

STUDY OF NUCLEOLAR VACUOLATION AND RNA SYNTHESIS IN EMBRYONIC ROOT CELLS OF *ZEA MAYS*

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SUMMARY

The process of nucleolar vacuolation has been quantitatively studied in root cells of the embryo of *Zea mays*. During germination, we have estimated the percentage of vacuolated nucleoli, the number of vacuoles per nucleolus and the volume density of the vacuoles, the latter by application of the stereological principles of morphometry. In the dormant embryo, no vacuoles can be detected in the nucleolus. When germination occurs at 22 or 16 °C, there is a rapid increase in the percentage of vacuolated nucleoli and in the volume density (V_A), with a maximum after 4 and 8 h. At 6 °C, a temperature which does not permit emergence of the root, the percentage of vacuolated nucleoli increases regularly but never reaches the level observed at 16 and 22 °C. In parallel with the appearance of nucleolar vacuoles, the size of the nucleolus decreases statistically and shows a minimum when vacuolation is maximum. This suggests that the appearance of vacuoles within the nucleolus is the result of loss of material from this organelle. Indeed, electron micrographs show that granular components are lost from the nucleolus during the first 8 h of germination at 16 °C. According to the literature, these granular components are probably ribosomal subunits.

An autoradiographic study of tritiated uridine incorporation shows that the nucleolus does not synthesize rRNA at the early beginning of germination. Hence the nucleolus is unable for several hours to rebuild the lost granular elements.

Application of actinomycin D, 5-fluoro-uracil and 2-thio-uracil to the seeds, during the first 48 h of soaking, inhibits rRNA synthesis, the vacuolation process, the loss of granular components and the reduction in size of the nucleolus.

These results allow us to determine some of the physiological conditions characterizing the appearance of the nucleolar vacuoles.

INTRODUCTION

In electron micrographs, the nucleolus of eukaryotes generally appears as a compact and dense organelle. It is essentially made up of 2 distinct zones: one characterized by a higher percentage of fibrillar structures and the other composed of granular elements. Fibrils and granules are enclosed in a clear matrix called fundamental substance (Busch & Smetana, 1970).

In both the animal and plant kingdom, one can observe in the nucleolus an important and temporary appearance of 'zones' which in light microscopy are optically empty, and in electron microscopy are only slightly electron-dense. These zones are called 'nucleolar vacuoles' (Chouinard, 1966; Johnson & Jones, 1967; Barlow, 1970). The origin, the content and the cellular function of these formations have never been fully analysed, even in the most recent studies (Johnson & Jones, 1967; Johnson, 1969; Barlow, 1970; Rose, Setterfield & Fowke, 1972).

Balbani (1864) and Luehr (1928) have observed their appearance *de novo* inside the nucleolus. Jordan & Chapman (1971), who made electron-microscopic studies of artichoke cells going from a quiescent to an active state, observed the formation of nucleolar L-zone by the intrusion and the subsequent dispersion of rDNA fragments in the nucleolus. This L-zone is very similar to vacuoles.

Electron microscopy provides relatively poor information on the content of the nucleolar vacuoles. Chouinard (1964) and Johnson (1969) note within the vacuoles the presence of fibrils and granules which constitute only a very small part of the total vacuolar volume.

Cytochemical tests have not revealed the presence within the vacuoles of metal ions, enzymes, proteins or lipids (Macary, 1954; Brachet, 1953, 1957; Albertini, 1959; Johnson & Jones, 1967). It is nevertheless possible that vacuoles contain specific material inside the nucleoli or particular cells (Favard, 1962). In *Vicia*, Chouinard (1964) and Johnson & Jones (1967) have shown the existence of ribonucleo-proteins in the vacuoles. In *Arbacia* eggs, Esper (1965) has found granules which stain with azur B and Sudan black B. Moreover, Kordan (1969) and Kordan & Preston (1968) have indicated that the vacuole content could be liquid crystals, and other authors have suggested the presence of RNA within the nucleolar vacuoles (Esper, 1965; Love & Walsh, 1968).

Various hypotheses have been proposed to explain the cellular function of nucleolar vacuoles. Several authors have observed a relationship between the restart of RNA synthesis in the nucleolus after a natural or an induced stoppage on the one hand, and the appearance of significant nucleolar vacuolation on the other (Johnson, 1969; Zybina, 1968; Deltour & Bronchart, 1971). A correlation is established between both facts: vacuoles could be absent from the nucleolus at rest and would reappear just before the restart of the ribosomal RNA synthesis. This simple correlation, however, is not confirmed in all examples of nucleolar vacuolation (Barlow, 1970). Observations on living cells have proved that the nucleolar vacuoles are not always static structures but that, in several species, they can be moved by cyclic pulsations or movements in the nucleolus (Johnson & Jones, 1967; Rose *et al.* 1972). The pulsations would induce the discharge of the vacuolar contents out of the nucleolus. These observations suggest that the vacuoles play a role in the transport of nucleolar substances into the cytoplasm. It is interesting to compare this hypothesis with the one proposed by Harris (1970) which postulates that the presence of an actively rRNA-synthesizing nucleolus is required to allow the transfer of chromatinic mRNA into the cytoplasm. But this transporting function played by the nucleolar vacuoles does not agree with all the data. According to Rose *et al.* (1972) 'nucleolar vacuoles do not appear to be the sole, or even main route of exit for material leaving the nucleolus but when present are probably involved in transport of some types of nucleolar RNA'. An autoradiographic study (Deltour, 1970) complemented by an ultrastructural study (Deltour & Bronchart, 1971) have revealed that after about 8 h of imbibition significant nucleolar vacuolation occurs during germination in radicular cells of *Zea mays*. This vacuolation immediately precedes the restart of nucleolar RNA synthesis and greatly decreases when the nucleolus incorporates [³H]uridine. The situation is

particularly favourable for observing possible correlations between the nucleolar ultrastructure, the vacuolation process and the nucleolar RNA synthesis. The purpose of this study is to establish those correlations.

MATERIAL AND METHODS

Germination conditions

Kernels of *Zea mays* L.cv. 'Inra 258' were soaked in Petri dishes in darkness in an Ehret temperature box at 16 °C. In these conditions the percentage of germination was always over 90.

