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ELECTRON MICROSCOPIC STRUCTURE OF THE ALEURON CELLS OF BARLEY DURING GERMINATION

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ABSTRACT

Germinating barley aleuron cells were studied electron-microscopically.

The *aleuron grains*, after $KMnO_4$-fixation and embedding in Vestopal W, contain a protein matrix, electron-dense, and electron-transparent bodies. Evidence is presented that the electron-dense bodies are globoids which consist of phytin. The nature of the electron-transparent bodies is discussed. During germination the protein matrix disappears and the globoids are decomposed.

The *rough-surfaced er* develops extensively in the first 8 days. It forms protein vesicles which seem to discharge their contents in the vicinity of the cell wall. The latter appears to be subject to chemical change. In the course of the process of germination the er makes connections with the spherosomes.

The *spherosomes* are for the greater part found around the aleuron grains. During germination their contents gradually diminish.

In the first 8 days the *mitochondria*, very undeveloped at the start, obtain more and longer cristae.

The cytological changes observed reflect the known physiological activities of the aleuron cells.

INTRODUCTION

The cells of the outer layers of the cereal endosperm contain a great number of large aleuron grains. They are called aleuron cells. In *Hordeum*, the aleuron layer, which is approximately three cell layers thick at the convex side of the seed, completely envelops the endosperm except where it is adjacent to the embryo. Besides protein, the aleuron layer contains fat; starch is absent. During germination the cell walls and the starch of the endosperm are broken down and used by the developing embryo. This process starts in the vicinity of the scutellum and proceeds in an apical direction along the aleuron layer (BROWN and ESCOMBE, 1898) since the necessary enzymes are produced and secreted by both these tissues.

In recent years much research has been done on the production of enzymes by the aleuron layer (YOMO, 1958, 1960; YOMO and IINUMA, 1962, 1963, 1964; PALEG, 1960a, b, 1961; PALEG, SPARROW and JENNINGS, 1962; PALEG, COOMBE and BUTTROSE, 1962; MACLEOD and MILLAR, 1962; BRIGGS, 1963, 1964; VARNER, 1964; LAZER, BAUMGARTNER and DAHLSTROM, 1961). It was established that a

[1]) The junior author, at present Mrs. A. A. van der Linden-van der Eb, has made this study in partial fulfilment of the requirement for her Master's degree.

gibberellic hormone stimulates the production of enzymes by the aleuron layer. This hormone is secreted by the embryo (YOMO, 1960) and penetrates the endosperm through the scutellum. The enzymes must be synthesized *de novo*, since they were found to be heavily labeled if labeled amino acids had been supplied at an early stage (VARNER, 1964). Accordingly, respiration appears to be indispensable for enzyme synthesis (MACLEOD and MILLAR, 1962; VARNER, 1964).

The changes in the structure of the protoplasm which accompany the production of enzymes by gibberellic acid-treated aleuron cells have been studied to some extent with the aid of the electron microscope. These cells contain aleuron grains – which are large vacuoles filled with reserve-protein (DANGEARD, 1922; BUVAT, 1962; POUX, 1962) – and lipid droplets called spherosomes. The aleuron grains are supposed to have originated from dilations of the endoplasmic reticulum (POUX, 1962; BUTTROSE, 1963a, b). They are not homogeneous since they may contain one or more electron-dense as well as some electron-transparent globules (HYDE and PALEG, 1963). So far, the character of these structures is not quite clear. Possibly owing to the use of acid media for fixing and rinsing (see Discussion), POUX (1963) observed no electron-dense, but only more or less electron-transparent globules in the aleuron grains of wheat aleuron cells. Since she could demonstrate that acid phosphatase was localized especially at the inner surface of the delimiting membrane of the globules, she concluded that the latter might be phytin-containing globoids.

HYDE and PALEG (1963) and PALEG and HYDE (1964) observed both electron-dense and electron-transparent round bodies in the aleuron grains of barley aleuron cells, after $KMnO_4$-fixation as well as after OsO_4-fixation. The bodies which were electron-dense after $KMnO_4$-fixation were electron-transparent after OsO_4-fixation and would contain protein, while the spheres which were electron-transparent after $KMnO_4$-fixation were dense after OsO_4-fixation and would contain lipid.

MACLEOD, JOHNSTON and DUFFUS (1964) call the $KMnO_4$-fixed electron-transparent bodies in their material vacuoles, and the dense ones, inclusion bodies.

So far, no changes in the ultrastructure have been reported that might be more directly related to the extensive *de novo* enzyme synthesis. Therefore, we studied this point in particular. Further, we tried to elucidate the character of the electron-dense and the electron-transparent spheres in the aleuron grains.

METHODS

The methods and materials used were primarily those of NIEUWDORP and BUYS (1964). Grains of *Hordeum vulgare* cultivar 'Proctor' were germinated at 11° C. Nearly all specimens were fixed with $KMnO_4$, which gave the best results, and were embedded in Vestopal W.

We always attempted to obtain sections from an area several cells above the apical end of the scutellum (Figs. 1 and 2). This is rather important since the greater the distance from the scutellum, the later the cell will be triggered into activity by the scutellum hormone. However, we were not always successful in this respect, and thus we sometimes found a younger stage than expected with regard to the number of days germination had lasted.

We found but little difference in ultrastructure between the outer and the inner cells of the same radial row of aleuron cells. Therefore, later on we did not try to localize the cells in this respect.

Results: The electron micrographs

Figure 3. The protoplasm in the resting stage. Spherosomes (s) and aleuron grains (a) are conspicuous, but no endoplasmic reticulum is apparent. The spherosomes are so electron-transparent that they may be considered empty. Their sole contents may have been lipid which was dissolved in the acetone used for dehydration of the specimen. The majority of the spherosomes are part of a single layer surrounding each aleuron grain. The aleuron grains contain globular bodies of two kinds, which appear in the micrographs as black (electron-dense) and white (electron-transparent) circular areas. The black ones often show irregularities due to sectioning, which suggests hardness. The white areas have a distinct black envelope.

Figure 4. The protoplasm of an aleuron cell of a seed germinated for 5 days. Some plasma membranes and some mitochondria (m) with inconspicuous cristae are visible. The dark envelope of the electron-transparent circular area in the aleuron grain now has a greyish, granular inner margin. The spherosome membranes have slightly shrivelled and their content has an irregular light-greyish hue.

Figure 5. The protoplasm after 6 days germination. The section has been contrasted with Pb, to which the wide, grey border within the spherosomes is evidently due. The electron-transparent globules in the aleuron grain have expanded at the expense of the protein matrix. Their outlines are now often undulated and they contain some granular material partly arranged in circles.

Figure 6. After 8 days germination. The electron-transparent globules in the aleuron grains have further dilated. The electron-dense body now shows an indistinct outline, and electron-dense material is scattered in the protein matrix. The spherosome membranes have shrivelled still more and their contents are now denser. In this electron micrograph the endoplasmic reticulum (er), with local swellings, is distinctly visible. The mitochondria (m) have more cristae and are outlined more sharply. Small globular bodies – probably protein vesicles – appear in the cytoplasm (pr).

Figure 7. Part of a cell of a seed which was also germinated for 8 days. This cell has passed through further changes. The upper half of the micrograph shows part of a nucleus (n) with nuclear envelope (ne) with pores (np). In the lower part is a comparatively

small aleuron grain, the contents of which have almost completely dissolved. It is still surrounded by spherosomes (s) which are transparent as usual after $KMnO_4$-fixation if the section has not been contrasted with Pb. A few spherosomes in the vicinity of the nucleus have reached a more advanced stage of development as appears from their contents being more electron-dense.

