REPLICATION OF NUCLEOLUS-ASSOCIATED

DNA DURING "G₂ PHASE"

IN *PHYSARUM POLYCEPHALUM*

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ABSTRACT

In the myxomycete, *Physarum polycephalum*, the bulk of nuclear DNA replication occurs during a period of a few hours immediately following upon mitosis. During the remainder of the intermitotic period, incorporation of thymidine-^{3}H continues at a low rate in the region of the nucleolus (radioautographs). A few nuclei incorporated thymidine-^{3}H into the extranucleolar chromatin at a high rate at all times of the intermitotic period. These nuclei were exceptionally large and they frequently contained several small nucleoli of different sizes rather than the one, central nucleolus which is characteristic of a normal interphase nucleus.

INTRODUCTION

With respect to nuclear DNA replication, the cell cycle is subdivided into several well defined periods (19): M (mitosis), G_1 phase (nonsynthetic period following mitosis and preceding DNA replication), S phase (period of DNA replication), and G_2 phase (nonsynthetic period following completion of DNA replication and preceding the next mitosis). The duration of these periods varies for different cell types to the extent that either the G_1 phase or the G_2 phase, or both, may become either extremely short or nonexistent. In the coenocytic myxomycete, *Physarum polycephalum*, it has been shown, with conventional biochemical (28) and radioautographic (4, 15) procedures, that nuclear DNA replication occurs within a short period of a few hours starting immediately upon mitosis (there is no G_1 phase), and that this period is followed by a G_2 phase which may last for several hours (31). More recently, it has been found that, during the latter half of the intermitotic period, thymidine-^{3}H is incorporated, at a low rate, into a small DNA fraction of higher density than the principal DNA (3, 17). We report here the incorporation, at a low level, of thymidine-^{3}H into DNA which is related spatially to the nucleolus. This incorporation occurs throughout the second half of the intermitotic period, when extranucleolar DNA replication in most nuclei has come to an end.

MATERIALS AND METHODS

Culture Methods

The organism was grown in agitated culture in form of microplasmodia (7). Mitotically synchronized surface plasmodia (13) were prepared as described previously (12).

Cytological Techniques and Radioautography

For morphological observation of the nuclei, ethanol-fixed smear preparations of small explants from the periphery of the plasmodia were inspected under phase contrast (12).

At different times before the 2nd and 3rd post-fusion mitosis, pieces of the plasmodia were placed, for periods of 20 min, on nonnutrient balanced salt solution (NBS) containing thymidine-^{3}H (Schwarz BioResearch, Inc., Orangeburg, N. Y., specific activity, 3.0 c/mmole, concentration, 25 μc/ml).

After incubation, small explants were washed for a few seconds in NBS not containing thymidine-³H, placed in droplets of isolation medium for nuclei (25) on microslides, and cut with a sharp blade into a few pieces. The pieces were moved about in the liquid for approximately 15 sec and then removed from the slide. The remaining isolation medium containing nuclei and other cytoplasmic constituents which had oozed from the plasmodial fragments after cutting was spread over the microslides and allowed to air dry. After fixation with 95% ethanol, the slides were rinsed briefly with tap water and immersed, for 5 min, in 5% TCA at room temperature. After removal of the trichloracetic acid by rinsing for 30 min with running tap water followed by distilled water, the slides were processed for radio-autography (Kodak radioautographic stripping film). Exposure of the radioautographic emulsion in the dark lasted for periods varying between 84 hr and 32 wk. The radioautographs were developed with Kodak D-19 developer (10 min at 20°C). During exposure for periods of more than 2 wk, the preparations were stored in nitrogen atmosphere.

Control slides were treated with DNAse (from Worthington Biochemical Corp., Freehold, N.J.) and RNAse (from Sigma Chemical Co., St. Louis, Mo.) according to the procedure of Plaut and Sagan (30), with the modification that incubation with both enzymes was for 7 hr.

