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Formation of Protein Storage Bodies during Embryogenesis in Cotyledons of *Sinapis alba* L.

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Abstract. An electron microscopic investigation of fine structural changes in post-meristematic cotyledon mesophyll cells during the period of storage protein accumulation (16–32 d after pollination) showed that the rough ER, the Golgi apparatus and the developing vacuome are intimately involved in the formation of storage protein bodies (aleurone bodies). At the onset of storage protein accumulation (16–18 d after pollination) storage protein-like material appears within Golgi vesicles and preformed vacuoles. At a later stage (24 d after pollination) similar material can also be detected within vesicles formed directly by the rough endoplasmic reticulum (ER). It is concluded that there are two routes for storage protein transport from its site of synthesis at the ER to its site of accumulation in the vacuome. The first route involves the participation of dictyosomes while the second route bypasses the Golgi apparatus. It appears that the normal pathways of membrane flow in the development of central vacuoles in post-meristematic cells are used to deposit the storage protein within the protein bodies. Thus, the protein body can be regarded as a transient stage in the process of vacuome development of these storage cells.

Key words: Aleurone bodies – Embryogenesis – Protein storage bodies – *Sinapis* – Storage protein.

Introduction

The ontogeny of protein bodies of seed storage tissues is not yet known in great detail. Most workers agree that the synthesis of storage proteins in maturing seeds takes place at the rough ER and that the completed protein bodies are homologous to the vacuolar system of the cell (see reviews of Matile 1975; Millerd 1975; Pernollet 1978). However, with respect to the intermediate pathway of transport from the rough ER to the vacuole there is no commonly accepted concept. Khoo and Wolf (1970) suggested a direct developmental relationship between the ER and protein bodies in the endosperm of young kernels of *Zea mays*. More recently similar opinions have been expressed for a variety of other species (e.g. Bain and Mercer 1966; Harris and Juliano 1977; see Pernollet 1978 for further reports). Burr and Burr (1976) found that the protein bodies of *Zea mays* endosperm are at their outside covered with ribosomes and that the polysomal fraction extractable with the protein bodies carries the mRNA for storage protein synthesis (Burr et al. 1978). Thus, in this case the ER membranes giving rise to the storage bodies (Khoo and Wolf 1970) obviously continue to synthesize the storage protein even after they have separated from the lamellar ER.

In other electron microscopic investigations, especially with legumes, it has been implied that the Golgi apparatus is included in the route of storage protein transport from the ER to the protein body (e.g. Harris and Boulter 1976; Davey and Van Staden 1978). Neumann and Weber (1978) working with *Vicia faba* however interpret their results to indicate that a direct participation of dictyosomes is not demonstrated using the electron microscope. Furthermore these workers deny a developmental continuity between the vacuole of the presumptive storage cell and protein bodies although previous papers (e.g. Harris and Boulter 1976) consider the subdivision of the central vacuoles as an important step in protein body formation at least in legumes.

In the present paper we report on a systematic fine-structural investigation of protein body development and morphological changes of cellular constituents which may be involved in protein body formation of storage cotyledons in maturing embryos of

Abbreviation: ER = endoplasmic reticulum

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Sinapis alba. This study extends the previous work of Rest and Vaughan (1972) with mustard embryos. A preliminary report has been published (Schopfer et al. in press).

Material and Methods

Material

The seeds of mustard (*Sinapis alba* L., harvest 1972) were obtained from Asgrow Company (Freiburg-Ebnet). Embryos were prepared from pods of synchronously pollinated plants raised in a phytotron chamber (Bergfeld et al. 1978).

Determination of Protein

Batches of 10 seeds were ground with mortar and pestle in 1.5 ml 50% ethanol. Protein was precipitated in a 100 µl aliquot of the homogenate by adding 100 µl of 20% trichloracetic acid. The precipitate was washed free from coloured substances with ethanol and assayed by the Biuret procedure using bovine serum albumin as a standard (Layne 1957). The same homogenates were used for the determination of total protein (present paper) and of triacylglycerol (Bergfeld et al. 1978).

Electron Microscopy

The electron micrographs were obtained from the same tissue samples as has been used in an earlier paper (Bergfeld et al. 1978) using the same methods. The observations reported in the present paper are based on the examination of 20–50 electron micrographs per stage taken from different sections through the palisade layer of embryonic cotyledons.

Results

1. Time Course of Protein Accumulation in Maturing Seeds

Under favourable environmental conditions the development of the mature mustard seed from the zygote takes about 40 d. The period of cell devision and organ formation (period of histo-differentiation; Walbot 1978) in the young embryo is finished less than 12 d after pollination. Subsequently the synthesis of storage materials (triacylglycerol and storage protein) commences rather abruptly and their accumulation in the embryo is accompanied by a dramatic expansion of the cotyledons due to cell growth. These organs contain about 90 per cent of the storage fat and protein (about equal amounts) in the mature seed. Figure 1 shows that the accumulation of protein in the developing seed starts about 16 d after pollination and comes to completion about 15–20 d later. The accumulation of triacylglycerol shows a very similar time course (Bergfeld et al. 1978).

