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Morphological aspects of starch and cell wall material mobilization in developing lupine cotyledons and the effect of kinetin on these processes

FORTUNAT MŁODZIANOWSKI AND MARIA WESOŁOWSKA

Department of General Botany, Institute of Biology, A. Mickiewicz University, Poznań

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Abstract

In the cotyledons of dry lupine seeds the presence of starch was not demonstrated. Its formation during seed imbibition in darkness is accompanied by a reduction in the thickness of cell walls containing hemicelluloses. It is believed that the products of hemicellulose hydrolysis, particullarily in isolated cotyledons, are the main source of materials for the synthesis of starch. In the process of cell wall decomposition the invaginations of plasmalemma appear to be involved. Kinetin enhance the hydrolysis of cell walls and the mobilization of starch in isolated cotyledons.

INTRODUCTION

The main storage material for lupine seeds is protein, and starch appears only during the imbibition of the seeds (Młodzianowski and Gezela 1974). As can be seen from the data reported by Krietowicz (1965) and Meyer and Poljakoff-Mayber (1963) in the cell walls of lupine seedlings there occur considerable quantities of storage hemicelluloses which constitute the main substrate in the respiration process and play a basic role as a source of materials for the building of new cells. According to Krietowicz (1955) the main products of the hydrolysis of lupine hemicelluloses are glucose and galactose. In the cotyledons of other Legums, which are rich in hemicelluloses, a mobilisation of this component of the cell wall has been reported for peas (Flinn 1969) and recently Smith (1974) has found that in the cotyledons of beans there is first a swelling and then a reduction in the thickness of the cell walls during germination.

A utilisation of cell wall materials as storage substances has been reported also for monocotyledonous plants (Meier 1958, Mac Leod and Sandie 1961). The thinning of cell walls during germination of Yucca seeds has been observed by Horner and Arnott (1966).

In the present paper, on the basis of the changes observable in the morphology of cell walls containing hemicelluloses (Krietowicz 1955) and in the content of starch during the development of lupine cotyledons, an attempt was made to evaluate mutual relationships between these two carbohydrates.

In the study the effect of kinetin on the metabolism of hemicelluloses and starch was also investigated. The function of this growth regulator is primarily known to stimulate the synthesis of nucleic acids and proteins (Kende 1971), however recently an increasing number of papers indicates that it participates also in the regulation of carbohydrate metabolism (Gepstain and Ilan 1970, Berridge and Ralph 1971, Tetley and Thimann 1974).

MATERIALS AND METHODS

The seeds of lupine (*Lupinus luteus* L. cv. Express) have been placed for 24 hours in water and then for a further 24 hours onto a germinator in darkness. In this stage the cotyledons were severed from the embryo axes and placed with the inner surface (the side of palisade parenchyma) down onto Petri dishes containing water (control) or a solution of kinetin at a concentration of 10 mg/l. The dishes were exposed to weak light (14 lux), similarily as in the previous study (Młodzianowski and Gezela 1974). The solutions were changed every second day and material for study was taken after 3, 6 and 12 days of culture, always from the same places. In the electron microscopy observations also 4-day old cotyledons from etiolated seedlings were included.

For the needs of the paraffin method the cotyledons were fixed in FAA (90 ml 70% ethanol + 5 ml of acetic acid + 5 ml of 40% formalin). The material embedded in Epone was fixed in glutaral aldehyde in a 0.1 M phosphate buffer with a pH of 7 for 2 hours at room temperature and at a temperature of +4°C for the whole night, and after washing out of the fixative it was postfixed in 2% OsO_4 in a phosphate buffer for 2 hours at room temperature. In the paraffin method dehydration was accomplished through an ethanol series and xylene, before embedding in Epone the material was washed in the phosphate buffer and dehydrated in an ethanol and propylene oxide.

The thickness of paraffin sections was 12 μm and those of semi-thin Epone sections of about 1 μm, for staining with toluidine blue according to Kaya (1965). Ultra-thin sections were contrasted with uranyl acetate

and lead citrate according to Reynolds (1963) and examined in a JEM 7A electron microscope. Starch was identified in the light microscope with I_2KI.

RESULTS

I. *Formation and mobilization of starch*

A. Dry seeds. In dry seeds the presence of starch was not observed by any of the methods employed (Fig. 1, 18). Proplastids, which are the only organelles accumulating starch contained in the granular matrix only well formed plastoglobules (Fig. 18) and as was demonstrated earlier, phytoferritin (Młodzianowski and Gezela 1974).

B. Seeds germinated in darkness. After a period of imbibition and germination (two days) in darkness on water in all the cells of the cotyledons there appeared large grains of starch, which was observable in light (Fig. 2) as well as in electrone (Fig. 19) microscope.

C. Isolated cotyledons incubated in low light intensity. The observations were conducted after 3, 6 and 12 days of incubation of isolated cotyledons in water (control) and in kinetin solution.

After three days of incubation both in water (Fig. 3 and 14), and in kinetin solution (Fig. 4 and 15), in all types of cells except for the upper epidermis starch was observed. On the kinetin solution the starch grains were smaller and there were fewer of them. In further stages a gradual degradation of starch was observed. After 6 days of incubation there was still quite a lot of starch in the control material (Fig. 5) and after 12 days only sporadic grains were detectable (Fig. 7 and 9), whereas after incubation on kinetin solution there was very little starch after 6 days (Fig. 6) and after 12 days it was not found at all (Fig. 8 and 10).

II. *Changes in the cell walls*

A. Dry seeds and seeds during imbibition. The cell walls of lupine cotyledons are thickened. These thickenings form wide strips on some sections (Fig. 11), whereas on others they coccur in corners of cells, similarily as in collenchyma. In the conrners of cells adjacent to intercellular spaces the cell wals are thickest. Between the corner thickenings of cell walls there occur numerous pits, which in an electrone microscope turned out to be pit fields with plasmodesmata (Fig. 12 and 25). Along the cell wall a layer of lipid bodies (spherosomes) was observed (Fig. 18).

B. Seeds germinated in darkness. During germination of lupine seeds in the darkness the thicknenings of walls described above appear to be greater, and a profile of these walls from the side of the cytoplasm

is wavy (Fig. 20 and 21). In the swallen thickenings the layering of the cell walls is visible and a distinct gradient of the loosening of the swallen inner cell wall layer. Lipid bodies (spherosomes) are agglomerated close to the plasmalemma. It is difficult in this stage to see the differences in the thickness of the cell walls between seeds germinating on water and those on kinetin solution. From a comparison of Figs. 12 and 13 it can be seen that the effect of kinetin is only to enhance the hydrolysis of the storage proteins.

C. Cotyledons of etiolated seedlings. During the germination of seeds, and particularily in the 4-days old etiolated seedlings, numerous invaginations of the plasmalemma are observed, containing an electron-dense material in the form of delicate fibres and some membranous inclusions (Fig. 26—33). Such invaginations of the plasmalemma were observed primarily near the thickenings (Fig. 27). Not only in the sections perpedincular to the wall (Fig. 30—32) but also in the tangential ones (Fig. 28) it was possible to observe a loosening of the fibrous elements building a wall in the region of the formation of plasmalemma invaginations.