The endoplasmic reticulum (er) is strongly developed, forming stacks of parallel lamellae. In the vicinity of the aleuron grain the ends of the er are club-shaped. The contents are more electron-dense than the surrounding cytoplasm. This also applies to the contents of the 'protein vesicles' (pr) which may be transverse or oblique sections through swollen er.

Figure 8. Also after 8 days germination. The protoplasm near the cell wall (w). 'Protein vesicles' are present both in the vicinity of the cell wall and farther away from it. The cell wall is now electron-dense, whereas it is always transparent in samples germinated for only a few days. This is indicative of a profound change in the chemical composition of the cell wall.

Figure 9. Also after 8 days germination. The upper part of the micrograph shows an aleuron grain, most of the protein of which has dissolved. In the lower part a leucoplast (l), spherosomes and endoplasmic reticulum, the latter with electron-dense swellings. In between, are many vesicles with contents of similar density. Nearly all of the spherosomes in this micrograph are somewhat electron-dense though the section was not treated with Pb acetate.

Figure 10. After 10 days germination. The cell wall is severely attacked. In the adjacent cytoplasm many vesicles and tubules of the er occur, several of which seem to discharge their contents into the wall. Note the intercellular connection (ic) in the cell wall.

Figure 11. Also after 10 days germination. In the right-hand upper corner a portion of a cell wall with indistinct plasmalemma and in the right-hand lower corner an aleuron grain are visible. The spherosome membranes have shrivelled further. Protuberances of the spherosomes seem to trail into appendages connected with the endoplasmic reticulum (arrow). There are too many 'protein vesicles' to consider them all transverse or oblique sections through swellings of the endoplasmic reticulum (see p. 696).

Figure 12. Also after 10 days germination. A small aleuron grain is nearly filled by an apparently disintegrating electron-dense body. Spherosomes connected with the er through protuberances, and mitochondria are present.

Figure 13. From the same stage as Figs. 10–12, shows an obvious connection between the er and a spherosome (arrow).

Figure 14. After 17 days germination. The upper half of the micrograph shows an aleuron grain enclosing an undissolved electron-dense body (gl). To the left in the lower half is a completely dissolved aleuron grain (a) surrounded by small, very electron-dense spherosomes. The protein vesicles (pr) have increased in size. Active mitochondria are still present.

Figure 15. After 7 days germination. This specimen was OsO_4-fixed in order to show both the free ribosomes (ri) and those adhering to the er, which thus form the rough surface of the er. After OsO_4-fixation the contents of the spherosomes are not dissolved (cf. Fig. 3).

Figure 16. Part of an aleuron cell of a full-grown barley seed, 35 days after flowering. The seed was still ripening and beginning to yellow. Spherosomes are numerous. The developing aleuron grains consist only of a small vacuole which is nearly completely filled by an electron-dense globule.

Figure 17. After 8 days germination. This specimen was fixed with 5% glutaraldehyde, left overnight at 0° C, thoroughly rinsed and then treated with OsO_4. The electron density of the dense globules has increased so much that the electron beam caused heat deformations (boiling bubbles). The less dense sphere in the aleuron grain, which after $KMnO_4$-fixation is usually transparent, now is somewhat more dense than the protein matrix.

Figure 18. After 7 days germination. The specimen was fixed with glutaraldehyde only. Even so, the bodies which are electron-dense after fixation with $KMnO_4$ or OsO_4 are of a dark shade. This proves that their electron density is not entirely due to $KMnO_4$ or OsO_4. Besides the aleuron grains, spherosomes are faintly visible.

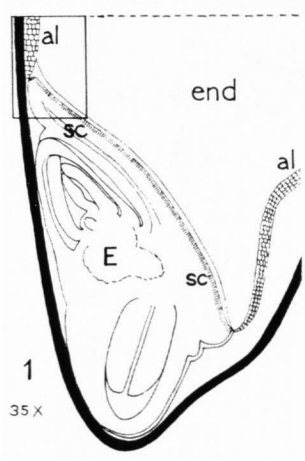
Interpretation of the micrographs and discussion

The changes in the structure of the diverse organelles during germination will now be discussed.

Aleuron grains

The aleuron vacuoles in the maturing barley seed are smaller than they will be in the mature seed. Each vacuole is for the greater part filled by an electron-dense globule, the globoid.

In the mature barley seed protein is the major component of the aleuron vacuoles, or aleuron grains, both in aleuron cells (Fig. 3) and in scutellum epidermis cells (Nieuwdorp, 1963). The aleuron grains contain a protein matrix enclosing, among other things, one or more electron-transparent, and one or more electron-dense globules. The former were called 'internal cavities' by Nieuwdorp (1963) and Nieuwdorp and Buys (1964). In dry seeds, they would be air-filled; in soaked seeds, they would be filled with an aqueous solution. They were assumed to have been formed during maturation by a process of precipitation of the protein from the liquid in the vacuole. During germination this process would be reversed, the internal cavities enlarging at the expense of the protein matrix which dissolves in the aqueous solution in the course of ca. 8 days (Fig. 7). During fixation of the specimen with $KMnO_4$ the dissolved protein probably precipitates in different forms (Figs. 4–7). After fixation with glutaraldehyde followed by OsO_4, however, the contents of the internal cavities seem to be homogeneous (Fig. 17).



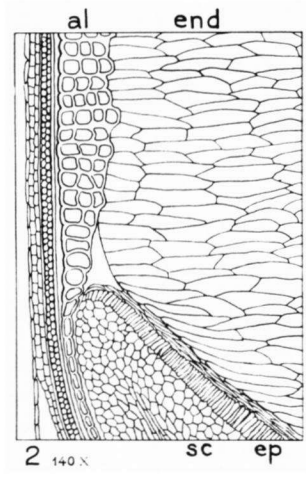


Fig. 1. Part of a longitudinal section of a barley seed.

Fig. 2. Part of Fig. 1 enlarged, to show the site of the ultra-thin sections.

PLATE I

ABBREVIATIONS TO FIGS. 3–18

a	aleuron grain (protein vacuole)	l	leucoplast
al	aleuron layer	m	mitochondrion
E	embryo	n	nucleus
end	endosperm	ne	nuclear envelope
ep	epithelium	np	nuclear envelope pore
er	endoplasmic reticulum	pr	protein vesicle
gl	globoid	ri	ribosome
ic	intercellular connection (plasmodesma)	s	spherosome
		sc	scutellum
		w	cell wall

