STUDIA FORESTALIA SUECICA

Nr 119 · 1974

Ultrastructural changes in the seeds of *Pinus sylvestris* L. during senescence

Förändringar i ultrastrukturen hos frön av Pinus sylvestris L. under åldrande

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Abstract

ODC 232.315 — — 015.28

The ultrastructure of three parts (endosperm, rootlets and cotyledons) of aged seeds of Pinus sylvestris *L. has been studied. In dry seeds no clear differences resulting from ageing are seen as compared with resting living cells, but during imbibition clear differences are evident. Inhibition of development of the DNA-containing structures, mitochondria and plastids, was the first indication of senescence. Lipolysis usually preceded proteolysis, in contrast to the course of events in living cells.*

Ms received 1974-08-15

Liber Förlag-Allmänna Förlaget
ISBN 91-38-02082-3
Berlingska Boktryckeriet, Lund 1974

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1 Introduction

Good seed years of *Pinus sylvestris* are not very frequent in the northern parts of Scandinavia and seed has to be stored for forestry purposes. Loss of the viability of seed is therefore a practical problem. Gymnosperm seeds generally have a short life span but they can be kept alive for many years in sealed cold storage (Kozlowski 1971). Loss of seed viability has been attributed to various internal changes. Reserve substances may alter so that they no longer furnish the nutritional requirements of the embryo. Proteins may denature and enzyme activities decrease. Yet no simple relationship exists between enzyme activity and ageing of seeds (Barton 1961). Chromosomal dislocations are more frequent in old than in fresh seed (Simak and Gustafsson 1968, Roberts 1972), but the changes may be the result rather than the cause of the loss of viability (Harrison and McLeish 1954).

Leakage of several substances (e.g. proteins, amino acids, sugars) has been found to occur in old seed (Roberts 1972). This has been used as an indication of deterioration of Scots pine seed (Pehap 1972). Leakage of substances from the seed points to severe membrane damage and to rapid autolysis of the cell contents. In senescing cotyledons of *Phaseolus vulgaris* permeability changes are an early sign of deterioration (Eilam 1965). In non-viable rye embryos abnormalities are seen in the plasmalemma and mitochondrial membranes (Hallam et al. 1972) and in artificially aged root cap cells of *Zea mays* (Berjak 1968, Berjak and Villiers 1972) morphological aberrations of mitochondria are considered to be the most critical. Some viability tests (TTC test) are based on biochemical reactions depending on mitochondrial enzymes. These tests have been standardized for seeds of several economically important conifers (Lakon 1950).

This work is part of the research project led by Professor Milan Simak at The Royal College of Forestry, Stockholm, concerning the viability of seeds of economically important Scandinavian forest trees. The aim of this study was to clarify what ultrastructural changes are first found in the different parts of pine (*Pinus sylvestris*) seeds during ageing. In this way the most sensitive structures or processes of the cells may be mapped at cellular level and this evidence may be applied to resolving the storage problems of the seeds. The biochemical changes of the same seed material, as used in the present work, have been studied by Pehap (1972).

2 Material and methods

Old seeds of *Pinus sylvestris* L. (collected in Borgsjö, Sweden, in 1945—1946) were used. This material did not germinate (cf. Pehap 1972) but its stainability in the TTC test indicated that dehydrogenase activity varied.

The seeds were surface-sterilized with 70 % ethanol for three minutes, rinsed with sterile distilled water, placed on sterile filter paper discs and incubated like the living seed material as described earlier (Simola 1974). This procedure prevented microbial infection, to which aged seeds are very prone. Samples were removed after 1, 2 and 5 days' imbibition; no necrotic drops were visible on the seed coat. The micropylar end of the endosperm, cotyledons and root tips were fixed in Karnovsky's fixative in cacodylate buffer or in 3 % glutaraldehyde in phosphate buffer (pH 7.2, 0.1 M) for 1.5 hours. Dry seeds were immersed in fixative (+4°C) for one hour, and the embryos dissected out in it and fixed for a further 1.5 hours at +4°C. The material was postfixed with 1 % osmium tetroxide in phosphate buffer (0.1 M) for 2 hours, dehydrated via acetone and propylene oxide and embedded in the epoxy medium of Spurr. The sections were cut with a diamond knife, stained with lead citrate and viewed with a Philips 200 electron miscroscope.

3 Results and discussion

The viability of different parts of the seeds, as shown in the TTC test, varied considerably in the seed material of *Pinus sylvestris* used in this work. A clear correlation existed between the stainability of the part of the embryo and the part of the endosperm next to the embryo. A corresponding correlation has been found by Lakon (1950). The hypocotyl proved to be the part of the seed most resistant to ageing in the present material. If a young embryo of *Triticum* is transplanted to an aged endosperm, the latter has deleterious effects on the embryo, possibly because of exchange of toxic metabolites between embryo and endosperm (Floris 1970). In a naturally aged seed sample loss of viability occurs gradually and unevenly, and one part of the seed may die earlier than another. How large a part of a seed must be healthy if a good seedling is to be produced is not known exactly, but presumably the main part of the embryo and endosperm have to be viable and only part of the cotyledons may be dead. Usually only those seeds of *Pinus* which have a well-developed and fully TTC-positive embryo and endosperm are considered viable (Proc. Int. Seed Testing Assoc. 1966).

The seeds of *Pinus sylvestris* were surface-sterilized in this experiment. Therefore the changes seen in the fine structure were really results of ageing processes and autolysis of the cells, not of attacks by microbes, which readily infect dead cells because of leakage of organic substances from the seeds. In surface-sterilized seeds no necrotic drops were found after imbibition. This suggests that this drop may be a result of microbial infection. No clear alterations are seen in dry senescent cells as compared with corresponding living cells. On the other hand, the great natural variation in living cells of ungerminated dry seeds (Simola 1974) prevents recognition of the changes in fine structure that results in ageing. The only clear difference is in the endosperm cells, where the spherosomes seem to be exceptionally electron-dense and the surrounding membrane possibly broken (Fig. 3).

In rootlets examined on the first day some initials seem to be developing between the spherosomes. Proteolysis is beginning at the edges of the protein bodies but the spherosomes have become electron-dense (Fig. 5). Two days after being soaked the cells may contain some weakly developed plastids and mitochondria and there are numerous ribosomes in the cytoplasm (Fig. 6). Proribosomes are also visible in the nucleus. These cells are only slightly damaged and it has been demonstrated that some metabolic processes like incorporation of ^{3}H-uridine and ^{3}H-leucine may continue in aged embryos of *Zea mays,* although no cell divisions occur (Berjak and Villiers 1972). The cotyledon cells (day 2) contain several free ribosomes between the electron-translucent spherosomes (Fig. 7). The dormant cotyledon cells of *Pinus banksiana* contain RNA in the cytoplasm, and the amount of RNA is higher in the nuclei of dormant cells than after germination (Durzan et al. 1971). The activity of ribonuclease is known to increase during ageing (Hanson et al. 1965). In the case of *Pinus sylvestris* the cytoplasmic ribosomes seem to be rather resistant towards the function of this enzyme but after five days' imbibition no ribosomes are visible in the cytoplasm (Fig. 12).