RESULTS

Figs. 1–10 show radioautographs of nuclei from plasmodia which were incubated at different times of the mitotic cycle. As the nuclei incorporated thymidine-³H heavily during the first few hours (S phase) of the intermitotic period, the radioautographic film emulsion was exposed to preparations from plasmodia of this stage of the intermitotic period (Fig. 1) for only 84 hr before development. Longer exposure times (up to 32 wk) were necessary for the majority of the nuclei in plasmodia which were incubated at later stages of the intermitotic period (Figs. 2–7). During the first few hours of the intermitotic period, the nuclei were predominantly labeled in the extranucleolar (peripheral) area (Fig. 1). In radioautographs which were exposed for only 84 hr prior to development the central, nucleolar area of the nuclei appeared almost unlabeled. When developed after longer exposure, numerous silver grains were also found over the nucleolar area. However, the technique employed, i.e. radioautography of whole, mounted nuclei, did not afford the degree of resolution necessary to determine whether all, or some, of these silver

grains were indeed due to the presence of underlying labeled nucleolar DNA.

Around mid-interphase (Fig. 3), while the nucleolus was still occupying the center of the nucleus, incorporation of thymidine-³H into most of the nuclei had declined to such a low level that an exposure time of 32 wk before development was necessary in order to obtain sufficient label above background. The few silver grains found over these nuclei and over most of the nuclei from here on throughout the remainder of the intermitotic period (Figs. 4–8) were concentrated over the nucleolar area.

Occasionally, we found a nucleus which was broken up during the mounting procedure, and the nucleolus of which was located outside the nucleus, adjacent to the nuclear membrane. In that case (Fig. 5) the label was associated with the free nucleolus, while the remainder of the nucleus was unlabeled.

Uptake of thymidine-³H was found until early prophase, approximately 15 min prior to metaphase (Figs. 2 and 6). After this time, the nuclei appeared unlabeled with the level of thymidine-³H employed and after the exposure times used so far. Incubations ending at ana- or telophase yielded no conclusive results. We could not with certainty distinguish, in the radioautographs, the small daughter nuclei from the slightly labeled, poorly preserved mitochondria which were of approximately the same size as the nuclei at that stage. Fig. 7 shows a preparation, from the same set of slides as those used for Fig. 6, which was treated with DNAse after fixation and before processing for radioautography. The number of grains found over the nuclei after this treatment was negligible. Treatment with RNAse did not noticeably reduce the number of silver grains over the nuclei.

Table I gives the results of grain counts over nuclei of the same preparations as those used for Fig. 1 (early interphase), Fig. 3 (mid-interphase), Fig. 4 (late interphase), and Figs. 2 and 6 (early prophase). In this group of experiments the rate of incorporation of thymidine-³H into nuclei which were incubated during mid-interphase (Fig. 3) was 2.0% of that found at early interphase, declined to 1.1% with approaching mitosis (fixation 50 min before metaphase, Fig. 4), and was higher again (1.4%) in preparations which were fixed at 15 min before metaphase (Figs. 2 and 6). This increase in labeling shortly before mitosis was not
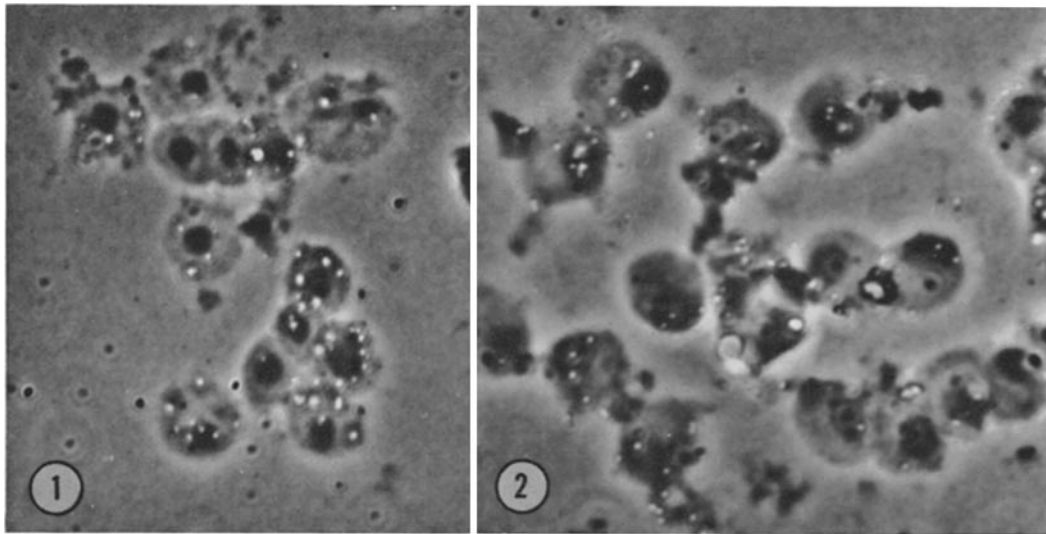
FIGURES 1-7 Radioautographs of "normal" nuclei after incubation of plasmodial sectors with thymidine-^{3}H at different times of the mitotic cycle. Exposure of radioautographic film emulsion: Fig. 1, 84 hr; Figs. 2-7, 32 wk. Magnifications: Figs. 1-2, × 2500. Figs. 3-7, × 4100. Figs. 1 and 2 are shown with phase contrast only; Figs. 2-7 were made with (a) and without (b) phase contrast.

