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Effect of Hyperthermia on Normal and Neoplastic Cells *in Vitro*[1]

Robert Love, Ramon Z. Soriano, and Richard J. Walsh

Department of Pathology, Jefferson Medical College, Philadelphia, Pennsylvania 19107

SUMMARY

The effects of hyperthermia on 3 lines of HeLa cells, 3 lines of human diploid cells, and BHK 21 cells were similar. Exposure of exponentially growing cells to 45–46° for periods of 15 to 60 min resulted in disappearance of nucleolar DNA and diffusion of nucleolinar material, as demonstrated by toluidine blue-molybdate, acid phosphatase, and lead staining throughout the nucleolus. Ultrastructural studies of the heated cells indicated that the fibrillar component of the nucleolus, like the nucleolini, became diffusely distributed throughout the nucleolus and that the granular component of the nucleolus almost completely disappeared. Hyperthermia led to the formation of cytoplasmic inclusions in 100% of cells with nucleoli. The inclusions were cytochemically similar to the body of the nucleolus observed by light microscopy and consisted of aggregations of ribosome-like granules in electron micrographs. Evidence is presented to indicate that the inclusions were derived from the nucleolus and possibly from the granular component. Hyperthermia also resulted in disaggregation of polysomes. The effects of heating to 46° for 15 min were completely reversible.

INTRODUCTION

During the last half century there have been numerous reports based on experimental and clinical evidence that tumor cells are more sensitive to heat than normal cells (3, 24). Little is known about the mechanisms involved in heat-induced cell injury. Cavaliere *et al.* (3) noted that the oxygen uptake of Novikoff hepatoma and Ehrlich ascites cells was decreased by increasing the temperature from 30° to 42°, although there was little difference in respiration at these 2 temperatures in normal and regenerating liver. McCormick and Penman (13) observed a breakdown of polysomes in HeLa cells heated to 42° for 10 min and noted that this process was slowly reversed during continued hyperthermia. They attributed the initial polysomal disaggregation to the failure of synthesis of a species of RNA which was slowly synthesized after longer periods of hyperthermia. The electron microscopic observations of Simard *et al.* (19, 20) have provided further information about the mechanism by which heat interferes with cellular metabolism. They noted that the normal architecture of the nucleolus was disrupted by hyperthermia. The fibrillar component of the nucleolus was dispersed and the granular component and the nucleolar chromatin almost completely disappeared. There was also a selective inhibition of nucleolar RNA synthesis. Further studies by Amalric *et al.* (1) indicated that at temperatures of 44.5° the synthesis of 45 S and 30 S nucleolar RNA was inhibited while some incorporation of uridine-^{3}H persisted in a fraction of RNA sedimenting in a sucrose gradient at 8 to 10 S. These authors suggest that the inhibition of synthesis of nucleolar RNA is related to the disappearance of intranucleolar DNA. Most recently, Warocquier and Scherrer (6) have confirmed the partial inhibition of synthesis of 45 S ribosomal precursor RNA in HeLa cells heated to 42° and have demonstrated a failure of maturation of rRNA.

The present study is an extension of these observations and presents cytochemical and electron microscopic studies of the effect of heat on normal and neoplastic cells. In particular, changes in the nucleolini and the granular cytoplasmic RNP[2] (6, 7, 10) as observed by light microscopy are correlated with alterations in the ultrastructure of the cell, and the formation of cytoplasmic inclusion bodies, apparently of nucleolar origin, is described.

MATERIALS AND METHODS

Cells. Three lines of HeLa cells were used: JJH, from Dr. J. J. Holland, Freed, from Dr. J. Freed, and a clone (2B) from HeLa F (25). The normal cells comprised the WI 38 line (15) and male and female human embryo lung lines from Flow Laboratories, Rockville, Md. A few experiments were also carried out with BHK 21 cells (15). The cells were cultured in Eagle's basal medium containing 10% fetal calf serum and chlortetracycline (50 μg/ml). For light microscopy, the cells were grown in "ring" cultures as previously described (27) and, for electron microscopy, in Blake bottles. Some cultures were also grown in Leighton tubes.

Cytochemistry. Cells were routinely fixed in trichloroacetic acid and formol sublimate as described for the TBM method (10). RNP were demonstrated by TBM Method B after digestion with deoxyribonuclease (11), by TBM Methods C and D (10), and by staining for 10

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[2] The abbreviations used are: RNP, ribonucleoprotein; TBM, toluidine blue molybdate; Tris, trishydroxymethylaminomethane buffer containing 0.045 M $MgCl_2 \cdot 6H_2O$ and 5 mM $CaCl_2$.

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min in 0.5% toluidine blue in McIlvaine's buffer at pH 3.0 (14). Specificity of staining of RNA was demonstrated by failure to stain after digestion with ribonuclease (0.1 mg/ml in 0.02 M Tris, pH 7.3, for 18 hr at 37°). DNA was stained by the Feulgen reaction (14) and nucleolinar acid phosphatase by a modification of Gomori's lead sulfide method at pH 4.8 (9). Some preparations were stained by the lead precipitation method (23) and by the acrolein-Schiff stain for protein-bound groups (14).

Electron Microscopy. Monolayers were fixed at 4° in Blake bottles with 2% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 (17) for 20 min, followed by 1% osmic acid in the same buffer for 30 min. Cells were gently scraped off the glass and centrifuged at 1050 rpm for 5 min and the resultant pellets were embedded in Epon (12). Sections were stained with uranyl acetate and Reynold's lead citrate (16) and examined with an RCA EMU 4 microscope.

