CYTOLOGICAL STUDIES OF PROTEIN BODIES OF DEVELOPING WHEAT ENDOSPERM

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Summary

Fluorescence microscopy of thin sections of developing wheat endosperm previously fixed with osmium tetroxide and stained with acridine orange was used to study cellular development. Starch formation is predominant in the early period after flowering. The fine structure of developing endosperm cells was studied by electron microscopy of thin sections fixed and stained with osmium tetroxide or with potassium permanganate, or with formaldehyde followed by potassium permanganate. In addition to starch granules, numerous dense spheroidal bodies were observed. The staining reactions and the increase in numbers during growth indicated that the bodies probably consisted of storage protein. Observations on the occurrence of these protein bodies were made with three wheat varieties during two growing seasons (1959 and 1960). Based on the cytological evidence, the origin and intracellular localization of protein bodies is discussed.

I. INTRODUCTION

The storage protein of wheat grain is formed predominantly in the endosperm tissue from about day 12 to day 40 after flowering (Graham, Morton, and Simmonds 1963; Jennings and Morton 1963a). Hitherto little was known of the form in which the protein was stored nor of its intracellular localization. For several reasons it seemed unlikely that the protein would be uniformly dispersed throughout the endosperm cell. Among animal tissues, the secretory cells of the mammary gland form casein as particulate material, which may be directly observed only by electron microscopy (Morton 1954; Hodge and Morton 1956; Bailie and Morton 1958). Early in 1959 (Morton and Palk, unpublished data) numerous osmiophilic, electron-dense bodies were observed in wheat endosperm by electron microscopy. The process of formation and accumulation of these bodies was therefore investigated by using similar techniques with developing endosperm of three wheat varieties in two subsequent seasons (1959 and 1960); the results are described here. Since the isolated osmiophilic bodies have been shown to consist predominantly of protein (Graham, Morton, and Raison 1963), the granules are called “protein bodies” (Graham et al. 1962).

II. MATERIALS AND METHODS

(a) Wheat Grain

Heads of developing grain were taken from plants growing in plots at the Waite Institute, Adelaide. Three varieties were used: Triticum vulgare cv. Gabo and Insignia, and T. durum cv. Dural. Heads were labelled on the day of flowering and

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were harvested at specific periods after the mean flowering date, taken to the laboratory, and whole grain or endosperm (dissected by hand at room temperature) was immediately fixed.

(b) Fixing and Staining Procedures

A number of different methods were used. Initially, blocks of tissue were fixed for varying periods up to 3 hr in either (i) 1% osmium tetroxide in modified Locke's solution at pH 7·4 (Hodge, Martin, and Morton 1957), or (ii) 0·6% potassium permanganate in similar buffer. After fixing, the blocks were washed several times in modified Locke's solution at pH 7·4.

Later, cut heads of grain were placed in a beaker containing sufficient fixative so that only the base of the head and the small piece of attached stalk was immersed in liquid. The heads were placed in a glasshouse for about 3 hr. The fixatives were prepared in modified Locke's solution at pH 7·4 (Hodge, Martin, and Morton 1957) so that the solutions contained the following final concentrations of reactants: formaldehyde (1%) or potassium permanganate (0·2%) or osmic acid (0·2%). Grain thus fixed by uptake of fixative while metabolically active was subsequently dissected from the heads and blocks of endosperm tissues were further treated, if necessary, with formaldehyde (3·5%), potassium permanganate (0·6%), or osmic acid (1%) in modified Locke's solution, pH 7·4. In other experiments, endosperm tissue was transferred directly into 3·5% formaldehyde in modified Locke's solution at pH 7·4 and at 0°C for 24 hr, washed in Locke's solution, and then transferred to 2% potassium permanganate for 2 hr at 0°C.

(c) Electron Microscopy

(i) Dehydration and Embedding.—The fixed tissue was dehydrated by successive treatment for at least 30 min each in ethanol or in acetone solutions (50, 70, 80, 90, and 99% (v/v) at 0–3°C). The dehydrating agent was removed by washing with n-butyl methacrylate. Generally, the tissue was then suspended for 2 hr in n-butyl methacrylate–methyl methacrylate (80 : 20 v/v) containing 1% of benzoyl peroxide as catalyst, and then transferred to a similar solution in No. 5 gelatin capsules. The plastic was polymerized at 48°C for 12–24 hr. For some studies, tissues embedded in "Araldite" were also used.