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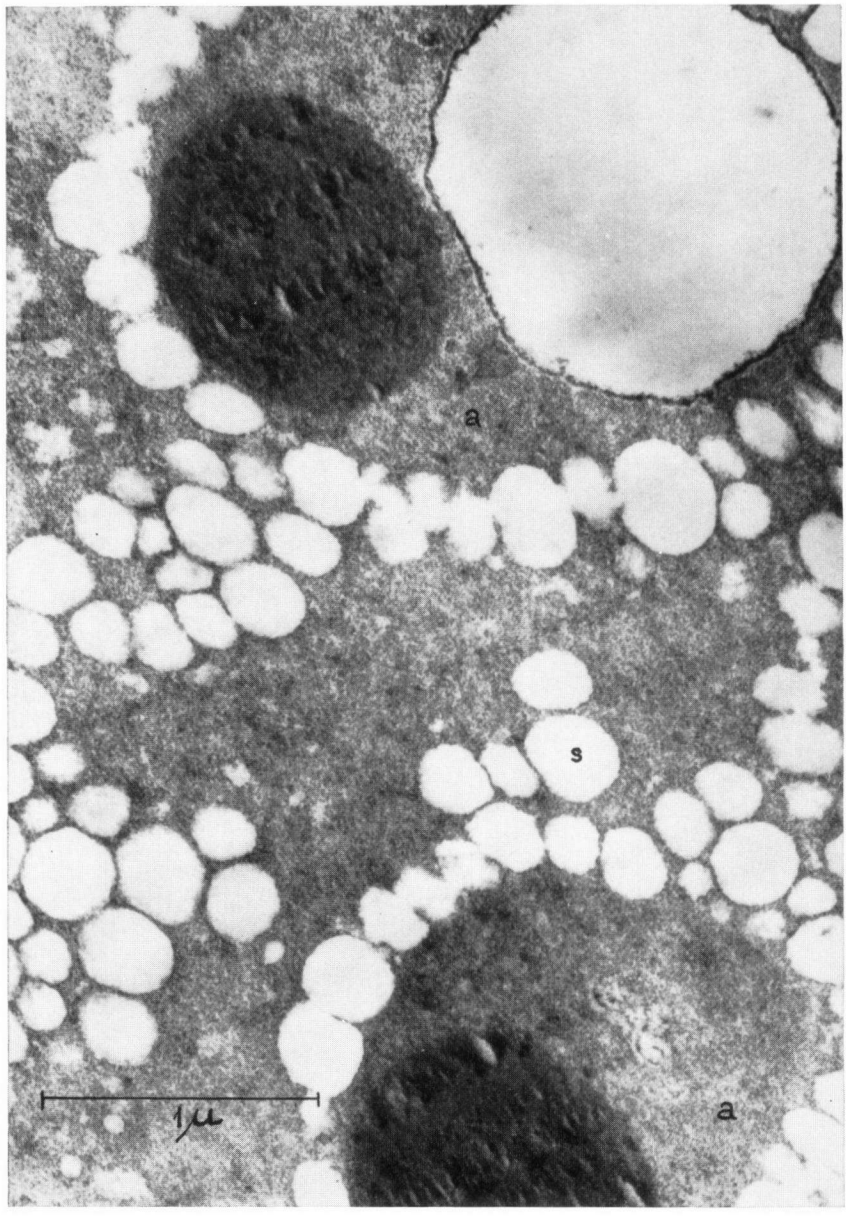


Fig. 3. Part of the protoplast of an aleuron cell in a barley seed soaked in water for 24 h at 0° C. $KMnO_4$-fixation.

PLATE II

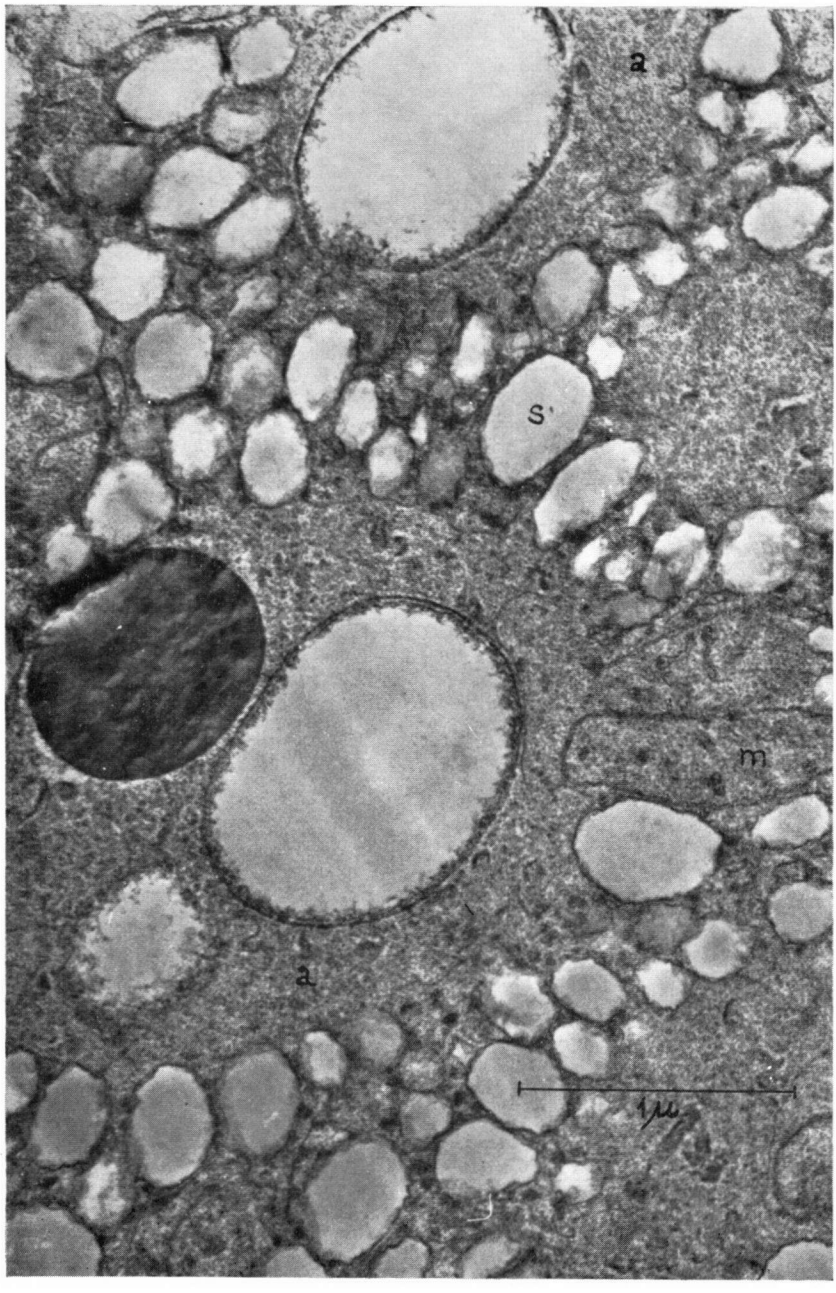


Fig. 4. Part of the protoplast after 5 days germination at 11° C. $KMnO_4$-fixation.