Hyperthermia. Ring cultures in Petri dishes were exposed to heat by floating the dishes in a circulating water bath at predetermined temperatures (±0.1°). Bottles and Leighton tubes were immersed in the bath. In preliminary experiments, inclusion bodies containing RNA were observed in the cytoplasm of a variable proportion of heated cells. Using the appearance of inclusion bodies as an index, it was determined that in order to produce inclusions in 100% of cells in interphase it was necessary to use a temperature 1° higher for ring cultures floating on the surface of the bath than for Leighton tubes which were completely immersed. Thus, the lowest temperature required to produce inclusions in 100% of WI 38 cells was 44° for Leighton tubes and 45° for ring cultures. For this reason, bottles and Leighton tubes were always heated at 1° lower than ring cultures. Control cultures were maintained at 37° without CO_2 for the same periods of time as the heated ring cultures without any detectable cytological or cytochemical effects.[3]

RESULTS

Cytochemical and Cytological Changes. The effects of hyperthermia at 46° on HeLa JJH cells are summarized in Table 1. There was some enlargement of the nucleolinar RNP bodies demonstrable by TBM, Method B, 5 min after heating. This was accompanied by a diminution in their number, suggesting fusion. The nucleolinar changes progressed with time and the intensity of staining of RNA decreased. At the end of 1 hr, the outlines of the nucleolini were ill-defined and the whole nucleolus was weakly, diffusely, and metachromatically stained by TBM Method B (*cf.* Figs. 1 and 2). Cells were completely unstained in preparations digested with ribonuclease and deoxyribonuclease and stained by TBM Method B.

The larger nucleolini can be visualized in living cells as vacuoles by phase microscopy (11). The same vacuoles can be observed in fixed preparations stained by TBM Method C or by toluidine blue (*cf.* Figs. 1 and 3). The vacuoles in nucleoli stained by these methods were either weakly stained or not recognizably stained and presented a negative image of nucleoli stained by TBM, Method B (*cf.* Figs. 1 and 3). During the course of hyperthermia, the vacuoles observed in preparations stained by TBM Method C or toluidine blue gradually disappeared until the whole nucleolus became almost homogeneously stained (*cf.* Figs. 3 and 4).

During the course of hyperthermia nucleolinar acid phosphatase appeared to diffuse from the nucleolini into the body of the nucleolus until it was uniformly and intensely stained (Table 1). This process has been described and illustrated elsewhere (9). Staining of nucleolini by the lead precipitation method (21) was similarly affected by heat and progressively decreased until it was replaced by diffuse weak staining of the body of the nucleolus after 15 min.

Nucleolar DNA was detectably reduced after 5 min of hyperthermia and was no longer detectable after 60 min (Figs. 5 and 6). As noted by Simard and Bernhard (20), the nucleolar-associated chromatin persisted (Figs. 5 and 6).

In preparations stained by TBM Method C or by toluidine blue, inclusion bodies having the staining characteristics of the body of the nucleolus appeared in the cytoplasm of cells in interphase, early prophase, and late telophase (Fig. 4). No nucleolinar RNP or acid phosphatase could be detected in the inclusions and, like the body of the nucleolus, they were weakly and orthochromatically stained by TBM Methods B (Fig. 2) and D. They were unstained by toluidine blue or any of the TBM methods in preparations that had previously been digested by ribonuclease. The inclusions, like the nucleoli, were also stained by the acrolein-Schiff method for protein-bound groups. No cytoplasmic inclusions were observed in cells that did not have nucleoli, *i.e.*, late prophase, metaphase, anaphase, and early telophase. Occasionally, extrusions of the nuclear membrane containing parts of nucleoli and actual rupture of the nuclear membrane were observed (Figs. 7 and 8). Inclusions were first observed in a few cells (10 to 20%) 3 min after the onset of heating and they increased in number until they were present in 100% of cells with nucleoli after 10 to 15 min. Multiple inclusions of variable size were present in the majority of cells (Fig. 4) and they were most numerous and largest after 15 min of hyperthermia. After 1 hr, they were slightly less numerous and smaller than after 15 min of hyperthermia.

Staining of a granular form of RNP in the cytoplasm by TBM Method D has been shown to be related to the state of aggregation of the polysomes (7). During the course of heating, this cytoplasmic granular RNP became finer and the granules were more numerous. Finally, this RNP in granular form was no longer recognizable and the cytoplasm was diffusely and metachromatically stained (Figs. 9 and 10).

At temperatures of 41–44° similar changes were ob-

[3] The thermometer used in these experiments was subsequently calibrated against a thermometer certified by the National Bureau of Standards. The temperatures in this paper are actually 0.15° higher.

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Table 1
Cytological and cytochemical changes produced by hyperthermia in HeLa cells in ring cultures

Duration of heat (min at 46°)	Nucleolinar RNP bodies	Nucleolinar acid phosphatase	Lead staining of nucleolini	Nucleolar DNA	Cytoplasmic inclusion bodies	Cytoplasmic granular RNA
3	No change	No change	No change	No change	Present in a few cells	No change
5	Decrease in number and increase in size	Decrease (increase in body of nucleolus)	As acid phosphatase	Decreased	Present in almost all cells	Granules become more numerous; finally not resolvable by light microscopy
10	Progression of changes at 5 min with some decrease in intensity of staining	Diffusion until whole nucleolus intensely stained	Entire nucleolus weakly stained	Further decrease until virtually absent in nucleolus	Present in all cells[a]	
15			Nucleolini not detectable			

[a] Except cells in mitosis which have no nucleoli.

served, but the nucleolar alterations were less pronounced and were not observed in all cells. Cytoplasmic inclusion bodies were fewer and smaller, and were present in a smaller and variable percentage of cells. Thus, in 2 replicate experiments when HeLa cells were heated to 41° for 15 min, 48% and 4% of interphase cells, respectively, contained cytoplasmic inclusions. For this reason, a higher temperature was used for electron microscopic studies, for which it was desirable to have inclusions present in every cell containing a nucleolus.

The effects of hyperthermia at 46° were completely reversible up to periods of 15 min. Twenty-four hr later the cells were cytologically and cytochemically indistinguishable from unheated controls.

The changes were essentially the same in all cells studied except that staining of nucleolinar RNA was more reduced in diploid and BHK 21 cells and the inclusions were smaller. All cells were cytologically and cytochemically indistinguishable from unheated controls 24 hr after 15 min of hyperthermia at 46°.

In electron micrographs of HeLa JJH cells heated in Blake bottles at 45° for 30 min, the changes in the nucleoli were essentially the same as those described by Simard and Bernhard (20). The granular component of the nucleolus almost completely disappeared and the morphology of the fibrillar component was disrupted so that almost the entire nucleolus consisted of fibrillar material (Figs. 13 and 14). In untreated cells most of the ribosomes and polysomes were not membrane-bound (Fig. 11). In cells subjected to hyperthermia, the number of ribosomes and polysomes throughout most of the cytoplasm was decreased (Fig. 12). In a few circumscribed areas, however, there were dense collections of ribosome-like particles (Fig. 12). These areas appeared to correspond in size to the inclusions observed by light microscopy.