On the basis of the electronograms presented it can be judged that the material in the plasmalemma hollows is separated by the formation of visicles and transferred into the cytoplasm. The vesicles were frequently in close proximity to the microbodies and mitochondria (Fig. 32, 33).

D. Cotyledons isolated and exposed to low light. In the isolated cotyledons the thickness of the cell walls declined substantially during the time of incubation, so that after 12 days of the experiment the cells walls are very thin (Fig. 14, 16 and 22). Kinetin markedly increased the process of cell wall thickness reduction (Fig. 15, 17 and 23).

In the morphology of the structures lining the walls no qualitative differences were observed between the material incubated in water and in kinetin solution. The morphological aspect of the kinetin stimulated hydrolysis of the cell walls could be only demonstrated by quantitative studies. In Figs. 24 and 25 the formation of the plasmalemma invaginations is shown near wall thickenings of cotyledons incubated on kinetin solution. Similar configurations of the plasmalemma, of the endoplasmic reticulum and of the vesicles have been observed at the same time though to a lesser extent in isolated cotyledons incubated on water as well as in cotyledons of etiolated seedlings (Fig. 26—33).

DISCUSSION

As was mentioned previously (Młodzianowski and Gezela 1974) and shown in detail in this study starch appears during the swelling of lupine seeds, and it is not a photosynthetic effect, since the process is observable in the darkness, in plastids without any thylakoids. The source

of material for the synthesis of starch in lupine cotyledons has to be sought in the existing storage materials of the seeds. The main storage material of lupine seeds are proteins appearing in the form of aleurone grains (Wesołowska and Młodzianowski — in print) and hemicelluloses present in the cell walls (Krietowicz 1955). The slight quantities of lipids cannot be considered as the source of materials for starch formation because their levels do not change significantly during the germination of seeds. (Wesołowska 1972). It appears therefore that hemicelluloses can be the source of sugars for the synthesis of starch. The studies of Hoffmanowa (personal communication) have shown that the quantity of proteins in isolated lupine cotyledons cultured in light is maintained at more or less the same level for 15 days of incubation, whereas the level of simple sugars rapidly increases in germinating seeds.

An increase in the level of simple sugars, coupled with the microscopically visible reduction in the cell walls that contain hemicelluloses, and the appearance of starch in the plastids seem to indicate the following sequence: hemicellulose, simple sugars, starch, occurring during the germination of lupine seeds. This conclusion, based primarily on morphological data presented in this paper would require confirmation by detailed biochemical analyses. From certain biochemical studies it is already known that hemicelluloses are forms of polysaccharides easy to mobilize during seed germination and easily to change into soluble sugars. On the germination of yellow lupine seeds about 90% of hemicelluloses are hydrolyzed to glucose as well as about 96% of polysaccharides that are polymers of galactose, and utilized in the processes of respiration and tissue building (Krietowicz 1955).

Since in the case of the isolated lupine cotyledons the products of hydrolysis of hemicelluloses cannot be transferred onto the embryo axes, the excess is accumulated primarily in the form of starch. This can be judged from a comparison of the starch content in excised and attached cotyledons (studies in progress).

It still remains to be clarified whether the globular and oval bodies densely lining the cell walls are only lipid bodies or they are also spherosomes containing some hydrolytic enzymes associated with the process of cell wall digestion. It might be mentioned here that in the lupine cotyledons it was possible to show in a light microscope both the lipids and the activity of the acid phosphatase in small, spherical bodies along the cell walls (unpublished data).

The numerous plasmalemma invaginations with some electron — dense substance osbervable during the reduction in the thickness of the cell walls in lupine cotyledons might be associated with the process of cell wall digestion. A similar phenomenon was described in longlasting cultures of protonemas of the moss *Funaria hygrometrica* which was

grown in the darkness on a medium containing glucose. In the early phase of the culturing of the protonema a thickening of the walls was observed and later, in starvation conditions, a reduction of the thickness. In that phase of the culture it was also possible to observe in the cells the vesicles and plasmalemma invaginations containing a fibrous material (Młodzianowski et al. 1972). Recently Mahlberg et al. (1974) have described a fibrous material similar to that forming the cell walls, which formed in the secondary vacuoles through an invagination of the plasmalemma, in tissue cultures of *Helianthus*. The observed invaginations of plasmalemma forming during hydrolysis of the cell walls suggest that the phenomenon is a common one.

A close association of the vesicles containing fibrous material with the microbodies that can be considered as glyoxysomes in view of the fact the seedlings grew in the darkness, suggest the possibility that they participate together with mitochondria grouped near these structures (Fig. 29) in the processes associated with carbohydrate turnover (Zurzycki and quoted literature, 1974).

The role of kinetin in the metabolism of cell walls has been frequently pointed out, indicating that it stimulates the process of cell wall lignification (Koblitz 1971, Halperin and Minocha 1973). The role of kinetin relative to the cell walls rich in hemicelluloses, appears to be associated with the stimulation of the mobilization of these reserve materials.

The later disappearance of starch grains also appears to be stimulated by the presence of kinetin.

The above interpretation of the results finds confirmation in the studies of Berridge and Ralph (1971) on leaf discs of *Brassica pekinensis* which have shown that kinetin mobilizes storage starch and increases the flow of carbohydrates needed for the synthesis of membranes building cellular structures and for the formation of ATP. As has been reported by Gepstain and Ilan (1970) kinetin has increased the amylase activity in bean cotyledons, and according to Sen and Sharma (1972) kinetin can compensate for the lack of the embryo axis in isolated cotyledons of *Merremia aegyptia* in the induction of amylase decomposing storage starch.

In conclusion it may be said that the effect of cytokinins is manifested not only in the maintainance of the level and increase of synthesis of nucleic acids and proteins (Kende 1971) but also in the carbohydrate metabolism, which is indicated by morphological observations presented in this paper and in a number of recent reports quoted in the introduction.

We wisch to thank Prof. Szweykowska for the facilities and critical reading of this report.