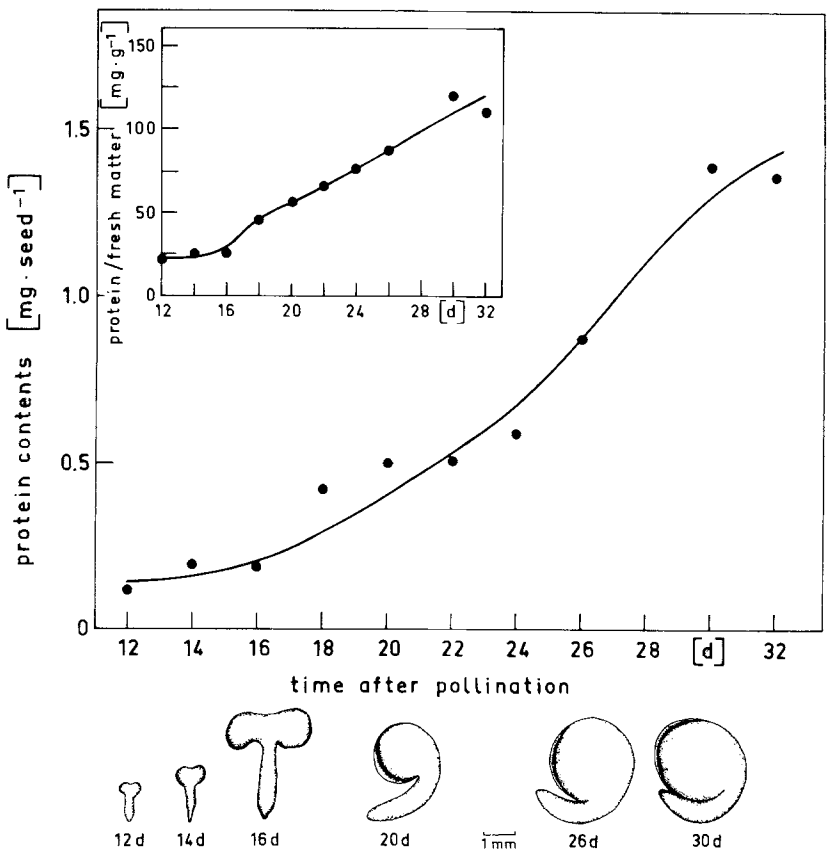

Fig. 1. Accumulation of protein and morphological development of the embryo in maturing mustard seeds

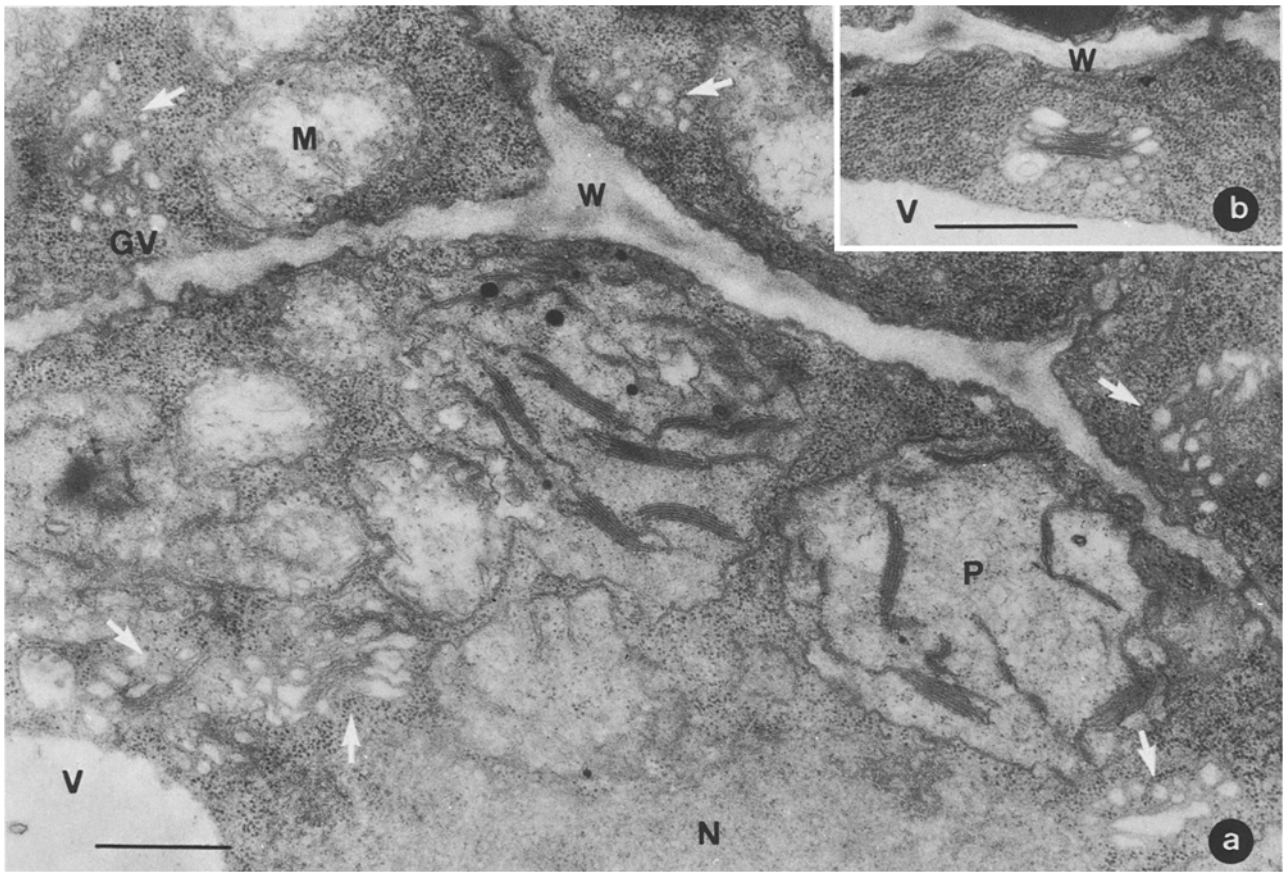

Fig. 2a and b. Fine structure of cotyledon mesophyll cells before the onset of storage material accumulation (**a** 14 d, **b** 16 d after pollination). Note the dense ground cytoplasm rich in ungrouped ribosomes (monosomes), chloroplasts with well-developed grana stacks, and the numerous dictyosomes (arrows) with many marginal dilatations surrounded by vesicles of similar appearance. These Golgi vesicles are characterized by an osmiophobic content (presumably cell wall polysaccharides). The bars represent 0.5 μm. *Abbreviations Figs. 2–9:* A = aleurone protein; D = dictyosome; GV = Golgi vesicle; L = lipid body (oleosome); M = mitochondrion; N = nucleus; P = plastid; PV = provacuole; rER = rough endoplasmic reticulum; T = tonoplast; V = vacuole; W = cell wall

2. Electron Microscopic Observations during Storage Protein Accumulation in Embryonic Cotyledons

This paragraph refers only to the ordinary mesophyll cells of the cotyledons ("aleurone cells").

The cotyledon mesophyll cells of 12–14 d old embryos are small and densely packed with monoribosome-containing cytoplasm (Fig. 2). At this age the mesophyll cells still show many of the characteristics of meristematic cells although the period of cell divison is definitely surpassed. Besides the nucleus and well developed chloroplasts, the most prominent organelles are numerous dictyosomes which are usually located close to the cell wall. These dictyosomes are composed of cisternae with large marginal dilatations obviously actively engaged in forming vesicles with an electron transparent, unstainable content (presumably polysaccharides exported into the cell wall space; see also Davey and Van Staden 1978).

