinard, 1971) and animal cells in culture (Goessens & Lepoint, 1974), it would appear that part of this so called persisting nucleolar material corresponds, in fact, to the nucleolar organizing segment of the nucleolar chromosomes.

A particularly interesting example of persisting nucleolar material has been observed during the division stages in macroplasmodia of the slime mould, *Physarum polycephalum*, where all mitotic nuclei, up to early interphase, exhibit several such remnants (Guttes, Guttes & Ellis, 1968). This study presents a detailed account of the structural evolution of nucleolar material in this organism and also furnishes radioautographic evidence for the presence of DNA within the remnants observed during the mitotic stages.

MATERIALS AND METHODS

The CL and M_3C strains of *Physarum polycephalum* were used for the present study. Microplasmodia of this organism were maintained in a semi-defined medium as described by Daniel & Baldwin (1964) and the surface cultures were obtained by fusion of these cells on Millipore filter paper (Guttes & Guttes, 1964). In order to identify the different stages of the cell cycle, small samples were excised from the surface cultures, smeared on a slide with a coverslip and then fixed for a few seconds in 4% glutaraldehyde adjusted to pH 7·2 with 0·1 M cacodylate buffer and examined under phase-contrast optics. For ultrastructural studies, samples were fixed in 1% osmium tetroxide in 0·1 M cacodylate buffer, pH 7·2, and embedded in a mixture of Epon-Araldite according to current procedures. Ultrathin sections were stained with uranyl acetate and lead citrate and observed with a Philips EM 300 electron microscope provided with an anti-contamination device.

For labelling purposes the whole surface culture or parts of it were transferred to a nutrient medium containing [6-^{3}H]thymidine (Schwarz-Mann, Orangeburg, N.Y.). This precursor was used at a concentration of 20 μCi/ml (sp. act. 8 Ci/mmol). Ultrathin sections for radioautography were deposited on collodion-coated slides and dipped in a solution of Ilford L4 emulsion diluted 1:1. These preparations were developed by the gold latensification-Elon ascorbic acid method (Wisse & Tates, 1968).

RESULTS

Electron-microscopic observations

The mature interphase nucleus consists of numerous, small, compact chromatin masses scattered within a fine, fibrillo-granular, nucleoplasmic material and of a single nucleolus generally occupying the central portion of the nuclear cavity (Fig. 1). This nucleolus is essentially constituted of large, irregular, particulate zones surrounding

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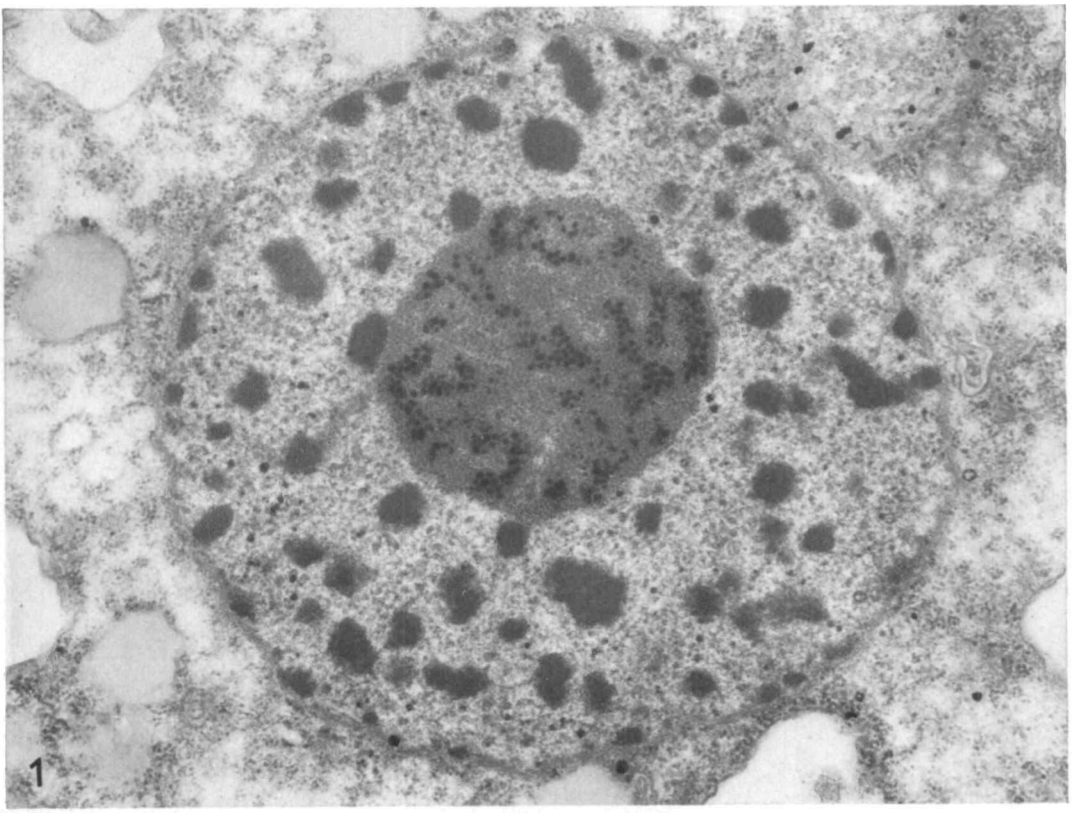
Fig. 1. Mature interphase nucleus of *P. polycephalum* characterized by the presence of numerous, compact chromatin masses, or chromocentres, scattered within a fine, fibrillo-granular, nucleoplasmic material and of a single nucleolus occupying the central portion of the nuclear cavity. The fibrillar zones of the nucleolus exhibit opaque particles which are grouped into thread-like arrays. ×42000.