In cotyledon cells lipolysis seems to be more rapid than proteolysis. In some cells the membrane surrounding the spherosomes is more resistant to lipolysis than the plasmalemma, which is usually one of the first structures destroyed (Fig. 12). Occasionally, however, the spherosomal membrane dis-

rupts and the contents of the spherosomes fuse (Fig. 11). Even in severely damaged rootlet cells, membrane damage and fusion of the cell contents into large droplets sometimes seem to occur (Fig. 9). It is possible that in such cells proteolysis starts earlier than lipolysis and leads to breakdown of the spherosomal membranes. The plasmalemma is among the least resistant parts of the cell.

The cells that have a moderately damaged ultrastructure have a well preserved nucleus and the protein bodies have not formed vacuoles but the spherosomes look electron-empty (Figs. 8 and 10). These cells seem to be in state of metabolic arrest. In dead cells the nucleus seems to be relatively resistant to autolysis, and lobing of the nucleus was rarely seen in *Pinus sylvestris* (Fig. 10). In several other plants the nucleus is one of the most resistant parts of the senescing cell (Shaw and Manocha 1965). Although possibly nuclear damages, due to accumulation of supposed automutagenic substances, might lead to chromosome aberrations, such changes would not be detectable in studies of this kind.

The endosperm cells of dry and imbibed seeds contain protein bodies with some phytate material in the globoid cavities (Figs. 3 and 4). Soon after imbibition (day 1), the globoidal material seems to lie at the periphery of or outside the protein bodies. After five days' imbibition no phytate is recognisable (Figs. 13 and 14) and it is possible that there is some phytase activity. This enzyme leads to the formation of free phosphate ions, which are not bound to new organic components, as in living cells. This may lead to some membrane damage. Some protein bodies may be intact even after a rather long imbibition period (day 5) but a great number of spherosomes and protein bodies may have fused together, and the plasmalemma has been destroyed. No new cell organelles are visible but reorganization of the cellular structure is slower in the endosperm than in the embryo in the living seeds (Simola 1974).

It is apparent that dry seeds of *Pinus sylvestris* contain lipases, but no DNAse or proteolytic enzymes and this explains the ultrastructural changes in old seeds during imbibition. In the endosperm of germinating Douglas fir seeds lipolytic activity is localized to the spherosomes (Ching 1968) and in *Pinus sylvestris* lipase activity is found even in unimbibed seeds (Nyman 1965). In several other fatty seeds lipases are also found at the resting stage, but it is possible that they need to be activated by some hormones or metabolites during imbibition (Black and Altschul 1965). In the starchy endosperm of germinating wheat there is no pre-existing lipase activity, but this requires induction and synthesis of RNA and protein (Tavener and Laidman 1969). The starchy seeds of *Pisum,* however, have higher phospholipase D activity (Quarles and Dawson 1969) at the resting stage than after germination.

Thus the degree of ultrastructural damage resulting in the ageing of seeds of *Pinus sylvestris* is about the same in the different parts of the seed. In slightly deteriorated cells some organelles and ribosomes may be visible (Figs. 5—7) but loss of the potentiality for development of DNA-containing organelles is a characteristic feature of naturally aged material. Degeneration of mitochondria and chloroplasts are among the first changes to appear in naturally or artificially aged seeds of several Angiosperms (Öpik 1966, Butler 1967, Treffrey et al. 1967, Berjak 1968, Berjak and Villiers 1972, Hallam et al. 1972). No dictyosomes, ER or polysomes are seen in dead cells of *Pinus sylvestris.* Similarly in artificially aged seeds of *Zea* the ER is almost degenerated, ribosomes occur as monosomes, and no dictyosomes are to be seen (Berjak 1968). Lipolysis is more rapid than proteolysis in severely damaged cells, in contrast to living seeds of *Pinus sylvestris* (Simola 1974). This uncontrolled lipolytic activity may lead to membrane damages, cause disorganization of metabolic processes, and end in the death of the cell. But in plants, as in animals, it is difficult to say when a cell is dead.

The degenerative changes seen during ageing of seeds of *Pinus sylvestris* correspond relatively closely to those seen in some Angiosperms, but the ultrastructural start-

ing-point is rather different in *Pinus*. Therefore, damage resulting from ageing does not become recognisable for a longer time after imbibition in this plant, and comparisons must be made with living germinating seed material (Simola 1974). As compared with the ultrastructural changes in imbibed dead seeds of *Picea abies* (Simola, in preparation), there is much more variation in the degree of deterioration in *Pinus sylvestris* but the main features (lack of development of new cell organelles and rapid lipolysis) are very similar.

4 Summary

The ultrastructure of three parts (endosperm, rootlets and cotyledons) of dry and imbibed aged seeds of *Pinus sylvestris* L. has been studied in order to clarify which part of the seed and what processes within the cells are the most sensitive to ageing during long-term storage. In dry seeds no clear differences resulting from ageing are seen as compared with resting living cells, but during imbibition clear differences are evident.

The stage of deterioration varied relatively greatly within a sample. Inhibition of development of the DNA-containing structures, mitochondria and plastids, was the first indication of senescence, and some cells remained in a metabolically arrested state although imbibed. In surface-sterilized seeds breakdown of storage material (protein and lipid) was slow. Lipolysis usually preceded proteolysis, in contrast to the course of events in living cells. Protein bodies might be intact even after 5 days' imbibition. In slightly damaged cells some mitochondria and ribosomes were visible. The nucleus seems to be morphologically one of the most resistant parts of the cell but the plasmalemma is more rapidly damaged than the membrane surrounding the spherosomes. There were no notable differences in the stages of deterioration of different parts of the seed.

Acknowledgements

My thanks are due to Professor Milan Simak for several kind discussions and for the seed material used in this work. I owe thanks to Docent Björn Walles, Ph.D., for his helpfulness during my visit to the Department of Forest Genetics, Royal College of Forestry, in 1971. The kind technical assistance of Miss Maija-Liisa Salonen, M.Sc., and Miss Pirkko Leikas, of the Electron Microscope Laboratory, University of Helsinki, is gratefully acknowledged. This work was supported by a grant from the Academy of Finland.

Sammanfattning

Ultrastrukturen i tre delar (endosperm, radikula och hjärtblad) av gamla frön av *Pinus sylvestris* L. har undersökts under viloperioden och efter imbibition för att studera, vilka delar av frön och vilka processer i cellerna som är de känsligaste för åldrande under långvarig lagring. Inga ultrastrukturella skillnader kunde påvisas mellan celler från levande och gamla ej grobara, torra frön. Tydliga sådana skillnader iakttogs emellertid i imbiberat material.