FIGURE 1 Early interphase, fixation 2.2 hr after metaphase. Note central nucleolus. Silver grains mainly over the extranucleolar area of the nucleus.

FIGURE 2 Early prophase, fixation approx. 15 min before metaphase. Nucleolus adjacent to nuclear membrane. Silver grains mainly over and near nucleolus.

found in another group of experiments involving a different batch of plasmodia.

Throughout the latter part of the intermitotic period we found a small percentage (less than 0.5%) of nuclei which were considerably above average in size and heavily labeled in pulse experiments (Fig. 8). More than half of these large nuclei contained several nucleoli of various sizes (Fig. 9). Some of them contained only one nucleolus. This nucleolus at early prophase moved close to the nuclear membrane, as do the nucleoli of the smaller nuclei (Fig. 10). In the large nuclei, the label was concentrated predominantly in the extranucleolar chromatin (Fig. 10).

DISCUSSION

The occurrence in *P. polycephalum* of a short S phase following immediately upon mitosis was first established by Nygaard et al. (28), who found that orotic acid-^{14}C was converted into acid-insoluble thymine-^{14}C during the first 90 min of the intermitotic period (S phase). A somewhat

longer duration of the S phase (approximately 3 hr) was found by a radioautographic study of the incorporation of thymidine-^{3}H into the nuclei (4). Sachsenmaier (32) observed a slow, continuous increase of the amount of total DNA during the second half of the intermitotic period, which he interpreted as being due to cytoplasmic DNA synthesis. More recently, incorporation of thymidine-^{3}H into a nuclear DNA fraction of higher density than the principal DNA (4, 14) has been found during the S phase (3) as well as during the latter half (3, 17) of the intermitotic period. Our own experiments show two types of nuclear labeling in pulse experiments during the latter part of the intermitotic period. Most of the nuclei were lightly labeled, and the label was concentrated in the nucleolar region. A few nuclei, which were above average in size, were heavily labeled. These nuclei were predominantly labeled in the extranucleolar chromatin.