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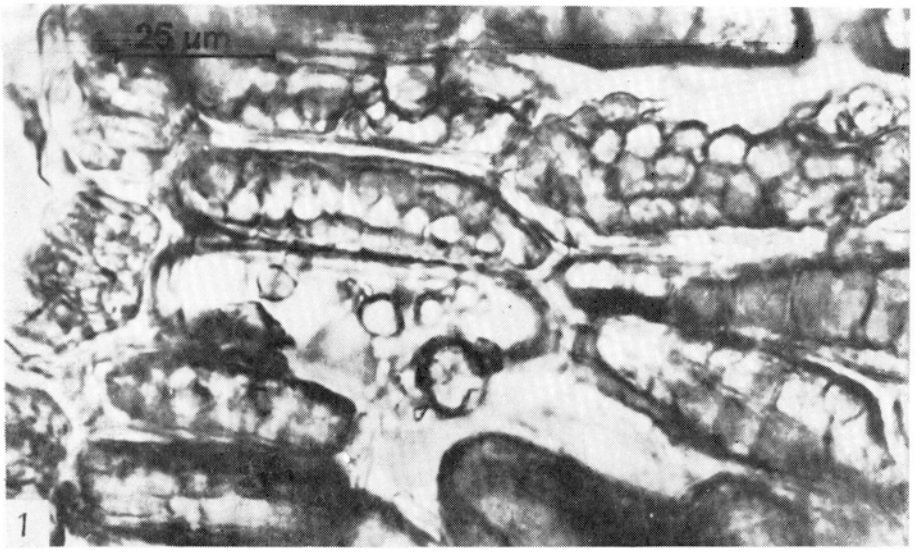
Authors' address:
Dr Fortunat Młodzianowski and
Dr Maria Wesołowska
Department of General Botany,
Institute of Biology A. Mickiewicz University;
Stalingradzka 14; 61-713 Poznań; Poland

Synteza i mobilizacja skrobi oraz zmiany ściany komórkowej w rozwijających się liścieniach łubinu z uwzględnieniem wpływu kinetyny na te procesy

Streszczenie

W liścieniach suchych nasion łubinu nie wykazano obecności skrobi. Jej tworzeniu się w czasie pęcznienia nasion w ciemności towarzyszy redukcja grubości ścian komórkowych zawierających hemicelulozy. Uważa się, że produkty hydrolizy hemiceluloz, szczególnie w izolowanych liścieniach, stanowią główne źródło dla syntezy skrobi. Udział w procesie rozkładu ściany komórkowej przypisuje się inwaginacjom plazmalemny.

Kinetyna przyspiesza hydrolizę ścian komórkowych i mobilizację skrobi w izolowanych liścieniach.



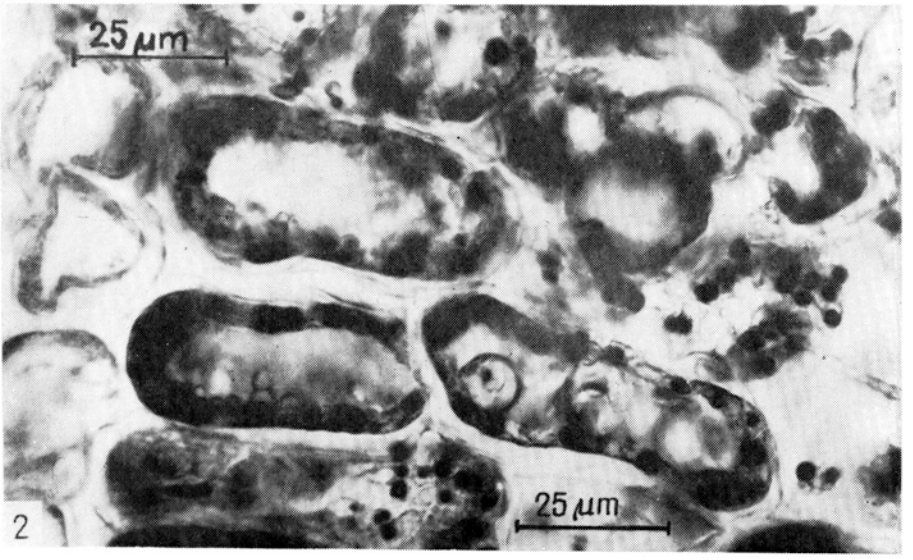


Fig. 1. Lack of starch in the cotyledons of air dried seeds (negative KI_2 reaction). Paraffin preparation.

Fig. 2. Starch in the cotyledons of seeds germinating in the darkness on water (positive KI_2 reaction). Paraffin preparation.

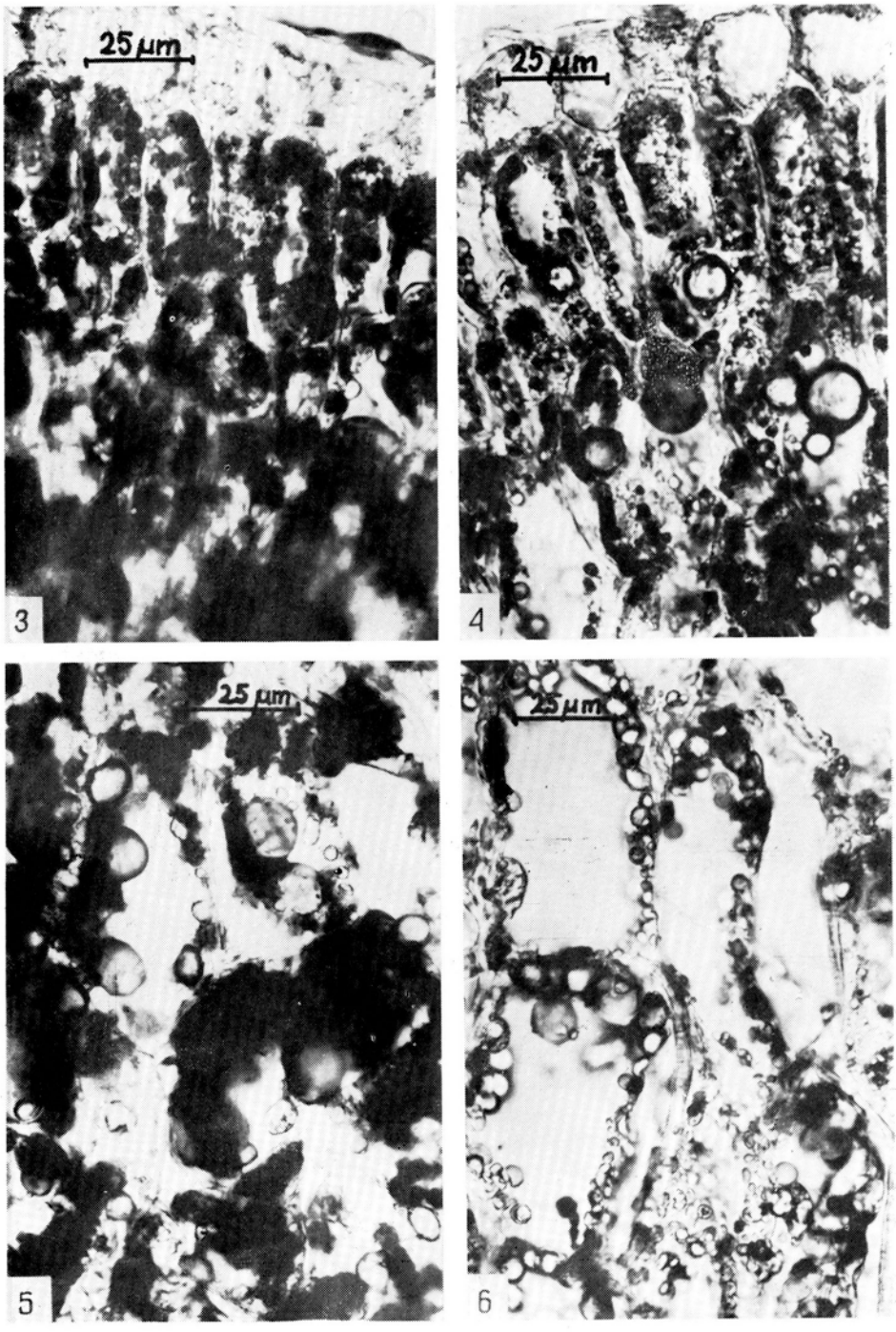


Fig. 3—4. Except for the epidermis there is a lot of starch in the cotyledons incubated for 3 days on water (Fig. 3) but less in those incubated on kinetin solution (Fig. 4). Paraffin preparation.