Graden av degenerationen varierade relativt mycket inom ett fröprov. Den första indikationen på åldrande är, att utvecklingen av de DNA-innehållande strukturerna, mitokondrierna och plastiderna, förhindrades och några celler stannade i ett metaboliskt blockerat stadium ehuru de imbiberade. I ytsteriliserade frön var nedbrytningen av lagringsmaterial (protein och lipid) långsam. Lipolysen sker snabbare än proteolysen i motsats till sekvensen av processer i levande celler. Proteinkorn kan vara intakta ännu efter 5 dagars imbibition. I de svagt degenererade cellerna är några mitokondrier, plastider och ribosomer synliga. Kärnan syns vara morfologiskt en av de hållbaraste delarna av cellen, men plasmalemmen blir degenererad hastigare än membranen omkring sfärosomerna. Det finns inga märkbara skillnader i degenereringsstadierna i olika delar av fröna.

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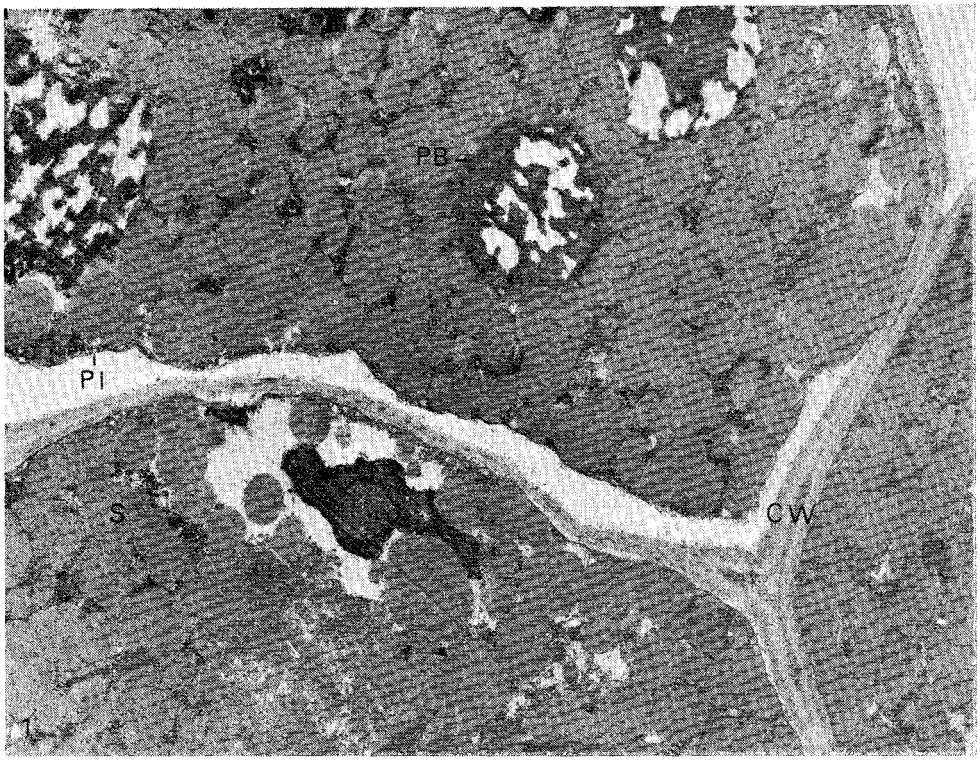


Figure 1. Rootlet cells of a dry embryo of Pinus sylvestris. Plasmalemma (Pl) loosened from the cell wall (CW). Fixation: Karnovsky—OsO_4. 6000×.

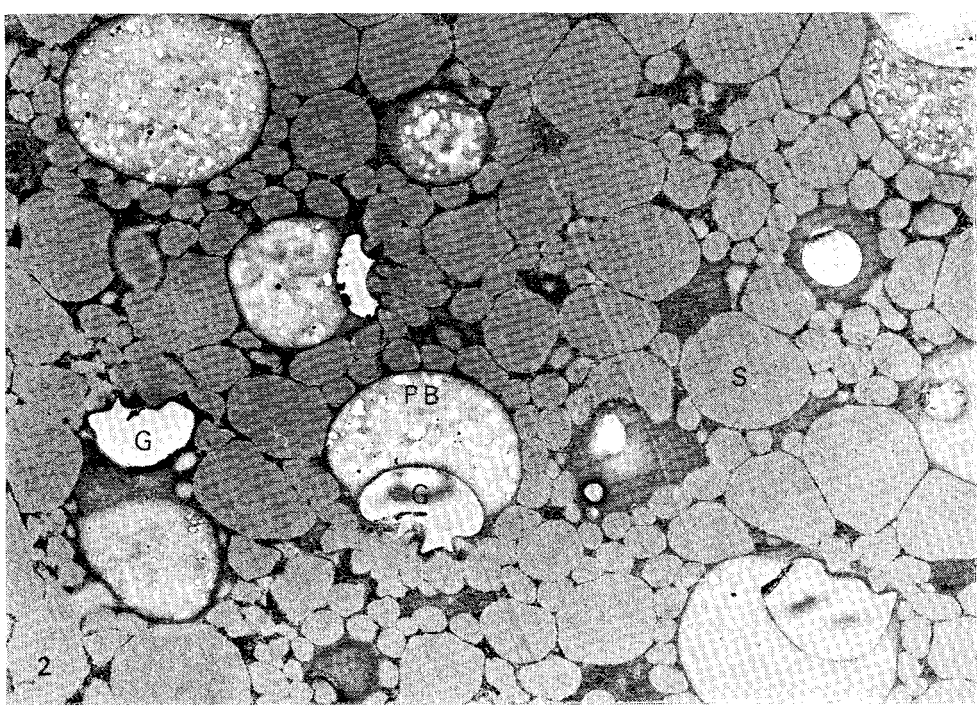


Figure 2. Cotyledon cells of a dry embryo. Protein bodies (PB) with large eccentric globoids (G). Fixation: Karnovsky—OsO_4. 3500×.