(ii) Microscopy.—Sections (10–30 mµ thick) were cut with glass or diamond knives by using a "Siroflex" (Fairey Aviation Co. Ltd., Salisbury, S. Aust.) ultramicrotome. The sections were collected in acetone (15% v/v), expanded with chloroform vapour, and transferred to copper grids coated with carbon–collodion film. They were examined without removal of the embedding material. Photographs were taken with an Elmiskop model I electron microscope (Siemens and Halske Ltd., Germany) at magnifications of 1000–20,000 times, and photographically enlarged.

(d) Fluorescence Microscopy

Tissue was fixed in osmium tetroxide solutions as described above; sections (50–100 µ thick) were cut, and then stained with 1% acridine orange (see Randles 1960). Tissue sections were viewed and photographed with a Leitz "Ortholux"
mircoscope equipped for fluorescence microscopy. Either a xenon or mercury arc lamp was used as the light source. Photographs were taken in colour with high-speed "Ektachrome" (A.S.A. 160) film (Kodak (Australasia) Ltd.).

III. Results

(a) General

The results presented here are selected from numerous observations made at various periods of grain development with three wheat varieties (Gabo, Insignia, and Dural) during two seasons (1959 and 1960). Some results obtained with cv. Kashmir in 1962 are also included. Qualitatively, similar changes in endosperm cells were observed in each variety in each season and hence the most pertinent photographs have been selected for presentation irrespective of variety or season. Quantitative differences in respect of rate of changes, sizes of cells, and sizes of intracellular components were observed between varieties in any season, and between seasons for the one variety.

(b) Initial Stages of Endosperm Formation and Development

Plate 1, Figures 1 and 2, shows the early stages of endosperm development after pollination as indicated by fluorescence microscopy. The endosperm cells arise from the "fusion nucleus" of the embryo sac; two male nuclei enter the embryo sac, one gamete fusing with the ovum, and one apparently with two "polar nuclei" to form the fusion nucleus. Subsequent divisions of the fusion nucleus gives rise to a peripheral layer of cells (aleurone layer; see Plate 1, Fig. 1) in the embryo sac; centripetal growth by cell division takes place during the following 12 days forming the endosperm tissue (Plate 1, Figs. 1–3) which occupies the space of the former embryo sac (see Percival 1921; Hector 1936; Sandstedt 1946). After treatment with acridine orange, starch grains show a green and cell walls a red fluorescence. As shown in Plate 1, Figure 1, at 2 days after flowering the endosperm cells are small and contain few starch grains, whereas some grains are present in the adjoining cells of the testa (seed coat) and the pericarp (fruit wall). The testa (see Plate 1, Fig. 1) arises from the inner integument, two cells in thickness, surrounding the ovule; it develops by cell enlargement rather than by cell division. The pericarp arises from the ovary wall and is comprised of several layers of cells (Plate 1, Fig. 1). The outermost epidermal cells enlarge and the walls thicken during development, and a cuticle layer develops on the outer surface. In the parenchyma adjacent to the inner epidermal cells of the pericarp are large, elongate, cylindrical cells which contain chloroplasts. Like the inner epidermal cells, they enlarge as the endosperm develops. Ultimately the inner epidermal cells of the pericarp are crushed against the testa and the adjacent parenchyma; they lose their chloroplasts and other components and are distorted during further growth of the grain (see Percival 1921). The very considerable expansion of the cells of wheat endosperm between day 2 and day 33 is apparent by comparison of Plate 1, Figures 1 and 3. Clearly, each photograph is of different material. The cells of cv. Dural at day 33 are generally somewhat larger than those of cv. Gabo and Insignia but, nevertheless, the photographs indicate the considerable expansion in cell size which occurs in wheat endosperm (see also Percival 1921).
Between day 5 and day 12 after flowering starch synthesis in the endosperm cells is initiated and this is accompanied by a rapid decline in the concentration of sucrose and of reducing sugars in the endosperm (Jennings and Morton 1963a). Protein synthesis also commences but lags somewhat behind starch synthesis; it is accompanied by a rapid decline in the concentration of free amino acids in the endosperm (Jennings and Morton 1963a, 1963c). Between day 12 and day 20, a considerable amount of starch fills the enlarging endosperm cells; Plate 1, Figures 2 and 3, shows both large ellipsoidal grains, which are formed first, and smaller spheroidal grains which are formed later during development. During this latter period especially, protein synthesis in endosperm is rapid (Graham, Morton, and Simmonds 1963; Jennings and Morton 1963a). However, neither by fluorescence nor by phase-contrast microscopy of fresh tissue, with or without staining with methylene blue, could the localization of protein in the cells be established.