Effect of Culture Conditions on Inclusion Body Formation. Inclusion body formation occurred in 100% of cells with nucleoli during logarithmic growth of HeLa and diploid cells. Heating during the first 24 hr of growth of diploid cells produced very small inclusions in a variable number of cells but usually in less than 100% of cells with nucleoli. The variability appeared to be due to the fact that inclusions were more frequently observed in cells forming contiguous sheets than in isolated cells. When diploid cells reached density-dependent inhibition of replication or HeLa cells cultures became very dense, with cells detaching from the glass, the percentage of cells with inclusions and the size and number of inclusions produced by heat decreased.

Effect of Actinomycin on Heat-induced Inclusion Formation. Treatment of HeLa cells for 72 hr with actinomycin (0.03 μg/ml) resulted in the loss of RNA from the majority of nucleoli. No RNA-containing cytoplasmic inclusions could be detected in cells without nucleolar RNA, but small inclusions did form in cells in which some nucleolar RNA persisted. Nucleolar fragments and cytoplasmic inclusions could be demonstrated by the acrolein-Schiff stain in the heated actinomycin-treated cells, indicating that the antimetabolite had not inhibited the mechanism by which the inclusions were formed.

DISCUSSION

The ultrastructural changes in the nucleoli observed here were similar to those described by Simard and Bernhard (20), who carried out most of their studies at 42°. In the present study, nucleolinar material, as demonstrated by TBM, lead precipitation, and acid phosphatase staining, diffused throughout the nucleolus. In electron micrographs, the granular component of the nucleolus almost completely disappeared and the nucleolus became largely fibrillar. The results confirm previous speculation (11) that the nucleolinar RNP probably corresponds to the fibrillar component observed in electron micrographs.

The basis of staining by the TBM method is that structures containing RNA which bind toluidine blue in such small amounts that they can be shown only by weak staining, or not at all, can be demonstrated by subsequent dye polymerization with molybdate (6). Thus

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nucleolini appeared as unstained or weakly stained vacuoles in preparations stained with toluidine blue (Fig. 3) (11). During hyperthermia, as the nucleolinar RNA or RNP diffused throughout the entire nucleolus, the nucleolar vacuoles became no longer recognizable and the whole nucleolus was stained by toluidine blue (Fig. 4). This could be explained by steric alteration in RNP resulting in the availability of increasing numbers of RNA phosphoryl groups for binding the cationic dye in the same manner as treatment with formaldehyde or nitrous acid (6). Furthermore, there is a decrease in protein synthesis during hyperthermia (13) so that there may be insufficient ribosomal protein available to form preribosomal particles or ribosomes in the nucleolus. In fact, Warocquier and Scherrer (26) have suggested that this may be the explanation for the disappearance of the granular component in heated cells. Thus, in the absence of adequate ribosomal protein, increasing numbers of RNA phosphoryl groups may be free to bind dye. The biochemical studies are consistent with the suggestion that the fibrillar component of the nucleolus, and, hence, nucleolinar RNA, is ribosomal precursor RNA since the 45 S RNA persists in the nucleolus during hyperthermia (see Refs. 1 and 26).

There is considerable evidence that the cytoplasmic inclusions are derived from nucleoli and, in particular, from the ultrastructural granular component, or the body of the nucleolus (11) observed by light microscopy. First, the inclusions only formed in cells with nucleoli. Second, various stages of extrusion of nucleolar material have been described by others (4, 5) and the effect of hyperthermia may be to accentuate this phenomenon. However, it should be emphasized that there was no evidence of massive extrusion of nucleolar material in the vast majority of cells so that extrusion of nucleolar material most probably occurred in submicroscopic amounts through nuclear pores. On the other hand, nucleolar extrusion may have occurred with such rapidity that it was only rarely observed. Third, the RNP of the inclusions was cytochemically similar to that of the body of the nucleolus. When the nucleolar RNA was absent in actinomycin-treated cells, the inclusions formed but did not contain RNA. Finally, the electron micrographs indicate that the inclusions consisted of aggregations of ribosome-like particles which may have been derived from the granular component of the nucleolus. Warocquier and Scherrer (26) described condensed particles in the nucleoplasm which they considered to have emerged from the nucleoli of the heated cells. It seems likely that these authors (26) and Simard *et al.* (19, 20) did not observe the inclusions because they heated their cells to lower temperatures than those used in the present experiments.

The possibility that the formation of cytoplasmic inclusions could be the result of activation of a latent viral infection in the heated cells is unlikely. In the 1st place, the inclusions were observed in all 7 cell lines. Second, extensive electron microscope studies failed to reveal any virus-like particles. Finally, the reversibility of the changes and the disappearance of the inclusions within 24 hr of the hyperthermic treatment are unlike the course of a viral infection.

The disaggregation of polysomes by hyperthermia accompanied by an inhibition of protein synthesis has been demonstrated biochemically in HeLa cells (13) and in *Physarum polycephalum* (18). Confirmation of the ultrastructural evidence of polysome breakdown is provided by the results of staining by TBM, Method D, since loss of staining of cytoplasmic granular RNP has been previously shown by a number of methods to coincide with polysomal disaggregation (7). Treatment of HeLa cells with puromycin, which is known to disaggregate polysomes and inhibit protein synthesis, also resulted in a disappearance of cytoplasmic granular RNP and produced nucleolus-like inclusions in the cytoplasm (21, 22). Further studies of inhibitors of protein synthesis should indicate whether there is any causal relationship between inhibition of protein synthesis and the formation of nucleolus-like RNP containing inclusions in the cytoplasm.