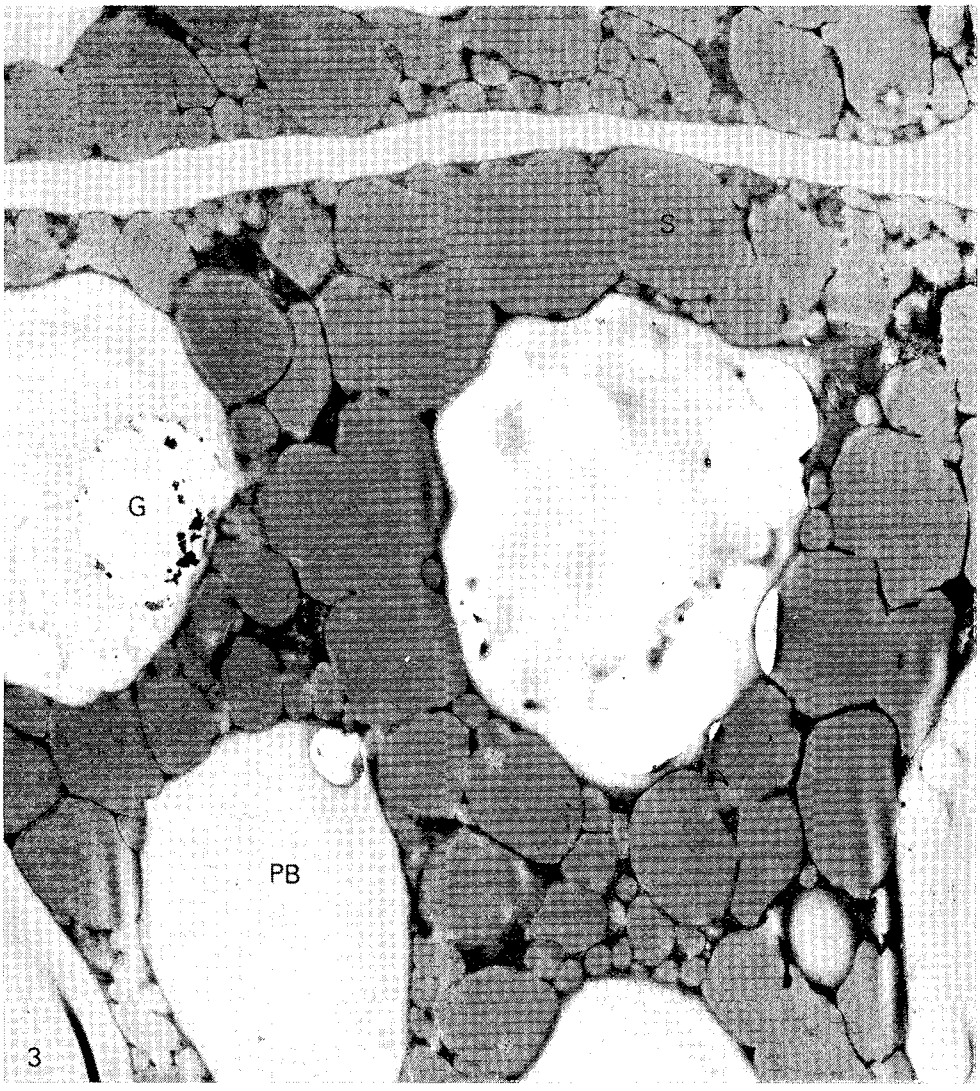


Figure 3. Endosperm cells of dry seeds. Globoids (G) containing traces of phytate. Some spherosomes (S) have fused. Fixation: Glutaraldehyde—OsO_4. 11,000×.

Explanation of abbreviations

CW = cell wall
G = globoid cavity
I = initial
M = mitochondrion
P = plastid
Pl = plasmalemma
R = ribosome
S = spherosome
V = vacuole

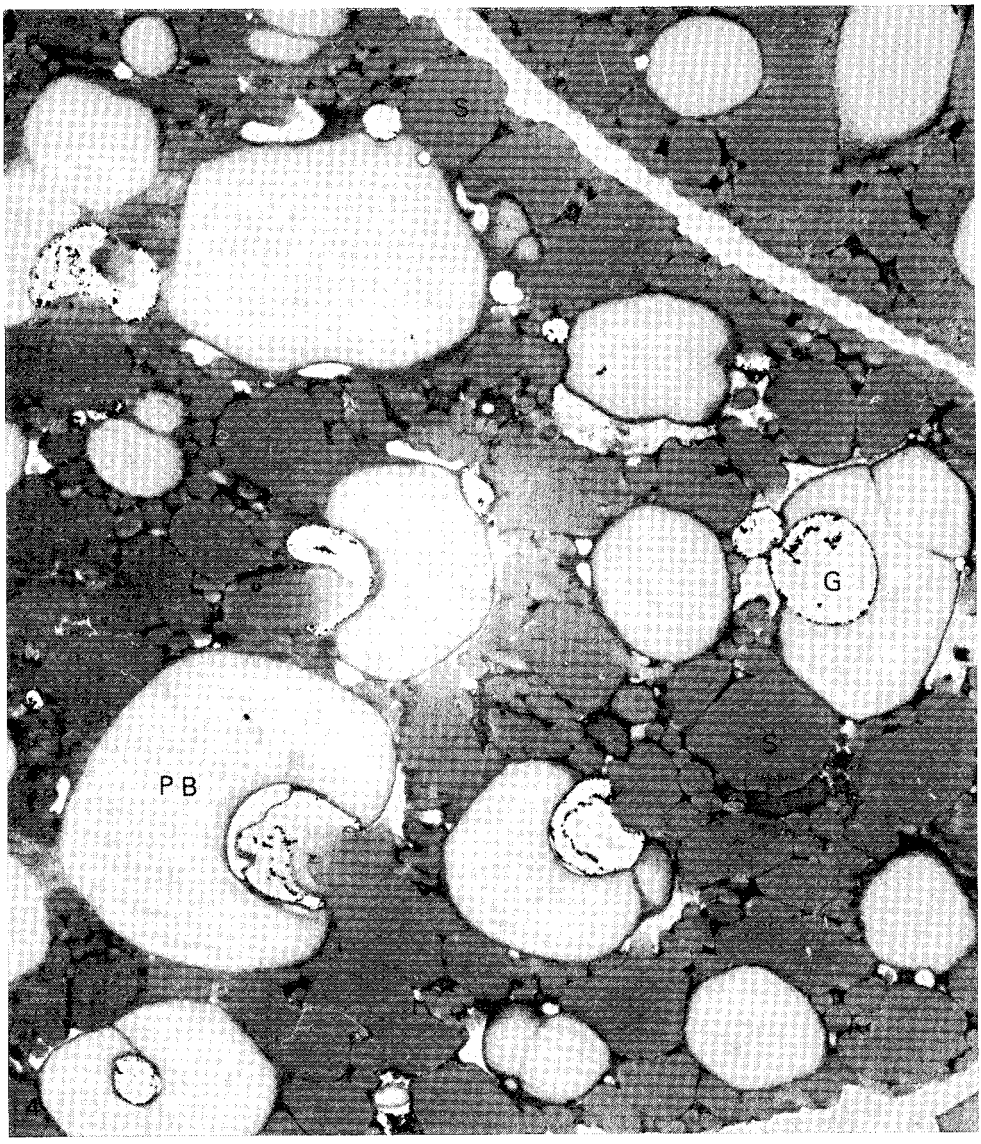


Figure 4. Endosperm cells after one day's imbibition. Fixation: Glutaraldehyde—OsO_4. 4000×.

