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Mobilization of Proteins in the Cotyledons of Germinating Soybeans (*Glycine max*)

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Abstract

The mobilization of proteins in the cotyledons of germinating soybean seeds (*Glycine max* [L.] Merr.) and seedlings was studied by using light microscopy and transmission electron microscopy. The cotyledon tissues of soybeans were packed with protein bodies(diameter 0.1-15μm) where storage protein of soybean is deposited. Degradation of protein bodies started in the epidermis and vascular tissues. After swelling of the protein bodies, autolysis of storage proteins began while the external membrane remained unbroken. Hydrolysis of proteins could be internal or peripheral and fusion might begin before complete protein degradation. Possible instances of vacuolar fusion were encountered in some cells. In all cases, the result of degradation was the same; the central vacuole of the cell. At the late stages of seedling growth, breakdown of tonoplast was observed in some cells.

Key words: mobilization, protein body, soybean, germination

Introduction

During germination and seedlings growth, the storage proteins of seeds are hydrolyzed into their constituent amino acids by endo- and exo -proteases. The liberated amino acids may be used for synthesis of new proteins, or to provide energy by oxidation of the carbon skeleton after deamination.

Several researches[1,2] have showed that total nitrogen content and water soluble protein nitrogen level were decreased gradually during germination and seedling growth. Son *et al.*[3] reported that the major reserve proteins of soybean (15s, 11s and 7s) was diminished in soybean sprouts and yang[4] reported the changes of amino acids composition in germinating soybean seeds and seedlings.

The storage proteins are located primarily in organelles called protein bodies which are restricted to specialized tissues of the seeds. Initially the cotyledon is storage organ of the seed, which is rich in protein and lipid(40% and 18%, respectively). In this study, particular attention has been given to the ultrastructural changes of protein bodies in cotyledons during germination and seedling growth. By using light microscopy and transmission electron microscopy, the ultrastructure of soybean seeds and seedlings and the pattern of protein body degradation in different tissues of cotyledons, such as epidermis, vascular tissues, and storage parenchyma, was observed. These structural changes will be discussed as far as possible with reference to the metabolism of germinating seeds and seedling.

Materials and methods

Plant material

Seeds of soybean (*Glycine max* [L.] Merr. variety 1984 AmSoy) were obtained from Dr. Ferr in Department of Agronomy, Iowa State University, Ames, U.S.A. and kept in refrigerator until used. Seeds were externally sterilized in 70% ethanol for 10 sec, rinsed with running tap water for 10 min, and soaked for 50 min in oxygen flushed bi-distilled water. Seeds were then layered on wet filter paper on cotton wool in a plastic box with a lid. The box was placed in the dark at 28±1℃.

Cotyledons of soybean seeds were selected ran-

domly from 1.5 hrs through 10 days of seedling growth. Time periods, where significant ultrastructural changes of protein bodies had been observed in preliminary experiments, were chosen.

Tissue processing

Cotyledons of germinated soybean seeds were placed in 4% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature. The tissue was cut with a razor blade into approximately 1mm^3 blocks and placed in fresh buffered fixative overnight at 4℃.

Fixation was followed by three buffer rinses, 10 min each, and post-fixed in 1% osmium tetroxide (OsO_4) for 4 hrs at room temperature using the 0.1 M sodium phosphate buffer (pH 7.4). The tissues were washed three times in buffer for 30 min followed by dehydration through a series of graded acetone solutions. Specimens were infiltrated with Spurr® resin (Polysciences, Inc., Warrington, PA) according to manufacturer's instruction for a "firm" block, cast in aluminum weighing pans, and placed in a 60℃ oven overnight.

Microscopy

Light microscopy. Thick sections were cut at 0.5-1.0μm with glass knives on a Reichert Ultracut E ultramicrotome. Sections were collected with a stick applicator and placed on a water droplet on a glass microscope slide. The slide was placed on a hot plate (50-55℃) to remove section wrinkles and to adhere the section to the slide. Sections were stained with 1% toluidine blue, washed, dried on warming tray, mounted in Permount, and dried on a warming tray for several days. Bright field observations were made on a Leitz Wetzlar Orthoplan microscope. Technical pan 2415 film was used in the attached Leitz Orthmat automatic camera.