Use of inhibitors of the nucleic acids synthesis

Three inhibitors of nucleic acids synthesis were used: actinomycin D, 2-thio-uracil (2-TU) and 5-fluoro-uracil (5-FU) at a concentration which gives a 50% inhibition of growth of the primary root if applied continuously during the first 48 h of germination. Reversion tests with orotic acid were made for 2-TU and 5-FU; they showed that the acid reverses the action of 2-TU on radicular growth but has no effect on the action of 5-FU.

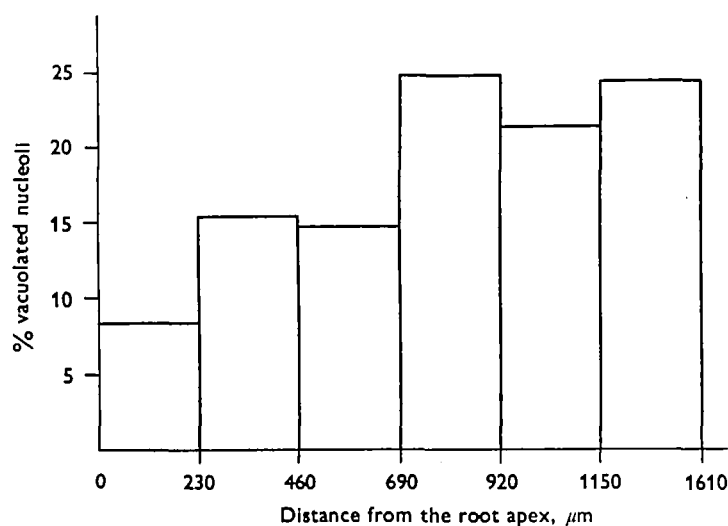


Fig. 1. Differences in the percentage of vacuolated nucleoli along the root. The longitudinal median root section (excluding the cap and the coleorhiza) is transversely divided into bands 230 μm wide (diameter of the microscopic field at the 50 \times magnification). The percentage of vacuolated nucleoli is observed in each sector. Every column represents the average of the same sector in 4 different roots.

Hence we consider that 2-TU acts with *Zea mays*, in our working conditions, as a specific inhibitor of RNA synthesis. Since the situation is different with 5-FU, it will be more difficult to find a correlation between its action and the nucleolar vacuolation.

At sampling times, the first millimetre of 10 radicules – the root cap being excluded – was cut off and fixed for 3 or 5 h at 4 °C in 6% glutaraldehyde, pH 7, buffered with sodium cacodylate, 0.1 M. After 3 washing periods of 30 min, the samples were postfixed 12 h at 4 °C in 2% osmium tetroxide and washed in distilled water. Dehydration was carried out in a graded ethanol series and the samples were finally soaked in propylene oxide. Embedding was done in a mixture of epoxyresins. Sections 0.5 μm thick were obtained on a Porter-Blum MT1 ultramicrotome. Staining was obtained by soaking the sections in a 0.1% toluidine blue

solution (E. Gurr) buffered with Na_2CO_3 (pH 11.3) for 12 min at 40 °C. With this stain, the nucleolus appears in intense dark violet blue. The nucleolar vacuoles which form a clear unstained zone can be easily identified (Fig. 7). The slides were dried overnight at 50 °C and mounted in a synthetic resin (HSR).

Our observations were made on transverse sections of roots 1000 μm away from the apex and essentially in the cortex tissue. Hence it is possible to investigate on one section a great number of cells equidistant from the radicular end. The choice for such a procedure is based on a preliminary study of longitudinal sections. This study deals with the distribution in the percentage of vacuolated nucleoli in the cortex as a function of the distance from the radicular end. Fig. 1 shows that after 8 h of germination, the percentage of vacuolated nucleoli reaches high values, 1000 μm away from the radicular apex.

Histoautoradiography

[G-³H]uridine (Radiochemical Centre, Amersham, England; specific activity 1–3 Ci/mM) was used at an activity of 250 $\mu\text{Ci/ml}$. Isolated embryos were submerged in 1 ml of the radioactive solution for 90 min and then washed in tap water for 30 min. The first millimetre of the radicle was cut off and fixed as described above. Before the osmium tetroxide postfixation, the samples were washed in sodium cacodylate buffer for 24–48 h in order to eliminate the non-incorporated marker (Monneron & Moulé, 1969). In darkness, the sections were covered with Kodak AR10 stripping film which was previously laid on distilled water at 28 °C. The slides were stored in boxes at 4 °C and exposed for a few weeks. After developing and drying, the autoradiographs were stained with 0.1 % toluidine blue, differentiated with 70 % ethanol and mounted in HSR.

One hundred cells in each series were used for the counting of silver grains; the results are given with the subtracted background. The background was always estimated from equivalent areas adjacent to the autoradiograph analysed, but lacking any sectioned material.

Recording methods

The sections were observed with a Wild M20 microscope at a final magnification of 1250. When the nucleolus was vacuolated in the cortical cells, the number of vacuoles and their size were variable. The smallest vacuoles distinguishable in light microscopy are 0.25 μm in diameter; we therefore took into account vacuoles of at least this size.

Determination of the percentage of vacuolated nucleoli. For each individual, 100 nucleoli were examined and the vacuolated nucleoli counted. Each series contains 10 individuals. Hence each point of a graph represents a percentage figure made from a total of 1000 nucleoli.

Determination of the number of vacuoles per nucleolus. The average number of vacuoles per nucleolus was determined in 100 nucleoli taken from the same section, and 10 individuals were observed.

Determination of the volume density of the nucleolar vacuoles by the stereological principles of morphometry. We determined the variation of the relative volume occupied by vacuoles in the nucleolus at various moments of germination. This was achieved using the stereological principles of morphometry (Weibel, 1969): for each sampling time, 100 different nucleoli were photographed on a Zeiss photomicroscope with a 100 \times immersion objective. Negatives were projected at a final magnification of 40000 on a screen made of a quadratic test system the interpoint of which was 8 mm, i.e. 2 mm less than the diameter of the smallest vacuole visible in projection. We have recorded the number of points of the quadratic system covered by the nucleolus (P_N) and the vacuoles (P_V). The relative volume of the vacuoles $V_A = P_V/P_N$ is expressed in per cent.