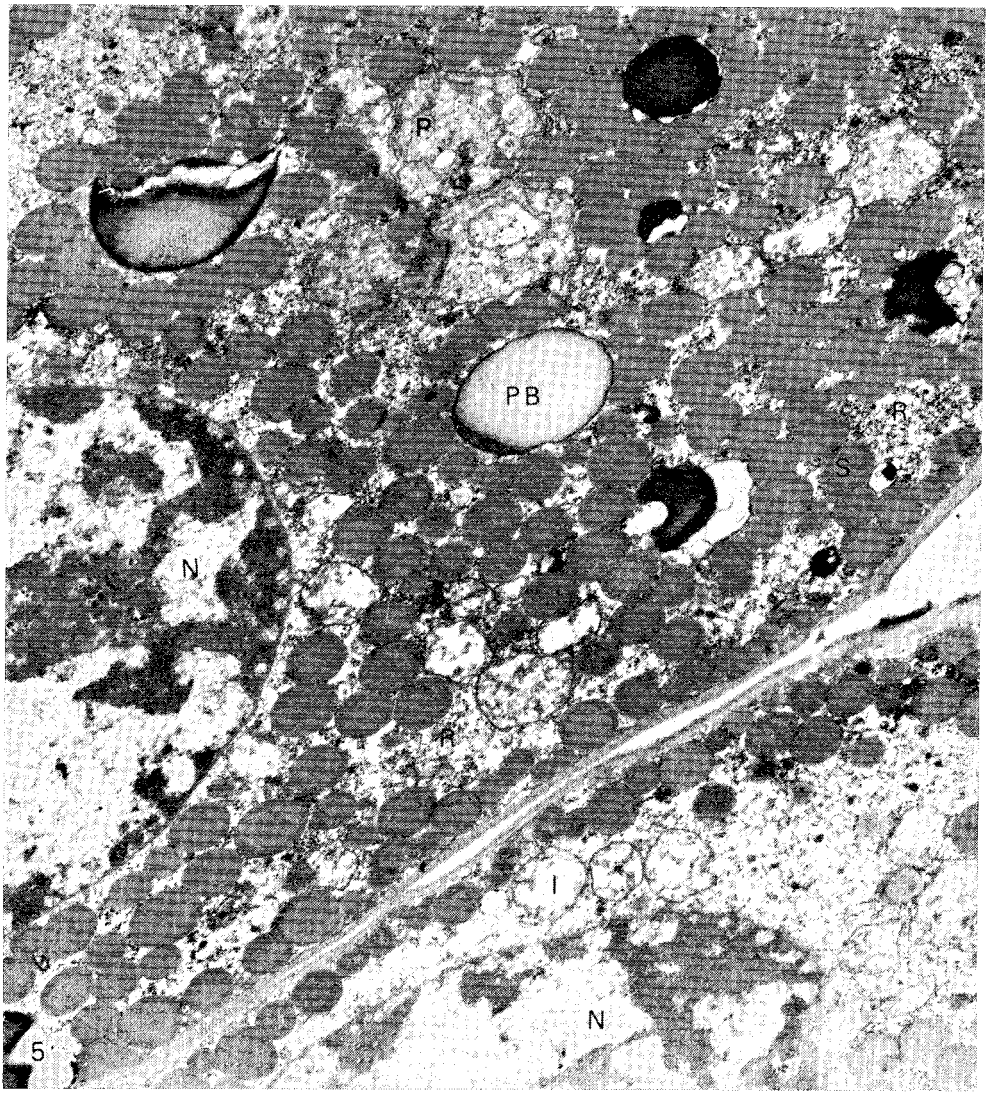


Figure 5. Rootlet cells after one day's imbibition. A slightly damaged embryo. Osmiophilic spherosomes (S). Organelle initials (I). Fixation: Karnovsky—OsO_4. 11,000×.

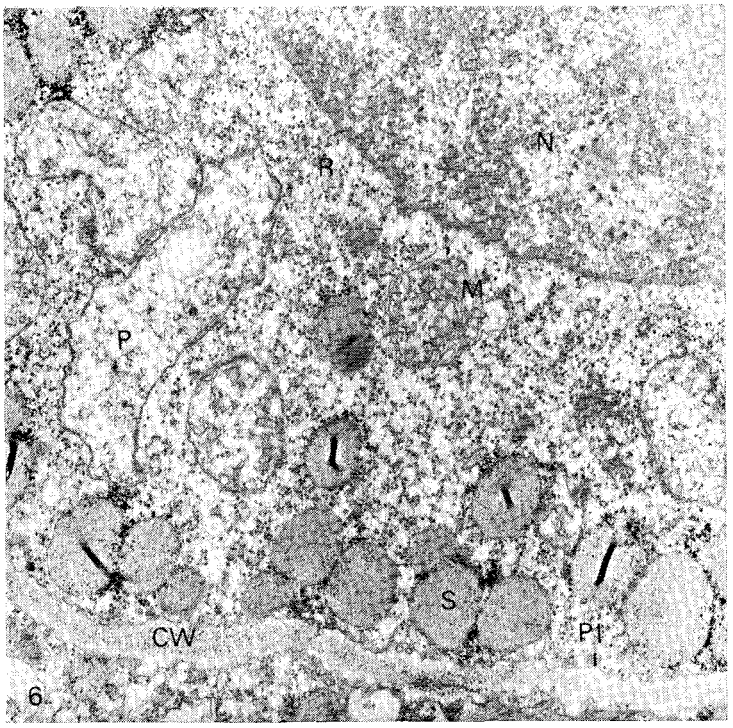


Figure 6. Rootlet cells after two days' imbibition. A slightly damaged embryo. Plastid with a starch grain (P). Fixation: Karnovsky—OsO_4. 17,000×.

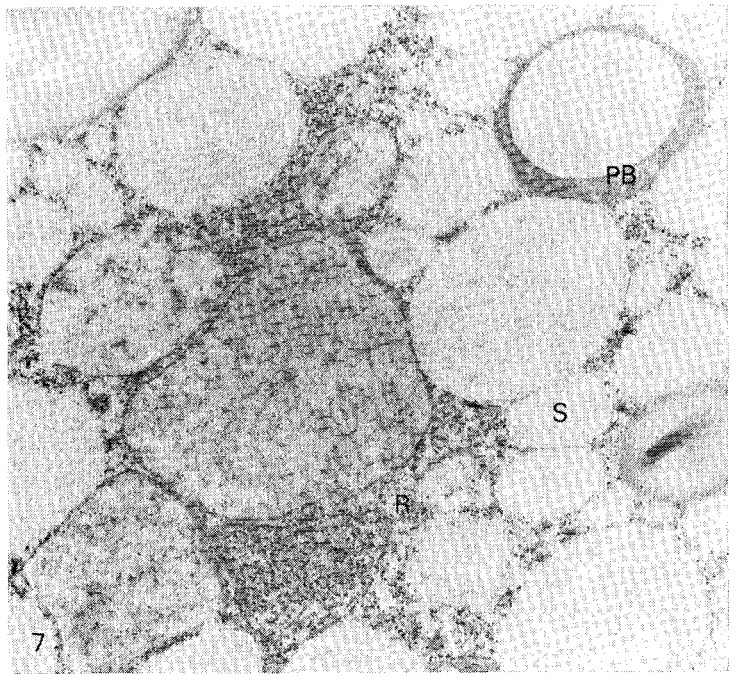


Figure 7. Part of a slightly damaged cotyledon cell after two days' imbibition. Lipolysis has been effective, but the cytoplasm contains numerous free ribosomes (R) and a body surrounded by a membrane and containing ribosomes is visible. Fixation: Glutaraldehyde—OsO_4. 42,000×.

