Protein body formation in the starchy endosperm of developing *Sorghum bicolor* (L.) Moench seeds

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Chemical analyses and transmission electron microscopical observations were carried out on developing *Sorghum bicolor* (L.) Moench seeds to determine when and how protein bodies are formed in the starchy endosperm tissue. Protein bodies were first visible at the milk stage and by late hard dough they completely filled the sub-aleurone cells. Between these stages of development there was an almost linear increase in the amount of prolamin protein in the seed. The protein bodies appeared to grow within envelopes of rough endoplasmic reticulum. It is, therefore, suggested that protein bodies in *S. bicolor* are formed as a result of prolamin polypeptides being synthesized on the ribosomes on the outside of the envelopes and then passing directly into the lumen of the rough endoplasmic reticulum. This mode of protein body formation is identical to that which takes place in the seeds of another member of the Panicoideae subfamily, *viz., Zea mays* L.

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Chemiese analises en transmissie-elektronmikroskopiese waarnemings is uitgevoer op *Sorghum bicolor* (L.) Moench saad om vas te stel wanneer en op watter wyse proteïenliggame ontstaan in die styselhoudende endospermweefsel. Proteïenliggame het vir die eerste keer sigbaar geword gedurende die melkstadium en toe die hardedeeg stadium bereik is, het die liggame die sub-aleuroonselle volledig gevul. Tussen hierdie ontwikkelingstadia was die toename in prolamin proteïen van die saad amper liniêr. Dit het gelyk asof die proteïenliggame gegroei het binne omhulsels van die growwe endoplasmiese retikulum. Die volgende teorie word dus voorgestel: Proteïenliggame in *Sorghum bicolor* ontwikkel as gevolg van prolamin polipeptiedsintese deur ribosome wat op die buitekant van die omhulsel geleë is, die proteïenliggame beweeg dan reguit na die lumen van die growwe endoplasmiese retikulum. Hierdie tipe van proteïenliggaamsintese is identies aan die sintese wat plaasvind in die sade van 'n ander lid van die Panicoideae subfamilie, nl. *Zea mays* L.

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Introduction

The bulk of the storage (reserve) protein of most seeds is located in organelles known as protein bodies (Ashton 1976). The formation of these organelles during seed development has been studied in a number of cultivated Gramineae species.

In the case of *Hordeum vulgare* (L.), Cameron-Mills & Von Wettstein (1980) suggested, on the basis of transmission electron microscopy (TEM) observations, that hordein storage proteins were first synthesized on the rough endoplasmic reticulum (RER). The hordein polypeptides were then deposited in the cell vacuole and gave rise to protein bodies. Smooth vesicles were suggested as a possible mechanism for the transport of the polypeptides from the RER to the vacuole. Also, most workers agree that a similar mode of protein body formation takes place in *Triticum aestivum* (L.) (Pernollet & Camilleri 1983). As with *H. vulgare* the exact mode of transport between the RER and cell vacuole is not known, although the Golgi apparatus has been implicated (Parker 1981; Bechtel *et al.* 1982). In both these species and in *Secale cereale* (L.), all members of the tribe Triticeae, the protein bodies are not extant at seed maturity (Pernollet & Mossé 1983), having merged to form a matrix of protein (Pernollet & Camilleri 1983).

In contrast to this theory which involves deposition of proteins within cell vacuoles, Miflin and co-workers have postulated a completely different mode of protein body formation in *H. vulgare* (Miflin & Shewry 1979) and *T. aestivum* (Miflin *et al.* 1981). They suggest that after synthesis of the storage proteins on the RER, they are deposited within the lumen of the RER where they aggregate. As the aggregate increases in size it breaks away from the RER and is deposited in the cytoplasm.