Starting at about day 14 each cell contains several "empty" irregularly shaped membrane-bounded lacunae which represent the first indications of the vacuolar system (Fig. 3a). These provacuoles increase in number and size after day 16. They may be formed from inflated parts of the ER which now becomes detectable in the cytoplasm (Fig. 3a, c). The subsequent growth and concentration of the vacuolar system may involve fusion of provacuoles as well as invagination of cytoplasmic material, including small provacuoles, in a phagocytosis-like process (Fig. 3b–d). Furthermore, inclusions bounded by a single membrane within the larger vacuoles indicate the occurrence of tonoplast invaginations (Matile 1975). In principle, the early process of vacuolation appeared very similar to that described for meristematic root

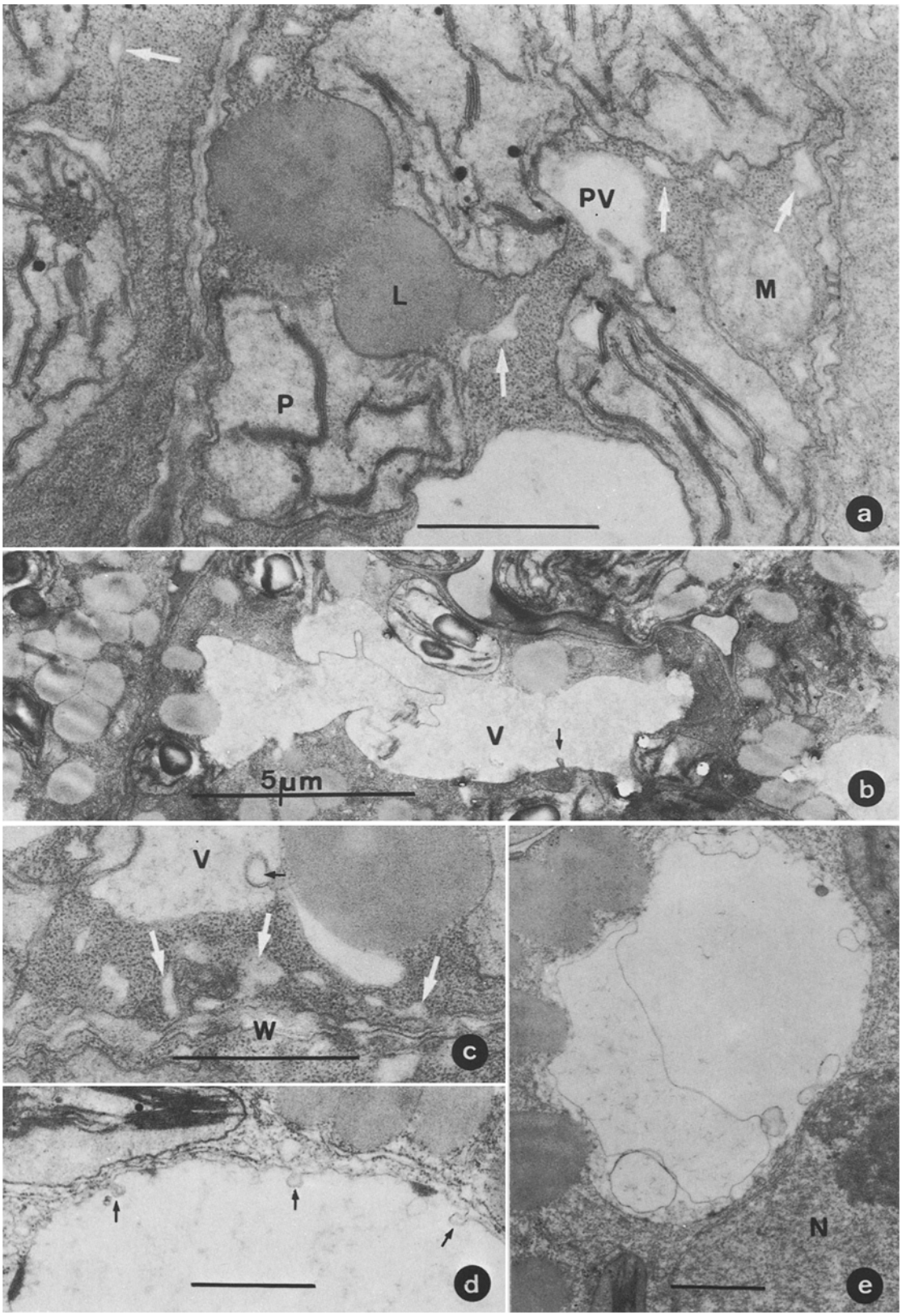

Fig. 3a–e. Fine structure of cotyledon mesophyll cells at the stage of vacuole formation (**a–c** 16 d, **d** 18 d, **e** 19 d after pollination). Note the continuous transition of dilated portions of the endoplasmic reticulum (white arrows in **a, c**) into larger vesicles (provacuoles) which may fuse to form central vacuoles (**b**). The tonoplast membrane shows invaginations (black arrows in **b–d**) containing cytoplasmic material, including small vacuoles (**c**). Larger vacuoles often contain extended membrane inclusions (**e**; see also Davey and Van Staden 1978). The bars in **a, c–e** represent 1 µm