By electron microscopy of thin sections of tissue fixed and stained with osmium tetroxide, Hodge and Morton (1956) and Hodge, Martin, and Morton (1957) observed vacuolar structures in developing wheat root cells. They also observed large, osmiophilic, electron-dense bodies (called “dense bodies”; Hodge, Martin, and Morton 1957) which varied considerably in size, and were detected not only in cells but among isolated cellular components. The nature of these dense bodies was not established but, by analogy with observations on casein particles formed in the lactating mammary gland (see Bailie and Morton 1958), it seemed not unlikely that the osmiophilic dense bodies were predominantly protein in composition. Hence similar structures were sought in developing wheat endosperm.

(c) Accumulation of Protein and of Electron-dense Bodies during Development

Cell division in the endosperm has practically ceased after day 14, and hence changes in amounts of components per endosperm also represent changes in amount per cell (Jennings and Morton 1963a, 1963b). Between day 14 and maturity, storage proteins (soluble in acetic acid or in sodium hydroxide) increase considerably as compared with buffer-soluble proteins (Graham and Morton 1963; Graham, Morton, and Simmonds 1963). To follow the related changes in intracellular structure during this period, comparable pieces of endosperm tissue were fixed in osmium tetroxide or in potassium permanganate; electron micrographs were made of suitable sections after both types of fixation.

Plate 2, Figures 1 and 2, shows areas of young endosperm cells after fixation with osmium tetroxide. A number of osmiophilic, electron-dense bodies are seen within vacuolar structures. There are also areas showing parallel arrays of lipoprotein membranes with associated ribonucleoprotein particles, identifiable with the endoplasmic reticulum of other tissues. Hodge and Morton (1956) found that casein particles of mammary gland are electron-dense in the electron microscope after fixation with osmium tetroxide, whereas globules of neutral fat were poorly stained. The rate of increase of endosperm lipid after day 14 is much less than the rate of increase of protein (see Jennings and Morton 1963a, 1963b). Moreover, washing unfixed tissue in xylol did not remove the electron-dense bodies. Hence it was
tentatively concluded in 1959 (Morton and Palk, unpublished data) that the osmiophilic, electron-dense bodies were predominantly composed of protein. This was confirmed by later studies of the isolated bodies, which showed that the bodies consist predominantly of storage proteins (Graham et al. 1962; Graham, Morton, and Raison 1963). These structures are therefore referred to subsequently as “protein bodies”.

Some of the bodies shown in Plate 2, Figures 1 and 2, have vesicular structures at or near their peripheries. The bodies are within vacuolar structures, and these are frequently appressed to starch grains, as in the lower portion of Plate 2, Figure 2.

(d) Intracellular Localization and Structure of Protein Bodies

The occurrence of bodies within the structure seen in Plate 2, Figures 1 and 2, could be due to modification of fine intracellular structure. In thin sections of tissue fixed with potassium permanganate protein bodies were observed either freely in the cytoplasm, or closely appressed to a fine lipoprotein membrane; Plate 3 is representative.

Further detail of the fine structure associated with the protein bodies was obtained after fixing endosperm in formaldehyde and then treating with potassium permanganate. Plate 4 shows a protein body and portions of two adjacent starch grains. The protein body is within a fine lipoprotein membrane, and the protein is appressed to a parallel array of lipoprotein membranes which form a lamellar structure. External to the fine lipoprotein membrane containing the body are a number of parallel double membranes which probably are part of the endoplasmic reticulum.

Plate 5 shows an area of a cell with a number of developing protein bodies adjacent to one another. The lamellar structures are clearly part of each protein body.

The considerable variation in the size of the protein bodies within the endosperm cells at any period of development, and the marked enlargement which takes place during development is seen by comparison of Plate 2, Figure 2, Plate 3, and Plate 5. In very young tissue (cv. Insignia (Plate 2, Fig. 2) at 14 days, or cv. Gabo (Plate 3) at 15 days) the larger bodies are only about 0·5 μ in diameter, whereas by day 22 (in cv. Kashmir No. 5 (Plate 5)) the largest body is almost 13 μ in diameter. However, Plate 5 also shows a body approximately 0·8 μ in diameter.