Fig. 5—6. Starch in the spongy parenchyma of cotyledons incubated for 6 days on water (Fig. 5) and kinetin solution (Fig. 6). Paraffin preparation.

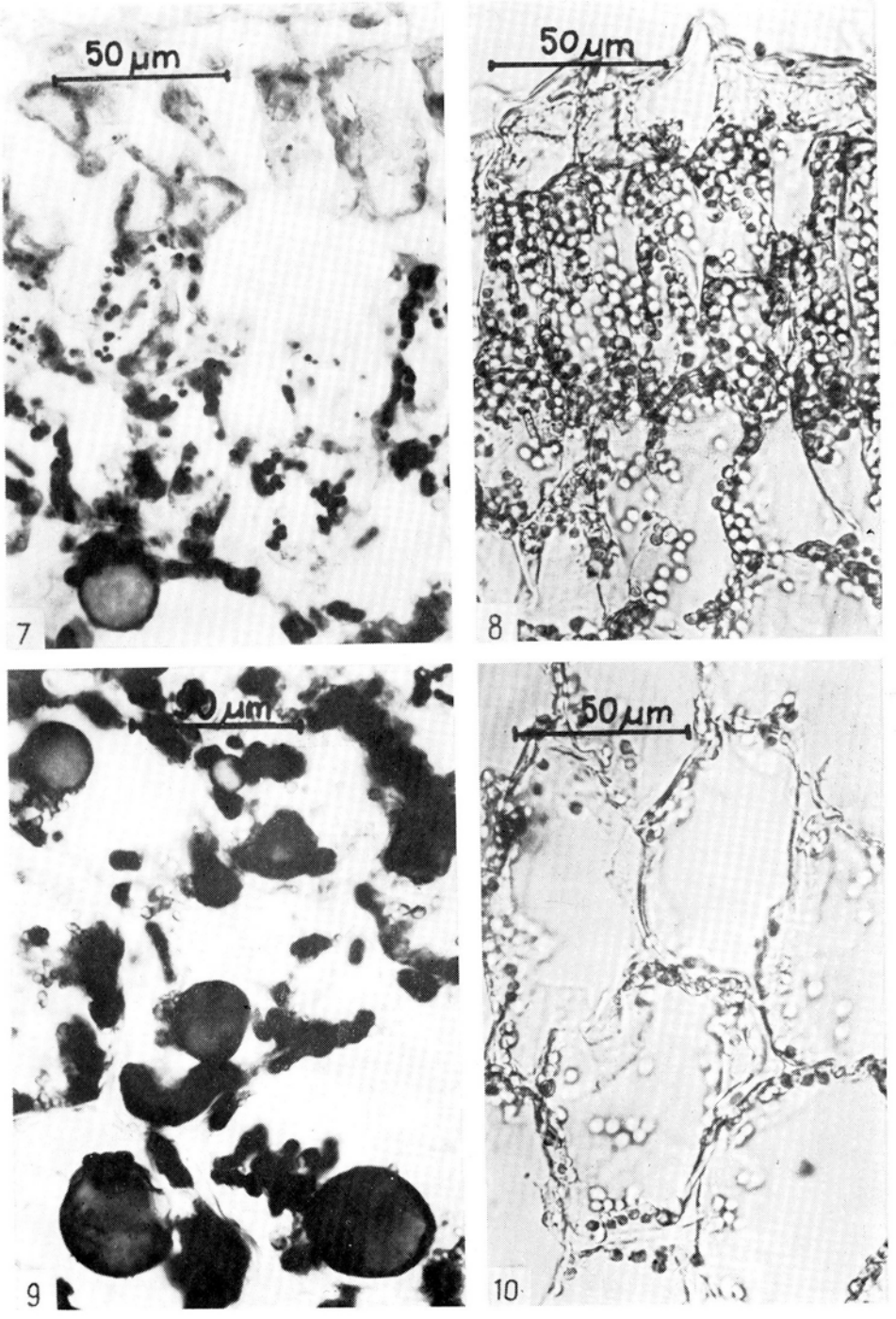


Fig. 7—10. Starch in the palisade (Fig. 7) and spongy (Fig. 9) parenchyma incubated in water, and the absence of starch in the material incubated in kinetin solution, both in palisade (Fig. 8) and spongy (Fig. 10) paranchyma. Paraffin preparation.