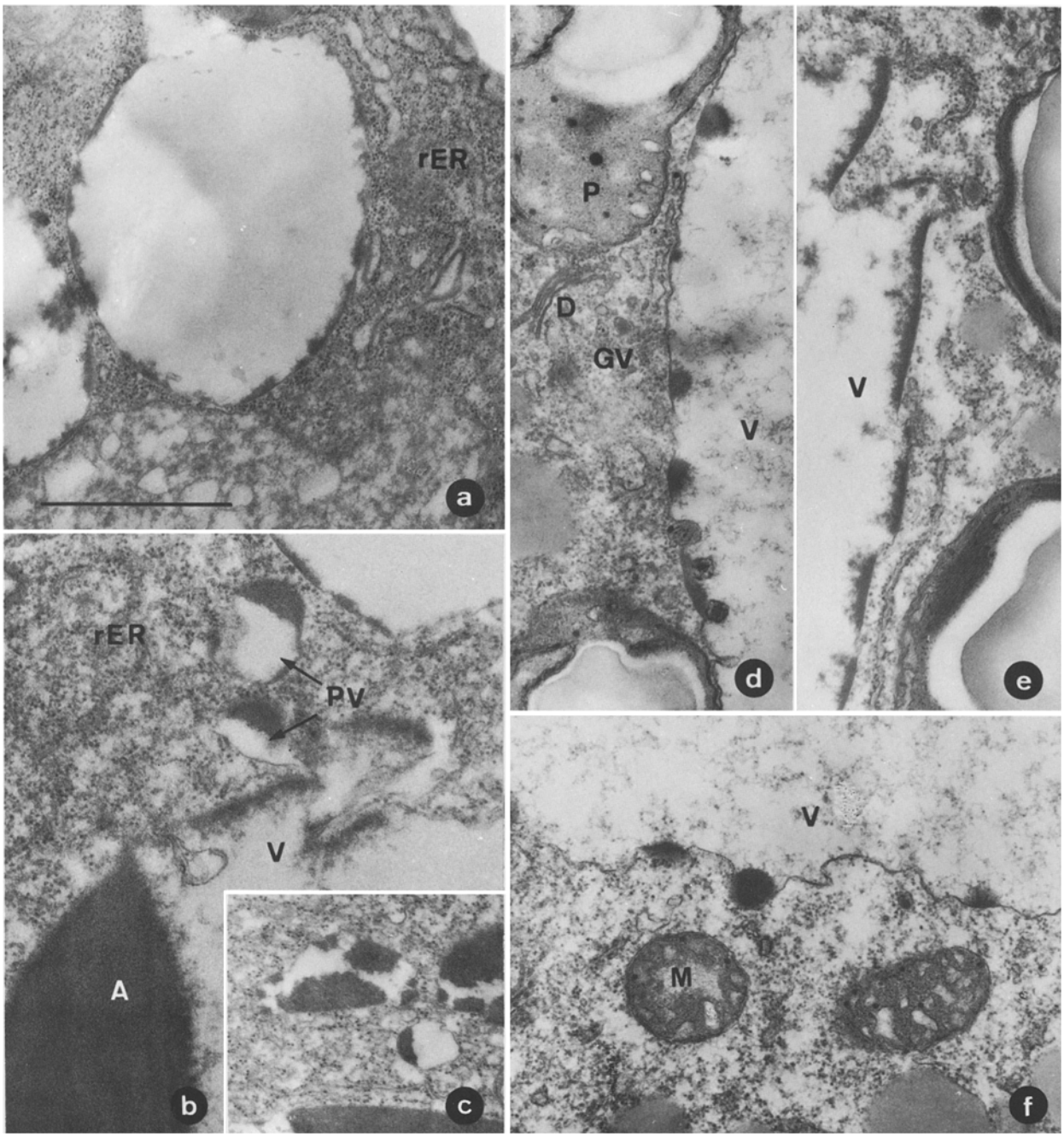

Fig. 4a–f. Fine structure of cotyledon mesophyll cells at the beginning of storage protein accumulation (**a** 18 d, **b–f** 24 d after pollination). Note the lumps of homogeneous osmiophilic material (aleurone protein) at the inner surface of all size classes of vacuoles. The lumen of the extended rough ER lacks an osmiophilic content (**a, b, e**). The bar represents 1 μm

cells by Berjak (1972). Other types of vacuole formation, e.g. by sequestration through encapsulation of portions of cytoplasm by ER cisternae (Nishizawa and Mori 1977; Marty 1978), have not been observed in mustard cotyledon cells.

In accordance with the time course of Fig. 1 the first structural indications of storage protein accumulation within the vacuolar system appeared at 16–18 d after pollination. Small lumps of osmiophilic material with a homogeneous fine granular appearance can be seen attached to the inner surface of the membrane of all size classes of vacuoles (Fig. 4). During the following days these deposits of storage protein rapidly increase in volume, leading finally to a contin-

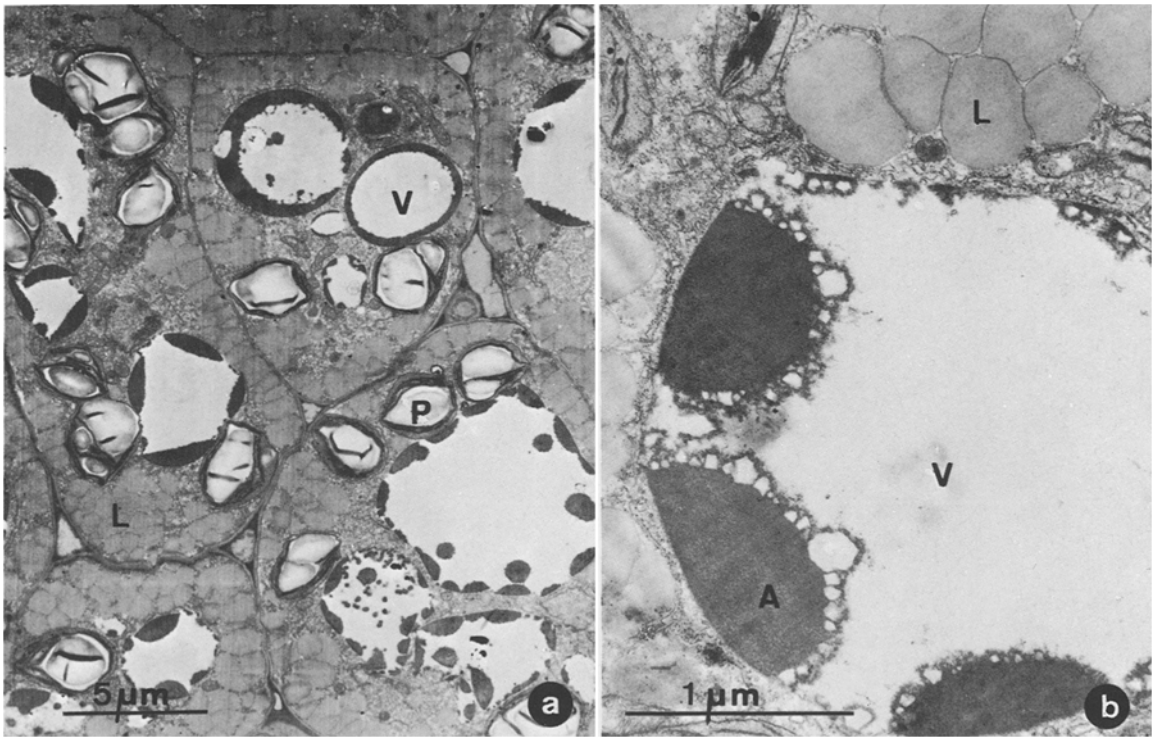

Fig. 5a–b. Fine structure of cotyledon mesophyll cells during the early period of storage protein accumulation (**a** 20 d, **b** 18 d after pollination). Note the lens-shaped masses of aleurone protein inside the vacuoles which may fuse into a centripetally growing layer (**a**). These particles are often covered with crystalloids (indicated by empty cavities; **b**). The plastids contain voluminous starch grains (**a**)