PLATE III

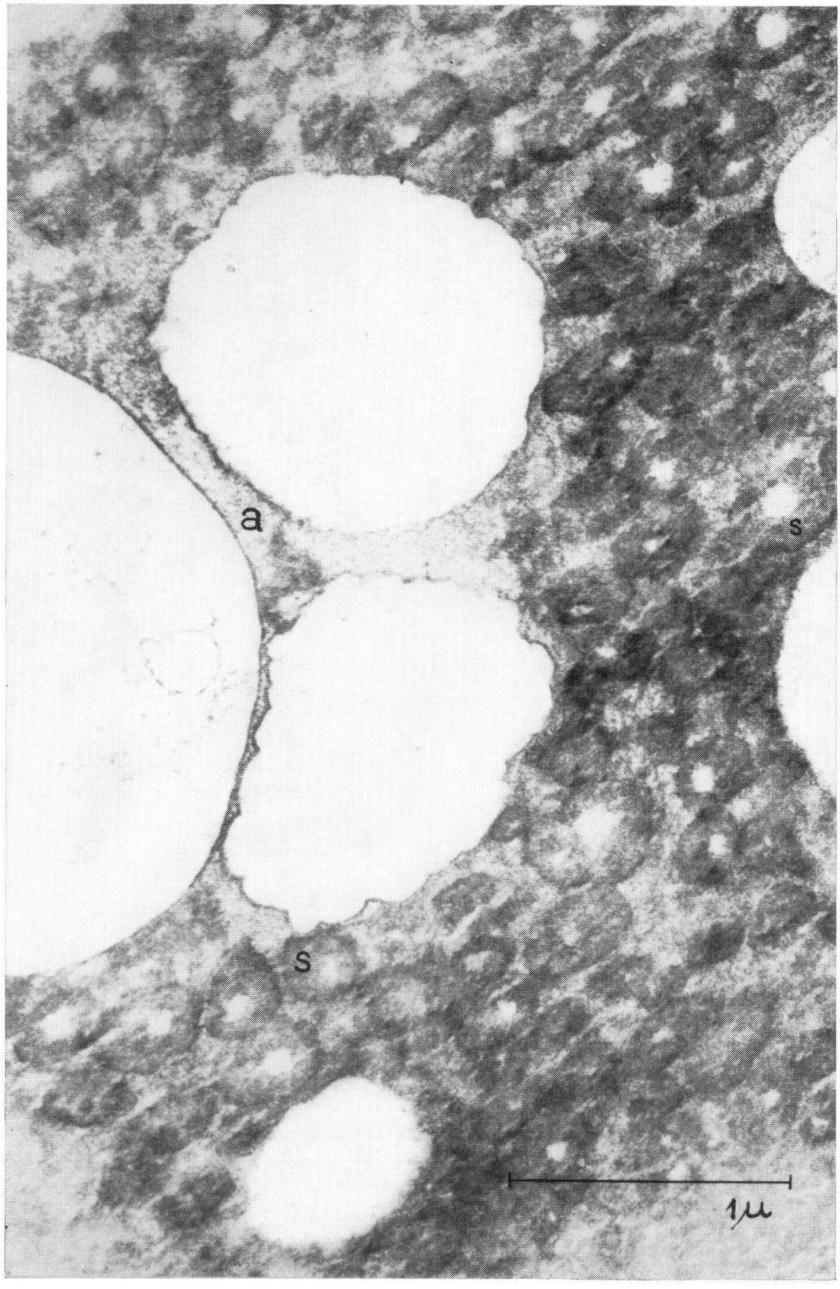


Fig. 5. After 6 days germination. $KMnO_4$-fixed and section contrasted with Pb.

PLATE IV

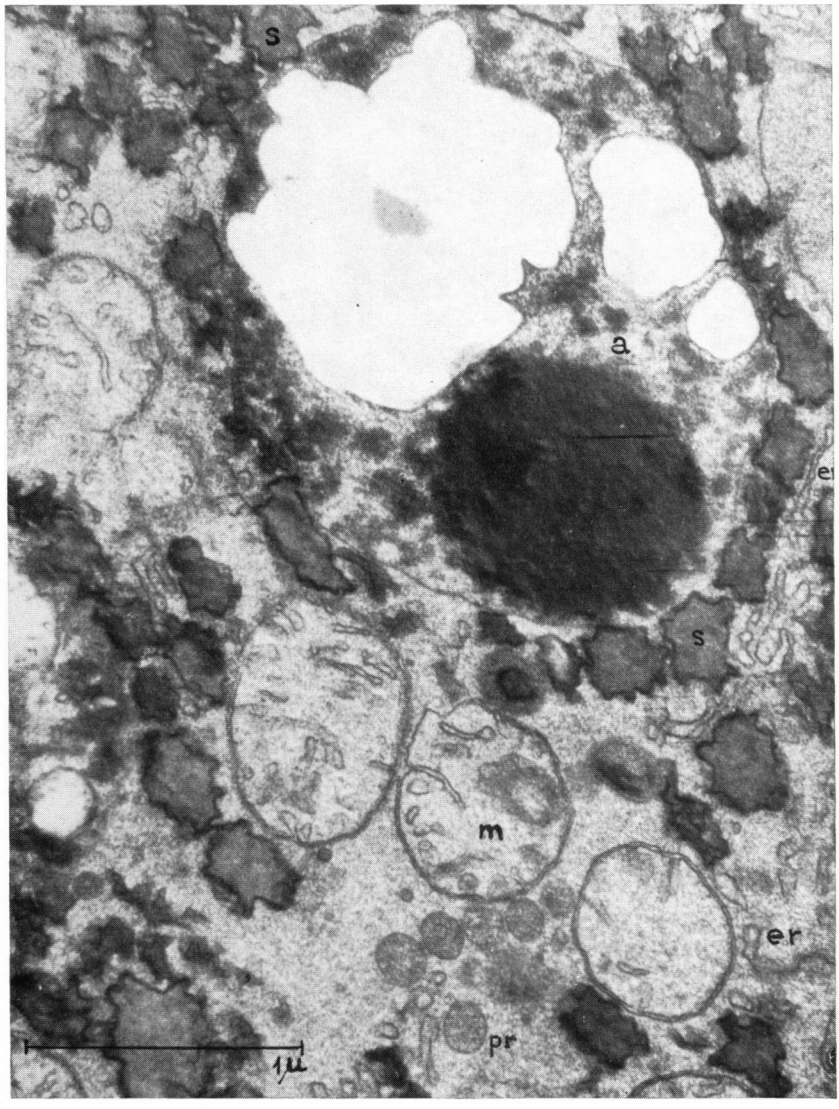


Fig. 6. After 8 days germination. $KMnO_4$-fixed and section contrasted with Pb.

PLATE V

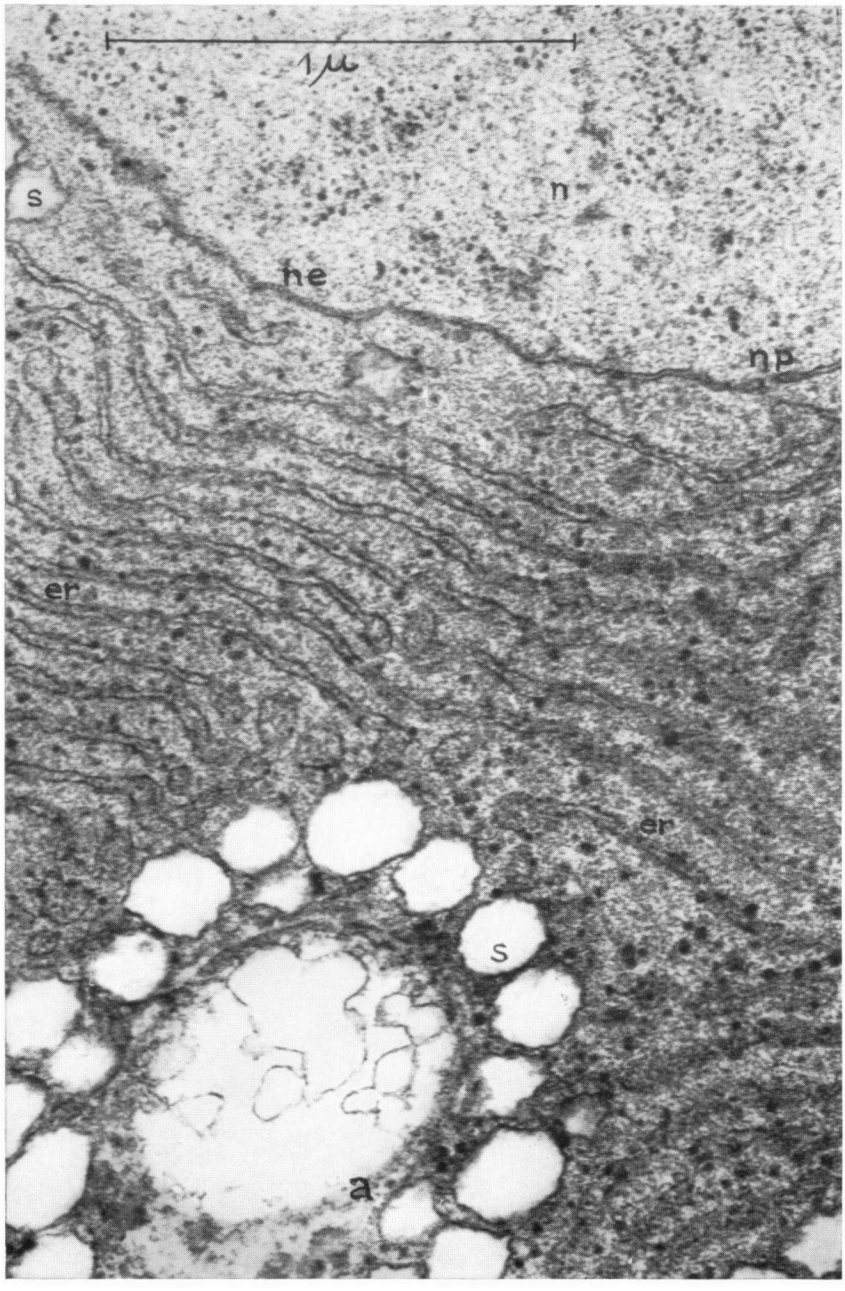


Fig. 7. After 8 days germination. $KMnO_4$-fixed.