Although there were no significant differences in the response of normal diploid cells and neoplastic cells to hyperthermia in the present experiments, more detailed investigation of the mechanism of the cytopathic effects of hyperthermia seems justified to determine whether conditions can be found to produce selective damage to the neoplastic cell similar to that reported by Cavaliere *et al.* (3). Changes in the nucleolus have been described in response to a member of antimetabolites (2, 8). Alteration of the nucleolinar RNP is frequently the 1st sign of cell injury in cells infected with a number of cytopathogenic DNA and RNA viruses (25). In the case of influenza virus infection of HeLa cells, there appears to be correlation between the proportion of cells with loss of stainable nucleolinar RNA and the resultant cytopathic effect. Further studies of the pathology of the nucleolus, and in particular the synthesis and processing of ribosomal RNA in response to hyperthermia and other cytotoxic procedures in normal and neoplastic cells, may throw light on the mechanism of cell injury and oncolysis.

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Fig. 1. Nucleolini of unheated HeLa JJH cells. Toluidine blue-molybdate, Method B after digestion with deoxyribonuclease. The nucleolini stain metachromatically and appear as roughly spherical dark bodies in the photomicrograph (*arrows* indicate nucleolini corresponding to vacuoles in Fig. 3). × 2200.

Fig. 2. Nucleoli of HeLa JJH cells heated for 60 min at 46° in ring cultures. The nucleolini are ill-defined and there is diffuse metachromatic staining of nucleolinar RNP. Some weak orthochromatic staining of cytoplasmic inclusion bodies (*arrows*) can be seen. Stained as Fig. 1. × 2200.

Fig. 3. Same cells as shown in Fig. 1 destained with 70% ethanol and restained with toluidine blue (0.5% in McIlvaine's buffer, pH 3.0 for 10 min). Unstained or weakly stained vacuoles (*arrows*) correspond to metachromatically staining (*dark* in the microphotograph) nucleolini in Fig. 1. × 2200.

Fig. 4. Same cells as shown in Fig. 2. The nucleolinar vacuoles have disappeared and the entire nucleolus stains homogeneously. Numerous inclusions are stained in the cytoplasm. Stained as Fig. 3. × 2200.

Fig. 5. Staining of DNA in untreated HeLa cells. The nucleolar DNA is weakly but definitely stained. Feulgen method, × 2200.

Fig. 6. Absence of staining of nucleolar DNA after hyperthermia at 46° in ring cultures for 1 hr. Nucleolar associated DNA persists. Feulgen method, × 2200.

Figs. 7 and 8. Apparent extrusions of nucleolar material into the cytoplasm of HeLa cells heated at 46° for 5 min stained with toluidine blue. × 2200.

Fig. 9. HeLa cells stained by TBM Method D. A diffuse form of RNP is stained in the nucleoplasm and the cytoplasm contains a granular form of RNP. × 2200.

Fig. 10. HeLa cells stained as Fig. 9 after heating to 46° for 1 hr. Note the absence of cytoplasmic granular RNP. × 2200.

Fig. 11. Untreated HeLa cells. Some rough endoplasmic reticulum is present and ribosomes and polysomes are fairly uniformly distributed throughout the cytoplasm. × 24,000.

Fig. 12. HeLa cells heated in a Blake bottle for 30 min at 45°. There is a decrease in polysomes and ribosomes throughout most of the cytoplasm and a dense collection of ribosome-like particles is present (*arrows*). × 19,600.

Fig. 13. Nucleolus of untreated HeLa cell containing granular and fibrillar (electron-dense) components. × 44,000.

Fig. 14. Nucleolus of HeLa cell heated as cells illustrated in Fig. 12. The granular component has almost completely disappeared and the nucleolus is almost completely fibrillar. × 44,000.

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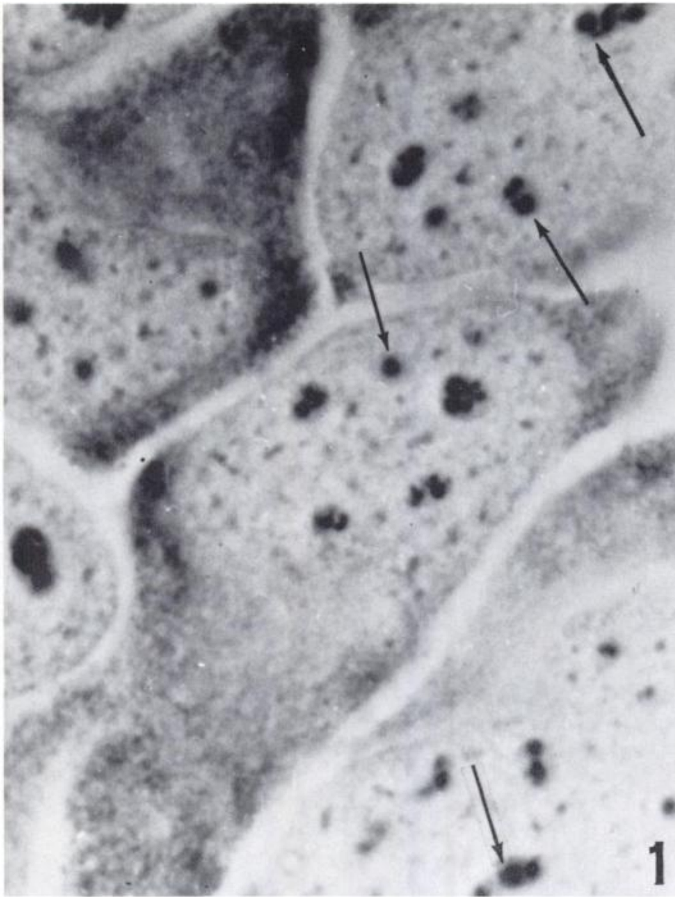