uous layer covering the inner surface of the large vacuoles (Fig. 5a). At an intermediate stage the storage protein forms characteristic lens-shaped masses which may represent the spread contents of smaller vacuoles after their coalescence with the larger vacuoles (Fig. 5). Often the tonoplast membrane forms small folds between more rigid patches of membrane which apparently originate from incorporated storage protein-filled vesicles (Fig. 4e). It appears that the storage protein deposition occurs simultaneously in all vacuoles and that the smaller vacuoles discharge their contents into the larger ones. Crystalloids can be frequently found at the surface of storage protein lumps within the larger vacuoles (Fig. 5b). Formation of these particles indicates the accumulation of a soluble "crystalloid protein" within the vacuole.

Two striking structural changes can be observed in the cytoplasm concurrent with the first appearance of storage protein deposits within the vacuoles at day 16–18: 1. The appearance of extended profiles of rough ER cisternae with an electron-opaque lumen (Fig. 4a). 2. The appearance of dictyosomes strikingly differing from those shown in Fig. 2 by producing Golgi vesicles filled with an osmiophilic material which is structurally indistinguishable from the storage protein lumps within the vacuoles (Fig. 6). The strict temporal correlation of these structural changes suggest a functional relationship between the organelles involved.

Up to 24 d after pollination there are no indications of other structures besides Golgi vesicles, provacuoles and larger vacuoles containing an osmiophilic material resembling storage protein. However, around 24–26 d after pollination there appears an additional type of storage protein-containing particle: spherical or irregularly shaped dilated cisternae which are studded with ribosomes. These vesicles can be found to be continuous with the rough ER (Fig. 7). Since there is no detectable accumulation of these structures – they rather seem to have disappeared at later stages when the large vacuoles have been completely filled with storage protein – it may be assumed that their contents are finally deposited within the large vacuoles.

At about 32–35 d after pollination the lumen of the vacuoles becomes completely occupied by storage protein (Fig. 8) and the seed enters the desiccation phase. The ground cytoplasm almost completely disappears (Fig. 8b). The cotyledon mesophyll cells have assumed the differentiation state of typical storage cells filled with a few storage protein bodies surrounded by a compressed mass of oleosomes (storage

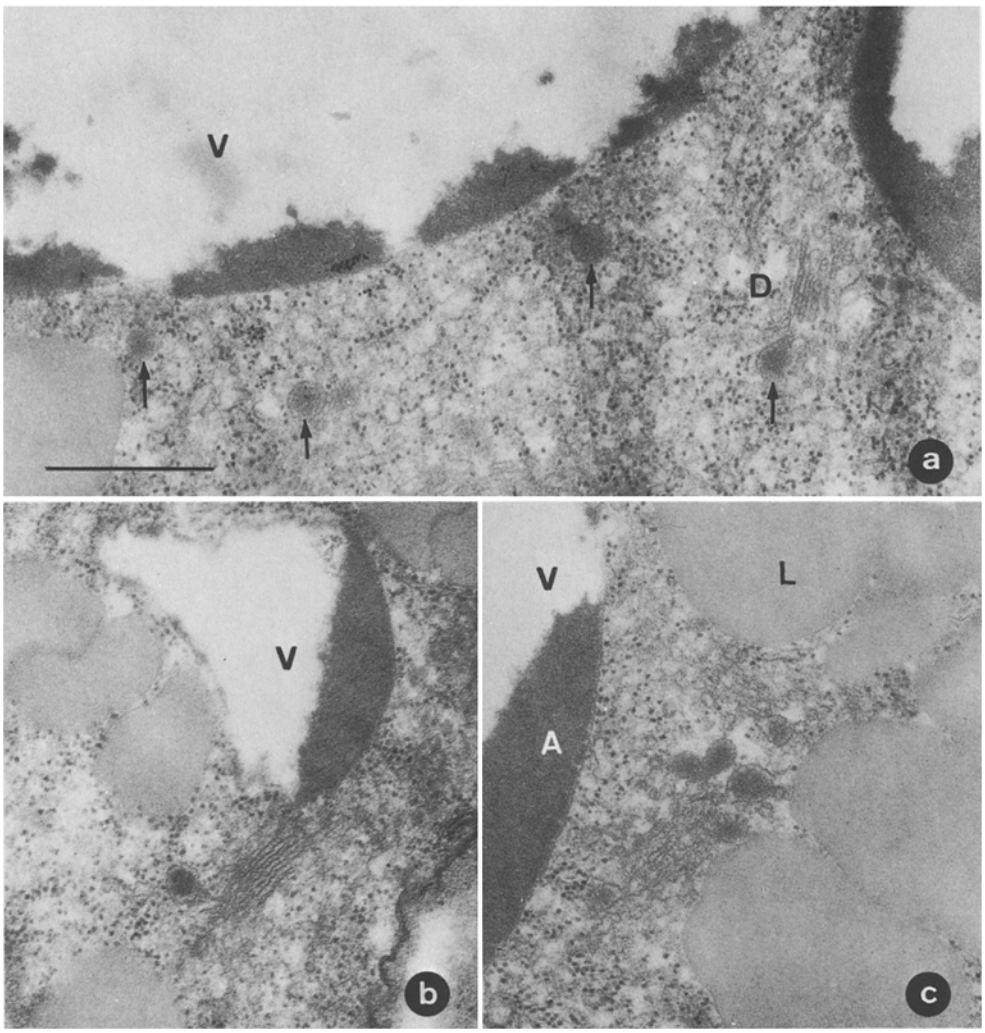

Fig. 6a–c. Fine structure of cotyledon mesophyll cells during the early period of storage protein accumulation (**a, c** 20 d, **b** 18 d after pollination). Note the dictyosomes producing Golgi vesicles with an osmiophilic material which resembles the aleurone protein within the vacuoles. The bar represents 1 μm

fat bodies), which are interspersed with a few organelles (nucleus, proplastids, promitochondria, microbodies). Dictyosomes, ribosomes and ER are no longer detectable. Due to the cessation of vacuome concentration at this point, a number of about 8–12 vacuoles of variable size are reached per cell. These vacuoles represent the aleurone bodies of the dry seed. We have never observed any subdivision of larger vacuoles (Rest and Vaughan 1972).

