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PROTEIN DEPOSITION IN DEVELOPING BARLEY ENDOSPERM

by

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A thesis submitted in accordance with the
requirements for the degree of
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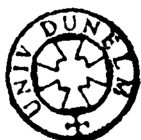
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ABSTRACT

The storage proteins of barley are both qualitatively and quantitatively important in determining the nutritional quality of the grain and its technological uses.

The development of the barley endosperm was followed from anthesis to grain maturity by light and electron microscopy. The deposition of storage proteins in the sub-aleurone and starchy endosperm was studied using conventional electron microscopy. Correlated studies using thick sections of zinc iodide-osmium tetroxide impregnated tissue have illustrated the three-dimensional interrelationships of the endoplasmic reticulum, Golgi apparatus and vacuoles during protein deposition.

To provide a clearer understanding of protein deposition in the wild-type barley, protein body formation was also investigated in mutant barley lines where a reduced and modified synthesis of storage protein is associated with an altered protein body structure.

Immunocytochemical localization of A, B, and C hordeins and the chymotrypsin inhibitors, CI-1 and CI-2, primary antibodies raised in rabbit and subsequently labelled with protein A-gold illustrated the storage protein in accumulating reserves in protein bodies of the wild-type barley, and the high-lysine mutant lines. Storage proteins were localized only in specific regions of both cytoplasmic and vacuolar protein deposits.

A comparison of specimen preparation techniques including different fixation and embedding protocols indicated that for barley endosperm, tissue post-fixed with osmium tetroxide and embedded in Spurr resin gave superior results to those embedded in LR White or Lowicryl K4M resin.

In situ hybridization was used to locate mRNA for CI-1 and CI-2 chymotrypsin inhibitors in barley endosperm using a biotinylated cDNA probe. The probe was localized at an ultrastructural level by incubation with avidin-peroxidase and subsequent DAB staining of the peroxidase activity.

The combined approach of thin- and thick- sectioning techniques for electron microscopy, in association with the molecular techniques of immunocytochemistry and *in situ* hybridization, has led to the development of a new model to illustrate the course of protein body development in barley endosperm. This new model also explains those previously published results used to support apparently contradictory earlier schemes.

DECLARATION

No part of this work has been submitted by me for any degree at this or any other University. All the work presented was done by me except where otherwise stated in the text.

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ABBREVIATIONS

(i) Abbreviations used in micrograph plates

In all micrographs:

μm Scale bars measured in (μm) micrometres.

Light micrographs:

(a) Toluidine blue: Plates 1-7

A	aleurone
cE	coenocytic endosperm
E	endosperm
Es	embryo sac
mP	matrix protein
n	nucellar layer
N	nucleus
P	pericarp
p	protein deposits, indicated by arrows.
S	starch granule
Sa	sub-aleurone
Se	starchy endosperm
V	vacuole
Vb	vascular bundle

(b) Fluorescence microscopy: Plates 8-9

A	aleurone
n	nucleus
P	protein deposits
S	starch granule
Sa	sub-aleurone
Se	starchy endosperm

Conventional transmission electron micrographs:

(a) Thin sections of developing Bomi barley caryopsis: Plates 10-17

A	aleurone
CW	cell wall
D	dictyosome
ER	endoplasmic reticulum
L	lipid body
M	mitochondrion
N	nucleus

P protein deposits; protein deposits can be singular, aggregated or complex, and are cytoplasmic or vacuolar.
pd 'electron-dense' component of protein complex
pf 'fibrillar' component of protein complex
pg granular component of protein complex
rER rough endoplasmic reticulum
S starch granule
Sa sub-aleurone
SM membrane of starch granule
V vacuole
Ve vesicles

(b) Thick sections of ZIO (zinc iodide-osmium tetroxide) impregnated Bomi barley caryopsis:
Plates 18-26

A aleurone
cER cisternal endoplasmic reticulum
CW cell wall
d)
D) dictyosome
m mitochondrion
N nucleus
NM nuclear membrane
P protein deposits; protein deposits can be singular, aggregated or complex, and are cytoplasmic or vacuolar.
S starch granule
Sa sub-aleurone
tER tubular endoplasmic reticulum
V vacuole
Ve vesicles
VM vacuolar membrane

(c) Thin sections of developing high-lysine barley caryopses:
Plates 27-32

CN cell wall
M mitochondrion
P protein deposit: protein deposits can be singular, aggregated or complex and are cytoplasmic or vacuolar.
Pd 'electron-dense' component of protein complex
Pf 'fibrillar' component of protein complex
Pg granular component of protein complex
pm membrane around protein deposit
rER rough endoplasmic reticulum
S starch granule
V vacuole
Ve vesicles
Vem vesicular matrix in protein vacuole.

(d) <u>Thick sections of ZIO (zinc iodide-osmium tetroxide) impregnated high-lysine barley caryopses</u>:
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cER cisterna1 endoplasmic reticulum
CW cell wall
D dictyosome
N nucleus
NM nuclear membrane
P protein deposit; protein deposits can be singular, aggregated or complex, and are cytoplasmic or vacuolar.
S starch granule
tER tubular endoplasmic reticulum
V vacuole
Ve vesicles

<u>Immunocytochemical localization in developing endosperm of low- and high-lysine barley</u>:
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CW cell wall
D dictyosome
ER endoplasmic reticulum
m mitochondrion
mP protein matrix
N nucleus
P protein deposit; protein deposits can be singular, aggregated or complex, and are cytoplasmic or vacuolar.
Pd 'electron-dense' component of protein complex
Pf 'fibrillar' component of protein complex
Pg granular component of protein complex
S starch granule
V vacuole
Ve vesicles.

<u>*In situ* localization of mRNA in the endosperm of the barley mutant Risø 1508</u>:
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CW cell wall
d dictyosome
ER endoplasmic reticulum
L lipid body
P protein deposit
S starch granule

(ii) Abbreviations used in Text

ANS	8-anilino-1-napthalene sulphonic acid.
CI-1,CI-2	Chymotrypsin inhibitors 1, and 2.
CTEM	Conventional transmission electron microscopy.
DAA	Days after anthesis.
DAB	Diaminobenzidine.
DNA	Deoxyribonucleic acid.
cDNA	DNA complementary to RNA, synthesized from RNA, and subsequently carried in a cloning vector.
EDTA	Ethylenediaminetetra-acetic acid, disodium salt.
ER	Endoplasmic reticulum.
LM	Light microscopy.
K_2HPO_4	Di-potassium hydrogen orthophosphate.
Mg^{2+}	Magnesium ions.
NADH	Nicotinamide adenine dinucleotide.
NH_4NO_3	Ammonium nitrate.
Poly A^+	Polyadenylated.
RNA	Ribonucleic acid.
mRNA	Messenger RNA.
SDS	Sodium dodecyl sulphate.
SDS-PAGE	Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis.
TCA	Trichloroacetic acid.
TEM	Transmission electron microscopy.
ZIO	Zinc iodide - osmium tetroxide complex.
Å	Angstrom unit.
µm	Micrometres.

Amino acids:

P	Proline
Q	Glutamine
F	Phenylalanine.
Y	Tyrosine.

CHAPTER ONE

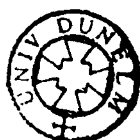
INTRODUCTION

1.1 General Background

Archaeological studies have indicated that man's exploitation of barley as a source of food began by the people of the Nile Valley 17,000 years ago (Wendorf et al., 1979) and today it is the world's fourth most important cereal after wheat, rice and maize, with a total yield of 1.78×10^8 metric tonnes in 1985 (FAO, 1986). In industrialized countries, such as the U.K., barley is produced primarily to feed livestock, but it also finds some use in malting and distilling (Hessayon, 1982). In the Third World countries, considerable quantities of barley are used for direct human consumption (Kent, 1978).

The nutritional value of barley for feeding to monogastric animals (such as pigs, chickens and humans) is limited by the low level of the essential basic amino acid lysine and threonine (Fuller et al., 1979a,b). This is determined by the prolamin storage proteins (hordein), which account for about half of the total grain nitrogen, but contain less than one mol% lysine (Shewry and Miflin, 1983a).

Storage proteins can be defined as those proteins which are present in protein bodies and function as a nitrogen store. They are laid down in one phase of development but function subsequently at a later metabolic phase to supply intermediary nitrogen compounds for biosynthesis in growing seedlings during the early growing period.



Seed proteins represent the major source of proteins for humans both directly (e.g. in bread) and indirectly by their use in livestock nutrition. As about half of the proteins in seeds are comprised of the storage proteins, the nutritional quality of the whole seed largely depends upon the amino acid composition of the storage proteins. In addition to their amino acid composition, the physicochemical properties of storage proteins are of importance in the food processing industry. Other seed proteins of importance in industry include enzymes, involved in food industries, lectins, which seem to play a role in recognition processes and are used in medical research, proteolytic enzyme inhibitors and proteins with allergenic properties. These various proteins may affect the nutritional quality not only of the seeds themselves, but also of other raw materials obtained from them such as starch and lipids used in the food industry.

There has been intermittent research to determine the subcellular locations of many of the storage proteins, and although some are known to be in protein bodies there is still controversy over the nature and mode of their deposition (Cameron-Mills and von Wettstein, 1980; Briarty et al., 1979; Miflin et al., 1983). This lack of knowledge creates a handicap in the understanding of seed development. Supported by world-wide pressure to increase and improve the production of plant proteins, considerable effort has been directed towards an understanding of the

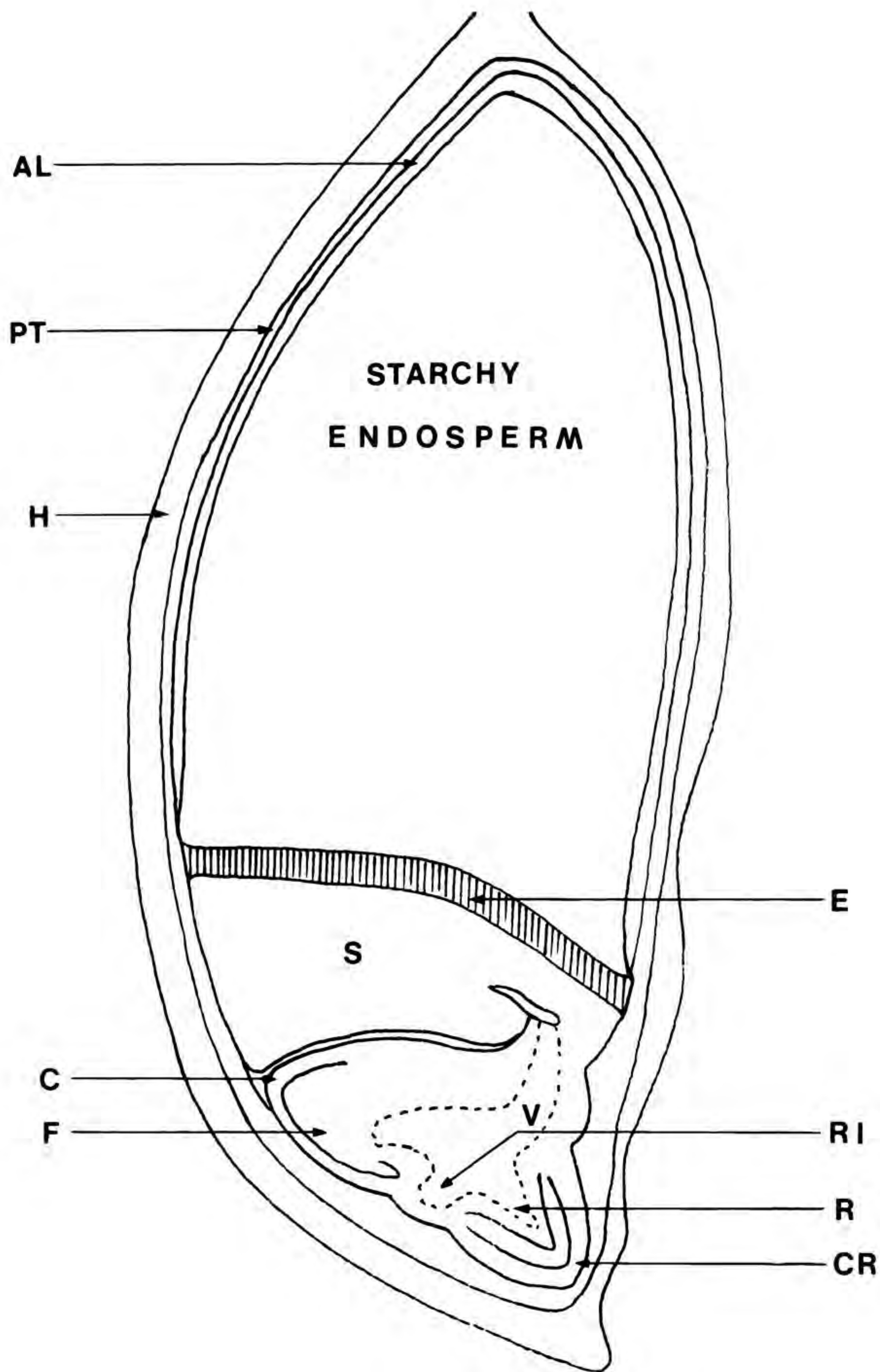
biochemistry and genetics of cereal grain development (Kreis et al., 1985). This is not only due to the economic importance of the crop but also because the developing grain is a system of considerable interest to biologists: the high rates of protein deposition in the grain during development make it a useful model for studying plant genome expression.

To understand the importance of storage proteins in determining the nutritional quality of the barley grain and its technological uses, it is desirable that existing controversy should be eliminated and the distribution and deposition of accumulating protein reserves in the barley endosperm be defined. Consequently, it is necessary to know how these proteins are synthesized and deposited in the grain, and relate morphological detail to molecular aspects of gene expression by an integrated approach to the problems of cell and tissue development and differentiation, utilising the recently developed techniques of cell biology and molecular biology and biochemistry.

1.2 Structure of the Barley Grain

The longitudinal structure of the grain is illustrated in Figure 1.1.

Although the husk (consisting of the lemma and the palea) generally adheres to the grain, naked or huskless varieties are known to exist (Briggs, 1978). The husk



C - Coleoptile
F - Foliar Shoot
AL - Aleurone Layer
RI - Root Initial
CR - Coleorhiza
PT - Pericarp-testa

R - Root
V - Vascular System
S - Scutellum
E - Scutellar Epithelium
H - Husk

FIGURE 1.1

LONGITUDINAL SECTION OF A MATURE BARLEY GRAIN

usually accounts for about 10% of the dry weight of the grain. Being a specialized leaf structure, it consists mainly of cellulose, hemicellulose, lignin and a small quantity of protein. The grain itself is a complex organ containing several types of tissue.

The testa and pericarp are the seed coat and fruit wall respectively, and it is their fusion which makes the barley grain a caryopsis or kernel. Anatomical studies (Palmer, 1969) of the pericarp show it to be a mass of compressed cellulosic cells, separated from the husk by a thin but complete waxy coating, and from the testa by an irregular alignment of large, flat, lignified cross cells running at right angles to the furrow of the grain. The testa and pericarp are both maternal tissues.

The endosperm is a triploid tissue arising from the fusion of a male gamete nucleus, from the pollen cell, with a diploid polar cell (containing the diploid polar nuclei) in the embryo sac. The endosperm makes up about 85% of the dry weight of the kernel (Kent, 1978). In common with other cereals, it is the endosperm of barley which is the principal site of reserve materials utilized by the embryo upon germination.

The outer layer of the endosperm, the aleurone, forms a jacket of living cells which lie between the testa and the starchy endosperm. The aleurone is three cells thick in the majority of barleys (Jones, 1969) and is developmentally related to the starchy endosperm it

encloses because both tissues differentiated from the same triploid fusion nucleus. However, the most obvious differences from the rest of the endosperm are that the aleurone contains fewer starch granules, and its protein bodies also differ in appearance.

The embryo represents only a small part of the kernel, 2-5% of the dry weight (Briggs, 1978), and is diploid, being formed by the fusion of the egg cell with a generative nucleus.

1.3 Development of the Grain

The barley ear, or inflorescence, consists of three spikelets which alternate on each side of the specialized stem (rachis) (figure 1.2). In six-rowed barley all the spikelets are fertile, but in the two-rowed type, this is only true of the median ones (Briggs, 1978).

Anthesis usually begins in florets near to the middle of the ear and can take from one to four days to complete. Prior to anthesis the pollen grains contain three nuclei: the vegetative nucleus and the two sperm nuclei (Cass and Karas, 1973). The embryo sac contains the egg cell, two synergid cells, two polar nuclei which are appressed to each other, and polynemic antipodal cells (Cass and Jensen, 1970).

At anthesis, the styles at the tip of the ovary diverge and the stamens rapidly elongate to shed the pollen contained in the anthers. Most of the pollen is

FIGURE 1.2

A : The barley ear (cv. Bomi) approaching maturity.
B : Inset shows a single grain with its awn, and the grain dissected from the husk with a distinct crease region.

shed within the spikelet, and some comes to rest on the stigmatic surfaces extended by the styles, where the pollen absorbs moisture and germinates. The emerging pollen tube extends over the surface of the stigmatic hair and penetrates the central lumen, continuing to grow downwards. Although several pollen grains may germinate, only one reaches the embryo sac where it deposits the vegetative nucleus, two sperm nuclei and some starch and cytoplasm into a degenerating synergid cell (Cass and Jensen, 1970).

The fusion of one of the sperm nuclei with the two polar nuclei, forms the triploid endosperm. This fusion takes place 5-6 hours after pollination (Briggs, 1978). The embryo is formed by the fertilization of the egg cell by the other sperm nucleus. This occurs 18-20 hours after pollination, by which time the endosperm has already divided 3-6 times (Briggs, 1978). Initially the endosperm is a coenocytic tissue, with hundreds of free nuclei in a common cytoplasm lining the edge of the embryo sac (Morrison and O'Brien, 1976). The tissue becomes multicellular 2-3 days after anthesis, and the endosperm nuclei cease to exhibit synchronous mitosis.

Percival (1921) described a five-cell stage in embryo growth in wheat, reached by a defined sequence of cell divisions, later workers such as Bennett _et al._ (1973) suggest more than one viable embryogeny. Following the five-cell stage, cell divisions occur in all directions,

and the embryo becomes a club-shaped mass of cells with a narrow, elongated base (Percival, 1921). Subsequent development leads to the formation of the various recognizable parts of the embryo, such as the scutellum, coleoptile and coleorhiza.

Cellularization proceeds by the growth of wall pegs which sporadically occur along the inner edge of the embryo sac (Morrison and O'Brien, 1976). These furrow inwards to partition the cytoplasm. After two days of growth, the pegs commonly branch and the growing ends turn towards each other and join. At this stage the endosperm cytoplasm contains numerous small vacuoles; there is also a large central vacuole limiting the endosperm on the inside of the embryo sac. After formation the peripheral cells enlarge considerably, and larger vacuoles are formed within. At about the same time, the nucleus in the cell divides and a cell plate begins to form between the daughter nuclei. The peripheral layer now begins to divide both radially and tangentially, and after approximately four days the entire embryo sac is cellular. Cell division continues tangentially for about sixteen days in wheat according to Evers (1970), although Radley (1978) and Donovan (1979) have suggested much longer periods. In support of the latter, Cochrane and Duffus (1981) have reported cell division continuing until 28-30 days in barley endosperm. Cell division is followed by cell expansion. Differential expansion of the dorsal and

ventral sides leads to the formation of a crease (Evers, 1970).

The endosperm contains almost all of the starch found in the grain (Briggs, 1978). Starch is deposited in amyloplasts, and the extent of starch deposition in a cell can be taken as a measure of its development since the cells die after starch deposition is complete. There appear to be two types of granules: large ($\sim$ 25 µm diameter) and small ($\sim$ 5 µm diameter) (Williams and Duffus, 1977). Approximately 10% of the total starch weight, but 90% of the number of granules is accounted for by the small granules.

Buttrose (1960, 1963a) studied the formation of organelles in the developing barley and wheat endosperm by electron microscopy. He reported the formation of small starch granules about two weeks after anthesis, although an earlier, unspecified, time was set for the initiation of the large grains. Different reserve cells are found to fill with starch at different times and rates. Deposition starts first in the cells towards the apex of the grain, and then in the central regions near the furrow. The last cells to initiate starch deposition are those immediately below the aleurone layer, while those next to the scutellum do not appear to contain any starch (Briggs, 1978). A possible explanation for this, is that the developing embryo uses some of the soluble carbohydrates which would otherwise be converted into starch (Percival,

1921).

A second class of organelles prominent in the endosperm are protein bodies containing the storage proteins. Although Buttrose (1963a) reported protein bodies as being visible one week after anthesis in wheat, Muntz (1982) has suggested that their formation should parallel the accumulation of storage protein. This is generally somewhat later (Bishop, 1930; Ivanko, 1971), although it is dependent on the growing conditions. The cells immediately below the aleurone layer appear to contain more protein than cells further into the starchy endosperm (Briggs, 1978). The protein bodies also differ in appearance.

The aleurone, although derived from the same triploid nuclear fusion, differs from the starchy endosperm in having very few starch granules (Buttrose, 1963b; Jones, 1969). It can be distinguished as early as ten days after anthesis, underlying the degenerating nucellus as peripheral meristematic cells which divide periclinally and from which the innermost cells differentiate to form the starchy endosperm and the outermost from the aleurone (Jones, 1969). At this stage, the aleurone layer does contain some minute starch grains. Buttrose (1963b) reports a few starch granules as being still visible at 18 days. By five weeks the aleurone layer has a characteristic appearance with tightly packed protein bodies almost completely surrounded by lipid droplets,

with the starch granules having disappeared (Buttrose, 1963b). The aleurone protein bodies differ from those in the starchy endosperm, and are about 4µm in diameter (Jacobsen *et al.*, 1971).

Surrounding the aleurone are the maternal tissues of the grain. Of these, the antipodal cells slowly degenerate as the embryo and endosperm develop, possibly contributing material to their growth (Bennett *et al.*, 1975). Similarly, the nucellus becomes absorbed as the ovule grows. Only the epidermis of the nucellus continues as a living tissue for some time. Ultimately the radial walls become absorbed and when the grain shrinks through water loss, the single cell tissue collapses leaving the upper and lower layers crushed together. This forms a delicate covering overlying the aleurone layer.

Three days after anthesis, the outer integument degenerates. The inner integument, consisting of two layers, retains its vitality until the grain reaches maximum size. At this stage, the cells in the outer layer collapse. The inner layer can be distinguished for some time later, and contains a yellow, resinous substance, which gives the grain its tint. Ultimately, the radial walls become disorganized, and the outer and inner layers are crushed together to form the testa (Percival, 1921).

The pericarp in barley consists of thin-walled parenchyma cells in between the inner and outer epidermis.

The cells next to the inner epidermis during development are the cross cells, and it has been suggested that these have an important role in the contribution of metabolites to the growing ovule (Jennings and Morton, 1963; Morrison, 1976). The cells of the inner epidermis and the parenchyma cells disintegrate by about 18 days, and the outer epidermis then lies next to the cross cells. The walls of the cross cells thicken and cytoplasmic degeneration follows. This layer of crushed cells then covers the testa (Percival, 1921).

1.4 Classification of Seed Proteins

1.4.1 On the Basis of Solubility

Early studies on the protein components of barley seeds were made by Einhof (1806), who showed that alcohol-soluble proteins were present. However the first systematic extraction and classification of barley seed proteins was made by Osborne (1895), and Osborne and Harris (1903). On the basis of similar studies of other plant and seed proteins, Osborne (1924) put forward his system of protein classification. He proposed the following groups of proteins, which were extracted sequentially:

Albumins: extracted in water.

Globulins: extracted in salt solutions.

Prolamins: extracted in aqueous alcohols (particularly 70% ethanol), rich in glutamine

and proline, and poor in basic amino acids.

Glutenins: extracted, after the above, by alkaline or acidic solvents.

This classification still remains in use today. Osborne and other contemporary workers originally gave names for all the different fractions of each species; the only one remaining in common use for barley is the term "hordein", originally used by Proust (1817), for the prolamin fraction. The term prolamin was suggested as a generic name because all of the alcohol-soluble proteins of cereal seeds have a large proportion of proline and amide-nitrogen, now known to be derived from glutamine.

The separation of proteins by their solubility results in fractions which differ from each other in their amino acid compositions and their physical properties. For this reason, Osborne's methods have retained their importance at the present time. However, the use of electrophoretic techniques has shown, as was suspected by Osborne himself and others for many years, and Preaux and Lontie (1975), that each of these fractions is made up of a heterogeneous mixture of proteins, and their compositions may overlap. For example, where extraction is done sequentially in the absence and then in the presence of reducing agents, the extracts are termed prolamin-I and prolamin-II respectively (after Sodek and Wilson, 1971). In wheat, the gliadins broadly represent the prolamin-I fraction and the glutenins the prolamin-II fraction. One

difficulty in defining the prolamin and glutelin proteins is that there may be a difference between solubility and extractability. Thus a polypeptide that is alcohol-soluble may not be extracted in aqueous alcohol because it is present in insoluble aggregates or bound to other insoluble proteins. The introduction of improved procedures for protein separation and analysis have led to modifications in the solvents used for extraction, so that fractions with the minimum of cross-contamination are obtained. Thus the prolamins have been recently defined as those proteins which are soluble (but not necessarily completely extractable) in alcohol in the presence of a reducing agent and at acid pH. As a result, many of the polypeptides once placed in the glutelin fraction are now considered to be prolamins (for detailed discussion see Miflin *et al.*, 1980a; Byers *et al.*, 1983).

With the variability between solubility groups classified by Osborne, there is a growing need for a nomenclature based on more exacting criteria. The need for such a classification provides a subject for much discussion and contention within cereal protein literature.

1.4.2 On the Basis of Function

According to our current knowledge of the biology of the developing grain, cereal proteins may be classified on the basis of their function. The three types are; cytosolic or metabolically active, structural,

and storage. Certain seed proteins appear to have evolved solely to store nitrogen and carbon and generally sulphur during seed development. These nutrients are then available to the growing seedling following their mobilization during germination. Seed storage proteins have been defined on the basis of the following criteria:

(i) tissue specificity: the proteins are usually only found in the seed tissues.

(ii) temporal accumulation: they accumulate during the later stages in the ontogeny of the seed, and this accumulation continues as the seed matures.

(iii) effect of N nutrition: their accumulation is disproportinately decreased relative to other seed proteins when nitrogen is severely limiting, whereas it is disproportionately increased with excess nitrogen.

(iv) subcellular location: they are present in discrete protein deposits within the cell.

(v) absence of any other function: those proteins normally considered as major storage proteins usually have no other known function; although in some cases other proteins e.g. enzymes, also appear to perform a storage function (e.g. sucrose synthetase in maize (Su and Preiss, 1978) and β-amylase in barley (Laberge _et al._, 1971: Hejgaard and Boisen, 1980)).

1.4.3 The Structure and Expression of Barley Storage Proteins

The prolamins of the Triticeae can be divided into three major sub-groups by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE): the S-rich, S-poor and high molecular weight prolamins (HMW) (Miflin *et al.*, 1983; Shewry *et al.*, 1984a; Shewry and Miflin, 1985). The general characteristics of these groups are summarized in Figure 1.3. In addition, at least two groups of low molecular weight (LMW) polypeptides are present, the CM Proteins and the LMW hordeins. (Salcedo *et al.*, 1980, 1982; Aragoncillo *et al.*, 1981). These two groups are together called A hordein.

The major group of the A hordeins of barley endosperm can be extracted in aqueous alcohol or chloroform-methanol mixtures; they are therefore called the CM-proteins. Some A hordeins can also be extracted by 0.5M NaCl and are therefore present in both the salt-soluble protein and hordein fractions extracted by sequential Osborne procedures. Using two dimensional isoelectric focusing polyacrylamide gel electrophoresis in aluminium lactate buffer at pH 3.2, Salcedo *et al.* (1980) demonstrated the presence of four major components, which they subsequently purified (Salcedo *et al.*, 1982a). These have molecular weights by SDS-PAGE of 12,000-16,000, and amino acid compositions similar to that of the CM proteins of wheat. They therefore labelled them CMa to CMd. Salcedo *et al.*

SDS-PAGE (cv. Sundance) Apparent Mol. Wt. [Kd]	Hordein Group	M_r by SDS-PAGE	SEU[a]	Partial Amino Acid Composition (mol %) Glx[b]	Pro	Gly	Phe	Lys	Cys	Met	% total fraction
	D	105,000	56,000	29.6	11.4	13.6	1.3	0.8	1.7	0.6	2-4
72	C2										
59	C1	55-75,000	52,000	41.2	30.6	0.3	8.8	0.2	0	0.2	10-25
46	B3	46,000	35,000	35.1	21.6	1.8	4.1	0.2	2.2	1.0	70-85
	B2	-	-	-	-	-	-	-	-	-	
35	B1	35,000	32,000	32.0	20.8	2.9	5.1	0.8	2.7	0.9	
	A hordein	12-15,000	-	13.6	10.1	8.7	3.3	3.1	6.4	1.9	1-2
	LMW hordein	10-16,000	-	25.5	17.0	5.5	4.4	0.1	5.3	3.7	?

[a] sedimentation equilibrium ultracentrifugation; [b] about 90% amidated in B and C hordein.

FIGURE 1.3. The characteristics of the groups of hordein polypeptides. (reproduced by courtesy of Dr. P.R. Shewry; in Shewry *et al.*, 1984).

(1984) have shown that CMa and CMc are encoded by genes on chromosome 1, and CMb and CMd by genes on chromosome 4. Salcedo _et al._ (1984) also identified a further LMW component, CMe, which was tentatively included in this group on the basis of its solubility and electrophoretic mobility. The structural genes for CMe were assigned to chromosome 3. Barber _et al._ (1986) have shown that CMa exhibits inhibitory activity against α-amylase from larvae of the insect _Tenebrio molitor_, while CMe corresponds to the major grain trypsin inhibitor (Lazaro _et al._, 1985) which was isolated and sequenced by Odani _et al._ (1983). Aragoncillo _et al._ (1981) showed that the LMW (under 25,0000) fraction from a chloroform-methanol (2:1) extract of barley endosperm contained a second group of components, present in smaller quantities than the CM proteins, that had low mobility on starch gel electrophoresis, and gave a major band on SDS-PAGE with a molecular weight of 18,000. More recently, Salcedo _et al._ (1982b) purified the major CMW hordein (LMW hordein-1 from barley). This had a molecular weight by SDS-PAGE of 16,500 and an amino acid composition similar to those of the low molecular weight prolamins of wheat and rye.

The high molecular weight prolamins are characterized by a particularly high content of glycine. They contain less proline and phenylalanine than other prolamins. Their abundance varies in amount, from about 2% (in barley) to about 10% (in wheat) of the total seed

prolamins. They are present exclusively in disulphide-linked aggregates and thus are extracted predominantly in the prolamin-II fraction. In barley, this HMW fraction is termed D hordein and in most cultivars migrates as a single band of molecular weight about 105,000 on SDS-PAGE (Shewry and Miflin, 1982).

In wheat endosperm, the high molecular weight seed storage proteins are termed the glutenins, and are coded for by several genes on each of the group 1 chromosomes of wheat - 1A, 1B and 1D (Thompson et al., 1983). Shewry et al. (1984b) purified a number of HMW subunits including at least one encoded by each genome. Further sequences have been deduced from partial cDNAs. Forde et al. (1983) reported two partial cDNAs which encoded the COOH-terminal parts of HMW subunits. They were closely homologous and encoded 101 and 39 residues. The central part of the polypeptide chain was shown to be based on repeated blocks of six and nine amino acids. The partial cDNA clone by Thompson et al. (1983) encoded over 200 residues, which consisted only of hexa- and nonapeptide repeats.

More recently, complete nucleotide sequences of genomic clones related to HMW subunits of wheat have been reported. Forde et al. (1985a) have sequenced an inactive glutenin gene derived from chromosome 1A. Two further nucleotide sequences of genes encoding for HMW-glutelin subunits have been determined by Thompson et al. (1985) and Sugiyama et al. (1985).

Nothing is known of the amino acid sequences of D hordein or the HMW secalins. The former is blocked to Edman degradation (Field et al., 1982) and the latter has not been studied. The two cDNA clones of Forde et al. (1983) also hybridized to a cDNA clone identified as related to D hordein of barley. This indicates the presence of a high degree of structural homology between the clones.

The sulphur poor prolamins contain no cysteine and so are almost completely extracted by aqueous alcohols without a reducing agent, and occur almost exclusively in the prolamin-I fraction. They constitute about 10-20% of the total prolamins in the seeds of plants grown under normal conditions of nutrition. In barley, this is called C hordein, and the fraction gives a number of bands on SDS-PAGE with molecular weights between 55,000 and 73,000. It is extremely rich in glutamine (about 40 mol%), proline (30 mol%) and phenylalanine (9 mol %). Amino acid sequences have been determined for the N-termini of whole proteins (Shewry et al., 1980a, 1981) and peptides (Tatham et al., 1985). Further sequence information has been derived from the nucleotide sequence of a C-hordein-related cDNA clone (Forde et al., 1985b).

At the N-terminus a distinct domain of 12 residues is followed by at least three repeated blocks of five residues (consensus PQQPY). The C-terminal domain is shorter being only 6 residues, and is preceded by repeated

blocks of 8 (PQQPFPQQ) rather than 5 residues. This octapeptide appears to correspond to the N-terminal pentapeptide plus a tripeptide. The ratio of amino acids in the octapeptide (4Q:3P:1F) is close to that in C hordein (40Q:30P:9F). Since glutamine (Q), proline (P) and phenylalanine (F) together account for almost 80% of the total residues, the octapeptide is probably the basic building block for most of the protein. The pentapeptide found in the N-terminal region may have been derived from this by deletion.

Tatham *et al.* (1985) used a combination of circular dichroism spectroscopy and computer prediction to show that C hordein has an unusual secondary structure with an absence of α-helix and β-sheet, but the presence of regularly repeated β-turns. This is associated with the repetitive primary structure. Their results indicate that the conformation is stabilized by strong hydrophobic interactions and by extensive hydrogen-bonding.

The S-rich prolamins vary in their extraction properties, some being present in the prolamin-I fraction and some in the prolamin-II. They account for 80-90% of the total prolamin fraction in seed grain grown under normal nutrient conditions, but decrease disproportionately under sulphur-stress. Their major characteristic is that they contain relatively large amounts (2-2.5m%) of S-amino acids. The sulphur-rich prolamins have less glutamine and proline, but more

cysteine (about 2.5m%) than C hordein. They can be sub-divided into different groups in wheat and rye but are generally treated as one group in barley; these are termed the B hordeins. With SDS-PAGE, they are shown to comprise of a number of polypeptides with molecular weights of 35,000 to 46,000. The B hordeins are the major storage proteins in the barley grain, accounting for 80-90% of the total hordein, and 30-40% of the total seed nitrogen (Kirkman et al., 1982).

Shewry et al. (1985) have reported the N-terminal amino acid sequence of fractions containing the 3 'B-hordein-like' bands from the barley mutant Risø 56. The mutant Risø 56 differs from the parental line Carlsberg II in a deletion of most (or all) of the genes at the *Hor 2* locus (Doll, 1980; Kreis et al., 1983a). Shewry et al., (1985) found the sequences are homologous with those of γ-type gliadins of wheat and γ-secalins of rye, indicating that they are not typical B hordeins. The identification of γ-type prolamins in barley has important implications for prolamin evolution. Kreis et al. (1985) made a detailed comparison of all the available amino acid sequences of S-rich prolamins and concluded that B-hordein was a derived type, most closely related to the low molecular mass subunits of wheat glutenin. The ancestral type of S-rich prolamin represented by the γ-type gliadins and γ-secalins did not appear to be present in barley. However, the study by Shewry et al. (1985) demonstrates

they are present, although as a small proportion of the total hordein in normal cultivars. Rasmussen et al. (1983) and Forde and co-workers (Miflin et al., 1983; Forde et al., 1985c) have reported the nucleotide and derived amino acid sequences of cDNA clones related to the B-hordein mRNAs. Rasmussen et al. (1983) determined the nucleotide sequences of four cDNA clones. The longest of these, the *pc hor 2-4*, encoded the 181 carboxy-terminal amino acids of a polypeptide that was homologous 61 identical residues out of 74 with the B1-hordein peptides sequenced by Schmitt and Svendsen (1980). A second cDNA clone, *pc hor 2-3*, encoded the 54 carboxy-terminal amino acids of a second hordein polypeptide, which differed from that encoded by *pc hor 2-4* in nine residues. The other two cDNA clones were shorter and encoded carboxy-terminal regions of proteins with the same amino acid sequence as that encoded by *pc hor 2-3*. The most notable difference between the amino acid sequences encoded by *pc hor 2-3* and *pc hor 2-4* was in the region close to the carboxy-terminus. Whereas the amino acid sequence encoded by *pr hor 2-3* was identical in this region to that reported by Forde et al. (1981) for a B3 type of hordein polypeptide, that encoded by *pc hor 2-4* differed in the sixth, seventh, eighth and ninth residues from the carboxy-terminus.

Forde et al. (1985b) have sequenced two longer cDNA clones related to B1 and B3 hordeins. Sequences (b) and

(c) were determined from complete genomic and almost complete cDNA sequences respectively of B1 (class I) hordeins from cv. Sundance, using hybrid-selection translations. A further sequence (d) was deduced from a partial cDNA from B3 (Class III) hordein from the same cultivar (Forde et al., 1985c). Brandt et al. (1981), deduced a sequence (a) from genomic DNA from a different cultivar, Carlsberg II, but suggested it may also correspond to a class I/II polypeptide.

The sequences all show two distinct structural domains. The N-terminal domain consists of 79 residues in the B1 hordein polypeptide from cv. Sundance (sequence b), but up to 23 residues less in the class I/II polypeptide from Carlsberg II (sequence a). This domain is proline-rich and based on repeated peptides. In the B1 (class I) hordeins (sequences a,b,c) these vary in length from five to eight residues, but are all based on a PQQP tetrapeptide.

The repeat motif is less conserved in the B3 hordein (sequence d), and there is no apparent sequence homology between the N-terminal part of this protein and the B1/Class I hordeins.

The C-terminal domains of the proteins consist of about 200 residues, are non-repetitive and contain less proline (11% in sequence "b" compared with 39% in the repetitive domain). With the exception of the B3 hordein, they also contain all the cysteine and methionine

residues.

The B, C and D groups of hordein polypeptides are encoded by single structural loci designated *Hor 2*, *Hor 1* and *Hor 3*, respectively. The chromosomal locations of structural genes for the prolamins of the Triticeae are illustrated in Figure 1.4.

Linkage data are taken from Shewry et al. 1983b, 1984c), Jensen (1983), Payne et al. (1982a,b), Jackson et al. (1985), and Singh and Shepherd (1984).

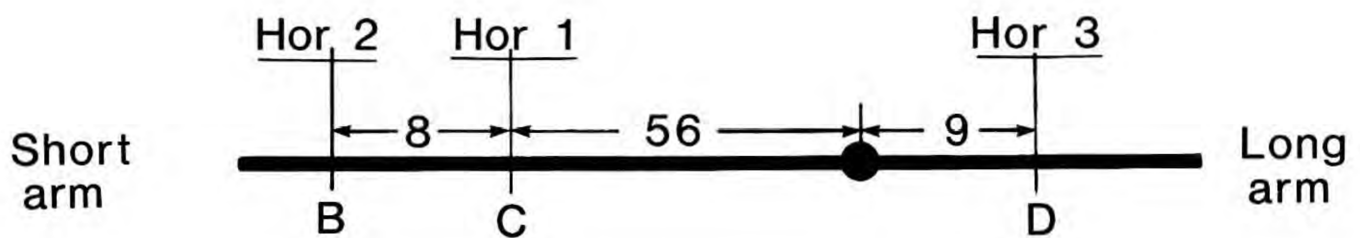
Hor 1, *Hor 2* and *Hor 3* are co-ordinately regulated in that they are only expressed in the endosperm and, at a certain stage of development. Also, they always appear to be expressed together, no one group of hordein polypeptides being synthesized without the others. However, within this broadly synchronized expression there are differences in the relative levels of synthesis and accumulation of the D-hordein, C-hordein and sub-families of B-hordein polypeptides. These occur as a function of the stage of endosperm development, in response to the availability of nitrogen and sulphur and as a result of the presence of mutant high-lysine genes.

Different classes of B-hordeins can be recognized by cyanogen bromide peptide mapping (Faulks et al., 1981), hybridization of cDNA and mRNA populations (Forde et al., 1981; Kreis et al. 1983a) and nucleotide sequencing of cDNA clones (Forde et al., 1985b), and are encoded by separate sub-families of mRNAs.

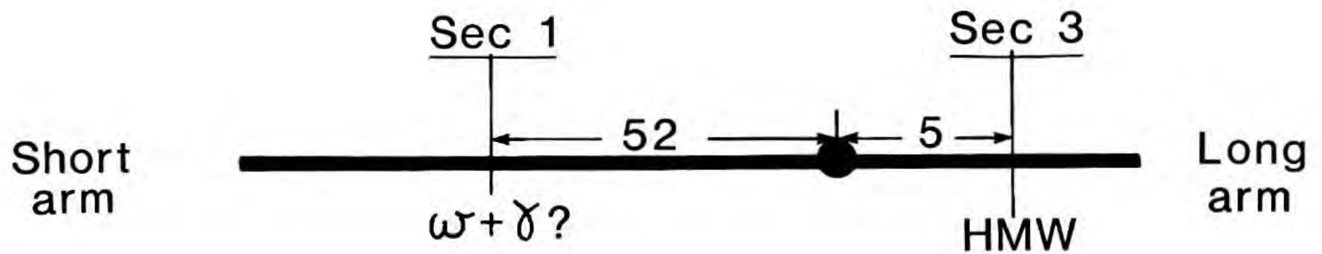
CHROMOSOMAL LOCATIONS OF PROLAMIN STRUCTURAL GENES

-ON GROUP 1 CHROMOSOMES

BARLEY -Chromosome 5



RYE -Chromosome 1R



WHEAT -Chromosome 1A,1B,1D

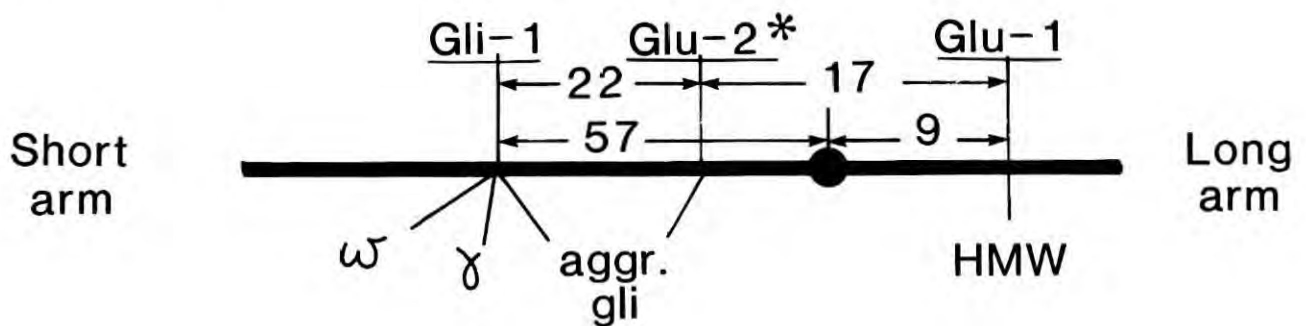


FIGURE 1.4

Chromosomal locations of structural genes for the prolamins of the Triticeae.
(reproduced by courtesy of Dr. M. Kreis; in Kreis et al., 1985).

1.4.4 The Non-Prolamin Proteins

The main storage protein in the barley endosperm, hordein, accounting for about 40% of the barley grain nitrogen has a very low content of the essential amino acid lysine. Attempts to improve the grain's nutritional value have thus centred on finding lines in which the lysine content is increased. The first high lysine line recognized in a cereal other than maize was Hiproly (Munck et al., 1970), which was found by screening the world barley collection using the dye-binding capacity (DBC) technique. This procedure compares the amount of Acilane Orange G dye bound to basic amino acids with the nitrogen (N) content of the sample, any deviant with a higher than average DBC being likely to have an increased amount of basic amino acids. The same procedure has been used by the research group at the Danish Atomic Research Station at Risø to screen a large number of seeds treated with either chemical or physical mutagens. From this, a number of mutants have been identified (Doll et al., 1974) including Risø 1508 which has the highest increase in grain lysine content yet recorded and was obtained by mutagenesis (Ingversen et al., 1973). A further high lysine mutant is Risø 56, induced by treatment of the cultivar Carlsberg II with γ-rays, and is characterized by a decrease in the amount of B hordein polypeptides, is believed to have a deletion in the structural genes coding for one of the hordein fractions, *Hor 2* (Doll, 1980).

Four major salt soluble proteins, β-amylase, protein Z and two chymotrypsin inhibitors CI-1 and CI-2 were identified in Hiproly derived lines (Hejgaard and Boisen, 1980). In this study it was shown that the four proteins accounted for 7% of the grain lysine in standard lines but for 17% in Hiproly lines. The same investigation also showed that only CI-1 was increased in Risø 1508. Giese and Anderson (1982) studied the course of protein synthesis during grain filling in Bomi and the high lysine barleys Hily 82/83 and Risø 56, and have shown that the four salt soluble proteins, protein Z, β-amylase and the chymotrypsin inhibitors, CI-1 and CI-2, are synthesized in greater amounts earlier in the high lysine lines than in Bomi. On the other hand, the hordeins are synthesized in greater amounts earlier during grain filling in Bomi than in Hily 82/83 and Risø 56. Giese and Anderson also found no indication of a significant reduction in total protein synthesis in the high lysine lines compared to the standard lines. They found Hily 82/83 and Risø 56 to be very similar in protein composition in that they have lower hordein content and higher levels of particularly β-amylase and chymotrypsin inhibitors than Bomi.

Kreis et al. (1983b) have analyzed the molecular nature of the Risø 56 mutation, which results in a depression of hordein accumulation and consequently a higher overall lysine content. In particular, the amount of B hordein, encoded for by the complex locus *Hor 2* , is

decreased by about 75% because of the absence of major components. Kreis et al. (1983a) found from the analysis of endosperm RNA, by *in vitro* translation and hybridization to various cloned cDNAs derived from hordein mRNA, that mRNA for the major B hordeins is not present in the endosperm. Furthermore, hybridization of a B hordein cDNA clone to gel-fractionated restriction digests of mutant and wild-type DNA indicates that at least 85kb of DNA has been deleted from the *Hor 2* locus in the high-lysine mutant.

In recent studies on the course of protein synthesis in the endosperm of the standard variety Bomi, the synthesis of the proteins, β-amylase and protein Z, was shown to occur a little later during grain filling than the synthesis of hordein. Also, protein Z was shown to be the protein in the salt soluble fraction that responds most to the increasing N (Giese and Hejgaard, 1984).

The chymotrypsin inhibitors CI-1 and CI-2, purified initially by Boisen et al. (1981), have been shown to be immunologically distinct, and to have slightly different amino acid compositions. Both inhibitors have a high lysine content and, unusually for protease inhibitors, devoid of cysteine. Both inhibitors have been sequenced (Svendsen et al., 1980a, 1982). The results support the original suggestion by Mikola and Suolinna (1971) that they are related to potato protease inhibitor I. More surprisingly, they are also homologous with eglin, the

elastase-cathepsin G inhibitor from the leech (*Hirudo iedicinalis*) (Svendsen et al., 1980a, 1980b, 1982). Amino acid sequence comparison showed that two of the CI-2 isoforms are the same proteins as the high-lysine proteins SPII A and B purified from the high-lysine barley line Hiproly (Svendsen et al., 1980b). CI-1 and CI-2 inhibitors have been shown to accumulate at about the same time as the major storage proteins - the hordeins (Kirsi, 1973; Giese and Andersen, 1984). However, they are not endosperm specific, in that they are synthesized in the embryo during germination, particularly in the growing root tips, and can be found in small amounts in the meristematic regions of the plant during growth (Kirsi and Mikola, 1971). In common with storage proteins, the amounts of these inhibitors accumulated in the endosperm are markedly increased by increasing nitrogen fertilizer (Kirsi, 1973; Hejgaard and Boisen, 1980; Giese and Andersen, 1984) and they are broken down relatively rapidly during germination (Kirsi, 1974).

Williamson et al. (1986a) have isolated several cDNA clones related to CI-1 and CI-2 using synthetic oligonucleotides as probes. Restriction mapping and sequencing show that there are at least two subfamilies of mRNAs for each inhibitor. "Southern blots" indicate that there are about 10 and 6 copies per haploid genome of the structural genes for CI-1 and CI-2, respectively. "Northern hybridization" experiments show that the

abundances of CI-1 and CI-2 mRNAs correlate with changes in the amounts of the proteins *in vivo* in normal and high-lysine barley lines. Figure 1.5 illustrates the relative amounts of CI-1 and CI-2 present in the low-lysine and high-lysine lines of barley.

Similar chymotrypsin inhibitors have been found in seeds of other plants. Hejgaard (1981) has shown that proteinase inhibitors, which are immunologically related to CI-2 in barley, are present in wheat and rye.

Cereal seeds have long been known to contain α-amylase inhibitors (Kneen and Sandstedt, 1943; Buonocore et al.,1971). Three major forms have been purified and analyzed from wheat (Shainkin and Birk, 1970; Redman, 1975, 1976; Granum and Whitaker, 1977; Kashlan and Richardson, 1981). These inhibitors have been found chiefly in the endosperm and can be extracted from the flour with water or aqueous alcohol (Silano et al., 1973). Complete amino acid sequences of α-amylase inhibitors have been determined (Maeda et al., 1985a; Maeda et al., 1983). Maeda et al., (1985b) produced monoclonal antibodies to demonstrate the structural relationship of numerous α-amylase inhibitors in wheat.

Parallel studies have shown that cereal grains contain large amounts of trypsin inhibitors (5-10% of the total water-soluble protein), present in both the embryo and the endosperm (Mikoli and Kirsi, 1972; Hochstrasse and Werle, 1969). Endosperm inhibitors have been

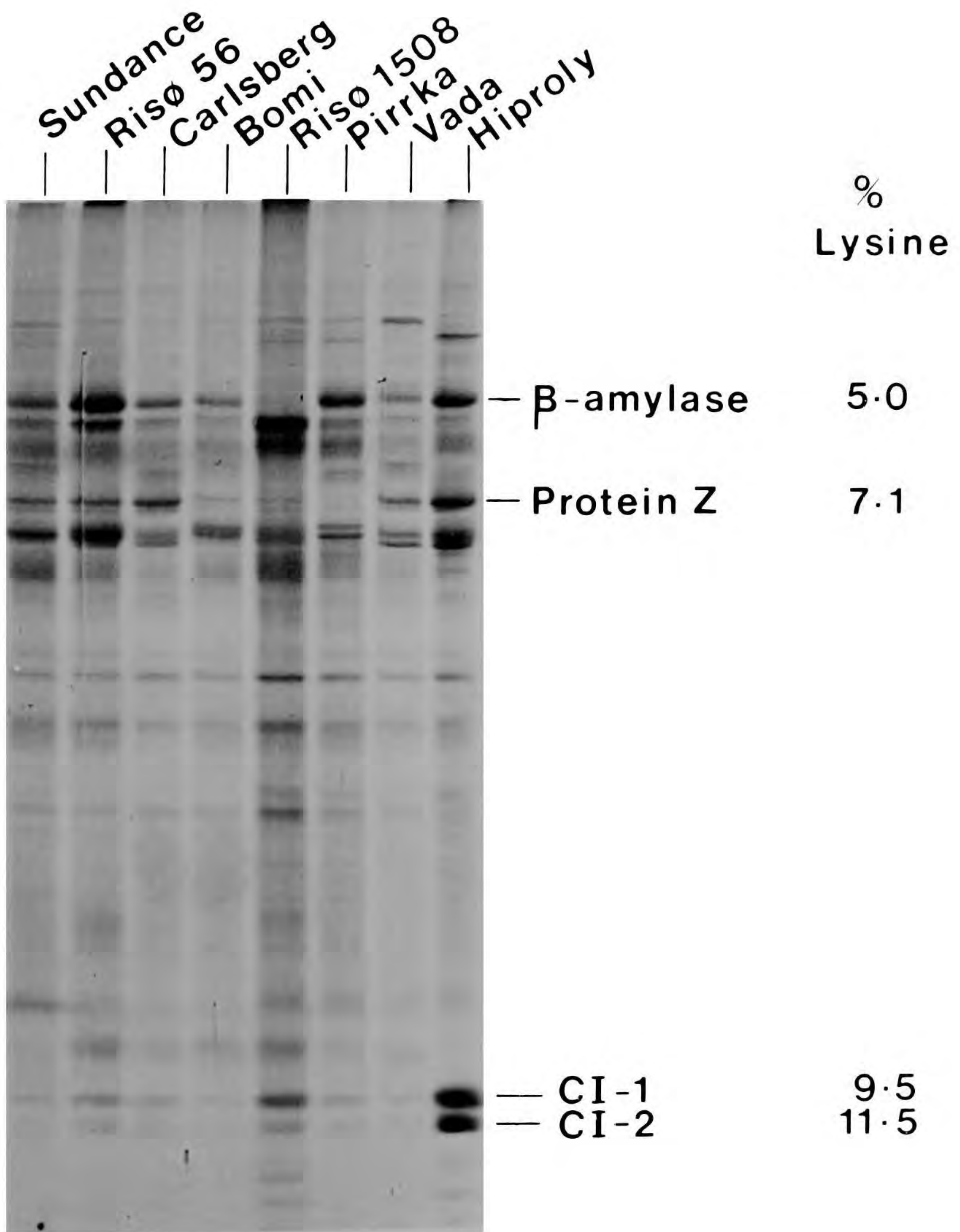


FIGURE 1.5

SDS-PAGE of Salt-Soluble Proteins in low and high-lysine barley lines.
(reproduced by courtesy of Dr. M. Kreis).

characterized for wheat, barley and rye (Boisen and Djurtoft, 1981a; Boisen and Djurtoft, 1981b; Boisen, 1983; and Shewry et al., 1984d). Besides being soluble in water, the trypsin inhibitors are also soluble in aqueous ethanol (50%) (Mikola and Suolinna, 1969).

Shivaraj and Pattabiraman (1981) have shown that one of the α-amylase inhibitors of Indian finger millet (*Eleusine coracana*) is also an active trypsin inhibitor. This inhibitor has been sequenced (Campos and Richardson, 1983). Odani et al. (1983a) also sequenced the barley trypsin inhibitor and noted the homology between their sequence and that previously reported for the wheat -amylase inhibitor (Kashlan and Richardson, 1981). There is considerable homology between the three α-amylase inhibitors of wheat, the trypsin inhibitors of barley and maize, and the bifunctional α-amylase trypsin inhibitor of finger millet. It therefore appears that the α-amylase and trypsin inhibitors belong to the same family and that some at least are bifunctional. Odani et al. (1983b) have also noted sequence homology between the barley trypsin inhibitor and castor-bean storage protein.

Many of the cereal protease inhibitors are bifunctional in that they also inhibit α-amylase activity. Mundy et al. (1984) characterized a bifunctional wheat inhibitor on endogenous α-amylase and subtilisin. The amino acid composition and N-terminal sequence (45 residues) show that the inhibitor is homologous with

cereal and leguminous inhibitors of the soybean trypsin inhibitor (Kunitz) family.

1.5 Endosperm Development

Certain aspects of the development of the endosperm of barley are particularly relevant to its technological uses: one such important process is that of protein deposition.

1.5.1 The Temporal Deposition of Proteins

Several studies have been made of the timing of protein deposition in cereal seeds. Bishop (1930) was probably the first person to study the developmental sequence in which the protein fractions of the barley grain accumulate.

Figure 1.6 illustrates the results of a recent study by Rahman *et al.* (1982) for the accumulation of protein fractions during the development of barley endosperm. These results emphasize the point that salt-soluble proteins are present from early in development but cease to accumulate when the endosperm has reached about half of its final weight. There may, however, be differences in the accumulation patterns of different components of the fraction, for example the globulins probably contain some storage proteins and their accumulation continues later in development than the albumins (Brandt, 1976). In contrast the hordeins only appear as the endosperm enters the phase of rapid growth and they continue to accumulate linearly until maturity.

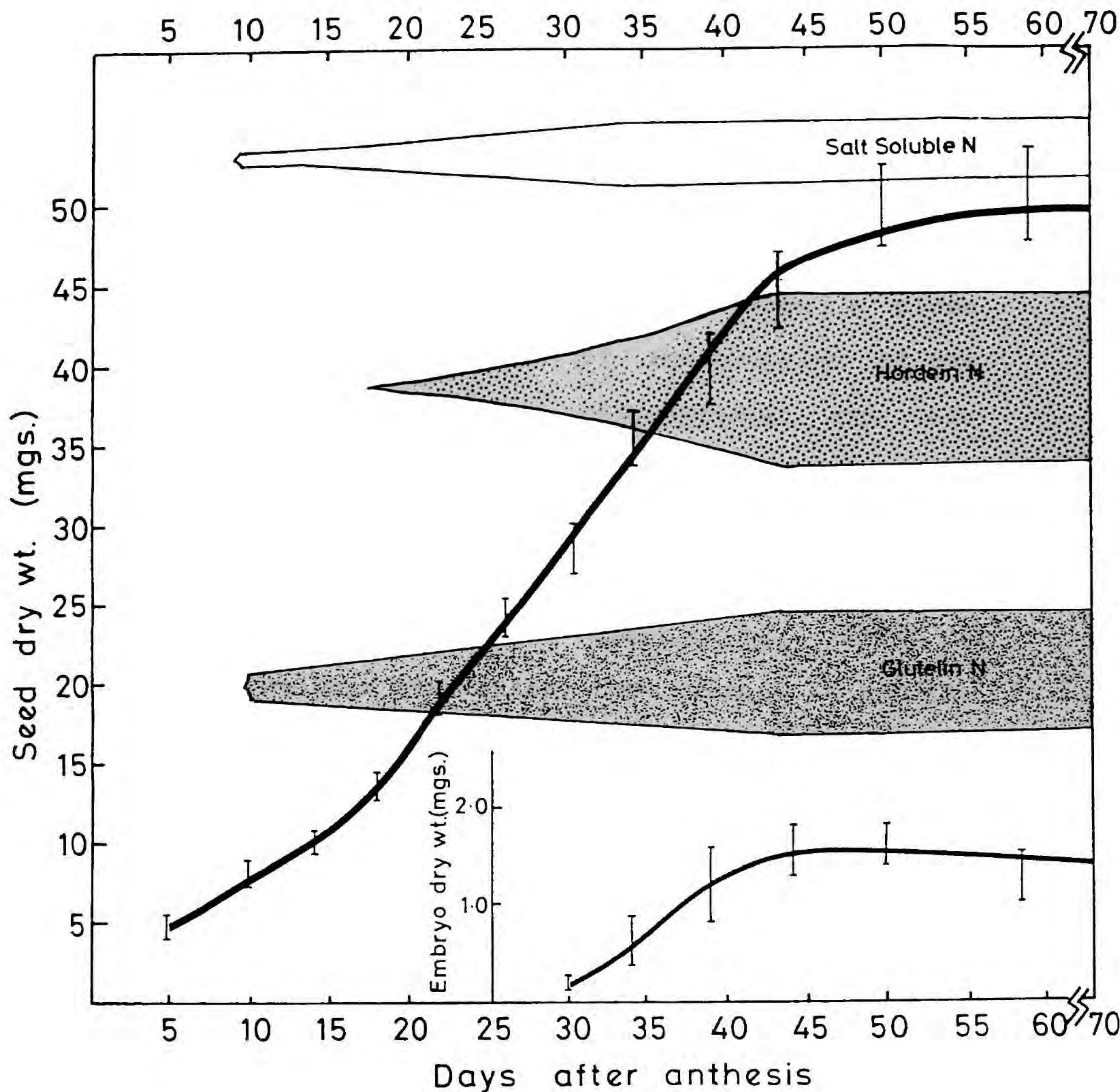


FIGURE 1.6

Accumulation of Protein Fractions in developing barley seed.

The accumulation of fractions during the development of the barley endosperm. The heights of the shaded blocks indicate the relative amounts of the various fractions. (reproduced by courtesy of Dr. B.J. Miflin; in Miflin *et al.*, 1983).

Rahman et al. (1982) have shown that within the total hordein fraction, the individual components accummulate at different rates. At the early stages, the C hordeins form a relatively greater proportion of the total hordein fraction, with the lower molecular weight B hordeins accumulating relatively later than the rest. Analyses of RNA fractions from such grain show a close correlation between the rates of accumulation of the different groups of hordein polypeptides and the populations of RNAs related to them (Rahman et al., 1983). This suggests that rates of hordein synthesis are determined largely by the abundances of mRNAs. There is also evidence from the studies of Rahman et al. (1984) on the effects of sulphur starvation, that changes in hordein composition may also result from differences in the efficiency of translation of mRNAs (for a more detailed discussion on the structure and evolution of seed storage proteins and their genes see : Shewry et al. (1984), and Kreis et al. (1985)).

The glutelin fraction, which probably contains mainly the structural proteins of the cell, is formed relatively early and continues to accumulate slowly throughout development.

Studies of wheat have shown a similar picture. Jennings and Morton (1963) found that wheat storage proteins were observable approximately 12 days after anthesis, and continued to accumulate until day 40. In

contrast, the buffer-soluble cytoplasmic proteins were present at the earlier sampling date (8 days), and continued to accumulate up to day 30, but not beyond.

1.5.2 The Origin and Development of Protein Bodies

Although it has long been recognized that the storage proteins of seeds are deposited in protein bodies, much controversy surrounds the origin of these protein bodies in cereal endosperm. Several lines of evidence show that the prolamin storage proteins are synthesized on the rough endosplasmic reticulum (RER). This evidence includes electron microscopy of the developing cereal grain and the *in vitro* synthesis of proteins using mRNA fractions extracted from isolated RER. Briarty et al. (1979) examined sections of wheat endosperms fixed at different stages of maturity and estimated the amount of endoplasmic reticulum (ER) by stereological analysis. The surface area of the RER was found to increase fourfold during the period of most rapid growth and protein deposition.

The RER may be physically isolated from cereal endosperms by homogenization followed by sucrose density gradient centrifugation. In barley and wheat, the alcohol-soluble proteins with electrophoretic characteristics of the prolamins are associated with the major peak of RER isolated from developing and endosperms (Miflin et al., 1981). Polyadenylated RNA, isolated from RER fractions of maize and barley, has been shown to direct the *in vitro* synthesis of prolamins (Cameron-Mills

and Ingversen, 1978; Larkins and Hurkman, 1978).