Fig. 2. Early prophase nucleus of *P. polycephalum*. The chromocentres have partly unravelled and the chromatin as a whole appears less structured than at late interphase. The nucleolus has migrated towards the outer portion of the nuclear cavity and now shows a looser organization. As a result, numerous, small whorl-like structures containing opaque granules may be seen scattered throughout the nucleolar mass. ×35000.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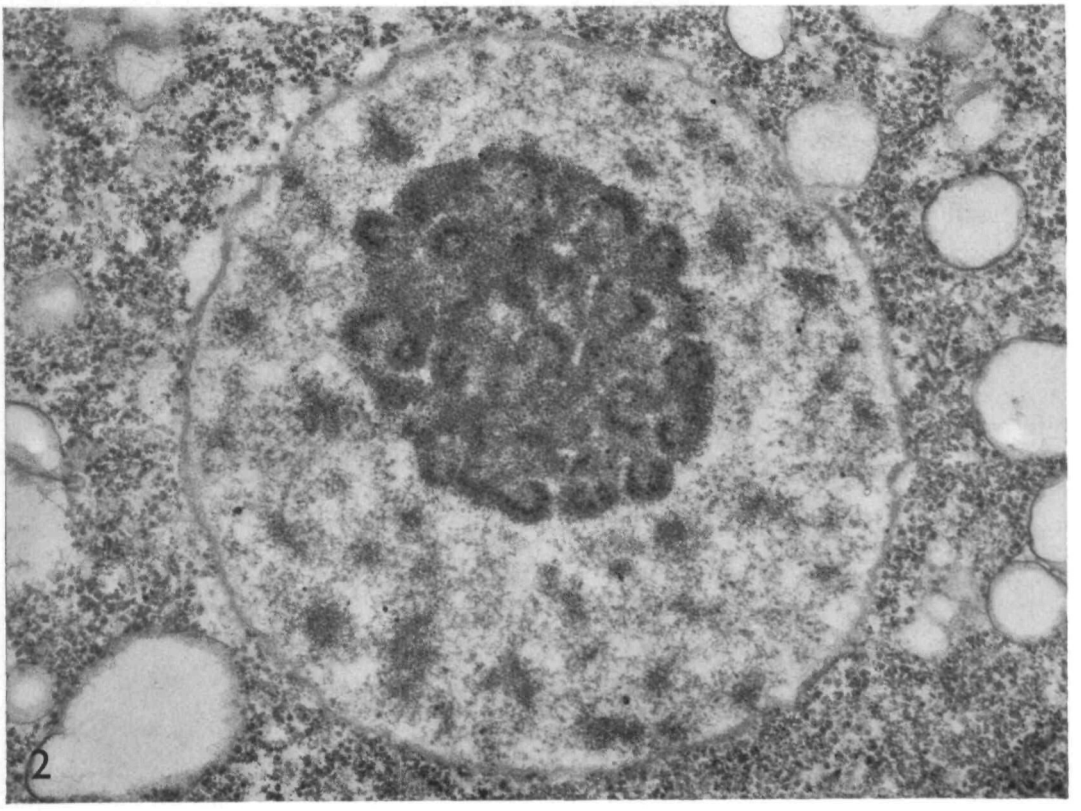
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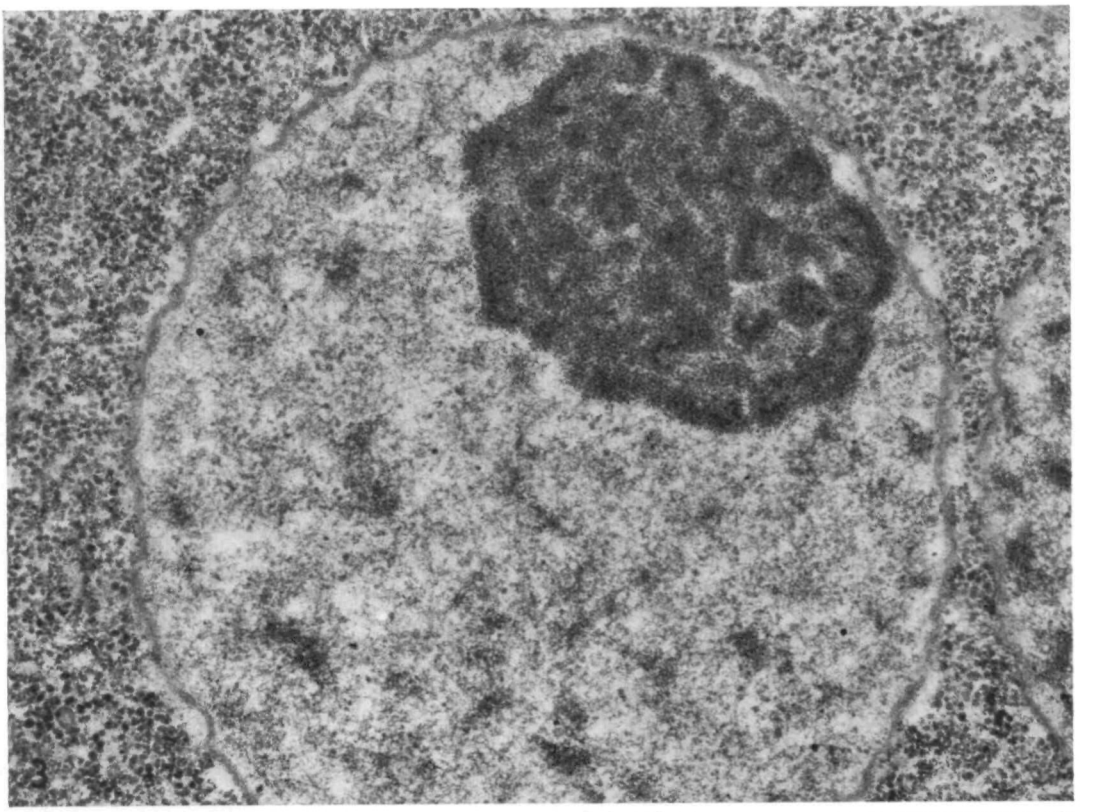
denser regions which are predominantly fibrillar in texture. Following fixation with osmium tetroxide only, the latter zones are further characterized by the presence of opaque granules approximately 25 nm in diameter which confer to the nucleolus a most heteregeneous appearance. Such particles are grouped into complex clusters which seem to reflect the presence of underlying thread-like structural elements.

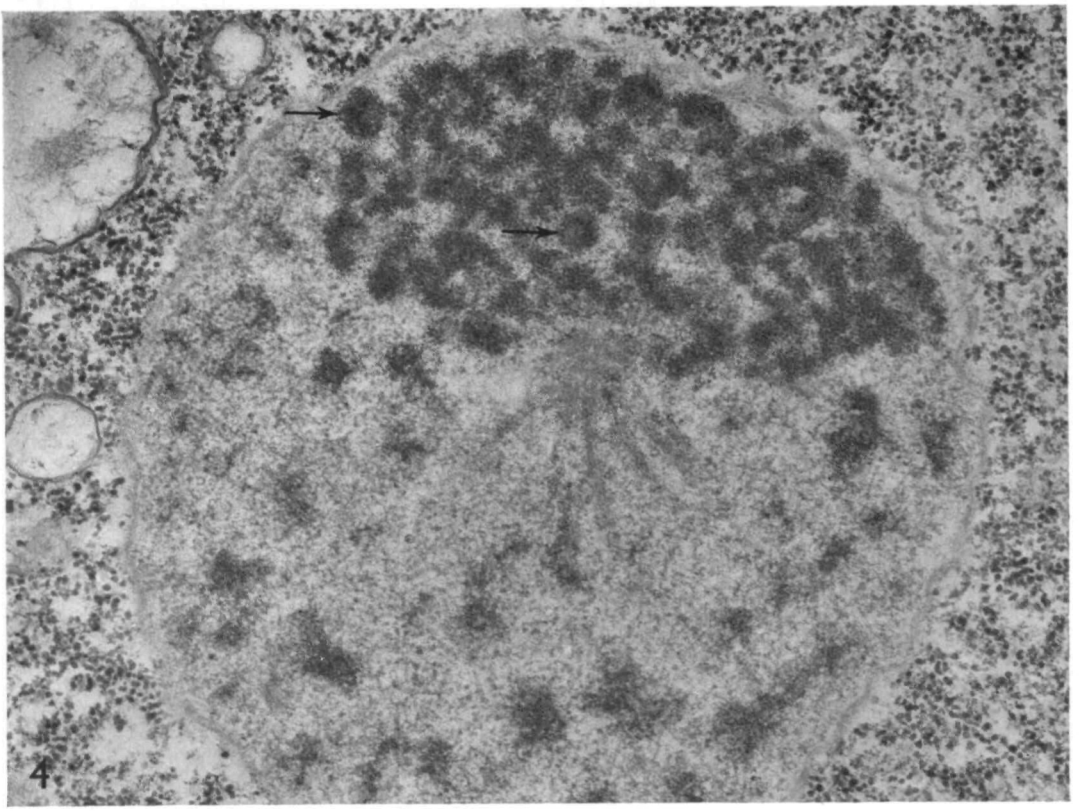
During the first half of prophase the chromosomes start condensing and, in ultrathin preparations, appear as slender irregular chromatin profiles coursing throughout the nucleoplasm. As prophase progresses, the nucleolus slowly moves towards the outer portion of the nuclear cavity (Guttes *et al.* 1968). During that stage the nucleolus takes on a slightly looser organization and numerous small, distinct, fibrillar zones can be recognized throughout its mass (Fig. 2). The opaque granules characterizing these nucleolar regions are now disposed in single rows in the form of short linear or curved arrays, the latter often appearing as partly opened circles or whorls and, less frequently, as small rings. The smallest of these apparent rings are rather similar in size within a given nucleolus or among equally advanced prophase nuclei and measure 0·1–0·2 μm in diameter. As migration of the nucleolus continues, the opaque granules often decrease in number or become much smaller with the effect that its fibrillar zones are not as clearly delineated as during earlier stages. Nevertheless, the remaining small opaque granules are still often disposed in rings and whorls (Fig. 3). Once the nucleolus has reached the peripheral portion of the nuclear cavity, it disperses partly, the remaining material forming a crescent-shaped mass closely appressed to the envelope (Fig. 4). At that stage the disorganizing nucleolar mass appears to consist mostly of coarse, convoluted, thread-like structures, roughly 0·1 μm in diameter, and showing a fibrillo-granular texture. A number of roundish bodies 0·1-0·2 μm in diameter, and generally containing very small opaque granules, may be recognized throughout this loose aggregate of nucleolar material.

As already reported in earlier publications (Guttes *et al.* 1968; Blessing, 1972), intranuclear microtubules appear in the course of prophase and, later on during that stage, bundles of these structures are seen diverging from the concave region of the nucleolus (Fig. 4).

Fig. 3. Late prophase nucleus of *P. polycephalum*. The nucleolus which has now reached the nuclear envelope still consists of numerous whorl-like and doughnut-like structures surrounded by granular material. The opaque particles are less numerous and smaller with the effect that these latter nucleolar portions are not as clearly delineated as during previous stages. × 34000.