This hypothesis that protein bodies are formed from distentions of the RER has, in the case of *Zea mays* (L.), been accepted as fact. Biochemical studies have shown that ribosomes isolated from protein body membranes can synthesize zein, *Z. mays* storage protein (Burr & Burr 1976) and that protein body membranes are continuous with the RER (Larkins & Hurkman 1978). These biochemical studies confirm two independent investigations into *Z. mays* protein body development using TEM (Khoo & Wolf 1970; Kyle & Styles 1977). However, the protein bodies in *Z. mays* seeds differ from those in the members of the Triticeae tribe which have been studied, in that they generally persist as individual entities at maturity, rather than merge to form matrix protein (Pernollet & Mossé 1983). This characteristic is exhibited in other members of the Panicoideae subfamily: *Sorghum bicolor* (L.) Moench (Seckinger & Wolf 1973) and *Pennise-*

tum glaucum (L.) R.Br. (Adams *et al.* 1976).

This study was carried out with the objective of determining when and how protein bodies are formed in the starchy endosperm tissue of developing *S. bicolor* seeds. The species is of particular importance in South Africa because of its drought resistance and ability to grow on marginal soils. However, as with most cereals, the seed protein is deficient in certain essential amino acids, particularly lysine (Jansen 1972). Therefore, an understanding of the process of seed protein synthesis and deposition is important if the quality of *S. bicolor* seed protein is to be improved, especially if this is to be done by direct genetic manipulation.

Materials and Methods

Two commercial *S. bicolor* cultivars were used in this study; SSK 52, a bird-resistant (high-tannin) cultivar and NK 283, a non-bird-resistant (low-tannin) cultivar. These two types of *S. bicolor* were chosen because they are physiologically different. Seeds of bird-resistant cultivars are characterized by having a testa between the pericarp and aleurone layer, whereas non-bird-resistant cultivars do not have a testa. Both cultivars were grown by the Plant and Seed Control Division of the South African Department of Agriculture at the Roodeplaat Experimental Farm during the 1980–81 and 1982–83 seasons, respectively. Seed samples were taken at different stages of maturity (Table 1); the same as those described by Vanderlip & Reeves (1972). Seeds were taken from the middle of panicles which had reached the desired stage of maturity.

Table 1 Developmental stages and times of sampling of immature *S. bicolor* seeds

	Sampling times (days post-anthesis)	
Developmental stages	Cultivar SSK 52	Cultivar NK 283
Post fertilization	5	7
Milk stage	10	14
Soft dough	15	21
Early hard dough	20	28
Late hard dough	25	35
Physiological maturity	73	63

For TEM, seeds from both cultivars were immediately immersed in 5% glutaraldehyde and sections were then prepared as described by Morrall *et al.* (1981). Seeds of cultivar NK 283 were also freeze-dried directly after collection and, after removal of the glumes, were subjected to a number of chemical analyses. Mean seed weight was determined on 100 seed samples in quadruplicate. Milled samples of the seeds were subjected to a modified Osborne protein fractionation procedure (Taylor 1983) to determine the amount of each of the following nitrogenous fractions: low molecular weight nitrogen (mainly amino acids and peptides), albumin plus globulin proteins, prolamin proteins and glutelin proteins. Nitrogen was determined by an automated Bertholet phenol-hypochlorite method (Thomas *et al.* 1967). The results were the