For the vacuoles with a diameter greater than 0.5 μm , the ratio (vacuole diameter)/(thickness of section) is greater than 1. In this case our measures are underestimated due to the 'Holmes effect' (Weibel, 1969). Consequently maxima could be slightly higher; minima are less affected.

Absolute measures of nucleolar size. Absolute measurements of nucleolar diameter were made on negatives projected on a screen at a final magnification of 40000. Each point of the graph (Fig. 4) represents an average score made from a total of 1000 nucleoli.

Electron microscopy

Sampling, fixation and embedding were similar to the procedures described for light microscopy. Ultrathin sections about 60 nm thick were cut using a Porter-Blum MT2 ultramicrotome, stained with saturated aqueous uranyl acetate and Reynolds (1963) lead citrate and finally observed with a Siemens Elmiskop 101 electron microscope.

RESULTS

Influence of the germination temperature on the process of nucleolar vacuolation

Vacuolation was studied at 3 different germination temperatures: 22, 16 and 6 °C. At the first 2 temperatures, kernels germinated normally (90%). At 6 °C, although a slow imbibition by the embryos was noted, the protrusion of the root was never observed. Anyway, only little cellular synthesis is possible in this condition (Table 2, p. 104).

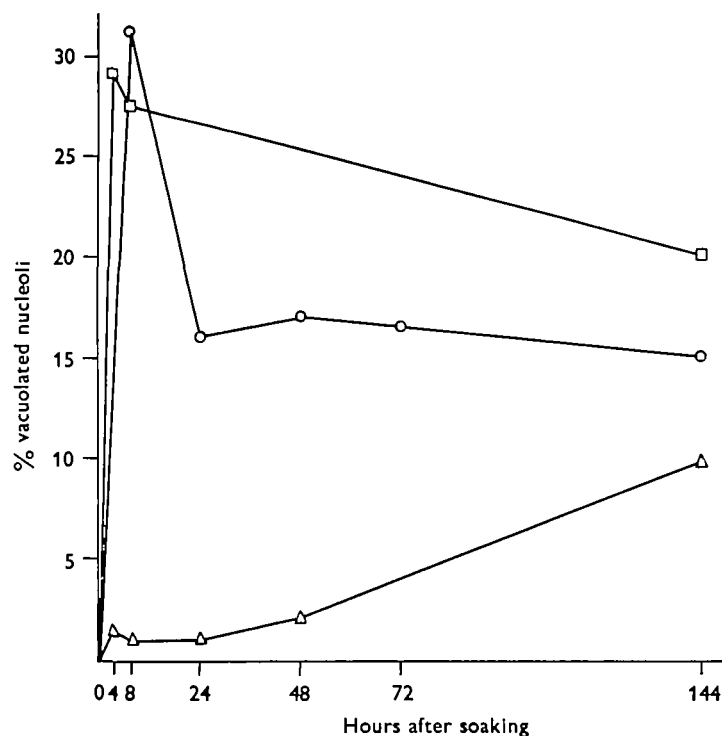


Fig. 2. Effect of different temperatures of germination upon the percentage of vacuolated nucleoli in the cortical cells. Δ — Δ , 6 °C; \circ — \circ , 16 °C; \square — \square , 22 °C.

16 °C. During germination at 16 °C, the percentage of vacuolated nucleoli in cortical cells varied as a function of the germination time (Fig. 2). It was zero after 30 min of germination but rose very quickly to reach a maximum of 31% after 8 h (Fig. 2). Between hours 24 and 144, approximately 15% of the nucleoli were

vacuolated. Comparison of the percentage of vacuolated nucleoli at different times of germination by means of statistical analysis showed a significant increase after 8 h ($P < 0.001$).

Table 1. Mean number of vacuoles in a vacuolated nucleolus within cortex cells or stele cells at different moments of germination

	Hours of soaking				
	4	8	24	72	144
Cortex	2.3	2.6	3.1	3.0	5.9
Stele	1.4	1.7	2.0	2.5	6.1

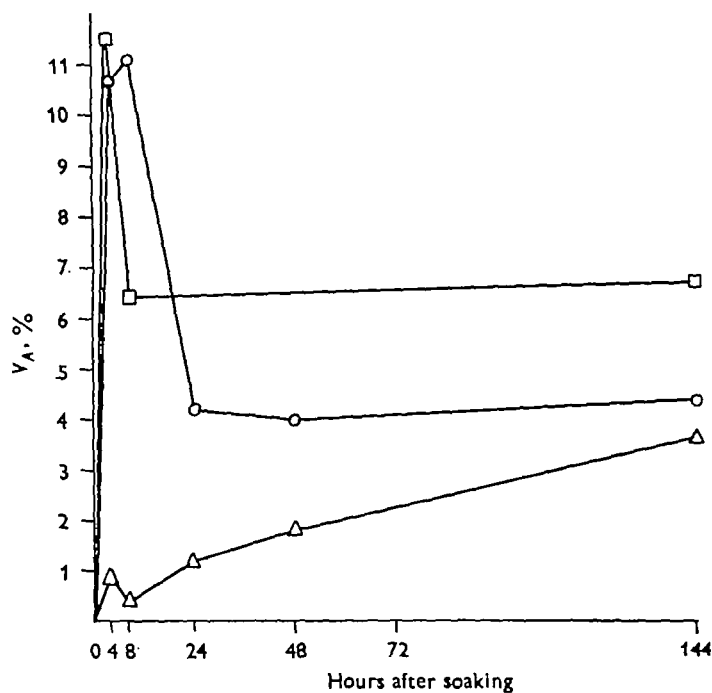


Fig. 3. Effect of different temperatures of germination on the volume density (V_A , in %) of nucleolar vacuoles within cortical cells. Δ — Δ , 6 °C; \circ — \circ , 16 °C; \square — \square , 22 °C.

The number of vacuoles per vacuolated nucleolus was also recorded (Table 1). An increase in the number of vacuoles was observed as a function of the germination time (Table 1). The same increase was observed in stele of the root cells.

The relative volume of vacuoles per nucleolus (V_A) was determined: as in the case of the percentage of vacuolated nucleoli, there is a maximum after 8 h (11% of the nucleolar volume) and a constant value of 4% of the nucleolar volume is reached after 24 h (Fig. 3).