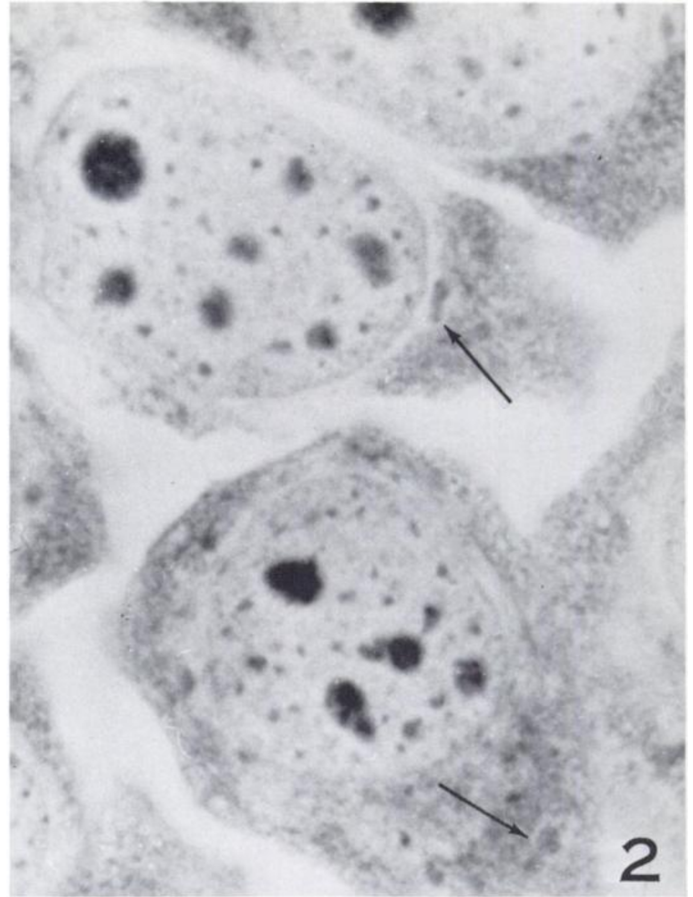

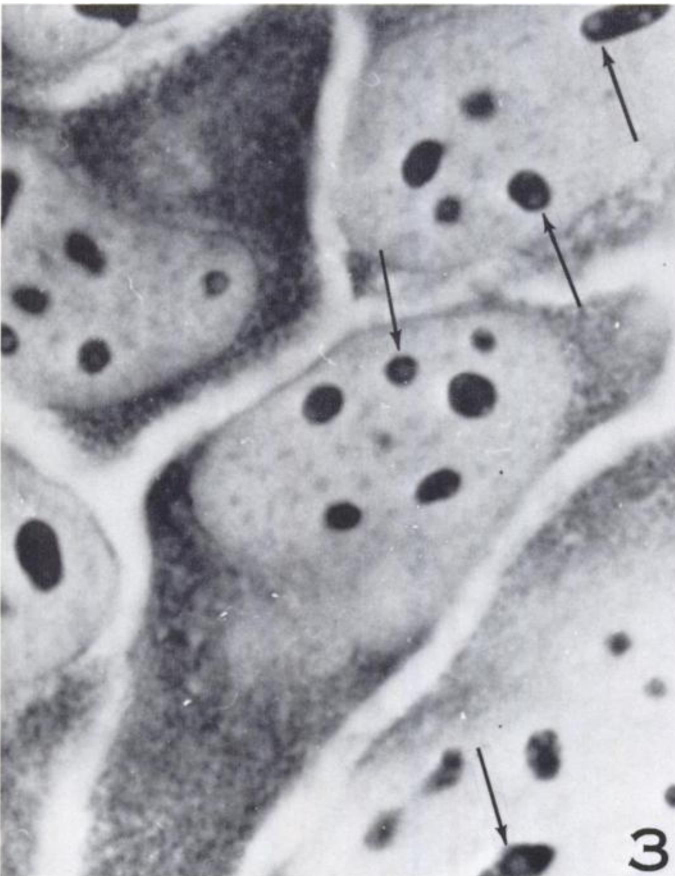

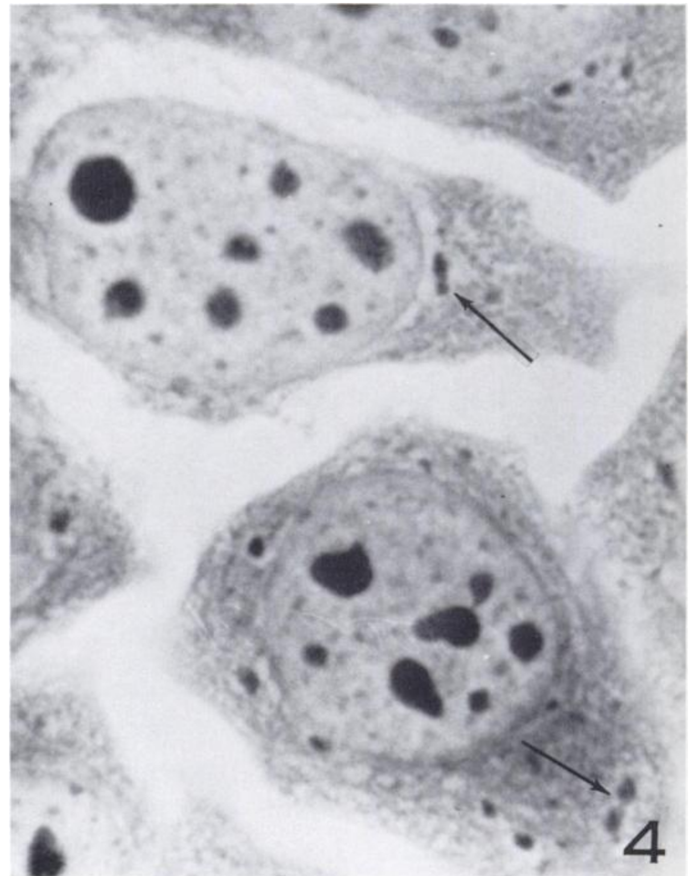

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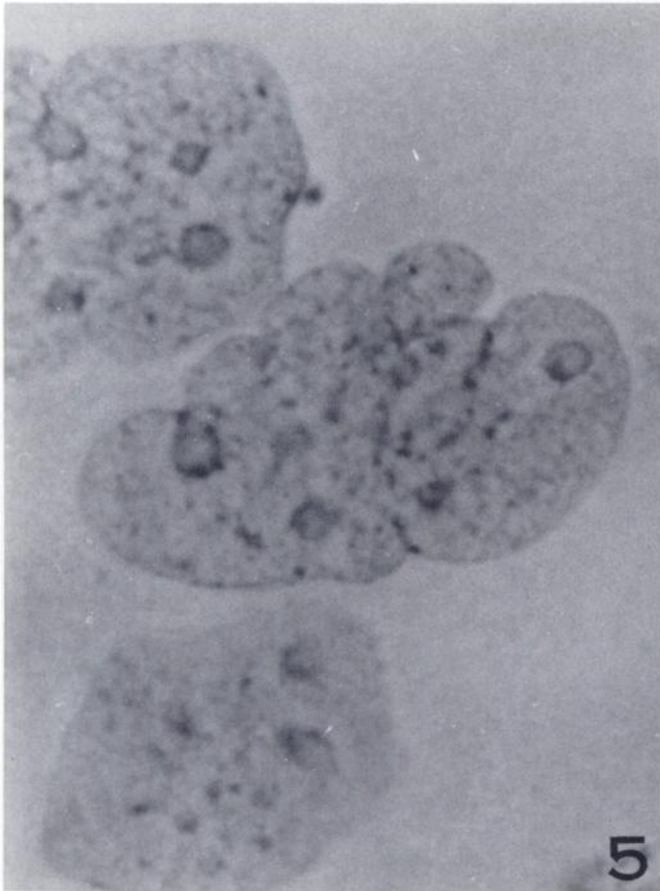