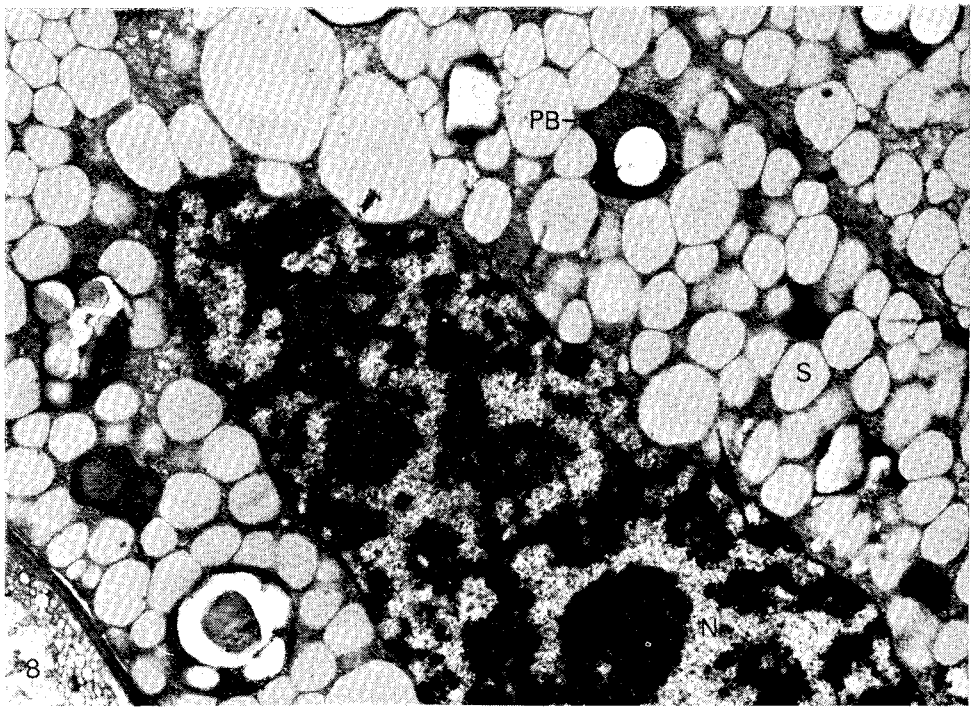


Figure 8. Part of a moderately damaged rootlet cell after 5 days' imbibition. Almost no ultrastructural changes. Nucleus (N) well preserved. Protein bodies (PB) and spherosomes (S) intact. Fixation: Glutaraldehyde—OsO_4. 11,000×.

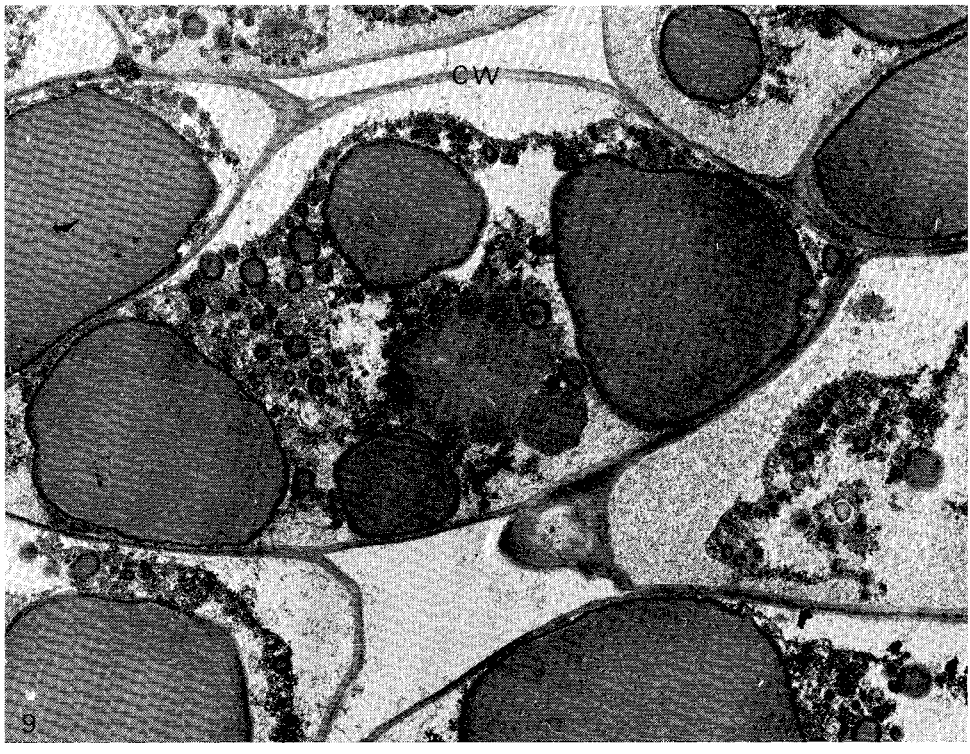


Figure 9. Rootlet cells of a severely deteriorated embryo after 5 days' imbibition. The storage material has fused into large vacuoles. Fixation: Glutaraldehyde—OsO_4. 3000×.