The spatial relationship to the nucleolus of the DNA which was labeled at late interphase suggests
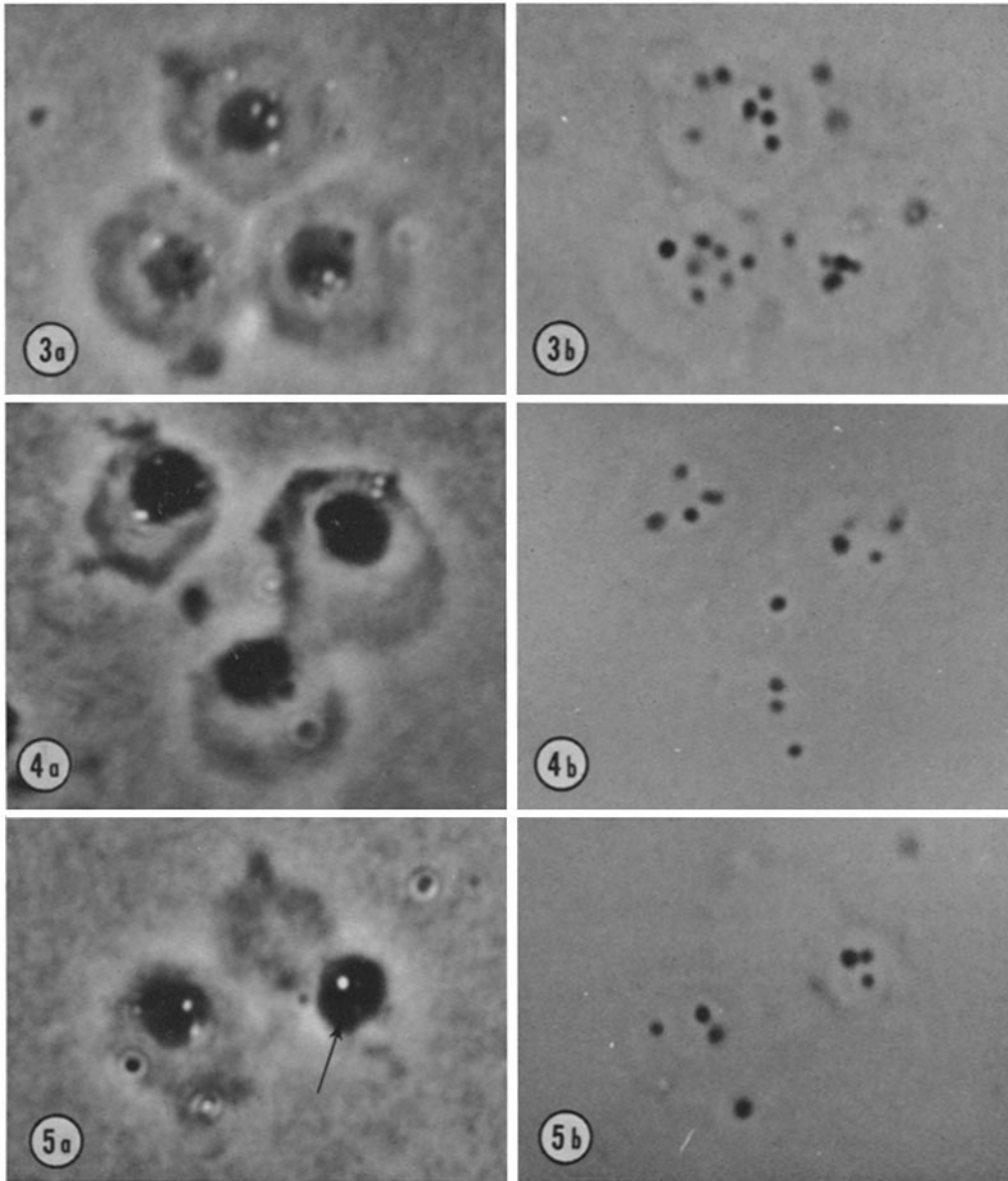
FIGURE 3   Mid-interphase, fixation 5.1 hr after metaphase. Nucleolus in center of nucleus. Grains in this and in the following pictures mainly over and near the nucleolus.

FIGURE 4   Late interphase, fixation approx. 50 min before metaphase at time when nucleolus began to move closer to the nuclear membrane.