The absolute measures of the nucleolar diameter are recorded in Fig. 4. Comparison of the nucleolar size at 0 and 8 h of germination showed a significant decrease after 8 h ($P < 0.05$). By comparing Figs. 3 and 4, we can see that at the time when the nucleolus is smallest the volume occupied by the vacuole is greatest.

22 °C. Vacuolation occurs at 22 °C as it does at 16 °C (Figs. 2, 3). However, the maxima in the percentage of vacuolated nucleoli and the relative vacuolar volume (V_A) occurred 4 h earlier. Such an acceleration in nucleolar vacuolation can be correlated with an acceleration in the resumption of RNA synthesis following a similar increase in temperature (Deltour, 1972).

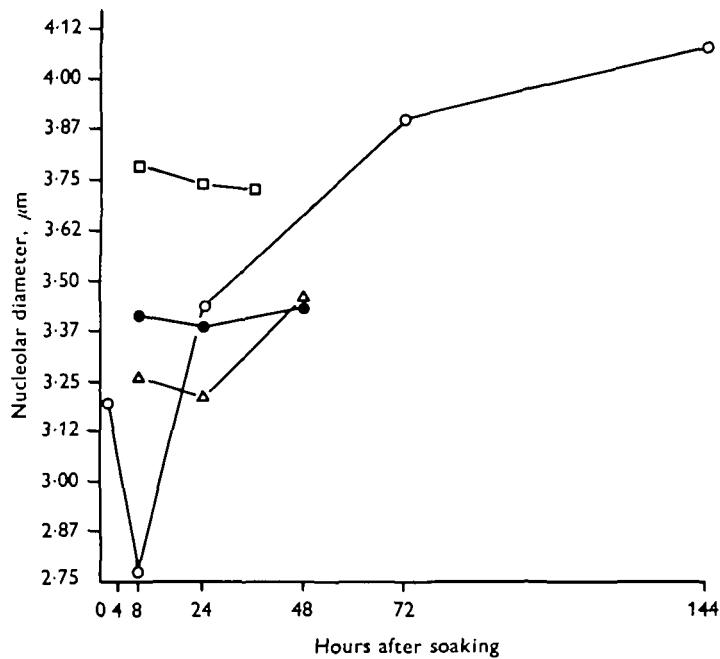


Fig. 4. Effect of actinomycin D, 5-FU and 2-TU on nucleolar size in cortical cells at 16 °C. ○—○, control; ●—●, actinomycin D; △—△, 5-FU; □—□, 2-TU.

6 °C. Vacuolation was almost absent during the first hours of imbibition at 6 °C. The percentage of vacuolated nucleoli slowly increased during the 144 h of observation but never exceeded 10% (Fig. 2). All variations in this percentage were reflected in changes in the vacuolar volume, though they both remained very slight (Fig. 3).

Hence with *Zea mays* we may observe a certain correlation between the vacuolation process and germination, since the best condition of germination corresponds with a maximum in vacuolation.

Influence of inhibitors of nucleic acid synthesis

The 3 drugs inhibited to various degrees the percentage of vacuolated nucleoli (Fig. 5). This decrease was always significant.

The action of actinomycin D was particularly drastic. The rise in the percentage of vacuolated nucleoli occurs in control seeds and those treated with drugs, despite the lower number of vacuolated nucleoli, and the maximum at the 8th hour remains visible except following actinomycin D treatment. That effect on the number of vacuolated nucleoli is reflected in the evolution of the relative volume of the nucleolar vacuoles (Fig. 6).

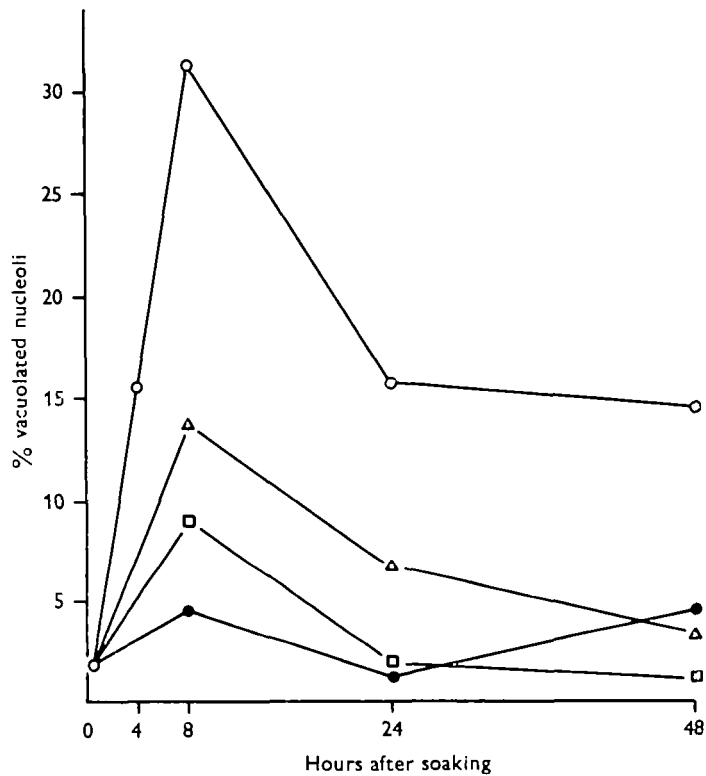


Fig. 5. Effect of actinomycin D, 5-FU and 2-TU on the percentage of vacuolated nucleoli in cortical cells at 16 °C. ○—○, control; ●—●, actinomycin D; △—△, 5-FU; □—□, 2-TU.

Besides their action on vacuolation, the inhibitors modified the increase in nucleolar size: they prevented the decrease in size which was normally observed during the first 8 h of germination (Fig. 4).

Ultrastructural study

The action of the inhibitors on the decrease in nucleolar vacuolation is important. Therefore we decided to investigate the problem of the ultrastructural modifications.

In the absence of inhibitors and at the start of germination, the nucleolus of *Zea mays* showed a fibrillogranular structure (Fig. 10). After 8 h, vacuolation occurred in a few nucleoli and the granular structures had practically disappeared (Fig. 11). After 48 h germination, the nucleolus had again a granulofibrillar structure with the

granules mainly localized at the periphery of the nucleolus (Fig. 12). This observation agrees well with the results obtained by Deltour & Bronchart (1971).