PLATE VI

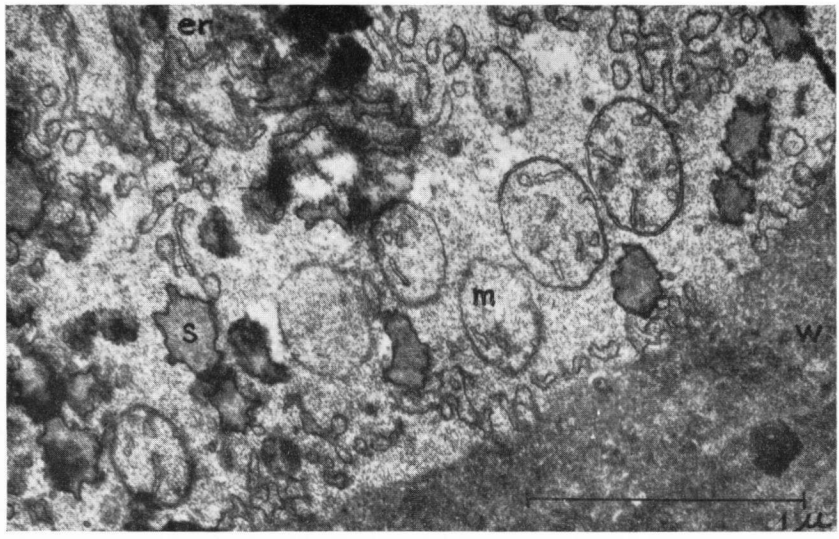


Fig. 8. After 8 days germination. Protoplast and cell wall. $KMnO_4$-fixed and section contrasted with Pb.

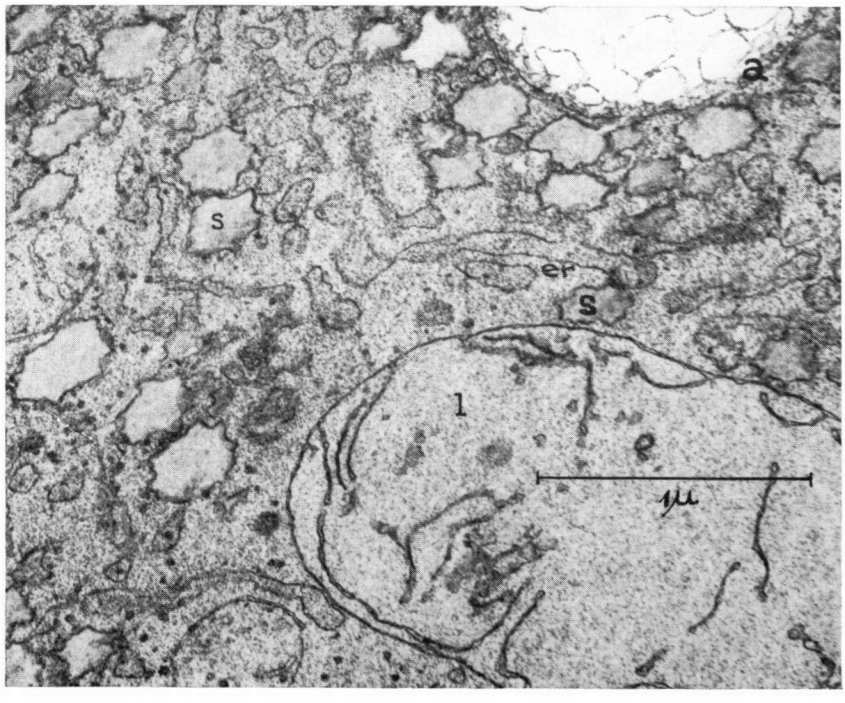


Fig. 9. After 8 days germination. $KMnO_4$-fixed.

PLATE VII

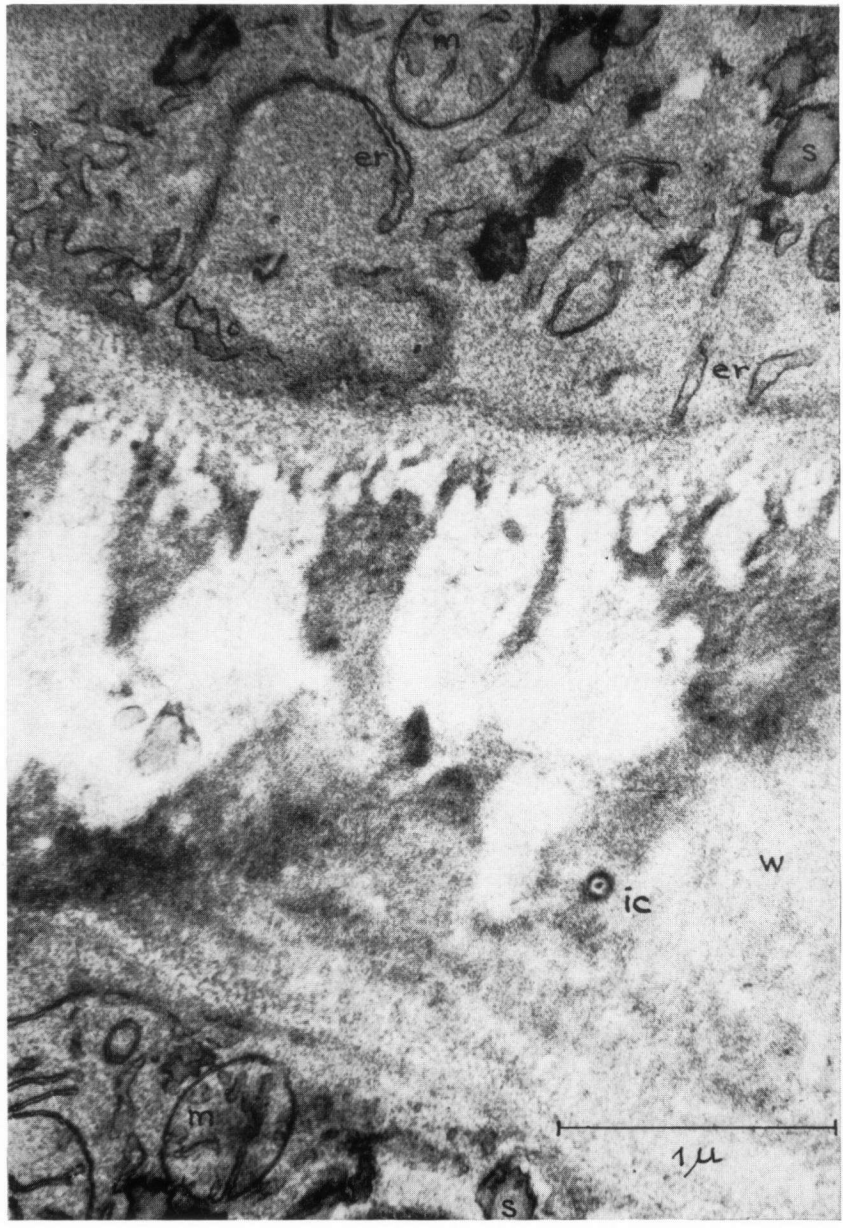


Fig. 10. After 10 days germination. Cell wall and adjacent protoplasm. $KMnO_4$-fixed.