Transmission electron microscopy. Ultrathin sections of approximately 60-85μm were cut with glass knives on a Reichert Ultracut E ultramicrotome, spread with chloroform fumes, and collected on 200 and 300 mesh copper grids. Sections were stained with 20% aqueous or 5% methanolic uranyl acetate, followed by 2% lead citrate.

Observations were performed on Hitachi HU-11C-1 transmission electron microscope at 50 KV accelerating voltage by using Dupont Cronar Ortho S Litho sheet films.

Results

The ultrastructure of dry seeds was difficult to preserve because there was little penetration of fixative due to the compactness of soybean cotyledon tissue. Cotyledons sampled after 1.5 hrs of germination were taken as tissue representative of the beginning of germination. It is recognized that the possibility of some changes during the 12-hour fixation period cannot be excluded. However, significant protein mobilization took place after 2 days of seedling growth. This 1.5 hrs period, therefore, is arbitrarily designated as the beginning of germination.

Anatomy of soybean cotyledon

The cotyledons are the site of reserve nutrient storage and account for almost 90% of the seed mass. Cotyledons of soybean comprise an epidermis, a distinct hypodermis, storage parenchyma, and vascular tissues (Figs. 1-2). Vascular tissues, surrounded by a parenchymatous bundle sheaths, are situated along the boundary between adaxial and abaxial parenchyma (Fig. 13). The cotyledons have an extensive system of intercellular spaces which can be observed as triangles at every cell wall junction of the parenchyma, when it is viewed as transverse sections (Figs. 9-10).

Mobilization of protein

Storage protein bodies was present in all cells of the soybean cotyledons including vascular tissues at the beginning of germination. In storage parenchyma cells, protein bodies were large (diameter: 2-15μm), and small protein bodies, mostly upto 0.1-5μm in diameter, occurred in epidermis and vas-