Fig. 4. Prophase nucleus that is slightly more advanced than that of the preceding figure. At this stage, the nucleolus is closely appressed to the nuclear envelope and follows its contour. Part of the granular material has apparently dispersed with the result that the nucleolar mass assumes a much looser organization. The remaining nucleolar material shows a fibrillo-granular texture and consists mostly of convoluted, thread-like structures approximately 0·1 μm in diameter and also of a number of spherical bodies (arrows) about twice as wide. Although these are not evident in the present micrograph, opaque particles are sometimes associated with portions of similarly advanced prophase nucleoli. Bundles of microtubules are seen diverging from the concave region of the disorganizing nucleolus. × 36000.

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At prometaphase, the nucleolar mass disperses completely and gives rise to numerous small bodies, usually still containing opaque granules, which encircle the condensed chromosomes grouped in the central portion of the nuclear cavity (Fig. 5). Most of these nucleolar remnants are more or less globular in shape but a few appear as short filaments.

The metaphase nucleus is characterized by a rather continuous equatorial mass of chromatin within which chromosomes cannot generally be recognized as distinct morphological entities. In appropriate preparations (Fig. 6), denser plaque-like structures, resembling the kinetochores recently described in *Dictyostelium discoideum* (Moens, 1976), are observed within these metaphase chromatin aggregates. At that stage, the microtubules have taken a spindle-shaped arrangement and converge to the envelope on opposite sides of the nuclear cavity. Although these microtubules are sometimes seen to extend to the nuclear envelope, the latter shows no particular structural differentiation at such polar sites of convergence. The distribution of the nucleolar remnants varies greatly at metaphase, partly as a result of the plane of sectioning, but also due to the fact that nuclei differ noticeably in this respect. Clear identification of these persisting nucleolar fragments is possible only in cases where they contain opaque granules. In certain longitudinal sections, these small bodies are mostly scattered within the interchromosomal spindle zone as well as at the poles of the nucleus. In other similarly oriented sections, the majority of the nucleolar remnants are grouped close to, as well as within, the chromosome mass; that many of these bodies are actually distributed throughout the equatorial region in such nuclei is clearly seen from transverse sections or from sufficiently oblique ones.

During anaphase the nucleolar remnants are also frequently observed in the immediate vicinity of the migrating chromosomes, some of them being located within the chromatin mass (Fig. 7). It is also evident from longitudinal sections that certain of these bodies may move to the nuclear poles ahead of, and therefore, independently of the chromosomes. As the chromosomes reach the poles of the nucleus they form a still more continuous chromatin aggregate than before, within which numerous remnant bodies are present (Fig. 8). This chromatin becomes intimately associated with the nuclear envelope which is then sometimes observed to disorganize partly. Since such disorganization is generally noted in cases where part of the spindle has persisted in the polar region of the nucleus, the impression is gained that these

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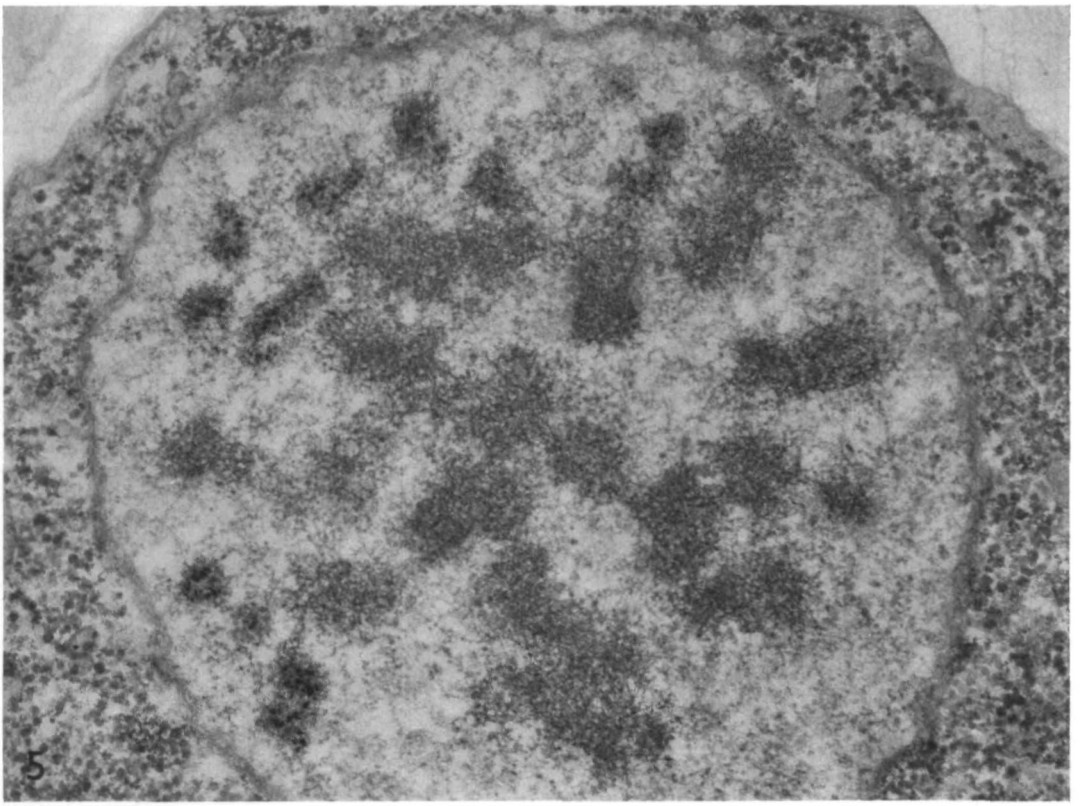
Fig. 5. In this prometaphase nucleus the nucleolar mass has completely dispersed and has given rise to numerous small bodies. Most of these nucleolar remnants are characterized by the presence of opaque granules and encircle the condensed chromosomes grouped in the central portion of the nuclear cavity. × 46000.

Fig. 6. Metaphase nucleus of *P. polycephalum*. The intranuclear spindle is complete and extends to opposite portions of the nuclear envelope. The chromatin forms a rather homogeneous equatorial mass within which chromosomes cannot be recognized as distinct morphological entities. Denser plaque-like structures, or kinetochores, are observed within these metaphase chromatin aggregates. In this section, most of the nuclear remnants are scattered in the immediate vicinity of the spindle zone, between the chromosomes and the poles of the nucleus. × 34000.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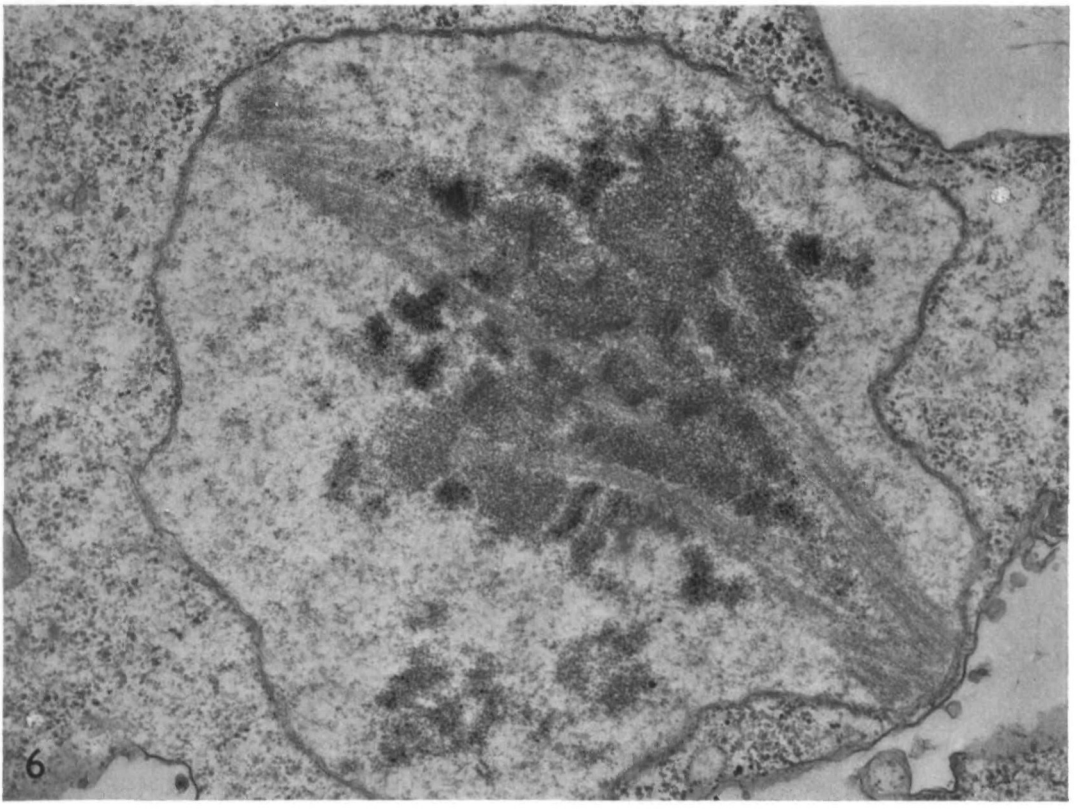
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local breaks result from pressure of the cone of microtubules against the envelope (Fig. 9).