Polysomes are either associated with membranes (membrane-bound) or free. The membranes associated with the bound fraction are presumed to be the ER. In species (wheat, barley and maize) in which the products of mRNA from free and bound polysomes have been compared, the membrane-bound RNA has been enriched with messenger RNAs coding for the prolamin storage proteins (Brandt and Ingvensen, 1976; Fox et al., 1977; Matthews and Miflin, 1980; Greene, 1981; Donovan et al., 1982; Okita and Greene, 1982; Forde and Miflin, 1983).

The final site of deposition of the prolamin storage proteins and the route by which this is reached are less clear. In maize, the protein body forms by dilation of the ER (Khoo and Wolf, 1970; Larkins and Hurkman, 1978). During development, the ribosomes can be seen attached to the outside of the protein body. These polysomes and the poly A RNA that they contain, can be isolated from the protein bodies and shown to direct the synthesis of zeins in an *in vitro* wheat-germ protein synthesis system. (Burr and Burr, 1976; Larkins and Hurkman, 1978; Viotti et al., 1978).

Furthermore, protein bodies from developing endosperms contain 40-50% of the total activity of the ER marker enzyme, reduced nicotinanide adenine dinucleotide phosphate (NADH) cytochrome *c* reductase (Larkins and Hurkman, 1978; Miflin et al., 1981).

There are divergent opinions as to what occurs in wheat and barley. Morton _et al._ (1964), suggested that protein bodies were independent organelles capable of internally synthesizing protein and containing ribosomes. However, some of the data upon which this conclusion was based has been questioned (Wilson, 1966). Many workers (e.g. Munck and von Wettstein, 1980; Parker, 1980; Bechtel, 1982a, 1982b) suggest that the prolamins are deposited in vacuoles, parallel to the situation in the legumes (Boulter, 1979). Other workers (e.g. Miflin _et al._, 1981, 1983) suggest that the important parallel is with maize, and that the deposits are associated with the endoplasmic reticulum. This conclusion is based upon micrographs that show polysomes closely associated with the outside surface of the protein body (Barlow _et al._, 1974; Campbell _et al._, 1981; Simmonds and O'Brien, 1981; Miflin _et al._, 1983). However, other micrographs appear to show protein deposits present in vacuolar-like bodies (Cameron-Mills and von Wettstein, 1980; Parker, 1980; Campbell _et al._, 1981; Bechtel _et al._, 1982a; Miflin _et al._, 1983).

In barley, Cameron-Mills and Ingversen (1978), and Brandt and Ingversen (1978), have shown that the hordeins, the barley prolamins, are vectorially discharged into the lumen of the endoplasmic reticulum. Contrary to the situation in maize (Khoo and Wolf, 1970), Cameron-Mills and von Wettstein (1980) have not been able to show any

continuity between protein bodies and the endoplasmic reticulum. They observed small vacuoles implicated in the transport of storage proteins from the endoplasmic reticulum to the protein bodies, although they were unable to determine the origin of these vacuoles.

Some progress has been made in the characterization and *in vitro* synthesis of barley storage proteins (Brandt and Ingversen, 1976; Brandt, 1976). In each case, the endoplasmic reticulum of the endosperm cells has been recognized as the site of storage protein synthesis, and the mRNA molecules coding for these proteins have been purified and translated *in vitro* (Brandt and Ingversen, 1976).

The application of electron microscopic techniques to developing endosperms is fraught with difficulties of fixation and sectioning (e.g. Campbell et al., 1981) and thus subject to artefacts. Miflin et al. (1983) show a very close relationship between the rough endoplasmic reticulum and developing protein deposits; similar conclusions can be drawn from micrographs pubished by Jennings et al. (1963); Parker (1980, Plate 3A) and those of Campbell et al. (1981). Miflin et al. (1983) show in an electron mirograph that protein may be being deposited in a vacuole also (e.g. see Cameron-Mills and von Wettstein, 1980), but they suggest this is due to poor fixation techniques. Careful examination reveals vesicles and fragments of what are apparently rough endoplasmic

reticulum present inside the "vacuole", and the general state of preservation within the cell is poor.

Miflin et al. (1983) suggest that this debate is unlikely to be resolved by electron microscopy alone, so they isolated protein bodies from several species and demonstrated that protein deposition in these bodies is predominantly storage prolamin including HMW subunits. They have also shown that there is an association between the storage proteins and the main fraction of the rough endoplasmic reticulum in wheat and barley. This is expected since it is possible to extract the membrane-bound polysomes and show that they direct the synthesis of the prolamins of barley (Brandt and Ingversen, 1976; Fox et al., 1977 Matthews and Miflin, 1980).

The alternative approach of subcellular fractionation has utilized both sucrose-(Ingversen, 1975; Larkins and Hurkman, 1978; Miflin et al., 1981) and silica sol-density gradient techniques (Cameron-Mills, 1980) to isolate protein bodies. In sucrose, protein bodies band at a density around $1.23\text{-}1.28 g/cm^3$, although this can change with age (Ingversen, 1975; Miflin et al., 1981). Isolated protein bodies of wheat and barley do not appear to be completely surrounded by an intact membrane, in contrast to those of maize and pea. Analysis of marker enzymes suggests that the maize protein bodies are surrounded by ER (Larkins and Hurkman, 1978; Miflin et al., 1981),

whereas legume proteins are not; in contrast, the legume protein bodies (but not those of maize) have marker enzymes characteristic of vacuoles associated with them (van der Wilden et al., 1980; Miflin et al., 1981). Wheat and barley protein bodies have a very small peak of reduced NADH-cytochrome *c* reductase associated with them, but no vacuolar marker enzymes (Miflin et al., 1981).

Protein bodies were exposed to protease by Miflin et al. (1980b) and Miflin and Burgess (1982) to assess whether or not they are completely enclosed by a membrane. Since this treatment almost completely digested the protein bodies, Miflin and his co-workers suggested that no such membrane exists. These studies led Miflin et al. (1983) to put forward three conclusions about prolamin-containing protein bodies:

(1) the prolamin-containing protein bodies of cereals are not vacuolar in origin,

(2) the prolamins are synthesized on the endoplasmic reticulum and pass through into the lumen where they aggregate,

(3) in maize, the endoplasmic reticulum remains and completely encloses the aggregate, whereas in wheat and barley the aggregate disrupts the endoplasmic reticulum and is not completely enclosed by this membrane.

Despite the controversy over where and how storage proteins are deposited in cereal endosperm, research has attempted to characterize the effects of mutation on

storage protein body formation. The developing protein bodies in the starchy endosperm of wild type barley consist of two components, the granular structured spheres and a fibrillar component in which the granular spheres are embedded (Munck and von Wettstein, 1976). The homogeneous spheres correspond to protein bodies in maize (Khoo and Wolf, 1970) and wheat (Buttrose 1963). Ingversen (1975) has isolated protein bodies which exhibit both these components. These components have an appearance similar to the protein bodies isolated from barley by Ory and Henningsen (1969). In the same work, Ingversen isolated protein bodies from the endosperm of the mutant barley Risø 1508 at a similar developmental stage to the wild type Bomi barley, and showed the protein bodies to consist mainly of a fibrillar component in which few granular spheres are embedded. A comparison of the nitrogen contents of the protein bodies of these endosperms showed a value of 7.4% for the mutant compared with 11% for the wild type.

Miflin and Shewry (1979) have shown that the protein bodies of the starchy endosperm of Risø 1508 are very different in appearance to those of Bomi wild type barley. The major effect of the high-lysine mutation in Riso 1508 (Ingversen _et al._, 1973; Miflin and Shewry, 1977) is to decrease the relative amount of hordein. Risø 1508 also has an increased component of non-protein N, and the endosperm is shrunken compared to Bomi. The hordein

fraction of Risø 1508 contains more lysine than in Bomi (2.5% compared to less than 1%) but the amounts of lysine in other fractions are unaltered (Shewry et al., 1978).

Investigation of the Risø 1508 mutation has shown it has a drastic effect on many aspects of the biochemistry of the endosperm including starch, lipid and protein metabolism (see Miflin and Shewry, 1979). Thus, the mutation causes changes in the sub-cellular structures, particularly of the protein bodies, and the elimination of small starch granules in Risø, indicating a general interference with the storage function of the endosperm.

1.5.2.1 The Non-Prolamin Proteins

Less work has been done on the synthesis and deposition of this group of proteins. Some of the high-lysine proteins of barley have been synthesized *in vitro* (Jonassen et al., 1981). The mRNAs for the chymotrypsin inhibitors and β-amylase appear to be present in membrane-bound polysomes, and the translation product precipitated by antiserum raised against chymotrysin inhibitor-1 appears to have a higher molecular weight than the product synthesized *in vivo* . Similarly, the CM-proteins of barley are synthesized by membrane-bound polysomes, and the initial translation products appear to be of higher molecular weight than the authentic proteins (Paz-Ares et al., 1983).

Rice endosperm contrasts with that of maize and barley since it has less than 5 per cent prolamin but over

80 per cent glutelin (Palmiano _et al._, 1968). Isolated protein bodies have the same ratio of protein fractions as whole milled rice protein (Mitsuda _et al._, 1967), indicating that glutelin is the major storage protein of rice protein bodies.

Electron microscopy of developing endosperm and subcellular separations have both shown that more than one type of protein body is present in the starchy endosperm of rice. Harris and Juliano (1977) and Bechtel and Juliano (1980) described three types of protein body - the small spherical, the large spherical, and the crystalline. Bechtel and Juliano (1980) proposed that both the large and small spherical protein bodies were formed from deposits directly within the rough endoplasmic reticulum, whereas the crystalline bodies were formed in vacuoles and contained protein that had been secreted by the Golgi apparatus. In contrast, Wu and Chen (1978) discounted the idea of vesicular transport and suggested that synthesis was by polysomes present on the outer membrane. More recently, Oparka and Harris (1982), using both thin and thick sectioning electron microscopy, confirmed Bechtel and Juliano's observations on the spherical bodies. They also showed that the membrane for the crystalline bodies was derived from the endoplasmic reticulum but did not have associated ribosomes. They proposed a different route of deposition for the proteins in the crystalline bodies which may involve the Golgi apparatus. Tanaka _et_

al. (1980) physically separated the spherical bodies (termed lamellar by them) and the crystalline non-lamellar bodies, using sucrose density centrifugation. They concluded from electrophoretic analysis of the proteins that the spherical, lamellar bodies contain the prolamins and, the crystalline, non-lamellar bodies the glutelins.

Bechtel and Pomeranz (1981) studied the ultrastructure of the starchy endosperm of mature oat grains in which the major storage proteins are the globulins and found evidence for only one type of protein body, although they did state that their results did not preclude some heterogeneity in composition. Protein bodies have been prepared from oat endosperms by both differential and sucrose density centrifugation (Pernollet et al., 1982; Peterson and Brinegar, 1983). Both prolamins and globulins were present in populations of protein bodies obtained. However, it is possible that two types of protein bodies are present, as in rice endosperms. Burgess and Miflin (1985) suggest from separation techniques that globulins and prolamins are in fact localized in different bodies in oats.

Polysomes and poly A^+ RNA have been isolated from developing oat grains and shown to direct the synthesis of 12S globulins (Brinegar and Peterson, 1982a,b; Matlashewski et al., 1982; Rossi and Luthe, 1983; Walburg and Larkins, 1983). The major products have been identified immunologically. These are a series of

polypeptides of molecular weight around 60,000 with no evidence of large and small sub-units. This is an exact parallel of the legume 11S globulins, which have been shown to be processed *in vivo* in the protein body shortly after deposition (for a review, see Chrispeels, 1984). Adeli and Altosaar (1983) used pulse-labelling methods to show that the large and small sub-units are deposited in oat protein bodies about four hours after application of the label. This compares with two hours for legumin processing in peas (Chrispeels et al., 1982).

1.5.3 Thick-Section Studies in Botanical Electron Microscopy

1.5.3.1 Introduction

Although thick-section studies can be carried out in conventional electron microscopes, the majority of such investigations have been undertaken using High Voltage Electron Microscopy. The term "High Voltage Electron Microscope" (HVEM) is generally applied to microscopes which can operate up to and in some cases exceeding one million volts. Although such microscopes have been in general use over the past decade, microscopists have tended to ignore their potential for the study of plant ultrastructure and most biological high voltage research has been confined to the fields of zoology and biomedicine.

van Dorsten et al., (1947) published the first images

of a biological specimen observed at accelerating voltages of over 100kV. They observed whole yeast cells at 400kV, and demonstrated that with increased accelerating voltages, increased penetration of the specimen by the electron beam could be achieved. The first one million volt electron microscope was introduced thirteen years later, and Dupouy et al. (1960) published images of bacteria observed at 650kV and 750kV. The first use of HVEM in a botanical study did not take place until comparatively recently when Gunning et al. (1970) published a 1 µm thick-section of a xylem transfer cell from the purple dead nettle, clearly revealing labyrinths of wall ingrowths in the cell. The limited list of publications for botanical projects is reviewed by Hawes (1981).

The apparent neglect of the use of high accelerating voltages for the study of thick biological specimens has been primarily due to the rapid advances in the development of the ultramicrotome and in the preparative techniques for material to be observed by conventional transmission electron microscopy (CTEM). This resulted in biologists consistently studying two-dimensional images derived from three-dimensional specimens, and an insight into the third dimension was only obtained by time consuming serial-sectioning and reconstruction techniques. However, sections up to 10µm can be observed in the HVEM without substantial loss of resolution by using high

accelerating voltages and appropriate preparative techniques, and three-dimensional information can be easily extracted by the use of stereoscopy.

Interpretation of the great quantity of information in a thick section can be facilitated by tilting the specimen and producing stereo-pairs. Quantitative depth information can be extracted from stereo-pairs by the use of measuring mirror stereoscopes or by direct measurement from each member of a stereo-pair. Serial thick sectioning can be used as an alternative to prolonged serial thin sectioning to aid in the reconstruction of large specimens. Stereo-images can be viewed in a variety of ways: with lenticular pocket stereoscopes, prismatic spectacles, or polarized spectacles when the image is projected onto a non-depolarizing screen or presented on TV monitors. For a review see Hawes (1981).

For practical and geographical reasons it is not always possible or necessary to use HVEM for the study of thick sections. As an alternative, thicker sections may be viewed in conventional transmission electron microscopes. At 100kV images can be obtained from specimens up to and exceeding 1 µm. As the three-dimensional complexity of most cells is so great, practical considerations of image interpretation often restrict HVEM studies to the use of 1-2µm sections, even though there is potential to penetrate 10µm specimens. A disadvantage of older generation conventional microscopes

with regard to the examination of thick sections, is that chromatic aberration corrections may be limited. More recent conventional microscopes generally give good results with 1-2μm sections although, as the beam current is increased to give the required brightness, the filament life is reduced.

Thick section work at conventional kVs has been particularly successful when used in conjunction with selective staining. The earlier studies of Porter and Machado (1960) used prolonged osmium impregnation to demonstrate the changes in distribution of endomembranes during mitosis in barley. Mollenhauer and Whaley (1963) used prolonged osmification for selective staining in a thin section of the Golgi apparatus. Other effective selective staining of the endomembrane system of plant cells in HVEM studies, utilising both prolonged osmium impregnation and Gomori lead localization of acid phosphatase in 0.5-4 μm sections of araldite-embedded tissue include: Poux (1973), Poux et al. (1974), Favard et al. (1971), and Favard and Carasso (1973). The concept of lyosomal plant vacuoles (Matile, 1975) was pursued in a series of HVEM studies of thick sections of selectively stained tissue (Marty 1973a,b; 1978). This work, using ZIO as the selective stain, demonstrated the interconnections of cisternal and tubular endoplasmic reticulum and their associations with the Golgi apparatus and to the GERL (Golgi-endoplasmic reticulum-lysosome),

and subsequent involvement of this in the formation of vacuoles.

More recently, HVEM and CTEM thin and thick section studies have revealed the relationship of the endoplasmic reticulum (ER) and dictyosomes to storage protein deposition in developing cereal grains and legumes. Parker and Hawes (1982) used HVEM to study the developing endosperm of wheat. Buttrose (1963) had suggested that the Golgi apparatus might be involved in protein deposition in developing wheat endosperm, but Barlow et al. (1974), Briarty (1976) and Briarty et al. (1979), using an electron microscopical stereological analysis of thin section micrographs, concluded that insufficient Golgi were present during the phase of protein deposition to support the dictyosomal role in storage protein transport. Parker (1981, 1982) suggested that membranes of dictyosomes and their associated vesicles rarely stain well in thin sections of wheat endosperm. However, in selectively stained thick sections, numerous dictyosomes are present in developing wheat endosperm during the period of protein deposition, so a dictyosomal role in the transport of storage protein between the endoplasmic reticulum and protein bodies cannot be discounted (Parker and Hawes, 1982).

Oparka and Harris (1982) used a combination of thin and thick sections to show that all types of rice protein body, characterized by Bechtel and Juliano (1980) were

initiated by dilation of the endoplasmic reticulum although protein accumulation occurred by different pathways. Protein deposition in developing legume seeds was examined with a similar combination of conventional and thick section techniques (Harris, 1979). Using such a combination of CTEM, thick-section CTEM and thick-section HVEM techniques, Harris and Chrispeels (1980) examined and quantified the changes occurring in the endoplasmic reticulum during mobilization in storage parenchyma cells of germinating mung-bean seed. Interconnections of endoplasmic reticulum and dictyosomes have also been studied in developing mung-beans, by Harris and Oparka (1982) using thick-section CTEM and, while results are in general similar to those obtained by Marty (1978) using HVEM, the lower kVs used have revealed previously uncharacterized fine tubular extensions from the peripheral reticulum of dictyosome cisternae.

Other thick section studies of botanical tissues also includes work on fungal hyhae and spores (Harris, 1975), germinating legume seeds (Harris and Chrispeels, 1980), hyphal tips (Howard and Ainst, 1981) haustoria (Finneran et al., 1978), and thallus tissue (Pechak, 1980). In all of these studies, the major advantage of HVEM imaging has been in the demonstration of three-dimensional inter-relationships in tissue ultrastructure.

1.5.3.2 Specimen Preparation for Thick-Sectioning

The same preparative techniques used for the fixation

of material for the CTEM are frequently employed for material to be observed in the HVEM. As with thin section studies, the most common fixatives used by plant microscopists are buffered aldehydes (either glutaraldehyde or a mixture of glutaraldehyde and paraformaldehyde), followed by osmium tetroxide post-fixation, although both osmium tetroxide without pre-fixation and potassium permanganate have also been used (review by Hawes, 1982). Osmium tetroxide has often been used as a post-fixative, although increasing use is being made of osmium complexes with zinc iodide or potassium ferrocyannide. The chemistry of these complexes is not fully understood, but they result in the accumulation of electron dense material in the lumen between double membranes and in membranous tubules. Hence, stain accumulates in the cisternal endoplasmic reticulum, nuclear envelope, perimitochondrial space, some dictyosome cisternae and chloroplast peripheral reticulum, although seldom in the chloroplast envelope. Similar dense aggregates form within the tubules of the endoplasmic reticulum and also in the tubular lattices of prolamellar bodies. These features contrast with a background in which single membranes, cell walls and cytoplasmic proteins etc. are not heavily stained.

A number of botanical studies have successfully used the complex of osmium tetroxide and zinc iodide (ZIO) after an aldehyde fixation (Dauwalder and Whaley, 1973;

Marty, 1973a; Harris, 1978, 1979; Harris and Chrispeels, 1980; Hawes, 1980; Parker and Hawes, 1982; Oparka and Harris, 1982: Harris and Oparka, 1982). Most workers avoid phosphate buffers and use only freshly prepared ZIO complex (recipes in Marty, 1973a; Harris, 1978).

Besides osmium, and complexes of osmium with zinc iodide or potassium ferrocyanide, acidified phosphotungstic acid has been employed to selectively stain the plasmalemma (Syrop and Beckett, 1972). Other techniques for selective localization of cell components include enzyme cytochemistry for acid phosphatase (Marty, 1978) and cytochrome oxidase (Davison and Garland, 1975). However, these techniques have not been fully exploited despite the enormous amount of information on three-dimensional ultrastructural inter-relationships that could be obtained.

The use of cytochemical and immunocytochemical staining procedures in association with HVEM has received much more attention from animal than from botanical electron microscopists (review, King *et al.*, 1980). King *et al.*, (1980) also review the advantages of thick section HVEM for autoradiographic studies.

1.6 Electron Microscopy and Molecular Techniques

1.6.1 Immunolabelling for Electron Microscopy

The localization of tissue antigens by electron microscope immunocytochemistry is a compromise between retaining sufficient antigenic activity within the

specimen to bind with antibody and maintaining adequate morphological detail within the tissue for reliable identification of the site of immunological reactions.

In transmission electron microscopy, the localization of cellular components requires a balanced fixation of the cell under study. Whilst antigenic determinants must not be denatured during fixation, the ultrastructural morphology of the cell should be well preserved. These two requirements tend to be mutually exclusive. Chemical fixation, organic solvent dehydration and embedding in resin adversely affects antigenicity. This general problem is clearly and widely recognized, and appropriate conditions have to be devised for each particular antigen.

1.6.1.1 Historical Aspects

A major development in cytochemical studies of cells and tissues at the light microscope level was the introduction of the use of fluorescent antibodies to identify sites of antigen-antibody reaction (Coons *et al.*, 1941). To take advantage of the much greater resolving power of the electron microscope, it was necessary to develop markers which render antibodies visible at the electron microscope level so as to identify antigens within or on the surface of cells.

The introduction by Singer (1959) of the iron-containing protein ferritin as an electron-dense marker which could be conjugated to other proteins such as antibodies opened a new field , immunolabelling for

electron microscopy, and thereby made a significant advance in the study of the molecular structure of cells and tissues. Antibodies so labelled can be individually located and identified at or near the site of interaction with the antigen. Singer's ferritin-labelled antibody technique was immediately accepted and quickly became widely used. An excellent review of the use of ferritin-conjugated antibodies in electron microscopy was published by Morgan (1972).

In 1966, Nakane and Pierce applied the reaction of horseradish peroxidase (HRP) and diaminobenzidine (DAB), previously introduced into histochemistry by Graham and Karnovsky (1966), to localize antigens by conjugating antibody to HRP. Ram _et al._ (1966) had also shown that the approach of conjugating enzyme (acid phosphatase) to antibody could be useful in ultrastructural studies. However, antibodies conjugated to acid phosphatase were found to give variable results, and this led Nakane and Pierce to try HRP. Thus, the introduction of the enzyme-labelled antibody technique became a second major landmark in immunolabelling for electron microscopy.

Feldherr and Marshall (1962) introduced colloidal gold particles as a tracer for electron microscopy, but it was nine years later that colloidal gold was applied as a specific marker for antisera by Faulk and Taylor (1971), and three years later for isolated antibodies by Romano _et al._ (1974). The immunogold staining technique has become

increasingly popular during the last ten years, and its use has also been extended to the scanning electron microscope by Horisberger _et al._ (1975). Reviews of the immunogold staining technique have been published by Horisberger 1979; Goodman _et al._ (1980); and Horisberger 1981.

1.6.1.2 Tissue Preparation for Electron Immunocytochemistry

Immunocytochemistry is probably the most demanding of all vizualization techniques currently performed for electron microscopy, and is prone to two main sources of problems. Firstly, there is the problem of the failure to preserve antigenicity in the tissue. A second difficulty concerns the steric hindrance between antibodies, conjugated or not, which can occur during the staining cycle. These problems also limit the choice of useful resins since careful attention must be paid to the chemical reactivity of the cured resin, and also to the method of curing and the degree of cross-linking then developed. Additionally, some of the resins used successfully at the light microscope level are excluded for electron microscopy because they do not exhibit beam stability. The resins that give the best results and tolerate the widest range of technique fall into two broad groups: the epoxy cross-linked systems, and the cross-linked hydrophilic acrylics. The epoxy resins which include Spurr resin (Spurr, 1969), the Araldites (Glauert

et al., 1956), and the Epons (Finck, 1960), have been used in many immunocytochemical studies (Bendayan and Zollinger, 1983; Hearn *et al.*, 1985).

Optimal ultrastrucural preservation is usually obtained using osmium tetroxide in the fixation procedure. However, this fixative almost completely inhibits labelling for the pancreatic proteins (Bendayan *et al.*, 1980, Roth *et al.*, 1981a). Bendayan and Zollinger (1983) presented a modification of the protein A-gold technique that allows labelling on osmicated tissues embedded in Epon. Their results suggest that fixation with osmium tetroxide does not destroy protein antigenicity, and that treatment of thin sections of osmium-fixed or post-fixed, tissues with strong oxidising agents such as sodium metaperiodate restores the possibility of revealing different antigens by the protein A-gold technique. The good ultrastructural preservation obtained led to superior resolution in the labelling.

The exact mechanism by which osmium tetroxide acts as a fixative is still poorly understood (Pearse, 1968) and thus it is difficult to analyse accurately the way by which oxidizing agents can remove the masking effect that osmium tetroxide exerts on antigenic sites. The restoration of the antigenicity was only observed after treatment with strong oxidizing agents. The treatment with oxidizing agents has been postulated to act as a reoxidation of the reduced osmium molecules, making them

soluble as tetroxides, and leading to their removal (Merriam, 1958). By reoxidizing the low osmium oxides, these might be removed from the cross-linked macromolecules, unmasking the antigenic sites. If this is the case, the fixative role of osmium, in contrast to its masking effect, is not reversed during treatment as the tissues remain well preserved.

Hearn et al. (1985) confirmed that osmium fixation does not prevent Ig immunolabelling. This conclusion was derived from a protein A-gold postembedding, immunolabelling method on tissues that were fixed in glutaraldehyde, post-fixed in osmium tetroxide, and embedded in epoxy resin; sections were pretreated with sodium metaperiodate. Hearn et al. could detect Ig in specimens without any section pretreatment, but found the use of sodium metaperiodate produced a significant unmasking of the antigen. The antigens could also be labelled in tissues that had been stored for many years in epoxy resin. Craig and Goodchild (1984) localized the legume seed reserve protein vicilin in developing pea cotyledons by immunogold labelling on sections of glutaraldehyde osmium-fixed, Spurr resin-embedded tissue. By treating sections with sodium periodate and hydrochloric acid before antibody labelling, a 20-fold increase in specific antibody binding was observed. These observations demonstrate the reality of successful immunocytochemical labelling on tissue prepared for

optimal fine structural preservation.

The acrylics that are used in electron microscopy are cross-linked to reduce sublimation of the lower homologues, and preferably aromatically substituted to minimize damage by electron beam bombardment. For immunocytochemistry the hydrophobicity of the acrylic is important. The acrylic can have hydrophilic groups built into them and thus avoid the need for peroxide or "etch" treatments. This is an advantage when dealing with sensitive antigens or unfixed tissue. The cross-link density of the acrylic can also be controlled, thereby reducing the need for "etching". Finally, the mildest curing conditions can be chosen, i.e. low temperatures in heat-cured systems, careful control of exothermy in amine/peroxide room temerature curing systems and the shortest possible irradiation times for light-cured systems.

The commercial aliphatic cross-linked acrylic embedding medium Lowicryl (Chemische Werke/Lowi, D-8264, Waldkraiborg, West Germany) is produced in a hydrophilic grade suitable for immunocytchemistry (Carlemalm et al., 1982). It consists of both hydroxypropyl and hydroxyethyl methacrylates cross-linked by an aliphatic glycol dimethacrylate to reduce sublimation in the electron microscope. The acrylic is cured by exposure to ultraviolet light using a benzoic alkyl ether as the activator. The resin is very mobile even at low

temperatures and is very suitable for techniques that require low temperature antigen preservation.

Embedding plant cells for immunoelectron microscopy presents infiltration problems due to rigid cell walls and highly vacuolated cytoplasm, and problems concerning adequate preservation of antigenic determinants. Lowicryl K4M, developed for low temperature embedding of animal tissues (Carlemalm et al., 1982) has also been found by Roth et al. (1978), to greatly improve immunochistochemical techniques. Rey (1984), reports that the low temperature embedding resin Lowicryl K4M appears to provide adequate morphological preservation of apple tissue and excellent retention of antigenicity. Wells (1985) devised a box cooled with liquid nitrogen and a simple tissue handling device to allow low temperature resin (Lowicryl K4M) embedding of biological material to become a convenient procedure. He reports excellent preservation of ultrastructure and antigenicity when using immuno-gold staining of thin sections of a wide variety of plant, animal and bacterial tissue.

LR White (London Resin company, Basingstoke, Hants.,) is a polyhydroxyaromatic acrylic resin formulated specifically to combine a high hydrophilic character with an electron beam stable cross-link. The resin can be either heat-cured at 60°C or room-temperature-cured using an aromatic tertiary amine accelerator. The cross-link density of the resin is critical for good

immunocytochemical results and Newman <u>et al.</u> (1983) have demonstrated that the best results are obtained using a slow heat cure at 50°C.

Craig and Miller (1984) combined LR White resin with post-embedding immunogold labelling to localize the storage protein vicilin within developing pea seed cotyledons by electron microscopy. Fine structural preservation was comparable to that obtained with Spurr's resin (Craig and Goodchild, 1984a), and antibody labelling is improved. More gold binds to protein bodies, to rough endoplasmic reticulum and to Golgi vesicles and in addition, vicilin was detected within Golgi cisternae, a site not previously observed.

Hence a suitable choice of tissue preparation is one which combines adequate morphological preservation along with excellent retention of antigenicity for the tissue and antigen of interest.

1.6.1.3 Electron-Opaque Markers for Immunocytochemistry

(a) Introduction

The labelling of cell surface and intracellular components for microscopical observation is an extremely important technique for studying molecular organization and cell function. Indeed, biochemical analysis alone would be incomplete since the data obtained represent average values and give little information regarding the distribution of cellular components. Hence, a large number of cytochemical techniques have been developed to

identify, localize, quantify and understand the dynamics of cell components at the ultrastructural level.

Cytochemical methods for transmission electron microscopy depend upon the product of reactive products which are opaque to the electrons, or the use of electron-opaque particulate markers. Ideally, markers should be easily recognized and quantified, and readily prepared from a variety of molecules recognizing ligands with a narrow specificity. Although diffuse markers are generally sensitive, because they are based on amplification effects, particulate markers are especially convenient for precise localization and quantification.

(b) The Immunoferritin Technique

A vast number of investigations have made use of ferritin as an electron-dense marker for transmission electron microscopy (e.g. Morgan, 1972; and Sternberger 1979). The iron-containing core of the ferritin molecule is electron dense and large enough, about 70Å, to allow visualization at relatively low magnification. The apoprotein coat permits chemical conjugation to other proteins such as antibodies by means of bivalent reagents. A major disadvantage of the immunoferritin technique is that it is dependent upon the chemical coupling of ferritin to antibodies. This can produce several problems: firstly, the loss of antibody activity, secondly, heterogeneity of products, so the active ferritin-antibody conjugates must be isolated, and lastly,

inefficiency of the coupling procedures. Initially, Singer (1959) employed metaxylene diisocyanate in the conjugation reaction, but a simpler and more adequate two-step cross-linking procedure using monomeric glutaraldehyde was suggested by Otto et al. (1973). The preparation of conjugates has been described by Hsu (1981).

The immunoferritin technique has been applied to a wide range of biological problems. Some examples of these studies are -the arrangement of fibrin in clots, red blood cell antigens, components of cell nuclei, and, the identification of components of bacterial or fungal origin.

(c) Immunoperoxidase Labelling for Electron Microscopy

Horseradish peroxidase can be used as an enzymic marker, and an important difference between these and the particulate markers such as ferritin and colloidal gold, is that the catalytic activity of enzymes can result in the accumulation of reaction products which diffuse away from the labelled site. This diffusion may be a positive feature because it aids detection of the antigenic site (Bretton, 1970), but can also be a drawback when fine localization of antigenic sites is required. Horseradish peroxidase can be chemically coupled to ligands such as antibodies or used as the unlabelled peroxidase-antiperoxidase (PAP) complex, (Sternberger et al., 1970). The unlabelled antibody peroxidase method was

found to be very sensitive for the histochemical localization of a variety of antigens, and quickly became very popular both in light and electron microscopy.

The preparation of antibodies conjugated to peroxidase was developed independently by Nakane and Pierce (1966) and Avrameas and Uriel (1966). Antibodies or other specific ligands are covalently linked to the enzyme by the periodate method, glutaraldehyde or other bifunctional coupling agents (reviewed by Sternberger, 1979). The marker can then be reacted with the tissues (both pre- and post-embedding techniques may be used) and the site of the enzyme is visualized by the formation of osmiophilic reaction products of the enzyme using 3,3'-diaminobenzidine or 4-chloro-1-napthol. The latter produces highly specific staining and less background staining than the former.

As with other labelling methods, the sensitivity of the immunoperoxidase procedures depends on the conditions of fixation and tissue processing. A compromise has to be made between the requirements for the preservation of the tissue and cell fine structure, good fixation, and the retention of antigenic activity. Since the introduction of peroxidase-antibody conjugates for ultrastructural studies (Nakane and Pierce, 1966), modifications of this immunolabelling technique have been applied extensively to detect viruses, enzymes, hormones and immunoglobulins in tissue sections.

Some particular problems inherent in the immunoperoxidase procedures are related to the need to inhibit endogenous peroxidase activity which may be achieved by the use of phenylhydrazine (Straus, 1972), and to the purity and toxicity of the commonly used chromogen diaminobenzidine (Pelliniemi _et al._, 1980). Also, conjugation of immunoglobulins to peroxidase with glutaraldehyde appears to impair the antigenicity more than the antibody site in multiple step marking (Tougard _et al._, 1979).

(d) Immunogold Labelling for Electron Microscopy

(i) General Aspects

Colloidal gold offers many advantages as a tracer for electron microscopy. Gold is particulate and very distinct, and a great advantage is that due to their high electron density, gold particles are easily detected by the electron microscope in contrast to the low opacity of the iron core of ferritin.

Gold markers were first employed in immunocytochemistry bound to immunoglobulins (Faulk and Taylor, 1971). The preparation of a stable Ig-gold is not as simple as the procedure to make protein A-gold, but has the advantage that it gives a higher yield of label than protein A-gold (Slot and Geuze, 1981). This, however, is not the result of more efficient recognition of antigens but a consequence of secondary amplification; hence, Ig-gold is a convenient marker in cases of low antigen

concentration.

The first application of collidal gold as an immunocytochemical marker for transmission electron microscopy was described by Faulk and Taylor in 1971. Gold labelling for scanning electron microscopy was introduced by Horisberger et al.(1975).

For transmission and scanning electron microscopy, monodisperse gold colloids are prepared by one of three procedures: the smaller particles, Au_5 - (the subscript of the gold indicates the mean diameter of the particles in nanometers (nm)), are obtained by reducing gold chloride (chloroauric acid, $HAuCl_4$) with yellow or white phosphorus (Faulk and Taylor, 1971; and, Horisberger and Rosset, 1977). Au_{12} particles are produced in the presence of sodium ascorbate as the reducing agent (Horisberger and Tacchini-Vonlanthen, 1983). For Au_{16} to Au_{150} particles, the reducing agent is sodium citrate (Horisberger 1979; and Horisberger and Rosset, 1977); the smaller the particle size, the higher the concentration of reducing agent required.

Colloidal gold has been labelled with a variety of molecules such as toxins, hormone polysaccharides, glycoproteins, proteins (such as protein A), lectins, immunoglobulins, enzymes and lipoproteins (reviewed Horisberger, 1981). The binding of such macromolecules to colloidal gold results from adsorption under specific conditions of pH, reagent concentration and ionic

strengths; no chemical conjugation is involved. Thus the method is easy, quick, and cheap because it requires only small amounts of specific macromolecules. Another advantage is the low non-specific adsorption of the coated gold particles.

As colloidal gold can be produced in a variety of sizes, multiple labelling can be achieved by the adsorption to different antibodies or macromolecules. Gold labelling with small particles, 5-20nm, has the advantage of being less susceptible to steric hindrance due to the small size of the label.

(ii) Immunogold Procedures

The preparation of a gold marker is based on he adsorption of macromolecules onto the surface of the gold particles. Upon adsorption, full biological activity of the secondary antibodies is preserved, and they remain globular (Bauer et al., 1975). Catalase is the only known exception to this rule, losing its activity when bound to gold particles (Horisberger and Rosset, 1977). As a result of the adsorption of macro-molecules onto gold particles, the hydrophobic negatively charged colloidal gold is stabilized and "protected" against subsequent aggregation by electrolytes since it becomes a lyophilic colloid.

Faulk and Taylor (1971) described method whereby the whole serum which contained the anti- *Salmonella* antibody was labelled with colloidal gold. This reagent was

demonstrated to be of value when investigating the distribution of antigens on cell surfaces by electron microscopy. Romano et al. (1974), described an indirect method in which affinity-purified horse antibodies to human IgG were labelled with colloidal gold and used to mark the distribution of Rh antigen sites on human erthrocyte ghosts. In later work, Romano et al. (1975) utilized a similar reagent to map the distribution of the A, D and C antigens of human red cell membranes under different conditions. This demonstrates that the gold-labelled reagent permits the study of the distribution of sites along with quantitative analysis.

Since 1975, many studies have been reported which used colloidal gold markers for labelling surface antigens of cells, viruses, bacteria, protoplasts, yeasts and also for marking intracellular antigens either with pre-embedding or post-embedding techniques on thin sections

(iii) The Staphylococcal Protein A-Gold Technique

As an alternative to gold coated with secondary antibody, the protein A-gold technique has become increasingly popular and it is now widely used for the localization of both surface and intracellular antigens.

Protein A is a polypeptide (molecular weight 42,000) isolated from *Staphylococcus aureus*, which binds immunoglobulin molecules without interacting at the antigenic site. This property permits the formation of

tertiary complexes consisting of protein A, antibody and antigen. The method is based on the ability of protein A to bind to the Fc portion of the IgG molecules of many animal species (Kronvall et al., 1970; 1974). This binding is rapid and reaches saturation level in about 30 minutes. The K_D values for human and rabbit IgG are similar to antigen-antibody reactions (Kronvall and Frommel, 1970; Myhre and Kronvall, 1980).

There are numerous reports of variation in binding to various IgG subclasses and interaction with other immunoglobulin classes (Vidal and Conde, 1980; Hjelm, 1975). In addition, there is also variation in reactivity of protein A with immunoglobulins from different animal species (Goudswaard et al., 1978). Despite this, protein A is a useful reagent for binding immunoglobulins from several animal species, a property rendering the protein of general applicability in immunocytochemistry.

Biberfeld et al.(1975), initially applied fluorescence-labelled protein A as a second layer reagent in the indirect immunofluorescence technique. Later, protein A conjugated with ferritin (Bächi, 1977) or peroxidase (Dubois-Dalcq et al., 1977) was introduced for antigen localization at the light and electron microscope level. Since the introduction of colloidal gold as an immunocytochemical marker by Faulk and Taylor (1971), colloidal gold coated with different classes of biological macromolecules has been applied to light and electron

microscopical cytochemistry (review Roth, 1983). Romano and Romano (1977) were the first to prepare a protein A-gold complex for the pre-embedding technique for the localization of antigens in the cell surface. The usefulness of the protein A-gold technique for post-embedding intracellular antigen localization in this resin embedded material has also been documented (reviews: Roth *et al.*, 1978; Roth *et al.*, 1980; Roth, 1982). Subsequently, the protein A-gold technique has been successfully adapted for the staining of frozen ultrathin sections (Geuze *et al.*, 1981).

1.6.1.4 Application of the Protein A-gold Technique for Post-Embedding Antigen Localization

The reliability of the protein A-gold technique has been demonstrated by the successful localization of different classes of proteins such as several secretory proteins and notably polypeptides in various cellular compartments - enzymes in mitochondria, peroxisomes and granules of eosinophilic leukocytes; cytoskeletal elements; extracellular space components; cytosolic proteins; membrane integral proteins etc. (review Roth, 1983).

Immunocytochemical localization has detected the presence of growth hormone in human pituitary by light microscopy of paraffin-embedded, formaldehyde-fixed tissue (Roth, 1982), and also at the electron microscope level in Epon thin sections of osmicated and non-osmicated tissue

(Bendayan and Zollinger, 1983). Similar studies on exocrine pancreatic enzymes confirmed established biochemical data on secretion (Palade, 1975) by demonstrating the presence of several enzymes in the rough endoplasmic reticulum, Golgi apparatus, condensing vacuoles, zymogen granules and acinar lumen (Bendayan et al., 1980). Roth et al. (1981b) obtained results suggesting the involvement of the whole Golgi stack in processing of the secretory products. Double labelling techniques have demonstrated the presence of different enzymes in the same cellular compartments (Roth, 1982; Bendayan, 1982). Quantitative evaluation of the labelling revealed differences in labelling intensity for the various enzymes and cellular compartments (Bendayan et al., 1980).

The synthesis and accumulation of proteins within developing seeds of cereals and legumes has been investigated intensively for several years. For legumes there is both biochemical and structural evidence that newly syhthesized seed proteins are sequestered within the endoplasmic reticulum and relocated into the vacuole or protein bodies, the ultimate site of storage (Bailey et al., 1970; Bollini et al., 1982). Using radiolabel incorporation and organelle fractionation, Chrispeels (1983) showed that newly synthesized *Phaseolus* lectin is modified within the Golgi compartment en route to the vacuole. Immunocytochemical methods give more direct evidence for the participation of the Golgi in seed

protein transport, for example, vicilin, one of the pea seed reserve proteins was shown within Golgi vesicles (Craig and Goodchild, 1984b). This suggests that at least some plant storage proteins are synthesized, packaged and transported via a pathway similar to that established for proteins destined to be secreted from animal cells, viz, endoplasmic reticulum ⟶ Golgi ⟶ vacuole. This supports the proposal (Chrispeels, 1976) that the plant cell vacuole may be considered as an "external" compartment, and that proteins destined to be sequestered within the vacuole may therefore be considered as secretory proteins. Most plant proteins for which the "ER ⟶ Golgi ⟶ vacuole" pathway is established are glycoproteins, but Harris (1984) demonstrated that the route through the Golgi apparatus is not restricted to those storage proteins that undergo post-translational glycosylation. They also demonstrated that the major storage proteins of *Pisum sativum* are transported by different compartments of the Golgi apparatus. In animal tissues however, all glycolsylated secretory proteins have been shown to pass through the Golgi apparatus (e.g. Yang et al., 1981).

Collectively, these data demonstrate the high degree of functional specialization within the Golgi apparatus, but on the other hand, clearly represent the potential of modern immunocytochemical procedures in cell biological research.

1.6.1.5. Preparation of Polyclonal Antibodies to Cellular Constituents - the Cereal Storage Proteins

Polyclonal antibodies have been used for many years as tools for analyzing various aspects of cell biology (e.g. Vincent, 1982; Cullimore and Miflin, 1984). The methods used for raising these antibodies are well documented in the literature. For the results of immuno-gold cytochemistry to be unambiguous, it is essential that the antibody preparation should be specific to the tissue constituent of interest. Any possibility that the antibody preparation is able to react with other components in the cell raises uncertainties in the interpretation of the data concerning localization of a particular antigen. Where proteins are involved, proof of specificity of the antibody can be achieved by various techniques involving electrophoresis (Chua and Blomberg, 1979), electroblotting and immunochemical staining (Towbin *et al.*, 1979; Hawkes *et al.*, 1982).

The use of controls in immuno-gold staining experiments is very important. A sample of preimmune serum is obtained from the animal in which the antibodies were raised. Other controls essential for establishing the authenticity of an antigen in thin sections when using protein A-gold staining, have been discussed by Roth (1982).

(i) Raising of Antisera and Solubility of the Antigens

Extracted prolamins are not usually soluble in aqueous solvents such as phosphate buffered saline (PBS). It is therefore necessary to dissolve them in a detergent or chaotropic reagent (e.g. urea) in order to study their immunological relationships. Such solvents may interfere with antibody-antigen reactions (Festenstein and Hay, 1982). The commonly used solubilizing agent SDS (sodium dodecyl sulphate) apparently produces a non-specific reaction with rabbit serum in double diffusion tests. This reaction, noted by others (Green *et al.*, 1975; and, Yen *et al.*, 1976), has been minimized by using 0.1% SDS-PBS to suspend the hordein antigens, but as a suspension rather than a solution ; the test is generally useful for qualitative rather than quantitative study.

Previous work emphasized the problems of working with proteins with such limited solubility (Festenstein and Hay, 1982) poor antigenicity (Daussant, 1977) and difficulty of obtaining purified fractions to raise antisera (Laurière *et al.*, 1983). Festenstein and Hay (1982) overcame the first two of these problems by developing a sensitive nephelometric assay with hordein dissolved in 0.1% SDS-PBS.

The nephelometric assay provides a quantitative measure of antigen-antibody reaction following SDS precipitation of non-specific reacting material, and hence

provided a suitable quantitative method for comparing different hordeins and different hordein antibodies.

(ii) Immunological Relationships of Barley Seed Storage Proteins

Common antigenic determinants have been shown to be present on the S-poor and S-rich prolamins. Kling (1975) found that the antisera to six hordein components gave confluent precipitation lines between all antisera and all antigens except one in double-diffusion tests. Festenstein and Hay (1982) demonstrated common antigenic deteminants for A and B hordeins and also B and C hordeins by double diffusion tests. They also concluded that B hordein contains extra determinants not present on A hordein and also not present on C hordein. The recent work of Laurière *et al.* (1983) also showed common antigenic determinants between B and C hordeins. However, in both cases, antisera used were raised against mixtures of prolamins containing both S-poor and S-rich hordeins (i.e. B plus C hordeins). More recently, Festenstein *et al.* (1984) used antiserum raised against a pure preparation of C hordein. Using this antiserum they were able to show clearly that B and C hordein contain a common antigenic determinant; furthermore, results with a total-hordein antiserum indicate that B hordein contains an additional determinant not present in C hordein.

Festenstein *et al.* (1984) also examined the antigenic relationships between the prolamins of barley, rye and

wheat by studying the specificity of an antibody to C hordein in a quantitative study using a laser nephelometer. They found the antibody reacts weakly with B hordein and strongly with 75-K dalton and 40-K dalton. γ-secalins from rye and γ_3 - and some ω-gliadins from wheat. Absorption experiments and immuno-diffusion tests indicated that there are shared antigenic determinants for most of the prolamins. All the species with reacting prolamins belong to the sub-family Festucoideae of the Gramineae. This agreed with the earlier results obtained by Kling (1971, 1975).

The prolamins of maize, pearl millet and sorghum, species of the sub-family Panicoideae, do not react (Ewart, 1966; and Dierks-Ventling and Cozens, 1982). The results obtained by Festenstein et al. (1984) show that the prolamins of the Panicoideae (Bietz, 1982) do not react with barley C hordein antiserum. Results also indicated differences within the sub-family Festucoideae ; whereas all the members of the tribe Triticeae (barley, rye and wheat) reacted, oats (tribe Aveneae) reacted only weakly.

1.6.2 *In situ* Hybridization with Nucleotide Probes - a Histochemical Tool

In situ hybridization, a procedure for the localization of specific polynucleotide sequences, was introduced in 1969 (Gall and Pardue, 1969; John et al., 1969). This technique identifies specific RNA or DNA sequences and also reveals their cellular or chromosomal

location providing information with a high degree of spatial resolution. This makes it a valuable tool for the study of gene expression at the morphological and cytological levels.

Frequently, molecular biologists have relied on methods that detect the RNA or DNA content of a specimen after homogenization and extraction. For example, Southern, Northern or dot blots detect the presence of nucleotide sequences in a specimen, and can even indicate the amount present. However each of these methods gives inferior spatial resolution as compared to *in situ* hybridization.

This technique has two important benefits. Firstly, the cell that contains the specific RNA or DNA of interest is identified precisely. Consequently, the exact location of the cells, their number and type as determined by morphology or function, is also known. This feature of the information provided by *in situ* hybridization is absolutely essential for the examination of heterogeneous tissues. Harris and Croy (1986) have used hybridization to locate mRNA for the storage protein legumin in cotyledon storage parenchyma tissue of developing pea (*Pisum sativum* L.) seeds.

The second advantage is that the subcellular location of the gene or gene product can be detected by this method, and is important in the determination of the chromosomal location of a particular gene, or the position

of a satellite fraction of DNA in a cell.

The sensitivity of *in situ* hybridization may equal that of any of the other techniques used to detect DNA or RNA in cells, e.g. Southern, Northern or dot-blot analysis. As a result it is possible to detect nucleotide sequences that occur with very low frequency in a specimen.

1.6.2.1 History and Rationale

Pardue et al. (1969) were among the first to use *in situ* hybridization, and their work was a keystone in the development of the technique as it is known today (Gall and Pardue, 1971). The technique was used to study the organization of the entire genome of *Drosophila* and *Xenopus* , including work on satellite sequences of DNA, and the 5s ribosomal gene (Mohler and Pardue, 1982; Pardue and Dawid, 1981; Bautch et al., 1982; and Fostel et al., 1984). This work provided a dramatic new approach to localizing DNA sequences within tissues, nuclei and chromosomes at the light microscope level.

Initially, many kinds of repetitive sequences were mapped (Eckhardt, 1976). Now recombinant DNA technology provides pure probes, which are required in order to map unique sequences. Nick translation (Rigby et al., 1977) and other enzymatic techniques allow nucleic acid probes to be labelled with tritium, thus giving the high specific activity and resolution needed for unique sequence mapping. Refinements in the hybridization protocols

(Brahic and Haase, 1978; Haase et al., 1982; and Gee and Roberts, 1983) enhanced probe access to target sequences, while the generation of probe networks (Wahl et al., 1979); Tereba et al., 1979) have increased the delivery of the probe signal. These improvements have combined to allow the mapping of single copy sequences in cells and chromosomes at the light microscope level (Harper and Saunders, 1981; Gerhard et al., 1981; Wahl et al., 1982; and Neel et al., 1982). In addition, since 1975, avidin-biotin interactions (Bayer and Wilchek, 1980) have been exploited to move *in situ* hybridization away from the constaints of autoradiography. This more recent methodology, based on the use of a biotinylated nucleotide analogue, provides a new, rapid, high resolution approach to *in situ* hybridization at both the morphological and ultrastructural level.

1.6.2.2 *In Situ* Hybridization using Biotin-Labelled Nucleotide Analogues

Until recently, radioactive probes and autoradiographic detection were essential for *in situ* hybridization. However, an inherent lack of resolution in tritium-labelled probes, due to the track of the decay particle and the thickness of the emulsion, makes the approach undesirable for precise morphological work. This induced the exploration of alternative approaches (Manning et al., 1975). A number of workers attempted the use of nucleic acid probes conjugated with fluorescent molecules

for the detection of *in situ* hybridizations (Cheung et al., 1977; Rudkin and Stollar, 1977; Bauman et al., 1981).

Ward and his colleagues pioneered the non-isotopic labelling of nucleotide probes and the use of *in situ* hybridization at the electron microscopic level (Singer and Ward, 1982; Manuelidis et al., 1982; Hutchinson et al., 1982; Lawrence and Singer, 1985). This involved the development of a biotinylated analog of dUTP, (which may be incorporated into DNA by nick-translation) containing an alkylamine linker arm between the biotin molecule and the pyrimidine arm.

Although initial hybrid detection schemes were based on avidin-biotin binding, recognition of strong background binding between avidin and DNA led to alternatives, primarily streptavidin, a fungal analogue of egg avidin, and antibodies specific for biotin. Thus, at the light microscope level, hybrid detection schemes now use fluorescence-tagged secondary antibodies or enzymatic reaction products such as immunoperoxidase staining for visualization (Langer-Safer et al., 1982; and Manuelidis et al., 1982).

Elaboration of this method for the electron microscope level requires the use of an electron-dense detection system. Hybridization probes can be visualized by using peroxidase (Langer-Safer et al., 1982; and Hutchinson et al., 1982), or proteins tagged with colloidal gold (Hutchinson et al., 1983; and Wu and

Davidson, 1981) or by avidin complexed with ferritin (Harris and Croy, 1986), to give extremely high spatial resolution under the electron microscope (Manuelidis et al., 1982; Hutchinson et al., 1982; Wu and Davidson, 1981).

1.6.2.3 Hybridization to RNA at the Electron Microscope Level

Hybridization to cellular RNA at the light microscope level (Brahic and Haase, 1978; Gee and Roberts, 1982; Venezky et al., 1981; Edwards and Wood, 1983) has generated a great deal of interest. However, at the ultrastructural level, there is a smaller body of literature. Recently biochemical analyses have suggested specific sub-cellular localization of mRNAs, particularly the idea that mRNAs being actively translated are attached to the cytoskeleton in regions where their translation products are to be used (Fulton et al., 1980). These ideas can be tested with the technique of ultrastructural *in situ* hybridization. Since the biotin nucleotide system has recently been used at the light microscope level for the localization of actin mRNA (Singer and Ward, 1982), the same technology can be utilized for RNA detection at the electron microscope level to identify particular active cells within a tissue. Harris and Croy (1986), published the first report of *in situ* hybridization of a biotinylated cDNA to mRNA in plant tissue at either the light or electron microscope level of resolution.

In situ hybridization was used to locate mRNA for the storage protein legumin in cotyledon storage parenchyma tissue of developing pea (*Pisum sativum* L) seeds. The mRNA was hybridized with a biotinylated probe consisting of cDNA inserted in the plasmid pBR 322, and subsequently located with avidin conjugates. Avidin-rhodamine was used for fluorescence microscopy localization at a tissue/cellular level, and avidin-peroxidase (with diaminobenzidine) and avidin-ferritin compared for localization at an ultrastructural level.

The results obtained by Harris and Croy illustrated that *in situ* hybridization is possible in plant as well as animal tissues, even in the presence of a plant cell wall, and that the technique is capable of giving information on the timing and position of specific events during cell and tissue differentiation that it would not be possible to obtain by either biochemical or microscopical techniques alone.

1.7 Aims of the Research

The subcellular location of many of the important proteins in barley are not known, and there is controversy over the nature and ontogeny of these deposits. This lack of knowledge creates a handicap in the understanding of seed development, particularly as the techniques of molecular biology are progressing quickly.

The purpose of this project was therefore to investigate the synthesis and deposition of various storage proteins in the endosperm of barley.

The work described in this thesis involved the following stages:

(a) the examination of the development of the barley endosperm from anthesis to maturity, using optical and conventional transmission electron microscopy.

(b) the investigation of the three-dimensional inter-relationships of the endoplasmic reticulum, the Golgi apparatus and the protein bodies during protein synthesis and deposition using thick-sectioning of zinc iodide-osmium tetroxide impregnated tissue in the CTEM.

(c) the determination and characterization of the sub-cellular locations of the storage proteins by immunocytochemistry at the transmission electron microscope level, with particular attention to organelles involved in their synthesis and transport as well as their deposition.

(d) a preliminary study of the location of mRNA for storage proteins in barley endosperm using *in situ* hybridization techniques to relate morphological detail to the molecular aspects of gene expression.

It was hoped that a combined approach using the techniques of electron microscopy in association with the molecular techniques of immunocytochemistry and *in situ* hybridization would yield information that biochemistry or microscopy alone cannot give, and so go some way towards eliminating the controversy surrounding the synthesis and deposition of accumulating protein reserves in the

developing barley endosperm.

CHAPTER TWO

MATERIALS AND METHODS

MATERIALS AND METHODS

2.1 Sources of Chemicals and Biological Materials

2.1.1 Source of Plant Material

Barley grains obtained from the Rothamsted seed store, were grown at Rothamsted Experimental Station, Harpenden, Hertfordshire.

The bulk of the research involved the spring cultivar of barley *Hordeum vulgare* L. cv. Bomi. Some of the work involved high-lysine barley lines, these were Hiproly, Risø 1508 (derived from Bomi), and Risø 56 (derived from Carlsberg II).

2.1.1.1 Growing Conditions

Barley plants for storage protein studies were either field-grown or raised in greenhouses at Rothamsted.

Field-grown plants were grown in plots at Rothamsted under conditions of normal farm practice (Rothamsted Field Reports 1984, 1985).

Greenhouse material was available all year round with plants grown under carefully controlled conditions. Seeds (10 per pot) were sown in Eff compost (Eff Products, Guildford, Surrey) in 7" pots, and thinned after emergence to 8 plants per pot. Plant pots were watered via wet benches comprising wet silver-sand which irrigates plant pots standing on the surface by capillary action through the holes of the pot. Plants were fed monthly with a

solution of 10% K_2HPO_4 and 10% NH_4NO_3 (commercial grade); 2 : 5 dilution with water per pot.

Temperature in the greenhouse was controlled day and night $18^oC/12^oC$, and this was maintained $\pm$ $1\frac{1}{2}^oC$ 90% of the time. A photoperiod of 16 hours was achieved using 400W SON-T high pressure sodium lamps giving 200 μ einsteins sec^{-1} m^{-2}.

Aphose (pirimicarb) was used monthly to control aphids, and Bayleton every 3-4 weeks controlled mildew infection.

2.1.2 Sources of Chemicals:

Unless otherwise stated, all chemicals used were of analytical grade and obtained from either:

BDH, Poole, Dorset, U.K; or

SIGMA CHEMICALS, Poole, Dorset, U.K.; or

POLYSCIENCE INC., Pennsylvania, U.S.A.

Where appropriate, certain specialized items used are listed with the name of the supplier.

2.1.2.1 Light Microscopy

Toluidine blue: Gurr microscopy materials, BDH.

Sodium tetraborate: BDH

2.1.2.2 Electron Microscopy

Reagents, Spurr resin, and other materials such as grids and embedding capsules were obtained from TAAB Laboratories, Reading, Berkshire, U.K.

LR White Resin, and Lowicryl K4M low temperature resin, were obtained from Agar Aids, Stansted, Essex. Absolute

ethanol was dried over molecular sieve type 5A, obtained from BDH.

PIPES (piperazine-N, N'-bis 2-ethane sulfonic acid) was obtained from the SIGMA Chemical Company.

Sodium metaperiodate was obtained from BDH.

2.1.2.3 <u>Fluorescence Microscopy</u>

ANS (8-anilino-1-napthalene sulphonic acid) was obtained from SIGMA Chemical Co. (hemi-magnesium salt, practical grade), and was prepared as a 0.1% solution in 0.1m Na_2HPO_4/0.05M citric acid buffer (pH 5.0).

2.1.2.4 <u>Protein A-Gold</u>

Protein A was obtained from the SIGMA Chemical Co.;

Sodium citrate and chlorauric acid were obtained from BDH;

Protein A-Gold (4nm), and goat - anti-rabbit secondary antibody/gold (15nm) both EM grade were obtained from Janssen Life Science Products, Wantage, Oxon, U.K.

2.1.2.5 <u>Subcellular Fractionation</u>

<u>from SIGMA</u>: Cytochrome-c-reductase; β-nicotinamide adenine dinucleotide, reduced form (β -NADH); hepes No. H-3375 (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid); sucrose (Grade 1, crystalline); and tricine (N-Tris hydroxymethyl methyl glycine).

<u>from BDH</u>: EDTA (ethylenediaminetetra-acetic acid, disodium salt).

<u>from Fisons</u>, Fisons Scientific Apparatus, Loughborough, Leicestershire : magnesium acetate; potassium acetate; and dipotassium hydrogen orthophosphate.

2.1.2.6 Gel Electrophoresis

Chemicals for gel electrophoresis were obtained from two sources;

SIGMA Chemicals: Urea; Bromophenol blue; 2-mercaptoethanol; Coomassie brilliant blue R250; Tricine (N-Tris hydroxymethyl methyl glycine); and Tris (2-amino-2-hydroxymethylpropane-1, 3-diol).

Those obtained from BDH were: sodium dodecyl sulphate (SDS); glycine; acrylamide; bisacrylamide; and N,N,N'-N'-tetramethylene-ethylene-diamine (TEMED).

2.1.2.7 *In situ* hybridization

Nick translation enzyme kit: Amersham International Ltd., Amersham, Bucks.

Biotin-11-dUTP: Bethesda Research Laboratories, Paisley, U.K.

G-50 Sephadex (fine): Pharmacia Fine Chemicals, Milton Keynes, U.K.

Heat-inactivated horse serum: Flow Laboratories, Irvine, Ayrshire, U.K.

Pronase B: Calbiochem, Bishop Stortford, Hertfordshire, U.K.

From SIGMA Chemical Co.:

Avidin-peroxidase;

4-chloro-1-napthol;

Herring sperm DNA;

Bovine serum albumin;

and all other reagents (analytical grade).

2.1.3 Antisera to the Storage Proteins of Barley

Antisera to A, B and C hordein were obtained from Rothamsted Experimental Station, by courtesy of Dr. G.N. Festenstein. Antisera to CI-1 and CI-2 were obtained from Dr. M. Kreis at Rothamsted; they were originally obtained from the Carlsberg Research Institute in Denmark.

All antisera were polyclonals raised by injection of the protein in rabbits.

2.2 Tissue Processing for Optical and Conventional Transmission Electron Microscopy

Material was embedded in epoxy or acrylic resin. Resin-embedded material processed for transmission electron microscopy was also used for light microscopy.

In any method of preparing material for microscopy, the possibility of artefact formation is a major problem. No one procedure can eliminate this problem entirely, but it can be reduced to an acceptable minimum. Hence, several fixation procedures were initially investigated to determine the best fixation protocol for the barley grain. The possibility of the observed image still being unrepresentative of the natural state of the specimen was always borne in mind.

2.2.1 Excision and Trimming of Tissue

Grains were immersed in fixative immediately following removal from the barley ear. Within 20 minutes,

slices (c.1-2mm thick) were taken across the central area of the barley grain using a sharp razor blade under fixative. These slices remained in fixative for a further 5 or 24 hours.

2.2.2 Fixation, Dehydration and Embedding

The processing schedule was as follows:

1. Fixation Several fixation procedures were initially investigated to select the most efficient protocol giving consistent results:

(a) 2.5% (v/v) glutaraldehyde, 1.5% (w/v) formaldehyde in 0.05M sodium cacodylate buffer, pH 7.0.

(b) 1:1 dilution of (a) with distilled water.

(c) 2.5% (v/v) glutaraldehyde, 1.0% acrolein in 0.1M phosphate buffer, pH 7.0.

(d) 1:1 dilution (a) with distilled water.

(e) 1.5% (w/v) formaldehyde in 0.1M phosphate buffer, pH 7.0.

(f) 2.5% glutaraldehyde, 1.5% paraformaldehyde, in 0.05M PIPES buffer pH 6.8.

(g) 2.5% glutaraldehyde, 1.5% paraformaldehyde in 0.05M phosphate buffer pH 7.0.

Initially, samples were fixed for five hours, later samples were immersed in fixative for 24 hours at room temperature on the bench.

(h) 5% potassium permanganate solution for 30 minutes.

Of all the fixatives investigated (g) (2.5% glutaraldehyde, 1.5% paraformaldehyde in 0.05M phosphate

buffer pH 7.0) gave the most consistent results with superior tissue preservation at all stages of development of the barley grain. Tissues were fixed for 24 hours at room temperature on the bench.