PLATE VIII

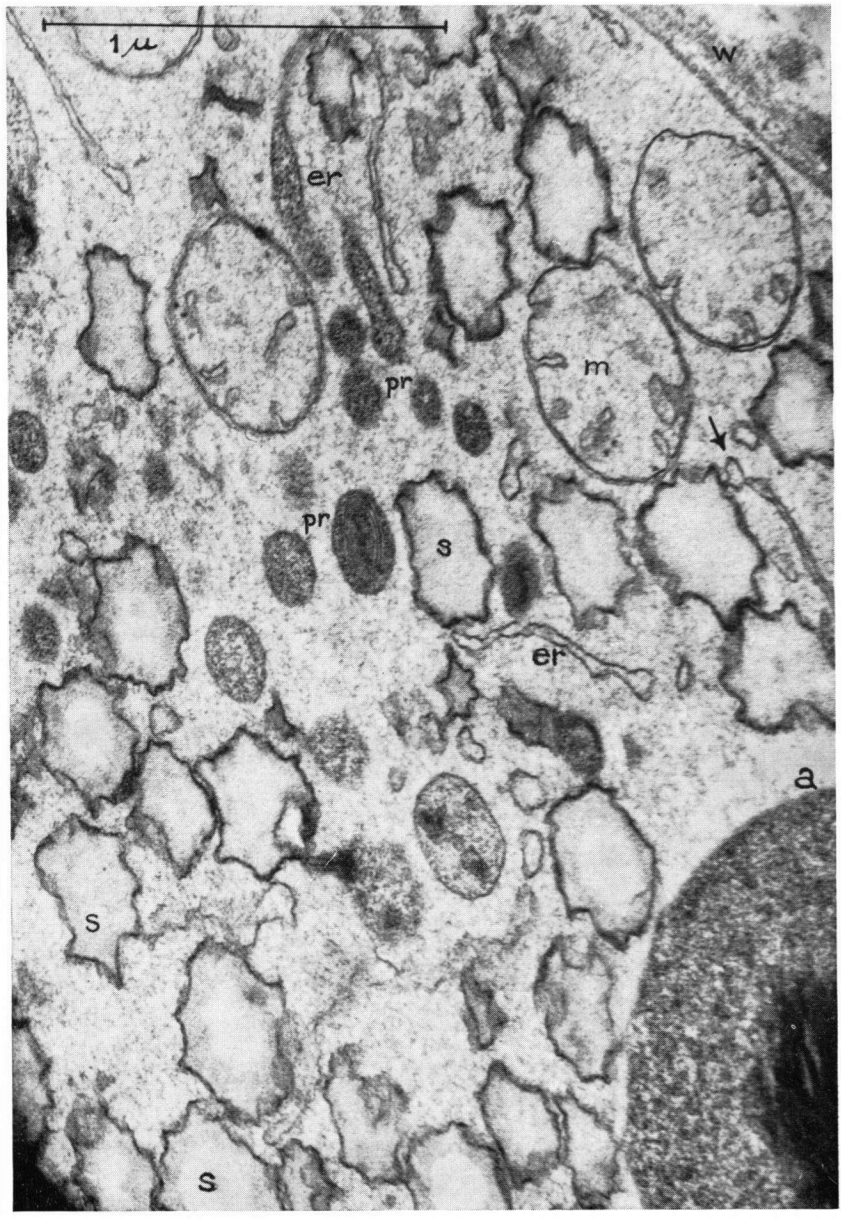
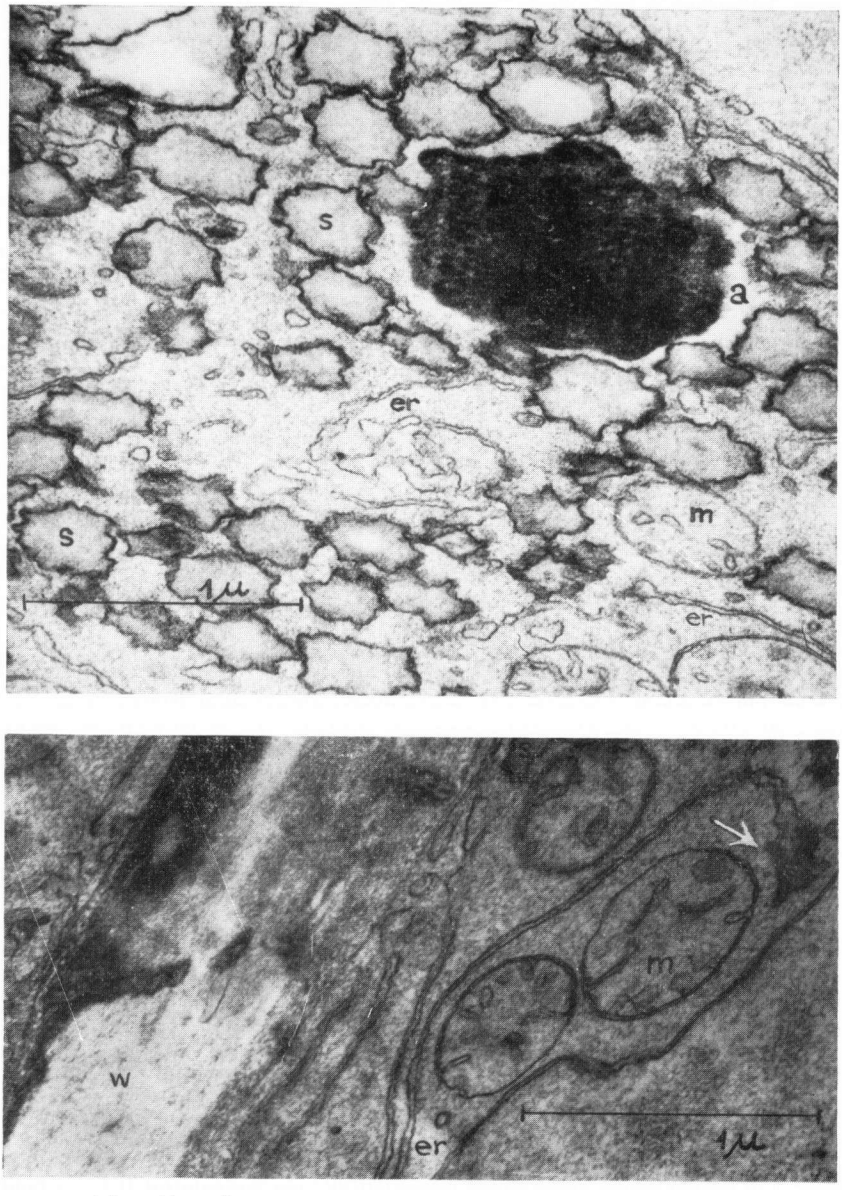


Fig. 11. After 10 days germination. $KMnO_4$-fixed.

PLATE IX



Figs. 12 and 13. After 10 days germination. $KMnO_4$-fixed.