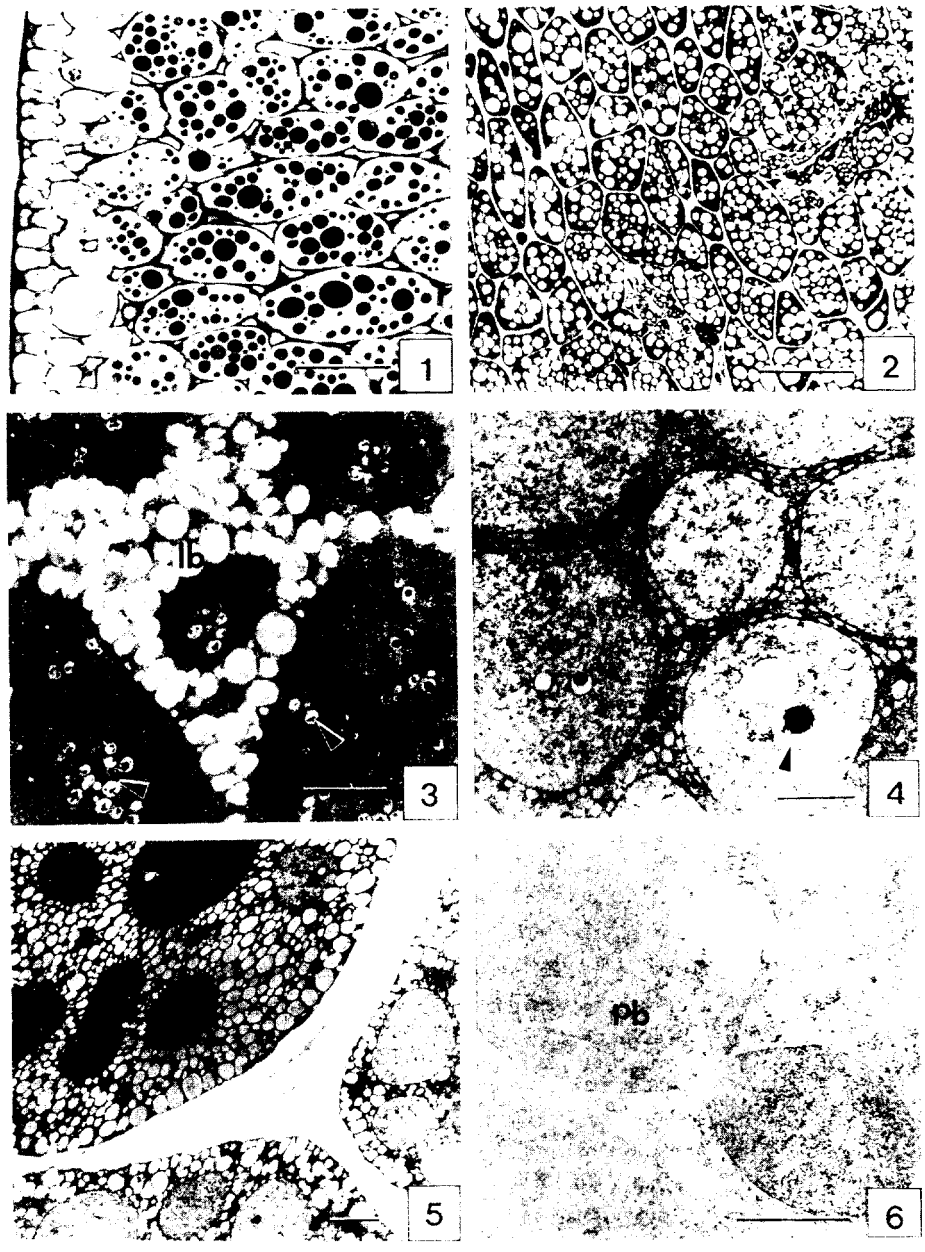

Fig. 1. 1.5 hrs cotyledon showing epidermis, hypodermis, and storage parenchyma; 280× (Bar=5μm)
Fig. 2. 1.5 hrs cotyledon showing procambium and storage parenchyma; 280× (Bar=5μm)
Fig. 3. 1.5 hrs. Storage parenchyma cells are packed with protein bodies (pb) and lipid bodies (lb). Note appearance of shattered globoids and globoid crest (arrows); 12,300× (Bar=0.1μm)
Fig. 4. 9 hrs. Globoids in reduced. Note a displaced globoid (arrow); 11,800× (Bar=0.1μm)
Fig. 5. 9 hrs. Protein bodies in storage parenchyma cells stain more intensely than ones in epidermis; 6,300× (Bar=0.1μm)
Fig. 6. 2 day. Fusion of protein bodies (pb) in storage parenchyma cell; 17,000× (Bar=0.1μm).

cular tissues (Figs. 1-2).

Each protein body was limited by a distinct single unit membrane and had an internal structure that was usually homogenous and without any inclusions (Fig. 5-6). Sometimes, however, small globoids were found mainly in protein bodies of storage parenchyma cells (Fig. 3) and radicle (Fig. 4). Sometimes protein bodies with aggregates of many small electron transparent regions or individually scattered electron transparent regions also were observed (Fig. 3-4). Protein bodies in the same cell varied in their density of staining (Fig. 1).

The shape of protein bodies was usually oval or round (Figs. 1-2, 4). As germination proceeded, the shape of protein bodies tended to vary due to changes induced by imbibition and pressure of adjacent protein bodies (Fig. 9-10). Changes in the internal structure of protein bodies were also observed, as evidenced by an increase in electron