2. Samples were then washed in phosphate buffer (2 x 15 minutes).

3. Where post-fixation was given, samples were immersed in 1.0% (w/v) aqueous osmium tetroxide for 24 hours.

4. Samples were then dehydrated:

   a. 12 % (v/v) ethanol; 2 x 15 minutes.

   b. 25% (v/v) ethanol; 2 x 15 minutes.

   c. 37 % (v/v) ethanol; 2 x 15 minutes.

   d. 50% (v/v) ethanol; 2 x 15 minutes.

   e. 75% (v/v) ethanol; 2 x 15 minutes.

   f. 100% dry ethanol; 4 x 15 minutes.

(the final two washes in (f) involved 100% ethanol dried over molecular sieve type 5A).

5. <u>Resin infiltration</u>.

Three types of resin were used to embed barley tissue namely, Spurr resin, LR white resin, and Lowicryl K4M.

(i) Spurr resin (Spurr, 1969) was prepared from TAAB premix kits. Tissue samples were rotated in 50:50 100% dry ethanol and Spurr resin for 3 days. The tissue samples were then transferred to 100% Spurr resin, and infiltration occurred over 20 days with daily resin changes. All stages were performed at room temperature with constant agitation of tissue in resin by rotating

vials on a rotator. Barley slices were flat embedded in TAAB capsules; resin was polymerized in an oven at c.70 °C for 10 hours.

(ii) Some samples were embedded in LR white acrylic resin. Infiltration times were the same as for Spurr resin. Samples were polymerized in dried, air-tight, gelatine capsules at c.60 °C.

(iii) Barley tissue was also embedded in Lowicryl K4M low-temperature embedding resin, using a temperature - controlled liquid nitrogen - cooled box devised from a design by Wells (1985).

Tissue processing involved dehydration of tissue (previously fixed in glutaraldehyde) with 30% ethanol at 0 °C, 50% ethanol at -20 °C, and the remaining stages of dehydration up to 100% ethanol and resin infiltration occurred at -35 °C. Polymerization also occurred at -35 °C in flat capsules, in the presence of ultra-violet light overnight. Specimens were rotated in a simple box devised to control low temperatures by using liquid nitrogen.

2.2.3 Sectioning

Thick sections (1 μm) for light microscopy and ultrathin sections (gold/silver interference colours - 150-60nm) for transmission electron microscopy were cut using glass knives on an LKB ultrotome (Type 4801A, Stockholm, Sweden), or on a Sorvall MT2-B ultramicrotome (Du Pont Instruments).

The sections were collected on the surface of

distilled water in a trough attached to the knife. All sections were flattened using a heat pen. Sections for light microscopy were picked up in a platinum wire loop and transferred to a drop of distilled water on a microscope slide. Ultrathin sections were picked up on either uncoated or formvar-coated copper grids (c.200 mesh).

Several replicate sections were taken both from the same sample and from other grain slices at the same developmental stage; micrographs in this thesis are those which show the most consistent observations.

2.3 Staining and Examination of Tissue for Optical Microscopy

2.3.1 Toluidine-Blue Staining.

A drop of 1% (w/v) toluidine blue solution made up in 1% (w/v) sodium tetraborate was added to 1μm sections which had been dried onto the slide over a hot plate. After warming over a hot plate for c.30 seconds, the dye solution was washed off with distilled water and the sections finally dried.

Toluidine blue-stained resin-embedded material does not exhibit the same degree of metachromasy found in wax-embedded material (O'Brien *et al.*, 1964). However, it serves to stain protein bodies a darker blue than cytoplasm, with starch remaining unstained.

Viewing and photomicrography were performed using a Leitz Ortholux Light Microscope with transmitted light.

Images were recorded using Kodak XP1, 400ASA, black and white film.

2.3.2 Fluorescence Microscopy

1μm sections of Spurr-embedded tissue were dried onto slides over a hot plate. A drop of 0.1% ANS in sodium citrate buffer pH5 was added to the sections on the microscope slide, and rinsed off with distilled water after 2-3 minutes. A coverslip was then added and sections viewed in a Nikon Diaphot-TMD inverted microscope (Nikon, Tokyo 100, Japan). Images were recorded using a Microflex AFX Nikon camera on Kodak Colour film 400 ASA.

2.4 Staining and Examination of Tissue for Conventional Transmission Electron Microscopy (CTEM)

For general ultrastructural observations, ultrathin sections on copper grids were double-stained as follows;

1. grids were floated on droplets of saturated aqueous uranyl acetate for 15 minutes,
2. thorough washing of grids with distilled water,
3. grids were then floated on droplets of alkaline lead citrate (Reynolds, 1963) for 15 minutes,
4. grids were thoroughly washed with distilled water.

Occasionally a saturated ethanolic solution of uranyl acetate was used for sections on uncoated copper grids. Dried grids were stored on filter paper in petri-dishes. Grids were viewed in a Phillips EM400 at 80 or 100 kV,

unless stated otherwise, and images were recorded on Kodak electron image film 4489.

Unless stated otherwise, all transmission electron micrographs are of double-stained sections.

2.5 Zinc Iodide - Osmium Tetroxide Impregnation for Thick-Sectioning of Material for CTEM

Material was fixed and processed as for conventional transmission electron microscopy, except that the osmication step was replaced with a zinc iodide - osmium tetroxide impregnation stage after fixed tissue was washed with distilled water. The zinc iodide solution was freshly prepared according to Harris (1978);

1.5g powdered zinc;

0.5g resublimed iodine;

10 ml water.

The solution was sonicated for one minute, stirred for a further five minutes and filtered. The zinc iodide-osmium tetroxide (ZIO) solution was made by adding an equal volume of 2% (w/v) aqueous osmium tetroxide to the zinc iodide solution.

Plant tissue was allowed to impregnate in the zinc iodide-osmium tetroxide solution for 24 hours and then dehydrated and resin-embedded as for conventional transmission electron microscopy. Sections were cut c.0.3μm thick on glass knives and viewed unstained in the transmission electron microscope operating usually at 80kV. In some cases the tilt facility of the TEM was used

in the preparation of stereo pairs.

2.6 Preparation of Protein A-Gold Complex

There are many different methods of producing gold sols of different sizes (reviewed, Roth, 1983). These are based on the controlled reduction of an aqueous solution of chloroauric acid using different reducing agents under varying conditions. According to Frens (1973), the assumption that all gold ends up in the reduced form is reasonable (at least for citrate gold). The size of the gold particles is determined by the number of nuclei formed at the beginning of the reaction, and the strength of the reducing agent.

Gold colloids were prepared by sodium citrate reduction of chloroauric acid following the method of Frens (1973). The heterodisperse sol was obtained without the repeated centrifugations and washing required to prepare monodisperse sols of particular sizes. The sols were coated and stabilized with Protein A following the method of Roth et al. (1978). This protocol yields a heterodisperse sol; after complex formation with Protein A, sols can be sized to homogeneity by density-gradient centrifugation (Slot and Geuze, 1981).

Freshly prepared Protein A-gold can be stored in autoclaved, screw-capped glass bottles for up to six months at 4°C without deterioration. Each preparation of gold marker, can be characterized by two methods (Goodman et al., 1981; Roth, 1983; Geoghegan et al., 1980): from the

absorption spectra of gold sols, and by direct measurement from transmission electron micrographs. As measurement of particle size from transmission electron micrographs is the only exact method of determination of particle size distribution, this was the one used to characterize gold markers.

2.7 Origin of antisera to the Storage Hordeins

Polyclonal antisera to A, B, and C hordeins were obtained from Rothamsted.

Hordein samples were prepared from *Hordeum vulgare* L. as described by Shewry et al. (1978, 1980b and 1981). They were successively extracted with water saturated butan-1-ol (to remove lipids), 0.5M sodium chloride (to remove albumins and globulins) and finally 55% (v/v) aqueous propan-2-ol + 2% (v/v) mercaptoethanol to provide the total hordein; extraction with 55% propan-2-ol followed by 55% propan-2-ol + 2% (v/v) mercaptoethanol gave hordein-I and hordein-II respectively.

Individual A and B hordein fractions of the barley variety Bomi were separated by preparative sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Miflin and Shewry, 1977). B and C hordeins of the variety Julia were separated by ion exchange chromatography and gel filtration (Shewry et al., 1980b).

Antisera to hordeins were prepared in rabbits (Festenstein and Hay, 1982; and, Festenstein et al., 1984). Antiserum to A hordein obtained from the mutant

barley line Risø 1508 was raised by injecting 4 mg hordein (previously separated by preparative SDS-PAGE, Miflin and Shewry, 1977) , containing SDS in complete Freund's adjuvant. The antiserum used was obained 8 weeks after injection. Antiserum to Julia C hordein was raised by injecting 20 mg hordein in 1ml 1% Triton X-100 in complete Freund's adjuvant (Festenstein et al., 1984). The Julia C hordein was stirred with 1% Triton X-100 for several hours to give the suspension for injection, and the antiserum was obtained eight weeks ater injection.

B1 hordein from the barley cultivar Julia was purified by preparative SDS-PAGE of reduced and pyridylethylated (PE) hordein using a Birchover MacroPAGE apparatus, essentially as described by Shewry et al. (1977). SDS was removed by chromatography on Sephadex LH30 in 70% formic acid. 50mg of protein was injected as a suspension in 6M urea with Freund's adjuvant.

Festenstein and Hay (1982) have shown common antigenic determinants for A and B hordeins and also for B and C hordein. B hordein contains extra determinants not present on A hordein and also not present on C hordein. Immunochemical relationships of the prolamin storage protein of barley complementing comparisons of the amino acid sequences of the proteins are given by Festenstein et al. 1986, (manuscript in preparation).

2.8 Immunocytochemical Localization of Storage Proteins with Protein A-Gold by Conventional Transmission Electron Microscopy

2.8.1 Protocol for Immunocytochemical Localization

Appropriate conditions have to be devised for each antigen and antisera depending upon which resin the tissue is embedded in and whether tissue is osmicated or not.

The basic protocol involved ultrathin sections of tissue collected on formvar coated, 200 mesh, copper grids, and incubated by floating on top of an antiserum droplet on parafilm at room temperature in a moist chamber for 30 minutes. Grids were then washed with a series of PBS droplets from a Pasteur pipette, and floated on a droplet of Protein A-gold for a further 30 minutes to localize bound antibodies.

The incubation period, and dilution of antibody and protein A-gold, were determined for each preparation.

The grids were finally washed in a gentle jet of distilled water, and carefully dried by absorbing the water from behind the forceps-held grid. Grids were then viewed in the transmission electron microscope.

Sections were also counterstained with uranyl acetate and lead citrate for 15 minutes each and viewed in the TEM.

2.8.2 Controls for Immunocytochemical Localization

Controls are essential for determining the

authenticity of antigen labelling with protein A-gold.

The first control involved incubation of grids of tissue sections on a droplet of protein A-gold followed by washings as described previously.

The second control involved incubation of grids of tissue sections on a droplet of pre-immune serum. Pre-immune serum was obtained from the rabbit prior to injection with the protein to which antibodies were to be raised. Incubation with pre-immune serum was followed by conventional washings and subsequent incubation in protein A-gold. Grids were washed as described in protocol.

2.8.3 'Etching' of Osmium-Fixed Tissues for Immunocytochemical Localization

Sodium metaperiodate was used to unmask protein antigenic sites on glutaraldehyde-fixed post-osmicated tissues following the method of Bendayan and Zollinger (1983).

Thin sections were cut and mounted on 200-mesh gold or nickel grids having a formvar film. Tissue was incubated for 30 minutes on a saturated aqueous solution of sodium metaperiodate. Grids were then thoroughly rinsed in distilled water and processed for immunocytochemical labelling.

2.9 Subcellular Fractionation of Endosperm Organelles

Barley (*Hordeum vulgare* L. cv. Bomi) plants were grown under controlled conditions in the greenhouse as

described previously. Freshly-squeezed endosperm was obtained from barley grains during the mid-phase of development when protein synthesis was at or about its fastest rate.

For subcellular isolations two isolation buffers were used; the first (Mg^{2+}-plus) consisted of Tricine (50mM); potassium acetate (100mM); magnesium acetate (10mM), pH 7.5, and the second (Mg^{2+} -free) consisted of Tricine (50mM); potassium acetate (100mM); EDTA (5mM), pH7.5.

Freshly-squeezed endosperms were chopped with a razor blade at a tissue to medium ratio of about 1:(1.5) in 15% (w/w) sucrose in extraction buffer until the material was finely divided (< 0.5mm cubes). A further 0.5 volume of medium was added and the homogenate filtered, with squeezing, through four layers of muslin. The homogenate was then layered on to sucrose density gradients (20-60% w/w) made up in either Mg^{2+}-free or Mg^{2+}-plus buffer as appropriate, and centrifuged at 25,000 rev. min^{-1} in an SW 27 rotor in a Beckman ultracentrifuge for 2.5 hours. The gradients were then fractionated using an ISCO density gradient fractionator model 640 at 2ml/min, collecting 1.2ml fractions with monitoring of absorbance at 280nm. Peaks were identified by position of the marker enzyme NADH-cytochrome *c* reductase (Tolbert, 1974) as described by Miflin et al. (1981). Sucrose content was determined by refractometry.

Fractions were analysed by SDS-PAGE; hordein was

extracted in propan-2-ol + 2% 2-mercaptoethanol by the methods of Shewry et al., (1978).

2.9.1 Electron Microscopy of Fractions

Aliquots of fractions from the density gradient were mixed with an equal volume of 5% (w/w) sucrose and stood overnight at $4^{o}C$. They were then diluted with an equal volume of buffer and centrifuged at 12,000 g for 15 minutes in a Beckman Microfuge. The pellets were washed with buffer then treated with osmium tetroxide overnight. After further washings the pellets were dehydrated in alcohol, embedded in Spurr resin, and sectioned as described for immunocytochemical localization.

2.10 Gel Electrophoresis of Proteins

Proteins were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the modified method of Laemmli (1970) (Forde et al., 1981). A 14% (w/v) separating gel was used, containing 0.34% bisacrylamide, 0.1% (w/v) sodium dodecyl sulphate (SDS), 4M urea, 0.05% (v/v) N,N,N'-N'-tetramethylene-ethylene-diamine (TEMED), 0.1% (w/v) ammonium persulphate, 1.0M Tris-HCl pH8.8. The stacking gel contained 3% (w/v) acrylamide, 0.4% (w/v) bisacrylamide, 0.1% SDS, 4M urea, 0.1% (v/v) TEMED, 0.06% (w/v) ammonium persulphate, 60mM Tris-HCl pH 6.8.

The proteins were prepared for electrophoresis by heating at 60° C for 20 minutes in gel loading buffer containing 8M urea, 4% (w/v) SDS, 10% (v/v)

2-mercaptoethanol, 50mM Tris-HCl pH 6.8, 0.005% bromophenol blue in glycerol. They were then centrifuged at 10,000 x g (r_{av} 82mm) at room temperature for 5 minutes prior to loading. The gel running (electrode) buffer was 192mM glycine, 25mM Tris pH 8.3, 0.1% SDS.

All gels were run using a Bio-Rad (Richmond, California, USA) Protean slab gel system with 16 x 8 cm cassettes. The separating gel was 12.5cm long, and the stacking gel was 1cm below the sample wells. Up to twenty samples could be separated on each gel.

Electrophoresis was at room temperature for 30 minutes at 20mA per gel, then at 30mA per gel for approximately 3.5 hours. After electrophoresis the gels were fixed and stained in 10% (w/v) trichloroacetic acid (TCA), 40% methanol and 0.1% Coomassie brilliant blue R250 for 16 hours with gentle agitation on a shaker, and then rinsed and destained in 10% (w/v) TCA for up to one week with gentle agitation; a small piece of packing foam was added to the destaining container to absorb excess stain.

Standards included a marker for Bomi storage hordein, and standards of known molecular weight purchased from SIGMA Chemical Company; Myosin (205,000), β-galactosidase (116,000), Phosphorylase B (97,400), Bovine Serum Albumin (66,000), Ovalbumin (45,000) and Carbonic Anhydrase (29,000).

2.11 *In situ* Hybridization using a Biotinylated cDNA Probe

Barley plants of the high-lysine line Risø 1508 were grown in greenhouses at Rothamsted as described previously (section 2.1.1.1).

2.11.1 Preparation of Biotinylated cDNA Probes

Chymotryptic inhibitor clones were isolated from a cDNA bank prepared using the mRNA from developing barley grains (Williamson and Kreis, unpublished; Williamson et al., 1986a,b manuscripts in preparation). cDNAs were cloned in the vector puc9, and cDNA libraries were screened using oligonucleotide probes.

The cDNAs for CI-1 and CI-2 were nick translated using an enzyme kit (Amersham International Ltd.) in a reaction mixture modified to include biotin-11dUTP in place of dCTP. A typical reaction mixture comprised 5 µg DNA, 10 units of DNA polymerase I, 100 pg of DNAase, 0.04mM each of dATP, dGTP, dTTP and biotin-11-dUTP (Bethesda Research Laboratories, U.K.) in a final volume of 50µl of 100mM Tris-HCl pH7.8 with 10mM magnesium chloride, 10mM mercaptoethanol and 10µg/ml bovine serum albumin (BSA). The labelled DNA was separated from unincorporated biotin-11-dUTP by passage through a 10ml column of Sephadex G-50 superfine equilibrated with 50mM Tris-HCl pH 7.5, 10mM EDTA, 100mM NaCl, 0.2% SDS. The excluded peak was collected and the DNA concentration determined spectrophotometrically and adjusted to 2-3 µg/ml

for subsequent hybridizations.

Incorporation of biotin into the DNAs was verified using a dot blot assay system. Aliquots of biotinylated DNA and controls of unlabelled DNAs and biotin-11-dUTP, were mixed with salmon sperm DNA to 250 µg/ml, heated at 95°C for 5 minutes then rapidly cooled on ice. One fifth of a volume of 20 x SSC (0.3M sodium citrate pH 7.0, 3.0M sodium chloride) was added and a range of volumes containing between 10 and 100 ng of plasmid DNA or 0.4 µM biotin-11-dUTP were applied onto a gridded nirocellulose disc equilibrated with 20 x SSC. The filters were then baked for 2 hours at 75°C under vacuum and washed several times at 22°C in 4 x SSC followed by PBS (50mM sodium phosphate pH 7.5, 0.7M sodium chloride). The filter was then incubated for 1 hour in horse serum buffer (PBS containing 10% heat inactivated horse serum) at 40°C, followed by 2 hours at 37°C in the same buffer containing 100µg/ml avidin-peroxidase, horse serum buffer and PBS . The bound peroxidase was detected by reaction with a freshly made chloronapthol reagent (60mg of 4 chloro-1-napthol in 20ml of methanol added to 100ml of PBS followed by 50µl of hydrogen peroxide (30% w/v)).

2.11.2 Tissue Fixation and Hybridization

Developing barley grains were harvested immediately into fixative and within 2-3 minutes, the grain was cut into 2-3mm slices and fixed in freshly made 4%

paraformaldehyde in P-S buffer (0.05M phosphate buffer pH7.0 with 0.05M sucrose) for 5 hours at 18 °C. Grains opposite those selected for fixation, were sampled to determine fresh weights. Tissue slices were washed in P-S buffer, and tissue pieces were cut to approximately 100 µm slices using a Mickle tissue chopper. The slices were washed in P-S buffer (4 x 15 minutes) and treated with pronase B at 25 µg/ml in P-S/EDTA (P-S buffer with 5mM EDTA) for 15 minutes at 18 °C. Pronase activity was stopped by incubation in 0.5M phenyl methylsulphonyl fluoride in P-S/EDTA with 4mg/ml glycine (1 hour at 18 °C).

Individual slices were then placed in siliconized Eppendorf tubes and equilibrated with 50% formamide in 0.3M NaCl/0.3M sodium citrate pH 7.0 (hybridization buffer) for 2 x 15 minutes. Hybridization was carried out in 150µl of 50% formamide hybridization buffer with 0.5µg/ml probe cDNA and 250µg/ml sonicated herring sperm DNA. After a denaturation step at 78°C for 3.5 minutes, the tubes were incubated for 40 hours at 37°C. Extensive washing with numerous changes of phosphate buffered saline (PBS) preceeded localization of the biotin using avidin-peroxidase. Control tissues were treated as above except that the probe DNA consisted of biotinylated plasmid without a cDNA insert.

2.11.3 Localization with Avidin-Peroxidase

After post-hybridization washing with PBS, tissue

slices were incubated in 5μg/ml avidin-peroxidase in PBS for 2 hours at 37°C. After a series of 15 minute washes in PBS the tissue slices were then washed overnight in a large volume of PBS, with constant agitation, before incubation with diaminobenzidine to localize the peroxidase activity. The reaction medium contained 10mg diaminobenzidine in 0.1ml 3% hydrogen peroxide and 4.9ml 50mM 2-amino-2-ethyl-1, 3 propane-diol pH 9.0. The tissue slices were incubated for 30 minutes at 37°C in the dark before PBS washing (3 x 15 minutes) and post-fixation in 1% aqueous osmium tetroxide for 30 minutes. After dehydration through a graded series of acetone the tissue was infiltrated and embedded in Spurr resin (Spurr, 1969). Thin sections were examined, after staining with uranyl acetate and lead citrate, at 60, 80 or 100 kV.

CHAPTER THREE

RESULTS

RESULTS

3.1 Morphology of the Developing Barley Grain (*Hordeum vulgare* L. cv. Bomi)

Individual ears of barley (*Hordeum vulgare* L. cv. Bomi) were tagged at anthesis in the field at Rothamsted in the summer of 1984. At various times after anthesis, a caryopsis was extracted from the middle portion of the ear to determine the fresh and dry weights.

Opposite grains to those used for fresh and dry weight measurements were fixed in an aldehyde fixative in phosphate buffer immediately upon harvesting. The fresh weight of each caryopsis was found to closely parallel their opposing number. Four treatments were employed: with osmium tetroxide, without osmium tetroxide, with zinc iodide-osmium tetroxide (these treatments were embedded in Spurr resin) and a final treatment without osmium tetroxide but embedded in Lowicryl K4M low-temperature-embedding resin. Four caryopses were fixed in each treatment.

Resin-embedded grain was sectioned for light and electron microscopy to study the development of the grain from anthesis to maturity with particular reference to storage protein deposition in the endosperm.

3.1.1 Fresh and Dry Weights of the Developing Barley Grain from Anthesis to Maturity

Figure 3.1 shows the average fresh and dry weights of 16 individual caryopses, and the average fresh weights of 16 individual endosperms of Bomi barley at various stages after anthesis.

Fresh weight of the caryopsis and endosperm increased with time until 32 days after anthesis and decreased thereafter. The endosperm was still liquid until 14 DAA which was the first determination of its fresh weight. The endosperm weighed 15 mg 14 DAA, and 66 mg 32 DAA, after which its fresh weight decreased. The weight of the caryopsis before the endosperm was formed was mainly due to the palea, lemma and pericarp; almost all of its subsequent increase in weight (from 3.5 mg at anthesis to 76 mg 32 DAA) can be ascribed to growth of the endosperm.

Figure 3.1 also shows the change in dry weight with time of the caryopsis of Bomi barley. Values increased rapidly from 12 DAA to 32 DAA, then increased more slowly thereafter. The dry weight constituted an increasing proportion of the fresh weight all through development of the caryopsis, and was about 80% of the fresh weight 45 DAA.

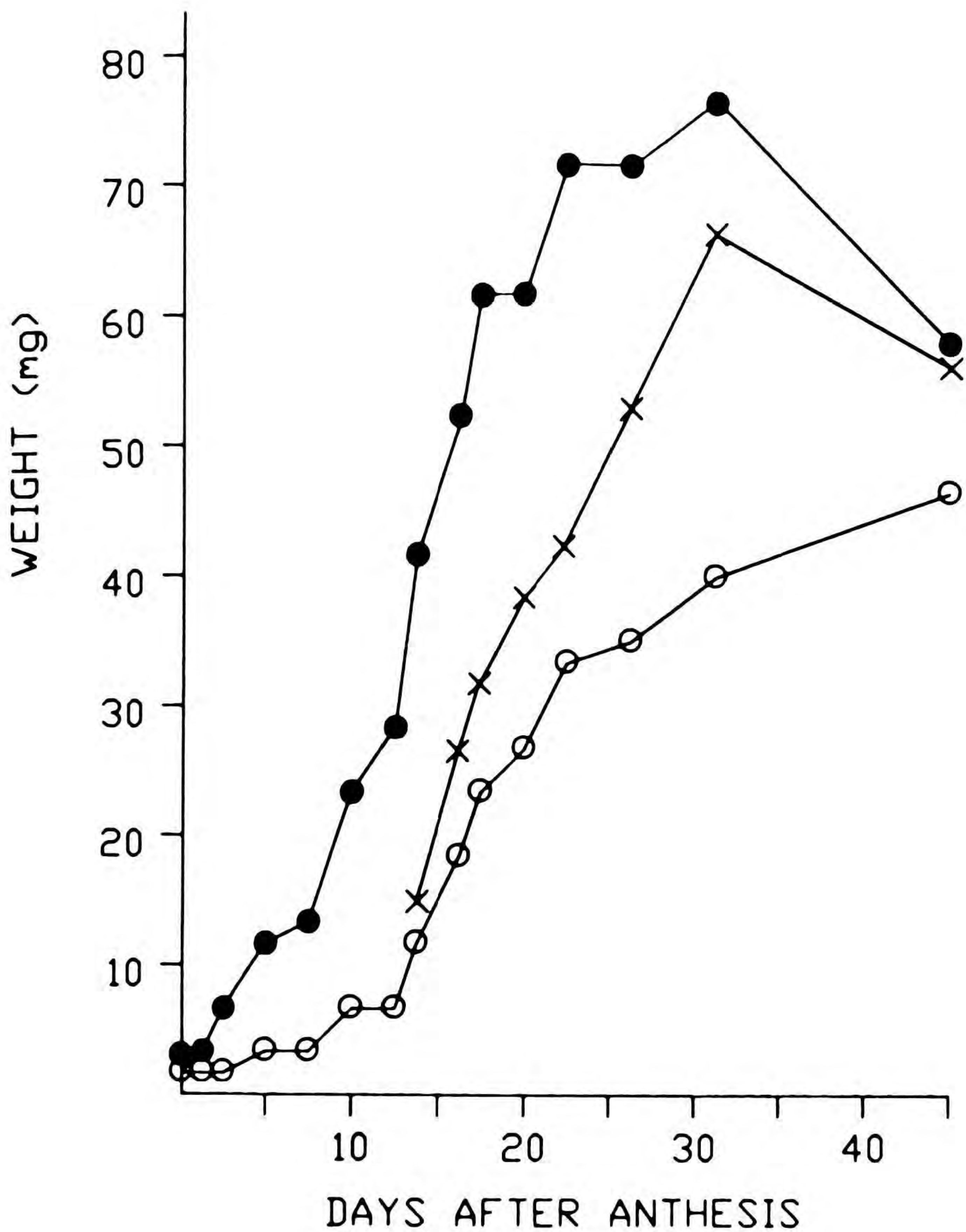


FIGURE 3.1

Fresh and dry weights of the developing grain

Fresh weight (mg) of caryopsis (●——●) and endosperm (×——×)
Dry weight of caryopsis (○——○)

3.1.2 Light Microscopy of the Developing Caryopsis from Anthesis to Maturity of Bomi barley

3.1.2.1 Transverse Sections of the Barley Caryopsis Stained with Toluidine Blue

At anthesis, there is no evidence of endosperm development (Plate 1a). The nucellar layer and seed coats surrounding the embryo sac, and the vascular bundle region are shown in micrograph 1b. By 3 DAA there is development of the coenocytic endosperm which lines the embryo sac into the central vacuole, (Plate 1c); the enlarged cells of the nucellar layer are also prominent. There is however, no evidence under the light microscope of any wall development in the coenocytic endosperm (Plate 1d).

5 DAA (Plate 2a, b) shows further development of the coenocytic endosperm. At this stage, from transmission electron micrographs (not shown), cell wall projections are evident from the cell wall surrounding the embryo sac. Between 5 DAA and 8 DAA, the embryo sac undergoes transformation from a multinucleate syncytium to a cellular endosperm (Plate 2c). In micrograph d (Plate 2), the endosperm cells are large and highly vacuolate with prominent nuclei.

By 10 DAA (Plate 3a, b), the outer three layers of the endosperm are differentiated to form the aleurone. During differentiation of the endosperm, the cells of the sub-aleurone are the last to be formed. Several

rows of radially elongated, prismatic endosperm cells extend inwards from the sub-aleurone to the inner, central endosperm which consists of larger, irregular-shaped cells. Plate 3c shows this tissue differentiation at 12 DAA. At this stage, starch begin to form in the starchy endosperm (Plate 3d).

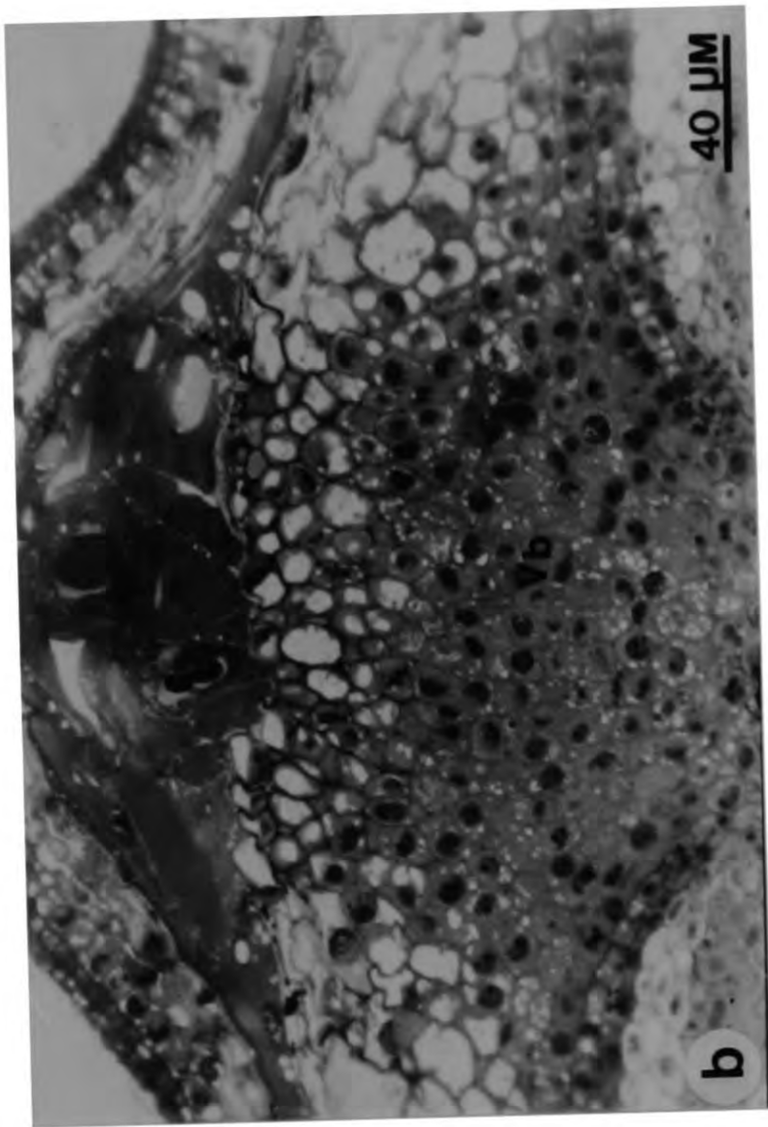
By 14 DAA (Plate 4a, b), further development has occurred. The sub-aleurone cells have many vacuoles, some containing protein deposits. Further into the starchy endosperm (Plate 4c), there are fewer protein deposits.

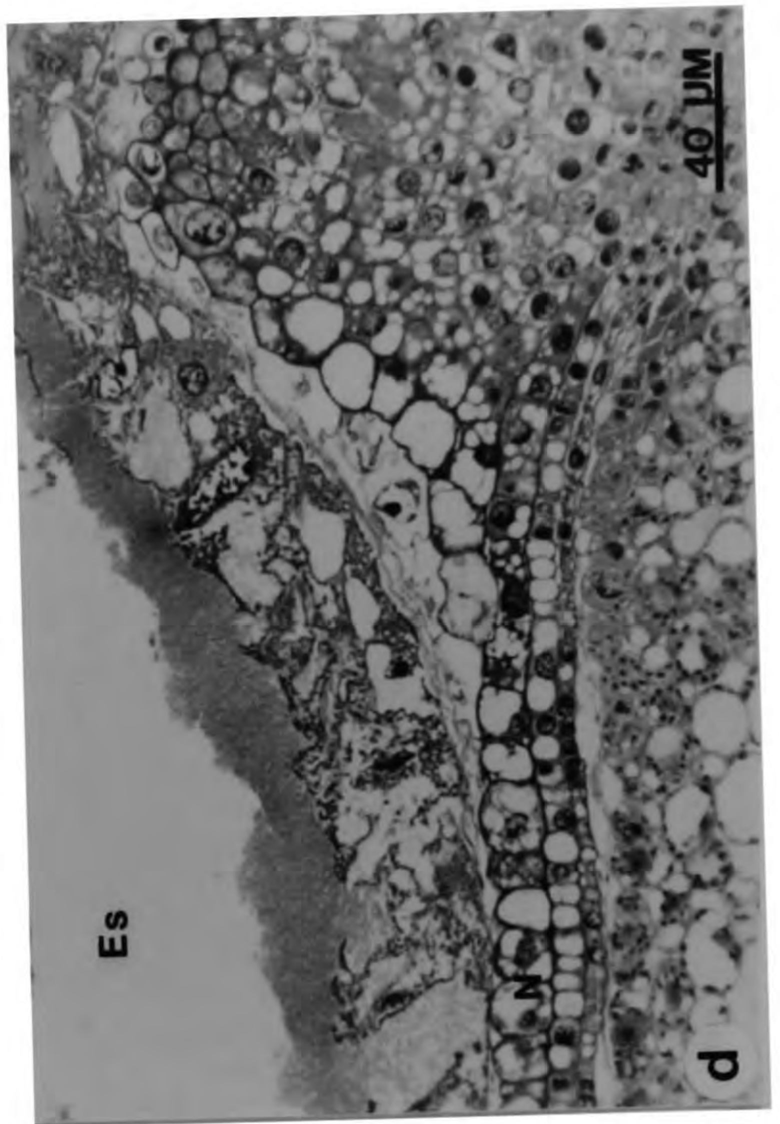
There is a marked increase in the density of protein deposits in the sub-aleurone 18 DAA (Plate 5a, b). These deposits are located in vacuoles. In micrograph c (Plate 5), the starchy endosperm cells are seen to have many large and small starch granules. There are fewer protein deposits further into the inner starchy endosperm.

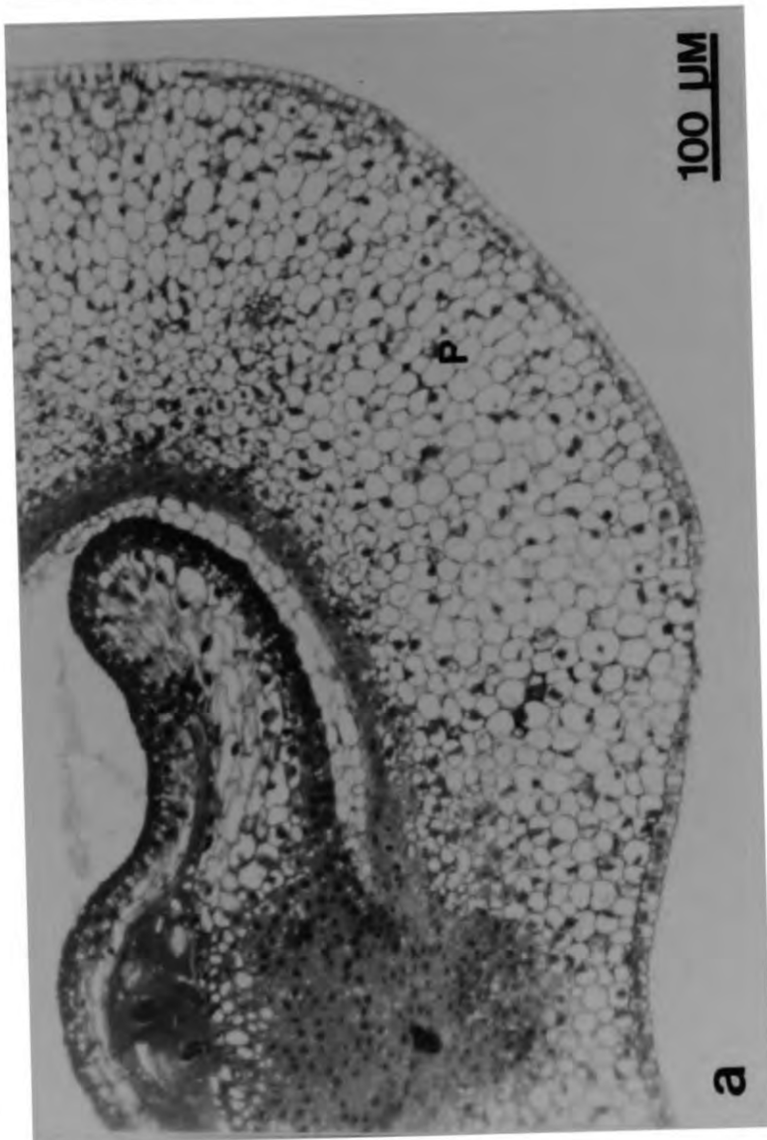
By 20 DAA, there is much starch deposition (Plate 6a). In micrograph b (Plate 6) there is a high density of protein deposits in the sub-aleurone. Further into the starchy endosperm, there is a high density of starch granules embedded in a protein matrix. In the inner endosperm, Plate 6c, cells have many large and small starch granules, and small protein deposits.

Plate 7 compares the later stages of development.

In micrograph a, 32 DAA, starch granules now evident in the sub-aleurone are embedded in a protein matrix. In the starchy endosperm (micrograph **b**), large and small starch granules are embedded in a protein matrix. The caryopsis 45 DAA (Plate 7c, d) shows little difference from 32 DAA caryopsis. The cells of the sub-aleurone and endosperm are packed with starch granules which are embedded in a dense protein matrix.







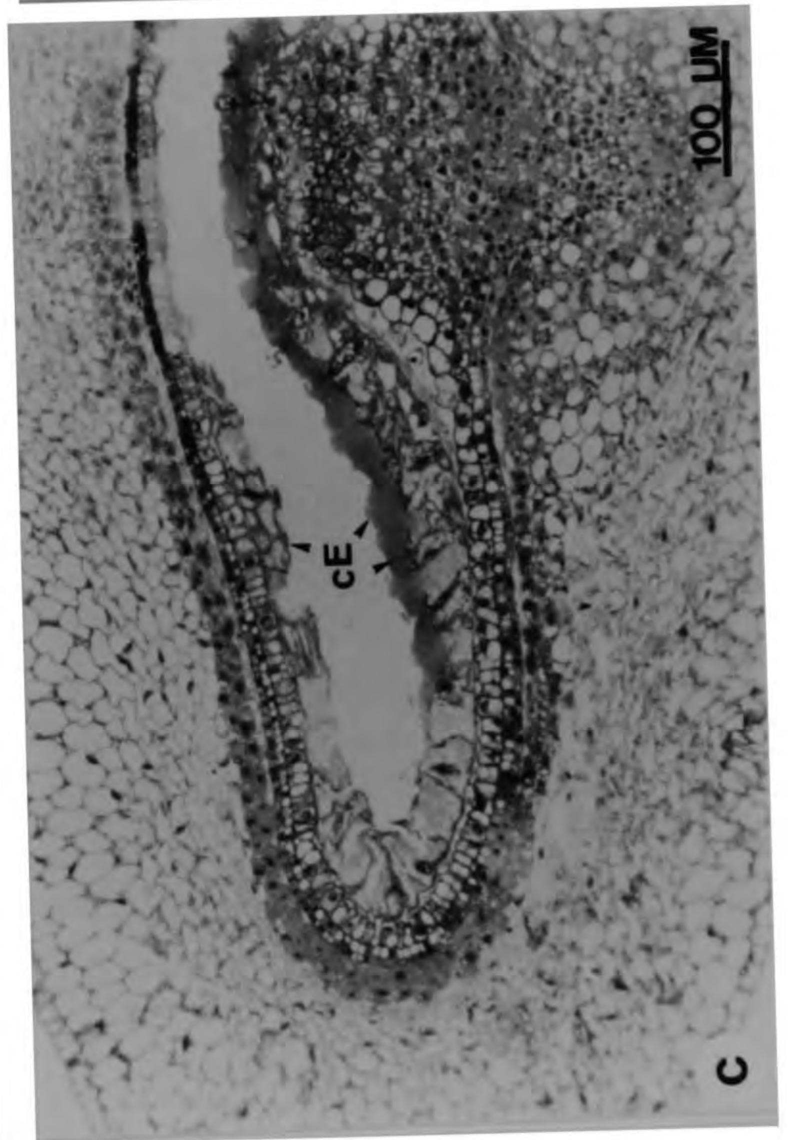
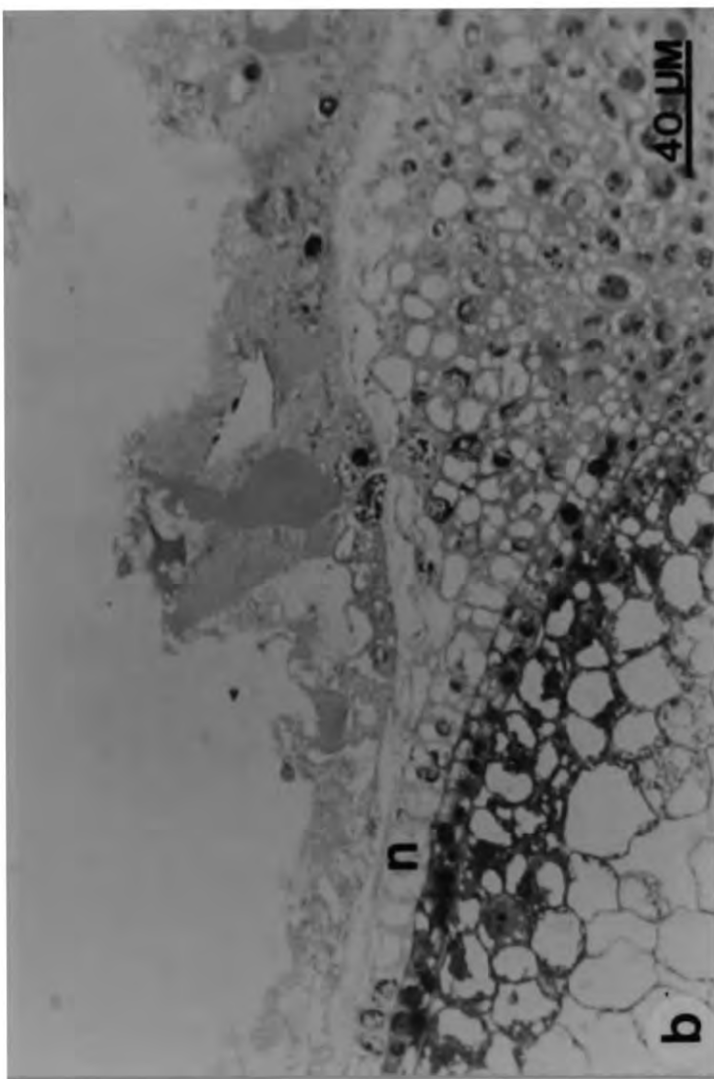
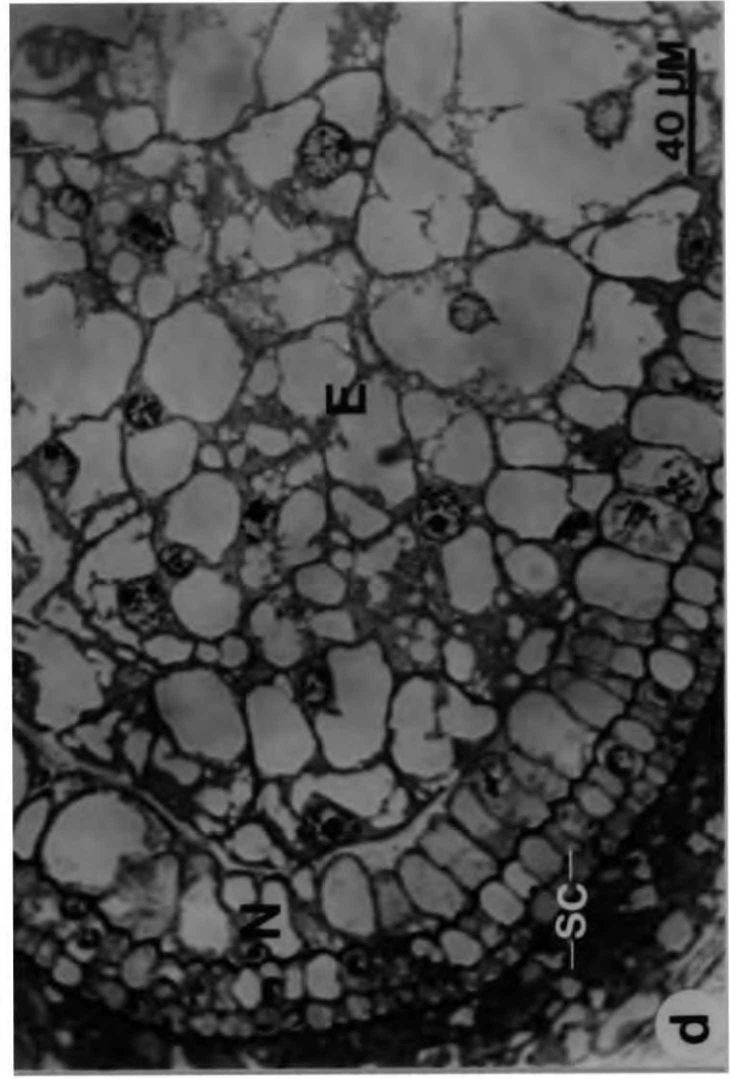
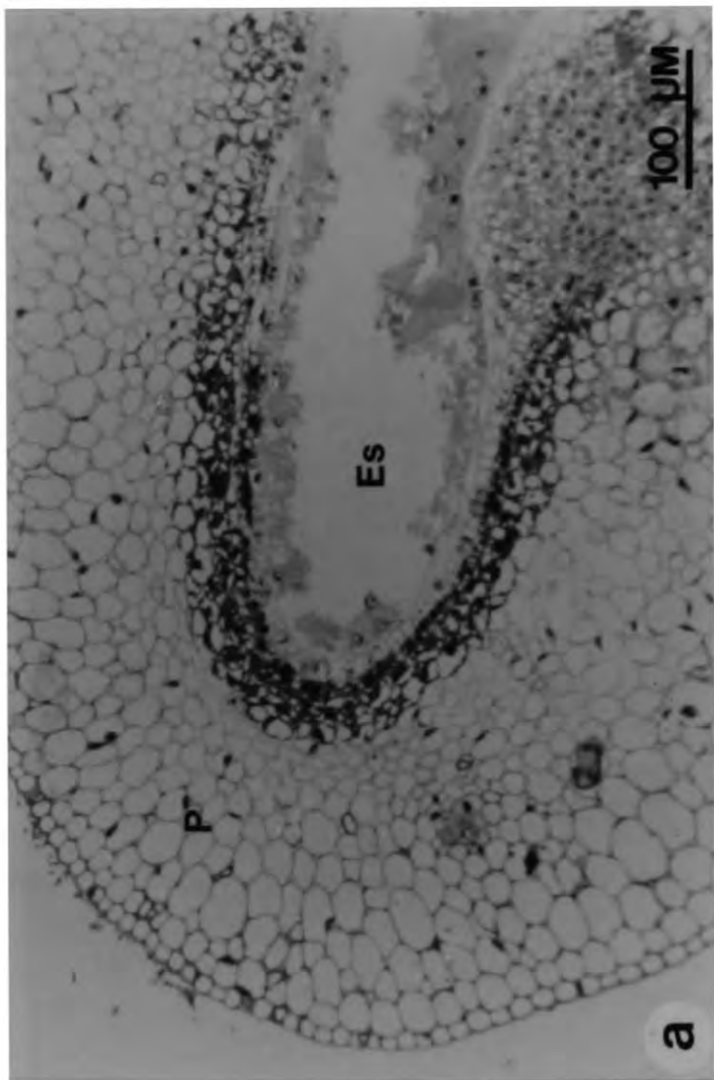


PLATE 1. Light micrographs a-d : transverse cross-sections through barley caryopsis, stained with toluidine blue.
a : Low magnification of the caryopsis at anthesis. b : Higher magnification of the vascular bundle region of a.
c : 3 DAA, showing coenocytic endosperm (arrows) lining the embryo sac. d : Higher magnification 3 DAA, showing the nucellus surrounding the embryo sac.







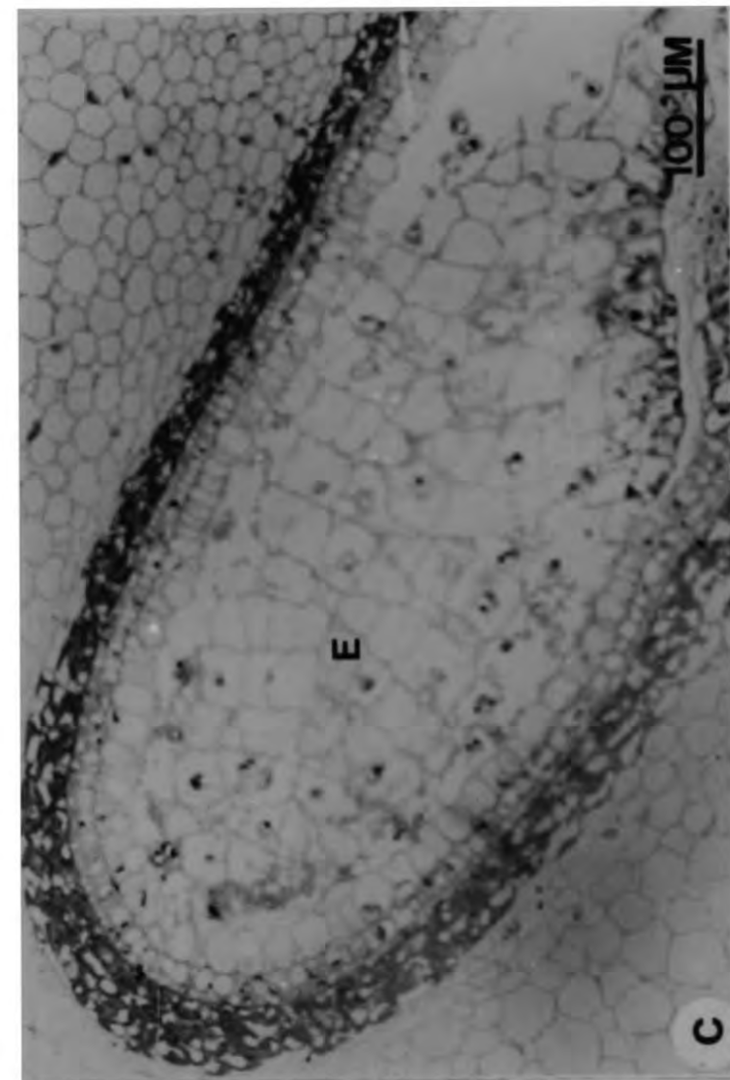
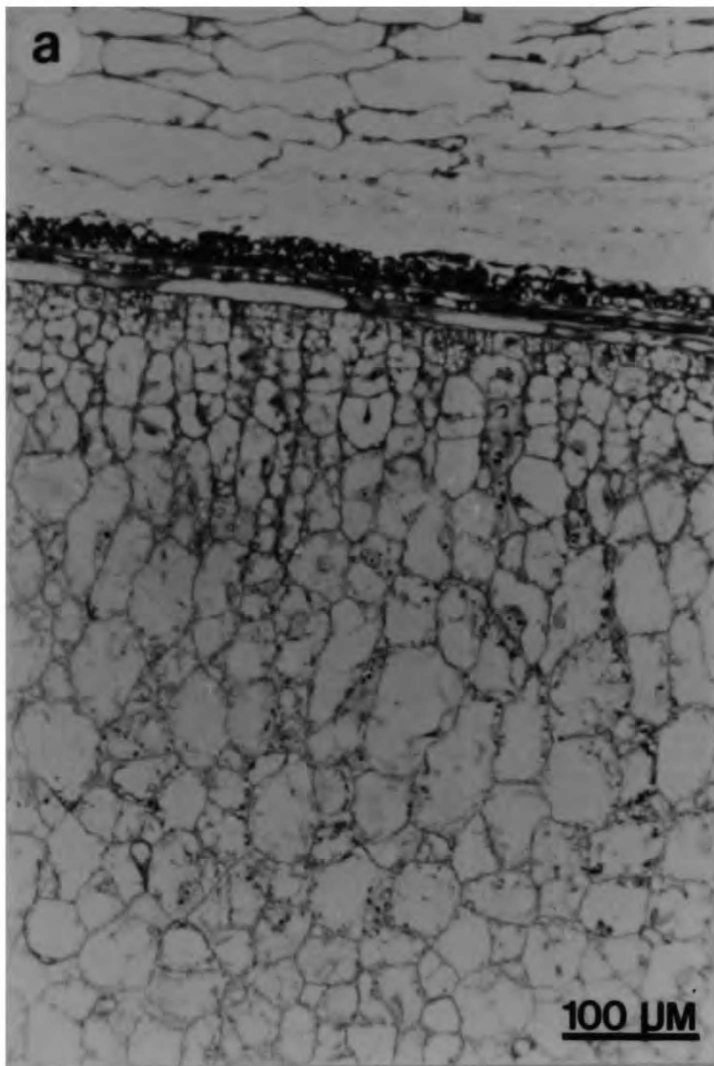
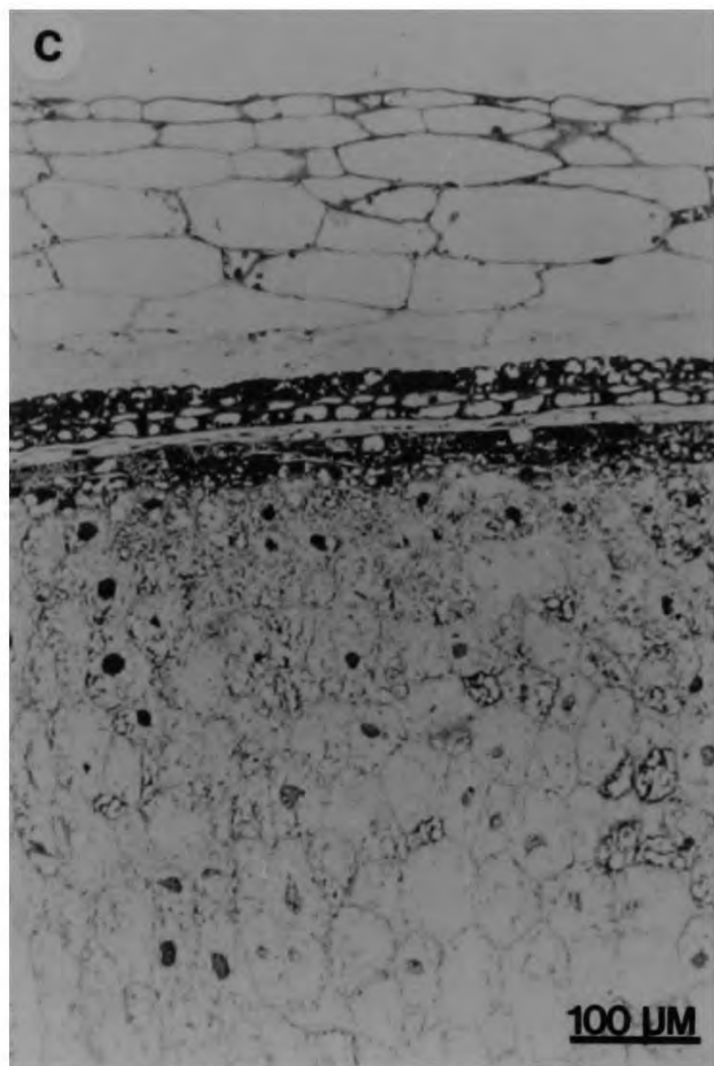
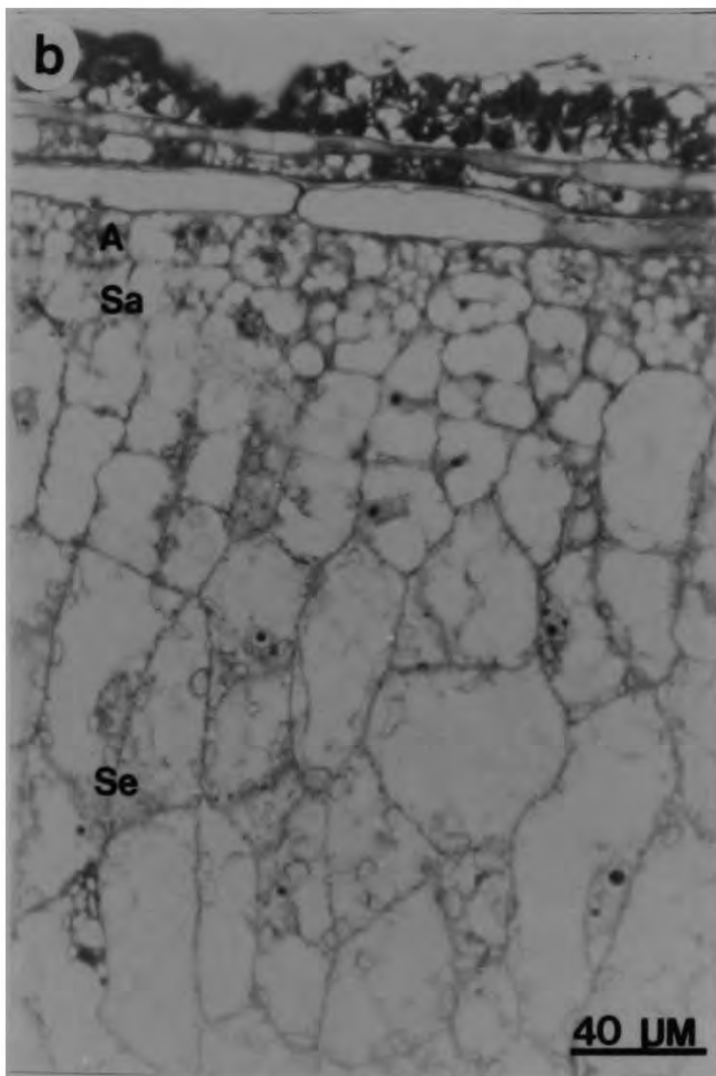


PLATE 2. Light micrographs a-d : transverse sections through the barley caryopsis, stained with toluidine blue.
a : 5 DAA, showing further development of coenocytic endosperm. b : 5 DAA, higher magnification showing development of coenocytic endosperm in the embryo sac.
c : Endosperm of 8 DAA caryopsis is completely cellularized. d : Higher magnification of cellularized endosperm. Nuclei are prominant in the cells.