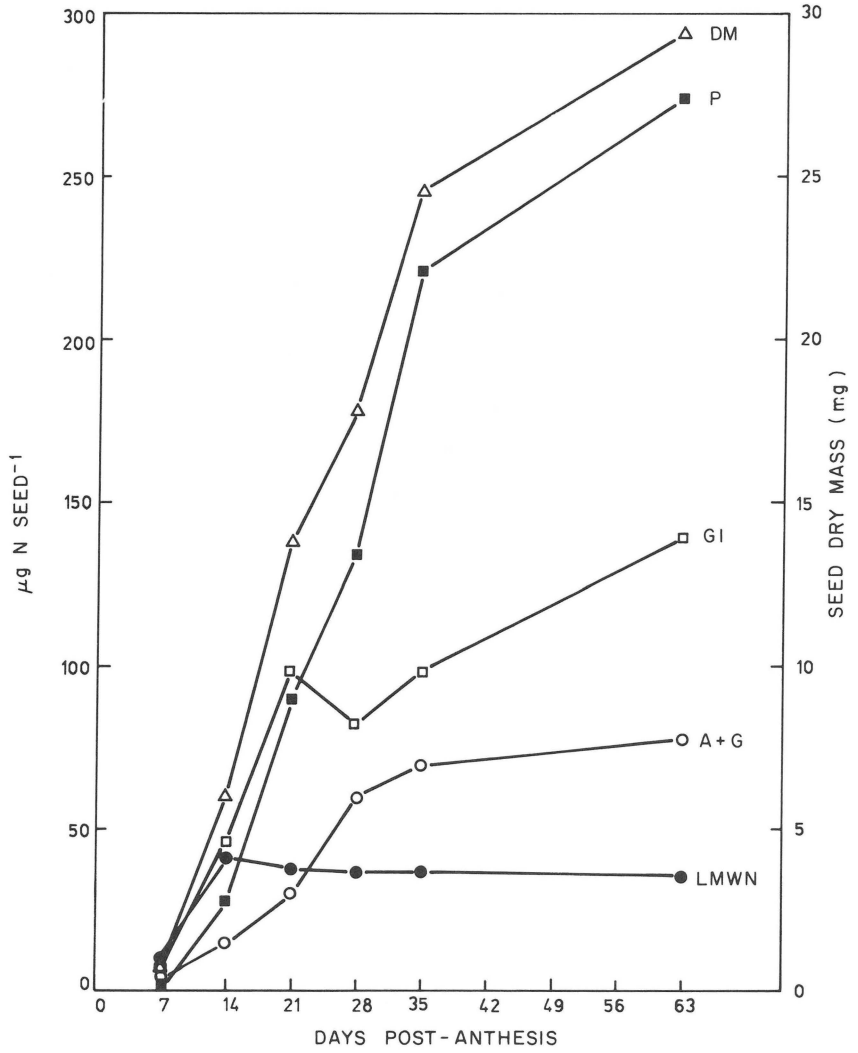

Figure 1 Change in dry mass and content of the various nitrogenous fractions during *S. bicolor* seed development (cultivar NK 283). A + G — albumin plus globulins; DM — dry mass; Gl — glutelins; LMWN — low molecular weight nitrogen; P — prolamins.

mean of two independent determinations assayed in duplicate. The prolamin fractions were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (Shewry *et al.* 1977).

Results

The change in the dry mass and content of the various nitrogenous fractions during development of *S. bicolor* seeds (cultivar NK 283) is shown in Figure 1. Seed dry mass increased almost linearly up to 35 days after anthesis (late hard dough) and then from 35 days to 63 days (physiological maturity) there was a further increase in mass, although the rate of increase was much slower.

Concerning the nitrogenous fractions, at 7 days after anthesis (post fertilization) low molecular weight nitrogen was the most abundant fraction; 39% of the total nitrogen in the seed. This fraction reached a maximum 14 days after anthesis (milk stage) and then declined gradually. This pattern reflects the probable utilization of amino acids and peptides in protein synthesis (Bewley & Black 1978). Protein synthesis is illustrated by the great increases in the amounts of all three protein fractions; albumins plus globulins, prolamins and glutelins; the greatest increase occurring in the case of the prolamins. Prolamins, which are the major storage proteins of *S. bicolor* seeds (Taylor 1983), are probably located exclusively in the protein bodies of the starchy endosperm tissue (Taylor *et al.* 1984). The rate of increase in this protein fraction, therefore, probably directly reflects the rate of protein body synthesis. It can be seen that at 7 days after anthesis (post fertilization), prolamins were the least abundant nitrogenous fraction in the seed; only 6% of the total nitrogen. However, from 14 days (milk stage) up to 35 days (late hard dough) there was an almost linear increase in prolamins. Thereafter, the rate of increase up to 63 days (physiological maturity) was much slower. By this time the prolamins had increased to almost 50% of the nitrogen in the seed.