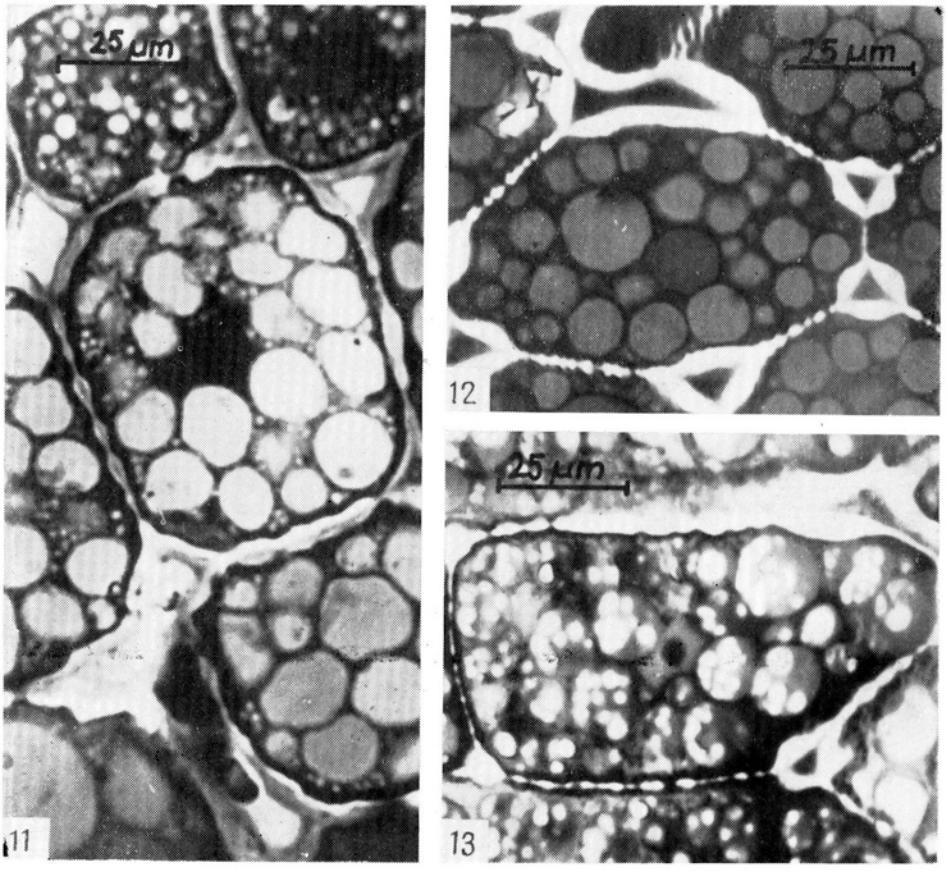
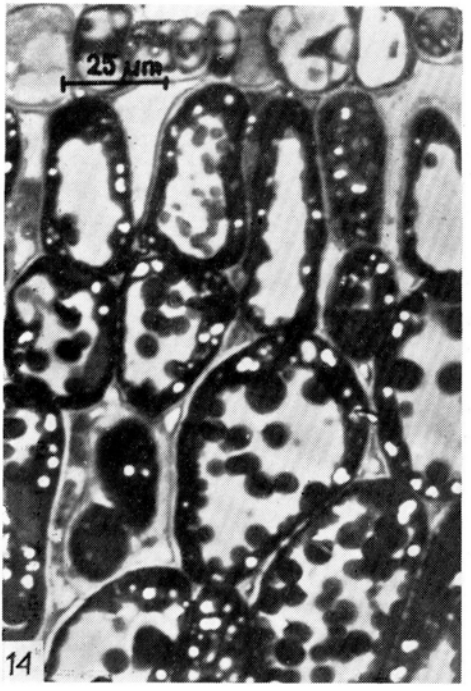
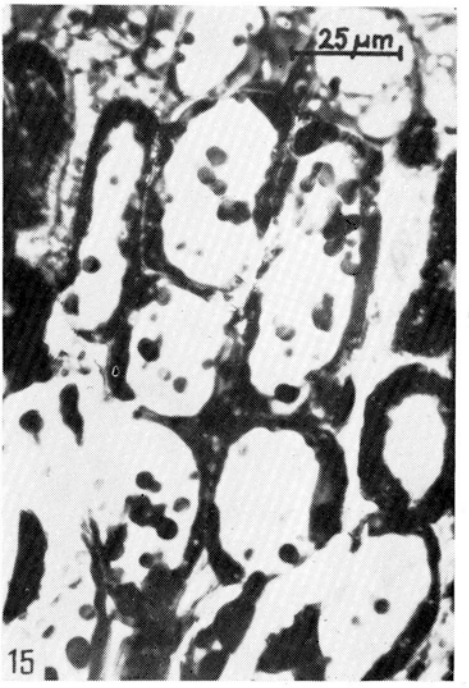
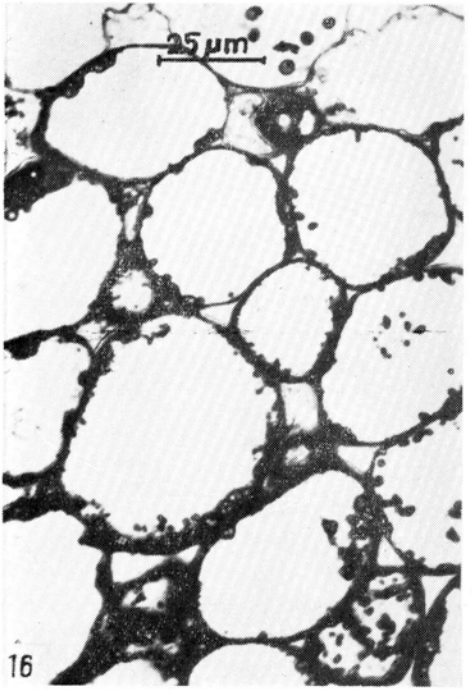


Fig. 11—13. Semi-thin Epone sections stained with toluidine blue. Visible thickenings in the cell walls of dry material (Fig. 11), after germination on water (Fig. 12) and on kinetin solution (Fig. 13).







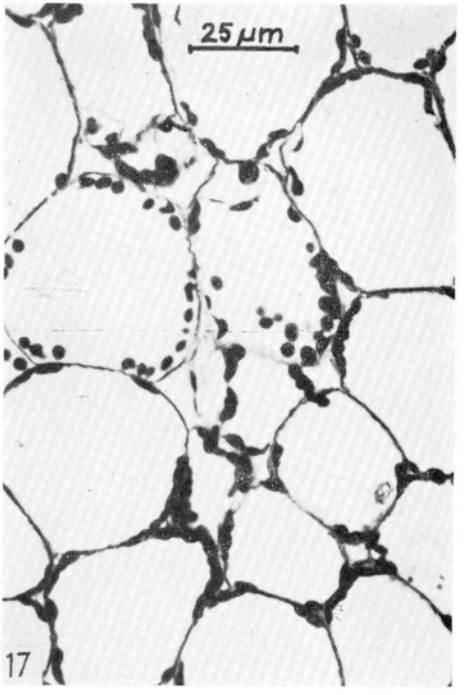
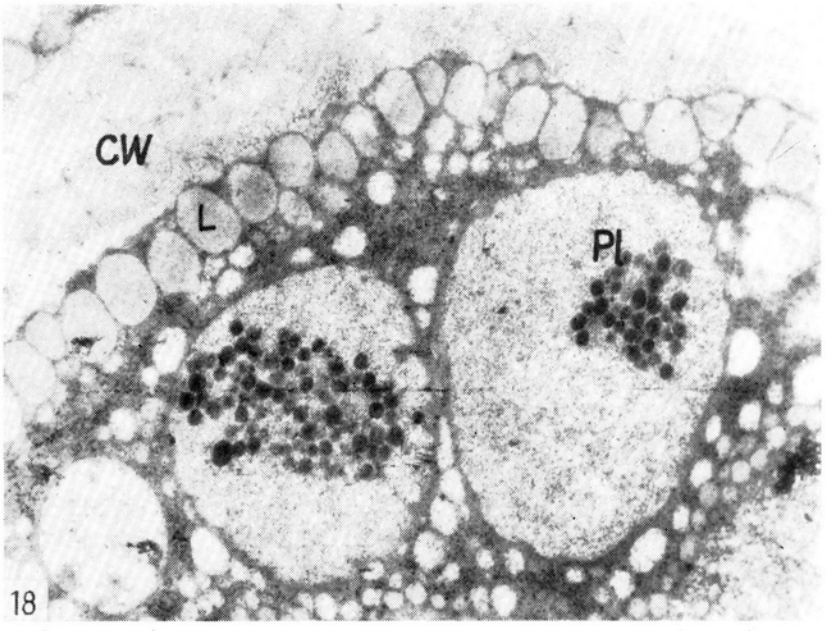


Fig. 14—15. Epone sections stained with toluidine blue. In cotyledons incubated in water (Fig. 14) the cell walls are thicker, and there is more storage protein (dark stains) and starch (bright stains) than in the material incubated in kinetin solution (Fig. 15).