PLATE X

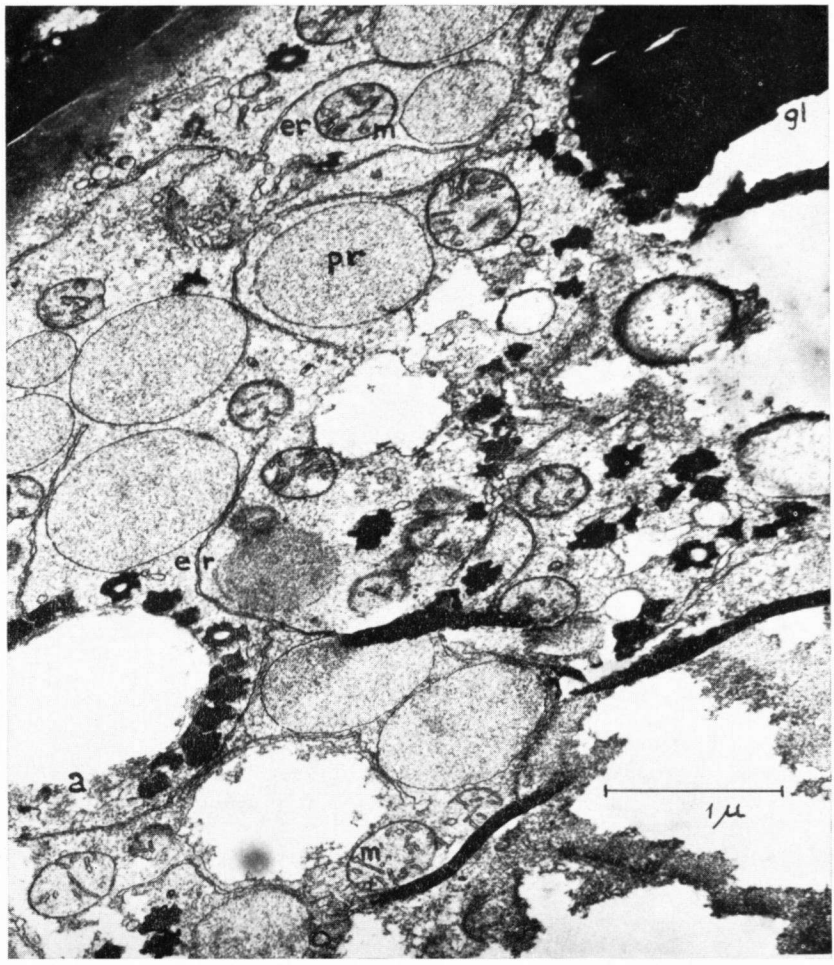


Fig. 14. After 17 days germination. $KMnO_4$-fixed and section contrasted with Pb.

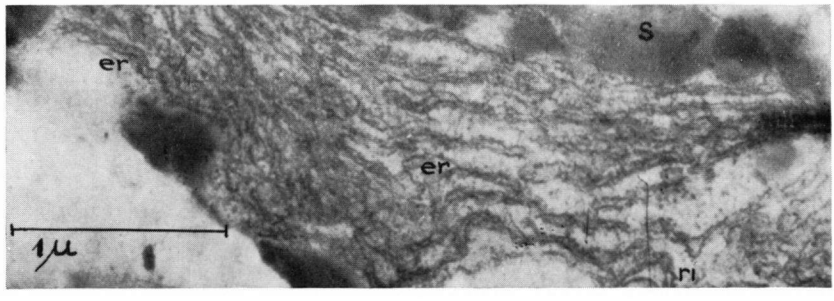


Fig. 15. After 7 days germination. OsO_4-fixed.

PLATE XI

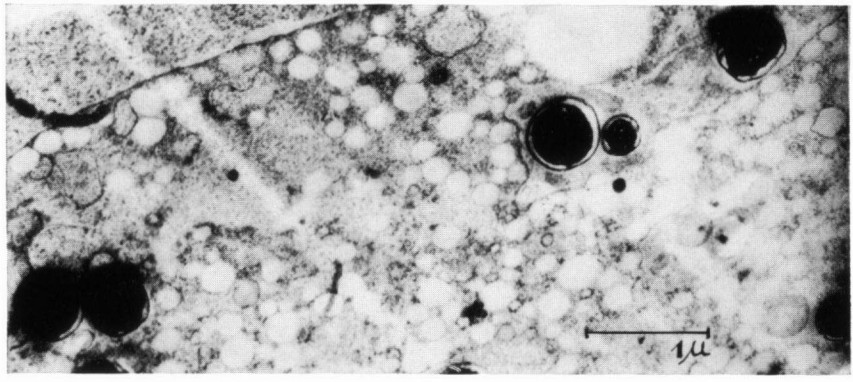


Fig. 16. Thirty-five days after anthesis. $KMnO_4$-fixed.

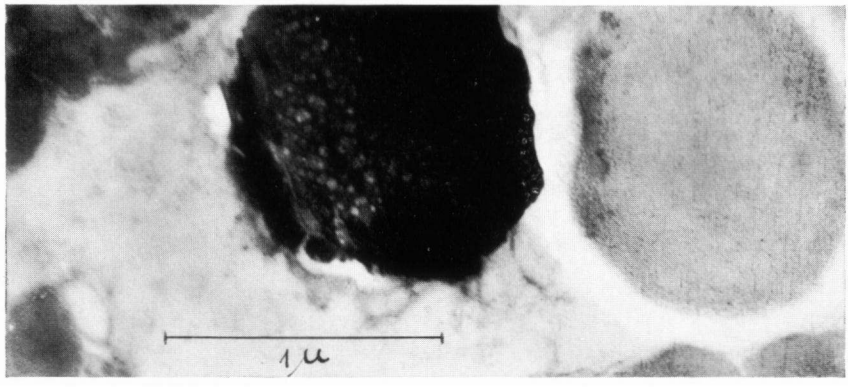


Fig. 17. After 8 days germination. Fixed with glutaraldehyde followed by OsO_4.

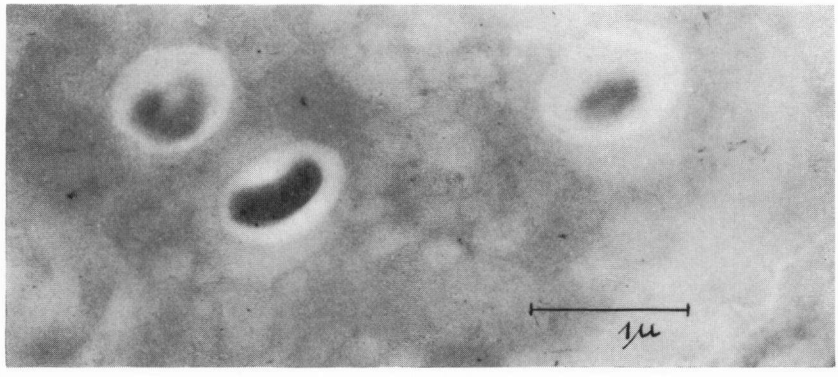


Fig. 18. After 7 days germination. Fixed with glutaraldehyde.

PLATE XII

Since POUX (1963) found acid phosphatase in aleuron grains one might suppose that the aleuron protein would consist mainly of enzymes which would merely need be dissolved and transported to the starchy part of the endosperm. However, since *de novo* synthesis, i.e. from amino acids, of at least α-amylase has been demonstrated (VARNER, 1964; BRIGGS, 1964) it seems more likely that the aleuron protein is a true reserve protein which is hydrolyzed into amino acids, the latter being the building units for the enzymes.