3. Vacuole Development in Idioblasts (Myrosin Cells) during Cotyledon Development

In addition to the ordinary mesophyll cells producing "aleurone grains" dealt with in Figs. 2–8, mustard cotyledons contain a small number of scattered idioblasts called "myrosin cells" which produce a different type of protein bodies ("myrosin grains"; Rest and Vaughan 1972; Werker and Vaughan 1974). It is unlikely that the proteinaceous matrix of myrosin grains serves as storage material since there is no structural evidence of breakdown after germination of the seed (when the "aleurone grain" material becomes completely digested). Histological and fine structural aspects of myrosin cell differentiation in mustard has been described by Rest and Vaughan (1972) and Werker and Vaughan (1976). These investigators provided evidence that the finely-grained, moderately electron-dense content of "myrosin grains" first accumulates in the lumen of fenestrated lamellar rough ER cisternae which then give rise to the myrosin-containing vacuoles. Figure 9 shows similar observations with the embryonic cotyledons. Si-

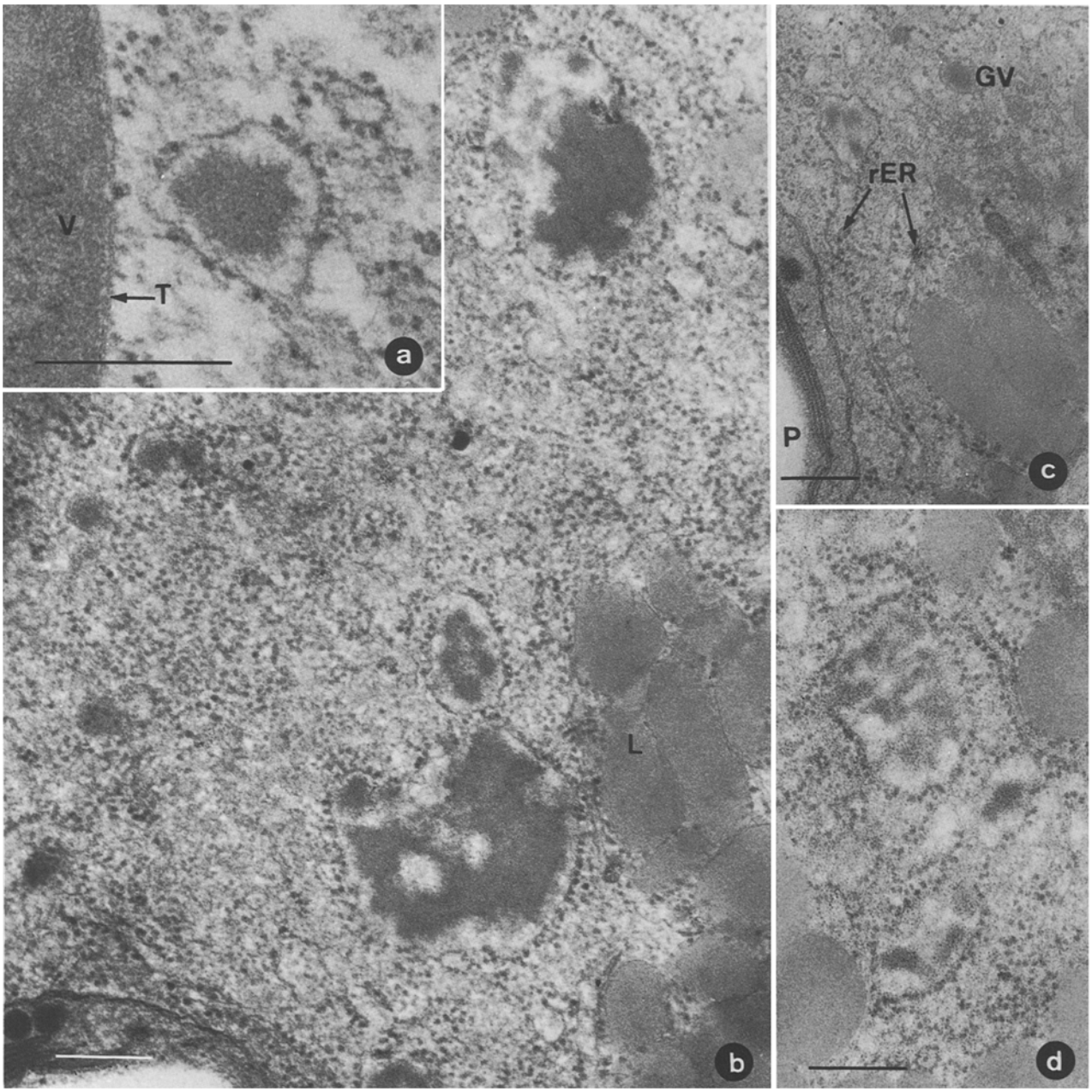

Fig. 7a–d. Fine structure of cotyledon mesophyll cells during the later period of storage protein accumulation (30 d after pollination). Note the ribosome-coated vesicles of irregular shape which are filled with an osmiophilic material resembling the aleurone protein within the vacuole. The bars represent 0.25 μm

multaneously with the aleurone body development the myrosin cells form vacuoles which appear to grow on the expense of a extended ER covered with polysomes (Fig. 9c). The lumen of the ER cisternae is filled with a material resembling the vacuolar content (Fig. 9b, d). In contrast to the vacuoles of "aleurone cells" the growing "myrosin vacuoles" are always homogeneously occupied by a matrix with a reticulate appearance which becomes granular later. Dictyosomes producing Golgi vesicles with electron-dense material have never been observed in myrosin cells.