Fig. 16—17. Spongy parenchyma of cotyledons incubated for 12 days in water (Fig. 16) and in kinetin solution (Fig. 17).



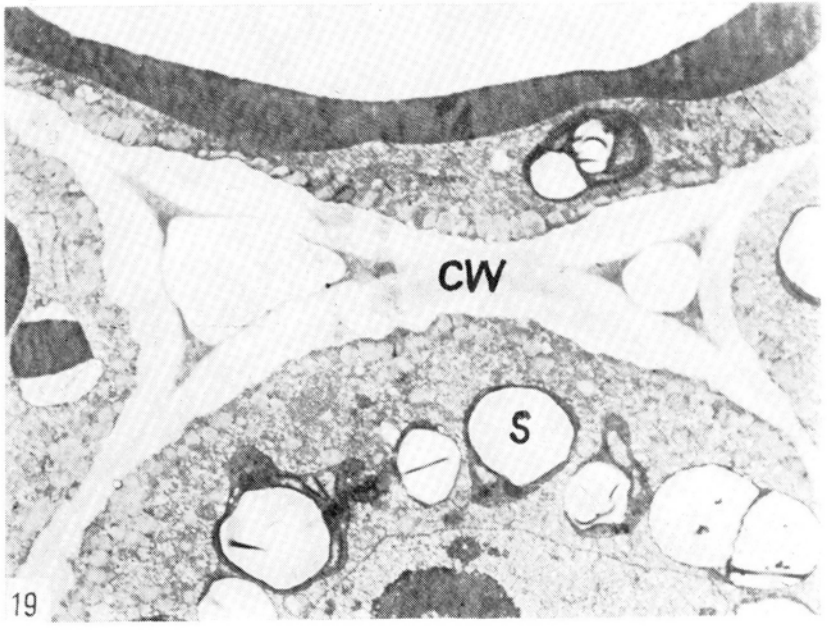
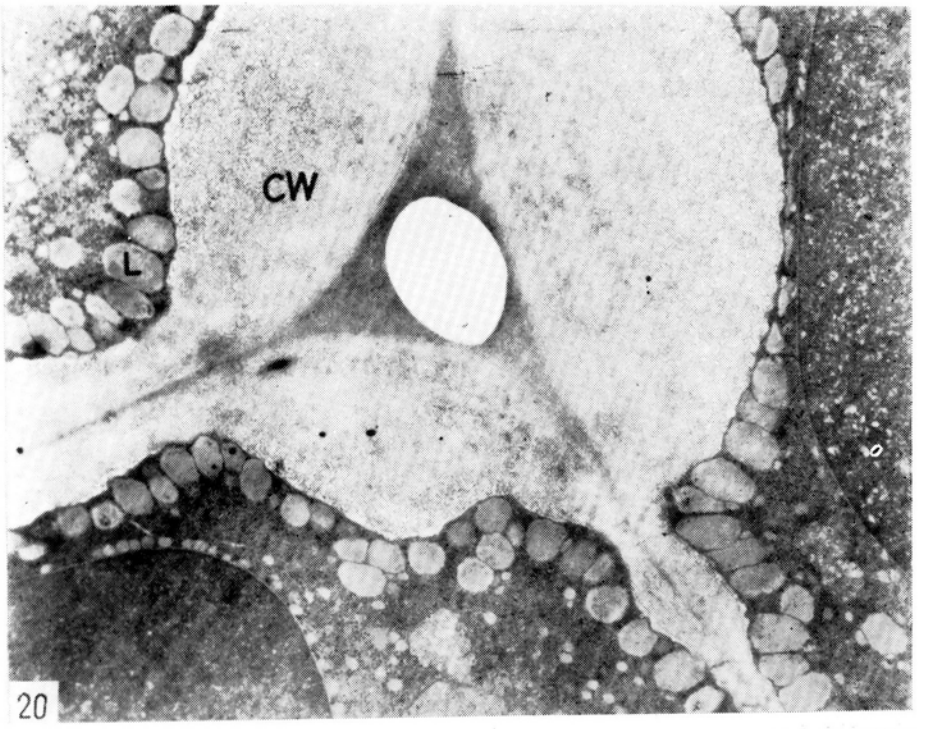


Fig. 18. Fragment of a parenchyma cell of an air dried cotyledon. In the proplastids starch is absent and plastoglobules (Pl) are visible. In the cytoplasm there are many vesicle-like structures. Near the cell wall (CW) there are numerous lipid bodies — L (spherosomes). × 15.000.

Fig. 19. Large grains of starch (*S*) in the cotyledons of seeds germinated in the darkness (24 hours) of soaking in water and 24 hours of incubation on wet filter paper on Petri dishes. Visible thickenings of the cell wall (CW). × 45.000.



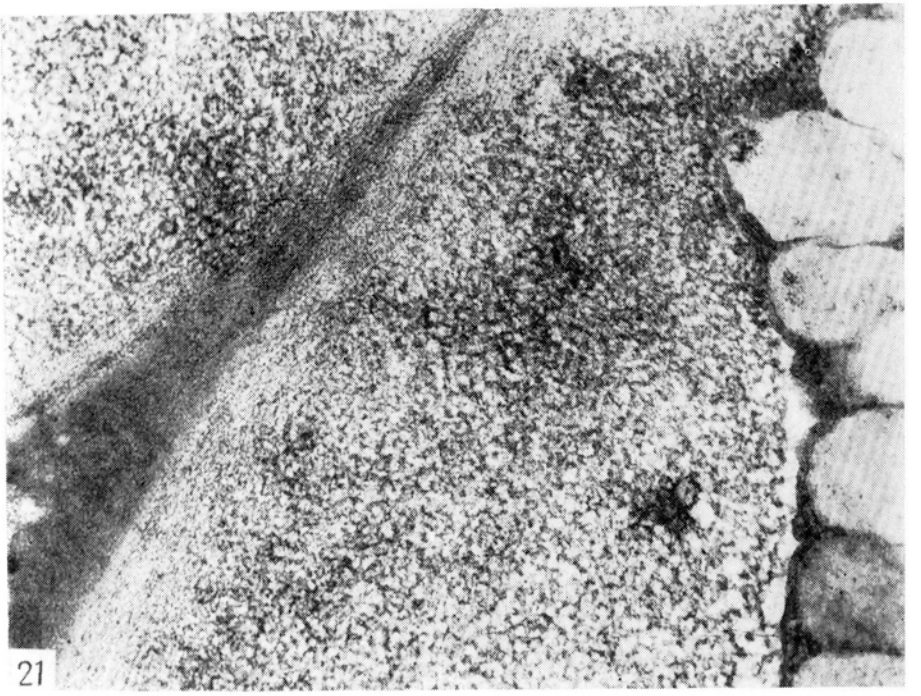


Fig. 20. Strongly thickened cell walls (*CW*) around intercellular spaces in the cotyledons of seeds germinated in the darkness on kinetin solution. The lipid bodies — *L* (spherosomes) are agglomerated near the cells walls. × 15.000.