From metaphase to late anaphase the nucleoplasm consists of very loose fibrillar material interspersed with a few granules which resemble those present within the cytoplasm. Once, however, the nuclear envelope partly breaks down at the poles and also becomes very irregular at the equator, the interchromosomal region of the early telophase nucleus shows a much larger concentration of granules presumably originating from the cytoplasm (Fig. 10). Shortly thereafter, the undulating nuclear envelope breaks down in the interzone and is reconstituted very rapidly since samples excised from a plasmodium only 2 min later show daughter nuclei each already delimited by a continuous envelope (Fig. 11). The observation that large fragments of membranes from the equatorial region remain attached to the nuclei as they separate suggests that they are involved in sealing the gap which is created in the proximal region of the daughter nuclei. Apart from a few, small, more transparent nucleoplasmic areas which appear at the time the envelope is reforming, the mid-telophase nucleus consists of a continuous mass of chromatin throughout which are scattered numerous bodies exhibiting opaque granules. These bodies having remained most conspicuous during the whole process of daughter nuclei formation, there is no reason to doubt that they correspond to the nucleolar remnants observed during the mitotic stages. In this respect, it should be noted that no evidence was ever obtained, in the course of the present investigation, to the effect that some of these remnants were not incorporated within the forming daughter nuclei.

During the following 10 min or so, the daughter nuclei increase rapidly in size and undergo extensive changes in appearance (Fig. 12). As the small transparent zones seen earlier increase in size, the continuous chromatin aggregate characterizing early and mid-telophase nuclei evolves into a very coarse reticulum. This relaxation of part of the chromatin is coincident with the onset of DNA duplication as can be demonstrated by high-resolution radioautography (authors' unpublished observations). Such early *S* nuclei still exhibit numerous clusters of opaque granules but it is not possible at this time to determine whether the material underneath possesses a texture different from that of chromatin. By the time the nucleus has become roundish in outline, the dense chromatin observed during the early *S*-period has largely disappeared and has presumably transformed into the diffuse fibrillar material present within the greatly enlarged nucleoplasmic zones (Fig. 13). The complex network

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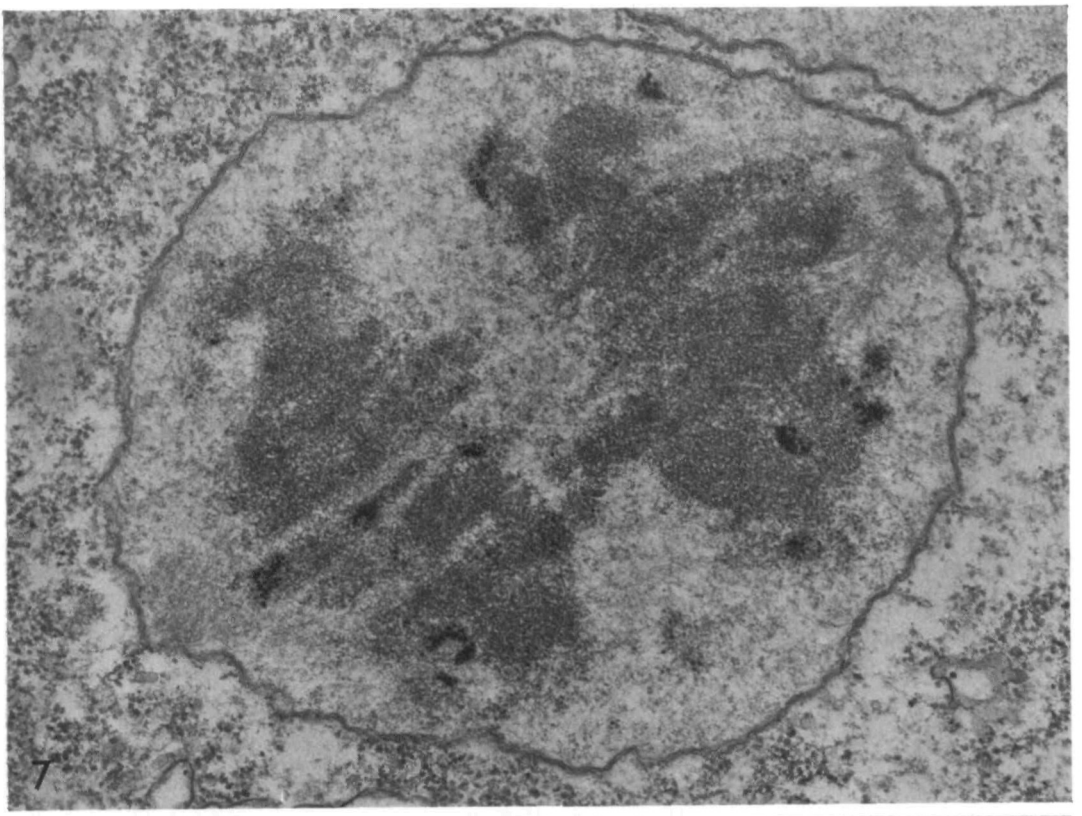
Fig. 7. Anaphase nucleus of *P. polycephalum*. The nuclear envelope remains intact as the chromosomes migrate towards the poles. A number of nucleolar remnants still containing opaque granules are observed at the periphery as well as within the 2 chromatin aggregates. Bundles of microtubules are present between these aggregates and the nuclear envelope. × 34000.

Fig. 8. Early telophase nucleus of *P. polycephalum*. The anaphase chromosomes have reached the poles and they form a still more continuous chromatin aggregate than before within which numerous remnant bodies are present. The chromatin mass has become intimately associated with the nuclear envelope and the latter is seen to disorganize partly in the polar region (arrows). × 35000.



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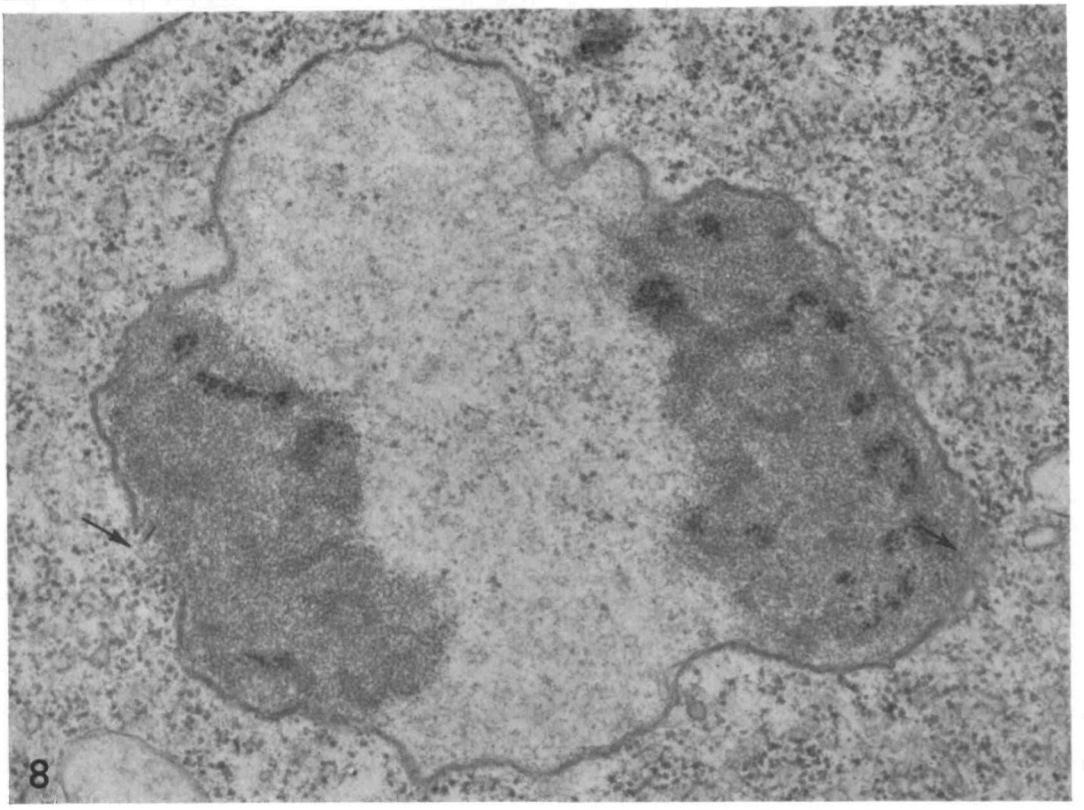

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observed in these early interphase nuclei exhibits a more granular texture than that of the chromatin reticulum present in younger nuclei, is further characterized by arrays of opaque particles and, to all appearances, consists partly of non-chromatin material. Subsequent growth of the interphase nucleus is accompanied by an apparent gradual coalescence of this fibrillo-granular material into a number of irregular nucleolar bodies all of which contain clusters of opaque particles. Thin granular zones eventually form at the periphery of these small bodies. Upon fusion of these bodies into one or two masses, the granular zones merge into larger ones and the resulting nucleoli then exhibit an organization indistinguishable from that of the mature organelles (Fig. 14).