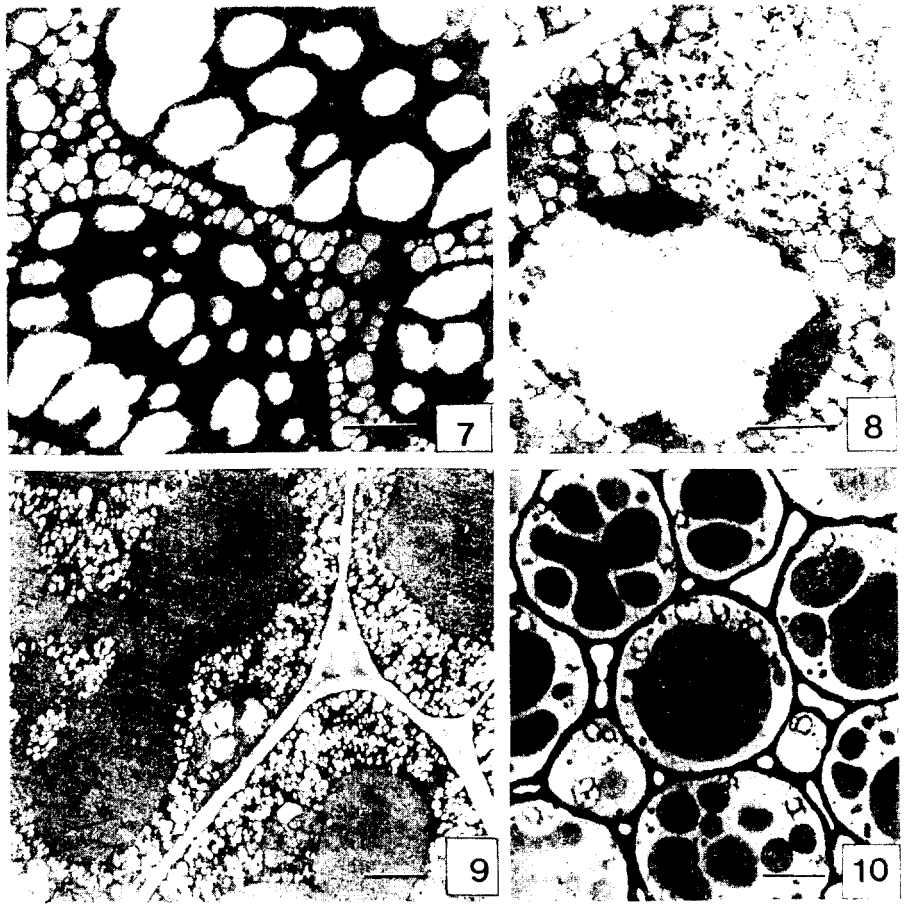

Fig. 7. 2 day. Internal degradation of protein bodies in adjacent cells of vascular tissues; 10,300× (Bar=0.1μm).

Fig. 8. 2 day. Internal degradation of protein bodies in vascular bundle cell; 13,200× (Bar=0.1μm).

Fig. 9. 3 day. Irregular and large protein bodies (pb) are seen in storage parenchyma cells. 4,500× (Bar=2μm).

Fig. 10. 3 day. View of figure 9 by light microscope; 450× (Bar=2μm).

transparent areas (Figs. 7-8) and a decrease in staining intensity (Figs. 4-5), both of which suggest partial hydrolysis of proteins. After 6 hrs of germination, protein bodies in epidermis stained less intensely than those in storage parenchyma cells (Fig. 5).

Between 2 and 6 days, protein bodies underwent several changes which differed within each cell and within different regions of the cotyledons. In storage parenchyma cells, fusions of the protein