Discussion

In studies with cereals and some legumes it has generally been assumed that the formation of protein bodies takes place directly through budding from the

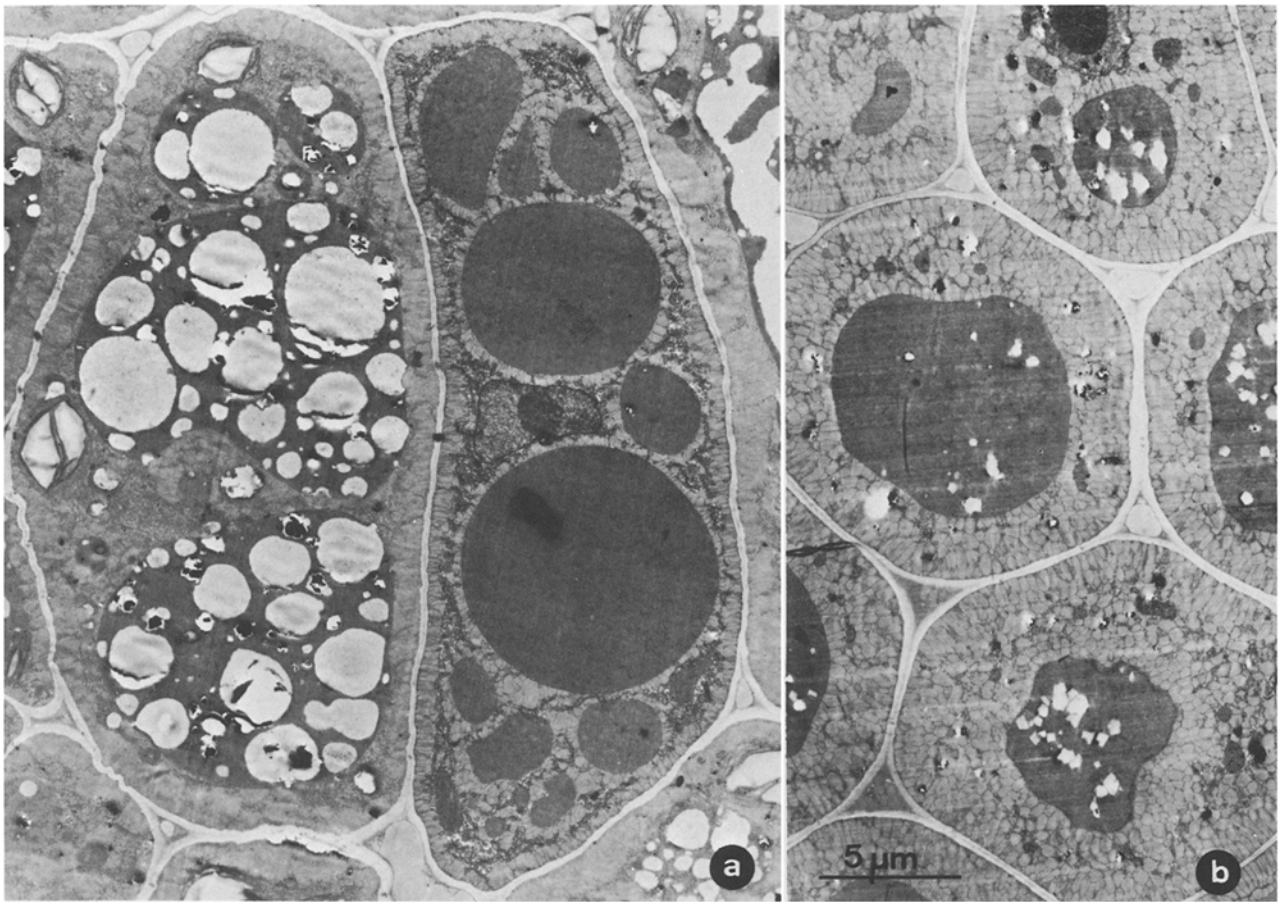

Fig. 8a and b. Fine structure of cotyledon mesophyll cells during the final period of storage protein accumulation (**a** 32 d after pollination, section normal to the cotyledon surface) and in the mature seed (**b** desiccated seed, soaked in 5 mg·l^{-1} of abscisic acid at 25° C for 12 d. This treatment prevents germination, see Schopfer et al., 1979; section parallel to the cotyledon surface). **a** shows an "aleurone cell" (left) and a "myrosin cell" (right)

ER in the absence of vacuoles and that these vesicles in fact represent the vacuome of developing storage cells (Khoo and Wolf 1970; Burr and Burr 1976; Harris and Juliano 1977; Neumann and Weber 1978). Rest and Vaughan (1972) clearly showed that the protein bodies ("aleurone grains") of mustard cotyledon mesophyll cells originate from preformed vacuoles into which the storage protein becomes discharged after its synthesis in the cytoplasm. The present paper bears on the fine-structural aspects of the transport pathway that the storage protein takes from its site of synthesis at the rough ER to the vacuole in mustard cotyledons.

The interpretation of our electron microscopic data rests on the assumption that the electron-dense material present in vacuoles, Golgi vesicles and rough ER vesicles specifically at the time of storage protein accumulation (Figs. 4–7) can be identified with storage protein. This material appears structurally indistinguishable from the matrix of mature protein bodies (Fig. 8) but biochemical evidence for this homology is still lacking. As reported for other plants, the storage protein of mustard seeds consists of several fractions (Kirk and Pyliotis 1976) which have not yet been biochemically sufficiently characterized.

With these limitations in mind we feel that our fine structural data are consistent with the following mechanism of protein body formation in the mustard embryo:

In the presumptive storage cells, vacuome formation from the ER takes place essentially as in other post-meristematic cells, including volume growth and concentration through fusion of provacuoles and incorporation (by fusion or phagocytosis) of Golgi vesicles and other cytoplasmic constituents (Berjak 1972; Matile 1975; Marty 1978). Autophagic activity of the vacuome is indicated by the occurrence of membrane invaginations (Fig. 3) and by the disappearance of most of the cytoplasm including ribosomes and ER membranes during seed maturation (compare Figs. 2 and 8b).