The 3 inhibitors have a similar action on nucleolar ultrastructure. The 2 main modifications they cause are a decrease in number of vacuoles and the persistence of granular and fibrillar zones during the 48 h of treatment (Fig. 13).

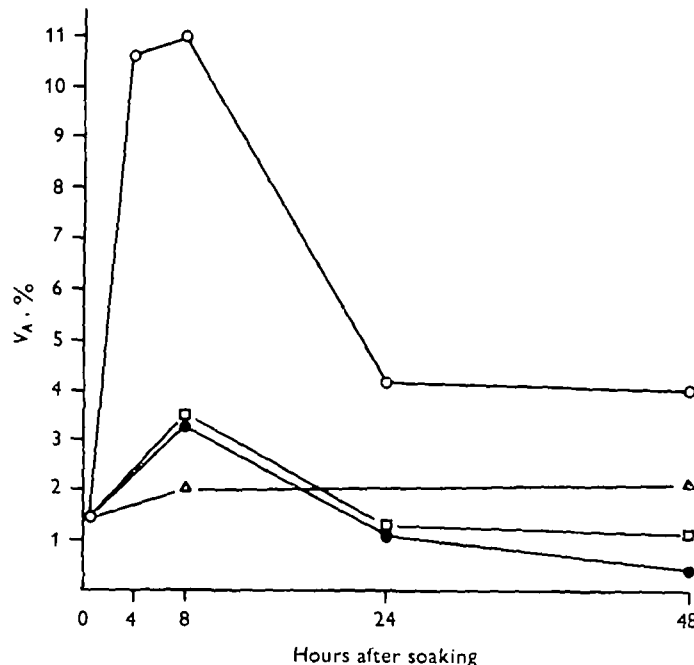


Fig. 6. Effect of actinomycin D, 5-FU and 2-TU on the volume density of nucleolar vacuoles of cortical cells at 16 °C. ○—○, control; ●—●, actinomycin D; △—△, 5-FU; □—□, 2-TU.

Histoautoradiographic study

Actinomycin D is a specific inhibitor of RNA synthesis. It has a strong action on the nucleolar vacuolation in *Zea mays*. Therefore, we studied tritiated uridine incorporation into the chromatin and the nucleolus at different times of germination, with or without the inhibitor (Figs. 8, 9; Table 2).

At 16 °C, in the absence of actinomycin D, very little radioactivity was found in the nucleolus after 8 h, i.e. at the very time when the nucleolar vacuolation was most apparent.

After 48 h, when vacuolation was less, a great amount of radioactivity was detected in the nucleolus. In the presence of actinomycin D, uridine incorporation in both the nucleolus and the chromatin was similar to the control after 8 h germination but practically non-existent after 24 h.

[³H]uridine incorporation was observed at 6 °C. At this temperature, only slight nucleolar vacuolation was recorded (Fig. 3).

Table 2. Number of silver grains above nucleolus and chromatin during germination in different conditions

Conditions of germination	Average number of silver grains above	
	Nucleolus	Chromatin
Control 16 °C, 8 h	1.3	6.6
Control 16 °C, 24 h	2.4	8.4
Control 16 °C, 48 h	6.3	11.4
Act. D 16 °C, 8 h	1.5	3.5
Act. D 16 °C, 24 h	0.0	0.9
Act. D 16 °C, 48 h	0.0	0.5
6 °C, 48 h	0.7	5.6

DISCUSSION

Johnson & Jones (1967) and Zybina (1968) have observed that vacuolation of the nucleolus occurs just before the revival of RNA synthesis by this organelle. However, these authors were unable to give any explanation of the appearance of these structures.

In *Zea mays* we described a very similar situation. Indeed, there is no vacuole in the nucleolus of quiescent cells. Vacuolation of the nucleolus occurs quickly during the imbibition by the seeds and reaches a maximum after about 8 h at 16 °C and after about 4 h at 22 °C. Subsequently, during germination, the vacuoles are still present in the nucleolus but their number has decreased. The amount of RNA synthesis in the nucleolus is small during the first 10 h of germination (Table 2). To understand the part played by the vacuoles, it is important to discover the exact origin of the vacuolation in the nucleolus which occurs at the beginning of germination. The data we have obtained on *Zea mays* have led us to explain this process by a quick and significant loss of granular components in the nucleolus. This loss cannot be immediately balanced, because at the beginning of germination the nucleolus is momentarily incapable of rebuilding this type of component. This explanation is based on the correlation of several observations.

(1) When the percentage of vacuolated nucleoli and the relative volume of the nucleolar vacuoles reach their maxima, the nucleolus shows its minimal size (Figs. 2-4). The reduction in nucleolar size during the early hours of germination can easily be explained by a loss of substance from this organelle (Girard, Penman & Darnell, 1964; Schwartz & Garofalo, 1967). Such a loss of substance accompanies the appearance of intranuclear vacuoles.

(2) Electron micrographs of the nucleolus show that the nucleolus loses nearly all its granular components during the first 8 h of germination.

(3) In the presence of inhibitors of RNA synthesis, nucleolar vacuolation does not occur; the granular components remain in the nucleolus and the size of this organelle does not change.

(4) We can consider, with Busch & Smetana (1970), Calarco & Brown (1969) and Hillman & Tasca (1969), that the granular components of the nucleolus are ribosomal

subunits. Since no rRNA is synthesized in *Zea mays* embryo root cells before the eighth hour of germination (Van de Walle, 1971 *a, b*), ribosomal subunits cannot be rebuilt at the beginning of the germination. Our autoradiographic study of [³H]uridine incorporation by the nucleolus has also shown that it is practically nil during the first hour of incubation.

With *Zea mays* we are now able to give the following theoretical explanation for the vacuolation process during germination at 16 °C.

Period from 0 to 8 hours. With the beginning of the soaking period, an important synthesis of proteins occurs in all the radicular cells (Deltour, 1972). This synthesis requires the presence of ribosomes, the elements of which are normally synthesized by the nucleolus. The latter, at this moment, is unable to synthesize RNA and supplies the cytoplasm with the ribosomal subunits accumulated during the embryonic maturation. The nucleolus becomes progressively free of its granular elements and subsequently a significant vacuolation of this organelle occurs together with a diminution of its size.