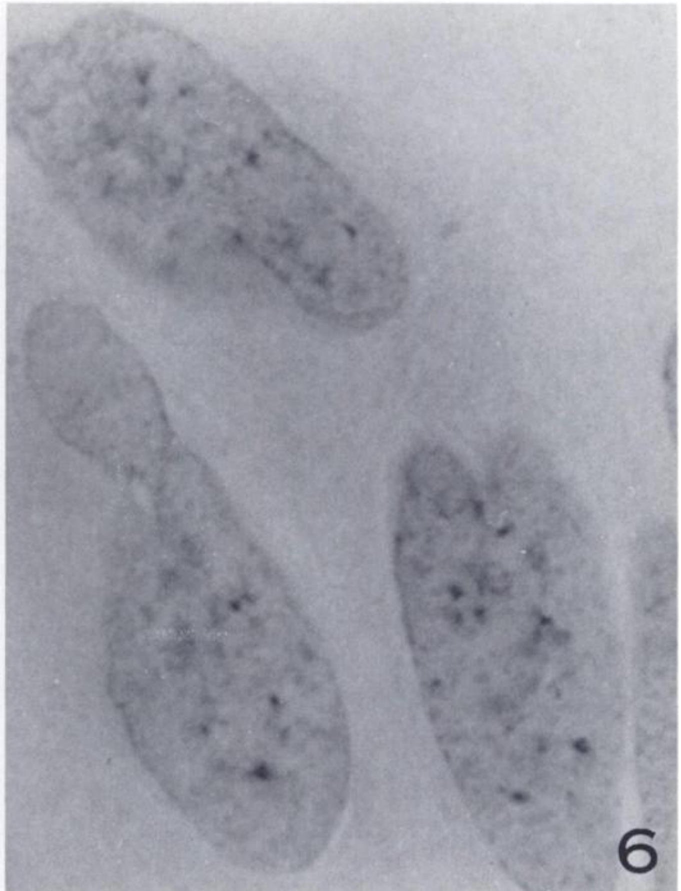

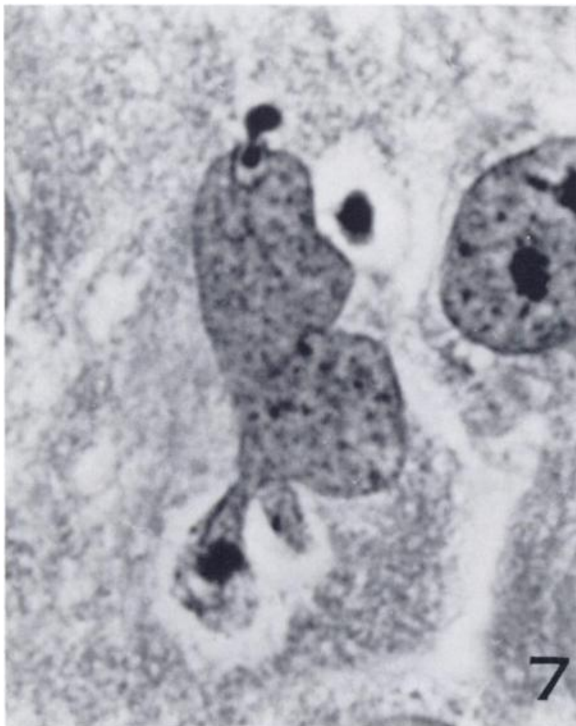

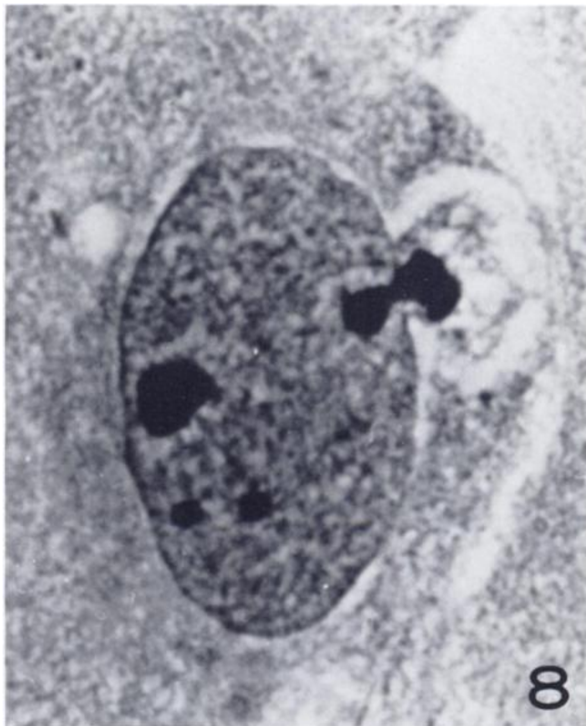

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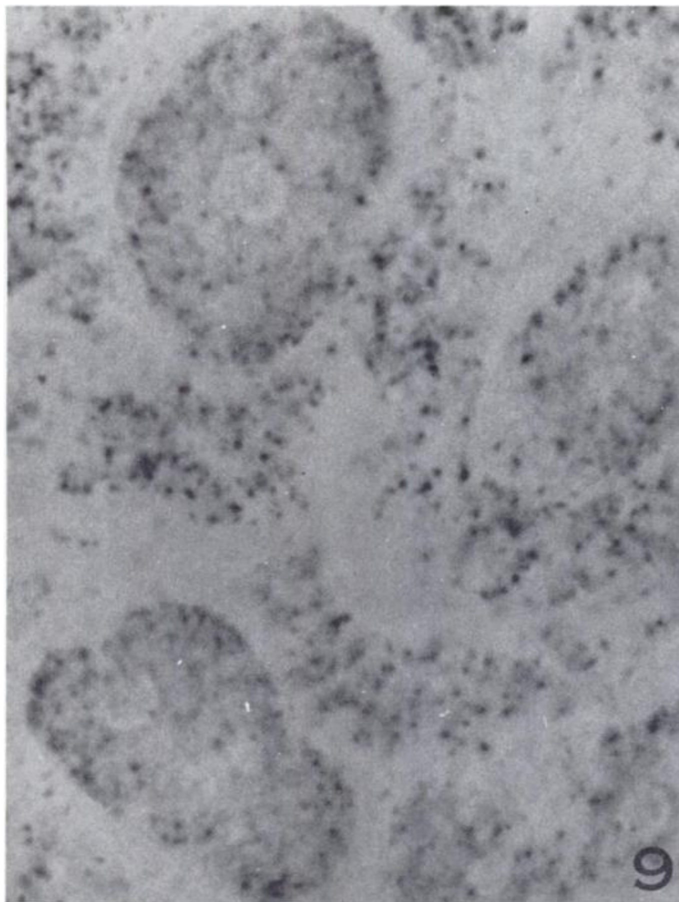