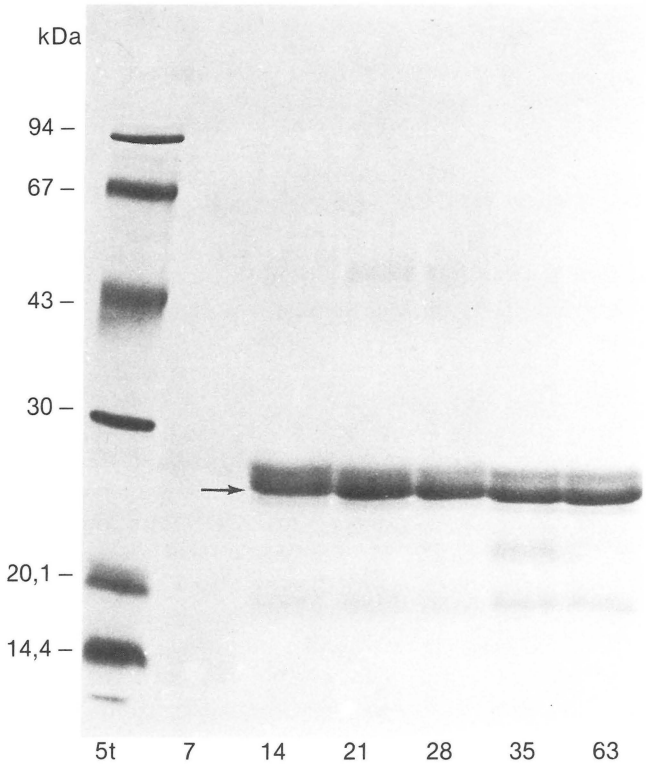

Figure 2 Sodium dodecyl sulphate polyacrylamide gel electrophoregram of the prolamin fractions from *S. bicolor* (cultivar NK 283) seeds at different stages of development. St — molecular weight standards, 7, 14, 21, 28, 35, 63 days after anthesis; arrow — major prolamin band.

Figure 2 shows the sodium dodecyl sulphate polyacrylamide gel electrophoresis patterns given by the prolamin fraction extracted at the different stages of *S. bicolor* (cultivar NK 283) seed development. It can be seen that a similar band pattern is exhibited from 14 days after anthesis (milk stage) to 63 days (physiological maturity). The apparent molecular weight of the major band (arrowed) is 26 kDa. This band pattern and molecular weight of the major band is characteristic of *S. bicolor* prolamins and has been observed in 10 other cultivars (Taylor & Schüssler 1984). No bands are visible at 7 days after anthesis (post fertilization), indicating that prolamin synthesis and, therefore, protein body formation did not start until after this stage. The nitrogen which was in the prolamin fraction at 7 days (Figure 1) could not therefore have been prolamin protein, or for that matter any type of protein. It was probably low molecular weight nitrogen carried over from the first stage of the Osborne-type protein fractionation procedure.

Protein bodies in the starchy endosperm tissue of both cultivars of *S. bicolor* were first observed by TEM at the milk stage; as was suggested by the electrophoresis results. They appeared as electron dense organelles of circular section, approximately 0,3 – 0,4 µm across (Figure 3). At this stage the

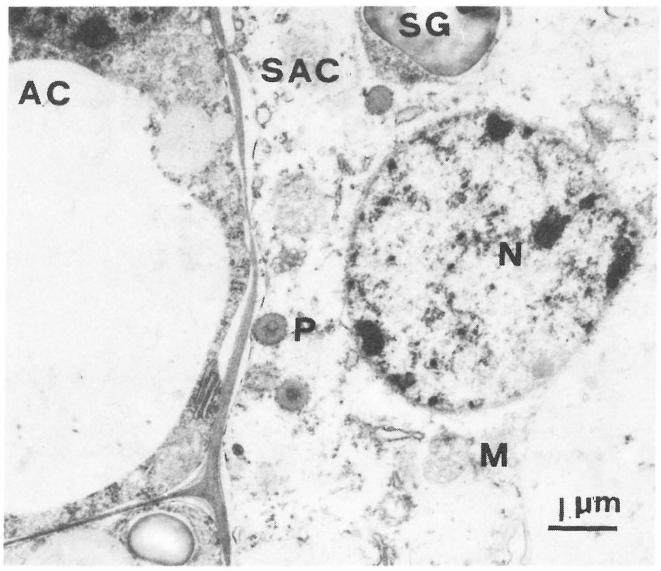

Figure 3 *S. bicolor* aleurone and sub-aleurone cells at milk stage of seed development (cultivar SSK 52). AC — aleurone cell; M — mitochondrion; N — nucleus; P — protein body; SAC — sub-aleurone cell; SG — starch granule.