IV. DISCUSSION

For reasons already presented, the structures described here are established as the sites of accumulation of storage protein in developing wheat endosperm; the term “protein body” is used to describe these structures. The apparent diameter of the bodies varies widely, but many from 1 to 15 μ in diameter have been observed. Such structures should be resolvable in the light microscope. However, as shown by Plate 1, Figures 2 and 3, the overlying and predominant starch grains probably obscure the bodies in the thicker sections required for light microscopy. With light microscopy, and on the basis of histochemical reactions, other workers have described protein granules in the seeds of maize (Duvick 1955, 1961), sorghum (Watson et al.
1955), and peanut (Altschul et al. 1961). The electron-dense bodies observed by electron microscopy in wheat root by Hodge, Martin, and Morton (1957), and later in barley endosperm by Buttrose, Frey-Wyssling, and Mühlethaler (1960), who considered that they were probably lipid in nature, are almost certainly analogous structures to the protein bodies described here and isolated and characterized by Graham, Morton, and Raison (1963). By electron microscopy, Morton and Palk (unpublished data) have observed similar structures in a number of plant seeds, including peanut and soybean.

As seen in tissue fixed with osmium tetroxide, during development the intracellular structure of endosperm cells is considerably modified as the products of synthesis accumulate. Protein bodies are observed frequently in young cells (15 days after flowering) which contain an abundance of elements of the endoplasmic reticulum and of mitochondria. At later stages of development, the nucleus may be much distorted in shape, and elements of the endoplasmic reticulum and well-formed mitochondria are less frequently observed. However, there are numerous protein bodies and starch grains. In the mature cell, adjacent large starch grains appear to distort the shape of the protein bodies.

Electron microscopy of thin sections of material fixed in osmium tetroxide led to the finding of the protein bodies which were not detected in the light microscope or by fluorescence microscopy (Plate 1, Figs. 1–3). However, the appearance of the protein bodies as seen in Plate 2, Figures 1 and 2, within vacuolar structures with lipoprotein membranes could be due to modification of fine structure caused by the fixation procedure. The occurrence of lipoprotein vesicles at the peripheries of a number of the protein bodies particularly indicated possible disruption of lipoprotein structures.

In material fixed with potassium permanganate directly, protein bodies are also detectable as electron-dense structures (Plate 3). Isolated bodies consist of 75–80% protein, based on nitrogen analysis (Graham, Morton, and Raison 1963) and they may therefore contain sufficient lipid to account for the electron-dense appearance after fixation in potassium permanganate. However, with potassium permanganate only (Plate 3) little further detail of the intracellular localization of protein bodies was established.

Plates 4 and 5 were obtained after fixation of the endosperm in neutral formaldehyde and treatment with potassium permanganate. With this novel procedure there was excellent preservation of fine structures as well as sufficient contrast to permit of high resolution in the electron microscope. The difference in appearance of Plates 4 and 5 to that of Plate 2, Figures 1 and 2, is due to the much better preservation of the fine structure as seen in the tissue fixed with formaldehyde. This establishes that protein bodies are indeed localized within lipoprotein membranes. Some of the lipoprotein structure is an integral part of the protein body (Plates 4 and 5).

The endoplasmic reticulum seen in Plate 4 lacks the ribonucleoprotein particles; these are not apparent in tissue fixed with formaldehyde and treated with potassium permanganate. However, the presence of the ribonucleoprotein particles is apparent in material fixed with osmium tetroxide (Plate 2, Figs. 1 and 2). The endoplasmic reticulum of plant cells is now well established.
The general appearance of Plate 2, Figures 1 and 2, may therefore be attributed to modifications brought about by fixation with osmium tetroxide which apparently causes the protein bodies to contract away from the appressed lipoprotein membranes. Similar modifications were observed in swollen liver cells by Hodge et al. (1956).

Protein bodies appear to be formed either singly (as in Plate 4) or in groups (as in Plate 5). The appearance of several bodies in a vacuolar structure (Plate 2, Fig. 2) is probably due to substantial disruption of the lipoprotein membranes separating several developing bodies. The close association of lipoprotein with the protein bodies may account for the necessity of addition of detergent for isolation of intact bodies (Graham, Morton, and Raison 1963).