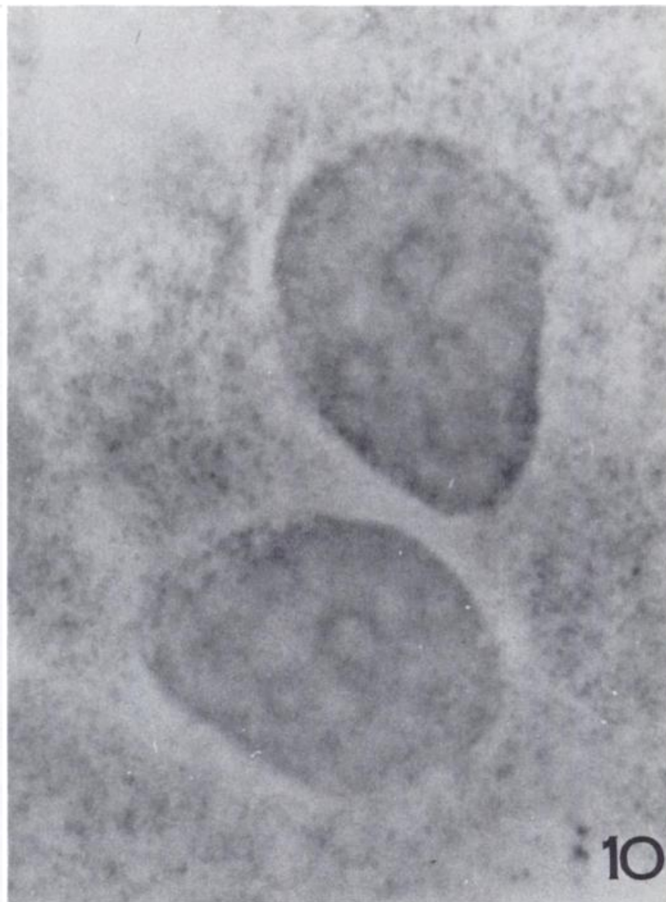

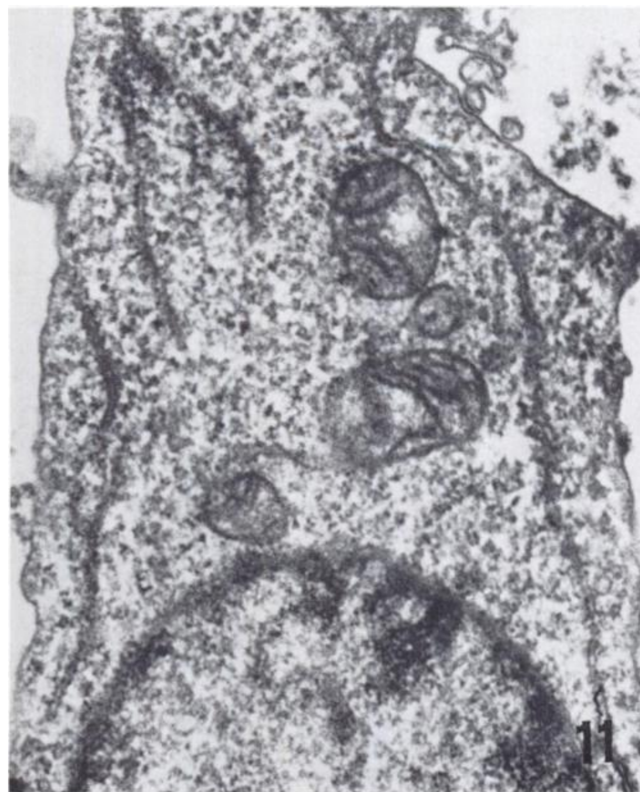

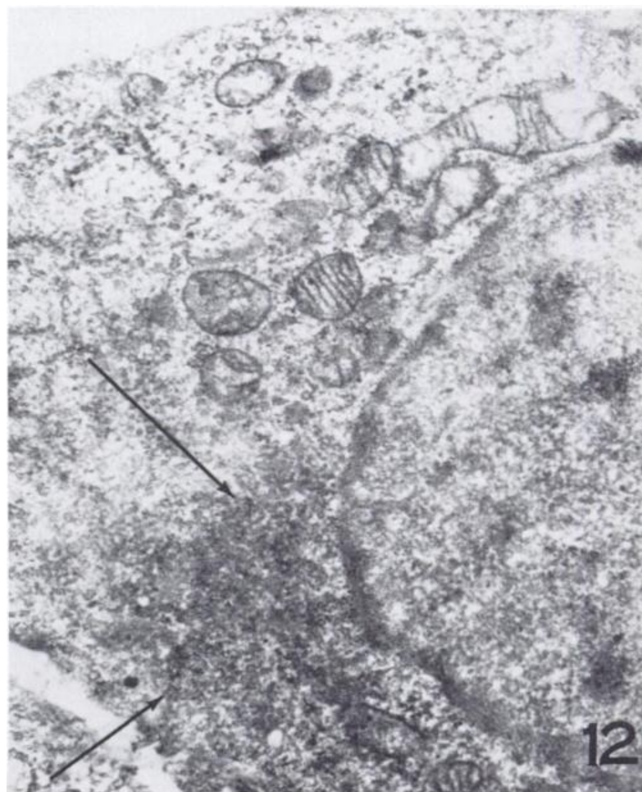

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