FIGURE 5   Late interphase, fixation approx. 90 min before metaphase. Note label over isolated nucleolus (arrow) adjacent to an empty nucleus.
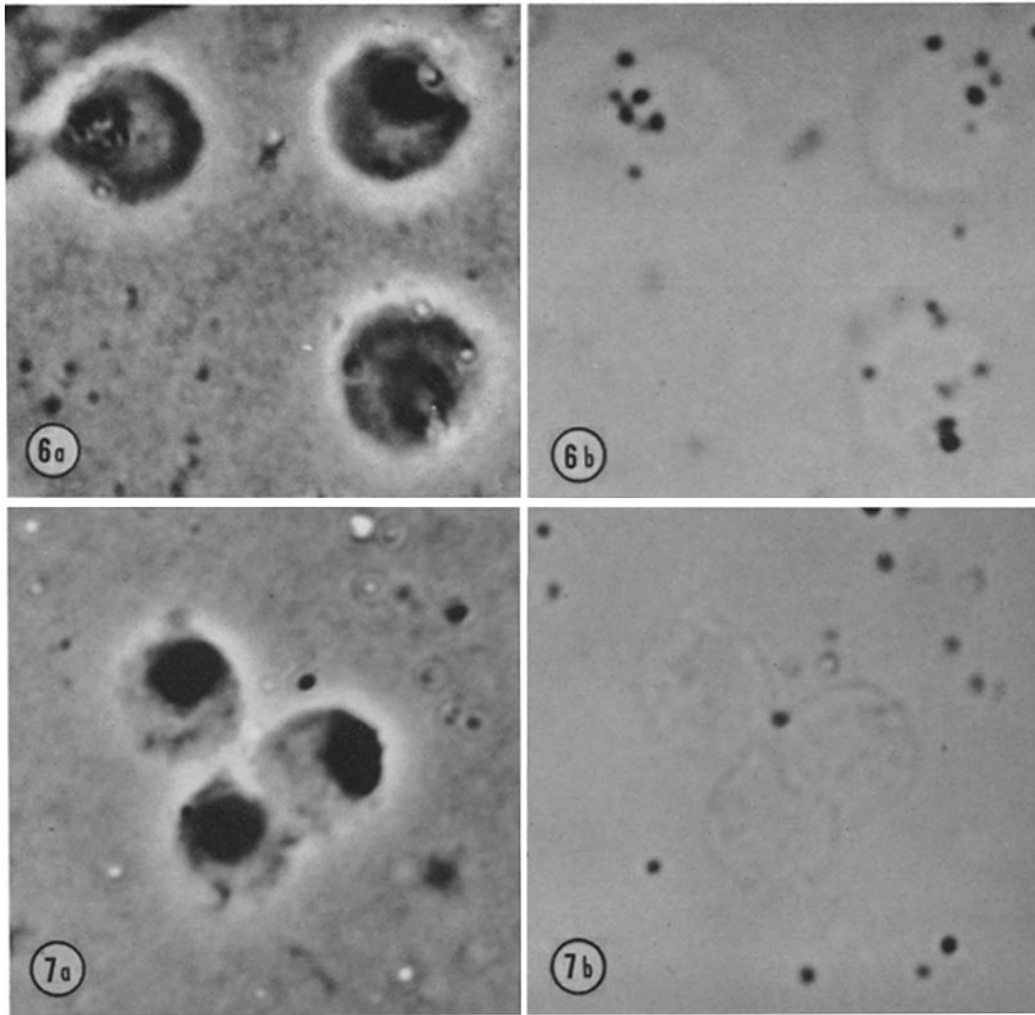
FIGURE 6 Same stage as Fig. 2. Very thin area of the preparation selected for better resolution.

FIGURE 7 Same stage as Figs. 2 and 6. Radioautograph made after previous incubation with DNAse.

that we are dealing with nucleolus-associated DNA. The low level of labeling raises the question of its significance. Cistrons with a high guanosine/cytosine (GC) content are predominantly transcribed during late interphase in *P. polycephalum* (6, 24). As nucleolus-associated DNA has a higher GC content than the bulk of the nuclear DNA (2, 8, 35), it is possible that the DNA which we find to be labeled in the region of the nucleolus at late interphase is identical with the DNA which was found to be predominantly transcribed during that time (6, 24). Hence, the possibility exists that the low rate of incorporation of thymi-

dine-³H in the nucleolar region could be due to repair replication following transcription (29) rather than to net synthesis. Preliminary experiments have shown, however, that mitosis in *P. polycephalum* is delayed by addition of inhibitors of DNA synthesis (deoxyadenosine, excess-thymidine) to the plasmodia at late interphase.[1]

Incorporation of thymidine-³H into nucleolar DNA, in the absence of extranucleolar labeling, has been reported recently by Lettré and Ghosh (22) for chicken fibroblasts. It was not determined,

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[1] E. Guttes and S. Guttes. Unpublished observations.

Stage of plasmodium at end of incubation	Correspond- ing fig.	No. grains over 100 nuclei
hr after M II		
2.2	1	564
		(36096)
5.1	3	734
min before M III		
50	4	421
15	2, 6	523

The counts were obtained from the same preparations which were used for the above Figs. 1, 2, 3, 4, and 6. Large nuclei (less than 0.5%) which were heavily labeled during the latter half of the intermitotic period were not counted. Exposure time before development was 84 hr for the nuclei which were incubated during early interphase (corresponding to Fig. 1), and 32 wk for all the others. The figure given in brackets indicates the theoretical grain count equivalent to exposure for 32 wk.

however, at what stage of the intermitotic period the nuclei were at the time of incorporation.