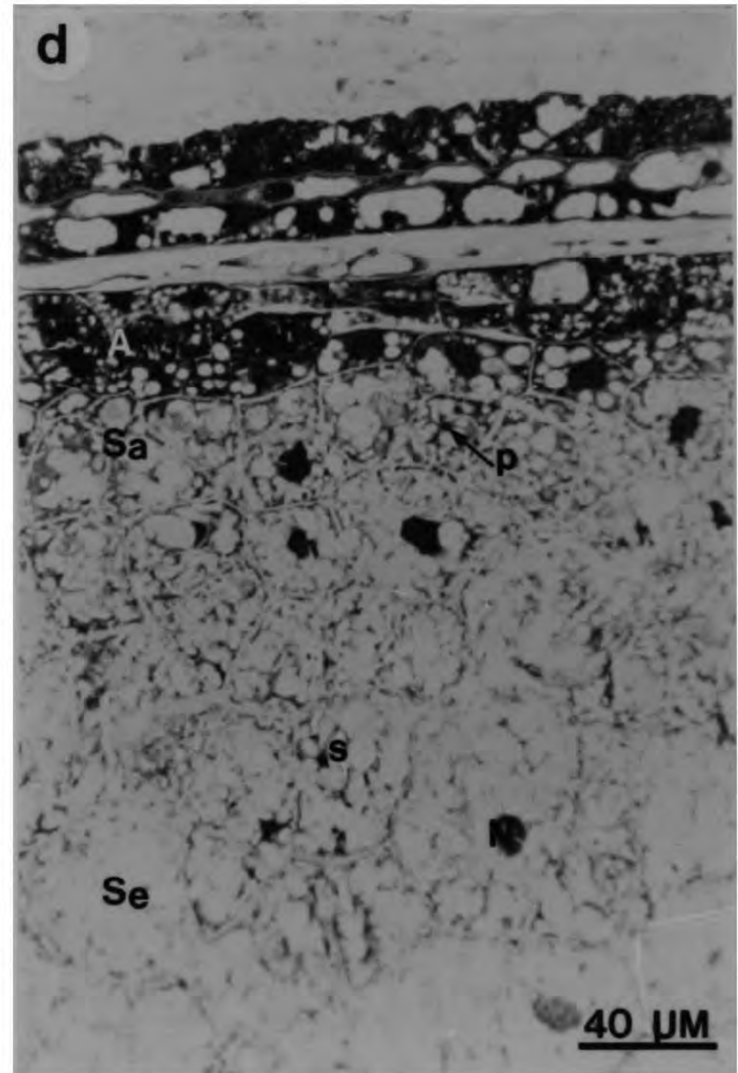
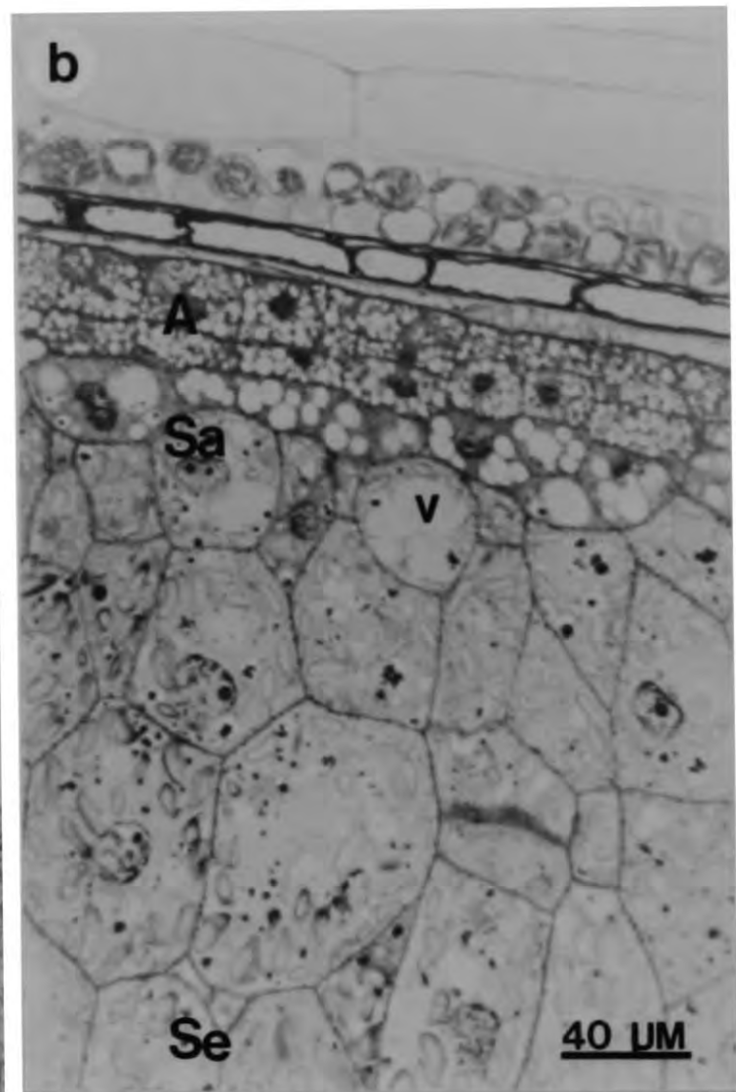
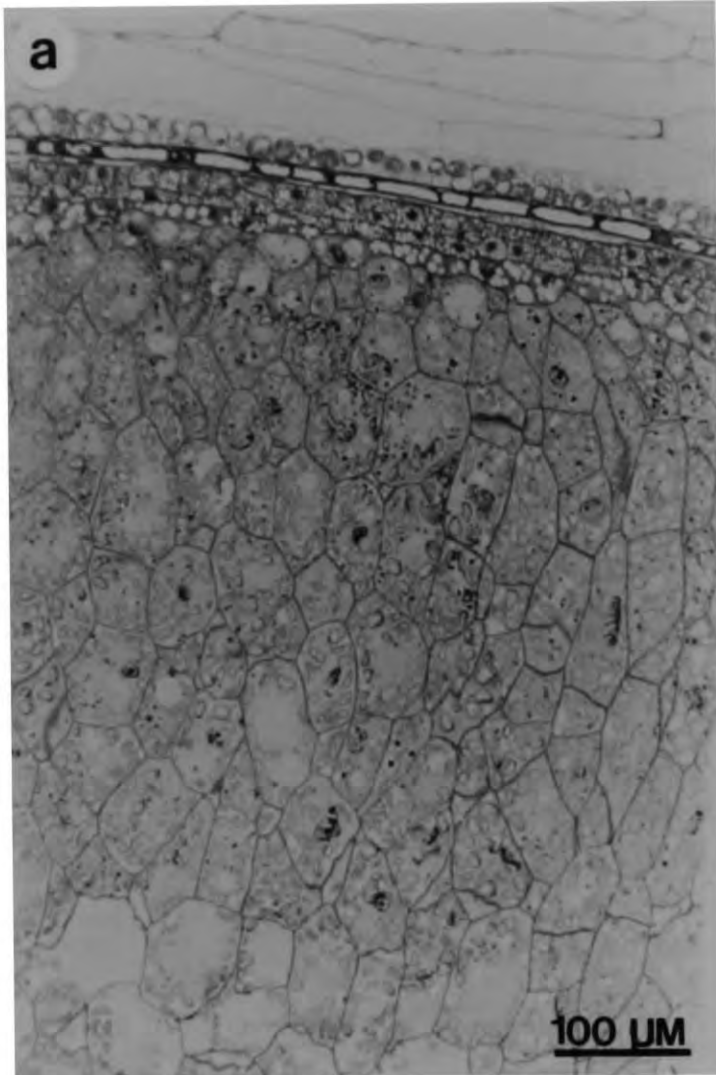


PLATE 3. Light micrographs of transverse sections through the developing barley caryopsis 10 DAA and 12 DAA, stained with toluidine blue.
a : 10 DAA shows differentiation into the starchy endosperm, sub-aleurone and young aleurone cells. **b** : 10 DAA, higher magnification showing development of cell organelles.
c : 12 DAA, aleurone consists of 3 cell layers. **d** : Higher magnification of 12 DAA shows some protein deposits in sub-aleurone. There are many starch granules in the starchy endosperm.





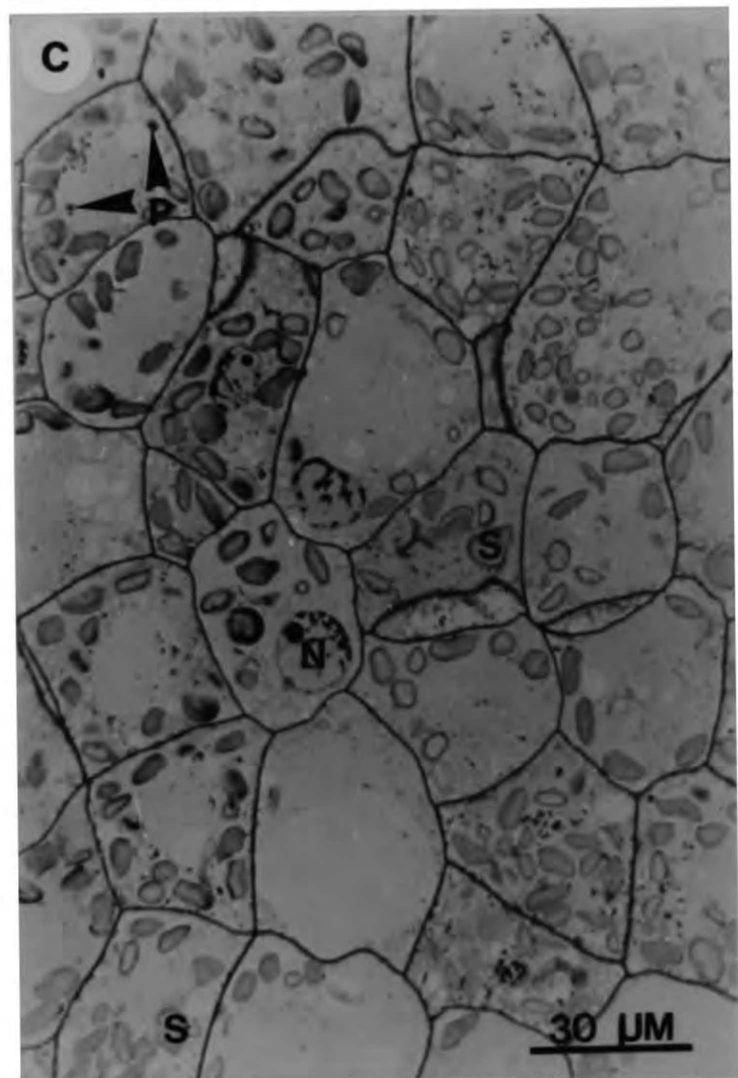
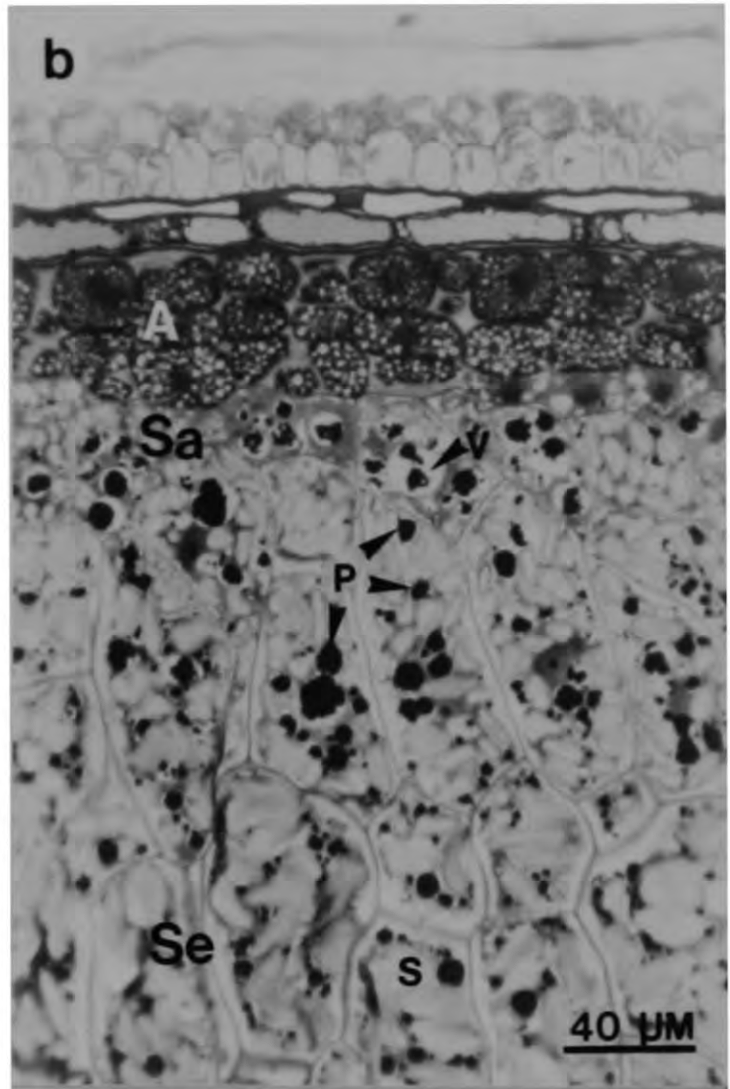


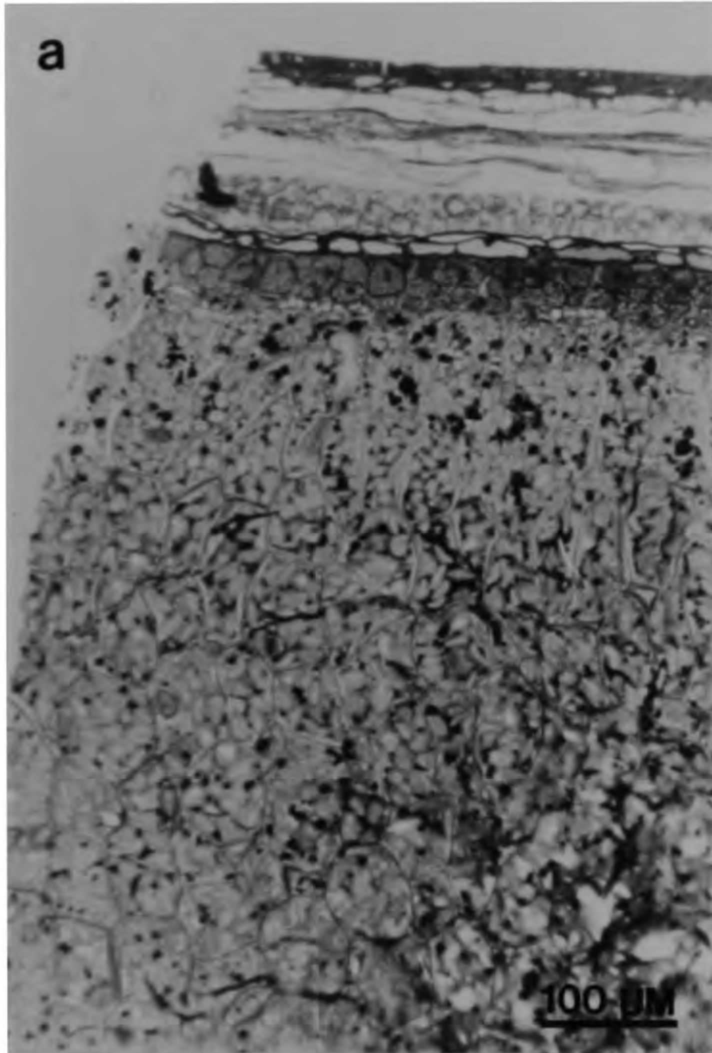
PLATE 4. Light micrographs of transverse sections through the developing caryopsis of barley 14 DAA, stained with toluidine blue.

a : Low magnification micrograph through the caryopsis of a developing barley grain, 14 DAA.

b : Higher magnification of a. Sub-aleurone cells have many vacuoles, some containing protein deposits.

c : High magnification of the starchy endosperm shows fewer protein deposits further into the starchy endosperm.





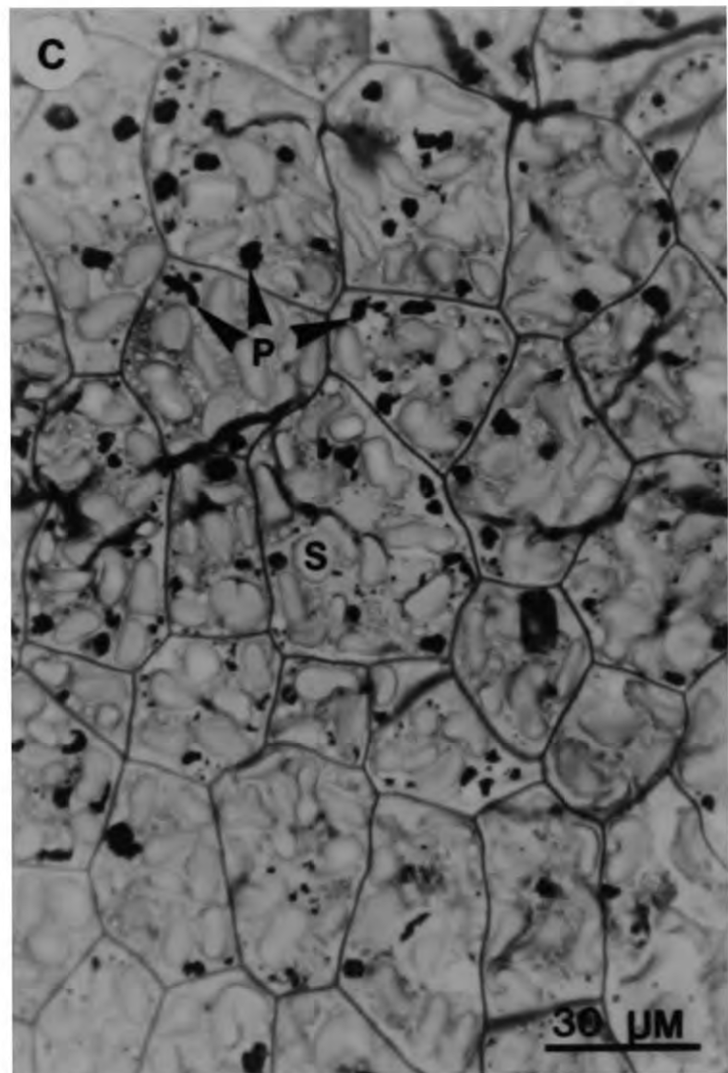
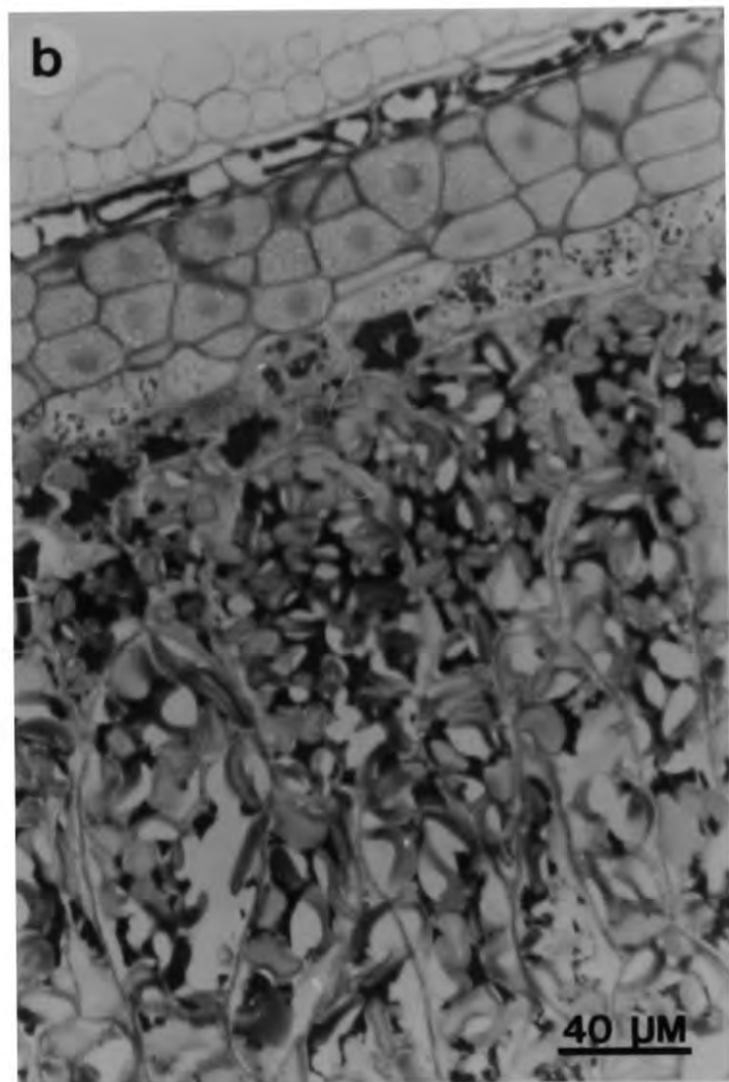
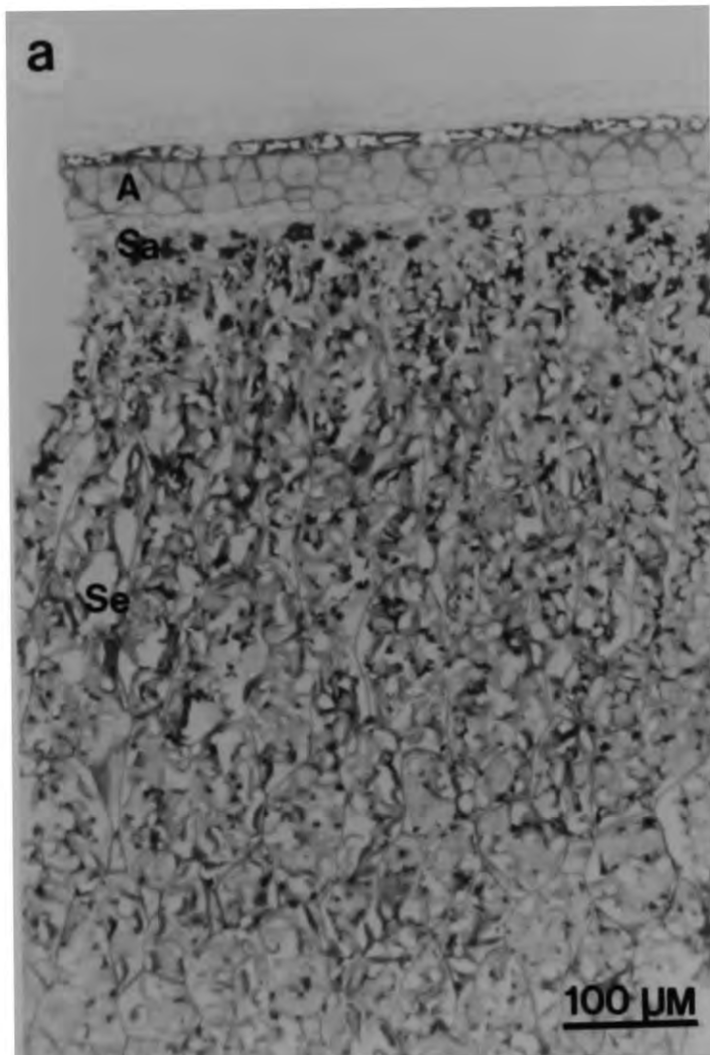


PLATE 5. Light micrographs of transverse sections through the developing barley caryopsis 18 DAA, stained with toluidine blue.
a : Low magnification of transverse section through the barley caryopsis at 18 DAA.
b : Higher magnification shows an increase in protein deposits at this stage in development which appear to be in vacuoles.
c : High magnification of the starchy endosperm shows large and small starch granules with fewer protein deposits further into the starchy endosperm.





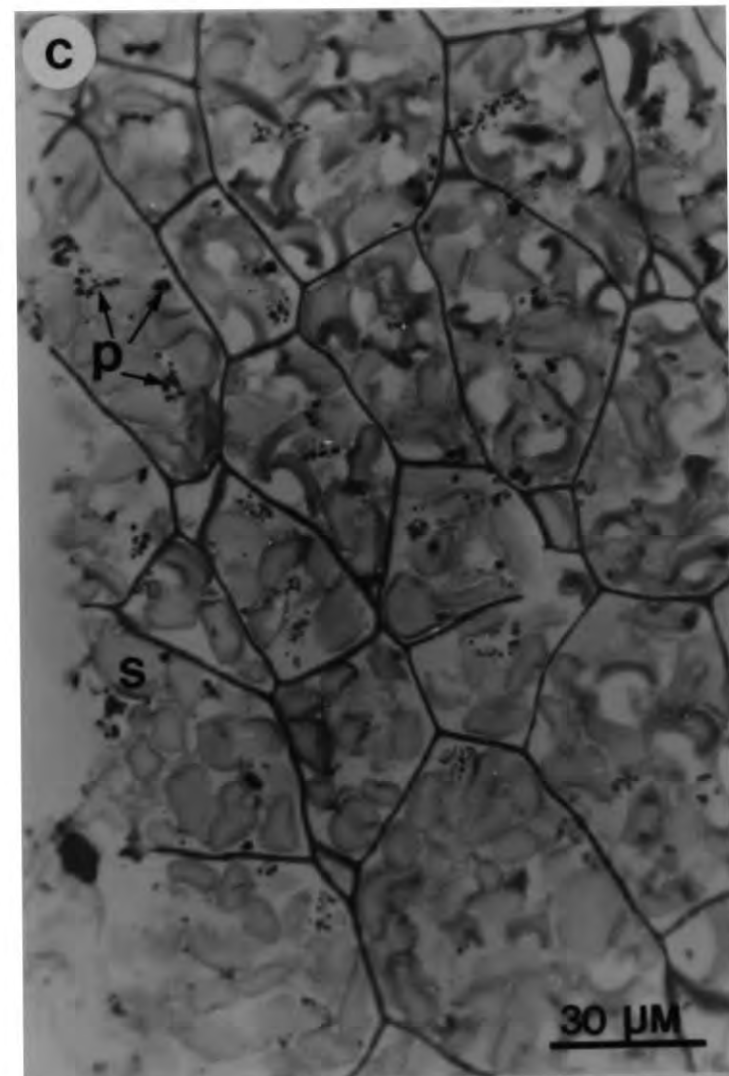
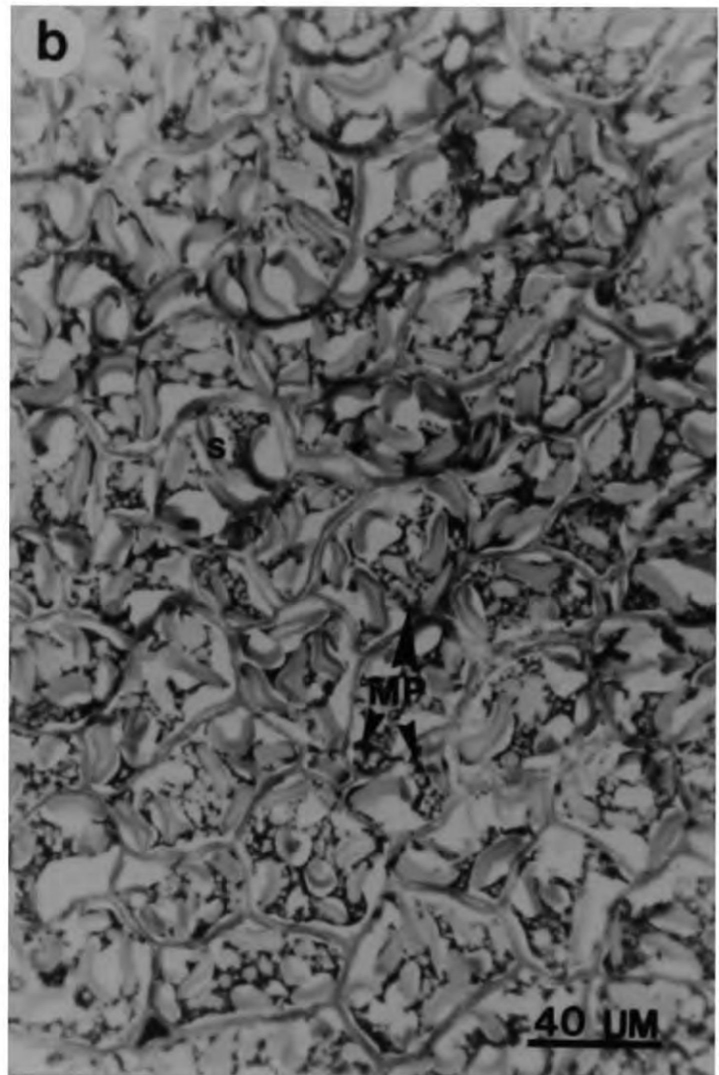
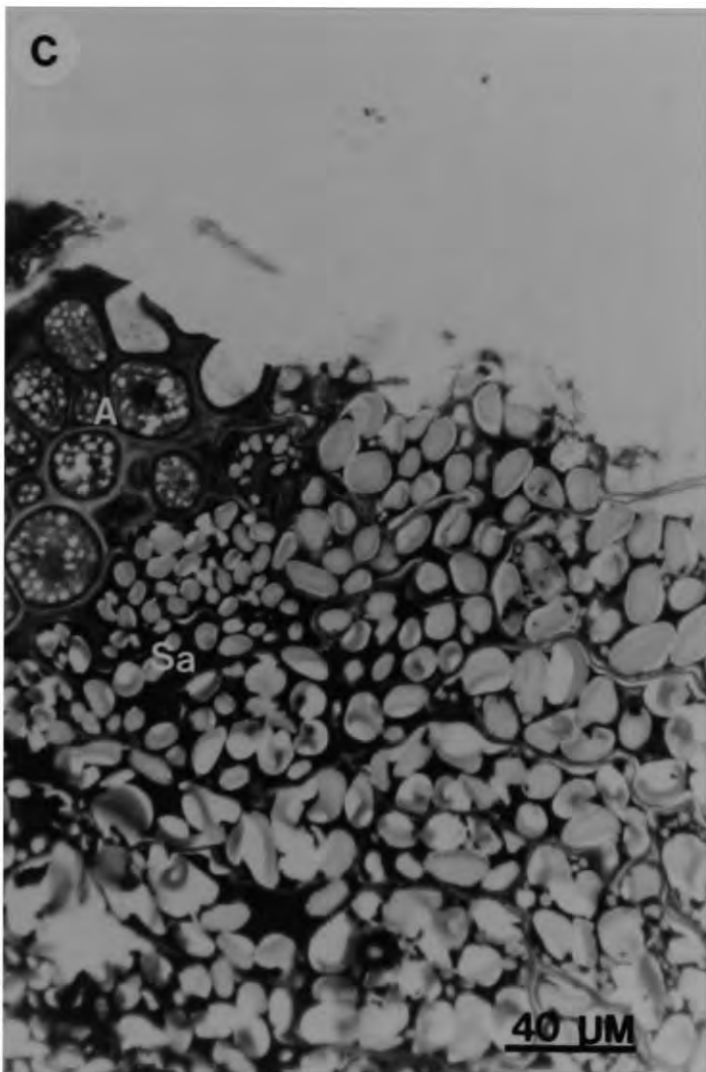


PLATE 6. Light micrographs of transverse sections through the developing barley caryopsis 20 DAA, stained with toluidine blue.
a : Low magnification of transverse section through a barley caryopsis shows much starch deposition 20 DAA. b : Higher magnification shows protein deposits in the sub-aleurone. Further into the endosperm there is a high density of starch granules embedded in a protein matrix.
c : High magnification of the starchy endosperm shows many small protein deposits with numerous large starch granules and small starch granules.





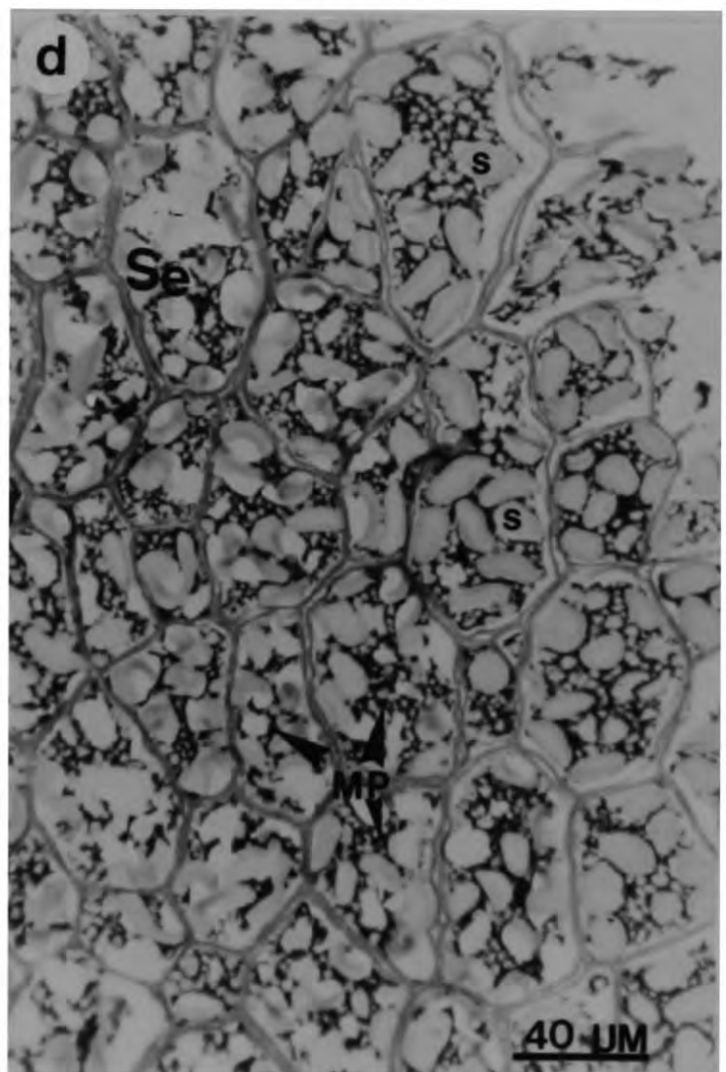


PLATE 7. Light micrographs of transverse sections through the developing barley caryopsis 32 DAA and 45 DAA, stained with toluidine blue.
a : 32 DAA, starch granules in the sub-aleurone are embedded in dense protein. b : In starchy endosperm 32 DAA, large and small starch granules are embedded in a protein matrix.
c : 45 DAA, shows little difference from 32 DAA. Starch granules are embedded in a dense protein. d : Large and small starch granules are embedded in a dense protein matrix. The cell walls are conspicuous.

3.1.2.2 Fluorescence Light Microscopy of Transverse Sections of the Barley Caryopsis Stained with 0.1% ANS (8-anilino-1-naphthalene sulphonic acid)

Plate 8 (a, b) and 9 (c, d) show transverse sections through a barley caryopsis 14 DAA stained with 0.1% ANS to illustrate the distribution of protein deposits and starch granules in the aleurone, sub-aleurone, and starchy endosperm.

The fluorescence micrograph distinguishes protein deposits from starch granules by colour. The former are visible as blue depositions, and the latter are white. There is an absence of protein deposits in the aleurone, but many in the sub-aleurone layer (micrograph 8 a). In micrograph 8b, the peripheral endosperm contains the highest density of protein deposits; there are fewer of such deposits further into the prismatic endosperm. In the central endosperm (micrograph 9 c), there are progressively fewer protein deposits towards the centre of the grain. The large cells of the central endosperm (micrograph 9 d) contain many large and small starch granules with only occasional protein deposits.

PLATE 8. Transverse section through a barley caryopsis 14 DAA stained with 0.1% ANS to show distribution of starch granules and protein deposits.
a : Sub-aleurone layer has many protein deposits and few starch granules. The peripheral starchy endosperm has many large protein deposits.
b : The peripheral starchy endosperm (LHS of micrograph) shows many protein bodies, but their numbers decrease further into the prismatic endosperm (RHS).

PLATE 9.

c : There are fewer protein deposits in the cells of the prismatic endosperm (LHS of micrograph) and central endosperm cells (RHS).

d : In the large cells of the central starchy endosperm there are many large and small starch granules with occasional protein deposits.

3.1.3 Ultrastructure of the Developing Barley Endosperm from Thin Sections

At 10 DAA the outer three cell layers of the endosperm are differentiated to form the aleurone (Plate 10A). These cells are roughly cuboid in shape with large nuclei, and the cytoplasm is densely packed with endoplasmic reticulum and organelles. At this stage, the endosperm differentiates to form the sub-aleurone, underlying the aleurone, and the outer and inner starchy endosperm.

At 10 DAA, although in the sub-aleurone (Plate 10B) protein bodies are both cytoplasmic and vacuolar, there is an absence of storage protein bodies in the aleurone. In the outer endosperm (Plate 10C) protein bodies are also vacuolar and cytoplasmic. These protein bodies are seen as individual granular deposits with vesicles around the periphery. Endoplasmic reticulum encircles many of the vacuoles. Further into the starchy endosperm, no protein bodies are evident at this stage in development. At a higher magnification (Plate 11A, C), endoplasmic reticulum surrounds an area in the cytoplasm, the contents of which appear denser than the cytoplasm. In Plate 11B, a dictyosome is evident in the cytoplasm. This is only noticeable in thicker-sectioning of a specimen subjected to osmium tetroxide for longer than in normal fixation regimes.

At 14 DAA (Plate 12A) further development of the sub-aleurone is seen. In particular, protein deposition increases, with many of these deposits located in vacuoles. Starch grains begin to accumulate within the cells of the sub-aleurone. Many mitochondria are aligned along the cell walls, and there is an increase in endoplasmic reticulum. In Plate 12B protein aggregates are seen in vacuoles surrounded by endoplasmic reticulum.

Plate 13 illustrates protein body development 14 DAA; micrograph 13A shows single protein bodies in vacuoles of the sub-aleurone. Mitochondria are regularly aligned along cell walls. A lipid body is evident in the cytoplasm, although such deposits are more numerous in cells of the aleurone (Plate 13A, bottom left; Plate 12A, top). Micrograph 13B illustrates that protein complexes are also found in vacuoles of the sub-aleurone. Endoplasmic reticulum surrounding the protein vacuole is prominent. The protein complex at this developmental stage consists of a large granular component and a smaller proportion of a fibrillar component, termed 'fibrillar' by Cameron-Mills and von Wettstein (1980).

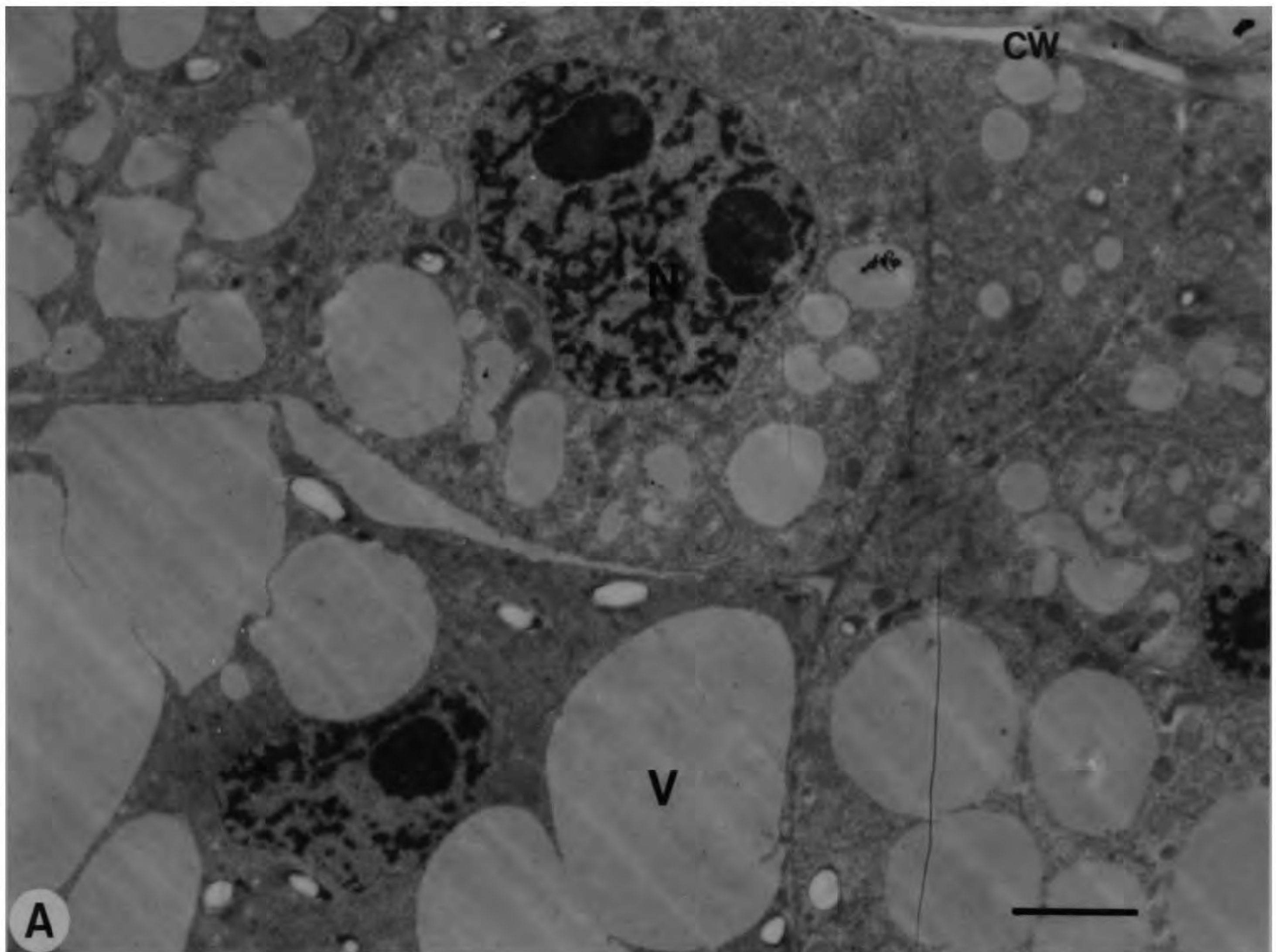
In the endosperm at 14 DAA (Plate 14A), protein bodies are both vacuolar and cytoplasmic. Both types are within close proximity to endoplasmic reticulum.

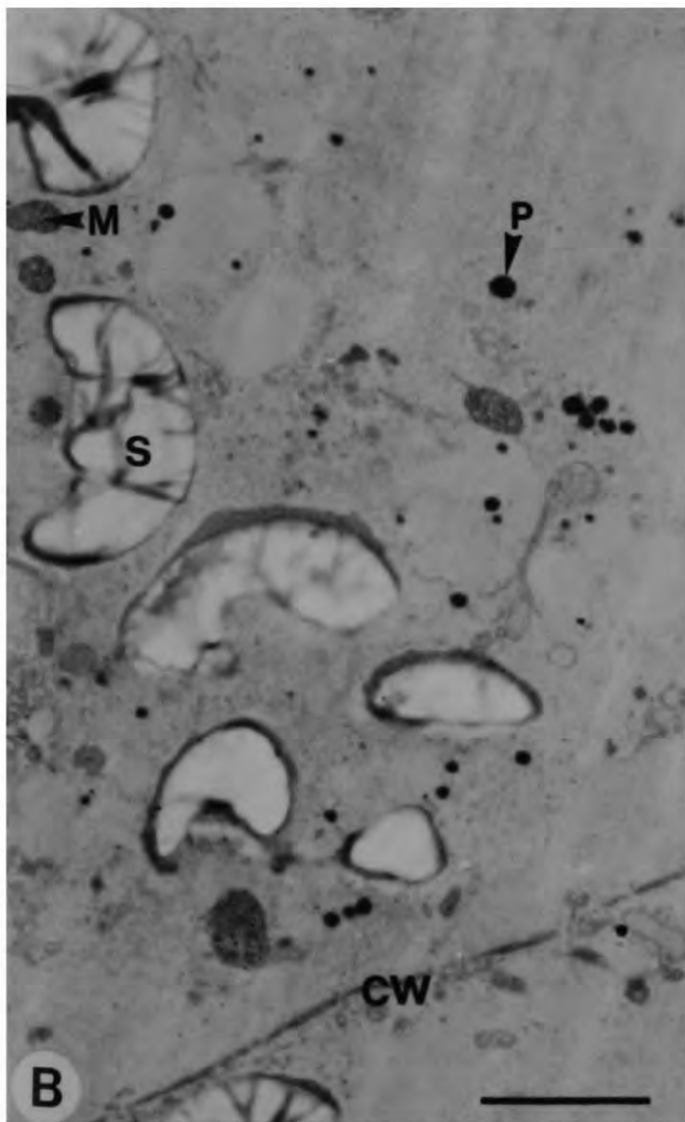
Plate 14B, shows endoplasmic reticulum surrounding a vacuole which contains a protein complex in an endosperm cell.

In Plate 15, micrograph A shows the aleurone and sub-aleurone 18 DAA. Within the aleurone cells there are numerous vacuoles each containing a dense deposit of a peripheral matrix with a transparent central core. In the sub-aleurone, large protein aggregates accumulate in the vacuoles of the cells. In the starchy endosperm (Plate 15B), most protein complexes are located in vacuoles. Vesicles surround the peripheries of the protein complexes.

Plate 16A illustrates sub-aleurone protein aggregates at 18 DAA, which are mainly vacuolar and consist of granular spheres embedded in a fibrillar matrix. In micrograph 16B the protein complex in the endosperm consists, at this time, of large granular areas with smaller fibrillar and electron-dense components. Complex protein bodies in the endosperm have vesicles around the periphery (Plate 16C).

At 20 DAA, the sub-aleurone structure (Plate 17A) shows little change from that observed at 18 DAA (Plate 15A), with protein aggregates located mostly in vacuoles. In the endosperm (Plate 17B), protein complexes are compressed into angular shapes between the large and small starch granules filling the cells.





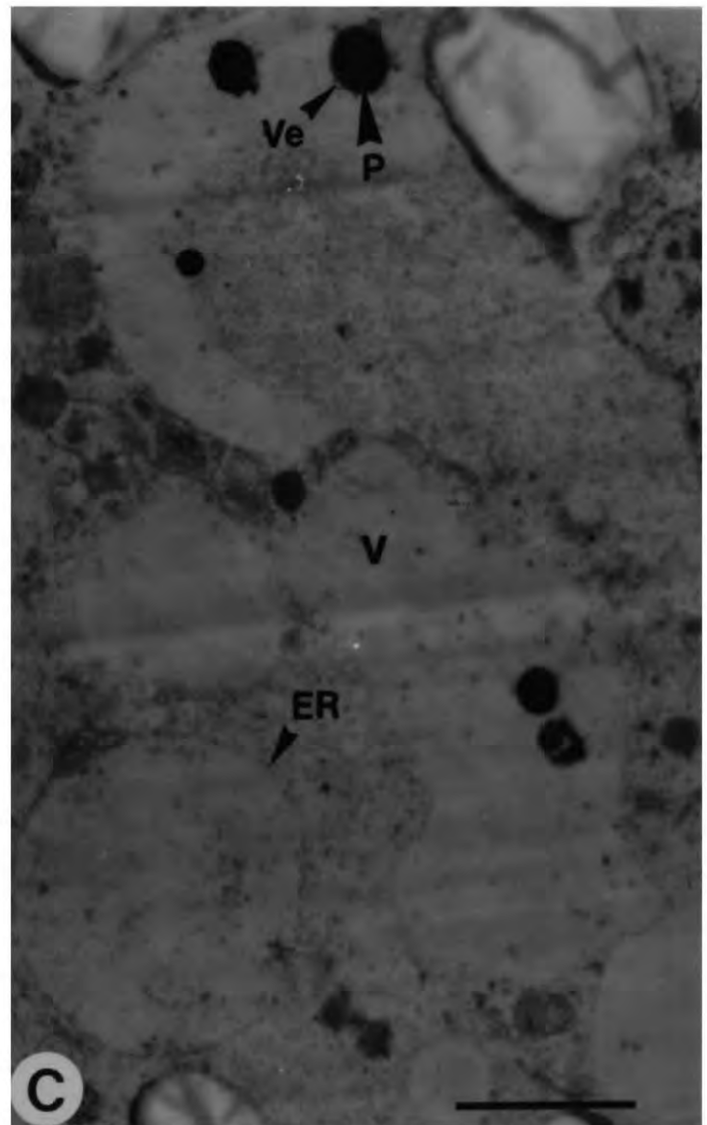
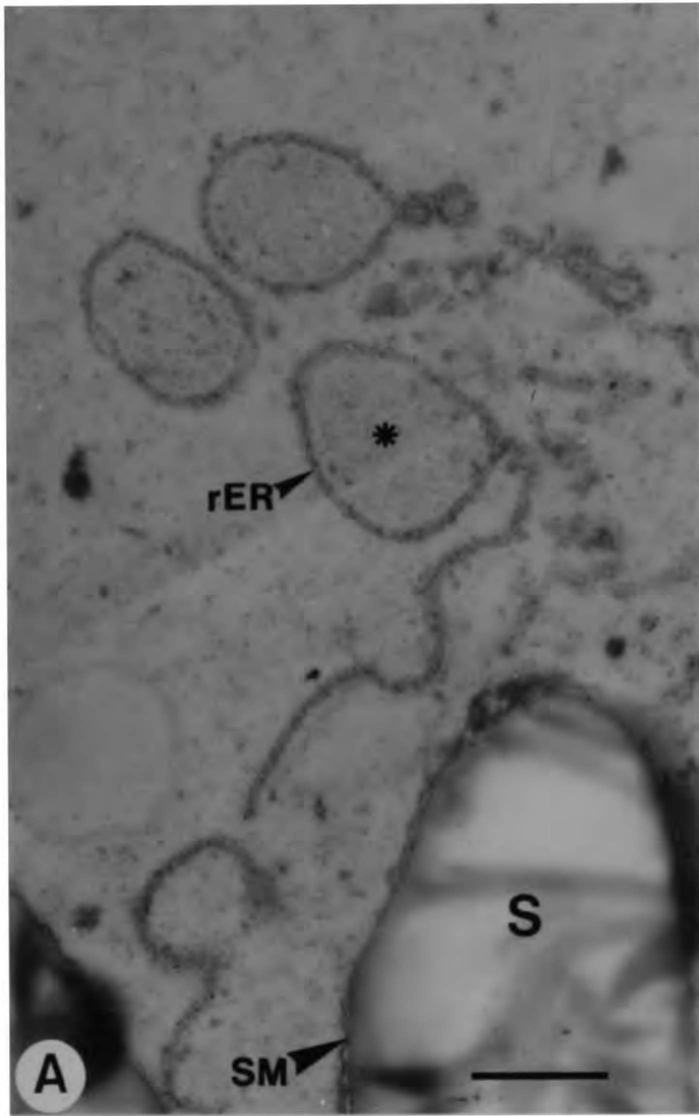
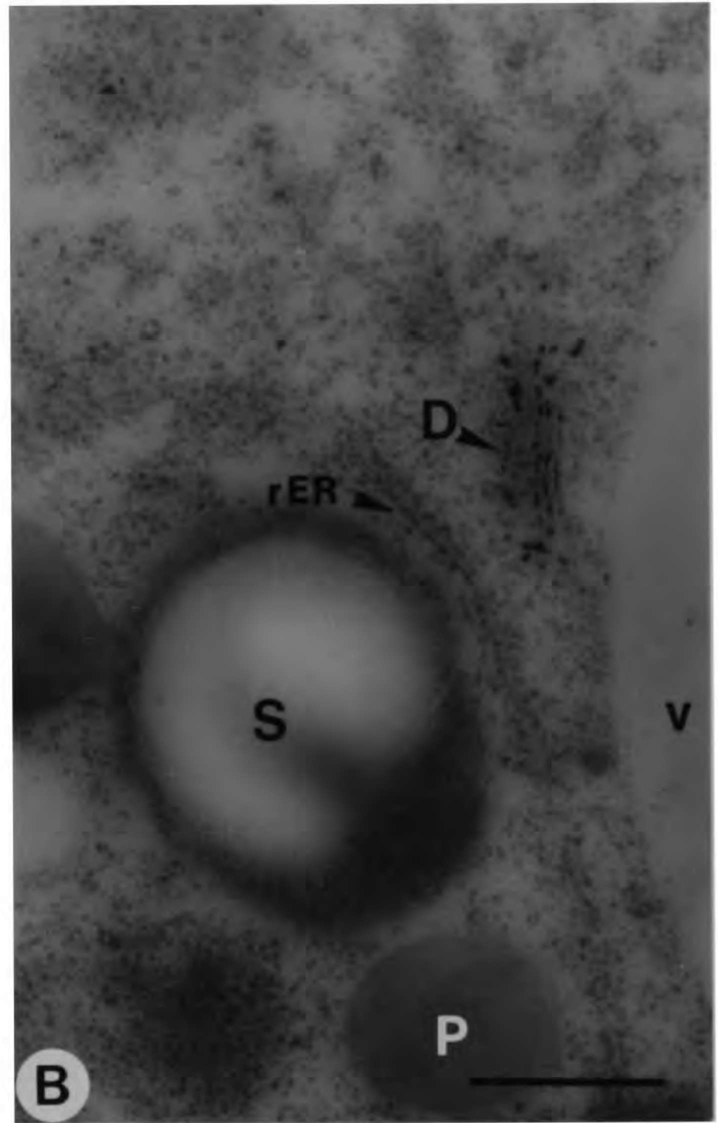


PLATE 10. Electron micrographs of an ultrathin section through a barley caryopsis 10 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.
A : Aleurone of 26 mg barley grain. Protein deposits are absent. Bar = 3 µm.
B : In the outer starchy endosperm, single protein deposits are vacuolar and cytoplasmic. Bar = 4 µm.
C : Higher magnification of the outer starchy endosperm shows single protein deposits in vacuoles. These protein deposits have many small peripheral vesicles. Bar = 2 µm.





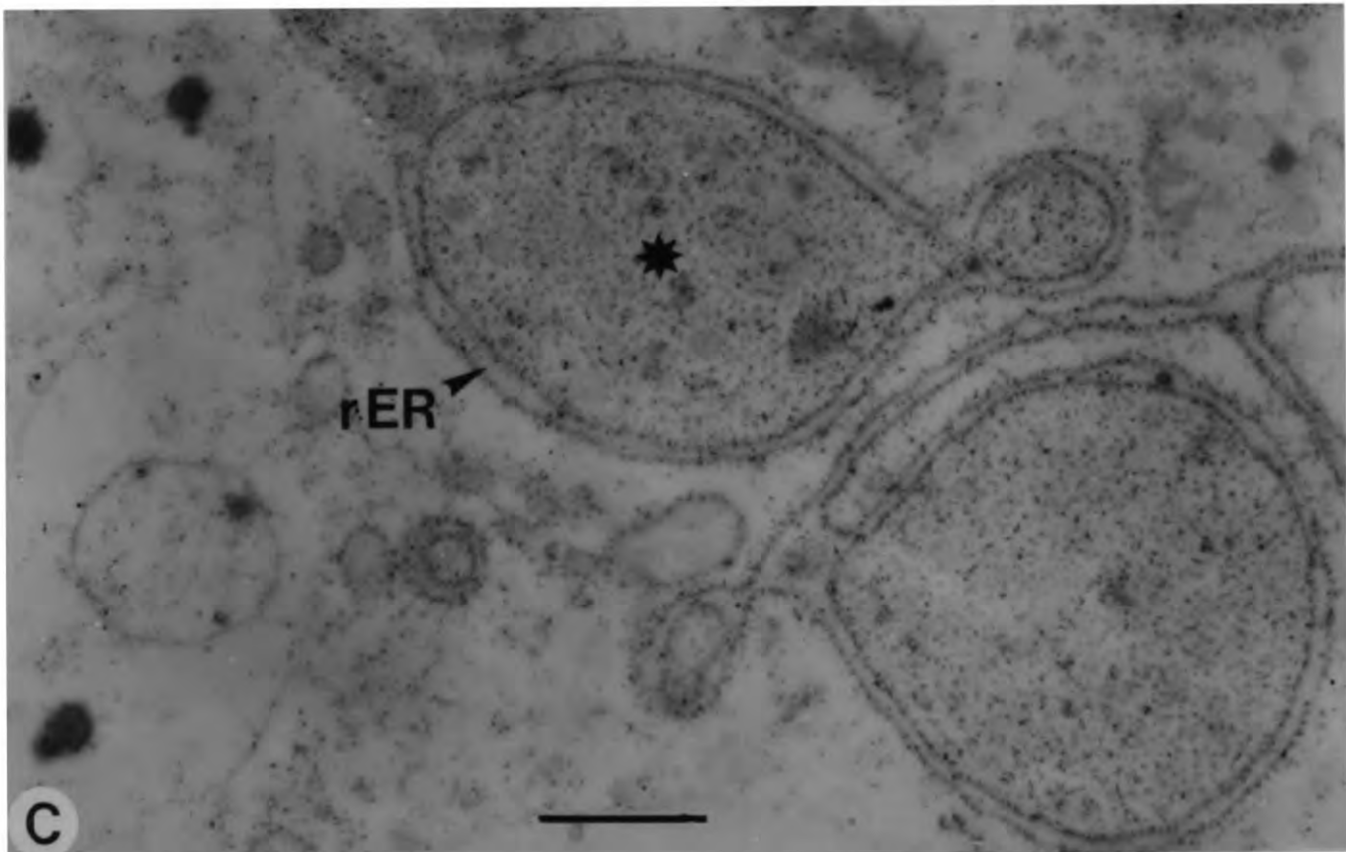
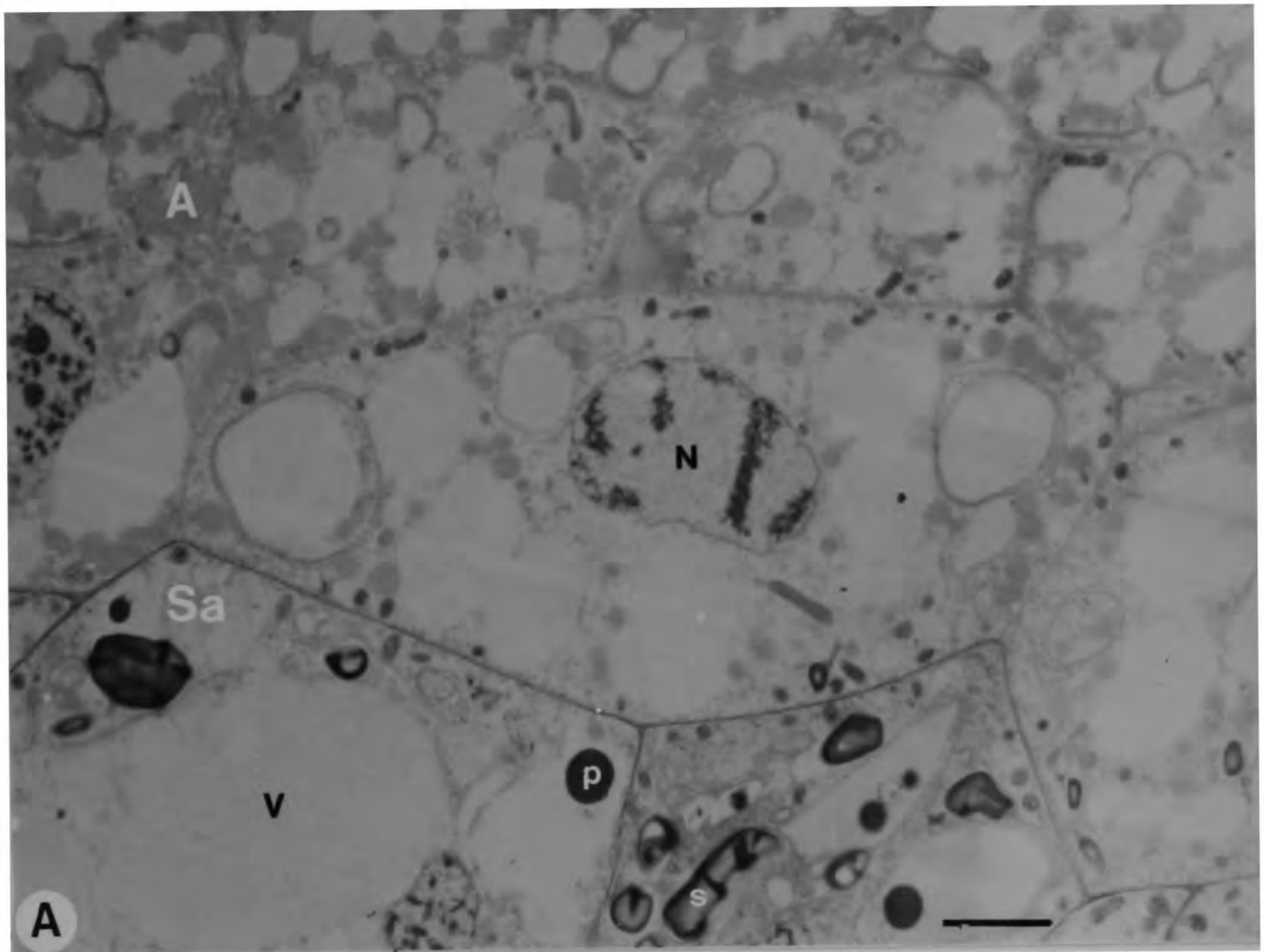


PLATE 11. Electron micrographs showing ultrastructure of the sub-aleurone of a barley caryopsis 10 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : Endoplasmic reticulum is studded with ribosomes and has a circular configuration. Membrane round starch granule is evident. Bar = 1 μm.

B : Single protein body in cytoplasm. Dictyosome is evident due to an "over-osmification" effect. Bar = 0.5 μm.

C : Contents of areas surrounded by rough endoplasmic reticulum (*) are denser than cytoplasm. Bar = 1.5 μm.



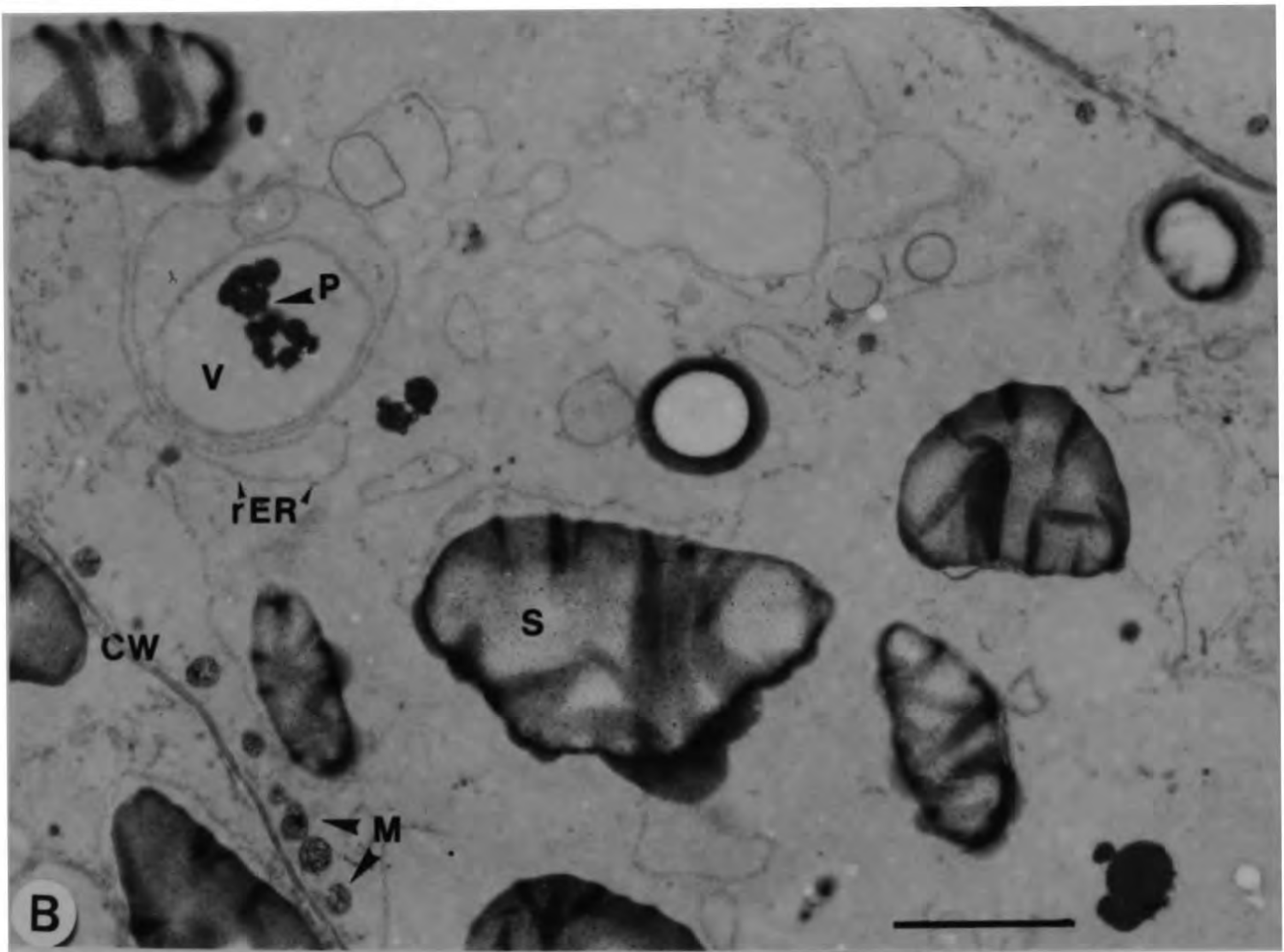
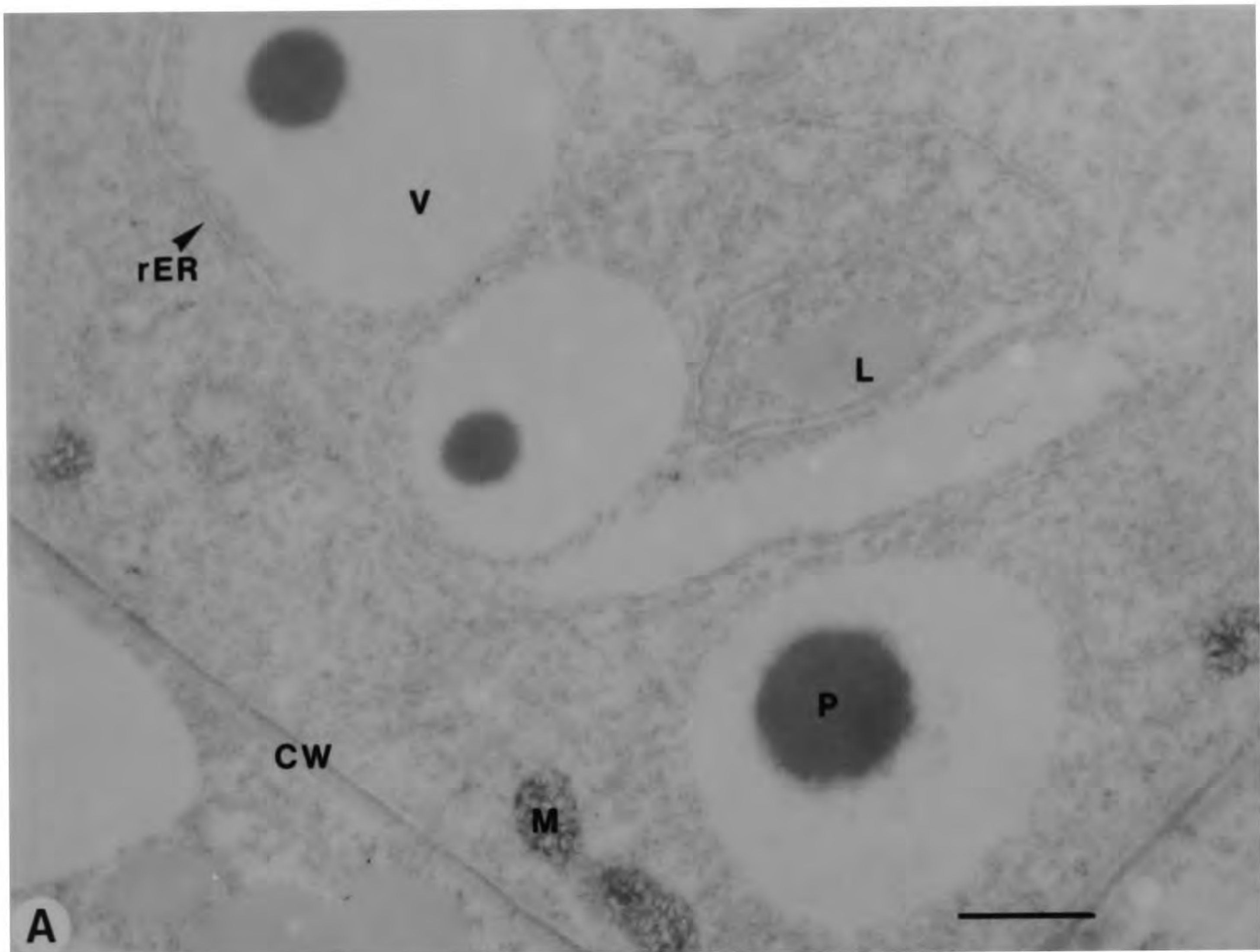


PLATE 12. Electron micrographs of an ultrathin section through a barley caryopsis 14 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.
A : Aleurone of 42 mg barley grain. Single protein bodies are seen in vacuoles of the sub-aleurone. Bar = 4 µm.
B : In the outer starchy endosperm, complex protein bodies are seen in the cytoplasm and also in vacuoles surrounded by rough endoplasmic reticulum. Bar = 4 µm.