endosperm cells contained both nuclei and mitochondria; these organelles degenerated during the later stages of seed development. Higher magnification of the protein bodies (Figure 4) showed them to have indistinct margins and be surrounded by many ribosomes. Some larger protein bodies, diameter 0,5 – 0,7 µm, were also observed (Figures 4 & 5). These protein bodies, which were presumably further developed, appeared to have a more distinct margin and be surrounded by RER. In a small minority of cases there was a gap between the RER envelope and the protein body (Figure 5). By the soft dough stage of seed development the starchy endosperm cells contained many more protein bodies with a diameter of up to approximately 2 µm. The protein bodies were surrounded by RER and new protein bodies appeared

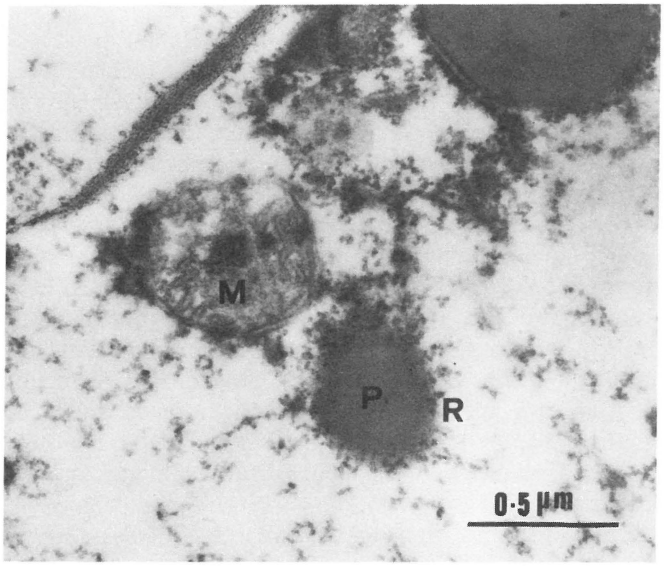

Figure 4 Small protein body at milk stage of seed development with indistinct margin surrounded by ribosomes (cultivar NK 283). M — mitochondrion; P — protein body; R — ribosomes.

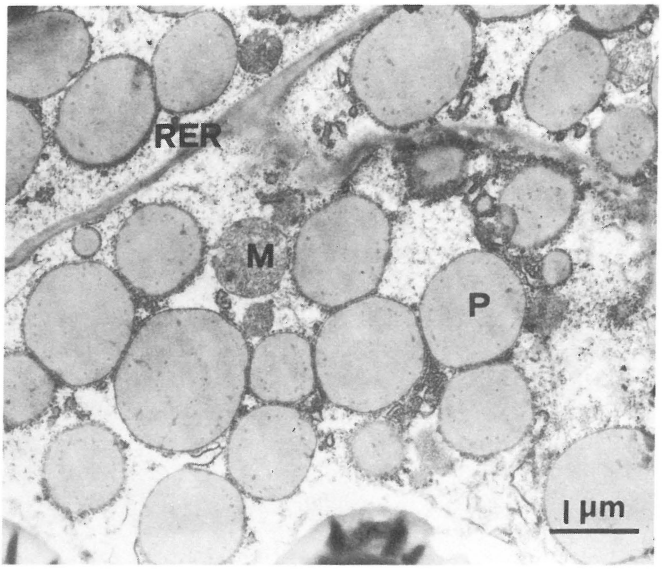

Figure 6 Protein bodies in envelopes of RER at soft dough stage of seed development (cultivar SSK 52). M — mitochondrion; P — protein body; RER — rough endoplasmic reticulum.