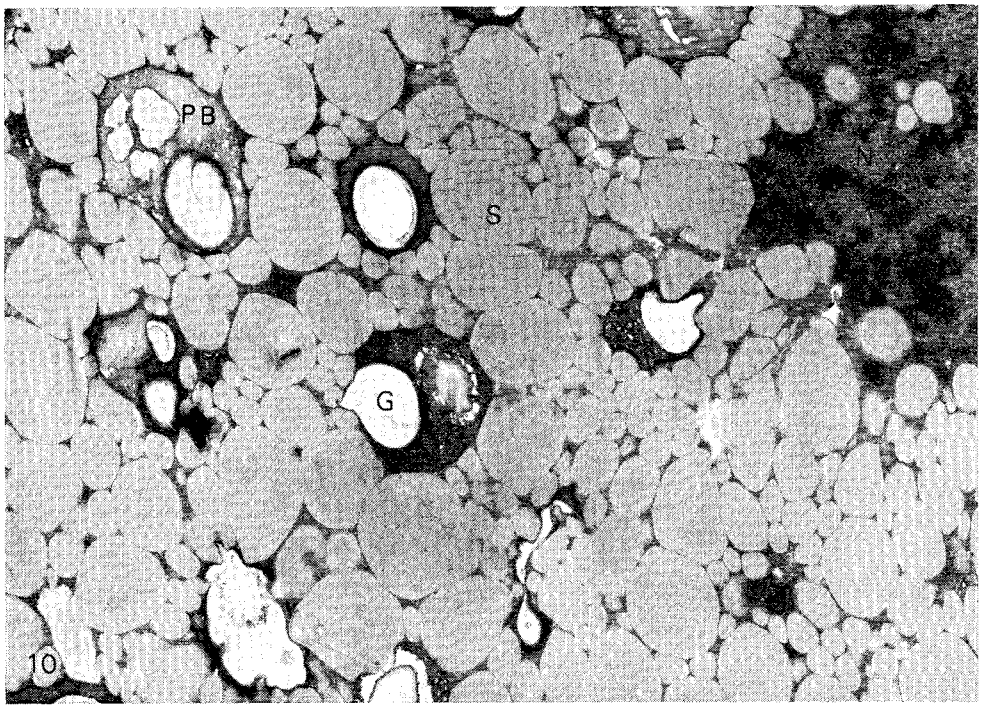


Figure 10. Part of a moderately damaged cotyledon cell after 5 days' imbibition. Only small ultrastructural changes as compared with cells of dry cotyledons. Nucleus (N) lobed. Protein bodies (PB) and spherosomes (S) intact. Fixation: Glutaraldehyde—OsO_4. 8500×.

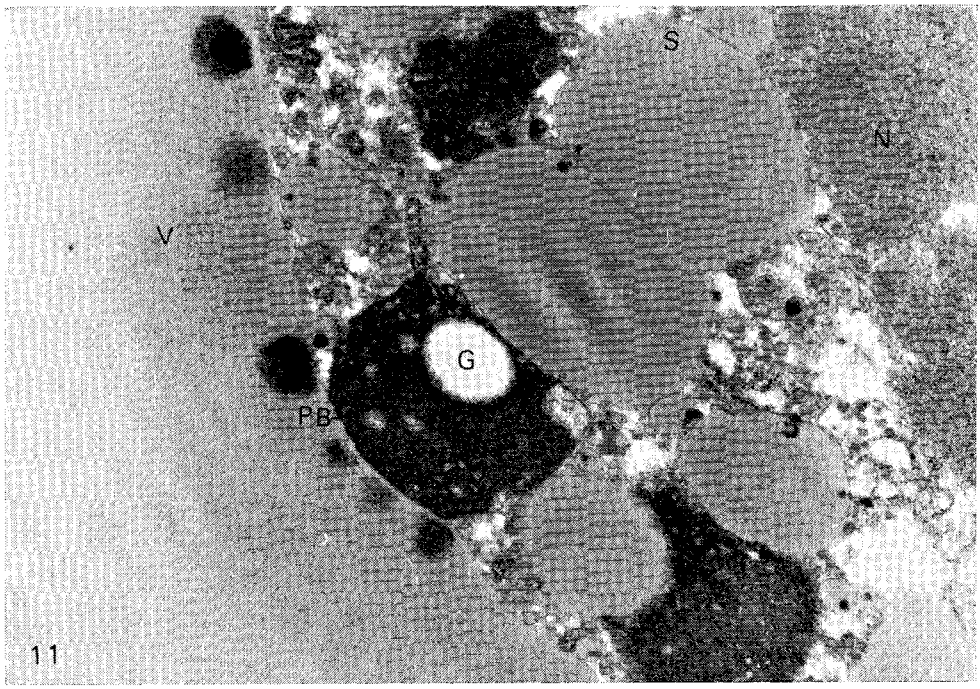


Figure 11. Part of a severely damaged cotyledon cell. 5 days' imbibition. Protein bodies (PB) have preserved their main structure. A great number of spherosomes and parts of protein bodies have fused together without changes in the electron-density of their content and formed a vacuole (V). Part of a nucleus (N). Fixation: Glutaraldehyde—OsO_4. 25,000×.