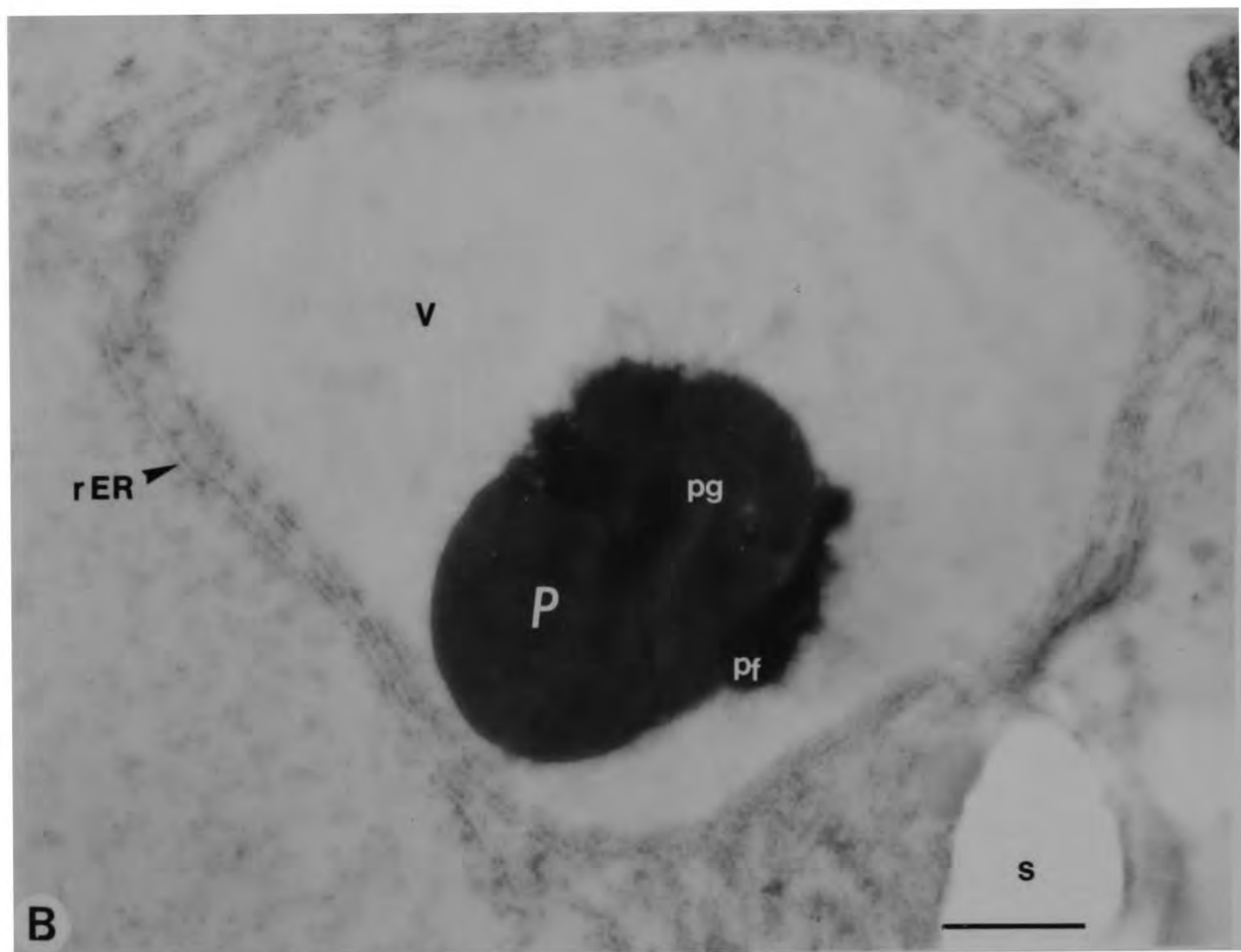
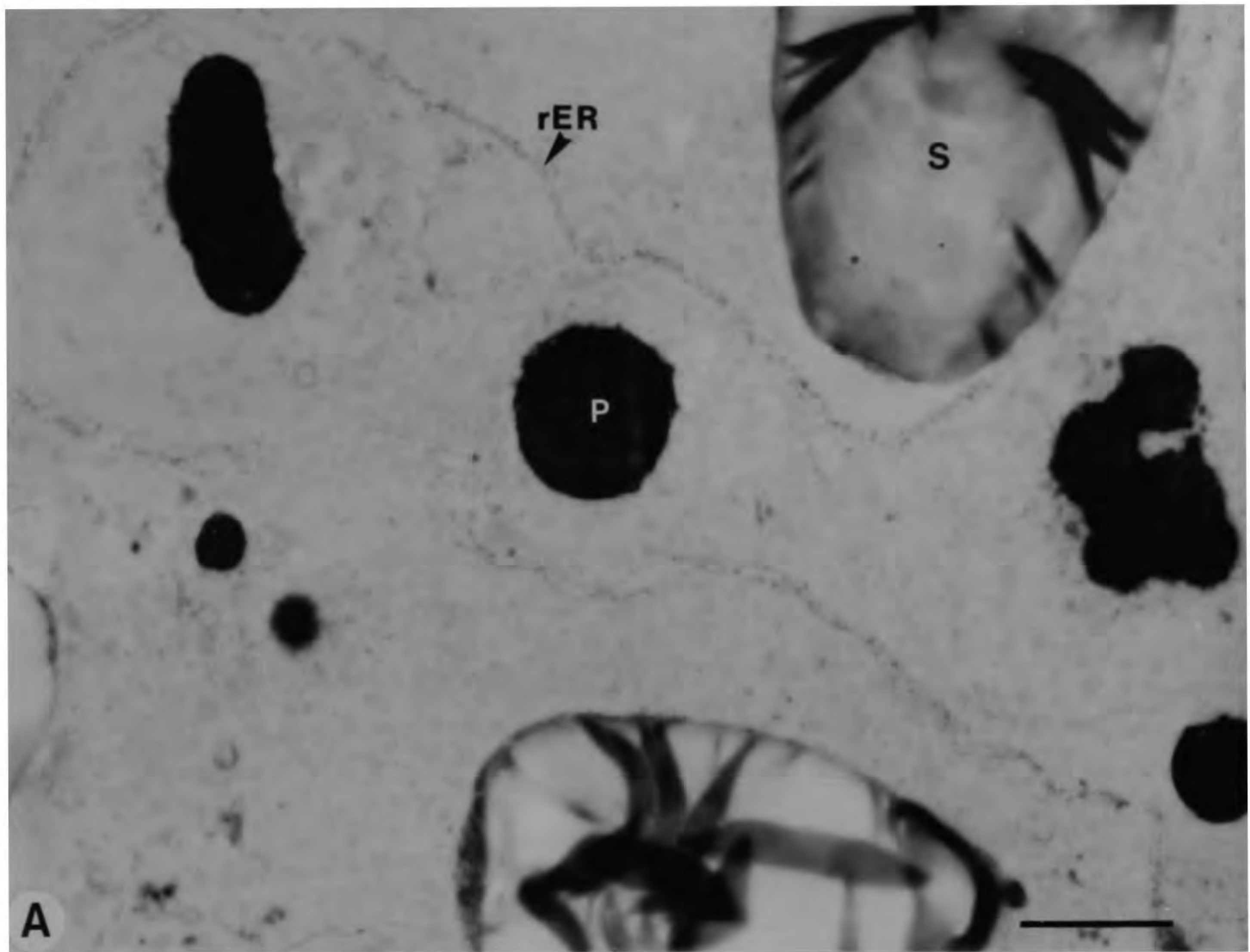


PLATE 13. Electron micrographs showing protein body ultrastructure of the sub-aleurone of a barley caryopsis 14 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : In the sub-aleurone, single protein bodies are in vacuoles. Bar = 1.5 μm.

B : Protein body complexes comprised of granular and fibrillar components. As with single protein bodies, these are located in vacuoles which are closely associated with rough endoplasmic reticulum. Bar = 1 μm.



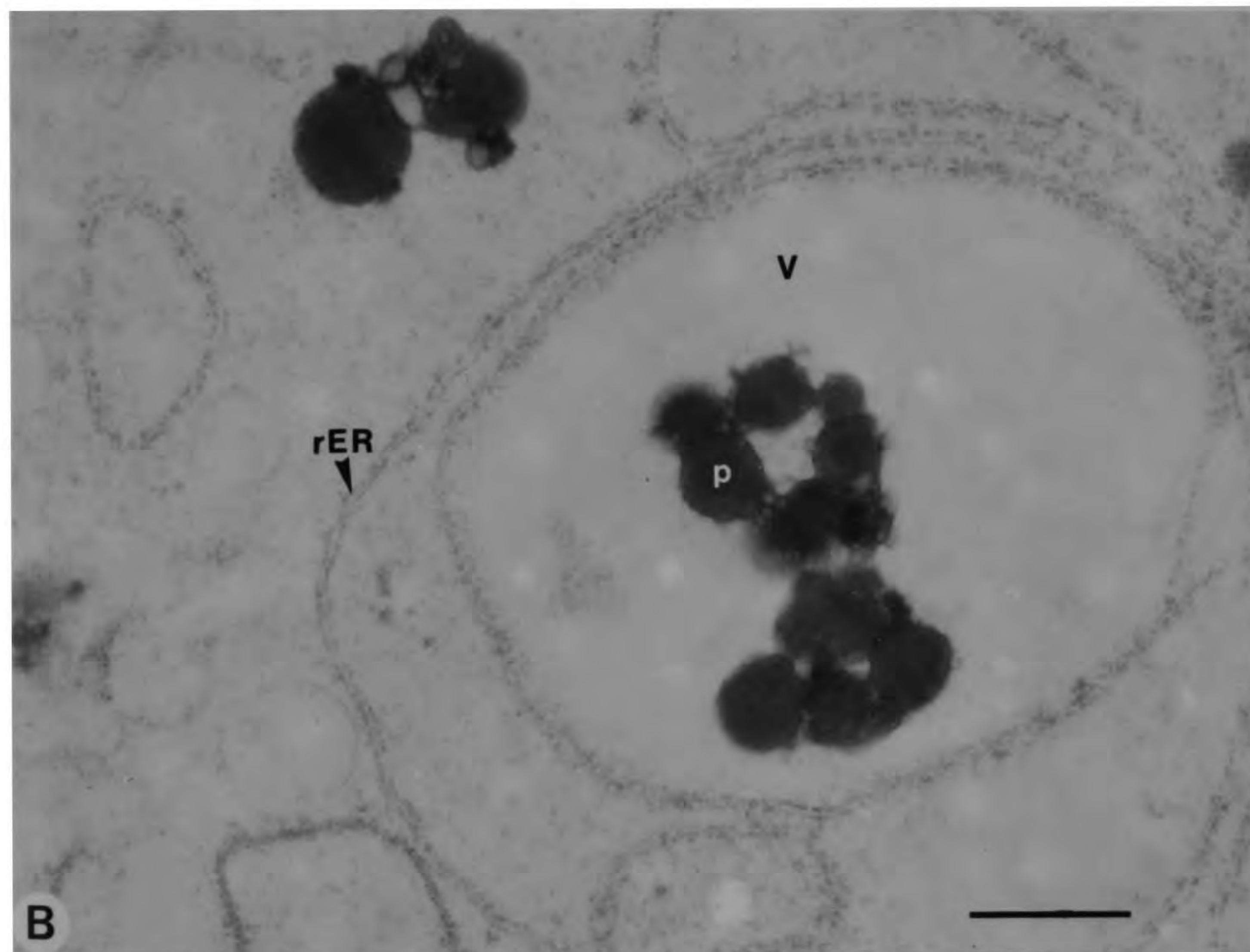
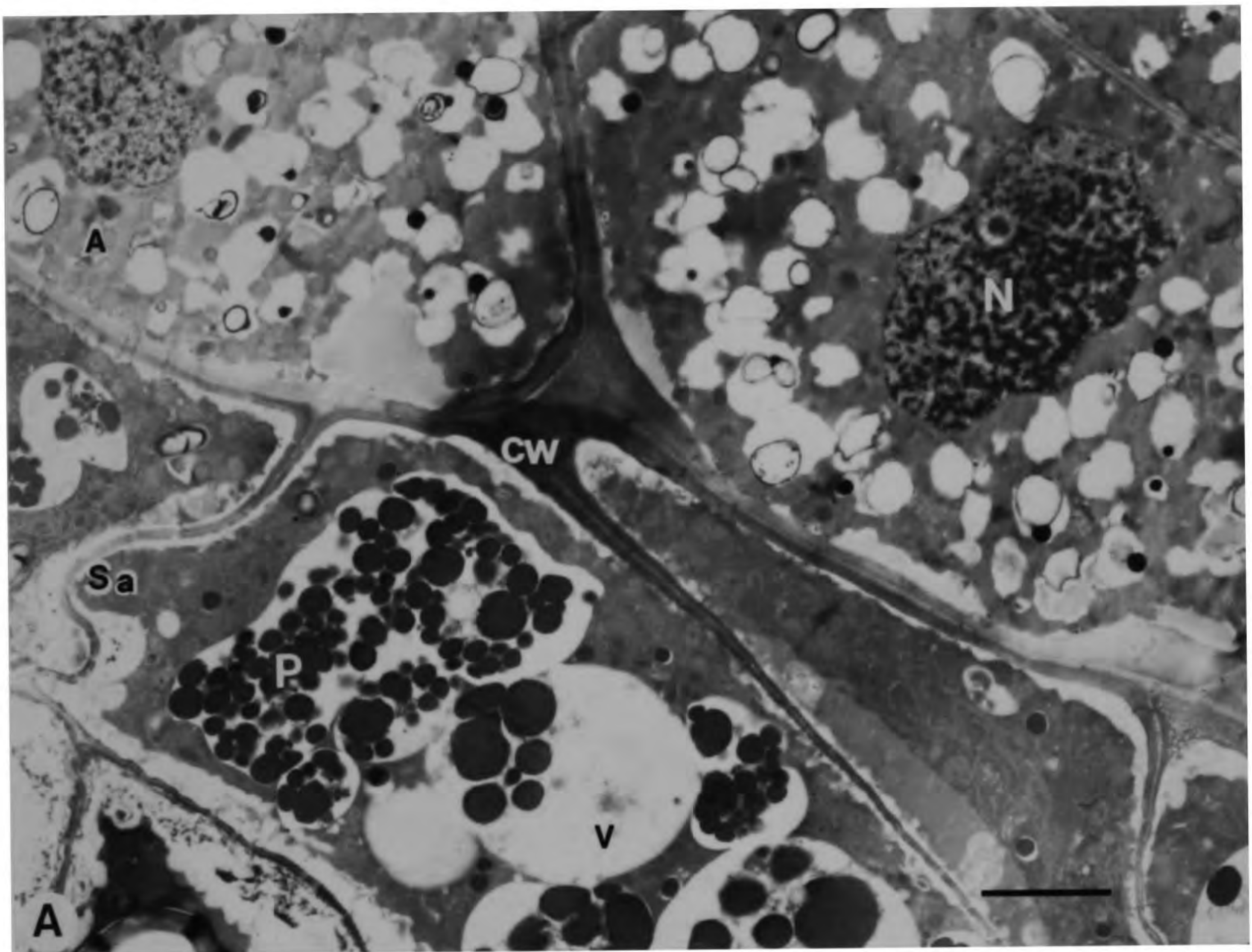


PLATE 14. Electron micrographs showing protein body ultrastructure in the endosperm of a barley caryopsis 14 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.
A : Single and complex protein bodies, both comprised of granular and fibrillar components, are found in vacuoles and in the cytoplasm of the starchy endosperm. Bar = 2 μm.
B : Detail of protein aggregates found in the cytoplasm and in vacuoles. Rough endoplasmic reticulum surrounds the vacuole. Bar = 1 μm.



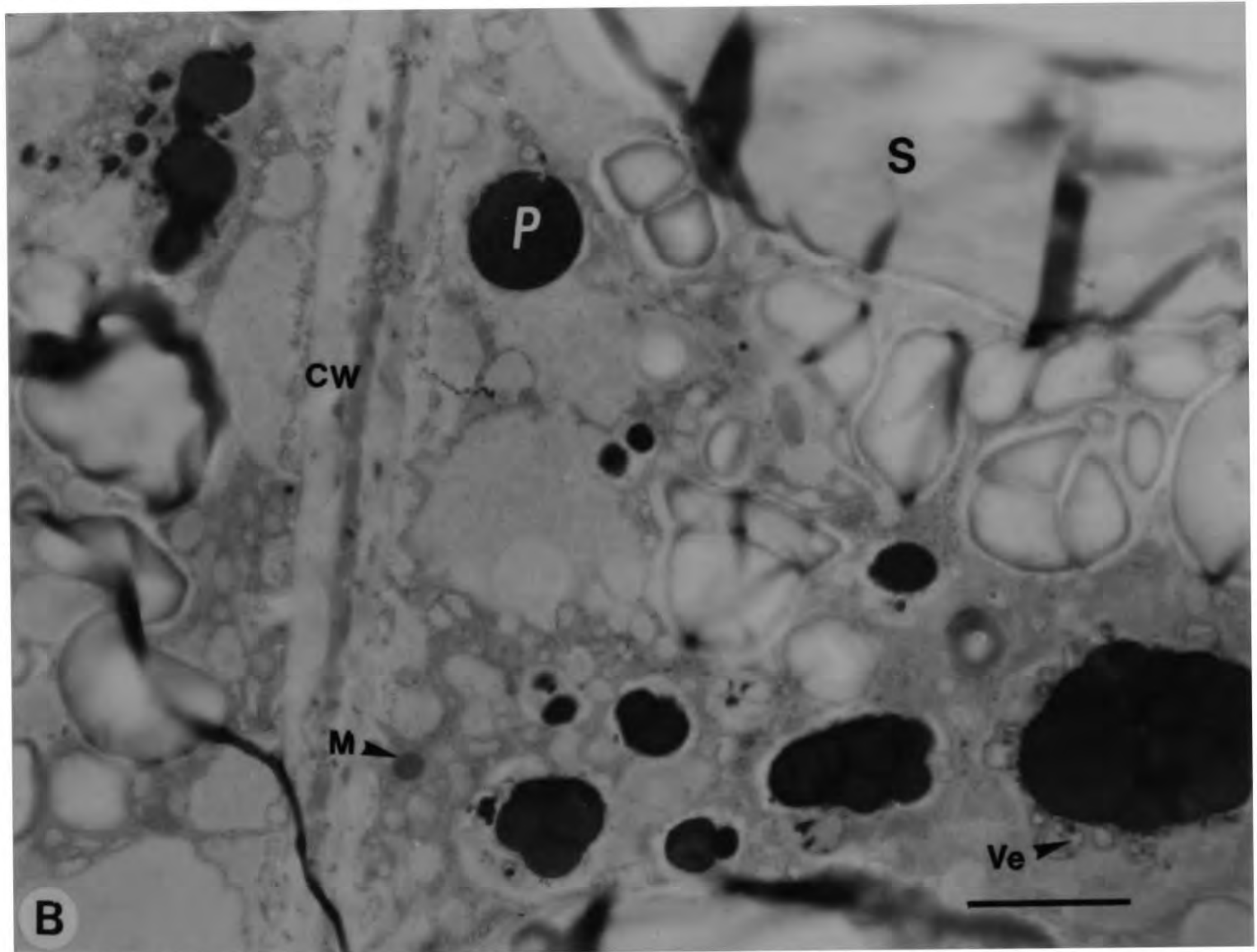
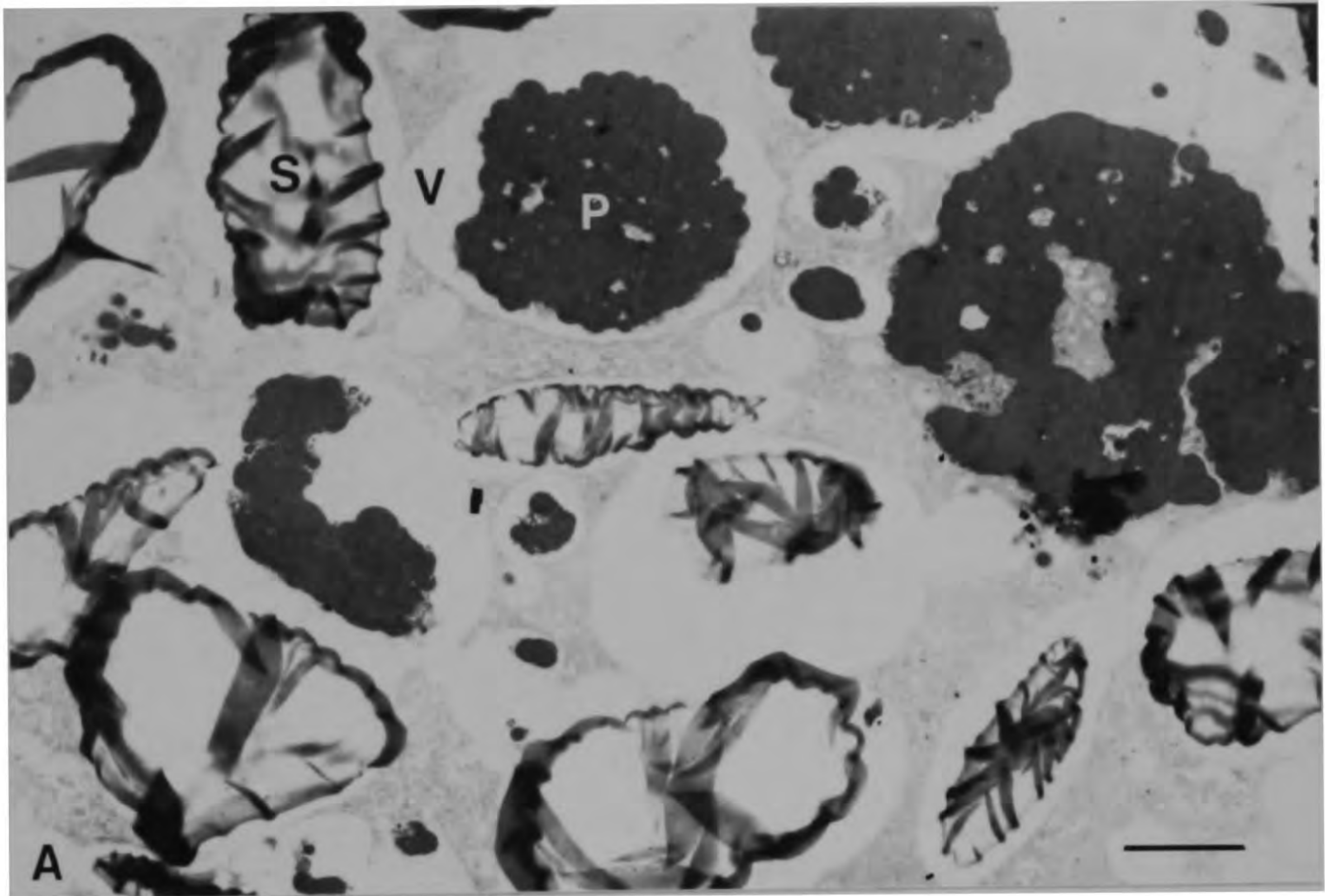
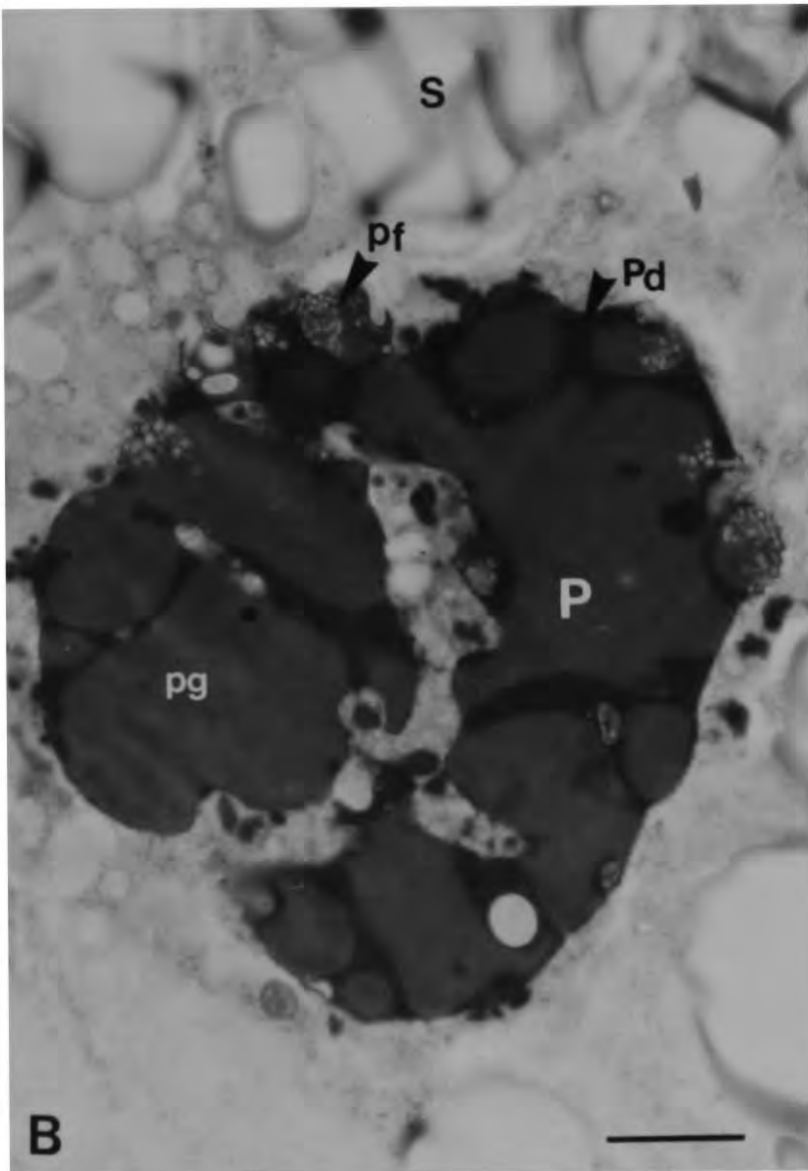


PLATE 15. Electron micrographs of an ultrathin section through a barley caryopsis 18 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : Large vacuoles of the sub-aleurone of a 58 mg grain are packed with many single protein bodies. Bar = 4 µm.

B : In the starchy endosperm, protein bodies are mainly complex, and are located in both the cytoplasm and in vacuoles. Peripheral vesicles surround multiple protein bodies. Large and small starch granules are seen. Bar = 3 µm.





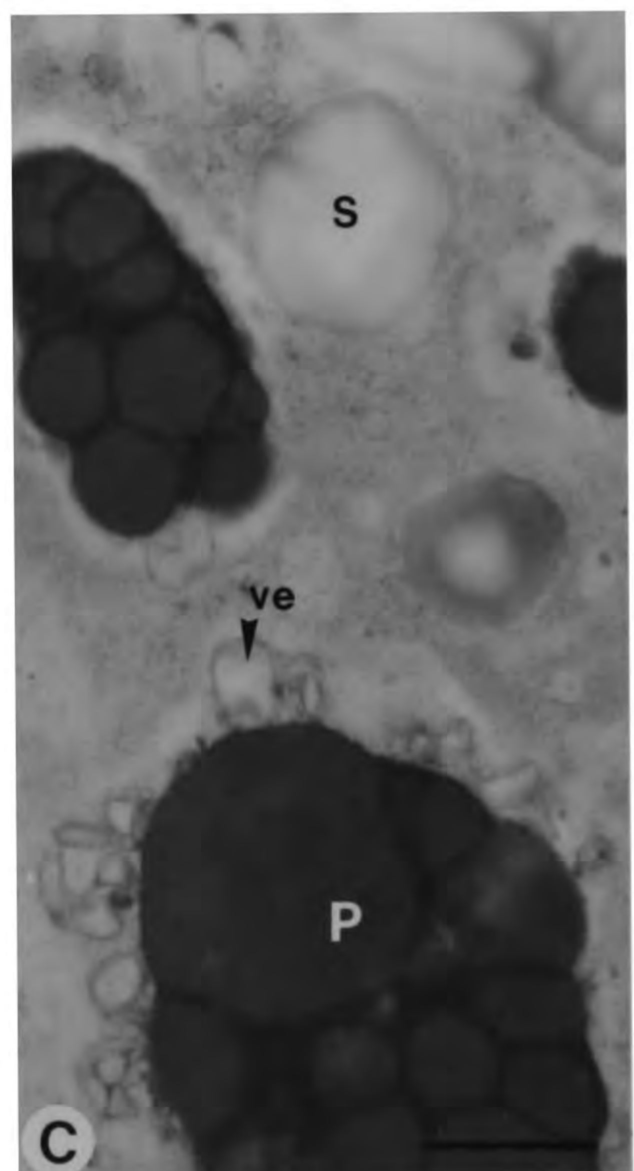
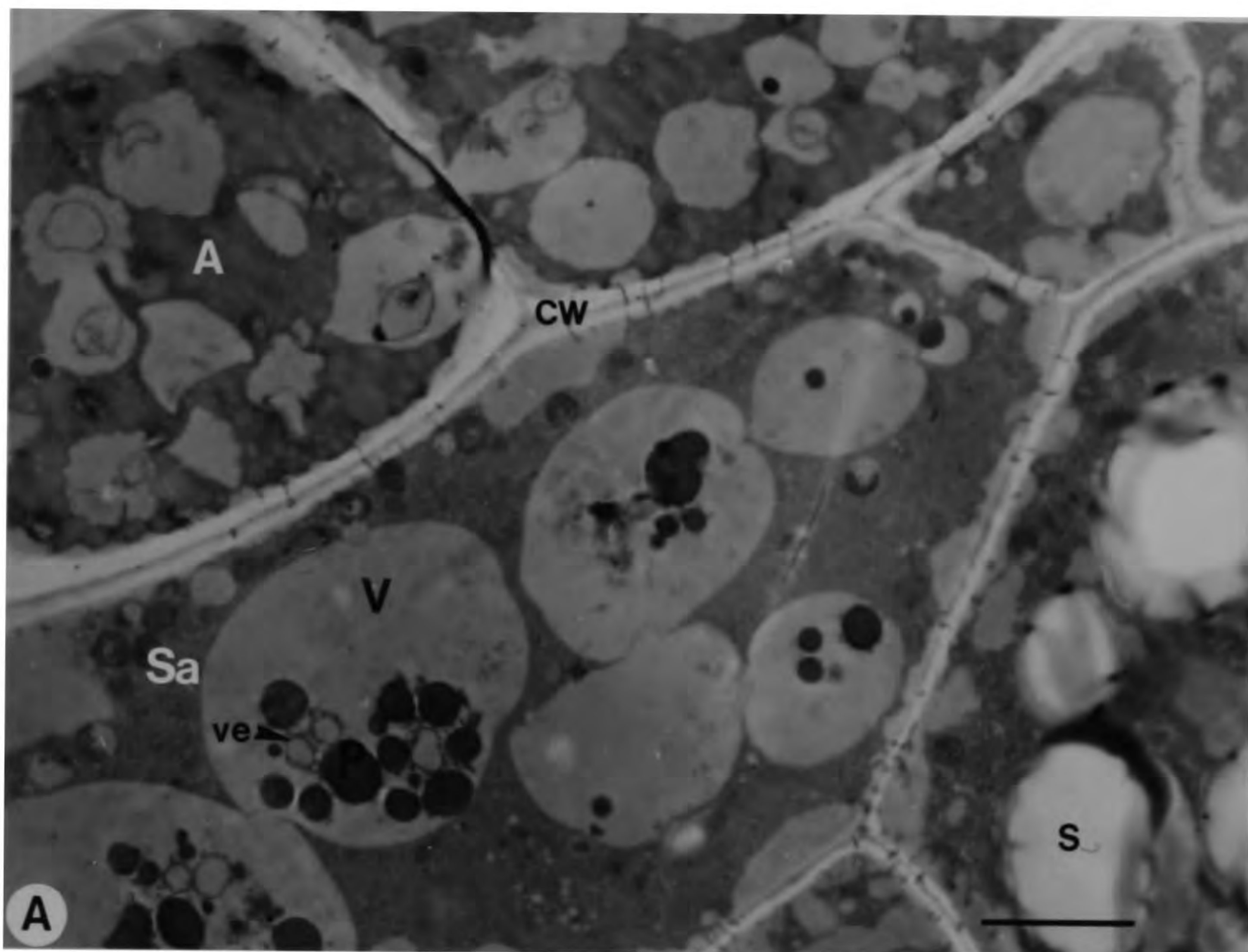


PLATE 16. Electron micrographs showing the ultrastructure of protein bodies of a barley caryopsis 18 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : In the sub-aleurone, large aggregates of protein are located in vacuoles. Bar = 4 μm.

B : A protein body complex in the starchy endosperm cytoplasm. Complex consists of fibrillar, granular, and electron dense components. Bar = 2 μm.

C : Complex protein bodies in the endosperm with associated peripheral vesicles. Protein complex is membrane-bound. Bar = 1.5 μm.



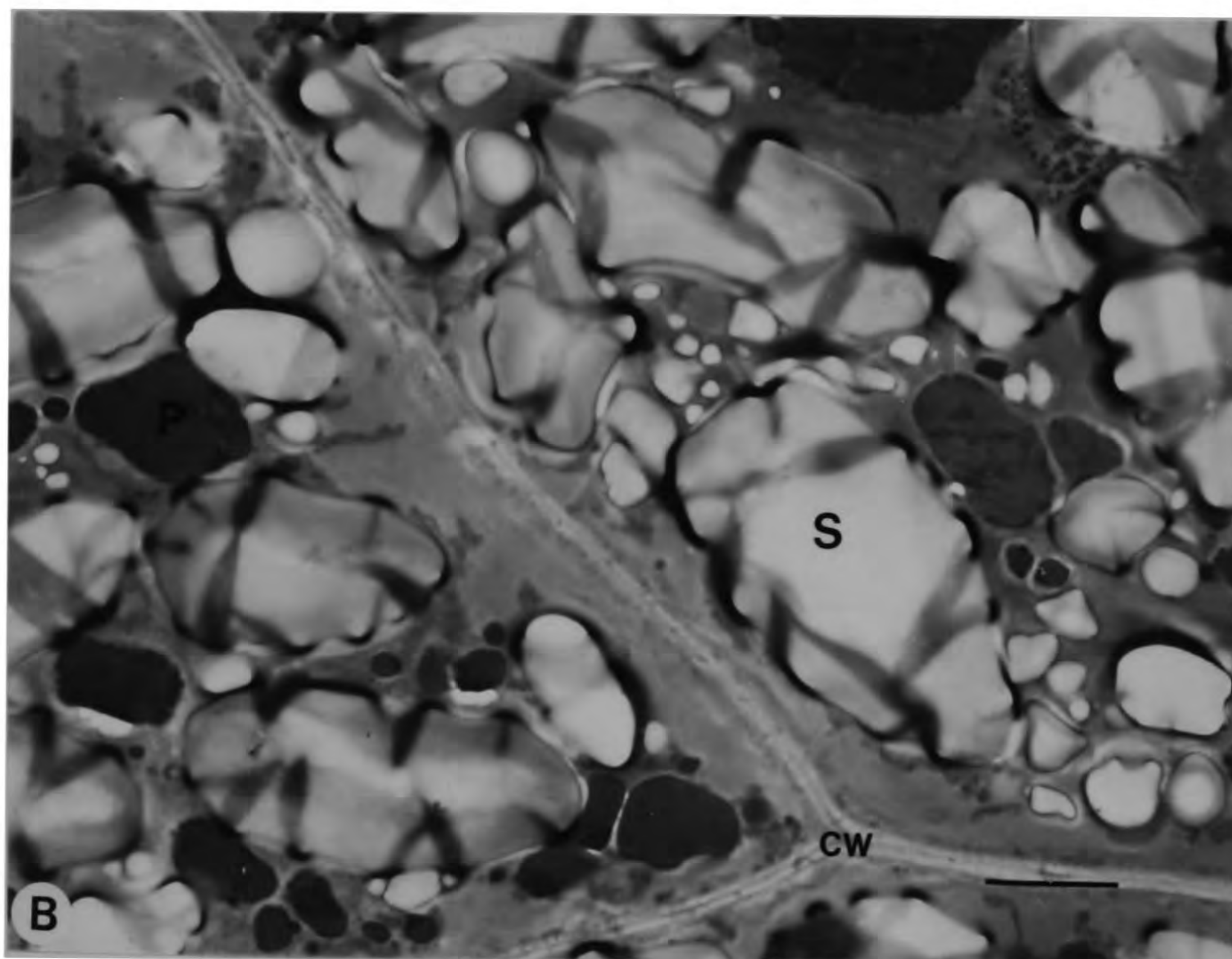


PLATE 17. Electron micrographs of an ultrathin section through a barley caryopsis 20 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.
A : Single protein bodies and vesicles are located in vacuoles of the sub-aleurone of a 64 mg grain. Bar = 3 µm.
B : In the outer starchy endosperm, single and complex protein bodies are located in the cytoplasm between large and small starch granules. Bar = 8 µm.

3.1.4 Ultrastructure of the Developing Barley Endosperm from the Study of Thick (0.3 μm) Sections by CTEM

The ultrastructure of Bomi barley was investigated from anthesis to maturity using a zinc iodide-osmium tetroxide staining complex, with subsequent thick-sectioning of Spurr resin-embedded tissue for conventional transmission electron microscopy.

Plate 18 shows a thick-section through the caryopsis of barley 10 DAA. Endoplasmic reticulum is accentuated following ZIO-impregnation. Cisternal and tubular endoplasmic reticulum extend around lipid droplets and vacuoles in the aleurone (Plate 18A). In the sub-aleurone 10 DAA (Plate 18B) there are many membrane-bound vacuoles, and large vacuoles contain several smaller membrane-bound vacuoles. Numerous dictyosomes are present in the cytoplasm; mitochondria tend to lie close to the cell wall. A higher magnification of the aleurone and sub-aleurone is illustrated in Plate 19. Micrograph 19A shows the distribution of cisternal and tubular endoplasmic reticulum in the aleurone. Numerous dictyosomes are seen in the cytoplasm in close proximity to the endoplasmic reticulum. There are numerous mitochondria, and membrane-bound vacuoles are in close association with the endoplasmic reticulum. In the sub-aleurone (micrograph 19B), membrane-bound vacuoles

are again surrounded by endoplasmic reticulum. Dictyosomes, with prominent densely-stained vesicles and a network of anastomosing tubules, are located in the cytoplasm.

In micrograph 20A 10 DAA, ZIO-staining shows the distribution of cisternal and tubular endoplasmic reticulum in the sub-aleurone. Dictyosomes are located in the cytoplasm close to cisternal endoplasmic reticulum, and the large vacuole is membrane-bound although not stained. Micrograph 20B, shows that cisternal and tubular endoplasmic reticulum, dictyosomes, and protein bodies are all in close proximity. The starchy endosperm 10 DAA is shown in Plate 21. Micrograph 21A illustrates the distribution of the cisternal and tubular endoplasmic reticulum and also of the large vacuoles, the membranes of which are not selectively stained by the ZIO complex. Several mitochondria are evident in the cell. At a higher magnification (micrograph 21B), several dictyosomes are seen in the cytoplasm in close proximity to cisternal endoplasmic reticulum; endoplasmic reticulum is predominantly cisternal.

Stereo-pair micrographs in Plate 22 illustrate the three-dimensional interrelationships of organelles in the sub-aleurone of a barley caryopsis 10 DAA. Micrograph 22A illustrates the relationship between the

protein body and cisternal and tubular endoplasmic reticulum, and tubular endoplasmic reticulum with the dictyosome. The interrelationship between the protein body, the cisternal endoplasmic reticulum and the dictyosome is shown in micrograph 22B. The cisternae of the dictyosome are accentuated by the ZIO-staining complex, and terminate in electron-dense sacs.

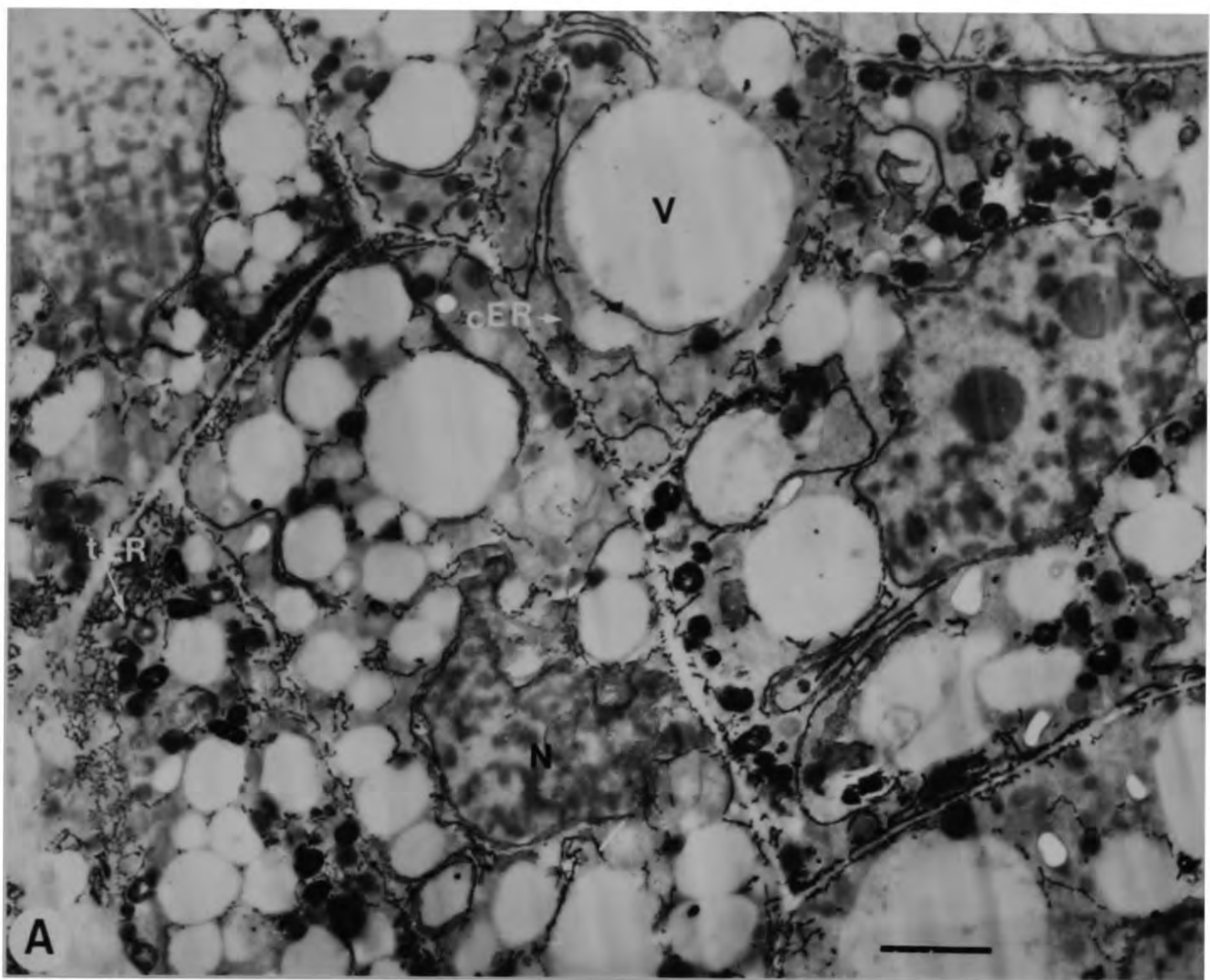
Plate 23 illustrates a low-power electron micrograph of the aleurone and sub-aleurone of a barley caryopsis 14 DAA. In the sub-aleurone, protein bodies are vacuolar. The distribution of cisternal and tubular endoplasmic reticulum is illustrated by the selective staining of the ZIO complex. Numerous mitochondria are regularly aligned along the cell walls of the sub-aleurone and, at 14 DAA, starch deposits are now more evident.

In the sub-aleurone 14 DAA (micrograph 24A), a higher magnification shows dictyosomes, still evident in the cytoplasm although not as numerous as at 10 DAA, having a more condensed appearance without the large network of vesicles and anastomosing tubules seen 10 DAA (compare micrograph 19B). In the starchy endosperm 14 DAA (micrograph 24B), large protein aggregates are located close to cisternal endoplasmic reticulum. Protein bodies have electron-dense peripheral vesicles.

A higher magnification of the sub-aleurone of a

barley caryopsis 14 DAA is shown in Plate 25. In micrograph 25A, the stereo-pair shows the interrelationship of cisternal endoplasmic reticulum with a protein body. Heavily-stained vesicles are located around the periphery of the protein body. A high power of a dictyosome in micrograph 25B shows many heavily-stained vesicles around a central cisternal plate. Dictyosomes differ in appearance from those seen 10 DAA.

At 20 DAA the sub-aleurone has large aggregates of protein (micrograph 26A). At this later stage in development of the grain, the cells of the sub-aleurone become filled with starch, and there is less endoplasmic reticulum. Micrograph 26B shows a stereo-pair in the sub-aleurone illustrating the relationship between a dictyosome and tubular endoplasmic reticulum. Occasional dictyosomes are seen at 20 DAA.



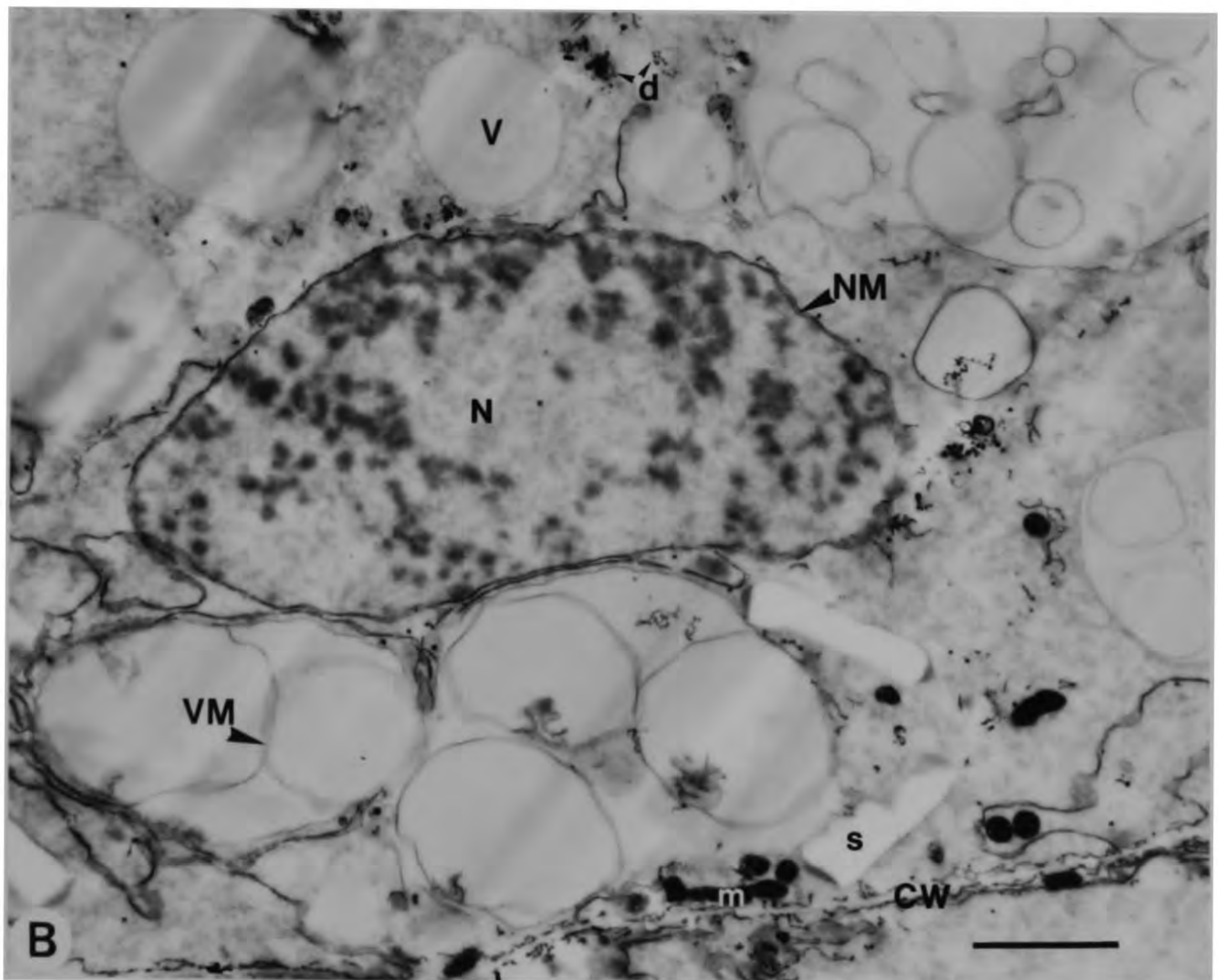
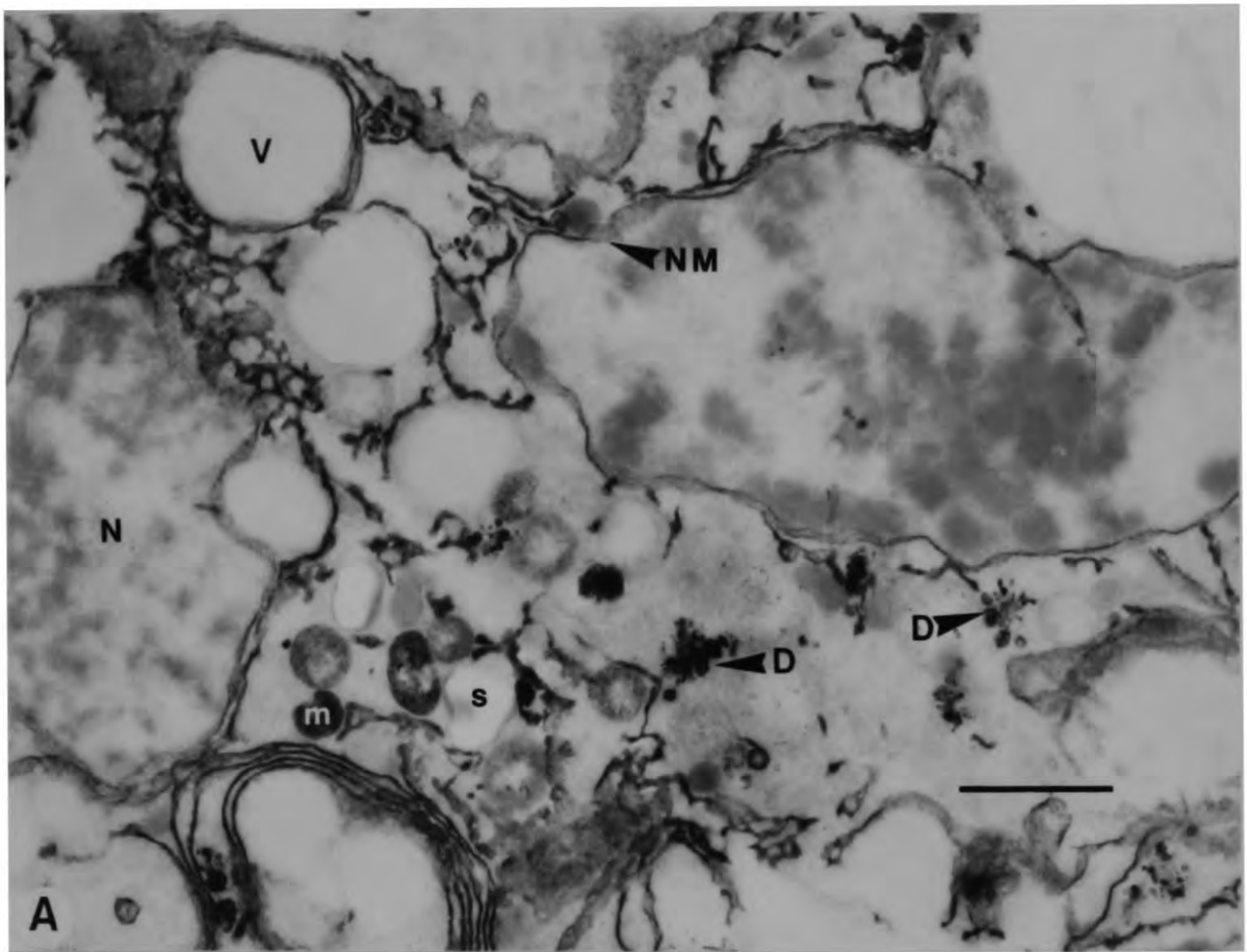


PLATE 18. Electron micrographs of 0.3 um sections of ZIO impregnated barley caryopsis 10 DAA.

A : Aleurone of barley grain showing distribution of cisternal ER and tubular ER. Bar = 3 µm.

B : Sub-aleurone cell highly vacuolate; large vacuoles contain several small membrane-bound vacuoles. Dictyosomes evident in cytoplasm, indicated by darts. Bar = 3 µm.



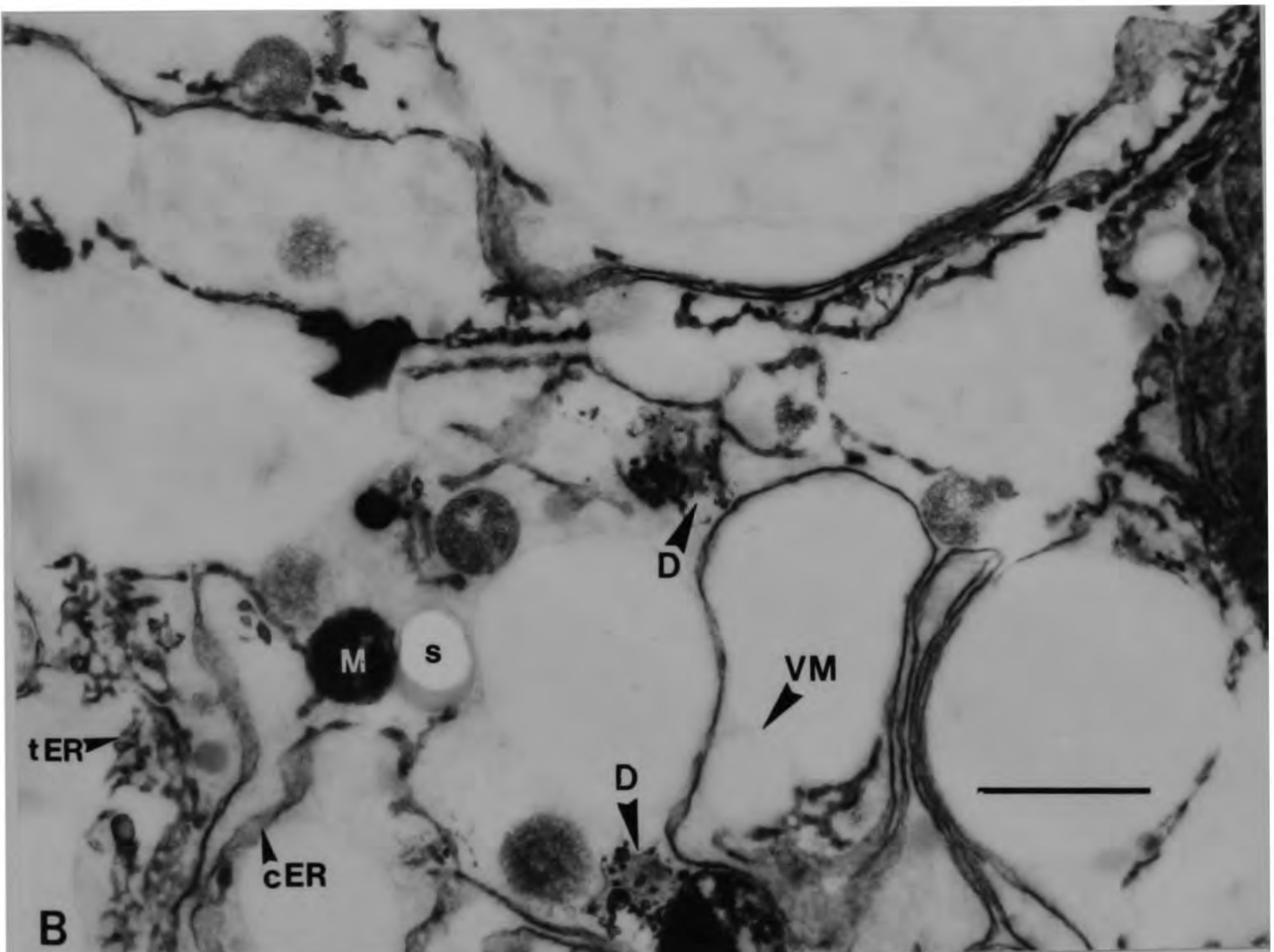
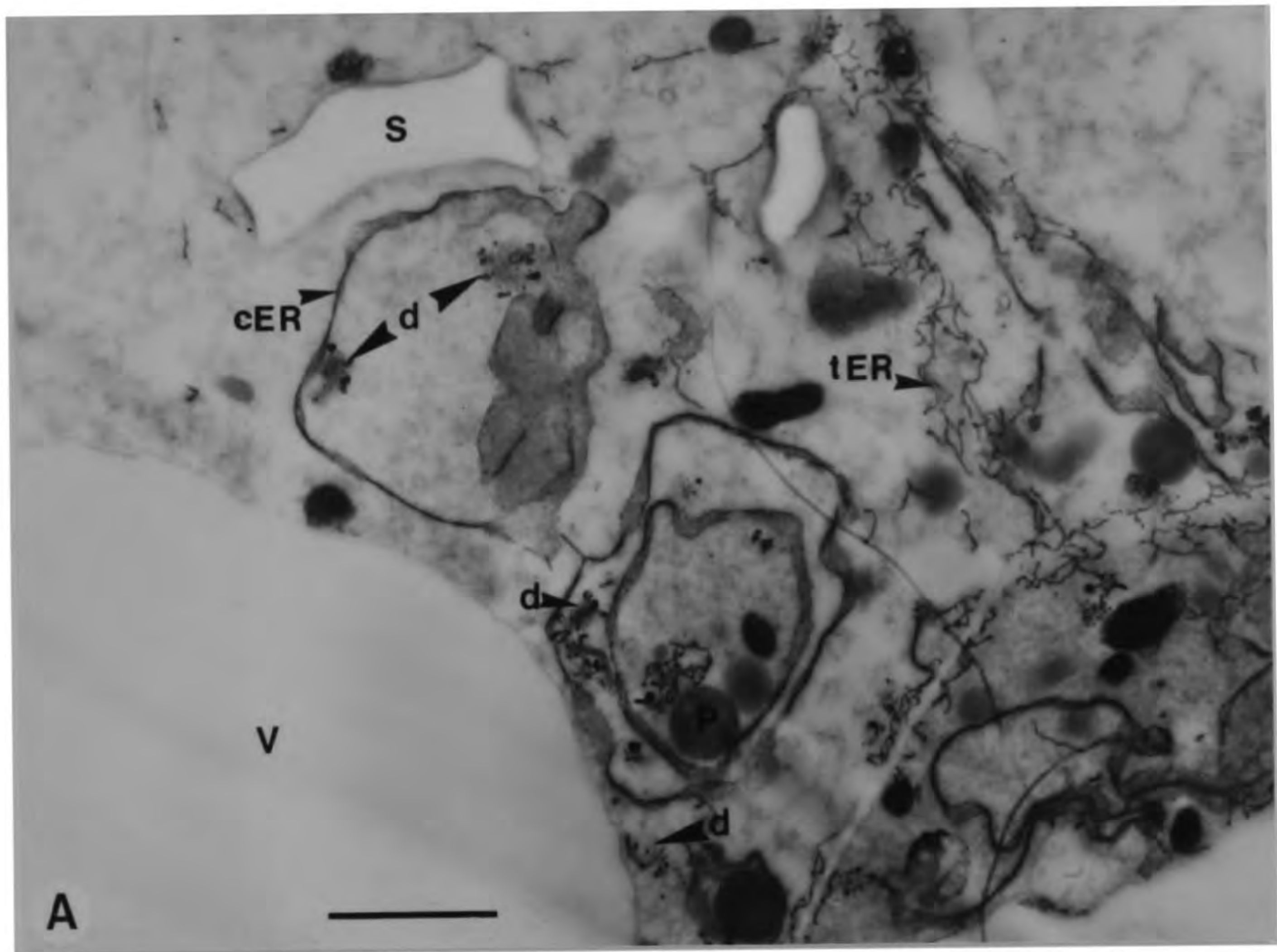


PLATE 19. Electron micrographs of 0.3 um sections of aleurone and sub-aleurone tissue of ZIO impregnated barley caryopsis 10 DAA.
A : Distribution of cisternal and tubular ER in the aleurone. Dictyosomes are indicated by darts. Bar = 2.5 μm.
B : In the sub-aleurone, membrane-bound vacuoles are surrounded by endoplasmic reticulum. Dictyosomes, with prominant cisternae and dense vesicles, are indicated by darts. Bar = 2 μm.