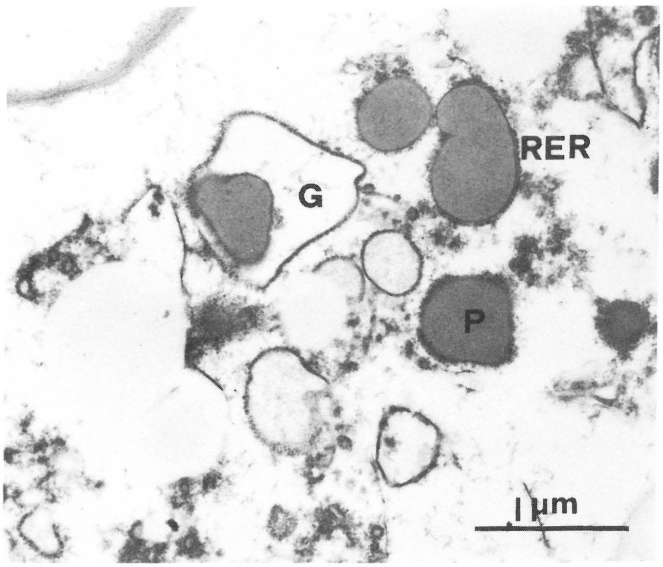

Figure 5 Large protein bodies at milk stage of seed development surrounded by rough endoplasmic reticulum (cultivar NK 283). G — gap between protein body and RER; P — protein body; RER — rough endoplasmic reticulum.

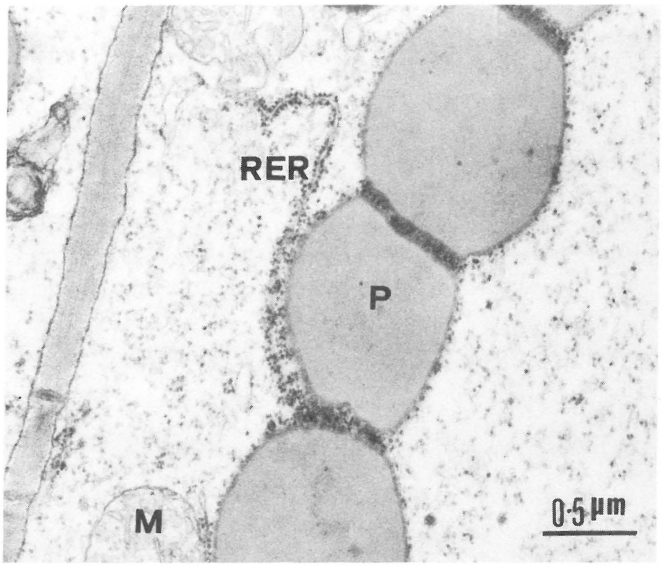

Figure 7 RER tubule in close association with protein bodies at soft dough stage of seed development (cultivar NK 283). M — mitochondrion; P — protein body; RER — rough endoplasmic reticulum.

to be in the process of being formed within envelopes of RER (Figure 6). RER tubules were frequently observed in close association with the protein bodies (Figure 7).

At high magnification it was evident that the RER was in fact continuous with the protein body membrane (Figure 8). At this stage of development many apparently intact mitochondria were observed amongst the protein bodies (Figures 6 & 7). In contrast, it was evident that the nuclei were degenerating as they were irregularly shaped and had no visible membrane.

At the early hard dough stage some of the sub-aleurone cells of the starchy endosperm had become full of protein bodies, whereas others only contained relatively isolated protein bodies (Figure 9). High magnification of the protein bodies did not reveal the presence of RER, although the ribosomes were still visible (Figure 10). Also, it was evident that in some cases the margins of individual protein bodies had become merged with each other. By the late hard dough stage the sub-aleurone cells had all become packed with protein bodies with diameters of up to approximately 2 µm (Figure 11); *cf.* with Figure 3. The fact that these protein bodies have a lower contrast compared with those in the earlier developmental stages has no physiological significance. This results from developing the Figure so that the more electron dense material around the protein bodies exhibits some detail. The darker staining material is known as matrix protein and consists of glutelin proteins (Taylor *et al.* 1984). All the stages of protein body development described above were identical in both cultivars of *S. bicolor* which were studied. Unfortunately, it was not possible to study the protein bodies in the seeds at physiological maturity because the kernels were too hard to section for TEM.