Throughout interphase the regions containing opaque particles remain sufficiently distinct for their number to be evaluated. Interestingly enough, these regions gradually increase in number from telophase to the late G_2-period, at which time approximately twice as many are observed (Fig. 15, p. 40). The values reported in this figure show that this increase is statistically significant (slope $= 0{\cdot}93$, $t = 17{\cdot}06$ for 204 d.f.).

Radioautographic observations

In view of the persistence, during metaphase and anaphase, of numerous bodies having certain morphological features in common with the fibrillar regions of the mature nucleolus and of their apparent incorporation within the forming telophase nucleolus, these remnants were further characterized by high-resolution radioautography using tritiated thymidine as a precursor. Taking advantage of the fact that part of nucleolar DNA is known to replicate during the G_2-period (Guttes & Guttes, 1969; Zellweger, Ryser & Braun, 1972; Newlon, Sonenshein & Holt, 1973), plasmodia were exposed to this precursor during late interphase so as to label specifically the nucleolus and samples were excised and processed for radioautography at various times, later on, during the cell cycle. These studies have revealed that the labelling observed within late interphase nucleoli is restricted to its non-granular zones. This specific labelling of the coarse, filamentous nucleolar component persists up to late prophase (Fig. 16), at which stage part of the granular material has already dispersed. At prometaphase, the radioautographic grains are largely found at the periphery of the nuclear cavity where nucleolar remnants also tend to be distributed (Fig. 17). Although

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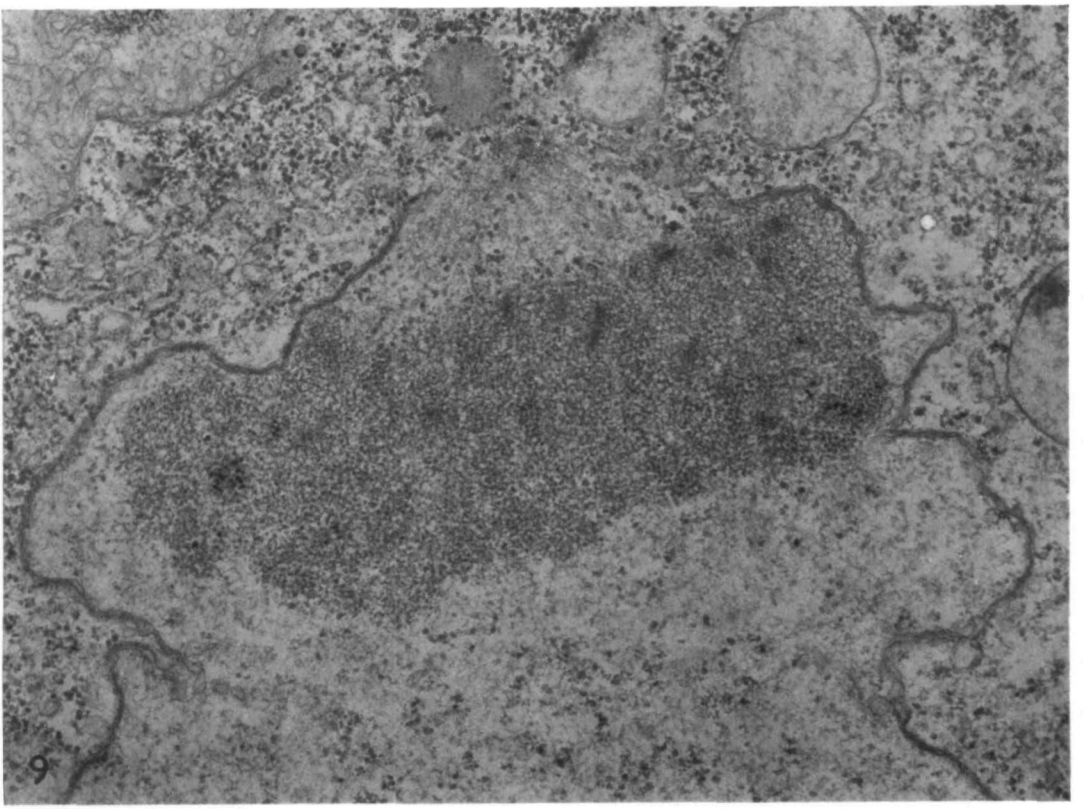
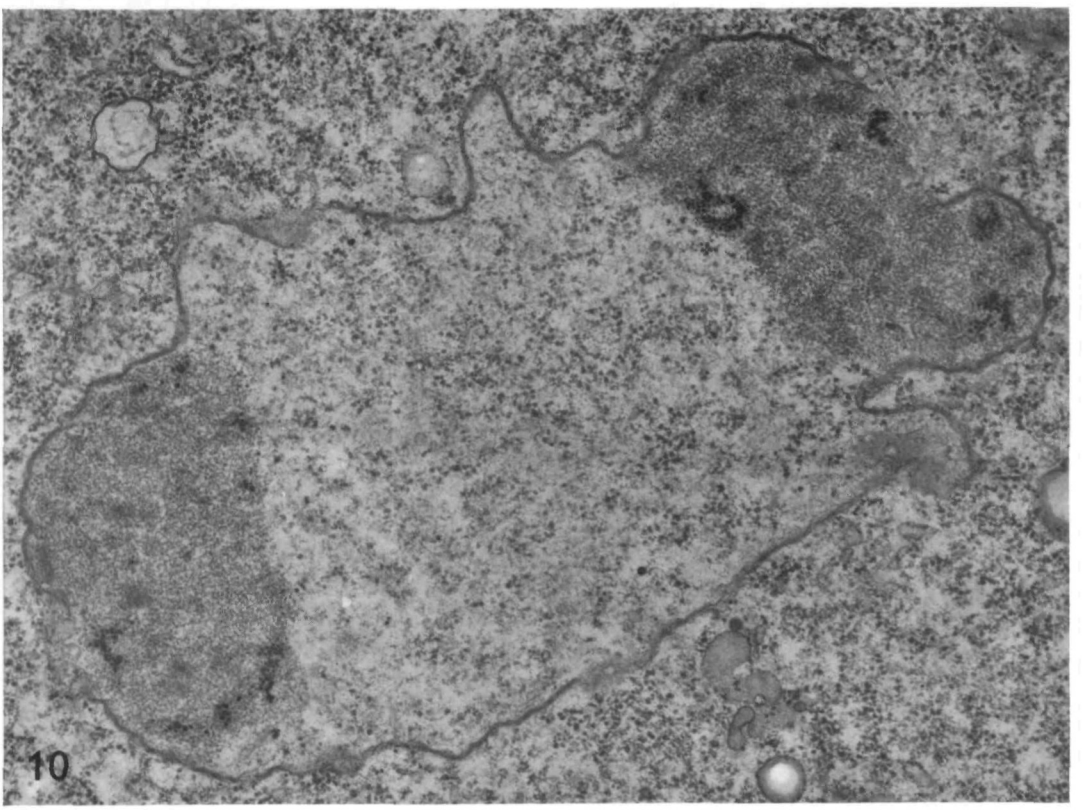
Fig. 9. The polar area of an early telophase nucleus of *P. polycephalum*. Spindle elements are seen in front of the chromatin mass and in close contact with the nuclear envelope which is disorganized at this point. The impression is thus gained that the local breaks of the nuclear membrane in the polar region result from pressure of the cone of microtubules against the envelope. Kinetochores are also visible within the chromatin mass. × 36000.