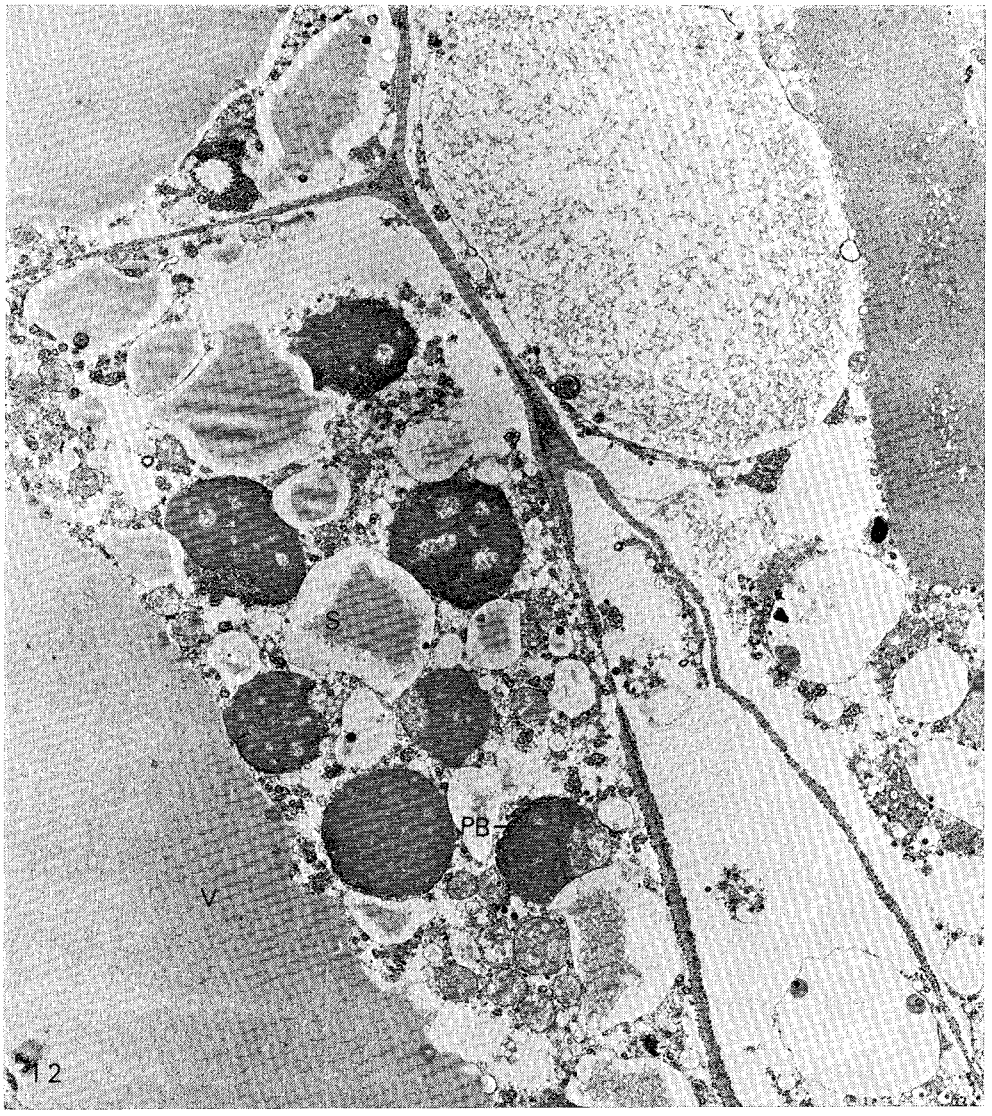
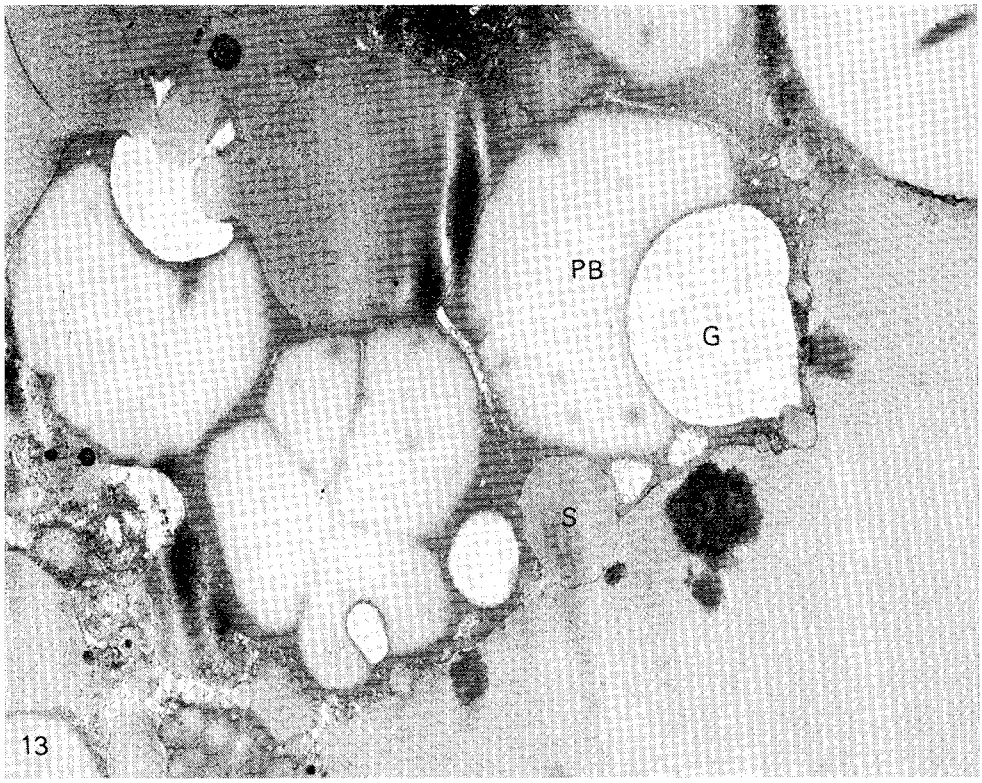
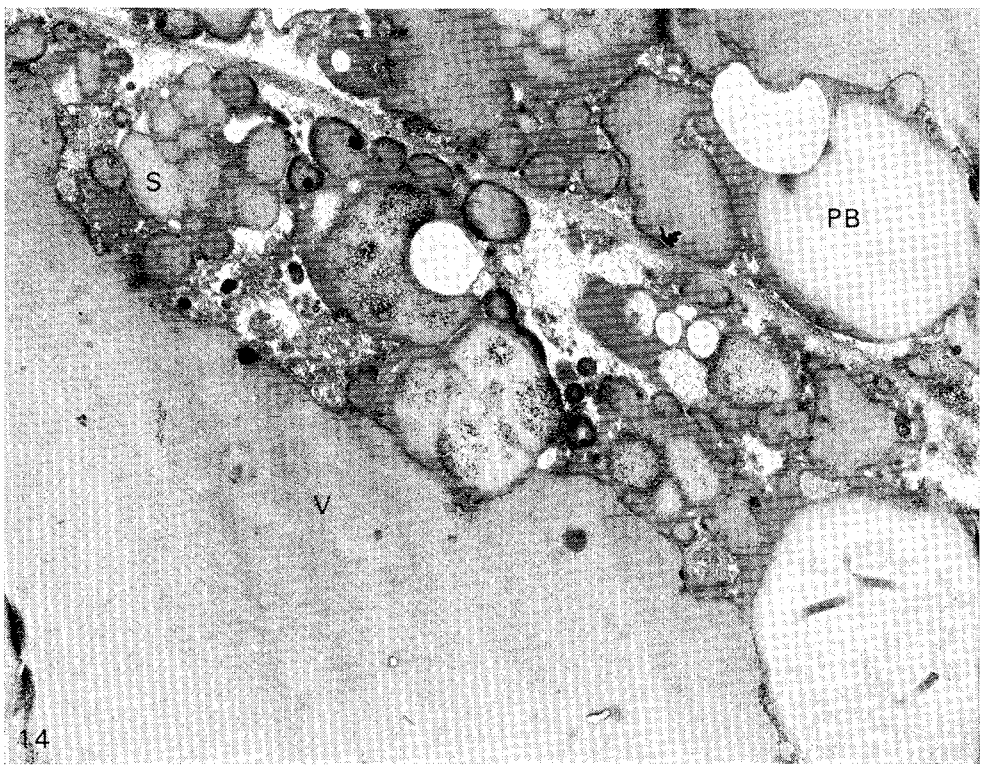


Figure 12. Cotyledon cells of a severely damaged embryo after 5 days' imbibition. Lipolysis has started at the edges of the spherosomes (S). Protein bodies (PB) (electron-dense but with some lighter areas) located at the edges of the cells. Storage material forms large central vacuoles (V) in the middle of the cell. No plasmalemma or organelles are recognisable. Fixation: Glutaraldehyde—OsO_4. 8500×.





Figures 13—14. Endosperm cells of an aged seed after 5 days' imbibition. Protein bodies (PB) with a large eccentric globoid cavity (G). No phytate visible. Large central vacuole (V) formed in the middle of the cell. Plasmalemma and organelles unrecognisable. Fixation: Glutaraldehyde—OsO_4. Fig. 13 17,000×. Fig. 14 11,000×.