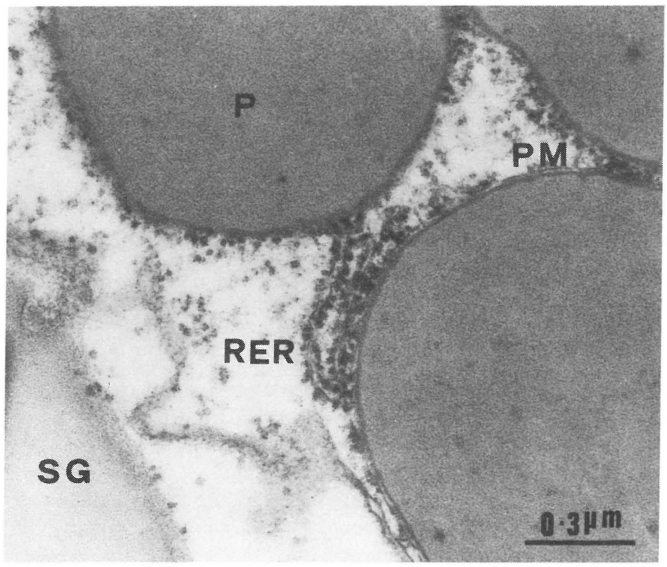

Figure 8 RER continuous with protein body membrane at soft dough stage of seed development (cultivar NK 283). P — protein body; PM — protein body membrane; RER — rough endoplasmic reticulum; SG — starch granule.

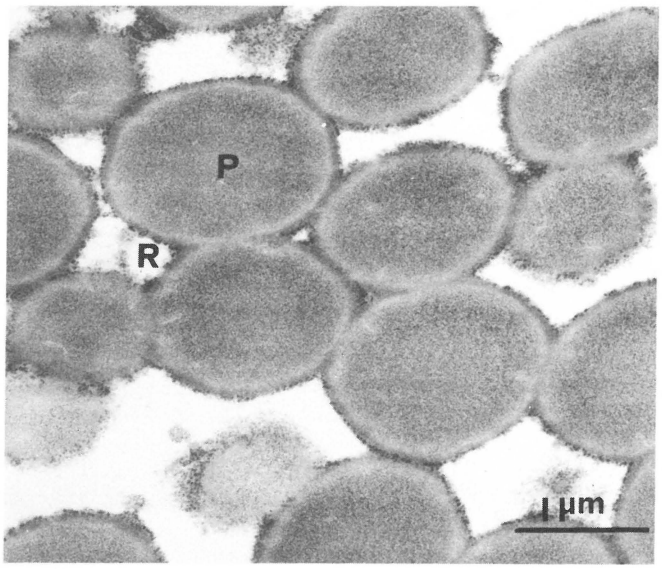

Figure 10 Protein bodies apparently without RER membrane at early hard dough stage of seed development (cultivar SSK 52). P — protein body; R — ribosomes.

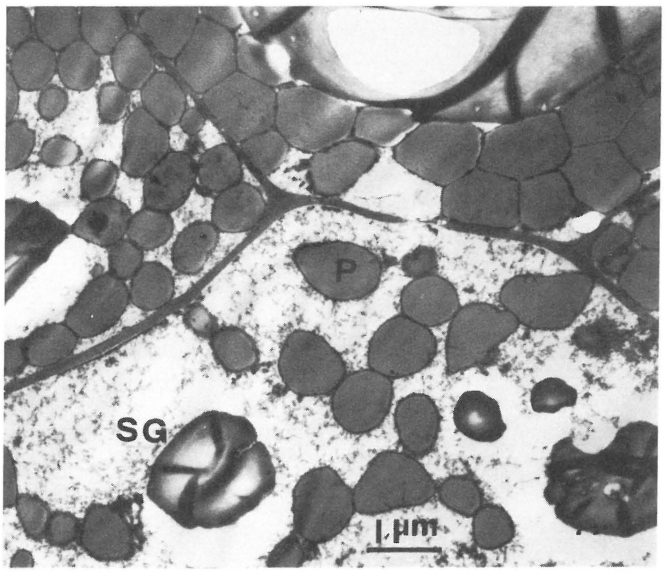

Figure 9 Distribution of protein bodies in sub-aleurone cells at early hard dough stage of seed development (cultivar NK 283). P — protein body; SG — starch granule.