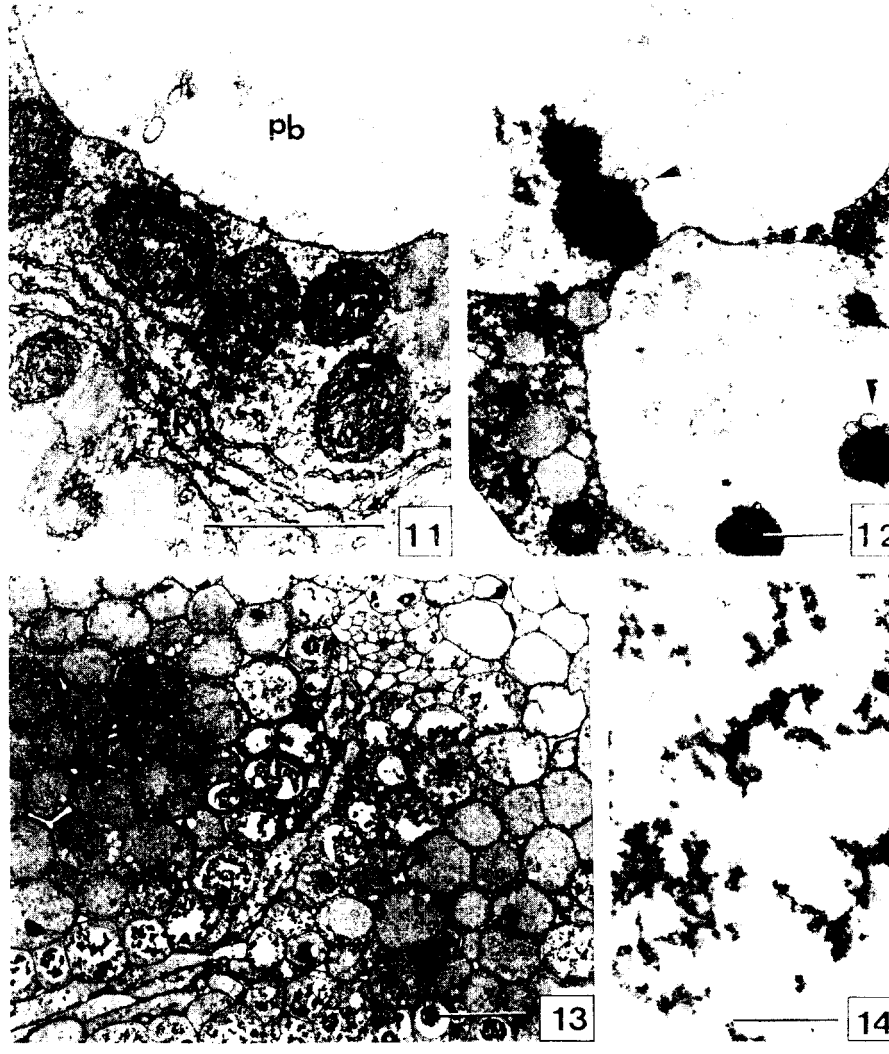

Fig. 11. 5 day. Parallel array of endoplasmic reticulum (ER) and numerous mitochondria (M) are seen in epidermis. Protein body (pb) is almost vacuolated. 27,300× (Bar=0.1μm).

Fig. 12. 3 day. Vesicles (arrows) in protein bodies appear as empty single membrane coated spheres which are interpreted as resulting from digestion of sequestered material leaving a digestion resistant limiting membrane. 12,700× (Bar=0.1μm).

Fig. 13. 6 day. Flocculent protein bodies in cells adjacent to vascular bundles are seen. 275× (Bar=5μm).

Fig. 14. 6 day. Flocculent protein matrix by transmission electron microscopy. 15,500× (Bar=0.1μm).