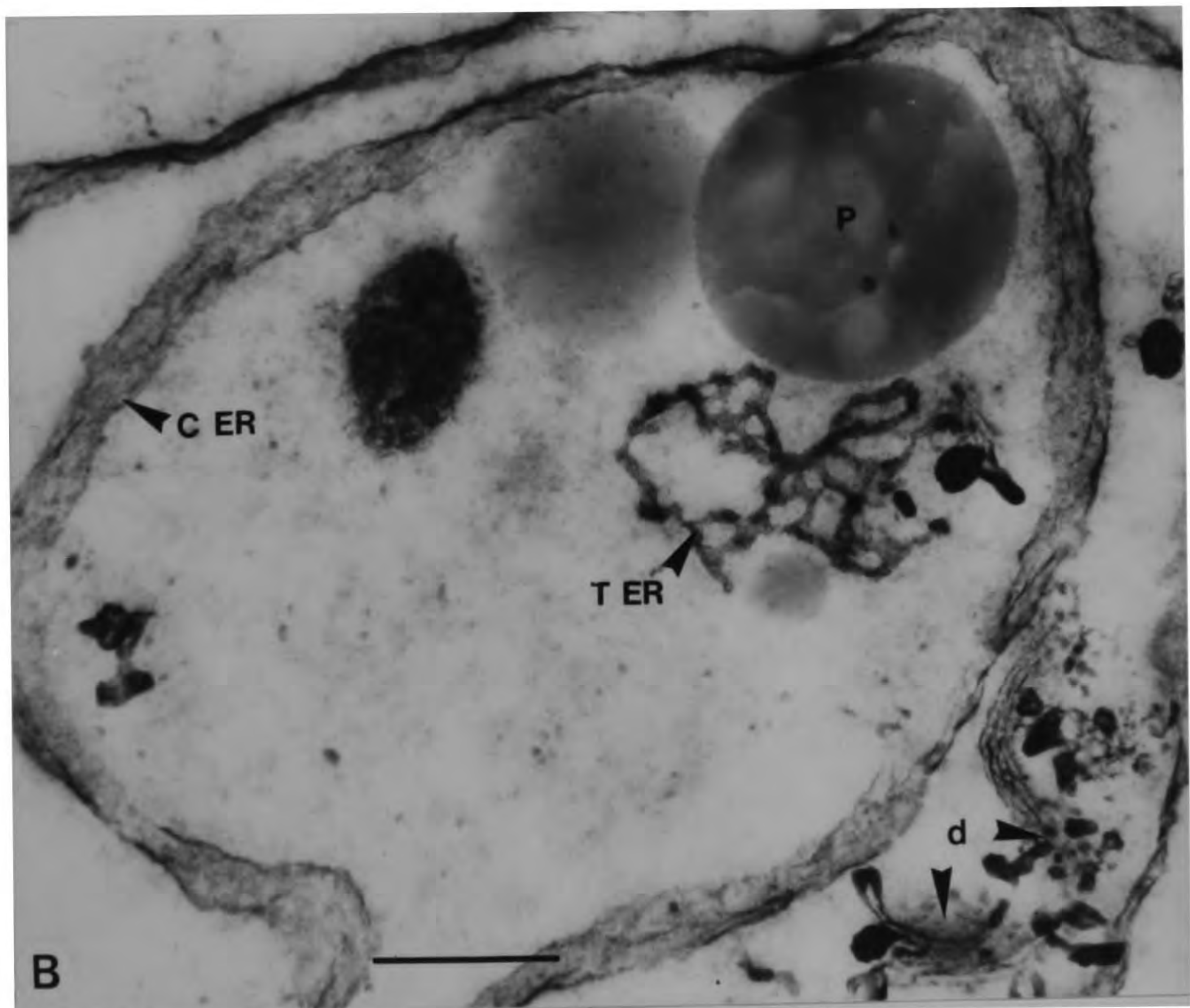
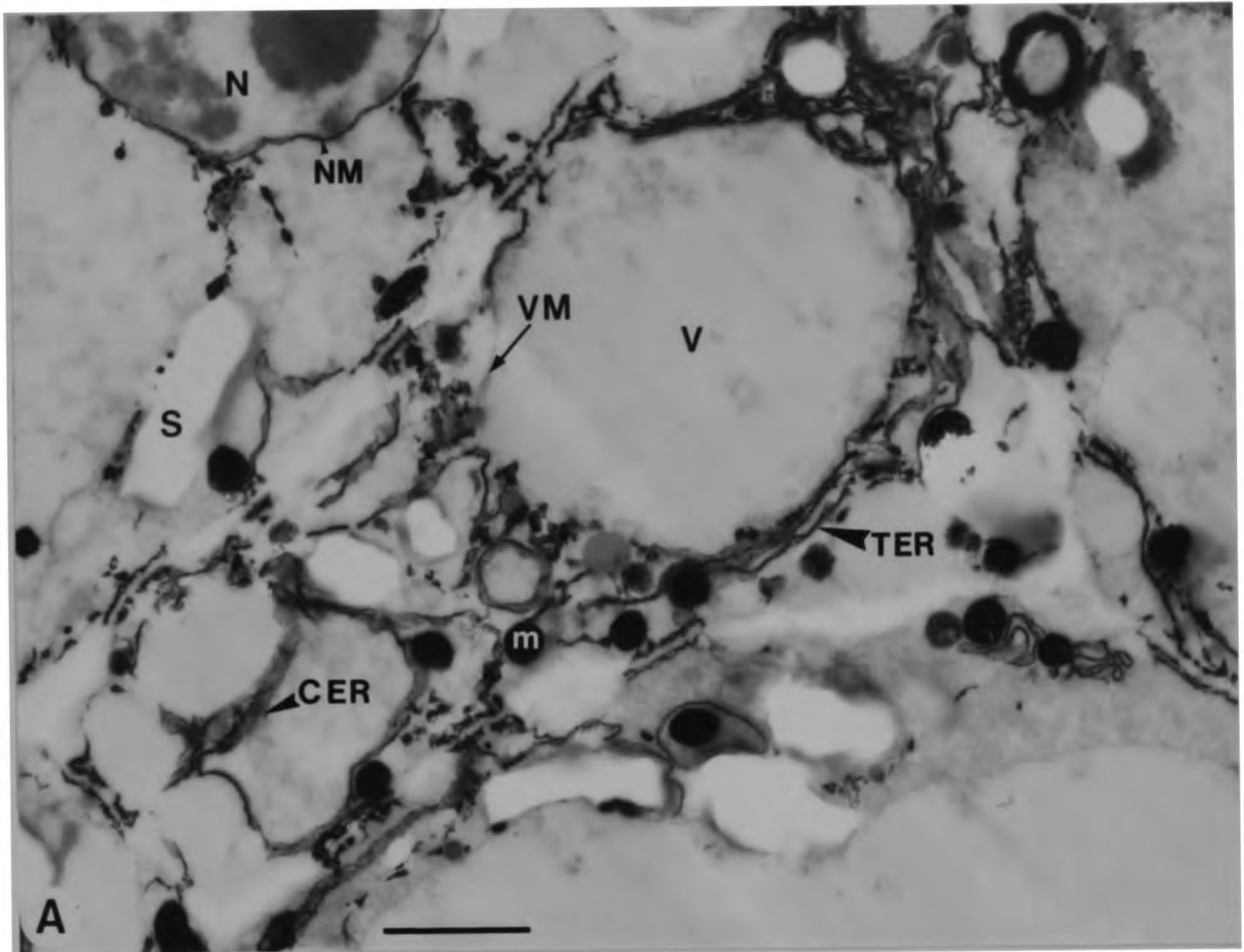


PLATE 20. Electron micrographs of 0.3 um sections of sub-aleurone of a barley caryopsis 10 DAA, impregnated with ZIO.
A : Sub-aleurone showing distribution of cisternal and tubular ER. Dictyosomes are located in cytoplasm close to cisternal ER. Bar = 2 µm.
B : Higher magnification showing cisternal ER surrounding a single protein body. Some tubular ER is evident. Dictyosomes are indicated by darts. Bar = 0.5 µm.



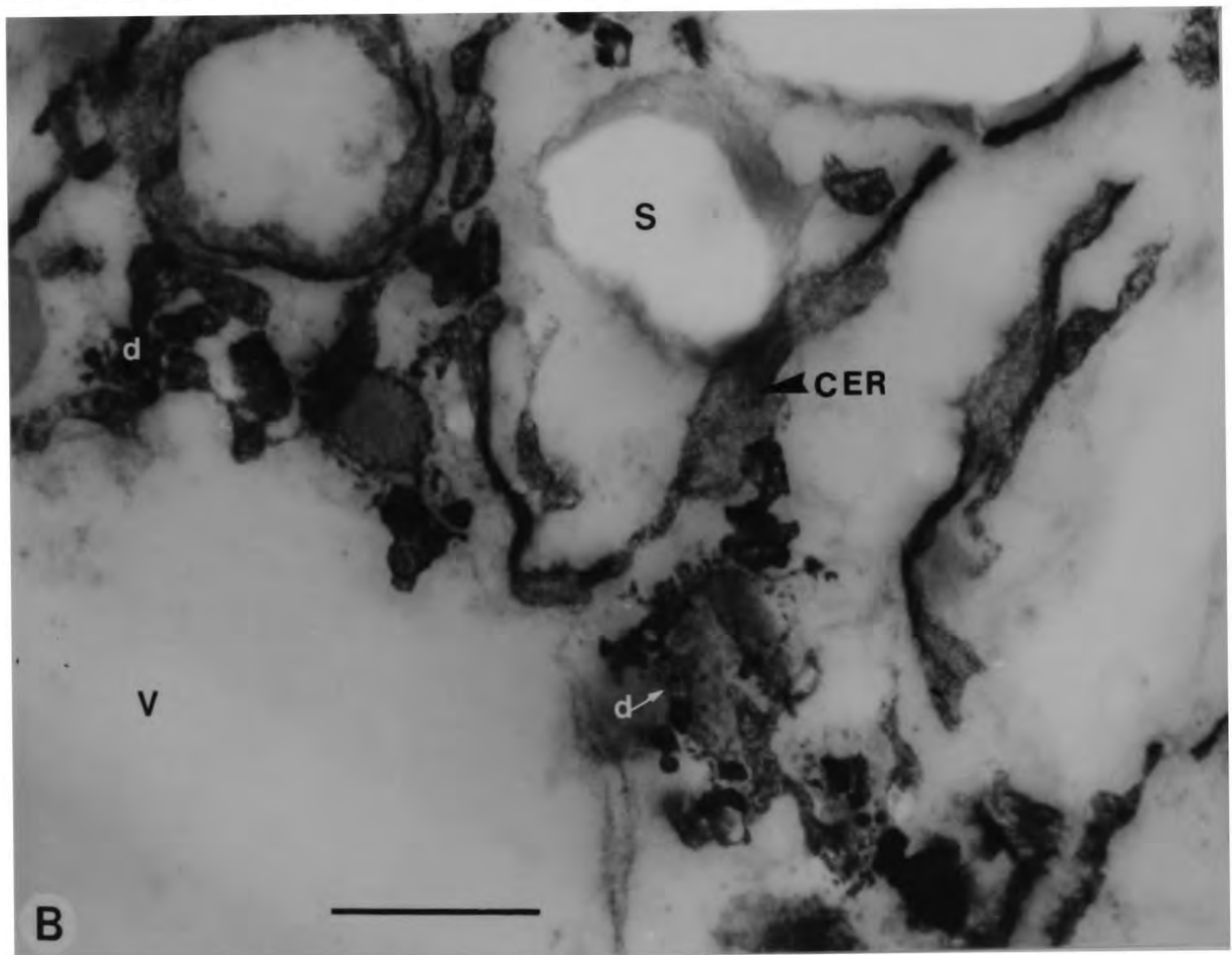


PLATE 21. Electron micrographs of 0.3 um sections of starchy endosperm of a barley caryopsis 10 DAA, impregnated with ZIO.
A : Starchy endosperm showing distribution of cisternal and tubular ER. Bar = 3 μm.
B : Higher magnification shows the interrelationship of the dictyosome to the ER (darts). Bar = 1 μm.

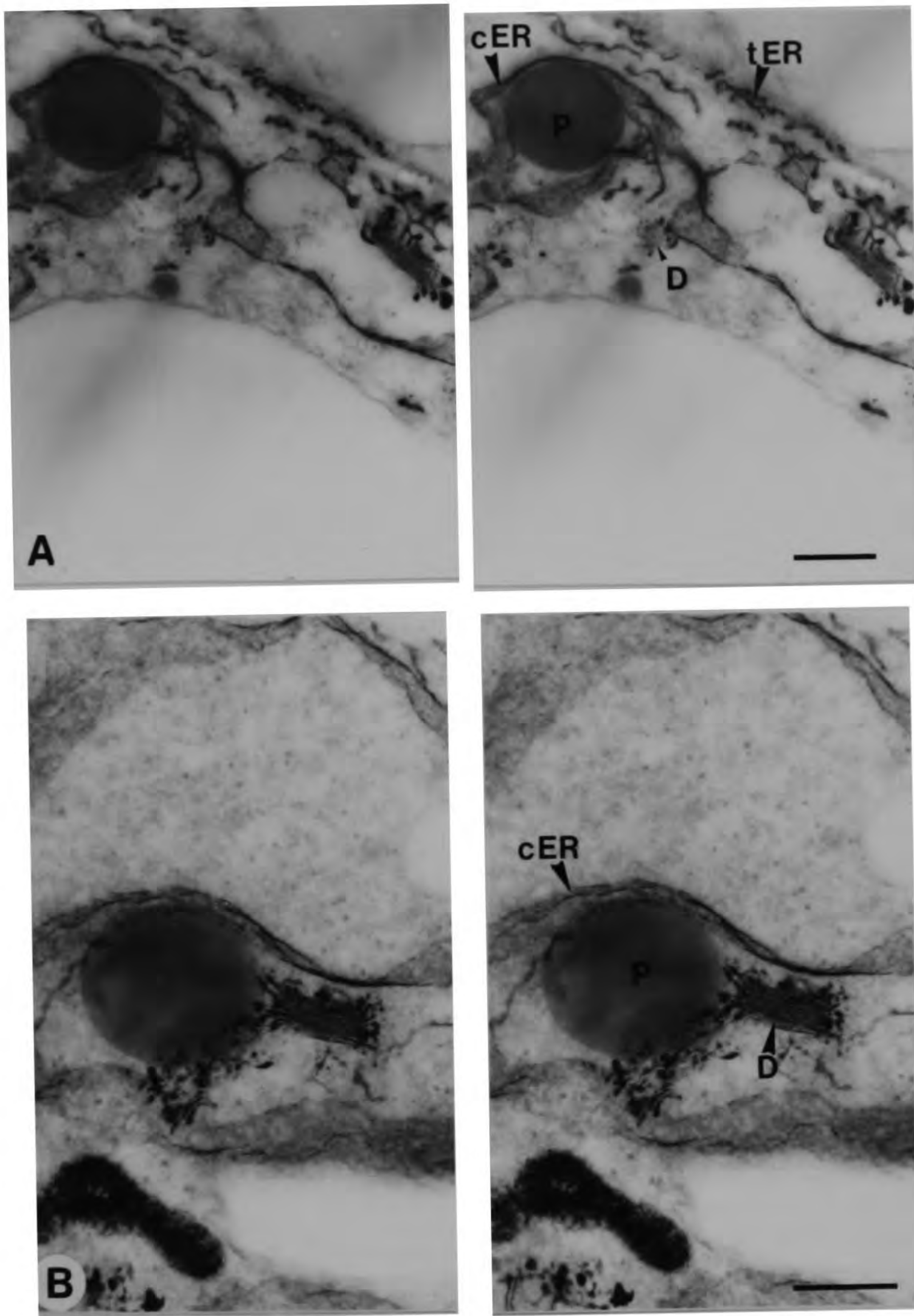


PLATE 22. Stereo-pair micrographs at 4° tilt of 0.3 um sections of a barley caryopsis 10 DAA, impregnated with ZIO.
A : Stereo-pair demonstrates interrelationship between the protein body, and cisternal and tubular ER, and the latter with the dictyosome. Bar = 0.5 μm.
B : Stereo-pair demonstrates interrelationships between the protein body, cisternal ER and dictyosome. Bar = 0.5 μm.

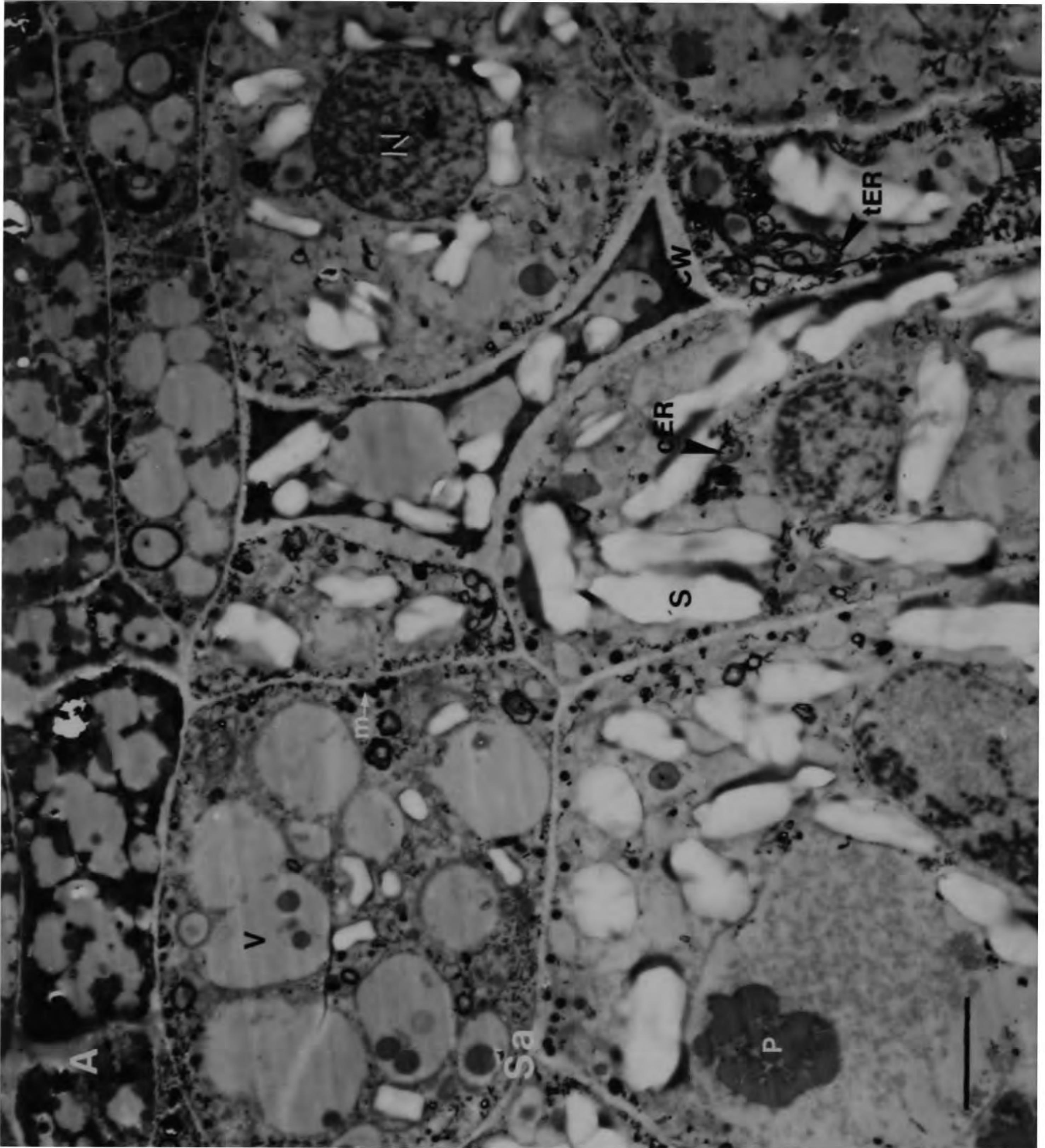
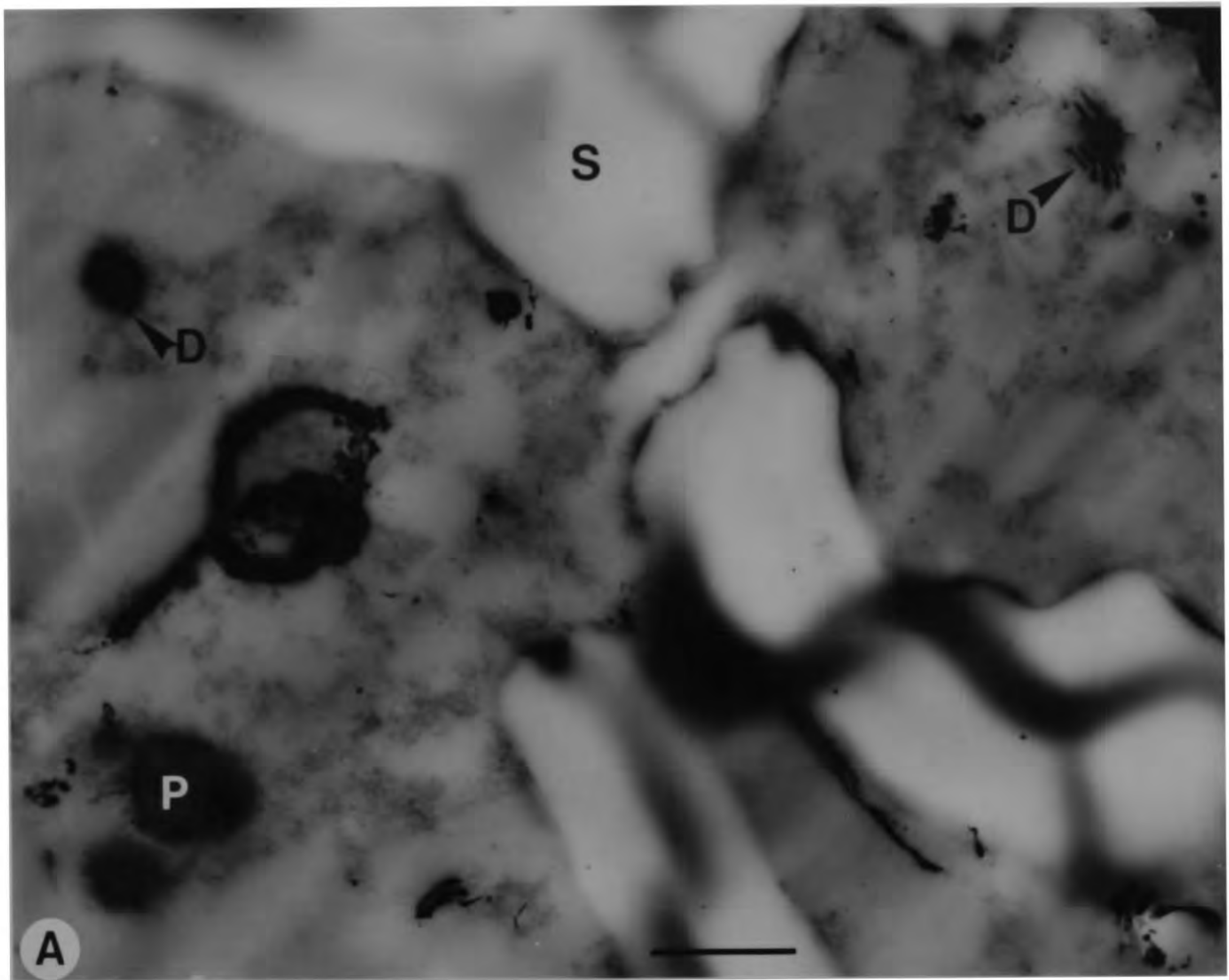


PLATE 23. Electron micrograph of 0.3 um section of the aleurone and sub-aleurone of a barley caryopsis 14 DAA, impregnated with ZIO.
In the sub-aleurone, protein bodies are vacuolar, and the distribution of cisternal and tubular ER is shown by the staining with ZIO. Bar = 6 μm.



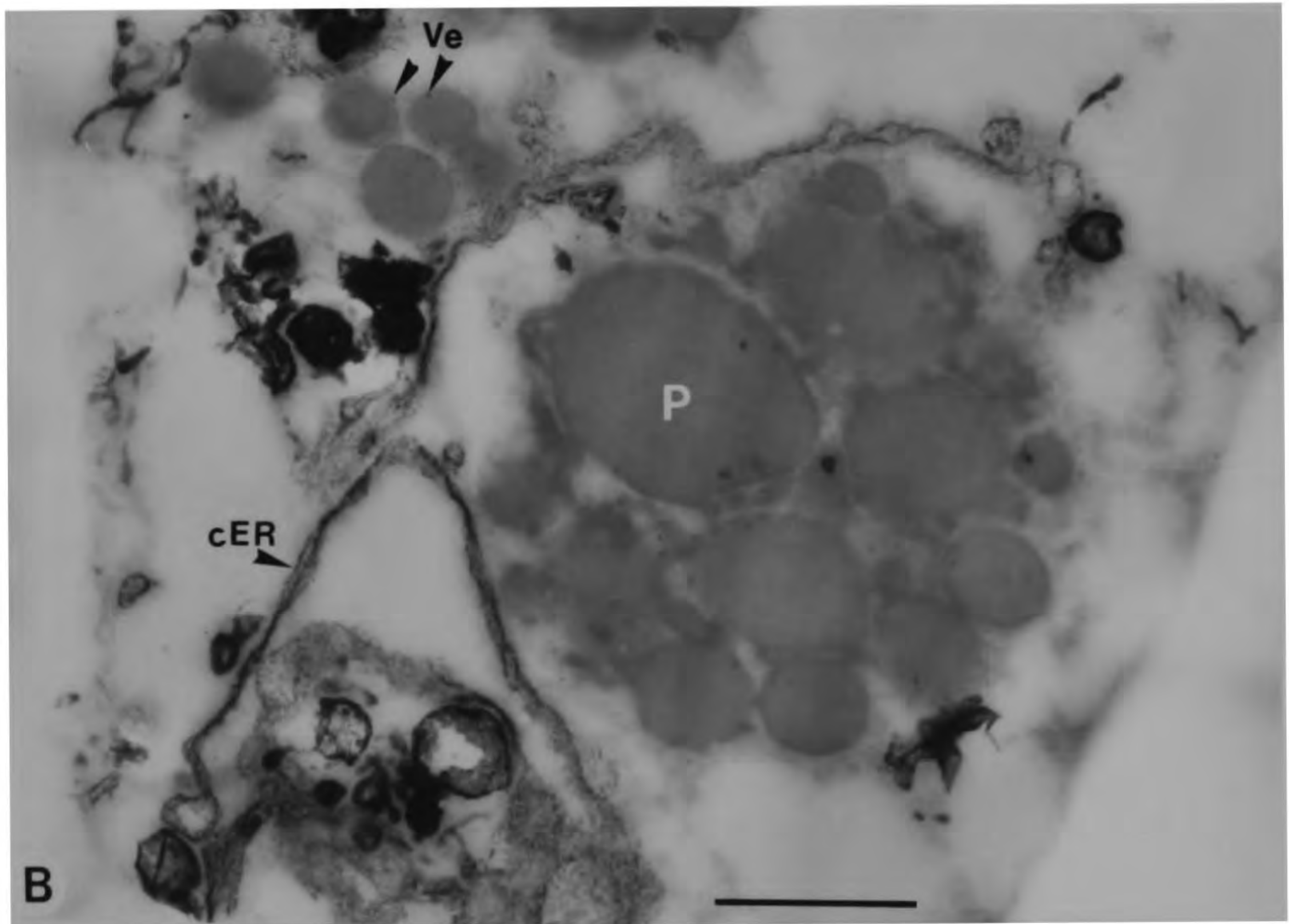


PLATE 24. Electron micrographs of 0.3 um sections of a barley caryopsis 14 DAA, impregnated with ZIO.
A : Sub-aleurone showing dictyosomes (darts) heavily-stained by ZIO. Bar = 1.5 µm.
B : Starchy endosperm showing a protein aggregate in close proximity to cisternal ER. Protein bodies have electron-dense peripheral vesicles. Bar = 1.5 µm.

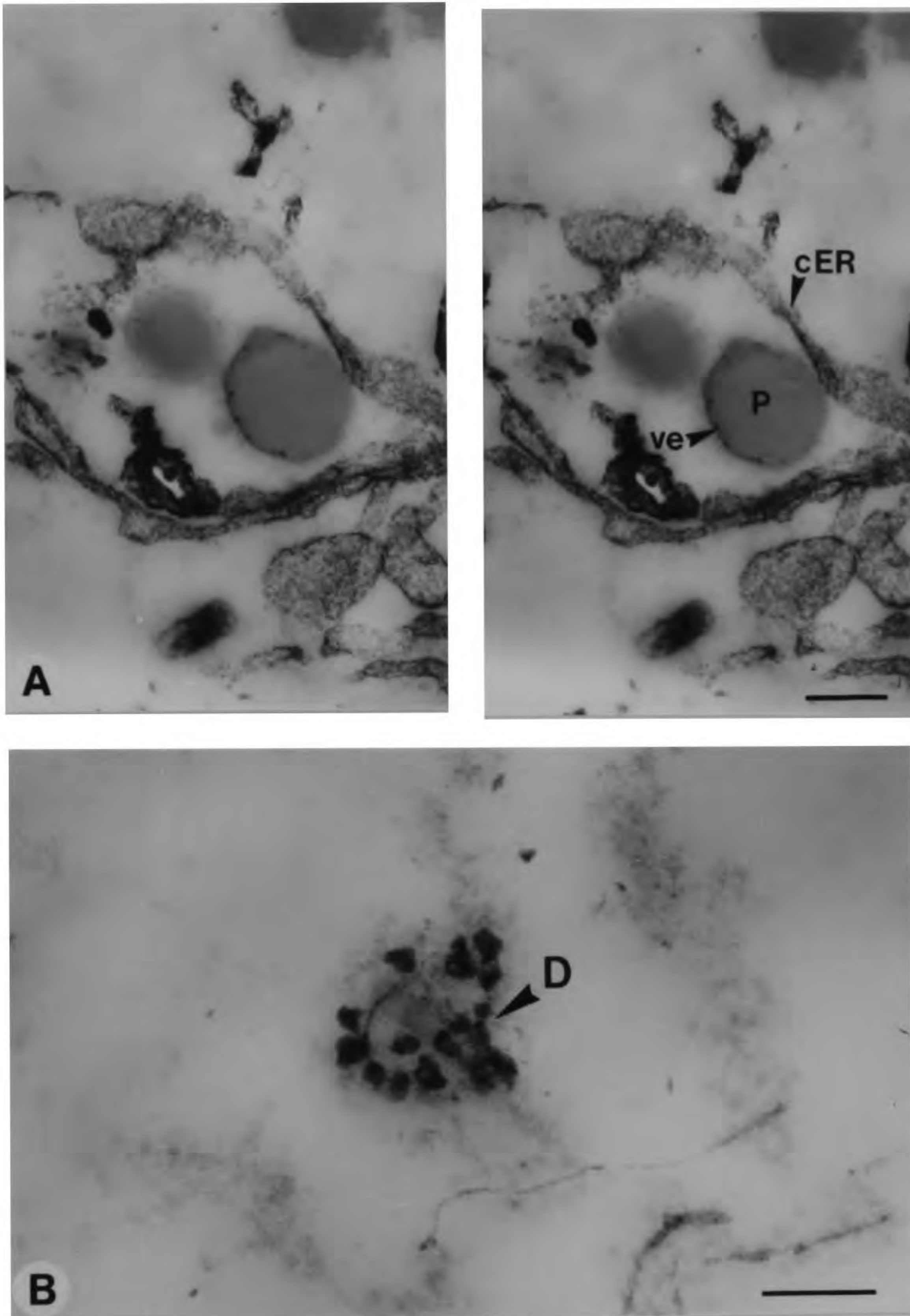
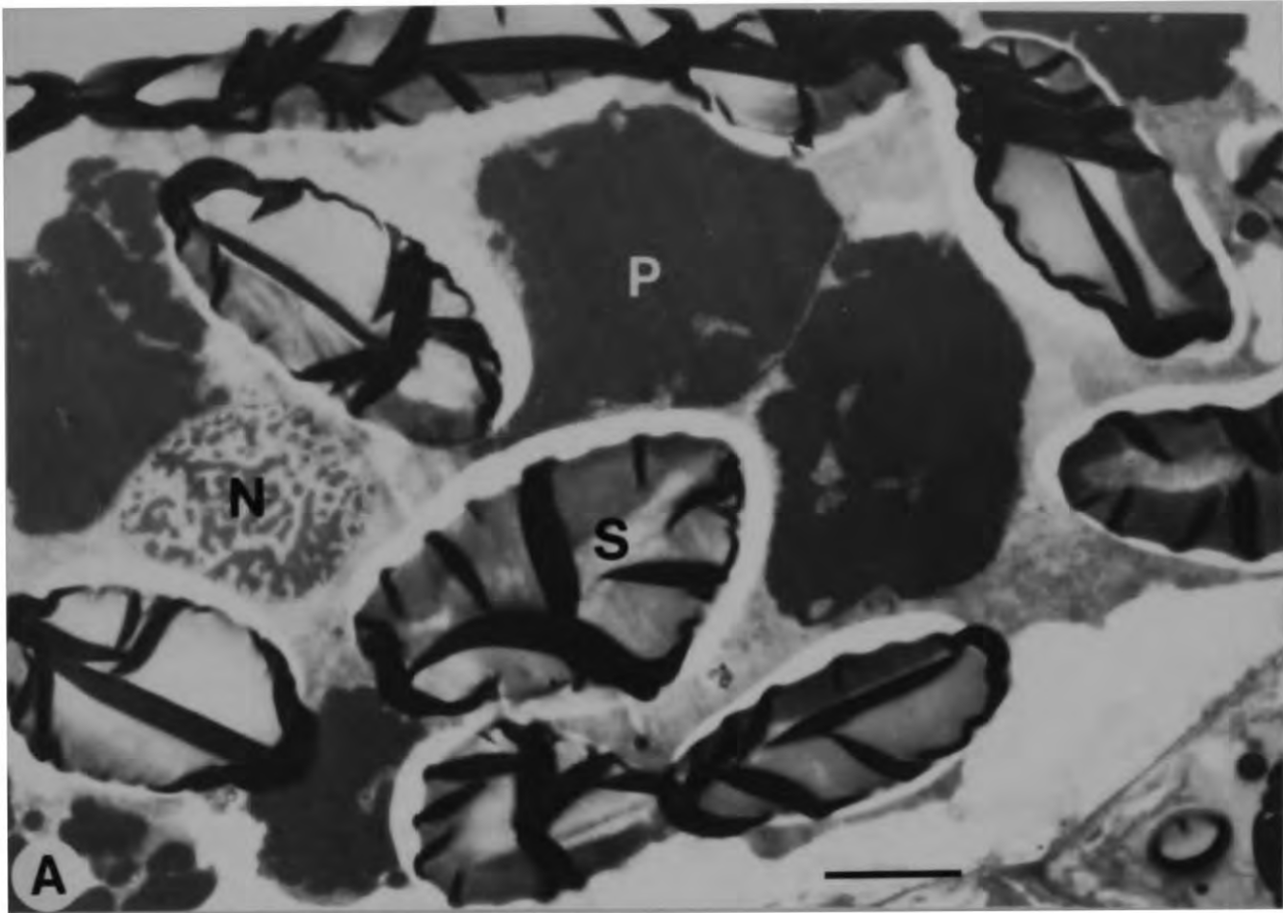
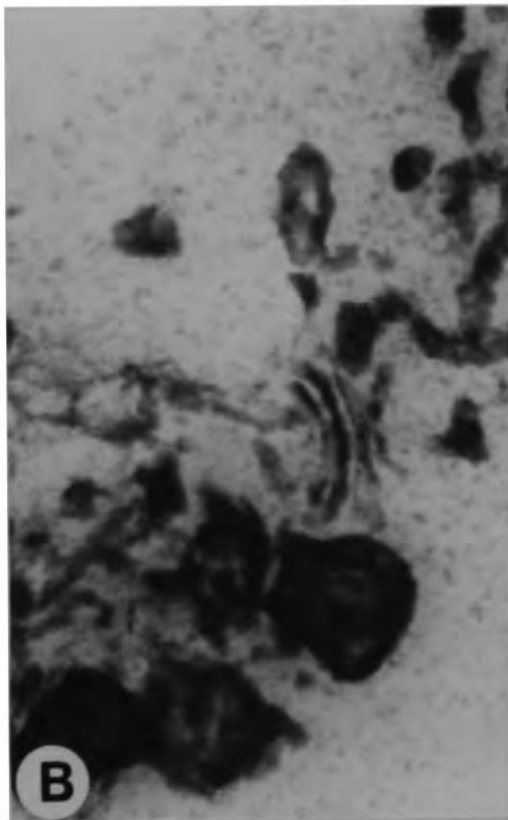


PLATE 25. Electron micrographs of 0.3 um sections of the sub-aleurone of a barley caryopsis 14 DAA, impregnated with ZIO.

A : Stereo-pair at 4° tilt showing interrelationship of the cisternal ER with a single protein body. Electron dense vesicles are seen around the periphery of the protein body. Bar = 0.5 μm.

B : Micrograph of the ZIO-stained vesicles of a dictyosome in the sub-aleurone. Bar = 0.25 μm.





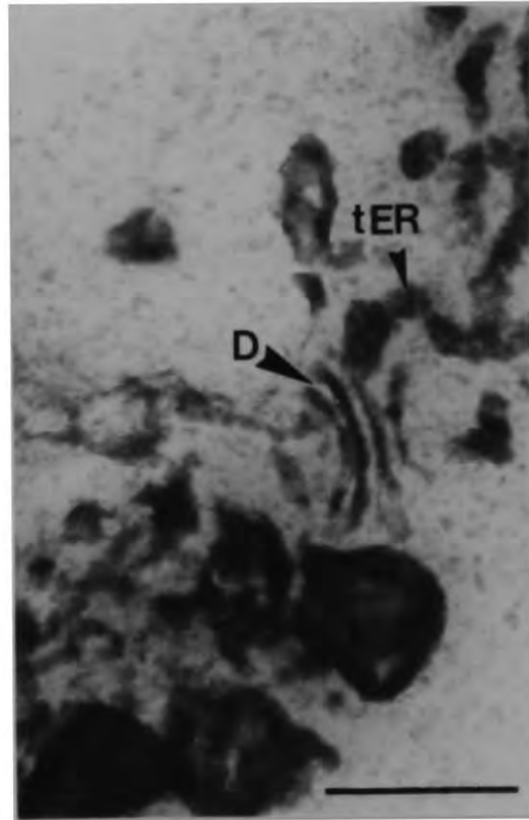


PLATE 26. Electron micrographs of 0.3 um sections of the sub-aleurone of a barley caryopsis 20 DAA, impregnated with ZIO.

A : In the sub-aleurone there are large aggregates of protein. Bar = 4 μm.

B : Stereo-pair at 4° tilt showing a dictyosome and tubular ER in the sub-aleurone. Bar = 0.25 μm.

3.2 Ultrastructure of the Developing Endosperm in the High-Lysine Barley Mutants

The previously described ultrastructure of the development of the grain from anthesis to maturity, shows that barley protein bodies can consist of three distinct components. These are the granular component, fibrillar component and electron-dense component.

Protein body formation was investigated in mutant barley lines, where a reduced and modified synthesis of storage protein may be associated with an altered protein body structure.

3.2.1 Ultrastructure of Developing Protein Bodies in the High-Lysine Barley Mutants; Thin-and Thick-Section Electron Microscopy

Three high-lysine barley mutants, Risø 56, Risø 1508 and Hiproly, were examined by thin- and thick-sectioning-microscopy. For thin-sectioning, the mutants were aldelyde fixed in phosphate buffer, with osmium tetroxide post-fixation, and subsequently embedded in Spurr resin. Zinc iodide-osmium tetroxide impregnated tissue for thick-section studies was also embedded in Spurr resin. Mutants were examined at two stages in development after anthesis; during the earlier part and towards the end of the period of storage protein deposition.

Plate 27 shows transmission electron micrographs of ultrathin sections through the caryopsis of the high-lysine line Hiproly 14 DAA. Single protein bodies

are located in the cytoplasm and in vacuoles of the sub-aleurone (Plate 27, micrograph A). Single protein bodies comprise of a granular component with many electron-dense areas; this can be contrasted with the wild-type Bomi barley at 14 DAA where protein bodies are mainly granular at a comparable stage in protein body development. In the starchy endosperm (Plate 27B), most single protein bodies are in the cytoplasm. By 21 DAA, Plate 28A, protein deposits increase in vacuoles of the sub-aleurone. Unlike Bomi barley at 14 DAA when protein bodies are at a similar developmental stage to those in Hiproly 21 DAA, there is a greater accumulation of vesicles and vesicular material in the vacuoles of the latter (Plate 28A). In the endosperm (Plate 28B) cytoplasmic protein bodies comprise large granular components with prominent electron-dense areas.

The mutant Risø 1508 is derived from Bomi barley. Hordein synthesis is depressed in this mutant, and protein body structure is considerably altered in endosperms of 17 DAA and 21 DAA barley caryopsis, compared with the wild-type Bomi barley. At 17 DAA (Plate 29A), protein bodies of the sub-aleurone are vacuolar. Unlike the wild-type protein bodies, the protein bodies of the mutant Risø 1508 are comprised predominantly of a fibrillar component. This component

has been termed "fibrillar" by Cameron-Mills and von Wettstein (1980), but under higher magnification these large fibrillar regions (see Plate 30B, protein bodies in the endosperm) are more like an interwoven network of fine tubules and vesicles. Plate 29B illustrates a dilated form of endoplasmic reticulum found in some cells of the sub-aleurone; this dilated endoplasmic reticulum is heavily studded with ribosomes.

By 23 DAA (Plate 30B) protein bodies in the vacuoles of the sub-aleurone of Risø 1508 are fibrillar with vesicles embedded in this matrix. Cytoplasmic protein bodies in the endosperm 23 DAA (Plate 30B) contain large fibrillar regions with electron-dense spheres round the periphery of the protein body. These spheres are larger and more common than in the wild-type, and vesicle inclusions in the protein body are also more frequent in the mutant Risø 1508.

In mutant Risø 56, derived from Carlsberg II, the hordein content is reduced from 60% to 45% of total seed nitrogen (Shewry et al, 1980). Comparing this mutant with the wild-type Bomi barley, it is immediately apparent that the mutation results, as with Risø 1508, in an altered protein body morphology. In the sub-aleurone of Risø 56 at 17 DAA (Plate 31A), the large vacuolar protein complex consists of many granular spheres embedded in a fibrillar component. In

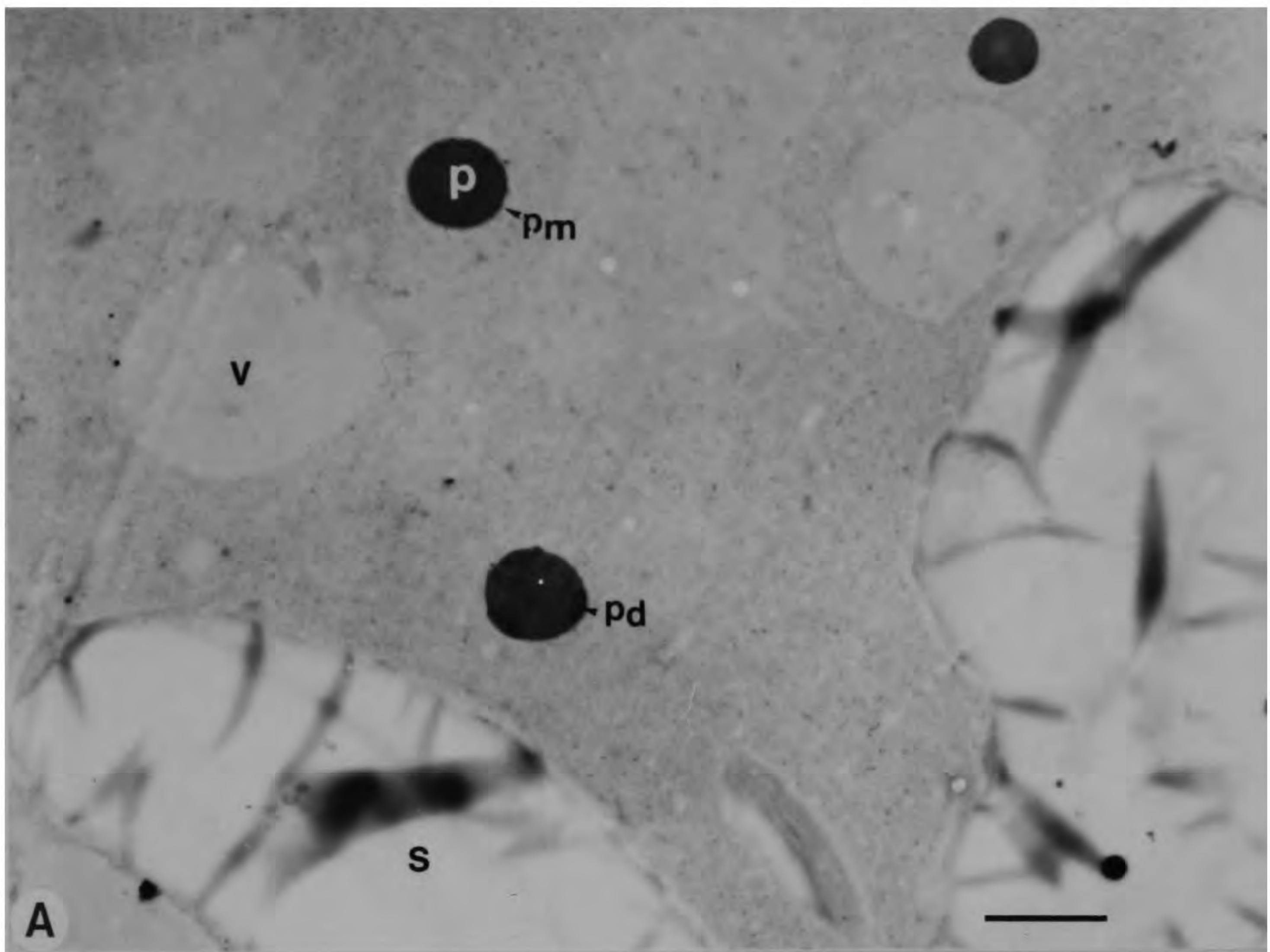
the endosperm however, the vacuolar protein complex consists of granular areas embedded in an electron-dense matrix (Plate 31B). Vesicles are also found embedded within the electron-dense region of the protein complex.

By 24 DAA, the protein deposits in the sub-aleurone of Risø 56 (Plate 32A) consist of many granular spheres embedded in a condensed fibrillar matrix. This micrograph also illustrates that in the vacuoles, there is an increase in vesicles and associated material. In the starchy endosperm (Plate 32B), protein bodies are both vacuolar and cytoplasmic. In both types, the protein bodies consist of large spheres of granular protein embedded in an electron-dense matrix. Within the vacuolar protein bodies, there are several protein deposits.

The ultrastructure of the sub-aleurone of Risø 56 and Risø 1508 was investigated using a zinc iodide-osmium tetroxide staining complex (Plate 33A, B). This demonstrates the distribution of both cisternal and tubular endoplasmic reticulum. In both Risø 56 and Risø 1508, tubular endoplasmic reticulum is predominant in the sub-aleurone 17 DAA.

The network of cisternal and tubular endoplasmic reticulum in the sub-aleurone of Hiproly 14 DAA (Plate 34A) is illustrated following selective staining with a

zinc iodide-osmium tetroxide complex. In Plate 34B, higher magnification shows a dictyosome in the sub-aleurone 21 DAA in Hiproly. The cisternae and vesicles of the dictyosome are selectively stained with ZIO. In the endosperm at the same developmental stage, the protein body has peripheral heavily-stained vesicles. In the cytoplasm there is also a network of dictyosome vesicles and anatomosing tubules.



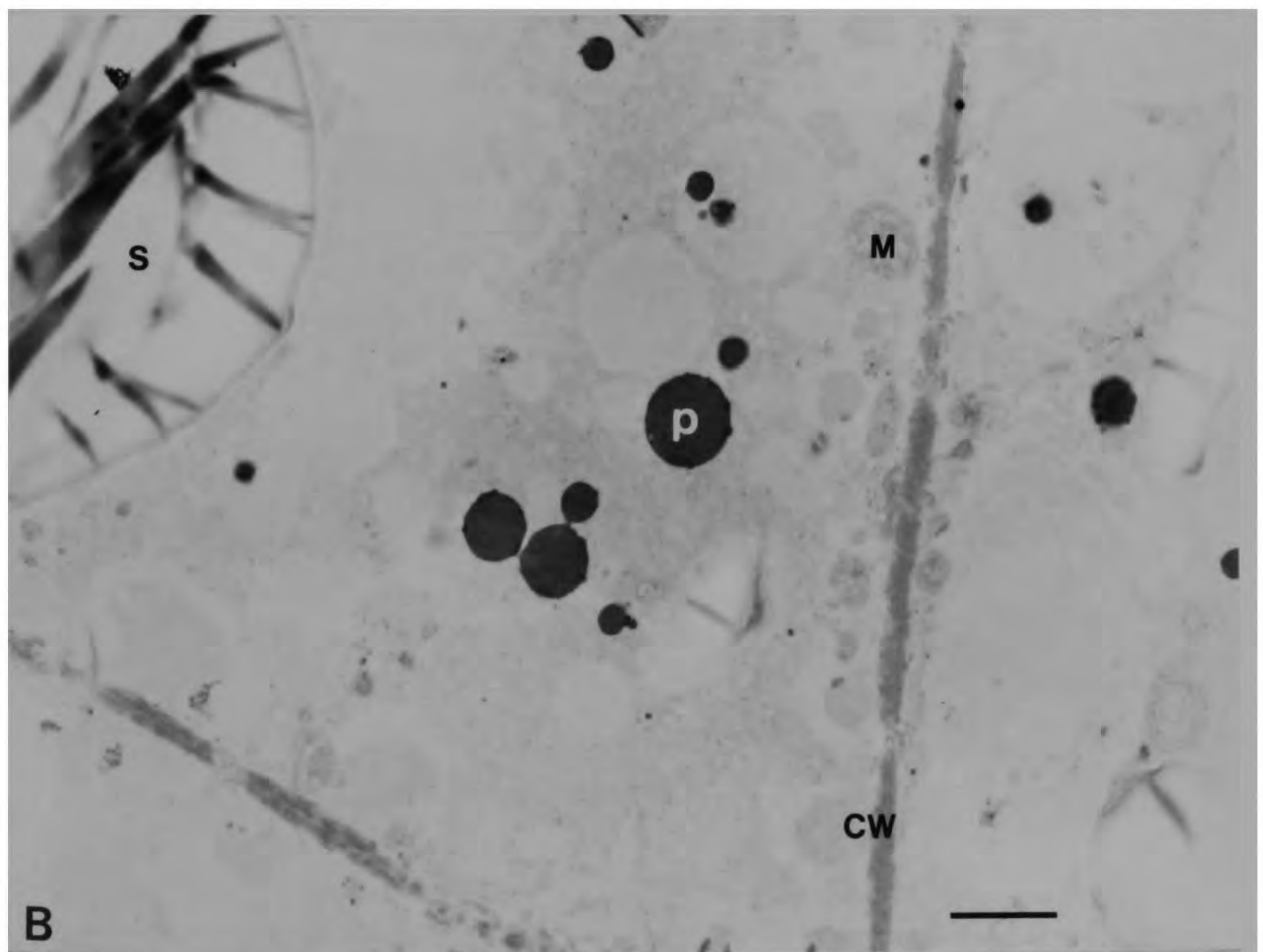
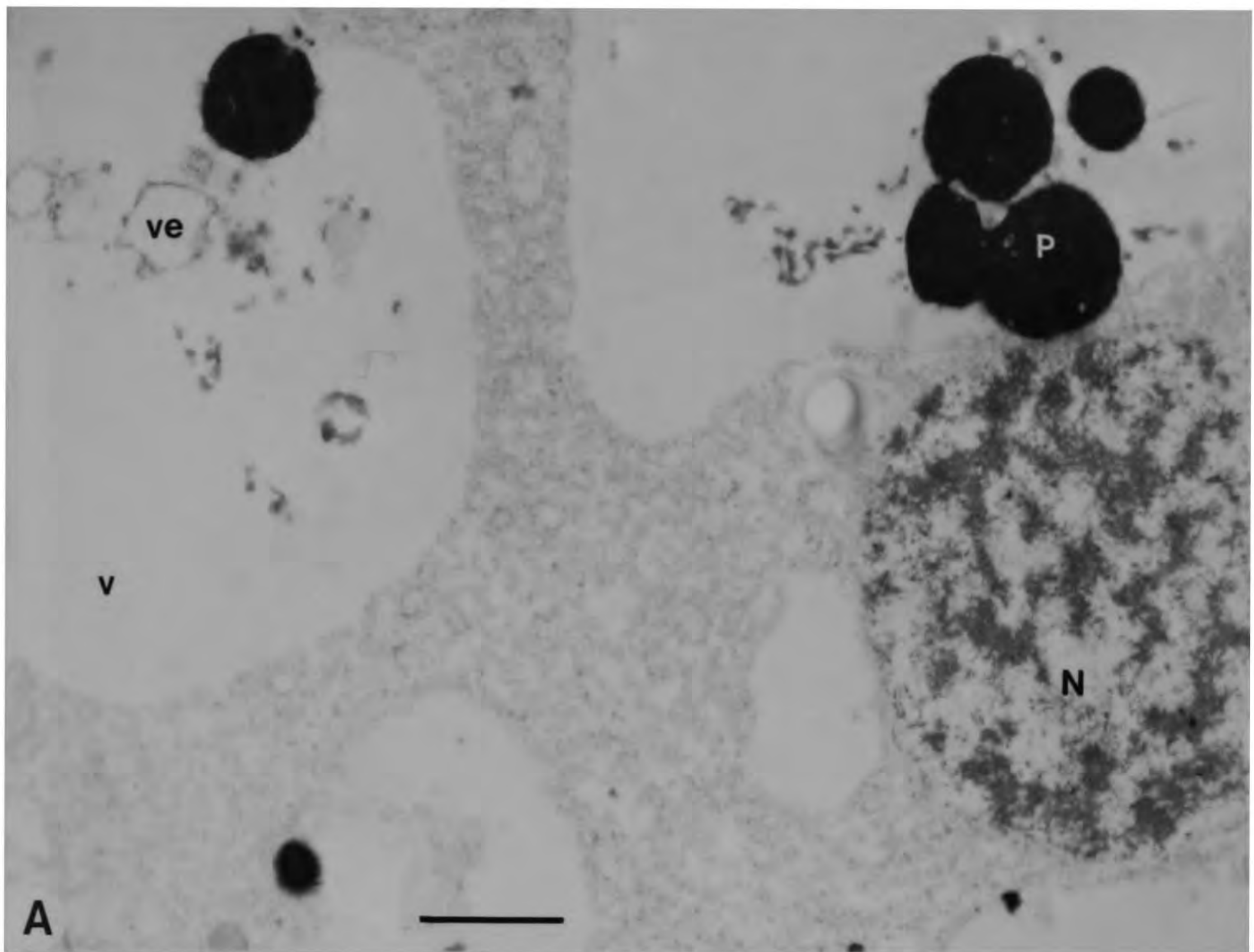


PLATE 27. Electron micrographs of ultrathin sections through the caryopsis of the high-lysine line Hiproly 14 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : Single protein bodies are located in the cytoplasm and in vacuoles of the sub-aleurone. Protein bodies are granular with electron-dense components. Bar = 2 µm.

B : In the starchy endosperm most single protein bodies are located in the cytoplasm. Bar = 2 µm.



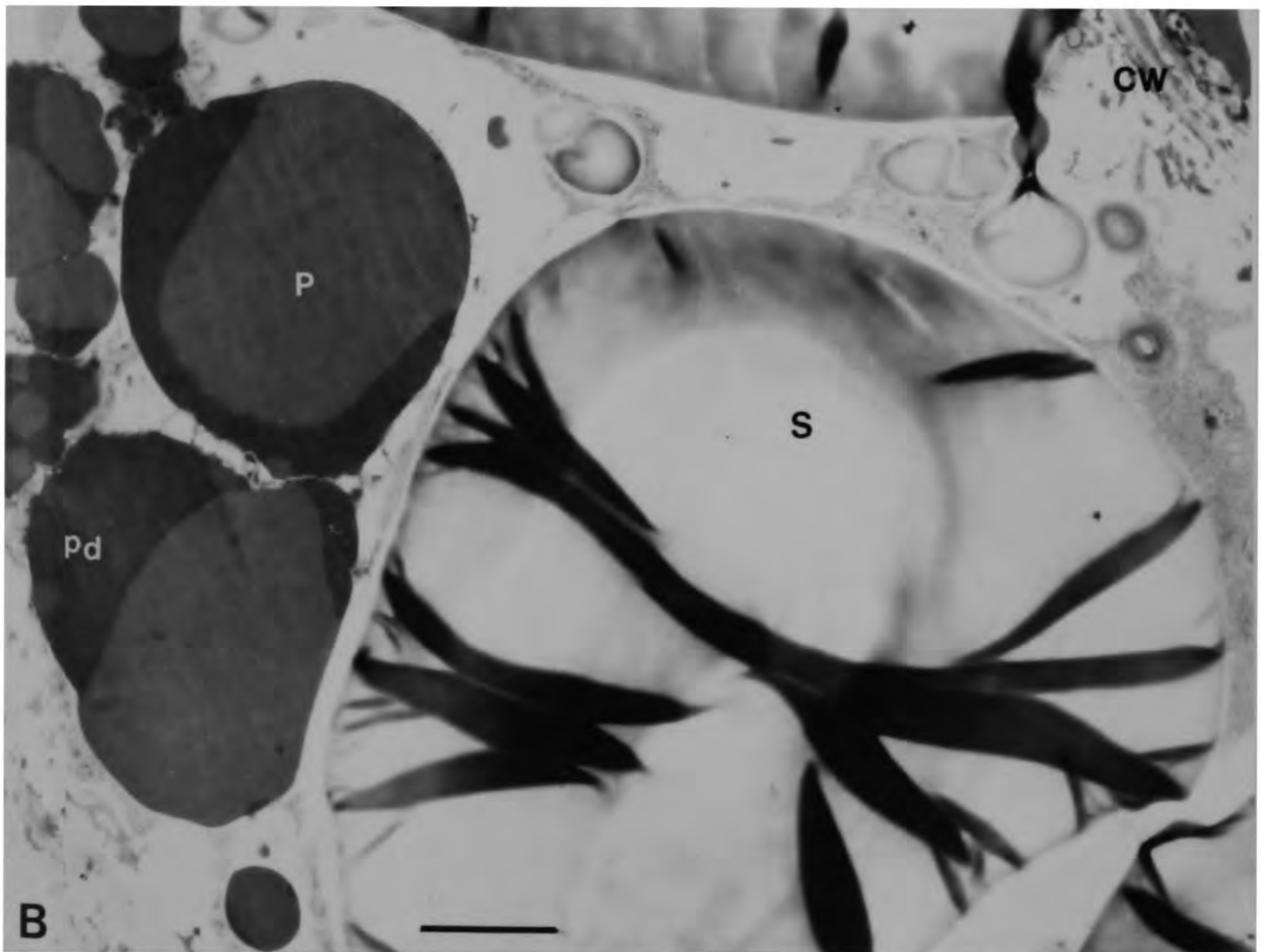
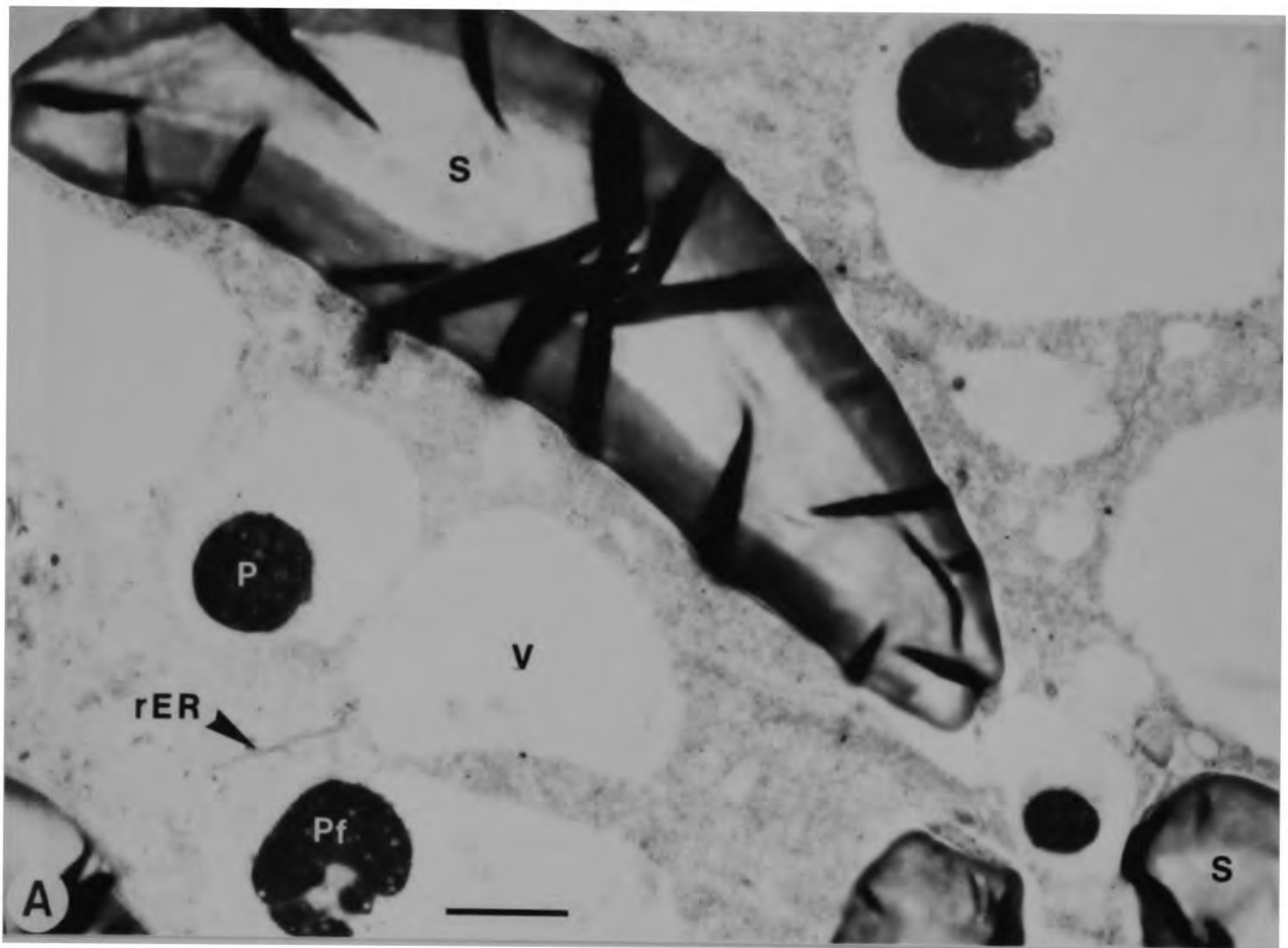


PLATE 28. Electron micrographs of ultrathin sections through the caryopsis of the high-lysine line Hiproly 21 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : Protein bodies in the sub-aleurone are located in vacuoles. Vesicles and vesicular material are also found in the vacuoles. Bar = 1.5 μm.