The established pathways of membrane flow from

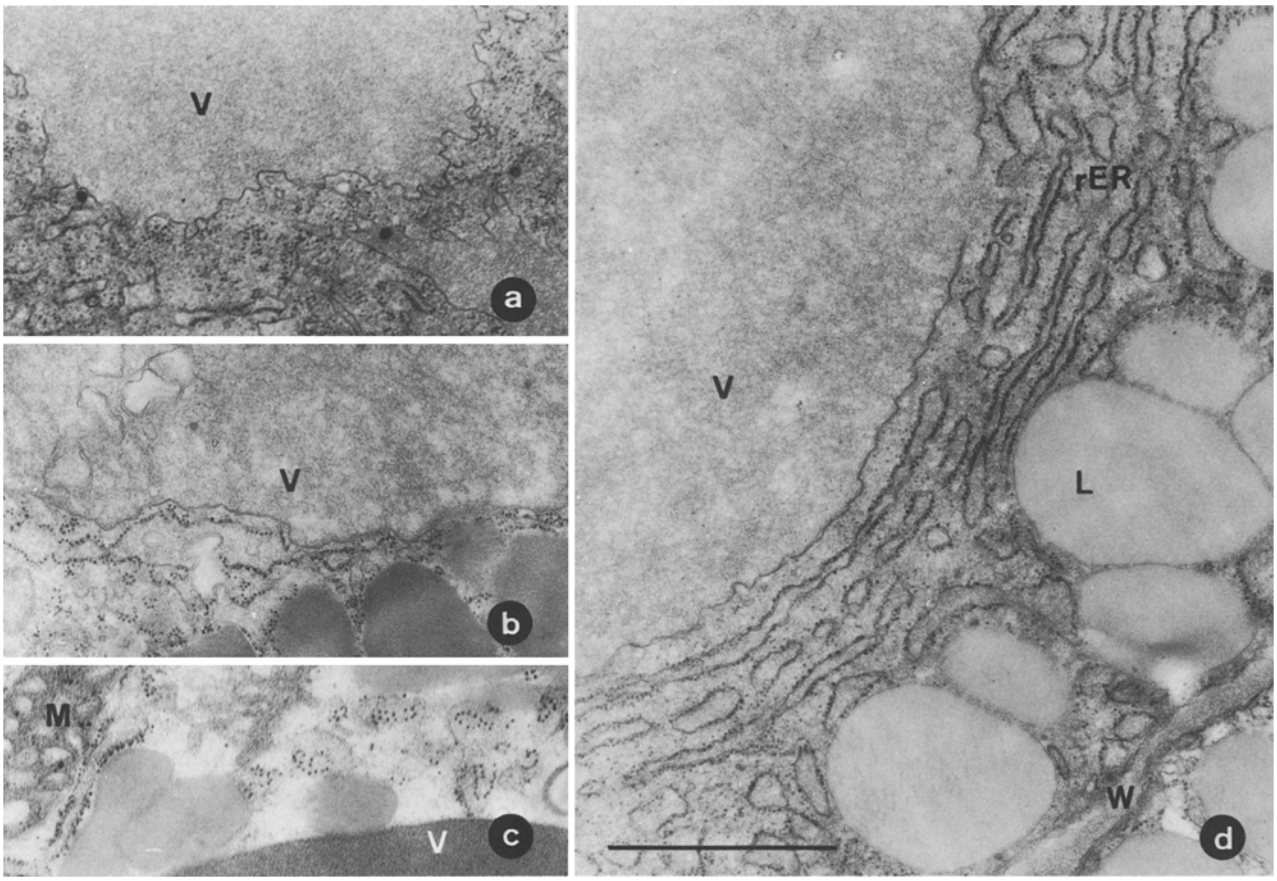

Fig. 9a–d. Details from cotyledon myrosin cells during storage protein accumulation (**a** 16 d, **b** 18 d, **c** 30 d, **d** 24 d after pollination). Note the loose, moderately osmiophilic matrix of the vacuoles which are surrounded by an extended rough ER containing a material of similar appearance. As in the case of "aleurone vacuoles" (Fig. 3*e*) the "myrosin vacuoles" may contain membraneous inclusions (**b**). The bar represents 1 μm

the ER may be used here to concentrate the storage protein within the vacuome. In accordance with the current views on vacuome development in post-meristematic cells we find evidence for two distinguishable routes of storage protein transport which are temporarily displaced. During the early period of storage protein accumulation the ER-synthesized proteins are passed on to the Golgi apparatus where they become concentrated (or otherwise rendered stainable). Golgi vesicles provide the vehicle for transport into existing vacuoles. Thus, this transport mechanism involves an inwardly directed flow of Golgi vesicles to the vacuome which is diametrical to the well-established plasmalemma-directed flow of Golgi vesicles used in the process of secretion. Golgi vesicles with an osmiophylic contents have previously been observed in storage protein accumulating cotyledons (Bain and Mercer 1966; Harris and Boulter 1976; Chrispeels 1977; Neumann and Weber 1978; Davey and Van Staden 1978). The involvement of these vesicles in storage protein transport is indicated by their occurrence in storage cells specifically during the period of protein body formation (compare Figs. 2 and 6) and the lack of any other detectable structure carrying a similar material. In a later stage of seed maturation, storage protein is synthesized and accumulates directly in inflated regions of the rough ER from where it is also transferred to vacuoles. It is conceivable that this route which bypasses the Golgi apparatus provides a special component to the heterogenous storage protein of the mature protein body. Similar rough ER vesicles have been observed in sub-aleurone endosperm cells of young maize kernels during storage protein accumulation (Kyle and Styles 1977). We could not detect any ribosomes attached to the outside of large vacuoles or protein bodies at different stages of development (Burr and Burr 1976).

The process of vacuome concentration is interrupted when the embryo assumes the quiescent state during seed desiccation. This process will continue at that point after germination, when the storage protein becomes digested and the depleted protein

bodies coalesce to finally from the single central vacuole of the cotyledon mesophyll cell (see Fig. 9 in Bajracharya and Schopfer 1979). Thus, the protein bodies can be regarded as a transient stage of the vacuolar development of storage cells which develop into ordinary assimilatory cells after their storage materials have been used up. In other plants (e.g. in cereals and legumes) which are characterized by an abundance of small protein bodies in the storage cells of mature seeds, the fragmentation of vacuoles into many small vesicles may be an additional step involved in protein body formation.

Our results imply that the various reports in the literature suggesting either ER- or dictyosome-derived vesicles as transport vesicles for storage protein may not be mutually exclusive. In mustard cotyledons both pathways can apparently be used within the same cell. An investigation on the origin of the proteinaceous "refractive spherules" which occur in sieve elements of the fern *Davallia fijiensis* has led to essentially the same conclusion (Fisher and Evert 1979).

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Note added in proof

The recent paper by S. Craig, D.J. Goodchild and A.R. Hardham [Austr. J. Plant Physiol. *6*, 81–98 (1979)] on vacuole and protein body formation in *Pisum* supports and extends the observations of Neumann and Weber (1978) with *Vicia*. The main difference between these legumes and plants such as *Sinapis* appears to be the appearance, concurrent with the import of storage protein, of extensive tonoplast folding (leading to an increase in the number of vacuole profiles in electron micrographs) in the legumes followed by fragmentation of the lobed vacuole into numerous small protein bodies. This morphological difference may result from a different volume/surface growth ratio of the vacuole in the two types of plants. Furthermore, Craig et al. (1979) provide evidence for the formation of an additional type of protein body from ribosome-studded vesicles at a late stage of storage protein accumulation in *Pisum* cotyledons.