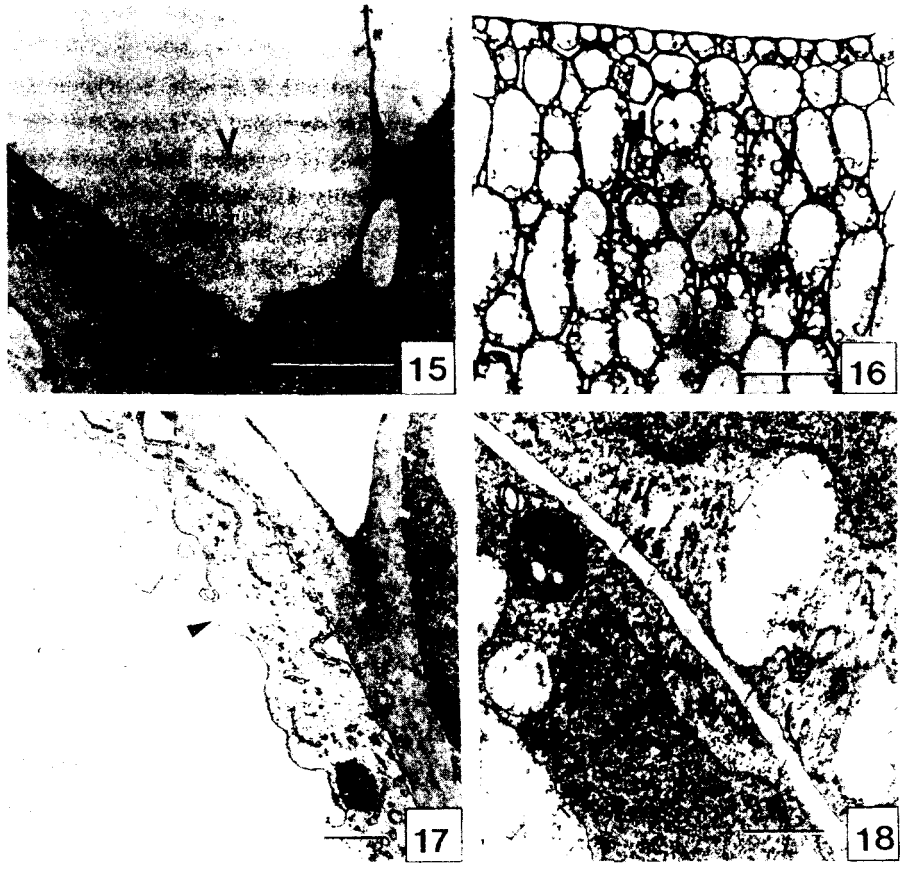

Fig. 15. 8 day. Vascular tissue showing various sizes of electron transparent vacuoles (V); 18,200 × (Bar=0.1 μm).
Fig. 16. 8 day. Epidermal cells contain one single vacuole in each cell and fusing protein bodies are still seen in storage parenchyma cells; 290 × (Bar=5μm).
Fig. 17. 9 day. Tonoplast breakdown (arrow) in epidermis; 9,500 × (Bar=0.1μm).
Fig. 18. 10 day. Some of vacuoles broke down in vascular tissue; 12,300 × (Bar=0.1μm).

bodies were often observed (Fig. 6). Subsequently, larger and more irregularly shaped protein bodies appeared by day 3 (Figs. 9-10). By day 6, these larger bodies showed a significant decrease in staining (Fig. 13). In vascular tissue, each protein body broke down in an irregular patten, usually beginning in the center of the matrix (Figs. 7-8). The entire matrix of each protein body eventually became flocculent (Figs. 12-14). The flocculent matrix was almost gone by day 8, leaving what have been termed as protein vacuoles (Fig. 15). In epidermis, vacuolating protein bodies were observed at day 5 (Fig. 11).

During the late stages of seedling growth, most cells were depleted of stainable storage protein, but the flocculent material remained visible (Figs. 16-18). At day 9, tonoplasts in epidermal cells appeared disrupted (Fig. 17), which was the case in vascular tissue at day 10 (Fig. 18). No tonoplast breakdown was observed in the storage parenchyma at this latter stage.

Discussion

The storage proteins of soybean seeds are found primarily in subcellular organelles called protein bodies[5-7]. Other terms such as protein storage vacuoles[5], and aleurone grains[8] are also used.

The abundance of protein bodies in the cotyledons of 1.5 hrs of germination was striking. The protein bodies appeared very similar to those observed by Tombs[9] with the exception of the existence of globoids. Two types of protein bodies were observed in the cotyledon cells, one with globoids and the other without any inclusions. This is in agreement with the results of Lott and Buttrose[10] and Lott[6].

Great size differences in protein bodies were observed; vascular tissue and epidermis have relatively small bodies, whereas storage parenchyma have very large ones. Similar observations were made by Lott *et al.*[11] in squash cotyledons. Many agree that the protein bodies vary in size from one plant to anothe and from one tissue to another[12-13].

The variability in the degree of staining of protein bodies in a cell may reflect simply differences in composition or in protein concentration. This can be partly explained by the finding of Tombs[9] who isolated two fractions of soybean protein bodies which have different densities of 1.29 and 1.30 g/cm^3. He also suggested that two fractions were mainly different in protein content (97.5 and 78.5%) and in the extent of non-proteinaceous materials such as phytic acids, RNA, and lipids.

A single limiting membrane was observed around each protein body prior to and during protein body degradation. This suggest that soluble products accumulate within protein bodies and then leak through the membranes into the cytoplasm[14], while protein bodies are undergoing autolysis[15].