B : Cytoplasmic protein bodies of the endosperm consist of large granular areas with electron-dense components. Bar = 2 μm.



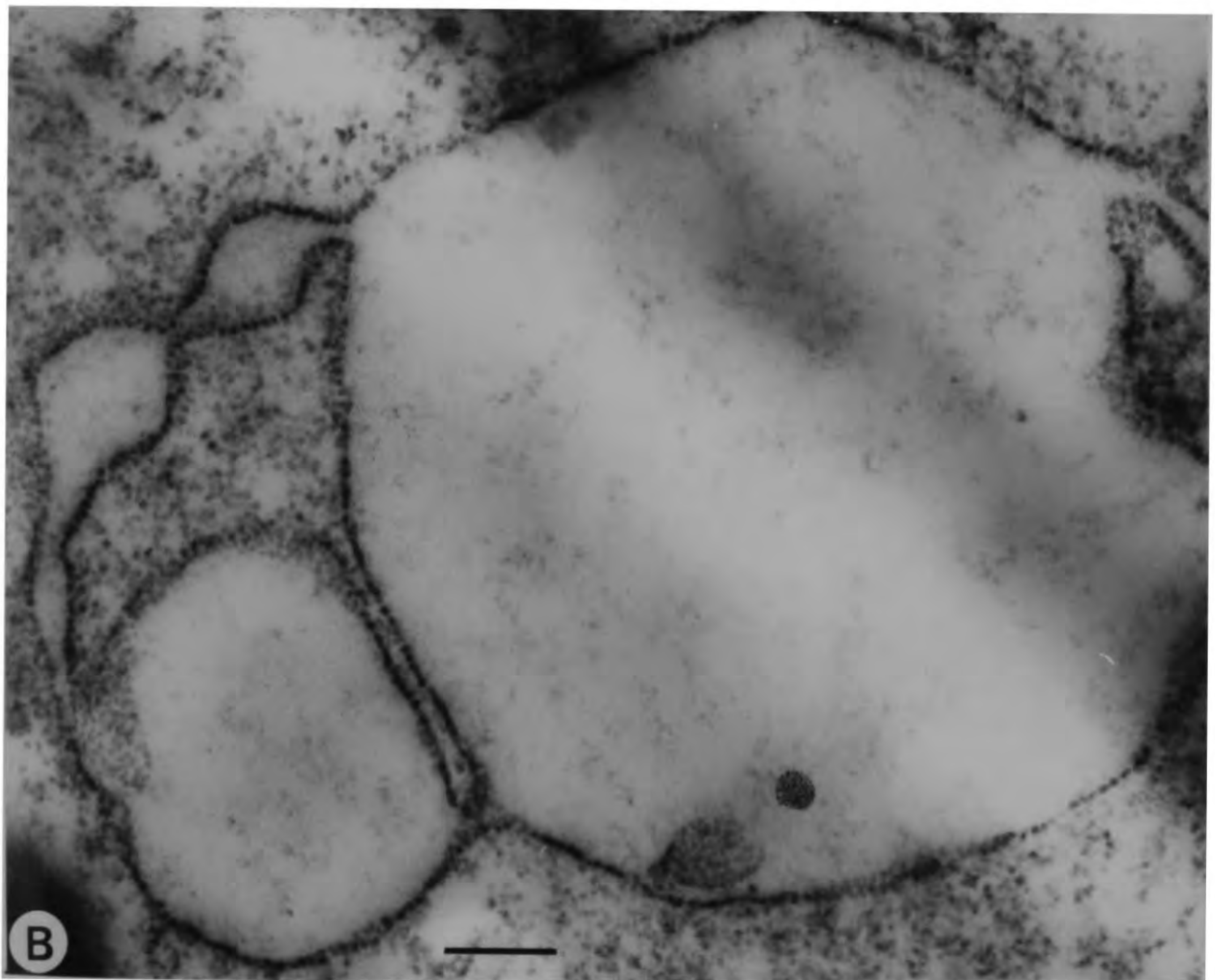
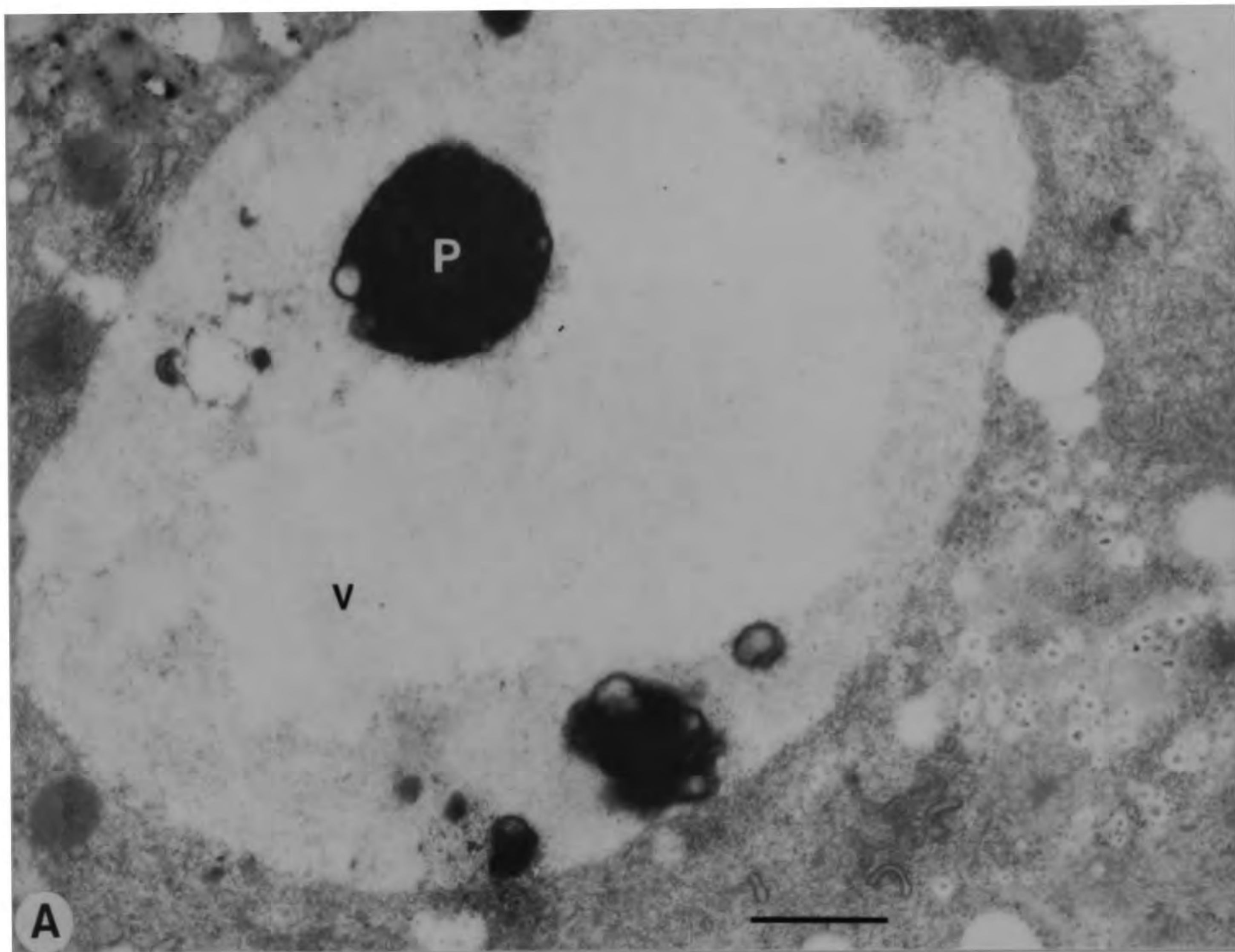


PLATE 29. Electron micrographs of ultrathin sections of the caryopsis of the barley mutant 1508, 17 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : In the sub-aleurone, protein bodies are vacuolar. These protein bodies consist of fibrillar components. Bar = 2 µm.

B : Endoplasmic reticulum in the cytoplasm of the sub-aleurone is dilated and studded with ribosomes. Bar = 0.25 µm.



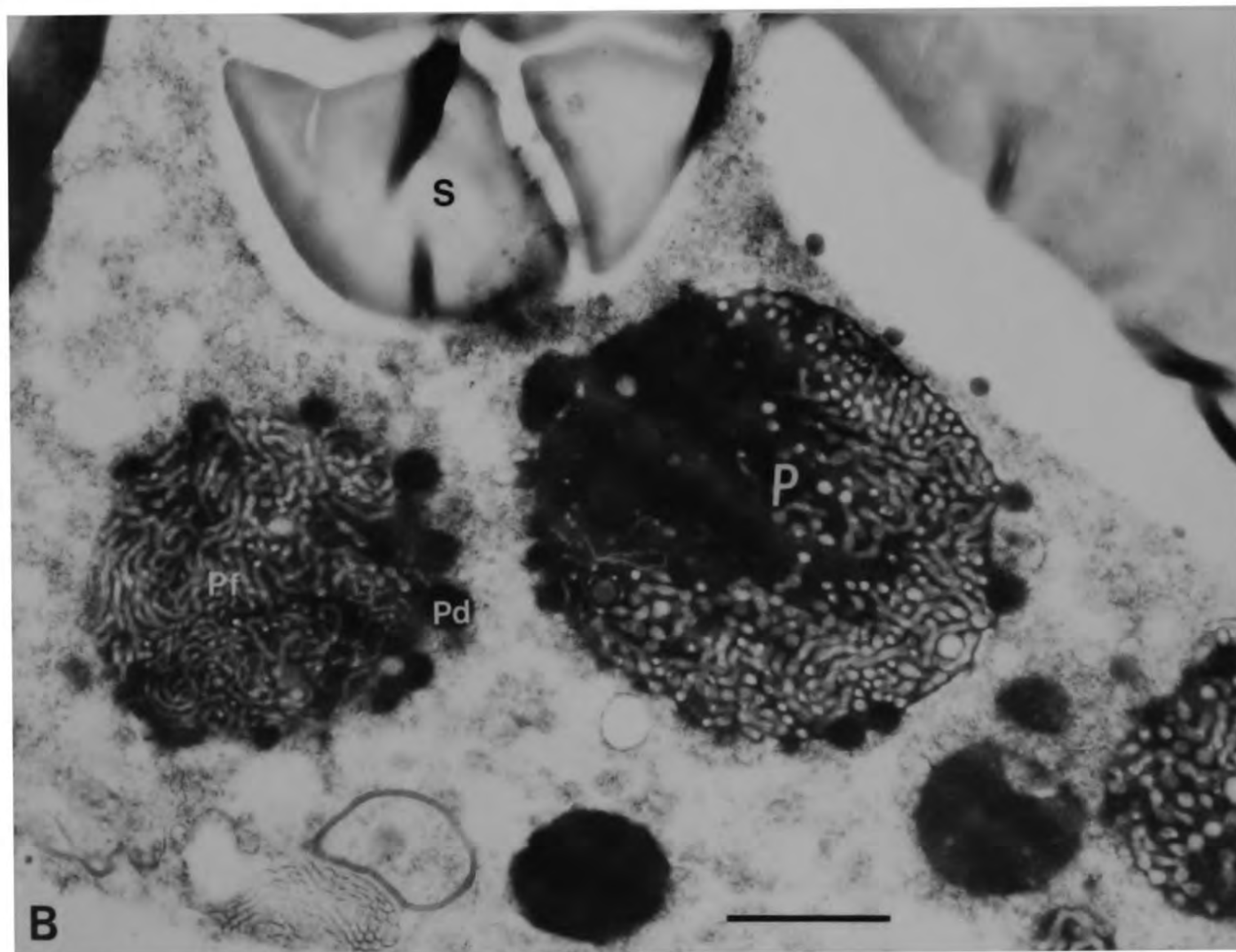
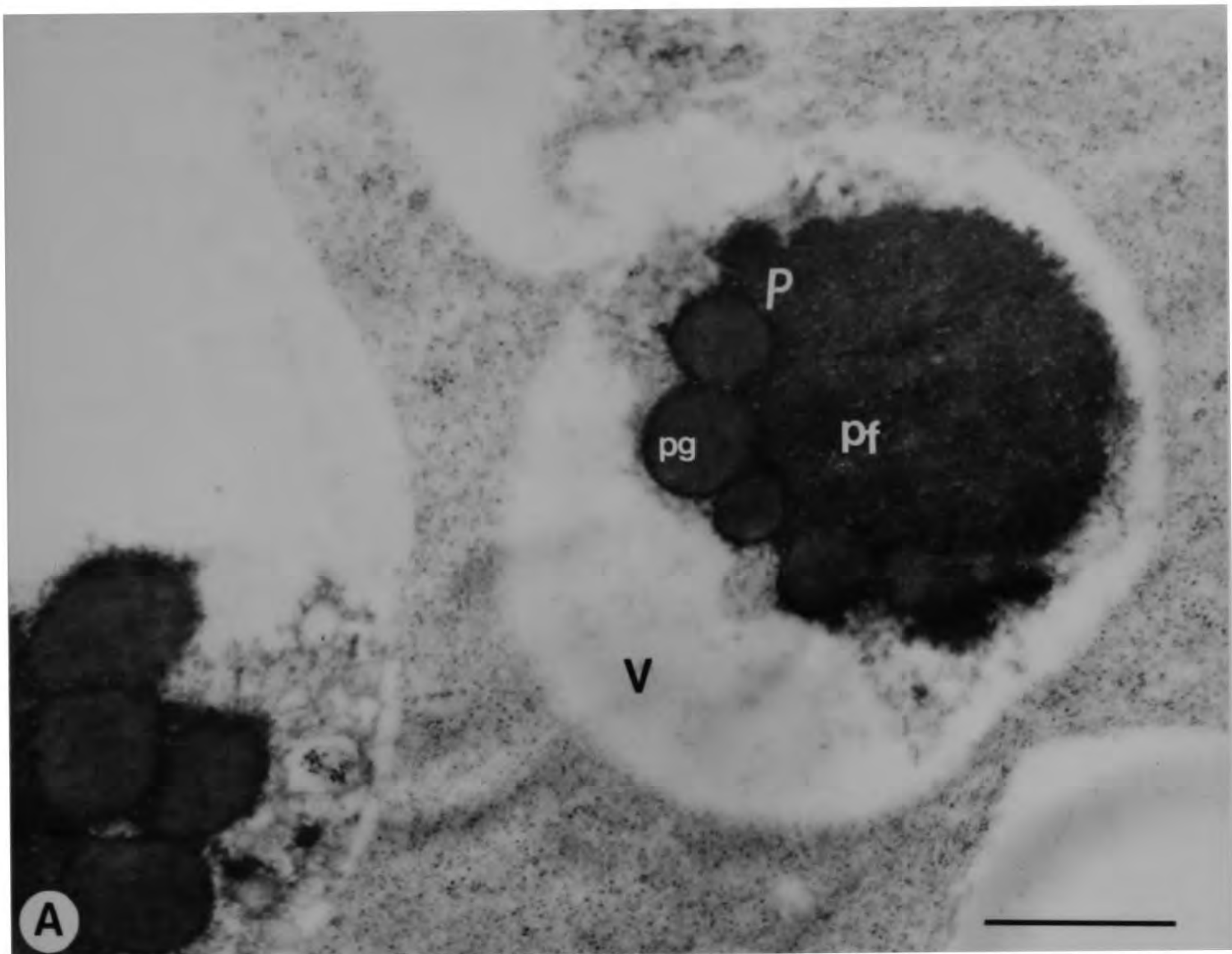


PLATE 30. Electron micrographs of ultrathin sections of the caryopsis of the barley mutant 1508, 23 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.
A : In the sub-aleurone, protein bodies are located in the vacuole. Bar = 1 µm.
B : Cytoplasmic protein bodies in the endosperm contain large fibrillar regions. There are electron-dense spheres round the periphery of the protein body. Bar = 1 µm.



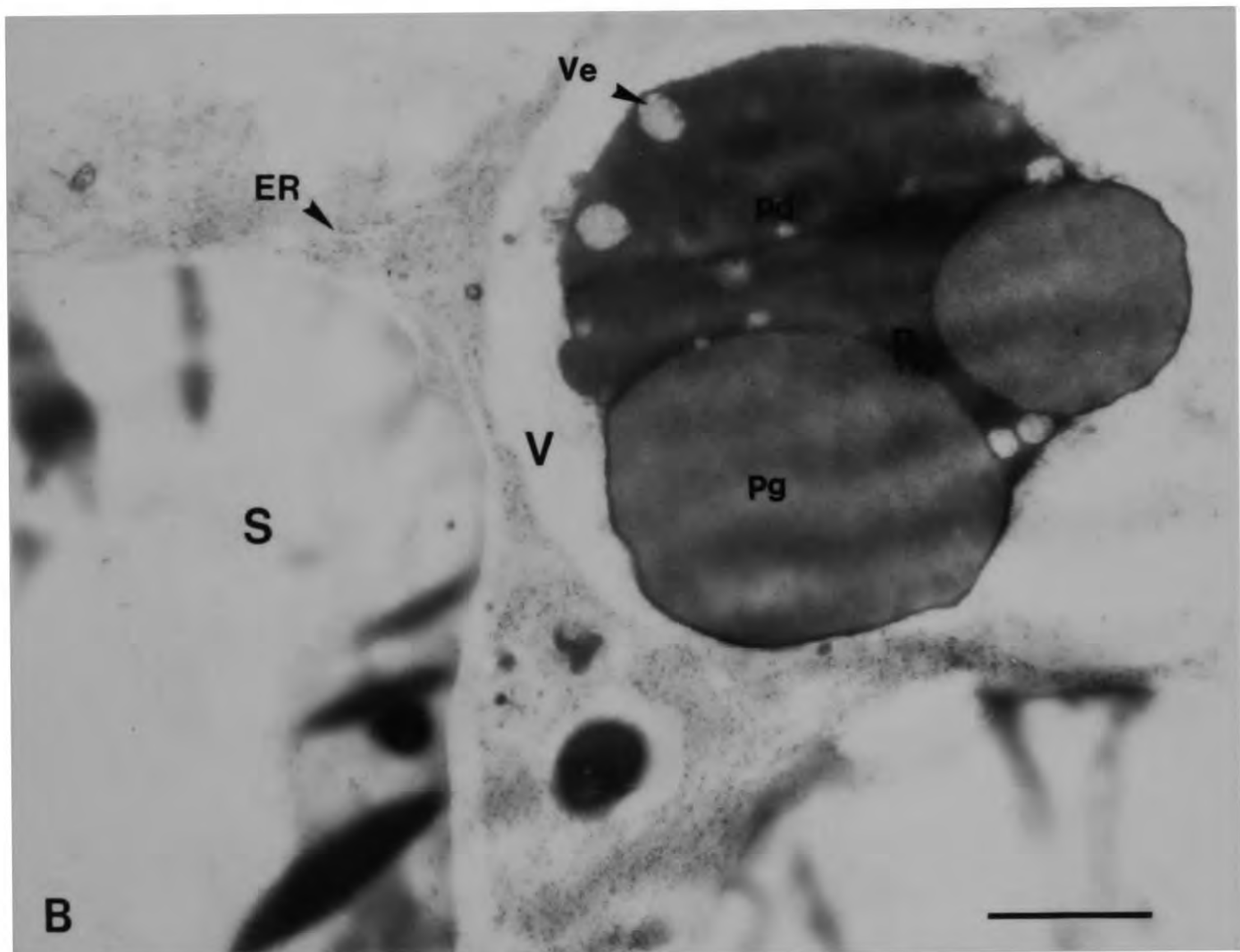
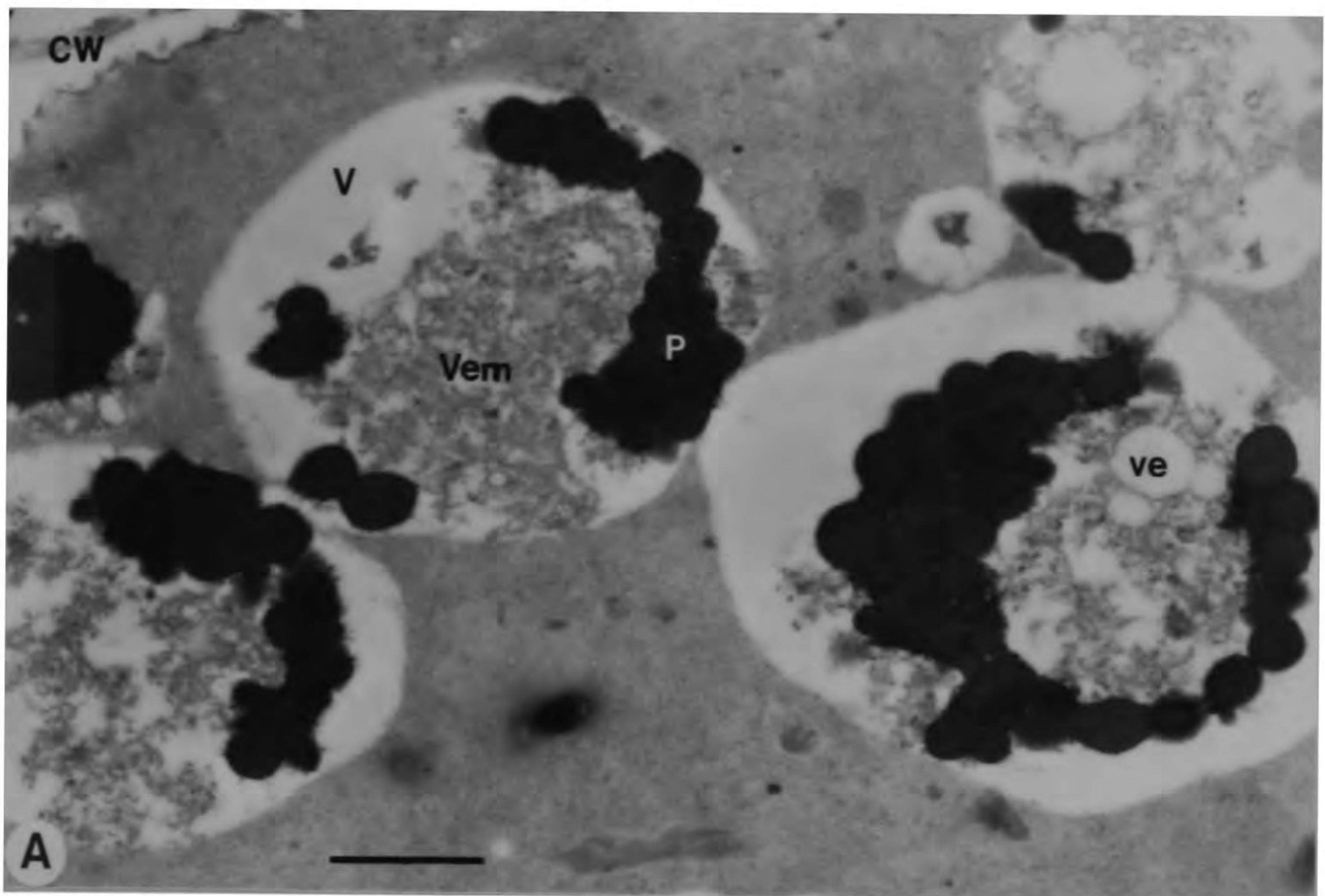


PLATE 31. Electron micrographs of ultrathin sections through the caryopsis of the barley mutant Riso 56, 17 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : Vacuolar protein complex in the sub-aleurone consists mainly of some granular components embedded in a predominantly fibrillar matrix. Bar = 1 μm.

B : In the endosperm, vacuolar protein complexes consist of granular areas embedded in an electron-dense matrix. Vesicles can also be seen in the protein body. Bar = 1 μm



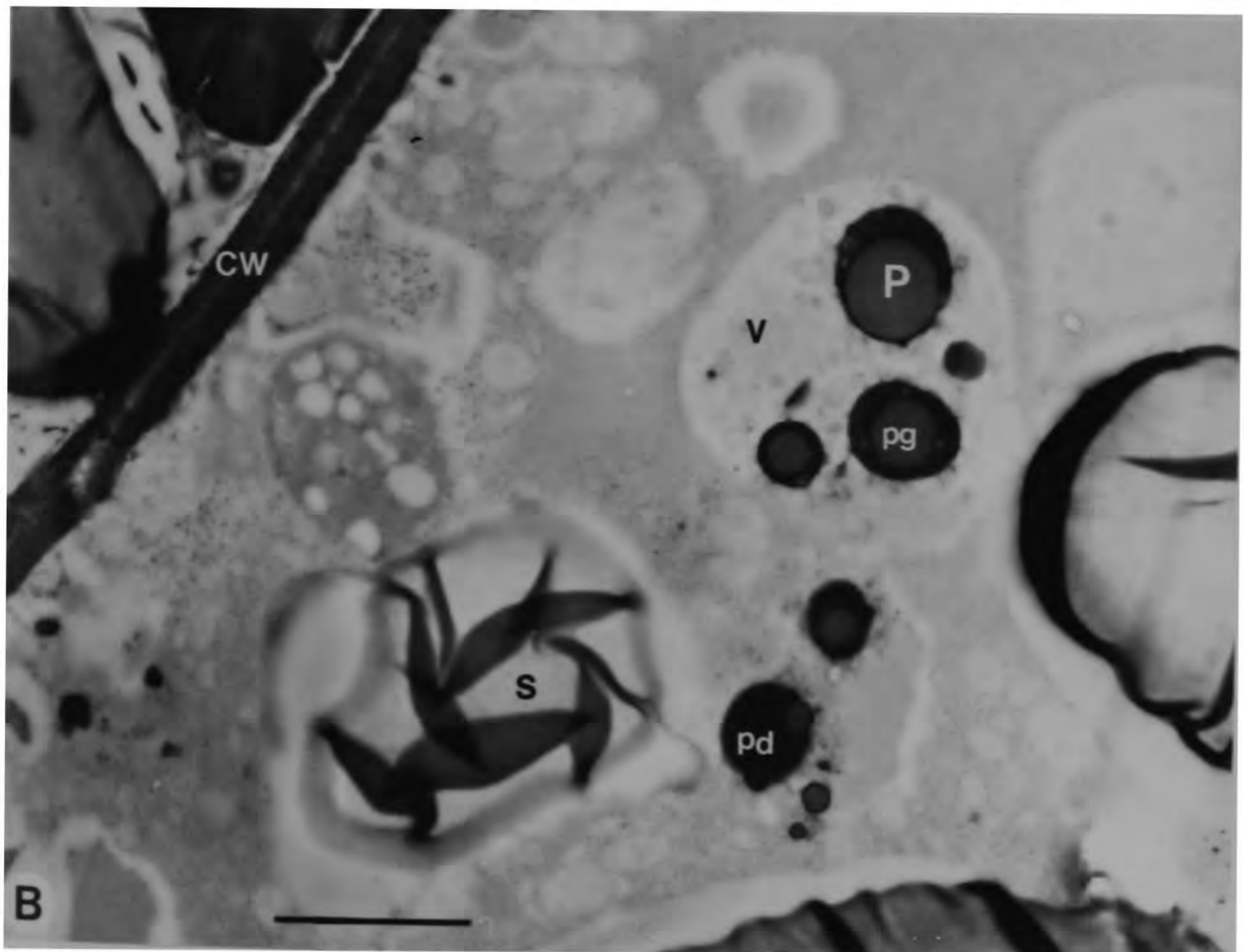
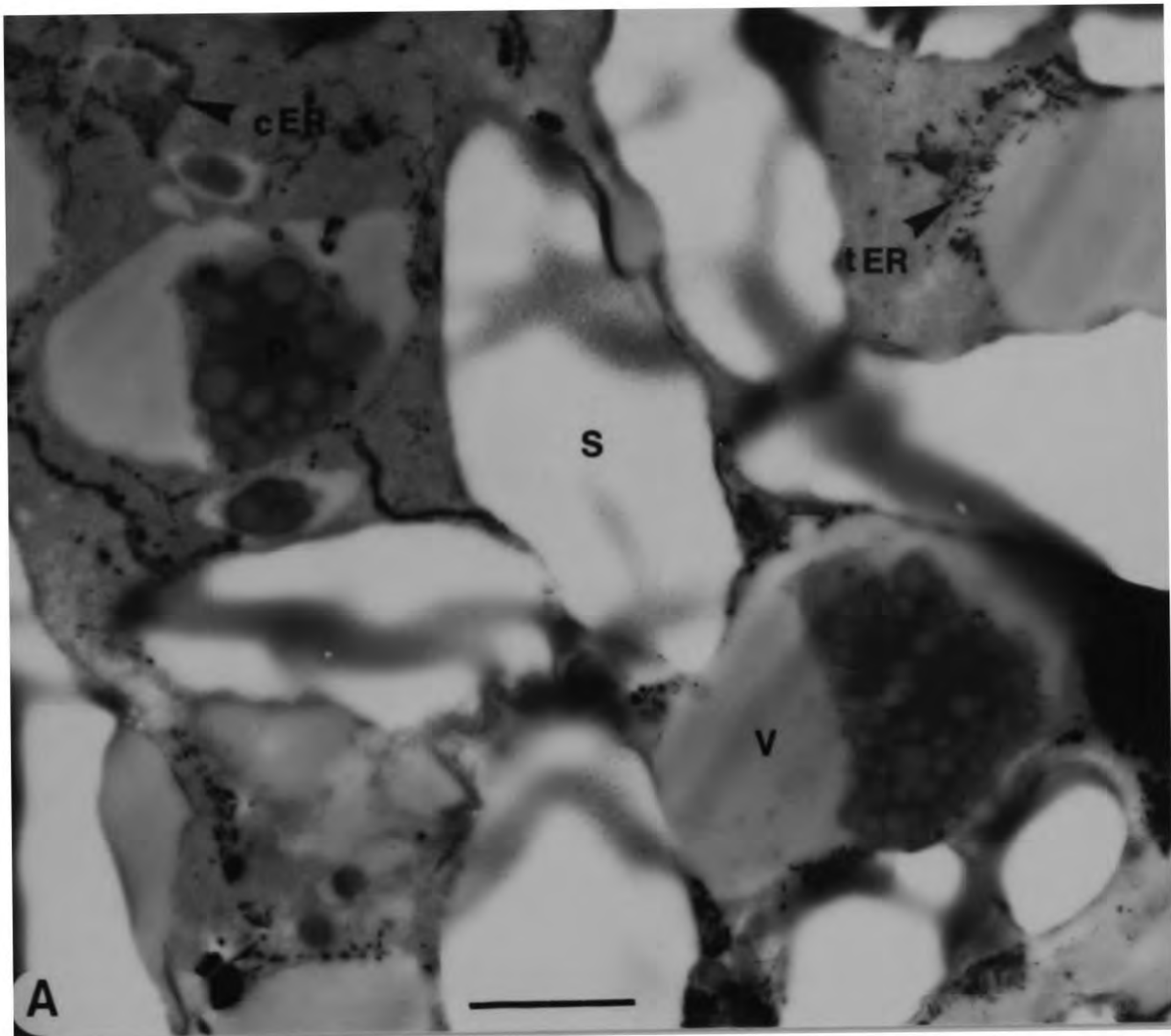


PLATE 32. Electron micrographs of ultrathin sections through the caryopsis of the barley mutant Risø 56, 24 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and post-stained.

A : In the sub-aleurone, vacuolar protein consists of many granular spheres embedded in a fibrillar matrix. Also in the vacuole there are vesicles in a vesicular matrix. Bar = 1.5 μm.

B : In the starchy endosperm, protein bodies are vacuolar and cytoplasmic. Protein bodies consist of large spheres of granular protein embedded in an electron-dense matrix. Bar = 3 μm.



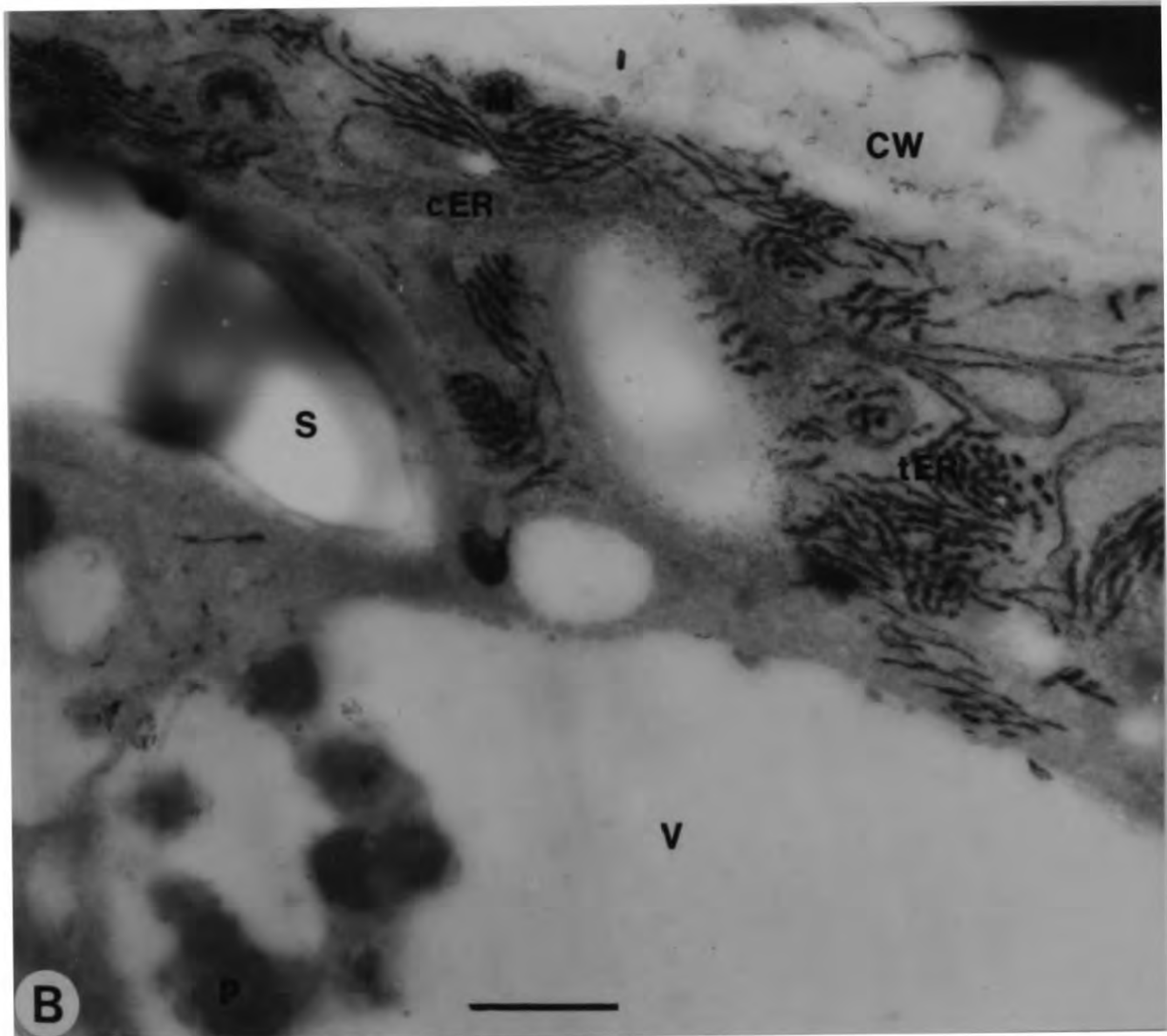
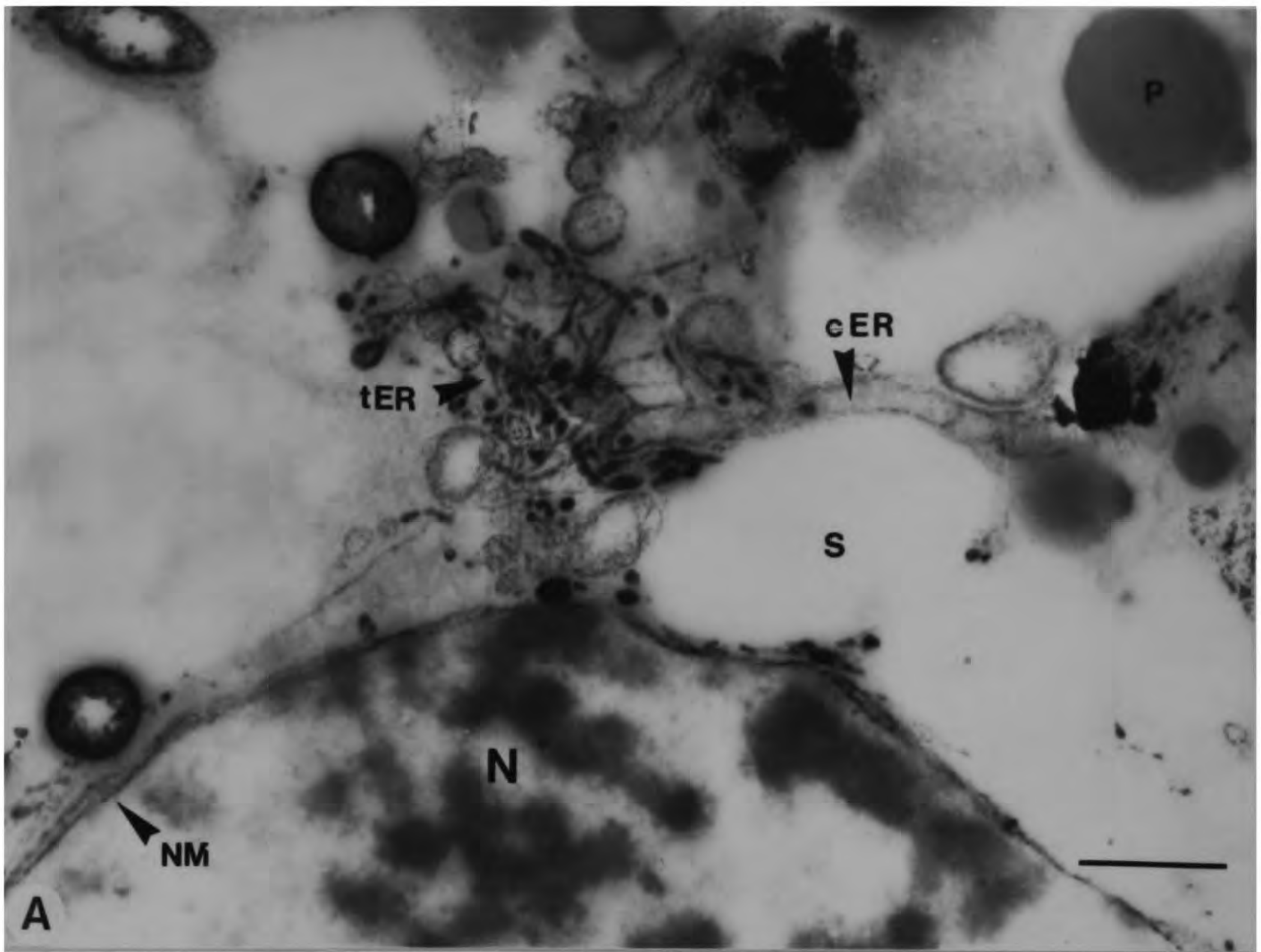
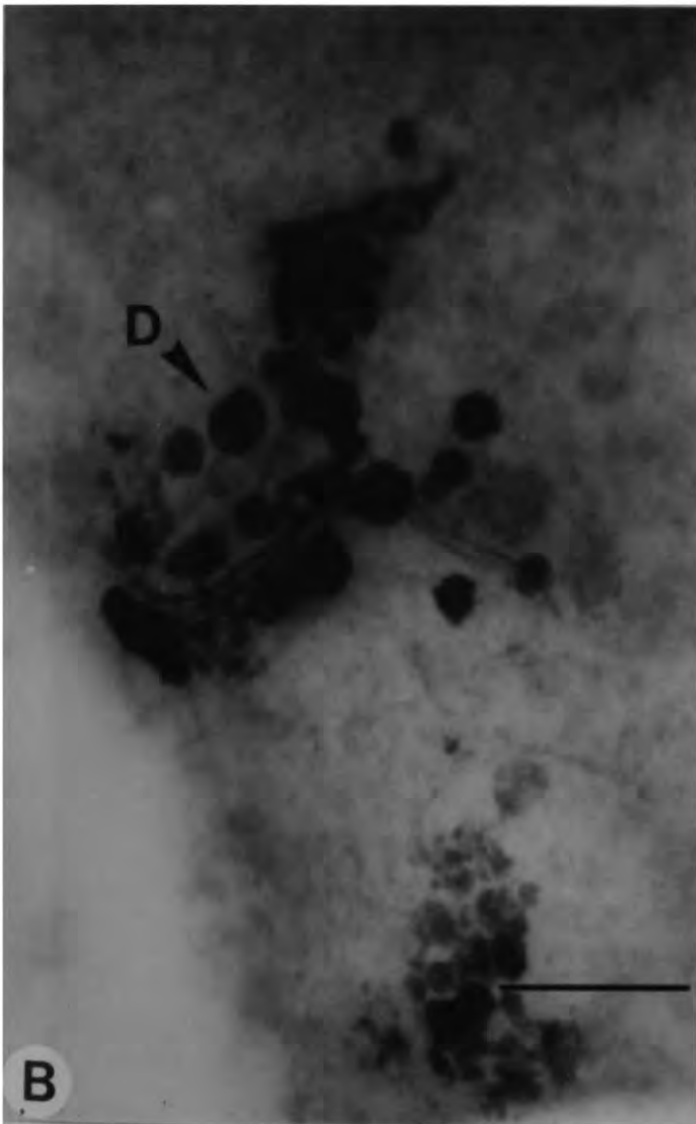


PLATE 33. Electron micrographs of 0.3 um sections of the sub-aleurone of the barley mutants Risø 56 and 1508, impregnated with ZIO.
A : Protein bodies in the sub-aleurone of Risø 56 (17 DAA) are located in vacuoles. ZIO staining shows distribution of cisternal and tubular ER. Bar = 3 µm.
B : In the sub-aleurone of 1508 (17 DAA), protein bodies are in vacuoles. Tubular ER is predominant. Bar = 1.5 µm.





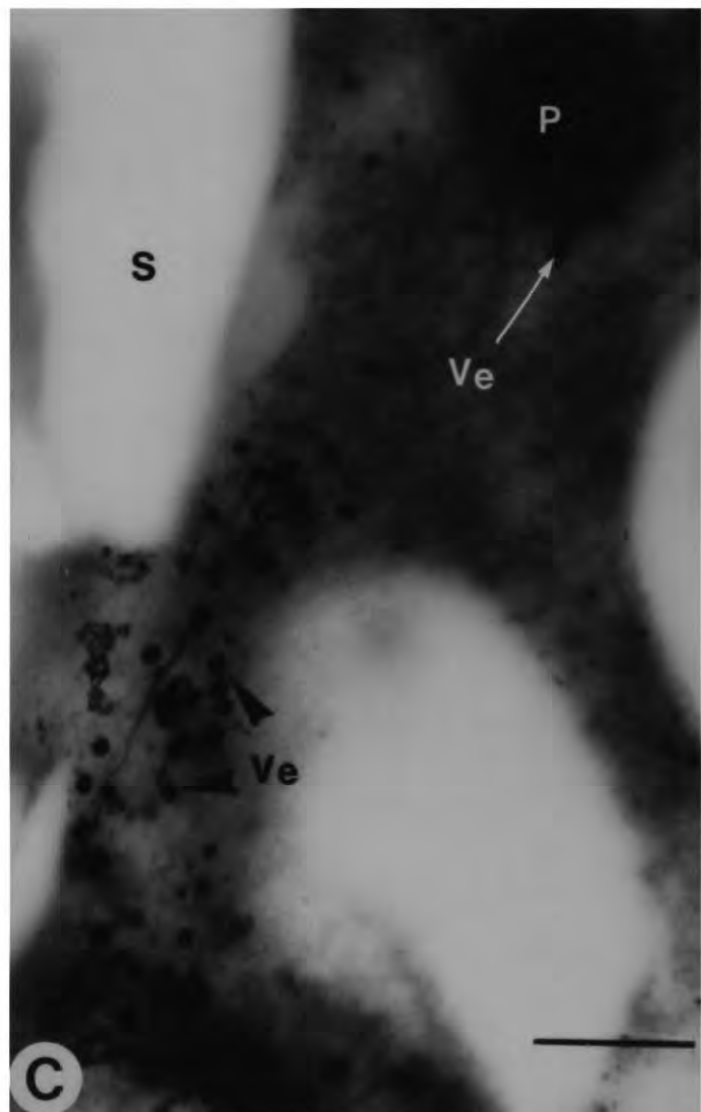


PLATE 34. Electron micrographs of 0.3 um sections of the Hiproly caryopsis, impregnated with ZIO.
A : Sub-aleurone of Hiproly, 14 DAA, shows a network of cisternal and tubular ER. Bar = 1 μm.
B : Heavily-stained cisternae and vesicles of a dictyosome in the sub-aleurone, 21 DAA. Bar = 0.3 μm.
C : In the endosperm 21 DAA, protein body has peripheral heavily-stained vesicles. A network of vesicles is also seen in the cytoplasm. Bar = 1 μm.

3.3 Subcellular Fractionations of Homogenates of Barley Endosperms

Homogenates were made of Bomi barley endosperms. Material was harvested from greenhouse-grown plants during the mid-phase of their grain development, when protein synthesis was at or about its maximum rate. These homogenates were separated on sucrose density gradients. Subcellular separations were made in the presence and in the absence of magnesium; these two conditions result in the retention or loss of ribosomes from the endoplasmic reticulum.

Protein body fractions were recognized by their content of storage proteins, their density, and their appearance under the electron microscope. The latter is described later (in Section 3.4.8) in conjunction with immunocytochemical localization techniques.

The distribution of the endoplasmic reticulum was followed by measuring the marker enzyme NADH-cytochrome *c* reductase.

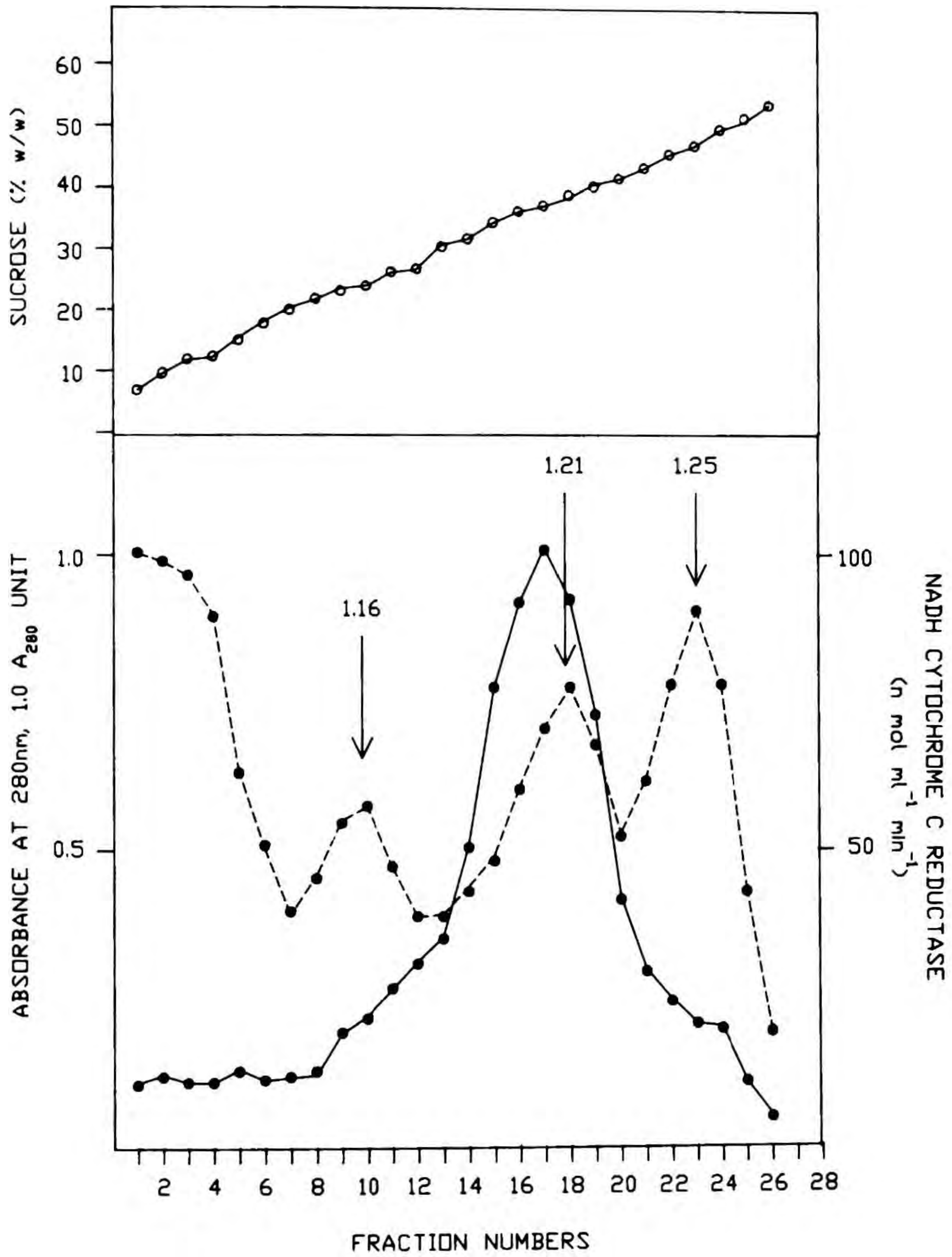
3.3.1 Sucrose Density Gradient Centrifugation Studies

Developing barley endosperms were chopped in two different media, one containing Mg^{2+} and no EDTA and the other EDTA and no Mg^{2+}. The resulting homogenates were then subjected to sucrose density gradient centrifugation. The gradients correspondingly also contained either Mg^{2+} or EDTA.

In both types of gradient (Figures 3.2 and 3.4) a prominent protein peak was found around a density of 1.25 g/ml. In the presence of Mg^{2+}, Figure 3.2, the endoplasmic reticulum marker enzyme NADH-cytochrome *c* reductase showed a major peak of activity at a slightly lower density than the smaller of the two protein peaks. When isolation and separation was carried out under stripping conditions (i.e. plus EDTA) the density of the NADH-cytochrome *c* reductase peak decreased (Figure 3.4), with a major proportion of the activity occurring around a density of 1.12 g/ml with a second peak around 1.14 g/ml.

The protein peak in the presence of Mg^{2+} (Figure 3.2) at 1.21 g/ml, disappeared in the absence of Mg^{2+}; in the absence of Mg^{2+}, there were two small peaks at densities around 1.12 and 1.14 g/ml.

The sucrose content of each fraction was determined by refractometry.



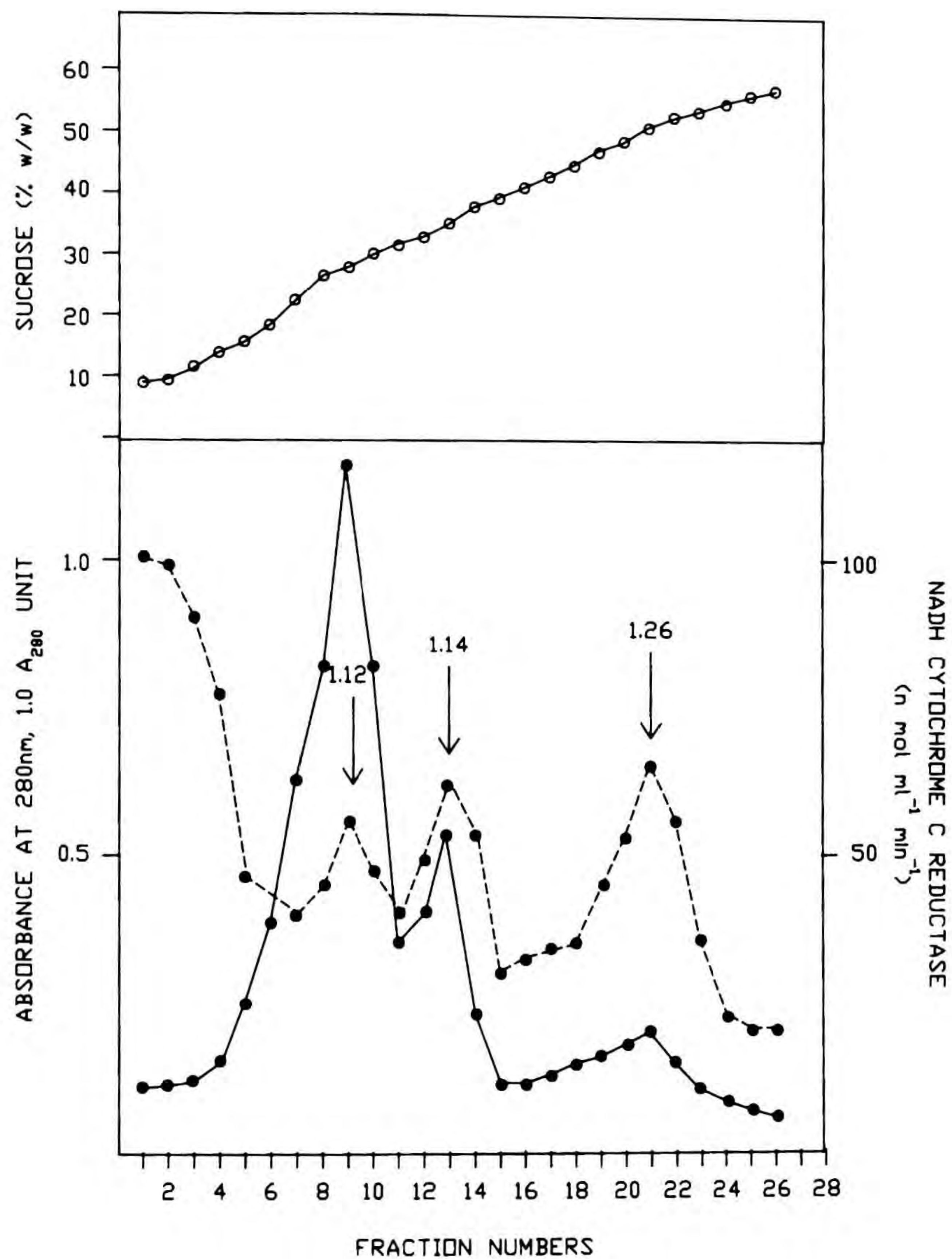
KEY :

(o——o) % Sucrose of the density gradient fractions

(•- - - -•) absorbance at 280 nm of fractions

(•——•) NADH cytochrome c reductase activity

FIGURE 3.2: FRACTIONS TAKEN FROM A SUCROSE DENSITY GRADIENT (20-60%w/w) SEPARATION OF HOMOGENATES OF BARLEY ENDOSPERMS WITH Mg^{2+} BUFFER. THE DENSITY (g/ml) OF THE PARTICULAR PEAKS IS GIVEN ABOVE THE ARROWS



KEY :
(o——o) % Sucrose of the density gradient fractions
(●- - - -●) absorbance at 280 nm of fractions
(●——●) NADH cytochrome c reductase activity

FIGURE 3.4: FRACTIONS TAKEN FROM A SUCROSE DENSITY GRADIENT (20-60%w/w) SEPARATION OF HOMOGENATES OF BARLEY ENDOSPERMS WITH Mg^{2+} - FREE BUFFER. THE DENSITY (g/ml) OF THE PARTICULAR PEAKS IS GIVEN ABOVE THE ARROWS.

3.3.2 Separation on SDS-PAGE of Fractions from Sucrose Density Gradient Separations

SDS-PAGE separations of fractions from sucrose density gradient separations of homogenates of barley endosperms in the presence and absence of Mg^{2+}, are shown in Figures 3.3 and 3.5.

The major protein peaks at densities around 1.25 g/ml showed mobility characteristics on SDS-PAGE equivalent to authentic Bomi marker hordein. A significant proportion of the protein in other peaks also had characteristics indicative of hordein, although the greatest proportion occurred in fractions from the protein peaks (1.21 and 1.25 g/ml in the presence of Mg^{2+}, Figure 3.2, and, 1.26 g/ml in the absence of Mg^{2+}, Figure 3.4).

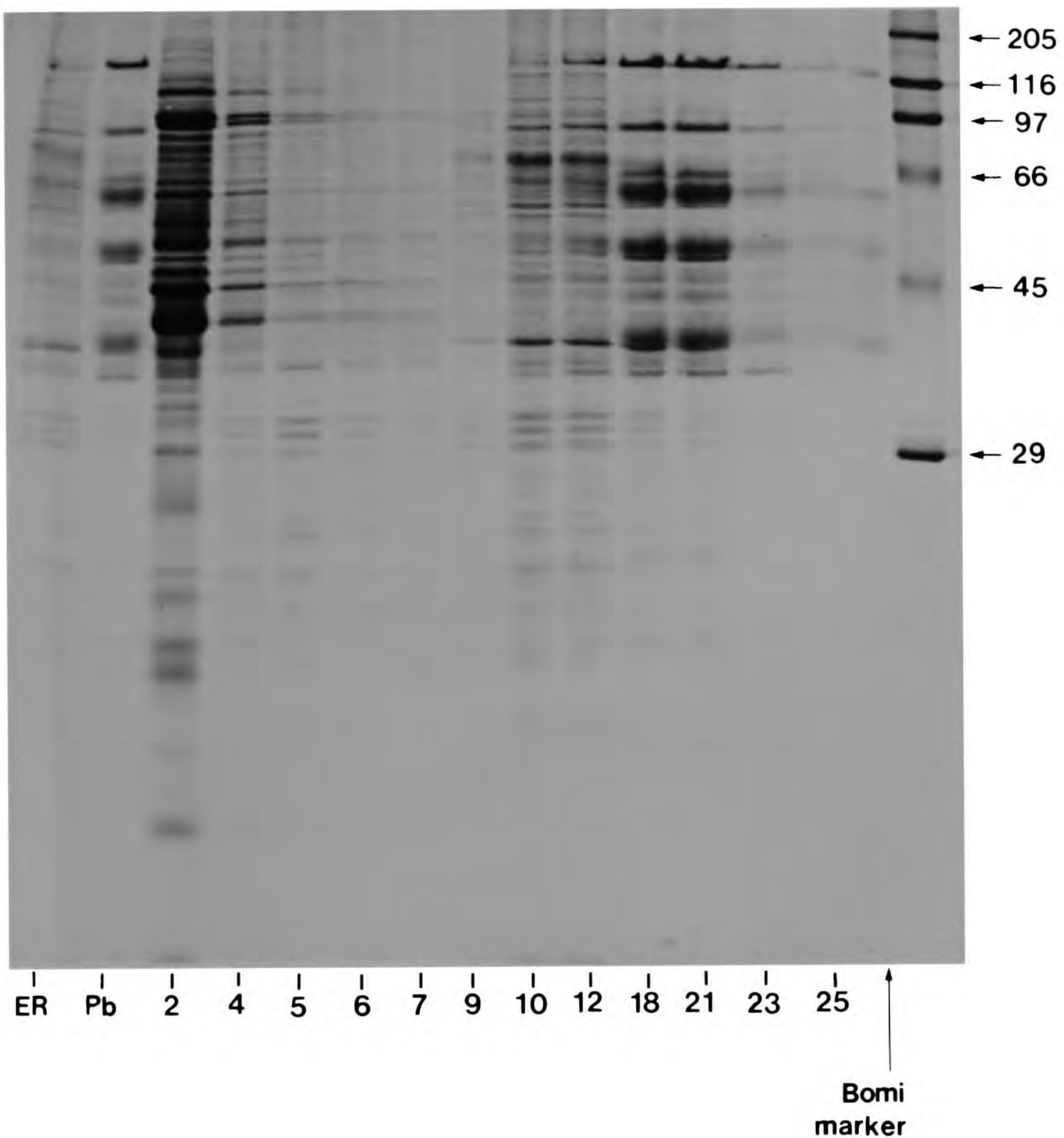


FIGURE 3.3

Separation on SDS-PAGE of fractions extracted from sucrose density gradient separations of homogenates of barley endosperms in the presence of Mg^{2+}.

Numbers correspond to gradient fractions (Figure 3.2); combined endoplasmic reticulum fraction (ER) was taken from around 1.16 g/ml, and combined protein body fraction (Pb) from around 1.25 g/ml. Arrows indicate approximate molecular weights in kilodaltons.