Fig. 10. Telophase nucleus of *P. polycephalum*. The chromatin aggregate still remains homogeneous in texture and shows nucleolar remnants scattered within its mass. The nuclear envelope is partly broken at the poles and is also very irregular at the equator. Local discontinuities in this latter region undoubtedly account for the presence within the nuclear cavity of a much larger concentration of granules presumably originating from the cytoplasm. × 27000.





this is not evident in the case of all such labelled peripheral bodies, a number of these are observed also to exhibit characteristic opaque granules. Similarly labelled bodies could be identified during metaphase and anaphase and were also observed to become incorporated within the reforming telophase nuclei.

DISCUSSION

As is generally the case for other organisms studied so far (Hay, 1968), the interphase nucleolus in *P. polycephalum* consists of granular zones interspersed with denser regions showing a predominantly fibrillar texture. As in higher plant cell nucleoli (Lafontaine & Lord, 1973) the latter zones exhibit opaque particles which are arranged into complex arrays. The fact that, in both types of cells, thymidine is incorporated in these fibrillar regions reveals that they contain the ribosomal genes which are known to be present within nucleoli (Miller & Beatty, 1969; Birnstiel, Chipchase & Speirs, 1971). In the case of plant cells, evidence has been presented that these genes are part of convoluted filamentous structures or nucleolonemata which correspond to loops extending from specific chromosome segments into the nucleolus (LaCour, 1966; Lord & Lafontaine, 1969; Lafontaine & Lord, 1974). Our electron-microscopic observations reveal that the interphase nucleolus in *P. polycephalum* contains numerous, coarse, filamentous structures which extend for rather short distances only throughout its mass and do not appear, therefore, to be part of a long convoluted thread. The distribution of the opaque particles within certain clusters would moreover suggest that, in this organism, the fibrillar nucleolar component also contains several distinct, doughnut-like structures.

This view that each of the small fibrillar zones present in *P. polycephalum* interphase nucleoli corresponds to a distinct structural entity would account, we think, for the rather unusual fragmentation of the late prophase nucleoli (Guttes *et al.* 1968) into numerous bodies of rather similar sizes. Our additional findings that the bodies which persist during the mitotic stages exhibit an ultrastructural texture similar to portions of interphase and prophase nucleoli and also remain labelled with thymidine strongly suggest that they correspond to well defined nucleolar structural subunits,

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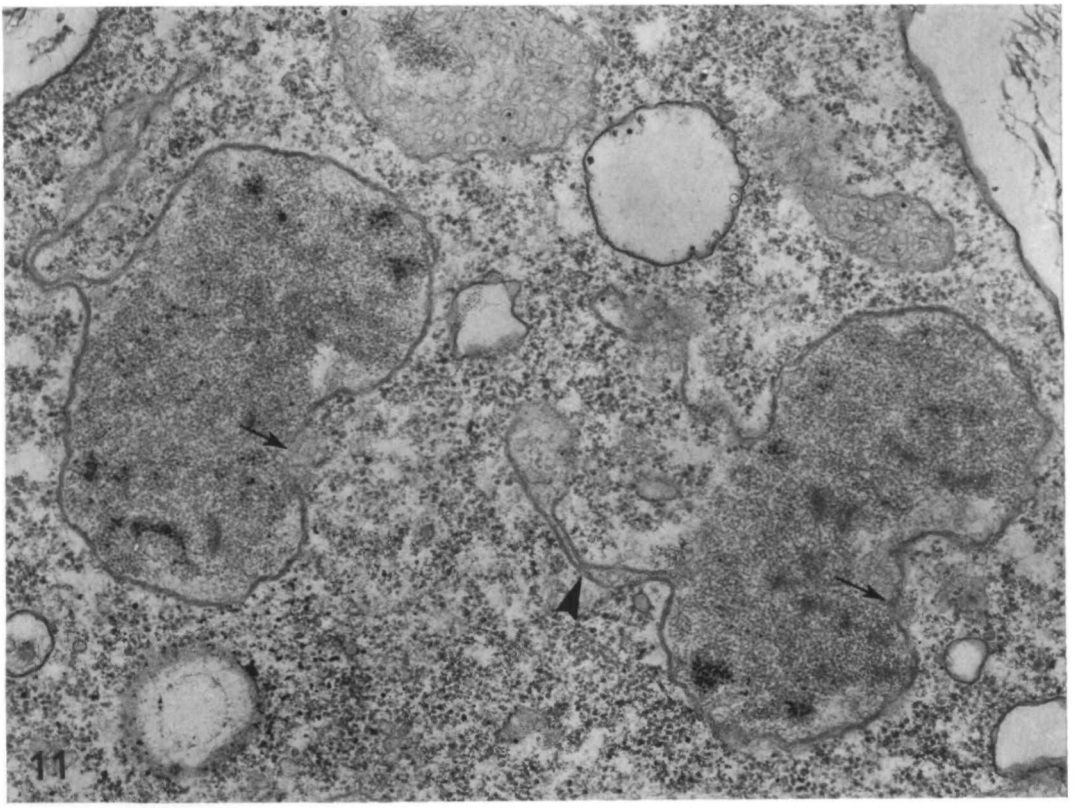
Fig. 11. Oblique sections through 2 late telophase nuclei belonging to different pairs. The plane of sectioning is such that the equatorial portion of these nuclei is oriented towards the left side of the micrograph. Restricted discontinuities are still evident in their distal region (arrows). Both nuclei exhibit, however, much more extensive gaps in their proximal region where the envelope has pinched off as the nuclei separated. Such gaps appear to be sealed eventually by large fragments of the envelope (arrowheads) which remained attached to that portion of the nuclei. Except for a few small transparent areas, the chromatin mass is still continuous and contains a number of small clusters of opaque granules. × 27000.

Fig. 12. In this early interphase nucleus the nuclear envelope has completely reformed and the continuous chromatin aggregate characterizing earlier stages has evolved into a very coarse reticulum. A few clusters of opaque particles are noted over certain regions of this reticulum. × 37000.



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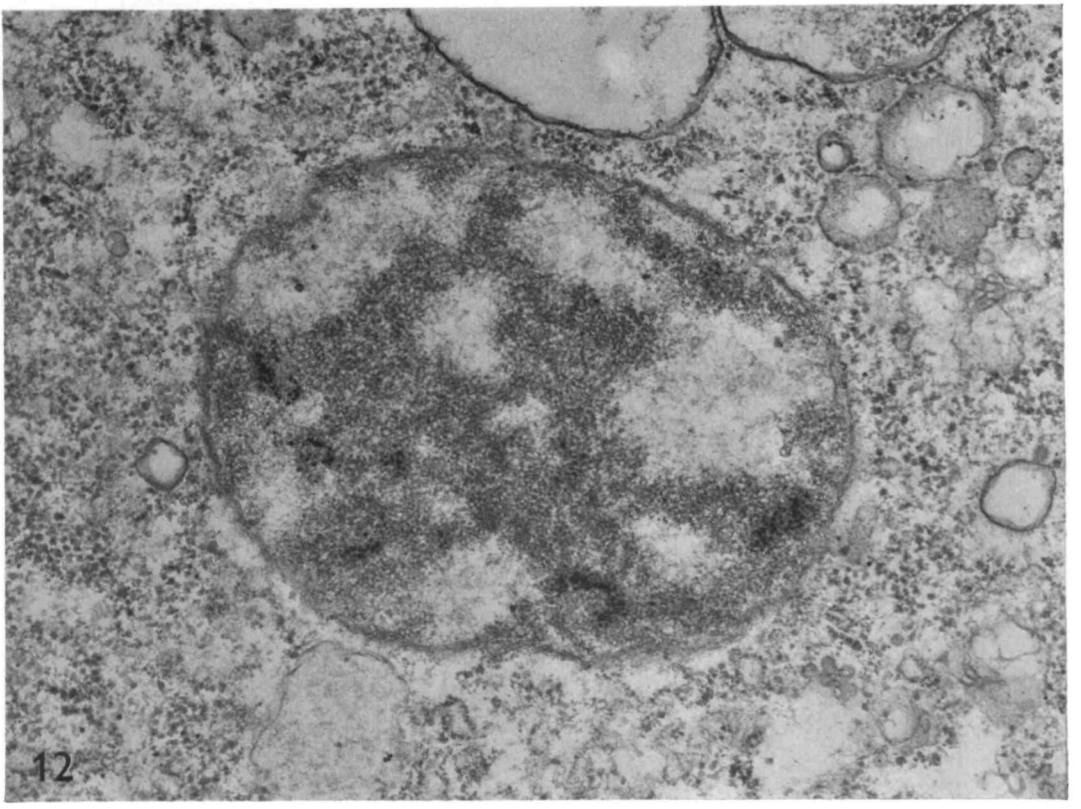