Fig. 21. Details of the structure of the cell wall from Fig. 20. The thickened hemicellulose layer has a loose spongy structure. A gradient of the loosening is observable towards the cytoplasm. It is also possible to distinguish the compact middle lamella. × 44.000.

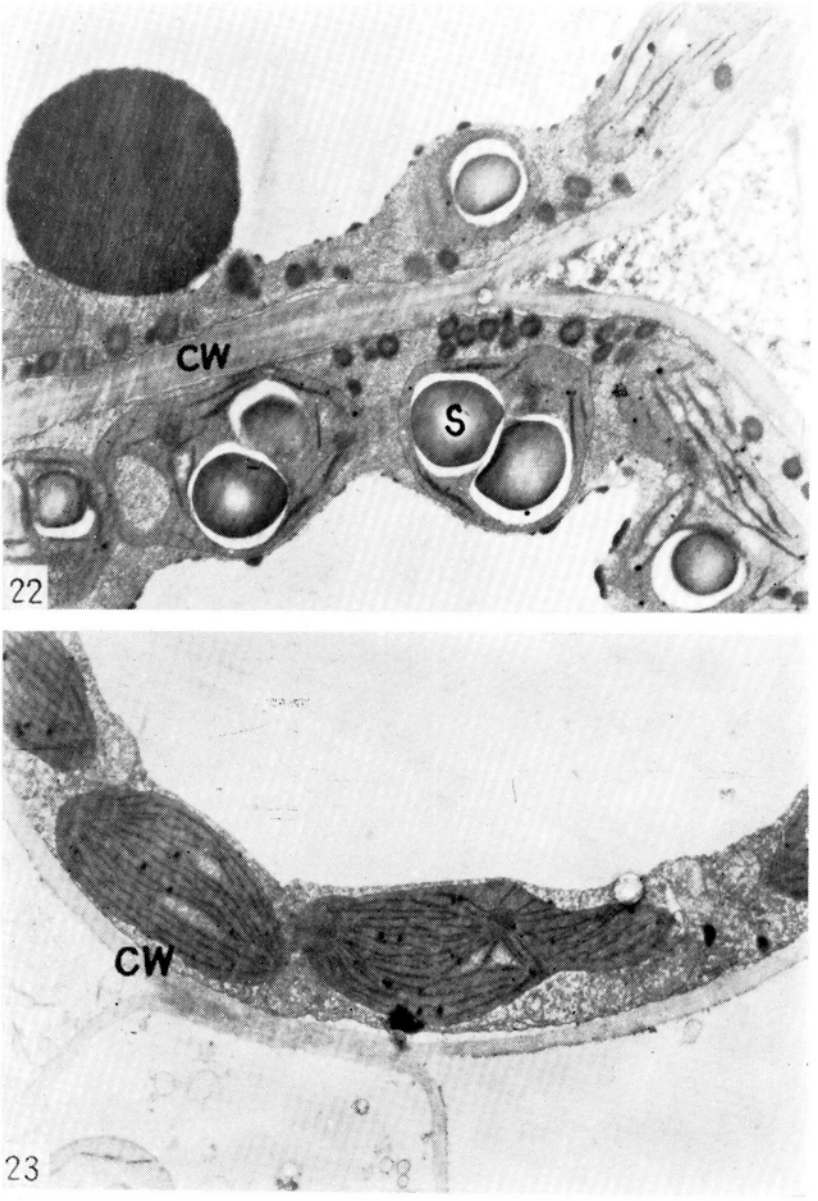
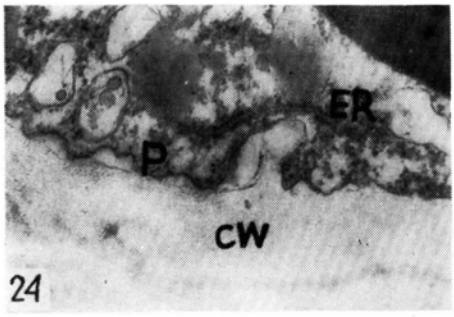
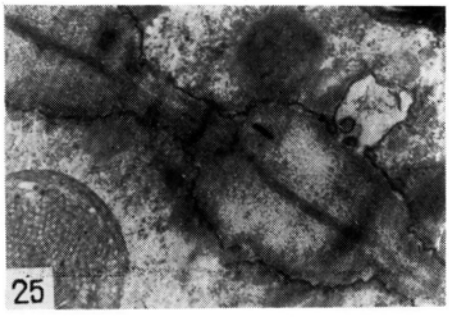
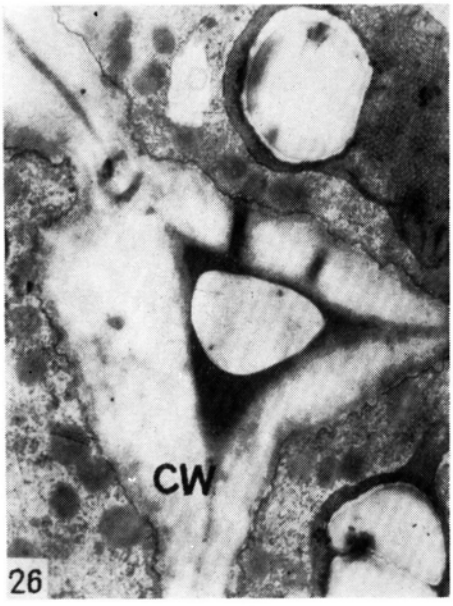
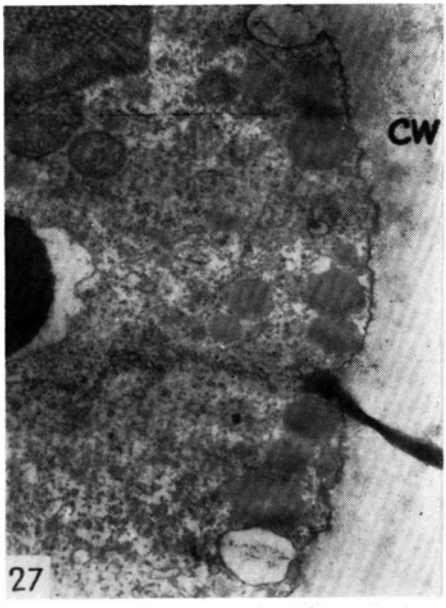


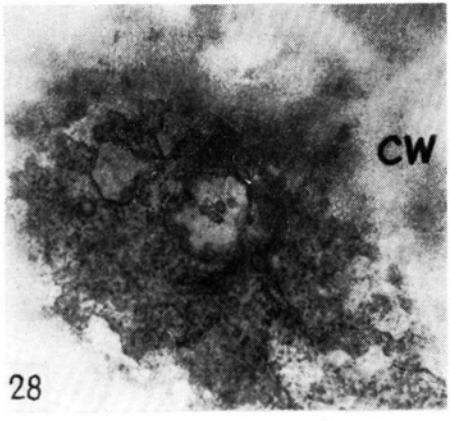
Fig. 22—23. Fragments of cotyledon cells isolated and incubated for 6 days on water (Fig. 22) and in a kinetin solution (Fig. 23). Differences are obsrvable in the starch content (*S*) and in the thickness of the cell walls (*CW*). × 10.000.