After the 8th hour of germination. The incorporation of [³H]uridine by the nucleolus increases regularly and there is rRNA synthesis. At that time the nucleolus rebuilds its granular components; nucleolar vacuolation is less obvious and the size of the nucleolus increases progressively.

Differences observed at the 3 tested germination temperatures

At 22 °C, the changes are similar to those observed at 16 °C (above) but for an acceleration of all the phenomena. Protein synthesis is more important and nucleolar RNA synthesis resumes earlier (2nd hour of imbibition) (Deltour, 1972).

At 6 °C, in contrast to 16 and 22 °C, a maximum in nucleolar vacuolation is not observed (Fig. 2). At this temperature the embryo is never characterized by any appreciable growth. Therefore, it is reasonable to assume that protein synthesis has not reached the level observed at higher temperatures when the embryo grows quickly. The exportation of granular components postulated above cannot take place suddenly and vacuolation never becomes conspicuous.

Effect of inhibition of nucleic acid synthesis

The 3 inhibitors tested have the same apparent effect on the nucleolus: they partially block the vacuolation process and the loss of granular elements. Curiously, in *Helianthus tuberosus* L., 5-FU has an opposite action: it induces the formation of giant vacuoles (Rose *et al.* 1972).

Finally, several conditions must prevail together at the cellular level to produce nucleolar vacuolation. These conditions are often present only in a transitory way and this explains quite well why numerous nucleolar vacuoles, in a given species, appear only in a fleeting manner or even sometimes never appear at all. On the other hand, the various observations recorded at the different temperatures show that the environmental conditions of the organism are important.

We can also wonder why the nucleolar vacuoles do not appear in all the nucleoli

at the same time. In this respect, the vacuoles could possibly be compared with mitoses which occur only in a few per cent of cells at the same time although all the cells are dividing.

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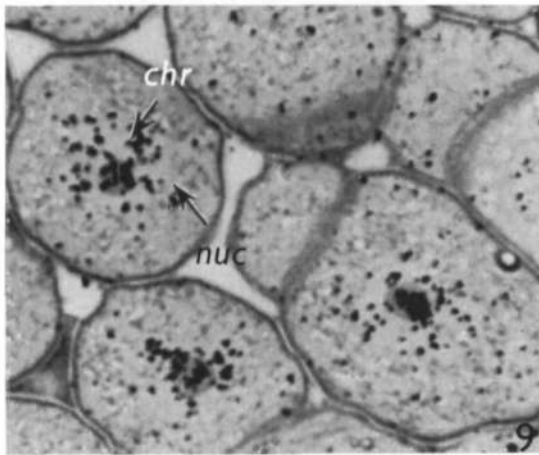
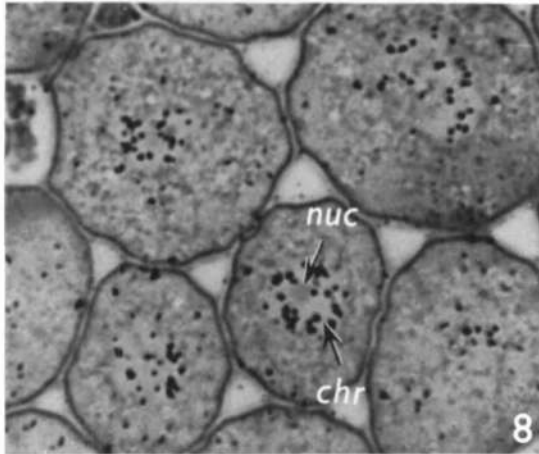
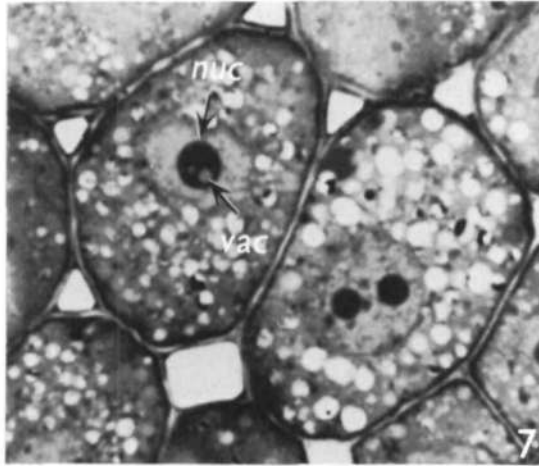
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Fig. 7. 0.5- μ m section of embryo root cells of *Zea mays* stained with toluidine blue. Eighth hour of germination at 16 °C. Nucleoli (*nuc*) with vacuoles (*vac*) are visible. $\times 1600$.

Fig. 8. Autoradiograph of cortical cells of *Zea mays* after 8 h germination. Silver grains are associated principally with chromatin (*chr*). Few grains occur above the nucleolus (*nuc*). $\times 1600$.

Fig. 9. Autoradiograph of cortical cells of *Zea mays* after 24 h germination. Silver grains occur above the nucleus. The nucleolus (*nuc*) is more radioactive than in Fig. 8. *chr*, chromatin. $\times 1600$.