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each containing DNA as a key component. Such an hypothesis appears most compatible with the recent demonstration by Bohnert, Schiller, Böhme & Sauer (1975) that *P. polycephalum* nucleolar DNA consists mostly of linear molecules 2·5 μm long and of a small percentage of circular ones. These circular DNA molecules were observed to be multiples of a basic unit measuring 3·9 μm in length. Taking these data into account, it is reasonable to assume that the thread-like and doughnut-like nucleolar regions observed in ultrathin sections of interphase and prophase nucleoli each contain a linear and circular DNA molecule, respectively. Since, however, the outside diameter of the smallest doughnut-like nucleolar regions (0·1–0·15 μm) is much less than that of the circular DNA molecules observed in spread preparations by these latter authors, it would appear that these molecules must possess some form of tertiary structure as a result of interaction with other nucleolar components. According to this hypothesis, our observation that the number of these DNA-containing nucleolar subunits increases up to late interphase is in complete agreement with the biochemical data showing that nucleolar DNA synthesis, in *P. polycephalum*, is not limited to the *S*-period but persists during the G_2-period (Guttes & Guttes, 1969; Zellweger *et al.* 1972; Newlon *et al.* 1973).

The existence within the mature *P. polycephalum* nucleolus of ribosomal DNA in the form of multiple linear and circular subunits would also account for the observation that its fibrillar regions do not undergo condensation throughout prophase as in higher plant cells where the long nucleolonemata have been observed to contract back to the chromosome axis (Lafontaine & Lord, 1974). Likewise, the persistence of DNA-containing remnants during the mitotic stages explains earlier observations (Guttes *et al.* 1968; Goodman & Ritter, 1969), as well as those reported here, to the effect that the initial stage of nucleolar formation involves a coalescence of these bodies. In this respect, therefore, the onset of nucleolar formation, in *P. polycephalum*, is strikingly different from the situation which prevails in most other eukaryotes. It is evident, however, from the present work that other material of a fibrillo-granular texture is soon added to the forming nucleolus and thus participates actively in growth of this organelle during interphase. Preliminary high-resolution radioautographic data indicate that one such new component is RNA and that its rate of synthesis is already quite high a few minutes only after formation of a continuous nuclear envelope

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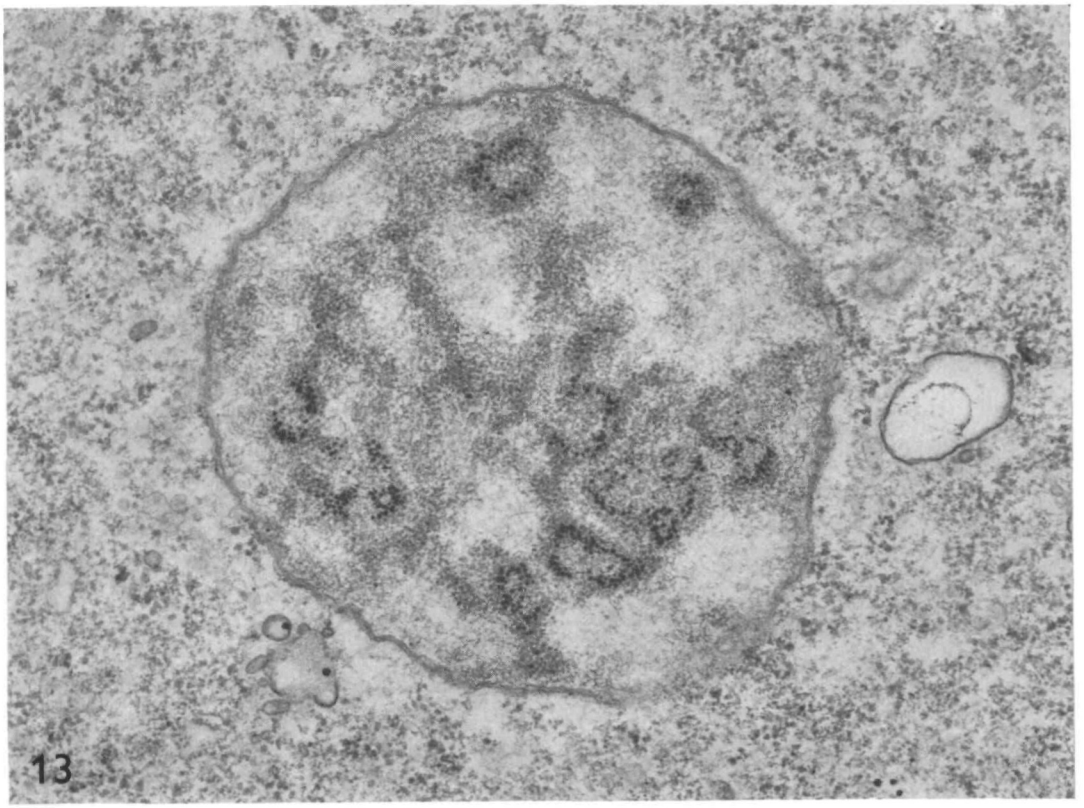
Fig. 13. Micrograph of an interphase nucleus which is slightly more advanced than the one illustrated in Fig. 12. This nucleus has become roundish in outline and the reticulum of chromatin noted in the preceding micrograph has largely disappeared, part of the dense chromatin having presumably transformed into the diffuse fibrillar material present within the greatly enlarged nucleoplasmic zones. The complex network observed in these nuclei is characterized by arrays of opaque particles and, judging from its fibrillo-granular texture, consists partly of non-chromatin material. × 38000.

Fig. 14. In this still more advanced nucleus coalescence of the fibrillo-granular material observed in Fig. 13 has given rise to a large and more compact body the ultrastructural organization of which is indistinguishable from that of mature nucleoli. Moderately dense chromatin masses, or chromocentres, have now appeared throughout the nuclear cavity. × 42000.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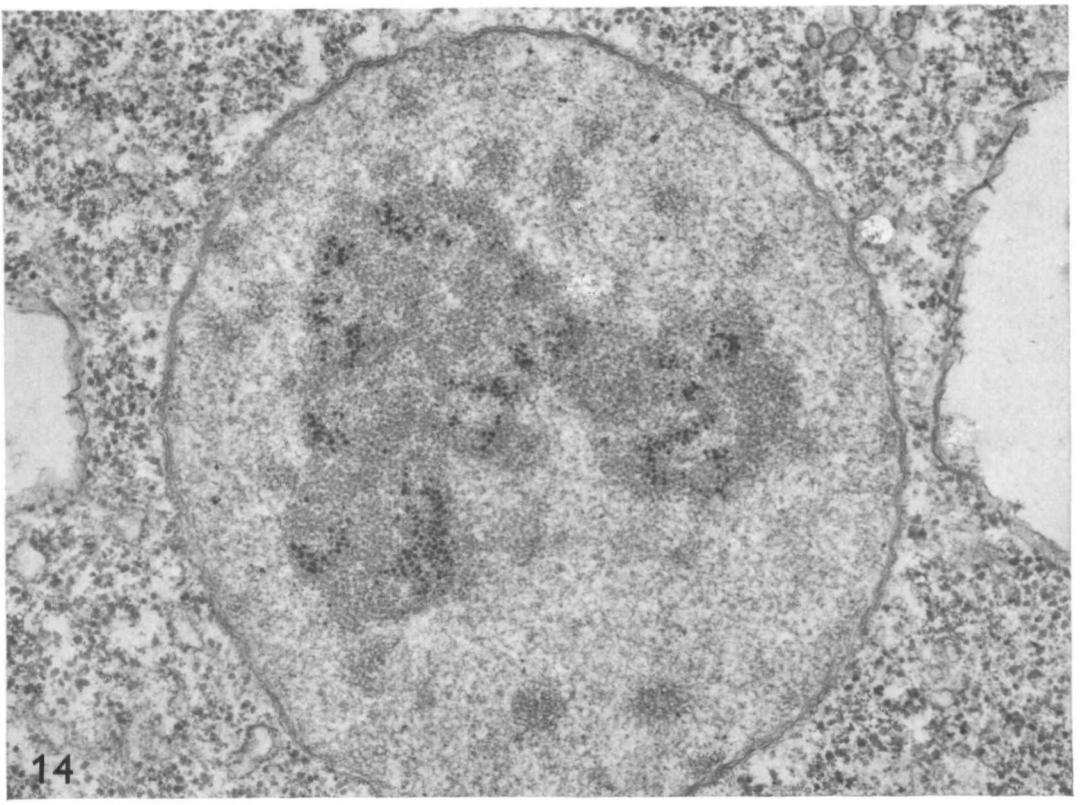
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(authors' unpublished observation). Since it is not known whether all of the nucleolar DNA is extrachromosomal in nature (Bohnert *et al.* 1975), further work will be required to understand better the process of nucleologenesis in this organism. Our results clearly indicate, however, that the later stage of nucleolar growth is an active process implying synthesis of new material. High-resolution radioautographic studies are being pursued to gain further information on this process.