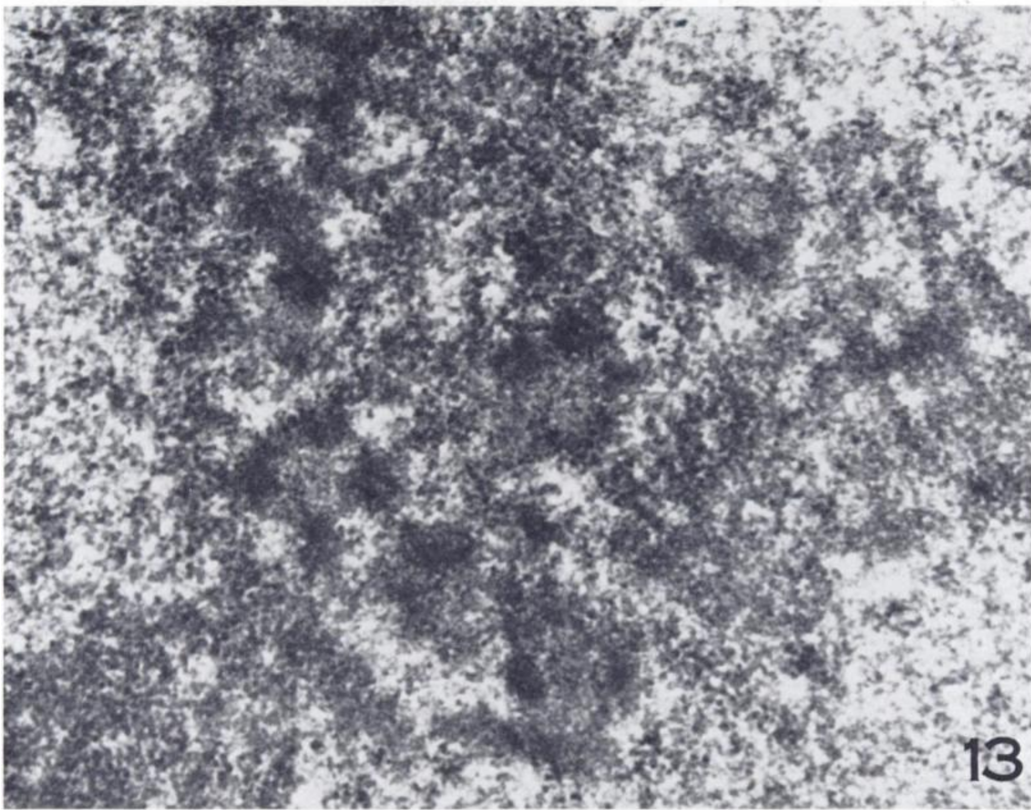

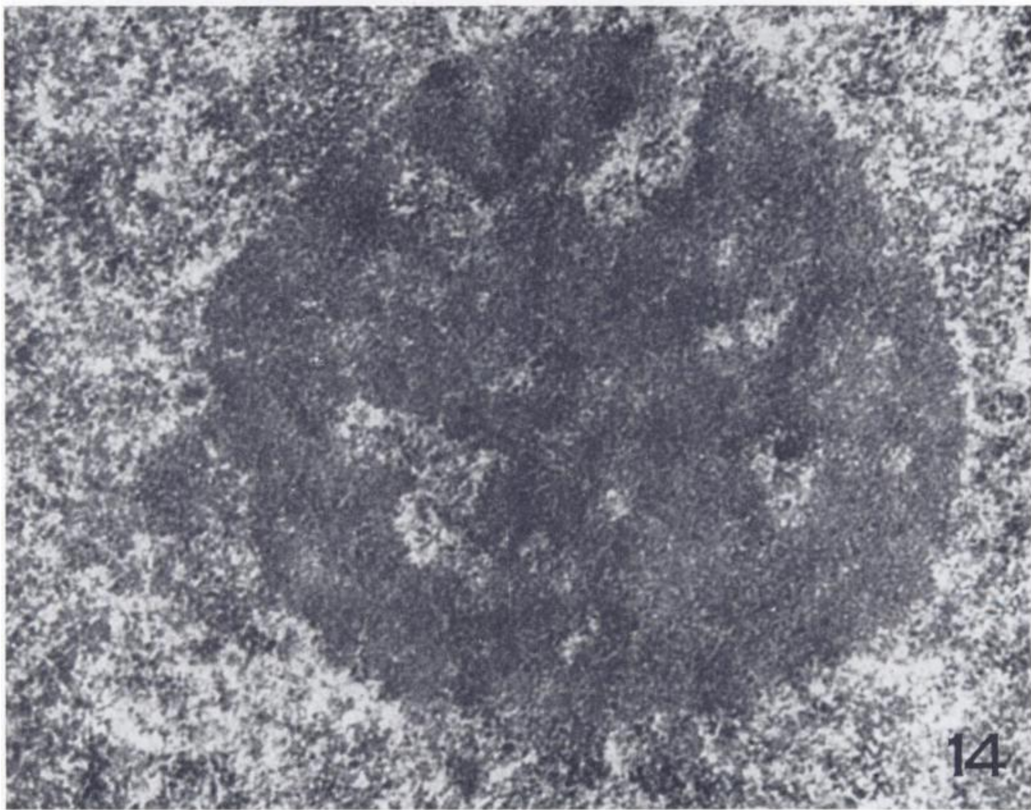

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