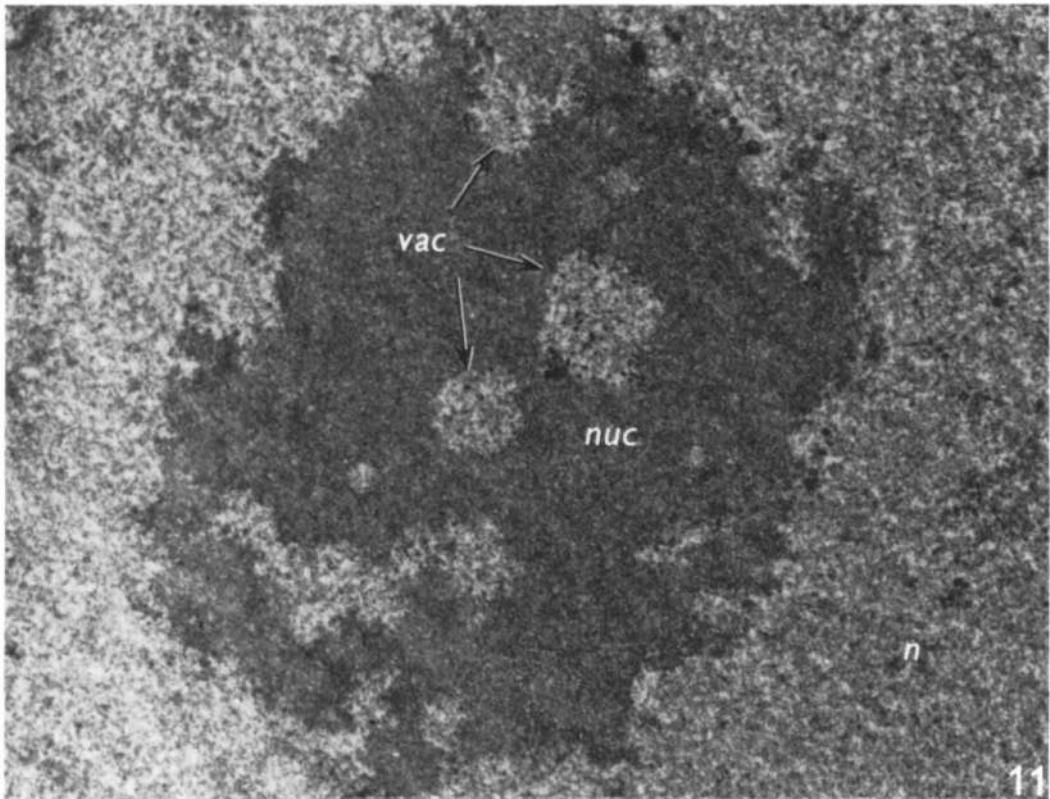
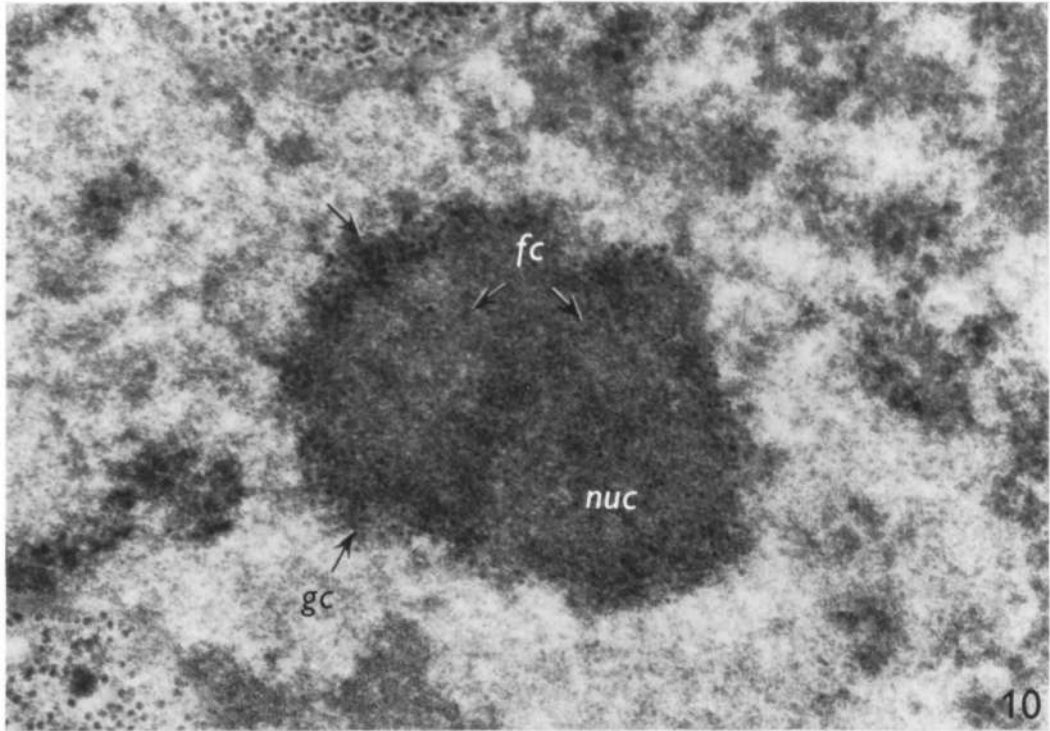


Fig. 10. Section of nucleolus (*nuc*) at 0 h of germination showing both granular (*gc*) and fibrillar components (*fc*). $\times 30000$.

Fig. 11. Part of nucleus (*n*) at the eighth hour of germination in normal conditions. The nucleolus (*nuc*), depleted of the granular components, possesses vacuoles (*vac*) with dense granules. $\times 30000$.