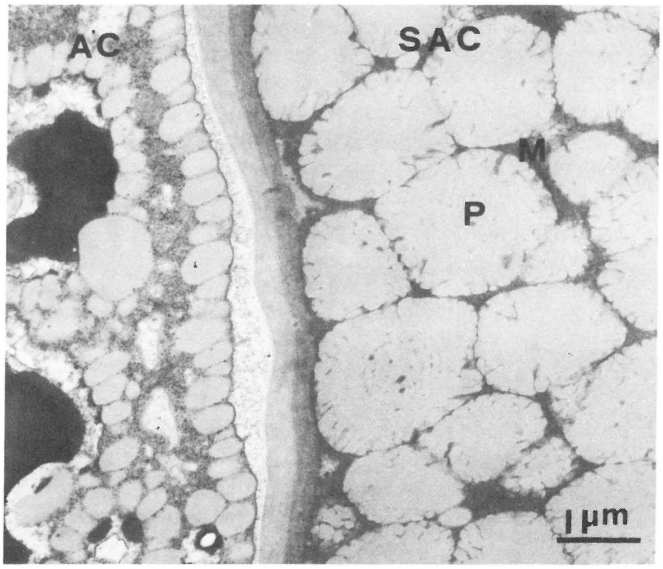

Figure 11 *S. bicolor* aleurone and sub-aleurone cells at the late hard dough stage of seed development (cultivar NK 283). AC — aleurone cell; M — matrix protein; P — protein body; SAC — sub-aleurone cell.

Discussion

The data in Figure 1 indicate that the majority of protein body synthesis occurs between the milk stage and late hard dough stage, as seed prolamin content increases almost linearly during this period. This is in general agreement with the TEM observations which showed that protein bodies are first present at the milk stage and by late hard dough they fill the sub-aleurone cells. Other work on protein synthesis in developing *S. bicolor* seeds (Johari *et al.* 1981) indicates that prolamin synthesis is more rapid later in seed development. However, direct comparison is not possible as these authors took samples at only three stages of maturity.

With regard to the mode of protein body formation, it is evident from TEM that during most of their growth they are enclosed by a membrane of RER. The apparent absence of RER around the protein bodies during the later stages of seed development was also noted in mature *Z. mays* seeds (Khoo & Wolf 1970). However, it is possible that the RER may still have been present but not discernible owing to the lack of contrast between it and the dark-staining matrix protein. It is, therefore, suggested that the prolamin polypeptides are synthesized on the ribosomes on the outside of the endoplasmic reticulum and then pass directly into the lumen of the RER. Thus, by this method, the protein bodies are formed within the RER membrane. This mode of protein body formation is identical to that which occurs in the other member of the Panicoideae subfamily which has been studied; *Z. mays* (Khoo & Wolf 1970; Burr & Burr 1976; Kyle & Styles 1977; Larkins & Hurkman 1978). It has also been suggested as being the mode of spherical protein body synthesis in another cultivated tropical grass, *Oryza sativa* (L.) (Bechtel & Juliano 1980). Interestingly, the protein bodies of *O. sativa*, like those

of *P. glaucum, S. bicolor* and *Z. mays*, remain as distinct entities in the mature seed (Horikoshi & Morita 1982).

It would, therefore, appear that the mode of protein body formation in tropical grasses is different from that which probably occurs in the temperate Triticeae, in that the storage proteins are merely deposited directly into the lumen of the RER and they are not subsequently transported to the cell vacuole.

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