The large increase in the size of the protein bodies between days 14 and 22 reflects the general development of endosperm tissue as shown by Plate 1, Figures 1–3, and as shown by the large increase in starch and protein per grain during this period (Jennings and Morton 1963a, 1963b). At late stages of growth (from day 22 onward) starch granules and protein bodies occupy almost all of the endosperm cell. Protein bodies up to 20 μ in diameter have been observed in mature tissue, but starch granules may be very much larger (see Plate 1, Fig. 3). However, mature tissue also has small protein bodies (about 0.5 μ in diameter, Plate 5), comparable to those seen in younger tissue (Plate 2, Figs. 1 and 2; Plate 3). Amino acid analyses (Jennings and Morton 1963c) show that small bodies may differ somewhat in protein composition from large bodies, the change in amino acid composition of the bodies reflecting the change in amino acid composition of the proteins of the whole endosperm during growth.

It is possible that the protein bodies are formed by “internal secretion” of protein within the lipoprotein membranes surrounding the bodies as discussed by Graham et al. (1962). However, other mechanisms of formation would also be consistent with the cytological evidence. By using the rates of incorporation of 14C-labelled glycine and of 35S-labelled sulphate into the storage proteins, as compared with the rates of incorporation into the supernatant (buffer-soluble) proteins of wheat endosperm, Graham and Morton (1963) and Graham, Morton, and Raison (1963) have obtained some evidence on the mechanism of formation of the protein in the bodies.

V. ACKNOWLEDGMENT

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VI. REFERENCES

Cytological studies of developing wheat endosperm 373


Explanation of Plates 1-5

Figures 1-3 of Plate 1 are photomicrographs obtained by fluorescence microscopy; all other figures were obtained by electron microscopy with an Elmiskop I electron microscope.

Plate 1

Fig. 1.—Section of developing wheat grain (cv. Gabo, day 2) showing pericarp (A), testa (B), aleurone layer of endosperm (C), and developing endosperm cells (D).

Figs. 2 and 3.—Sections of endosperm of developing wheat grain (cv. Gabo, day 16 (Fig. 2); cv. Dural, day 33 (Fig. 3)) showing enlargement of cells (compare with Fig. 1). Numerous large and small starch grains (St) are shown. By fluorescence microscopy after treatment with acridine orange the starch grains show green fluorescence and the cell walls (CW) red fluorescence.

Plate 2

Fig. 1.—Portion of endosperm cell (cv. Insignia, day 14) fixed in osmium tetroxide and showing a number of electron-dense protein bodies (PB) in vacuolar structures (V).

Fig. 2.—Portion of endosperm cell (cv. Insignia, day 14) fixed with osmium tetroxide, showing an accumulation of electron-dense protein bodies (PB) in vacuolar structures (V). A starch grain (St) is also shown enclosed in a membrane. Elements of the endoplasmic reticulum (ER) with associated ribonucleoprotein particles are apparent. Note the dense areas (DA) of some protein bodies, the distinct periphery, and small vesicles (Ve) associated with some of the bodies.

Plate 3

Portion of an endosperm cell (cv. Gabo, day 15) fixed with potassium permanganate, and showing electron-dense protein bodies (PB) and lipoprotein elements of the cell.
Plate 4

Portion of an endosperm cell (cv. Kashmir No. 5, about day 22) fixed with 3.5% formaldehyde at pH 7.5 for 24 hr at 0°C and then treated with 2% potassium permanganate at pH 7.5 for 2 hr at 0°C and embedded in "Araldite". A protein body (PB) is seen contained in a fine lipoprotein membrane. Within this membrane is seen a lamellar structure (LS) of lipoprotein layers with regular spacings. Outside the membrane containing the protein body is seen parallel double membranes of the endoplasmic reticulum (ER). Portions of two starch grains (St) are shown.

Plate 5

Portion of an endosperm cell (cv. Kashmir No. 5, about day 22), fixed as described for Plate 4, and showing a region of developing protein bodies (PB). A marked variation in the size of the protein bodies is apparent: PB1 is about 0.8 μ and PB2 about 13 μ in diameter. Lamellar structures (LS) consisting of parallel arrays of lipoprotein membranes with regular spacings are seen to be part of the structure of each protein body. Two small starch grains (St) are present.
Cytological Studies of Developing Wheat Endosperm
CYTOLOGICAL STUDIES OF DEVELOPING WHEAT ENDOSPERM
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