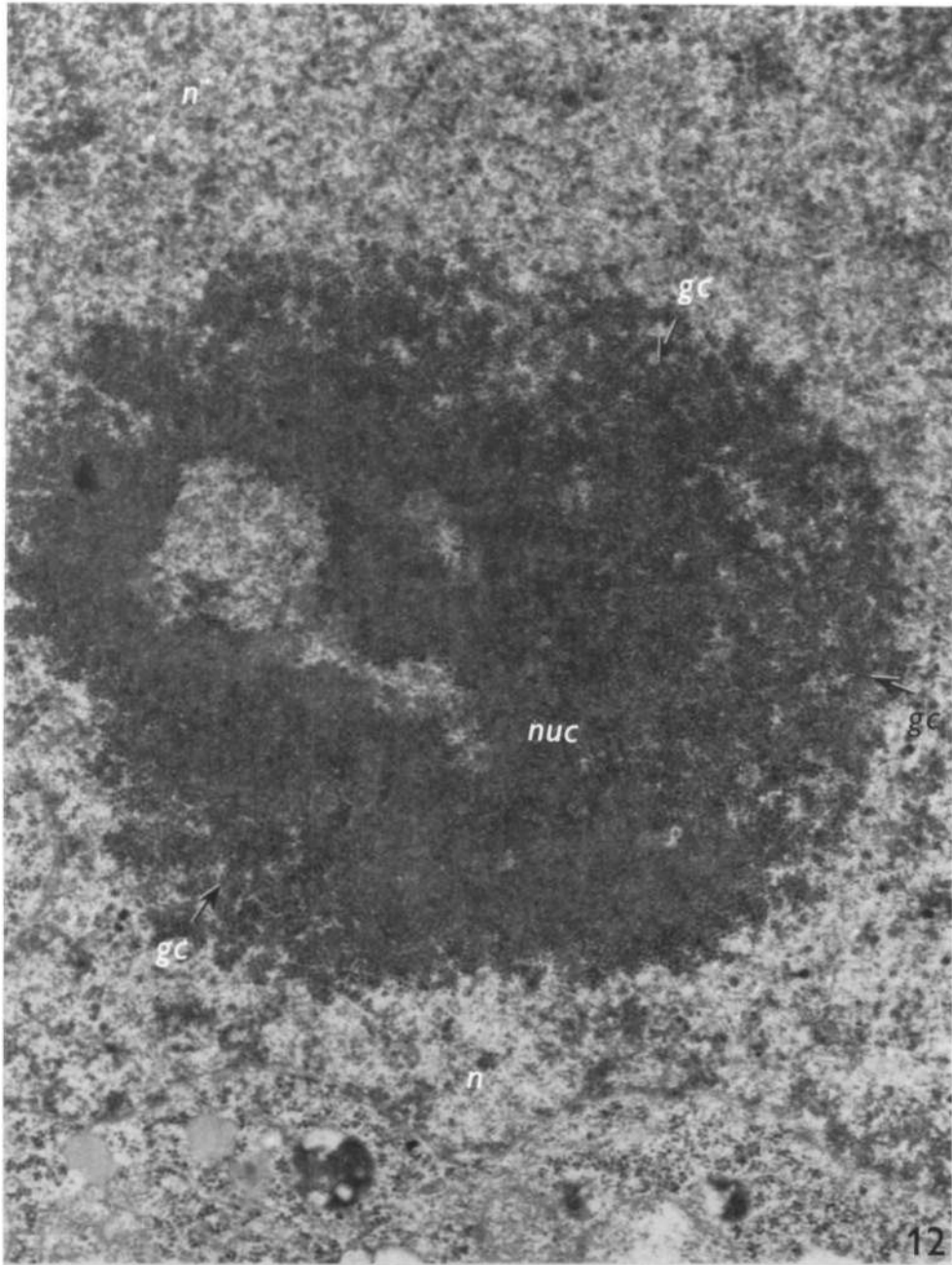


Fig. 12. Portion of the nucleus (*n*) at the forty-eighth hour of germination at 16 °C. The heterochromatin forms a loose network. A ring of granular structures (*gc*) is visible at the periphery of the nucleolus (*nuc*). × 30000.

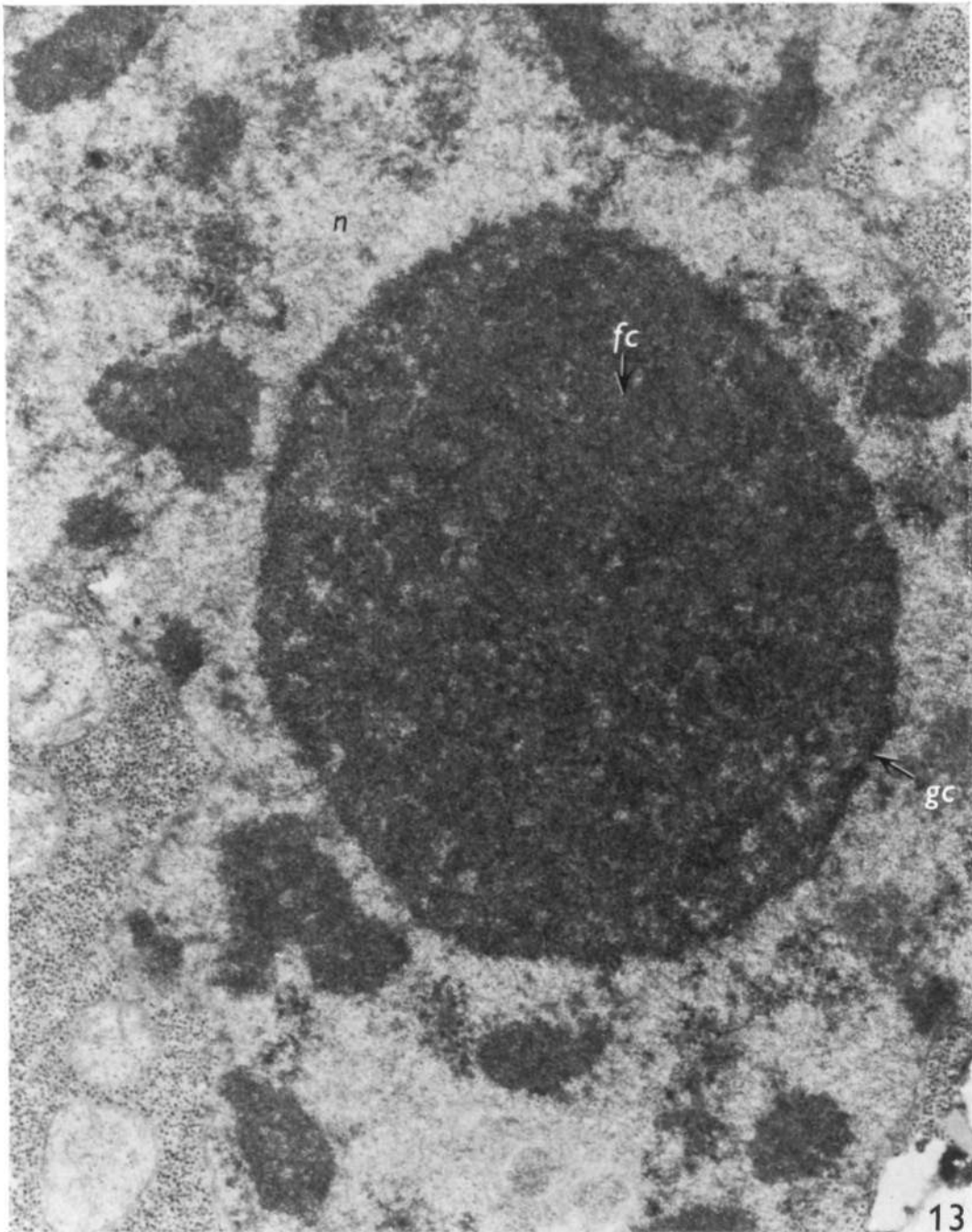


Fig. 13. Part of a nucleus (*n*) at the eighth hour of germination during continuous treatment with 2-TU. The nucleolus contains granular (*gc*) and fibrillar (*fc*) structures. $\times 30000$.