The observed pattern of protein body degradation was more complex than those described for *Glycine*[9,13] or other legumes, including *Pisum*[14], *Phaseolus*[16], and *Vigna*[15]. The pattern in the cell around vascular tissues, where protein was degraded with little or no coalescence of bodies, is like that described for *Pisum sativum*[14]. Possible instances of vacuolar fusion were encountered in these cells. The pattern in epidermis and storage parenchyma where the bodies swell, fuse, and then are vaculoated was the one most frequently described[9,16]. A similar pattern was found in seeds of *Yucca schidigera* where the protein bodies in the embryo coalesced before breaking down; however, the ones from the perisperm disappeared directly[17].

A completely different pattern of protein body degradation was reported by Taylor *et al.*[18] in germinating sorghum, where the protein bodies neither swelled nor formed a central vacuole. They were observed to undergo a progressive reduction in their size. Since the predominant storage protein in cereals is prolamine, which is hydrophobic and insoluble in water and salt solution, protein bodies should not swell like the ones in germinating legumes which contain salt-soluble globulin proteins[19].

Flocculent protein bodies were observed in most of cotyledons between days 2 and 10. In epidermis and vascular tissues, this type of protein bodies was observed earlier than in storage parenchyma cells due to earlier hydration and degradation.

Some technical difficulties were met in monitoring the degradation of globoids in protein bodies. Since globoids is mainly composed of insoluble calcium magnesium salt of inositol hexaphosphoric acid (phytin), they are not penetrated well by fixatives and resins[10]. As a result, they tend to shatter when cut[7], and fall out of sections. This occurrence left a hole or small fragments of globoids around globoid area, as shown in figure 3 and 4. Furthermore, globoids are easily displaced from the globoid area during microtoming and thus, are found in other places (Fig. 4; arrow).

However, Khokhlova[8] reported that globoids were the last material to be dissolved during protein body degradation in squash cotyledons because the phytin deposited in the globoids is the least soluble crystalloid[7].

Protein body degradation in soybean cotyledons occurred first from the epidermis and vascular tissues. In storage parenchyma, protein body degradation began in adjacent cells around vascular tissue and epidermis and gradually progressed to the central storage tissue.

A similar pattern of protein mobilization was reported in *Pisum*[20], where degradation began at the periphery of the cotyledons. This result did not seem to show any correlation with the distance from the vascular tissues. Different zonation of protein mobilization was reported in *Phaseolus*[16] and *Vigna*[15] where protein mobilization began in the cells farthest from the epidermis and from the vascular tissues. Yomo and Taylor[21] confirmed this observation by reporting that protease activity was highest in the cells farthest from the vascular tissues in cotyledons of germinating *Phaseolus vulgaris*.

With this understanding of the basic patterns of hydrolysis of protein, we may be better prepared to understand the complex metabolic controls which are associated with the breakdown and utilization of protein during germination and seedling growth.

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발아중인 대두 (*Glycine max*)에서의 단백질 유동

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발아 중인 대두와 두채 자엽부에서의 단백질 유동 현상을 광학현미경(LM)과 주사 전자현미경(TEM)을 이용하여 epidermis, vascular tissue 그리고 storage parenchyma에서 비교 관찰하였다. 발아 초기의 자엽에는 직경이 0.1-15μm에 해당하는 다양한 크기의 단백질 체(protein bodies)로 채워져 있었으며 epidermis와 vascular tissue의 단백질체는 parenchyma 세포보다 훨씬 적은 크기의 0.1~5μm였다. 발아가 진행됨에 따라, 단백질의 분해현상은 epidermis와 vascular tissue에서 먼저 시작되었다. 그 분해 유형은 먼저 침투한 수분에 의해 팽창된 단백질체의 외막에는 손상이 없어 단백질 체 내부와 외부로부터 가수분해가 일어났으며, 단백질이 완전히 분해되기 전에 단백질체 끼리의 융합 또한 관찰 되어졌다. 단백질체 분해의 결과는 세포내에 중심 액포를 만든다는 것이며, 두채 성장 말기에는 액포막의 파괴현상도 관찰되었다.