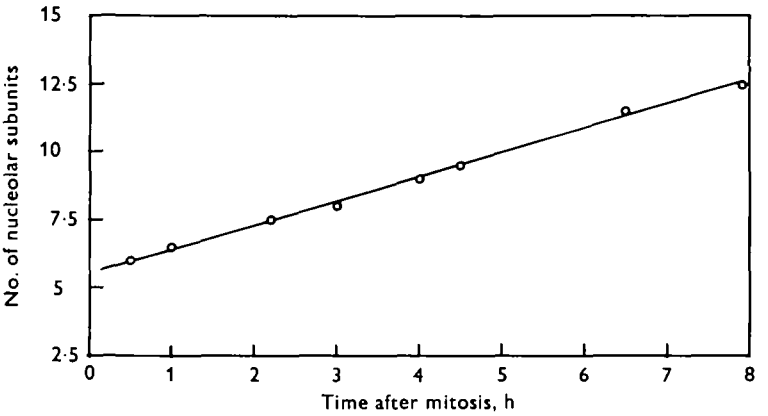

Fig. 15. Graph illustrating the evolution of the number of opaque granule-containing zones in growing nucleoli from samples fixed at regular intervals throughout interphase. The data obtained indicate that the number of distinct nucleolar zones such as these increases linearly during that stage and that G_2 nuclei contain twice as many as early interphase nuclei.

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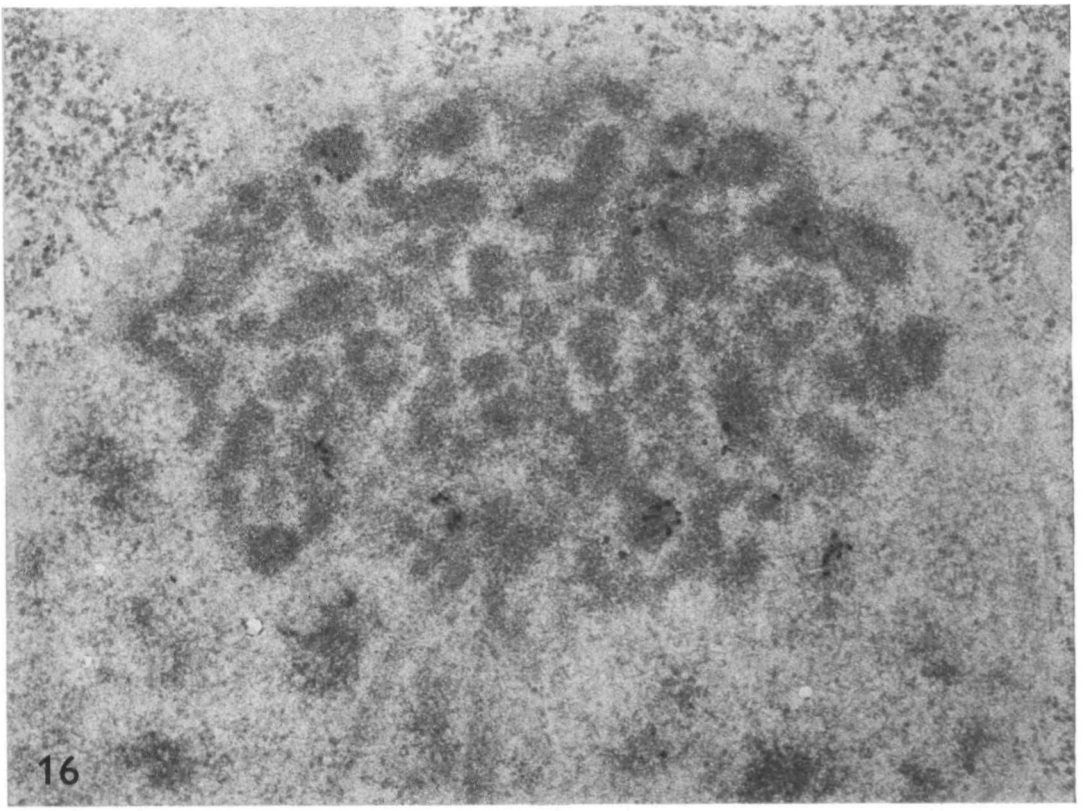
Fig. 16. Late prophase nucleolus of *P. polycephalum* labelled with tritiated thymidine during the G_2 period. Radioautographic grains are observed only over the nucleolus and their distribution is limited to its non-granular zones. × 43 000.

Fig. 17. Prometaphase nucleus labelled with tritiated thymidine during the G_2-period. The radioautographic grains are now largely found at the periphery of the nuclear cavity where the nucleolar remnants tend to migrate as shown by Fig. 5. × 31 000.



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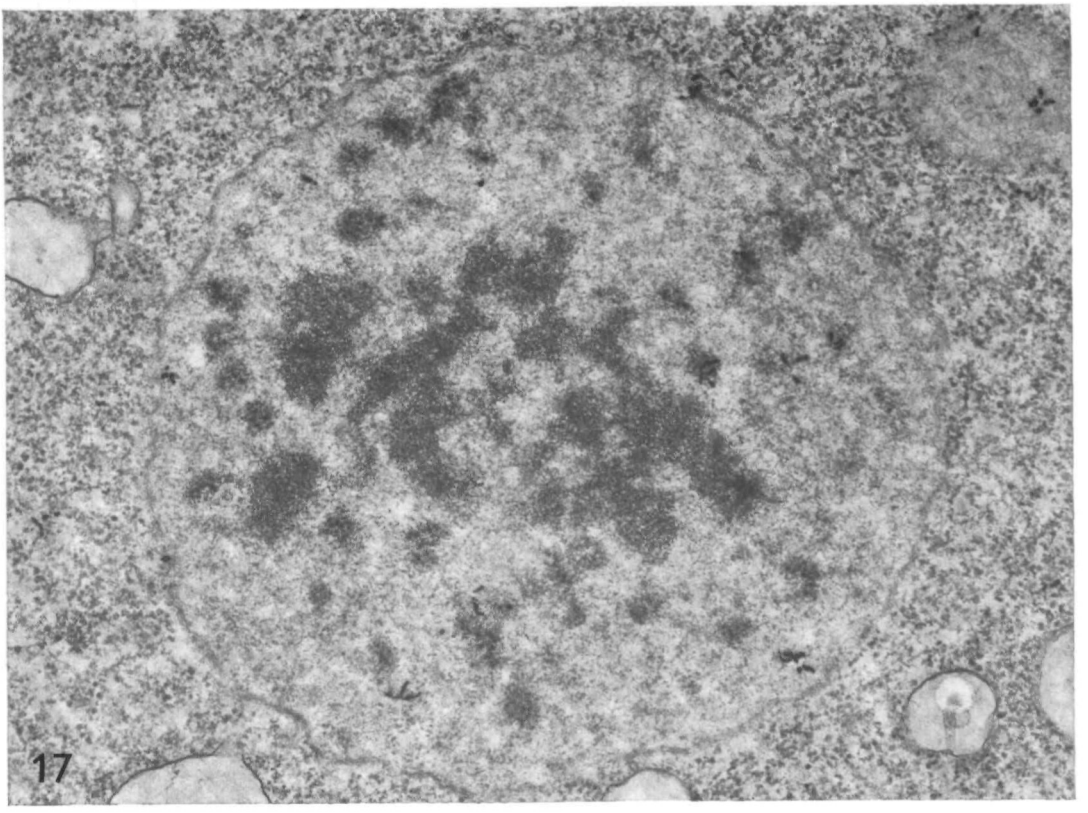

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