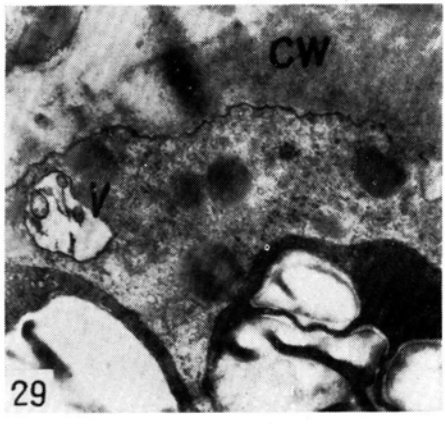
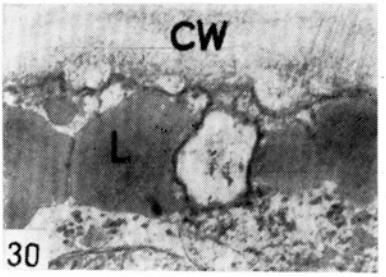
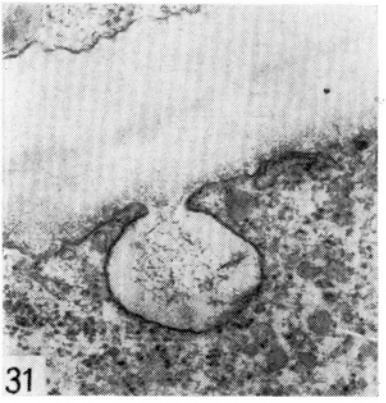
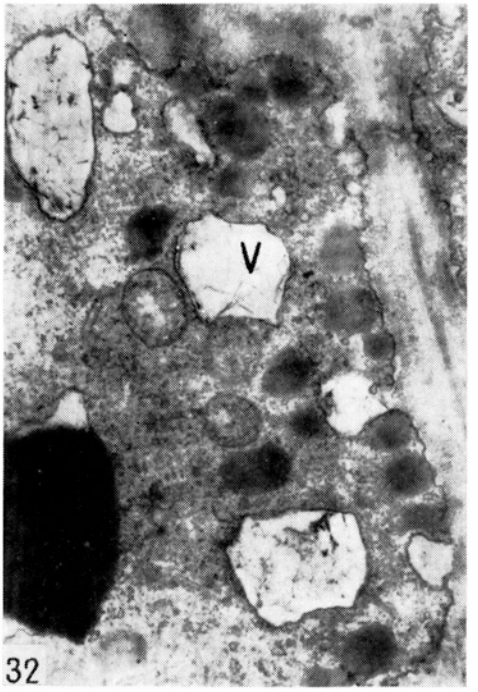


Fig. 24—28. Various cytoplasmic structures associated with the cell wall (*CW*) in isolated cotyledons incubated on a kinetin solution for 3 days (Fig. 24—25) and on etiolated 4-day old seedlings (Fig. 26—29). Fig. 24. Wavy plasmalemma (P) and its invaginations in close proximity to the endoplasmic reticulum (*ER*). × 28.000 Fig. 25. Considerable thickenings of cell with a wavy line of the plasmalemma. × 12.500. Fig. 27. Invaginations of the plasmalemma with finely fibrous content. × 12.000. Fig. 28. Tangential section through a cell wall and cytoplasm. The visible membranous configurations with the loosened elements of the cell wall. × 25.000. Fig. 29. Vesicles with fibrous and membranous structures (*V*) formed by the invaginations o fthe plasmalemma. (Compare with Fig. 25). × 14.500.







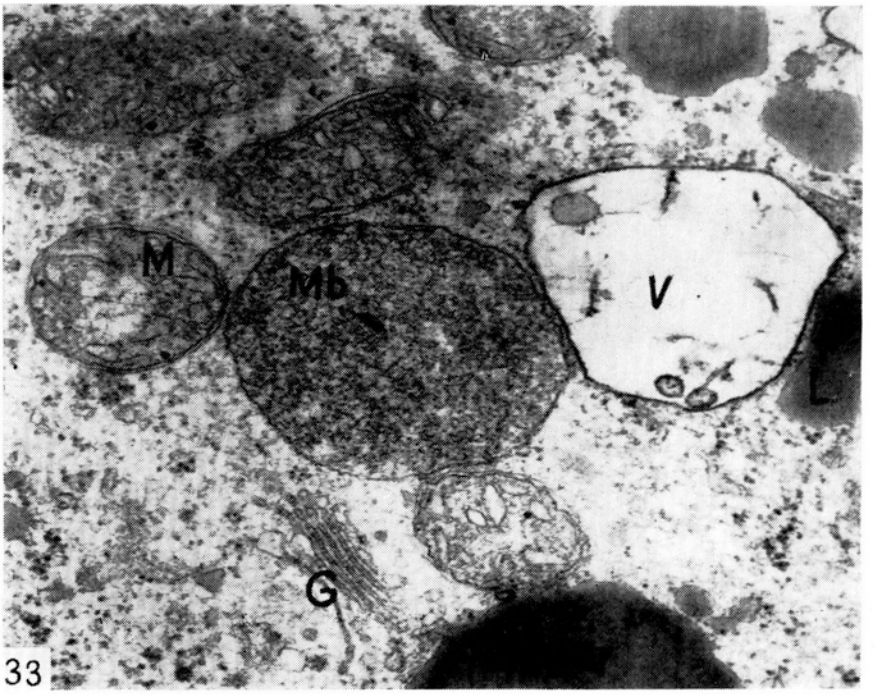


Fig. 30—33. Formation of the vesicular structures (V) in etiolated, 4-day old seedlings. Fig. 30. In the hollows of the plasmalemma diluted material of the cell walls (CW), and an intruding in between lipid-L droplets (spherosomes) vesicle with cell wall material can be seen. Fig. 31—32. Invaginations of plasmalemma and vesicles formed in this way with fibrous material. × 16.000. Fig. 33. A Complex: Microbody (*Mb*) mitochondrium (*M*), lipid body (*L*), Golgi apparatus (*G*), vesicle with fibrous material (*V*). × 32.000.