Incorporation of thymidine-³H into nuclei as late as prophase has been found in grasshopper neuroblasts (9) and, during metamorphosis, in epidermal cells of the milkweed bug *Oncopeltus fasciatus* (21). DNA synthesis also occurs during meiotic prophase in microsporocytes of *Tradescantia* (27), in male newts (36), in GC-rich regions of the chromosomes of lily anthers (18), and in oocytes of *Xenopus* (8, 23). In the latter, this DNA synthesis represents the production of extra copies of GC-rich DNA cistrons of the nucleolar region (5, 8) which code for ribosomal RNA.

Although in our experiments the label found during the latter part of the intermitotic period, until early prophase, was localized preferentially in the nucleolar region of the nuclei, we cannot exclude chromosomal segments other than the nucleolar organizer region from consideration. Late replication of nuclear DNA has been found for the centromeres of some chromosomes (1, 20, 26, 34), and there is indirect evidence in one case (33) suggesting that the GC-content of the cen-

tromeric region might be higher than the average GC-content of the chromosomes.

Heavy labeling during late interphase of a few nuclei which were above average in size has been observed previously (14). The origin of these nuclei is at present unknown. In normal mitosis, chromosomal division is preceded by disintegration of the one, central nucleolus and followed by multifocal reappearance of several, small pronucleolar bodies which gradually coalesce, within less than 90 min after mitosis, to form one central nucleolus again (13, 16). Except for their large size, which exceeds that of interphase nuclei in the same plasmodium, the multinucleolated, labeled nuclei resemble postmitotic nuclei. Their large size would suggest that they are polyploid. The high rate of incorporation of thymidine-³H into the extranucleolar chromatin throughout the latter part of the intermitotic period, and even during a period of time immediately preceding mitosis, seems puzzling. If these nuclei became polyploid during, and started DNA synthesis immediately after, the previous mitosis, one would expect DNA synthesis in the extranucleolar chromatin to be completed along with that in the other nuclei. The incorporation of thymidine-³H at a high rate during late interphase and the fact that many of the large nuclei have several small nucleoli could be accounted for by assuming that polyploidization occurred in these nuclei during the intermitotic period. We have no evidence, however, that such an event actually took place.

Incorporation of thymidine-³H into heavy nuclear satellite DNA in *P. polycephalum* has been found previously (3, 17). This DNA because of its higher GC content (2, 8, 35) could be identical with the DNA of the nucleolar region which is labeled at late interphase. The occurrence at the same time of heavy labeling in the extranucleolar chromatin of the larger nuclei could account for the low, variable level of incorporation of thymidine-³H into the principal nuclear DNA during late interphase which has been found by CsCl density gradient analysis (17).