Aleuron grains in aleuron cells always contain one or more electron-dense globules (Figs. 3, 4, 6, 16) which we consider to be identical with the light-microscopically observed globoids. These are known to consist mainly of phytin (Ca-Mg-inositol-hexaphosphate). Phytin, due to the elements Ca, Mg and P, may be expected to scatter electrons heavily. As a model experiment, we dried very small droplets of a phytin solution on a formvar membrane. In the electron microscope they were so electron-dense that they 'boiled'. In harmony with this result it was found that the globoids were electron-dense even when the specimen had been fixed only with glutaraldehyde. Subsequent treatment of the specimen with OsO_4 made the globoid so dense, that the boiling effect was observed (Fig. 17). This concept is also supported by the considerable hardness indicated by the unevenness of the sectioned globoids (Fig. 3). POUX (1963a, b), on the other hand, found electron-transparent globoids in embryonic wheat tissue, but this result may be explained by the fact that she used acid solutions for fixing and rinsing in order to be able to demonstrate acid phosphatase. These acid solutions may have dissolved and extracted the phytin. In a recent article POUX (1965) mentioned a same procedure applied to the seeds of *Linum* and *Cucumis*. In both these species the aleuron grains were found to contain crystalloids. The observations of PALEG and HYDE (1964) are consistent with our concept (though their conclusions are different from ours), and it is also supported by the finding of globoids in green malt (VAZART, 1960).

During germination the globoids are attacked, although much slower than the protein matrix. After ca. 8 days their outlines become irregular, some material lying scattered in the protein matrix (Fig. 6). Even after 17 days a major part of the original globoid is still found in the aleuron grains which by that time are devoid of protein (Fig. 14).

A similar phenomenon was observed in aleuron grains of scutellum epidermis cells where globoid-like bodies remained intact for a long time (NIEUWDORP and BUYS, 1964). In the corresponding cells of non-germinated barley, however, NIEUWDORP (1963) did not find globoids in the aleuron grains.

Aleuron grains are known to originate from endoplasmic reticulum. In scutellum epidermis cells we incidentally found a few connections, but unlike PALEG and HYDE (1964) we did not find any in aleuron cells. We assume that the connections observed by these authors were due to the treatment of the seeds, viz. soaking at 30° C for 1–2 days and subsequent separation of the aleuron layer from the starchy endosperm.

Endoplasmic reticulum (er)

Whereas in scutellum epidermis cells of barley seeds soaked in water for one day at 0° C some endoplasmic reticulum was found (NIEUWDORP, 1963) which rapidly increased during germination until it reached a peak within seven days (NIEUWDORP and BUYS, 1964), we did not find any er in resting aleuron cells. After 5 days germination some er appeared (Fig. 4) which further increased until the 8th day (Fig. 7). Thus the development of the er in the aleuron cells lags behind that of the er in scutellum epidermis cells. This may be due to one or more of the following factors: a) the absence of er in resting aleuron cells as compared to the presence of a small amount in scutellum epidermis cells, b) the need for stimulation, directly or indirectly, by gibberellin or some other hormone, and c) a difference in the rate of imbibition of the aleuron cells and the scutellum epidermis cells during the soaking of the seed.

When fully developed, the er in the aleuron cells forms stacks of parallel rough-surfaced lamellae, a structure typical for plant and animal cells which produce and secrete large quantities of proteins. Such structures are lacking in scutellum cells. Besides, free ribosomes occur (Fig. 15).

After the development of the er has reached its peak a decline already visible after ca. 2 days sets in (Figs. 10–13). At this stage, and later, the er shows many local swellings filled with relatively electron-dense material. At first, the swellings are formed mainly in the vicinity of aleuron grains (Fig. 7); later on they are formed in all parts of the er. Some enlarge and disconnect themselves, thus becoming what we called protein vesicles with a diameter of 0.125–0.2 μ (Fig. 11). The vesicles usually have granular contents indicative of protein; in some of them concentric structures occur. Since many such vesicles were found near cell walls which at the same time had become unusually electron-dense, it can be inferred that the vesicles discharge their contents into the walls which they must pass on their way to the starchy part of the endosperm. Burst vesicles are seen near the wall (Fig. 10). This suggests decomposition of the wall by the hypothetical enzymes of the vesicles (Figs. 8, 10). Protein vesicles were found up to the end of the germination process; in the latest stage many were considerably larger (Fig. 14).

In developing endosperm of wheat grains BUTTROSE (1963a, b) described protein vesicles containing protein bodies. In larger vacuoles GRAHAM *et al.* (1962) and JENNINGS *et al.* (1963) observed several protein bodies which had supposedly been secreted by the er; thus, there is a process of secretion within the endosperm cell. JENNINGS *et al.* (1963) described the occurrence of multi-layered sheets between protein bodies and vacuolar membranes. We have never observed similar structures in the aleuron cells of germinating barley, except in one body with concentric layers (Fig. 11).

The vesicles we found in aleuron cells did not occur in scutellum epidermis cells, but dilations of the er were found also in the latter

tissue. In scutellum epidermis cells, on the other hand, much smaller vesicles, probably derived from the Golgi apparatus did occur (NIEUWDORP, 1963; NIEUWDORP and BUYS, 1964). Neither the Golgi apparatus, nor the latter kind of vesicles were often found in aleuron cells.

Connections between the er and aleuron grains were never observed.

Spherosomes

In resting seeds and in those germinated for less than 5 days, the spherosomes are spherical vesicles of ca. 0.4 μ in diameter, with smooth surfaces; most of them are found surrounding aleuron grains. In $KMnO_4$-fixed specimens their contents have disappeared, probably because the lipid material has been dissolved in the acetone used in the embedding procedure.

In more advanced stages of germination the spherosomes were found to have altered in several respects. Firstly, the peripheral parts have become less soluble in acetone and showed up after $KMnO_4$-fixation (Figs. 10, 11) and more conspicuously after subsequent contrasting with Pb (Figs. 5, 6, 8). The change may be due to the formation of fatty acids as a result of lipolysis. Secondly, after 5 or more days of germination the smooth outline has become irregular, indicating a surface of troughs and ridges. This may be due to surface growth of the envelope without corresponding growth in volume, but a decrease in volume without diminution of membrane surface is more probable since the change in appearance coincides with the chemical change mentioned and since in still later stages many spherosomes have disappeared while the remaining ones have obviously decreased in size (Fig. 14). This is in accordance with the fact that after 10 or more days of germination connections have formed between the er and the spherosomes (Figs. 11–13). Their contents are likely to have dissolved. Still, a small number of spherosomes persist even after 19 days germination.

Mitochondria

In cells of the resting seed the mitochondria have few cristea and the latter are poorly developed. During germination the cristae increase in number and in size and their membranes become more electron-dense. This becomes visible after 8 days, whereas in scutellum epidermis cells the same stage is already reached after 3 days. Also the enveloping membranes of the mitochondria become more electron-dense. Double membranes are now clearly visible, but for considerable parts the outer membrane is lacking which makes the cristae look like open-mouthed tubules (Figs. 6, 8, 11, 13, 14). Is this an indication of increased metabolism? This would be in line with the observation of VARNER (1964) that respiration is essential for amylase synthesis. The mitochondria persist after 19 days germination (Fig. 14).

TABLE 1

Changes in the aleuron cells of germinating barley *as observed after* $KMnO_4$*-fixation*

	0 days	5–6 days	6–8 days	10 days	17–19 days
Cell wall	light; fibrillar	irregularly darkening (due to change in chemical compostion)			dark-grey; fibrillar
Er	not found	visible	increased in size; local dilations; contact with, and release of material to cell wall; contact with spherosomes		decreased
Spherosomes	electron-transparent; contents dissolved; spheres	greyish border; irregular spheres	dark; forming appendages	contact with er	decreased in number
Mitochondria	not found	becoming visible	clearly visible; increased cristae		reduced
Aleuron grain					
matrix	dark	more transparent		dissolved	
globoid	black	some dissolved			reduced
transparent cavity	clear	borders become granular; outlines undulated			vanished

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