With the exception of the highly labeled nuclei, we have not been able to find nuclear label during late interphase in radioautographs made from sections. The reason for this is probably that, in a preparation of whole, mounted nuclei which were dried and flattened before fixation, more of the labeled, nucleolus-associated DNA is within a distance of 1–2 μ from the radioautographic emulsion
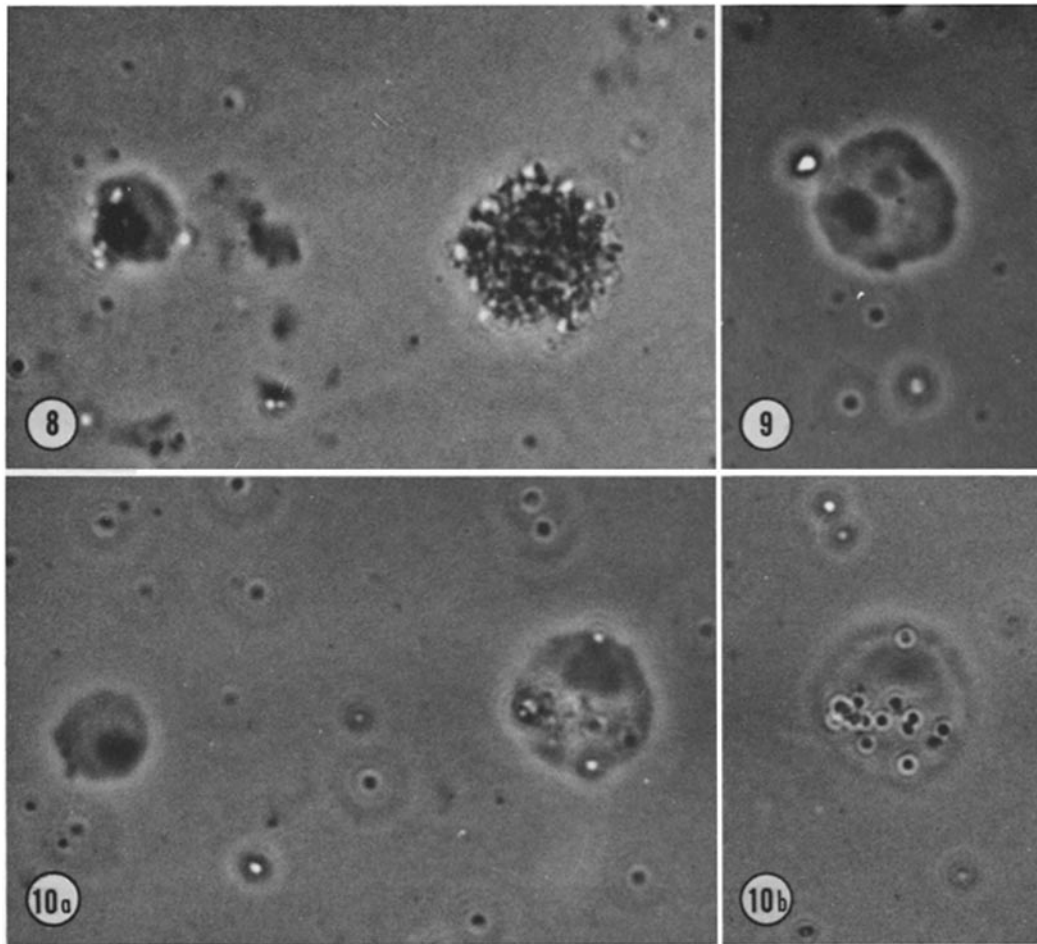
FIGURES 8–10 Radioautographs of large, heavily labeled nuclei. The plasmodia were fixed at early prophase after incubation with thymidine-^{3}H for 20 min. Exposure of radioautographic film emulsion: Fig. 8, 32 wk; Figs. 9–10, 84 hr. Magnification: $\times$ 2350. All photomicrographs were made with phase contrast. Fig. 10 was made at two different focussing positions (a and b) to show localization of silver grains.

FIGURE 8 Large nucleus obscured by numerous silver grains. Note size and labeling of "normal" nucleus.

FIGURE 9 Nucleus having several nucleoli of various sizes. Radioautograph made after previous incubation with DNAse.

FIGURE 10 Large nucleus with one eccentric nucleolus. Silver grains mainly over the extranucleolar chromatin. Note small, "normal" nucleus of the same stage.

than is the case in sectioned material. Hence, the number of low-energy (^{3}H) beta particles reaching the area of the film emulsion underlying a thymidine-^{3}H–labeled nucleus is probably higher, despite increased self-absorption, in preparations of mounted nuclei than in sections. In view of the

possible role in the initiation of mitosis of a few, late replicating cistrons, a reexamination would perhaps be warranted of those cell types for which the existence of a long-lasting G$_2$ phase (10, 11) has been established by radioautography of sections involving moderate levels of isotope and

comparatively short exposure times of the film emulsion prior to development.

Note Added in Proof: M. Alfert and N. K. Das (*Proc. Nat. Acad. Sci. U.S.A.* 1969. **63**:123.) have recently found that the rate of DNA replication in polyploid nuclei of snapdragon seedlings and mouse-liver cells is higher than that in diploid nuclei, but lower than one would expect from the increased level of ploidy. If this were a more general phenomenon, the incor-

poration during late interphase of thymidine-^{3}H into the giant nuclei of *P. polycephalum* could be due to prolongation of the time needed for DNA replication in these nuclei.

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