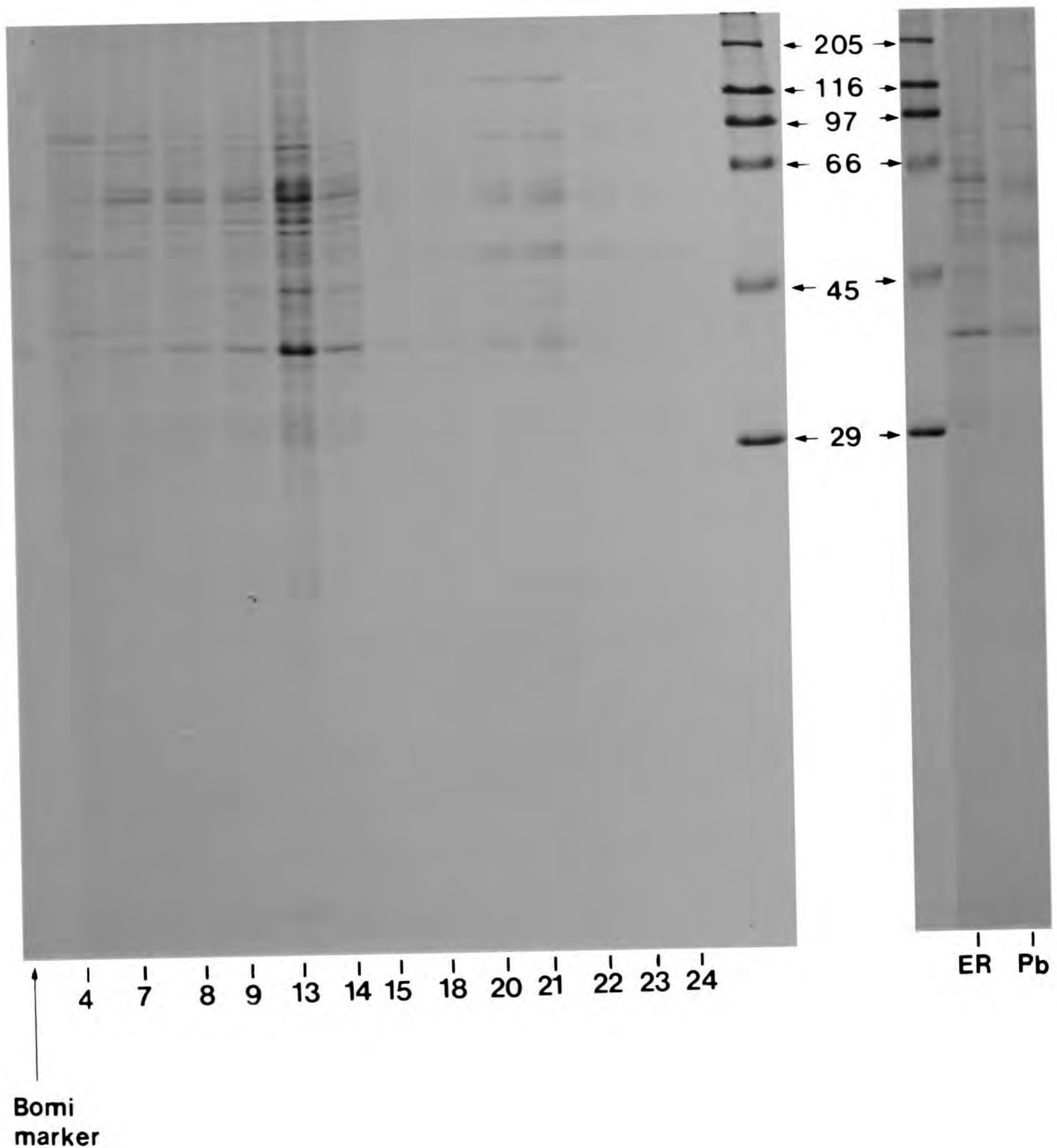


FIGURE 3.5

Separation on SDS-PAGE of fractions extracted from sucrose density gradient separations of homogenates of barley endosperms in the absence of Mg^{2+}.

Numbers correspond to gradient fractions (Figure 3.4); combined endoplasmic reticulum fraction (ER) was taken from around 1.14 g/ml, and combined protein body fraction (Pb) from around 1.26 g/ml. Arrows indicate approximate molecular weights in kilodaltons.

3.4 Immunocytochemical Localization with Protein A-Gold by Electron Microscopy

Polyclonal antibodies (as described in Section 2.7) were used to investigate the composition of the protein deposits in the sub-aleurone and starchy endosperm of the developing Bomi barley caryopsis.

Sections of resin-embedded tissue were incubated with antibodies, and the sites of specific binding were shown by subsequent incubation with protein A-gold.

A comparison of the acrylic resins (Lowicryl K4M and LR White), and an epoxy resin (Spurr resin) has been made. The effect of osmium tetroxide post-fixation (with Spurr resin) has been investigated, as has the effect of periodate 'etching'.

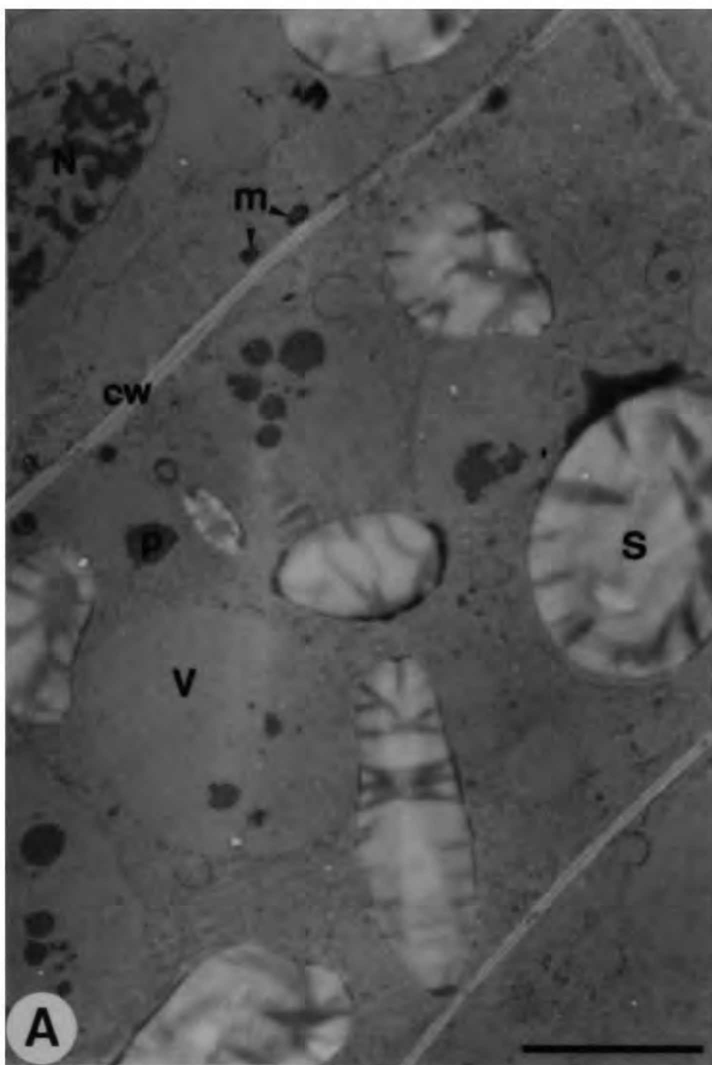
As the ultrastructural morphology of the protein bodies of the high-lysine mutants has been shown to differ considerably from the wild-type Bomi barley, the mutant barley lines were also investigated using immunocytochemical localization to determine if an altered protein body morphology results in a different protein distribution compared with the wild-type.

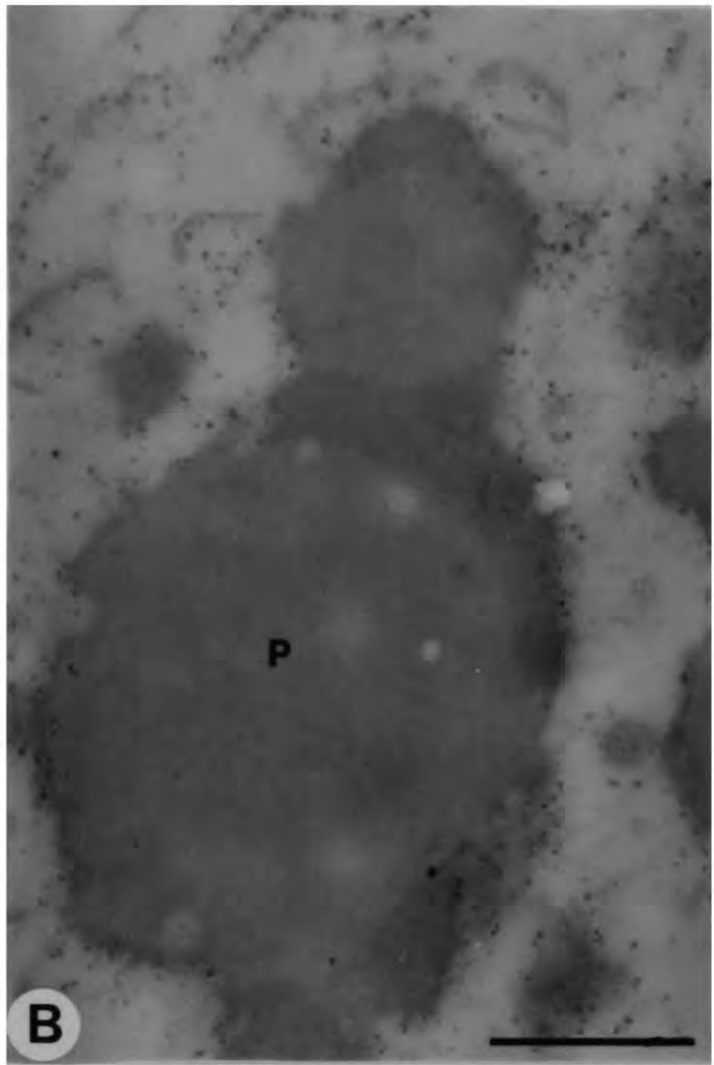
3.4.1 Immunocytochemical Controls

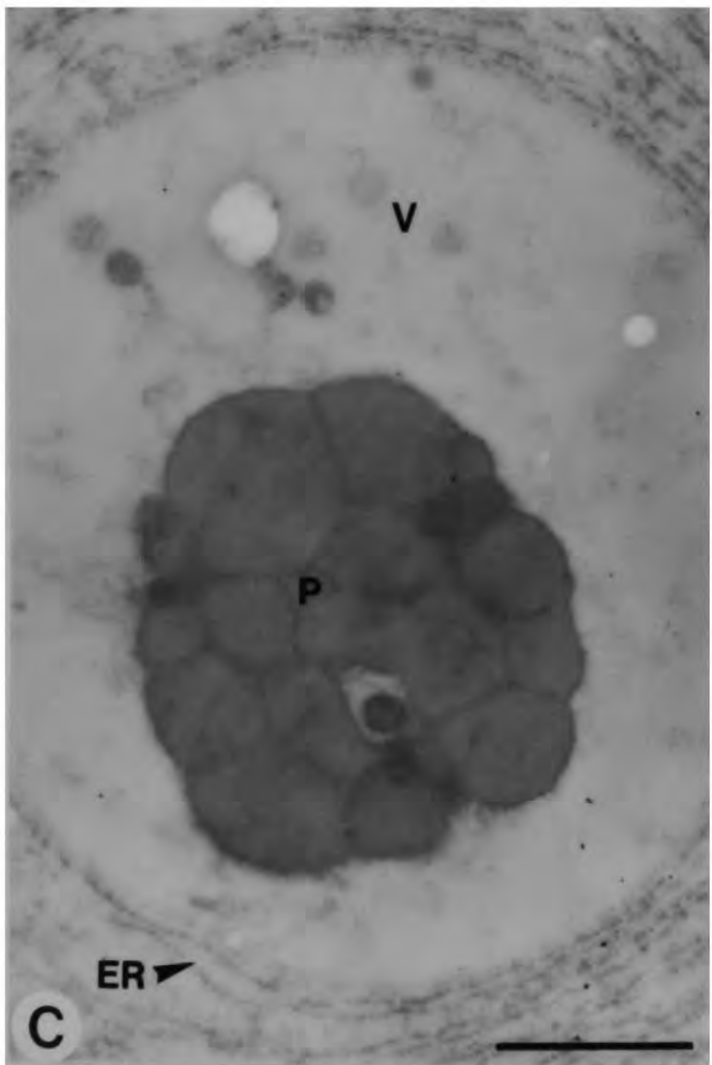
Immunocytochemical controls were employed to establish the validity of observations for each antigen used. Plate 35 illustrates the controls used in the immunocytochemical localization of C hordein.

The first control involved incubation with protein A-gold only (micrograph 35A). At a higher magnification, micrograph 35B, there is no non-specific binding of the protein A-gold complex to the surface of the section. Micrograph 35C, illustrates the section after incubation with pre-immune serum. This serum was extracted from the rabbit prior to injection with the protein for which polyclonal antibodies were to be raised. Following incubation with protein A-gold, a few antigenic sites are located randomly over the surface of the section.

Micrograph 35D shows a section of the sub-aleurone of barley labelled for C hordein by protein A-gold. Almost all of the labelling is in the granular region of the protein body. The occasional cytoplasmic gold particles may be non-specific, as indicated by a similar distribution in the pre-immune control (micrograph 35C).







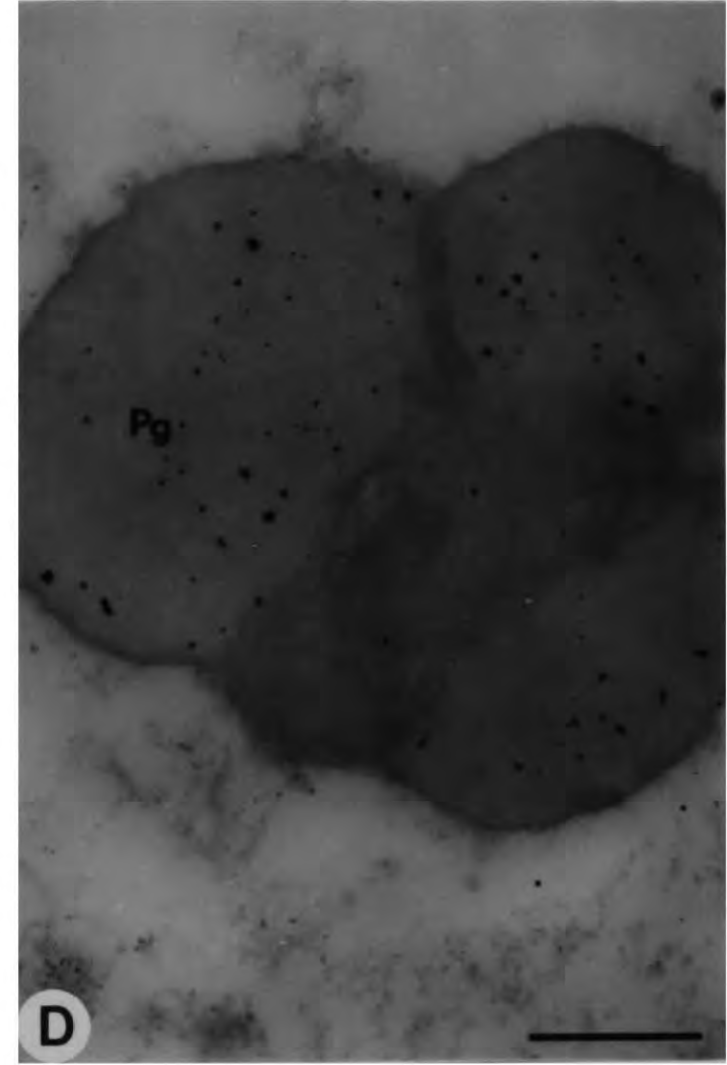


PLATE 35. Immunocytochemistry controls for C hordein in osmicated tissue, 14 DAA (Spurr resin).
A : Low magnification of barley incubated in PAG_h only. Bar = 0.5 µm.
B : Detail of (A). No gold markers are seen over section. Bar = 0.5 µm.
C : Sub-aleurone protein body incubated in pre-immune serum, followed by PAG_h labelling. Only occasional gold markers bind non-specifically. Bar = 1 µm.
D : PAG_h labelling of C hordein. Gold marker is associated with the granular area of the protein aggregate. Bar = 0.3 µm.

3.4.2 Immunocytochemical Localization of Hordeins in Barley Endosperm (cv. Bomi)

Polyclonal antibodies to A, B and C hordein, raised in rabbits were used to demonstrate, in an immunocytochemical study, the distribution and composition of developing protein bodies in barley (cv. Bomi) endosperm.

Micrograph 36A, illustrates immunocytochemical labelling of C hordein in small cytoplasmic protein deposits in the sub-aleurone of a 26 mg grain (10 DAA). By 14 DAA the barley caryopsis has increased in fresh weight to 42 mg, and vacuolar protein aggregates are seen in the sub-aleurone. These protein aggregates label densely with protein A-gold after prior treatment with antisera to C hordein. The sub-aleurone 32 DAA (fresh weight 76 mg) is densely packed with starch, and protein forms a matrix in the interstices between the starch grains (micrograph 36C). In micrograph 36D, protein A-gold labelling for C hordein shows a high density of immunogold labelling in the interstitial space between the starch grains. Very occasional gold particles bind non-specifically over the starch grains.

In micrograph 37A, immunogold labelling of antibodies directed against C hordein shows selective labelling of the granular areas of protein bodies in the sub-aleurone of barley at 14 DAA. Similarly, in

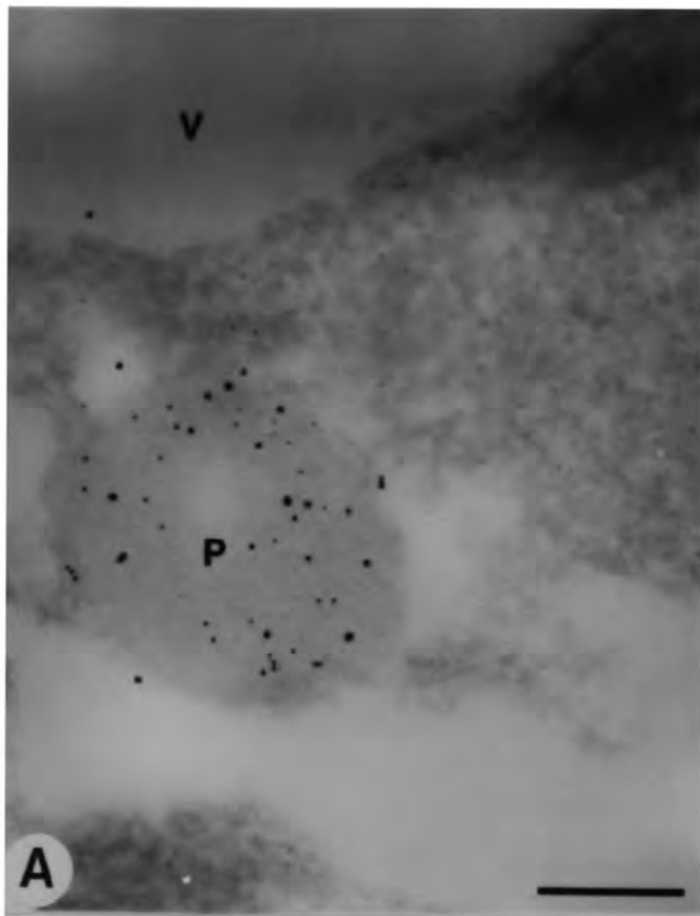
the endosperm (micrograph 37B), labelling shows C hordein confined to the granular areas of the protein complex. The electron-dense areas of the protein bodies, surrounding cytoplasm and starch granules have no label. Micrographs 37C and 37D compare the distribution of B and A hordein in the endosperm protein complexes at 14 DAA. As in micrograph 37B, localization of B and A hordein is in the granular regions of the protein complex. Associated vesicles of the protein complex (micrograph 37D) have no immunolabel.

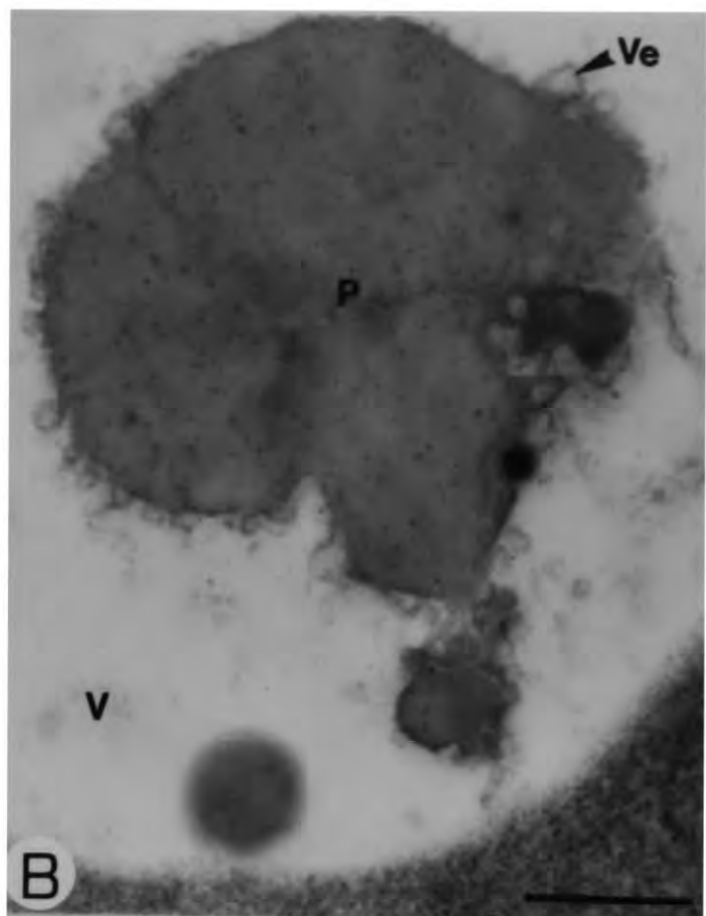
Plate 38 illustrates investigation of the effect of osmium tetroxide post-fixation upon antigenicity of Spurr resin-embedded tissue. Micrograph 38A, shows immunocytochemical labelling of C hordein by protein A-gold in the protein body, 14 DAA. Gold label is mostly confined to the granular region of the protein body, and surrounding vesicles and the vacuole are unlabelled. Periodate 'etching' of osmicated sections before incubation with antiserum to C hordein, produces a subsequently denser labelling of antigenic sites over the protein body (compared with micrograph 38A). In micrograph 38B, immunogold labelling is located over the fibrillar regions of the protein body as well as the granular regions. There is very little labelling in the cytoplasm.

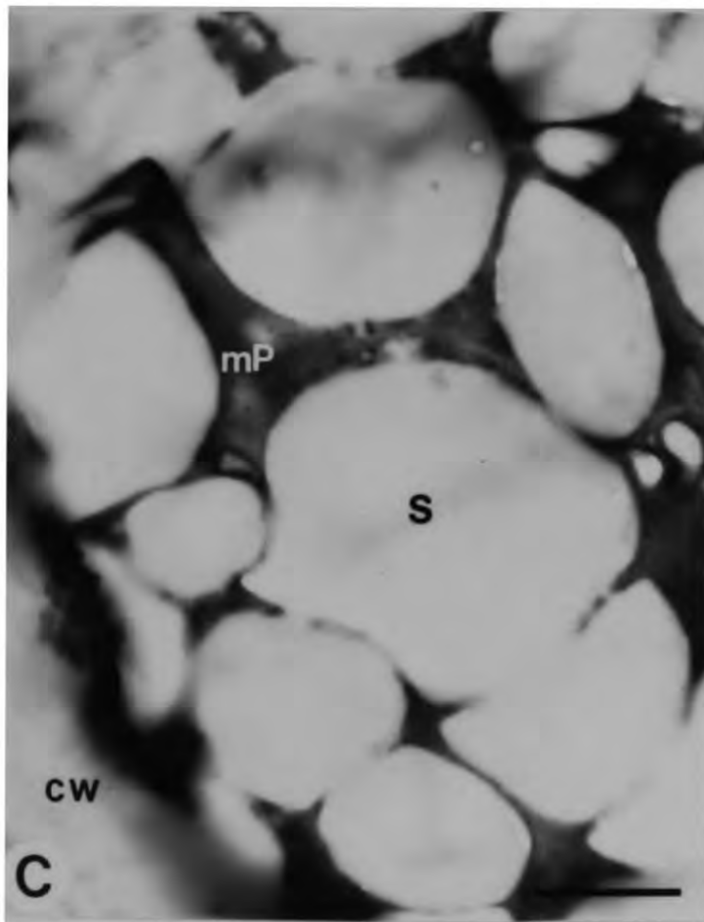
Micrographs 38C and 38D illustrate the effect of omitting osmium-tetroxide post-fixation with Spurr resin-embedded tissue on immunocytochemical labelling. Micrograph 38C shows a protein body with peripheral electron-dense spheres, post-stained with uranyl acetate and lead citrate. Immunocytochemical localization of C hordein with protein A-gold shows a high density of labelling over the granular area of the protein body. Electron-dense spheres are unlabelled, and occasional gold particles are seen in the cytoplasm. In micrograph 38D, localization of C hordein with protein A-gold shows a high density of labelling over the protein body. However, omitting osmium and post-staining, results in a poorer contrast.

The reduced antigenicity resulting from osmium-tetroxide post-fixation is again illustrated in Plate 39. A comparison was made using barley sub-aleurone tissue 14 DAA labelled with protein A-gold after incubation with antibodies to C hordein. In micrograph 39A, of osmicated tissue, C hordein is localized in compound vacuolar protein aggregates of the starchy endosperm. There are also some antigenic sites over the dictyosome and endoplasmic reticulum. In micrograph 39B where osmium post-fixation has been omitted, gold labelling for C hordein is localized over a structure in the sub-aleurone cytoplasm. Whilst this

has some resemblance to a dictyosome with its peripheral vesicles, it may also represent a tangential section through the periphery of a protein complex.







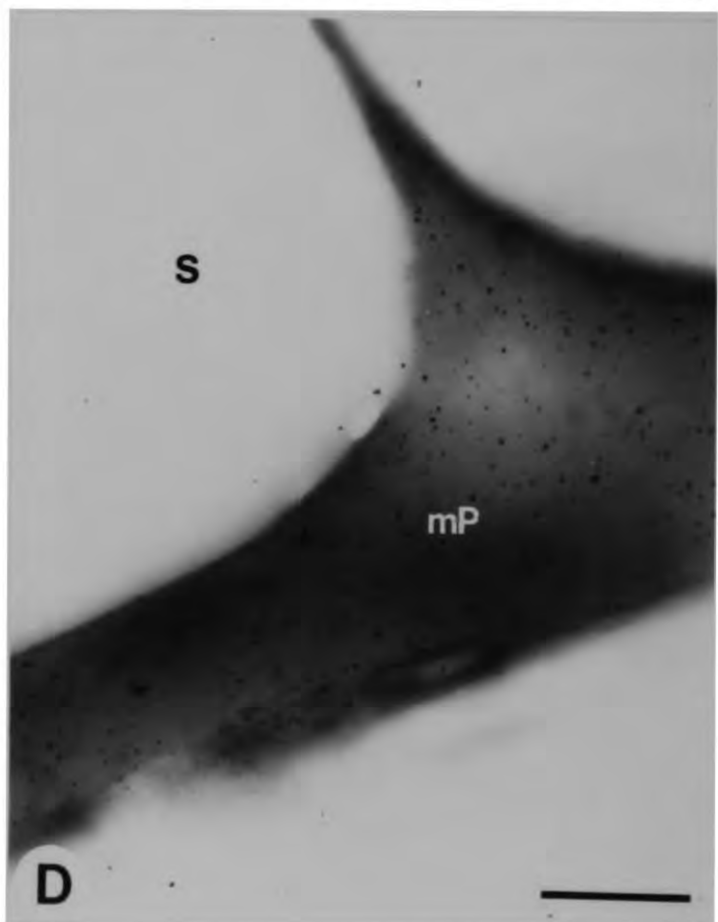


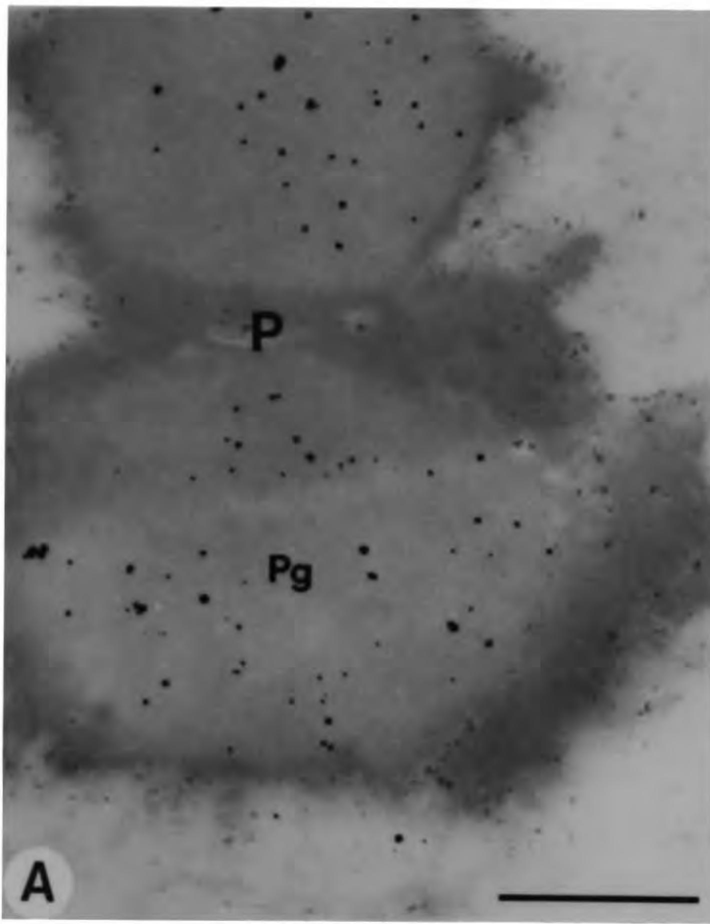
PLATE 36. Immunocytochemical localization of C hordein in developing barley endosperm. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin.

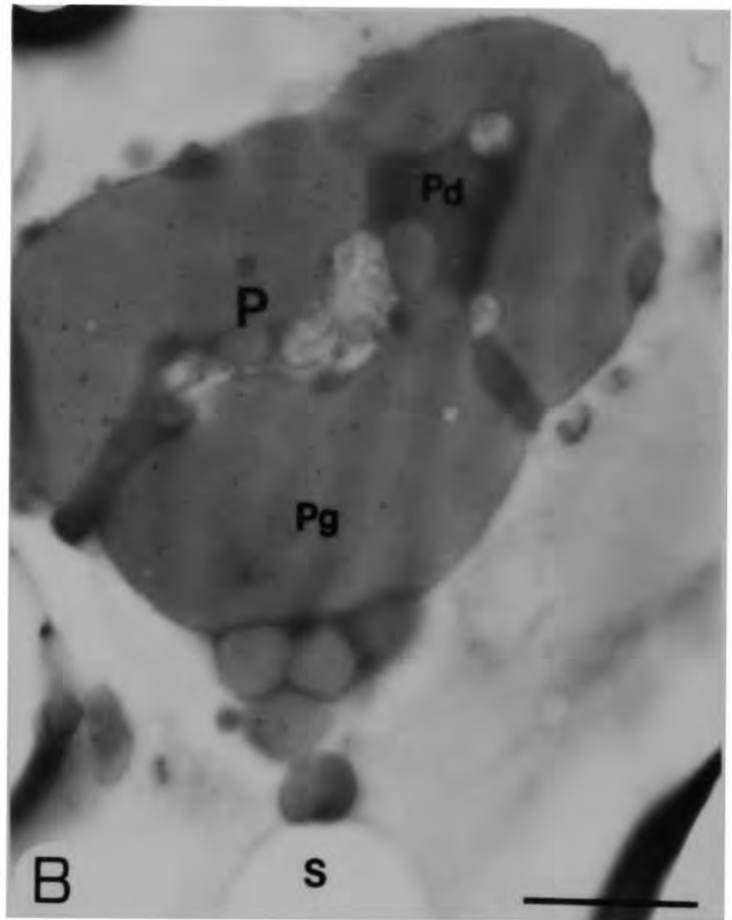
A : Immunocytochemical labelling with PAG_h of C hordein in small cytoplasmic protein deposits in the sub-aleurone of barley, 10 DAA. Bar = 0.25 μm.

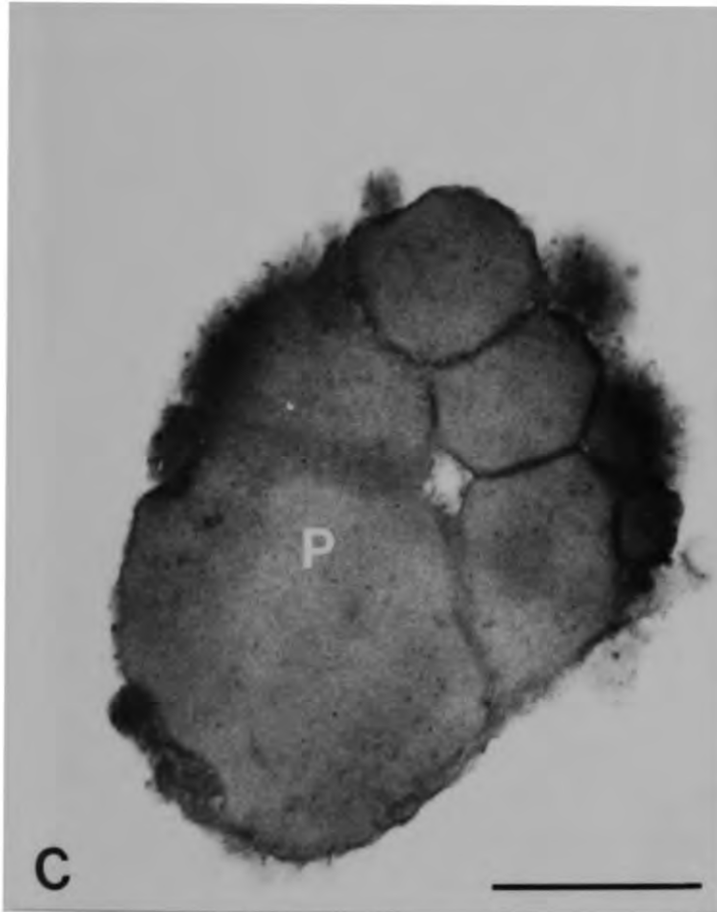
B : PAG_5 labelling of C hordein in vacuolar protein complex in the sub-aleurone, 14 DAA. Bar = 0.5 μm.

C : PAG_h labelling of C hordein in barley endosperm, 32 DAA. Bar = 2 μm.

D : PAG_h labelling of C hordein is located in the protein matrix between starch granules. Bar = 0.5 μm.







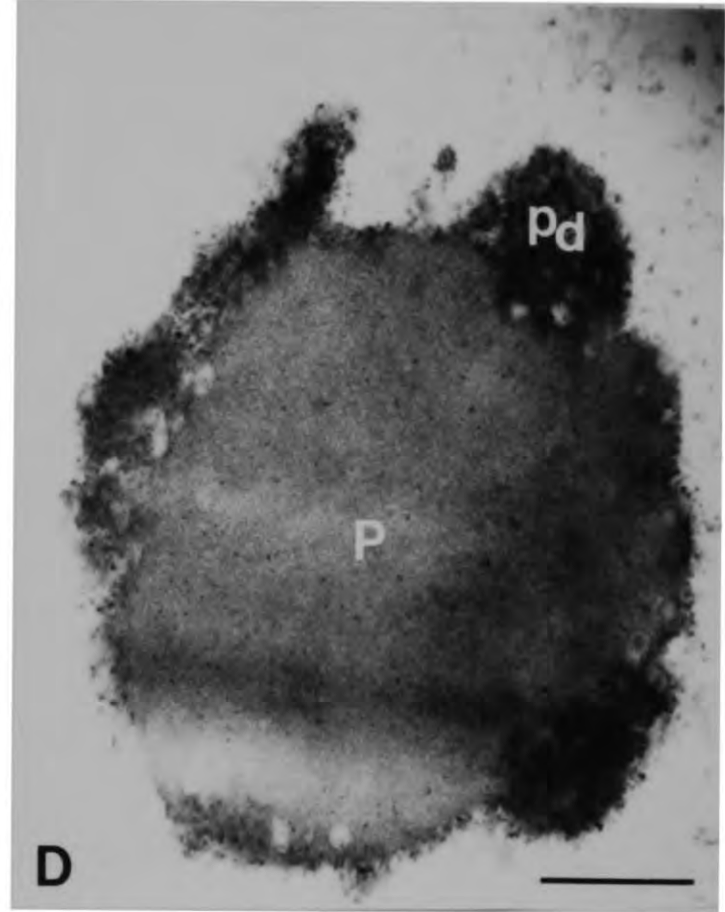


PLATE 37. Immunocytochemical localization of hordein in the protein bodies of barley caryopsis, 14 DAA. Tissue was fixed in aldehyde and osmium tetroxide, and embedded iin Spurr resin.

A : PAG_h labelling of C hordein; label is associated with the granular area of sub-aleurone protein body. Bar = 0.3 μm.

B : PAG_5 labelling of C hordein in an endosperm protein complex. Bar = 2 μm.

C : PAG_5 labelling of B hordein; label is associated with granular area of protein body in the endosperm. Bar = 0.5 μm.

D : PAG_5 labelling of A hordein, shows gold label associated with the granular area of the protein body. Bar = 0.3 μm.

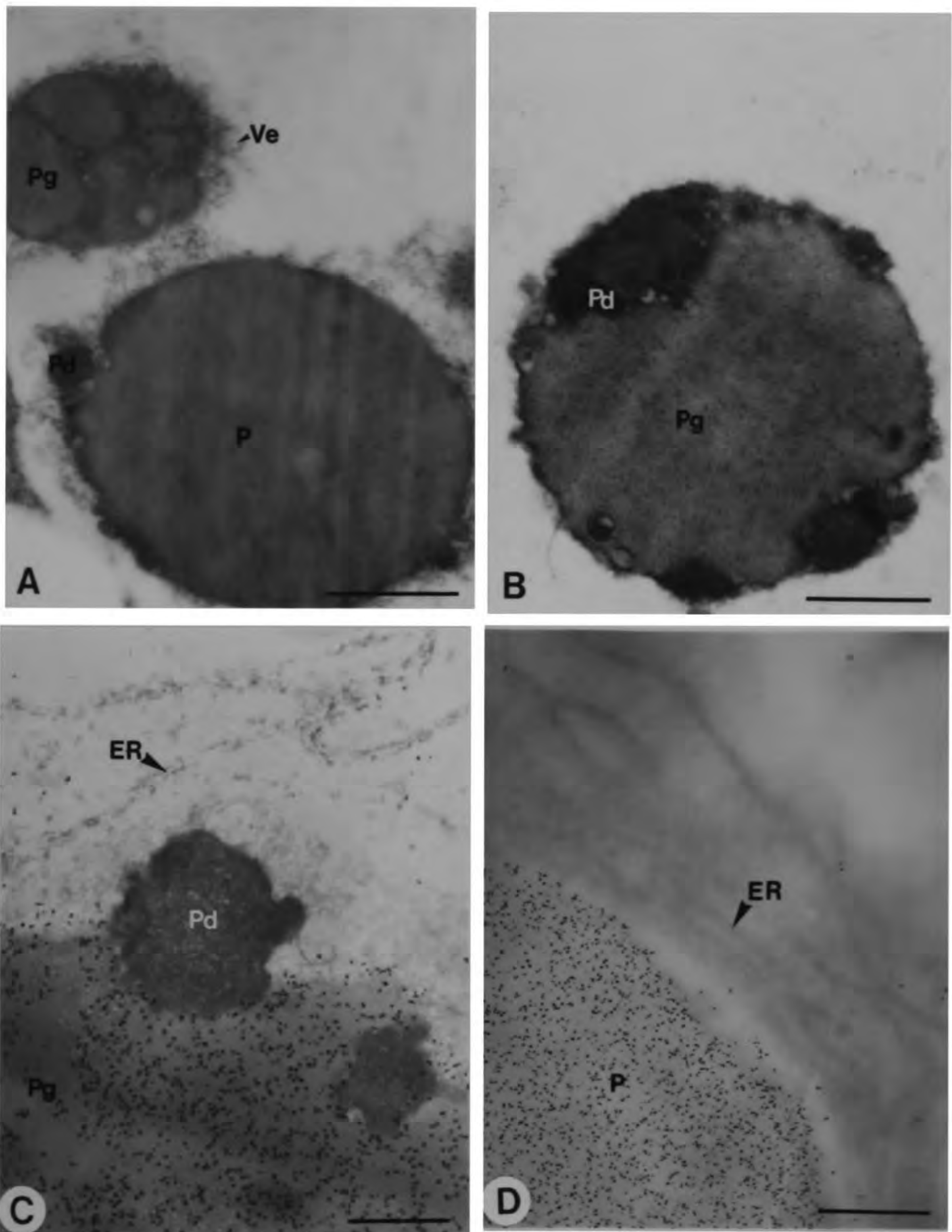


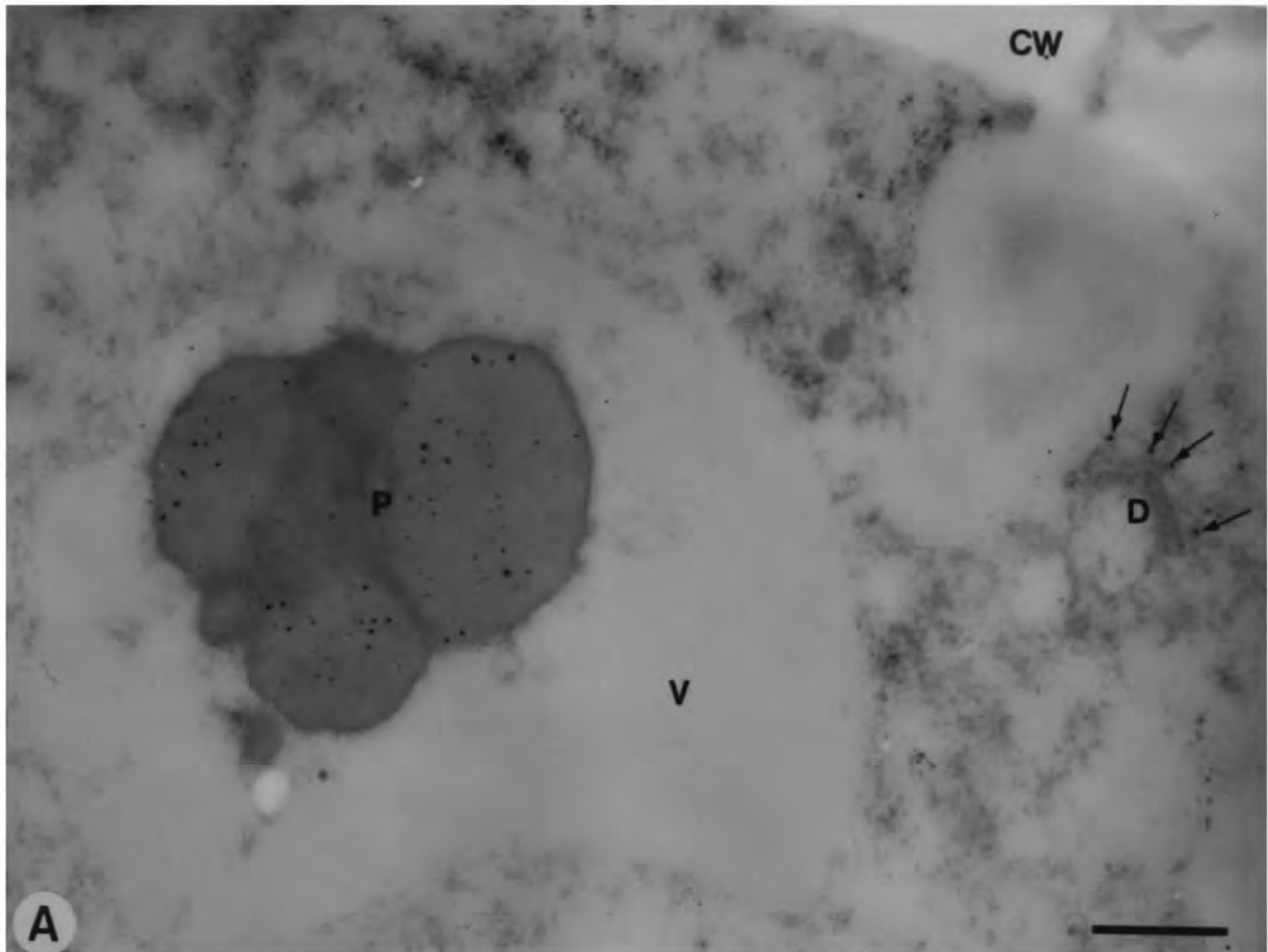
PLATE 38. Electron micrographs showing the effect of osmium tetroxide post-fixation upon antigenicity. Sections of barley endosperm, 14 DAA were incubated with anti-C hordein and protein A-gold.

A : PAG_5 labelling of C hordein in a vacuolar protein body in the sub-aleurone. Tissue was osmicated and post-stained. Bar = 1 μm.

B : PAG_5 labelling of C hordein was intensified in osmicated tissue "etched" with periodate before incubation with antibodies. Bar = 0.5 μm.

C : PAG_{15} labelling of C hordein is non-osmicated tissue, post-stained with uranyl acetate and lead citrate, shows a high density of gold label in the granular area of the protein body. Bar = 0.5 μm.

D : PAG_{15} labelling of C hordein in the protein body of non-osmicated barley sub-aleurone, not post-stained. Bar = 1 μm.



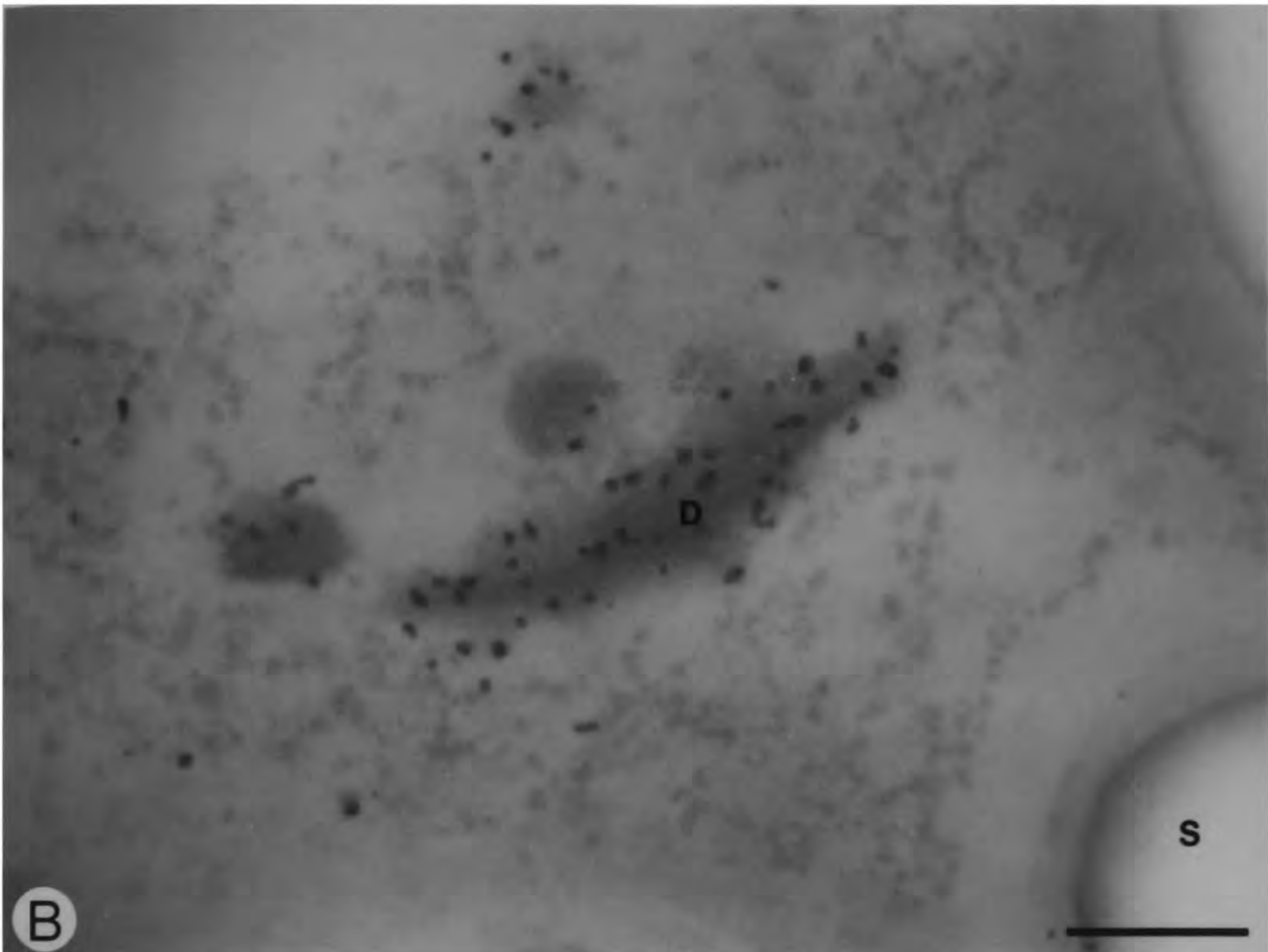


PLATE 39. Immunocytochemical localization of C hordein in barley sub-aleurone, 14 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin.

A : PAG_{15} label associated with the granular areas of the protein aggregate; gold label is also associated with the dictyosome. Section was post-stained. Bar = 0.5 µm.

B : PAG_{15} label associated with a? dictyosome (see text), and cytoplasmic vesicles. Tissue was post-stained. Bar = 0.25 µm.

3.4.3 Comparison of Embedding Media for Immunocytochemical Localization

Plate 40 and 41 illustrate the effect on tissue preservation of aldehyde fixation in a PIPES buffer. Bomi barley tissue was embedded in LR white resin following fixation in a PIPES-buffered aldehyde mixture. The aleurone and sub-aleurone of a barley caryopsis at 14 DAA was labelled with protein A-gold after incubation with B hordein antibodies. Micrograph 40 A and B show a very low level of non-specific binding of gold particles over the cell of the aleurone. In micrographs 40C and D of the sub-aleurone, there is a high density of label over protein bodies. Both the fibrillar and granular areas of the protein bodies are labelled with gold particles, although none are seen over the electron-dense regions. The low level of immunolabelling in the cytoplasm in the immediate vicinity of the protein bodies (Plate 40C), being unidirectional (indicated by white arrows), is probably the result of a smearing of protein during sectioning. In micrograph 40D, no label is seen over the endoplasmic reticulum.

Immunocytochemical localization of C hordein in LR white embedded Bomi barley tissue, shows a similar distribution to B hordein. In micrograph 41A, there is a low level of non-specific binding of immunolabelling over the aleurone. In micrograph 41B there is a high

density of gold particles associated with the vacuolar and cytoplasmic protein bodies. A unidirectional smearing effect is also seen close to the protein deposits (indicated by white arrows). In micrograph 41C the granular regions of protein bodies are densely labelled with protein A-gold which is marking C hordein; there is no immunolabelling associated with the endoplasmic reticulum. In micrograph 41D, immunocytochemical localization of C hordein shows gold label associated with the protein body. There is no label in the lumen of the endoplasmic reticulum, and there is a unidirectional lower level of label in the vacuole close to the protein deposit (indicated by white arrows).

Plate 42 illustrates immunocytochemical localization of A, B, and C hordein on Bomi barley endosperm embedded in Lowicryl K4M low-temperature embedding resin. Micrograph 42A shows C hordein in the sub-aleurone protein body of an unstained section immunolabelled with protein A-gold. The level of contrast is poor without post-staining after immunocytochemical localization. Improved contrast is obtained with uranyl acetate/lead citrate post-staining of the grid before viewing on the electron microscope (micrograph 42B). Labelling of C hordein is associated with the granular areas of the protein body. In

micrograph 42C immunocytochemical localization of A hordein in the sub-aleurone shows gold particles associated with the granular areas of the protein body. The low level of cytoplasmic label close to the protein body (indicated by white arrows) is a unidirectional smearing effect. In micrograph 42D, localization of B hordein with protein A-gold shows a high density of labelling in the sub-aleurone protein body. There is a low level of non-specific binding over the starch granules and the cell walls. A, B and C hordein show a similar distribution in storage protein deposits in the sub-aleurone and endosperm; immunolabelling is associated with the granular areas of the protein body.

The highest degree of antigenicity is retained in non-osmicated, Spurr resin-embedded tissue, and is closely followed by tissue embedded in LR white resin. However, with LR white embedding medium, a smearing effect is produced in the direction of sectioning. A smearing phenomenon also occurs with Lowicryl embedded tissue.

A compromise has to be reached with regard to the degree of contrast required, and the retention of antigenicity. For hordein antisera, osmification of Spurr resin-embedded tissue achieves a high degree of contrast in tissue ultrastructure, with some loss of antigenicity. Good ultrastructural preservation and

contrast is obtained with LR white embedding, along with a high degree of antigenicity. However, there is a smearing effect not apparent with Spurr resin-embedded tissue.

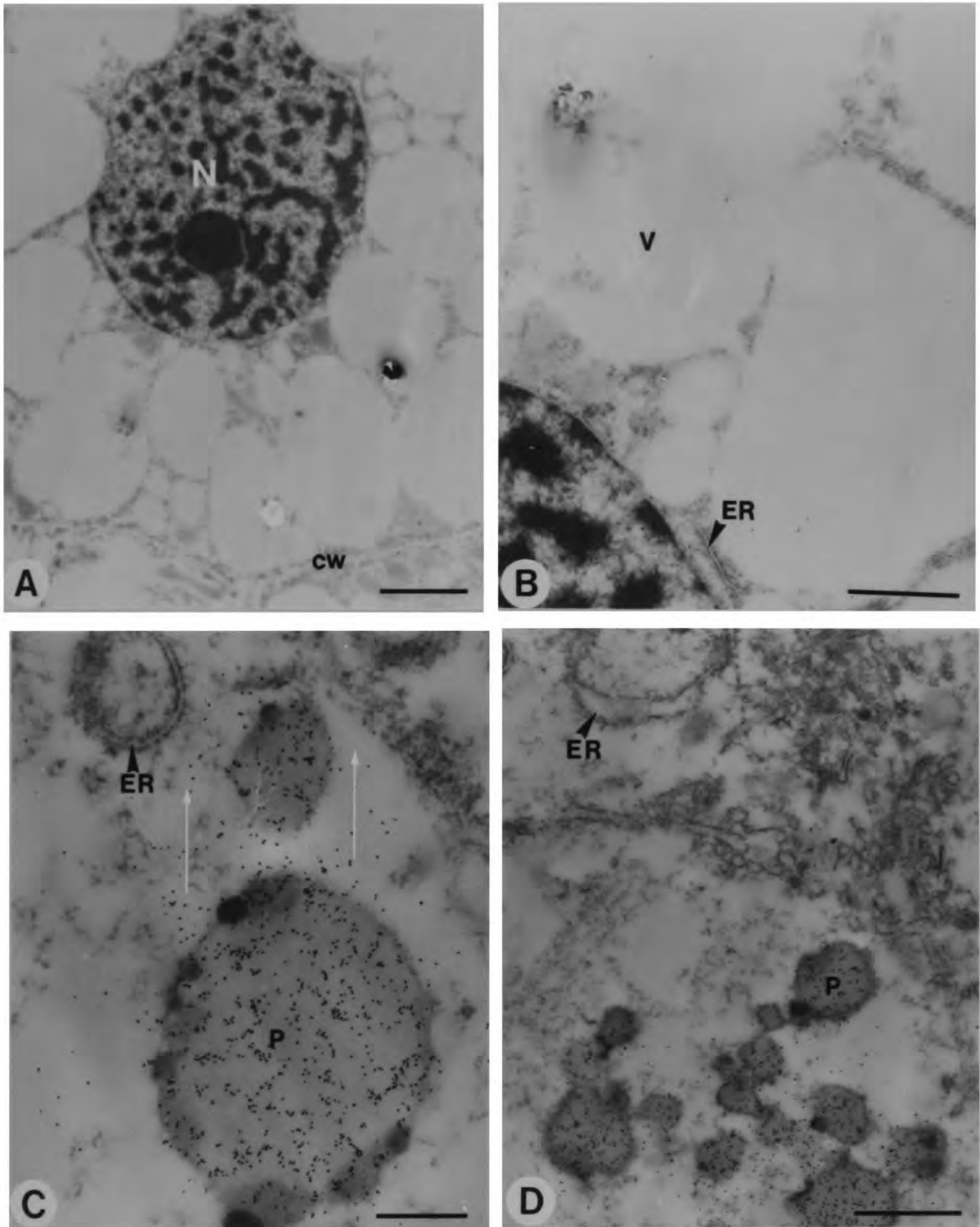


PLATE 40. Immunocytochemical localization of B hordein in non-osmicated barley caryopsis, 14 DAA, embedded in LR white resin and post-stained with uranyl acetate and lead citrate.

A : PAG_{10} labelling of B hordein in the aleurone. Bar = 2 µm.

B : Detail of (A); showing a very low level of non-specific binding over the nucleus, cytosplasm and vacuole. Bar = 1 µm.

C : PAG_{10} labelling of B hordein in sub-aleurone shows a high density of label in the granular area of the protein body. Bar = 0.5 µm.

D : Distribution of PAG_{10} labelling of B hordein in the sub-aleurone. Gold marker is associated with protein bodies. Bar = 1 µm.

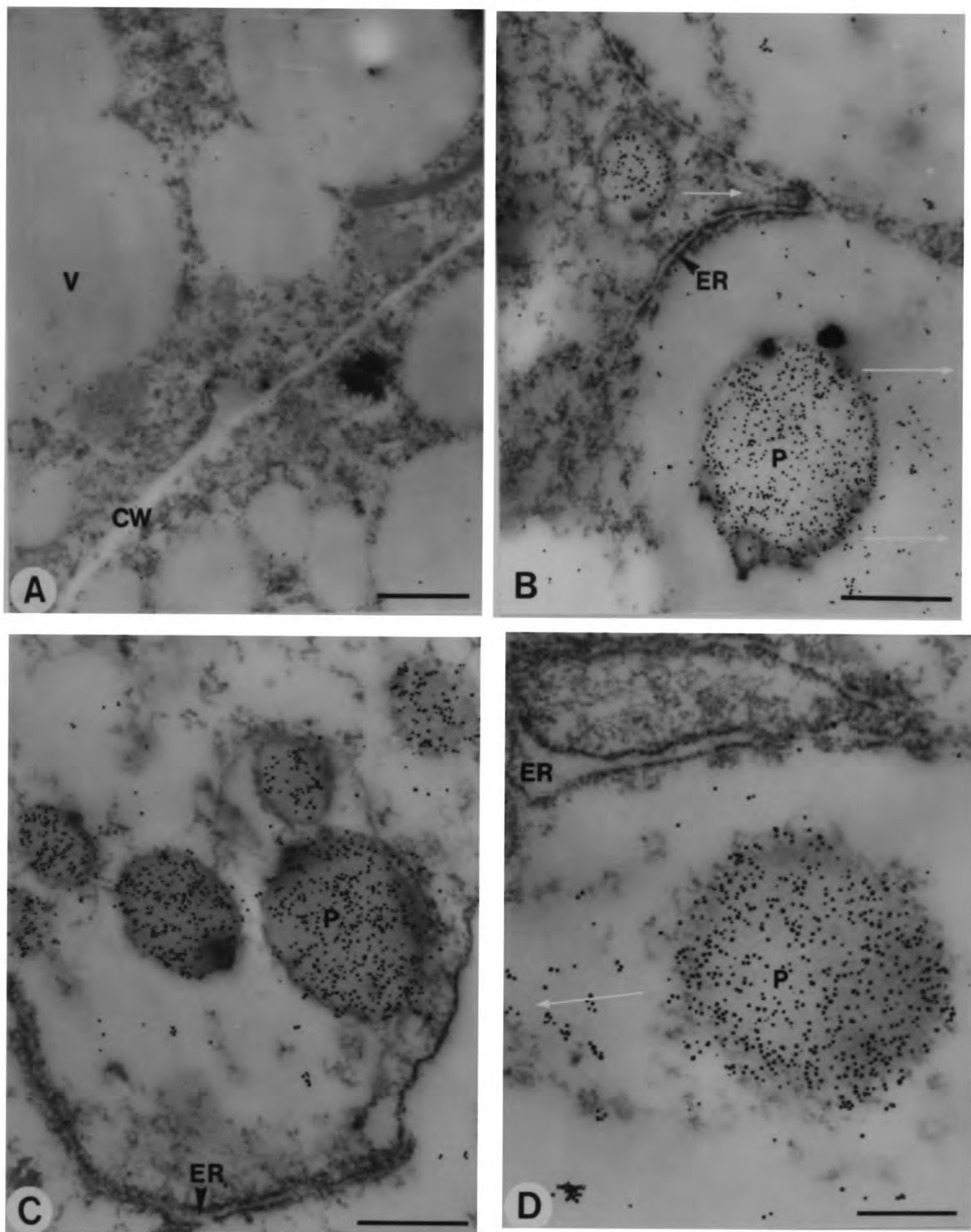


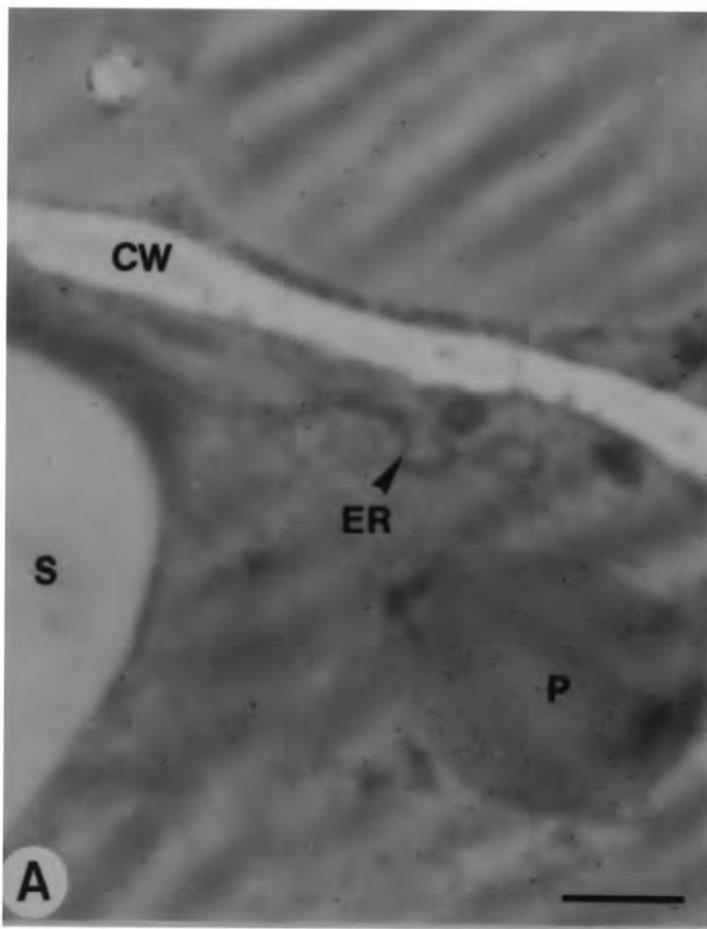
PLATE 41. Immunocytochemical localization of C hordein in non-osmicated barley caryopsis, 14 DAA, embedded in LR white resin and post-stained with uranyl acetate and lead citrate.

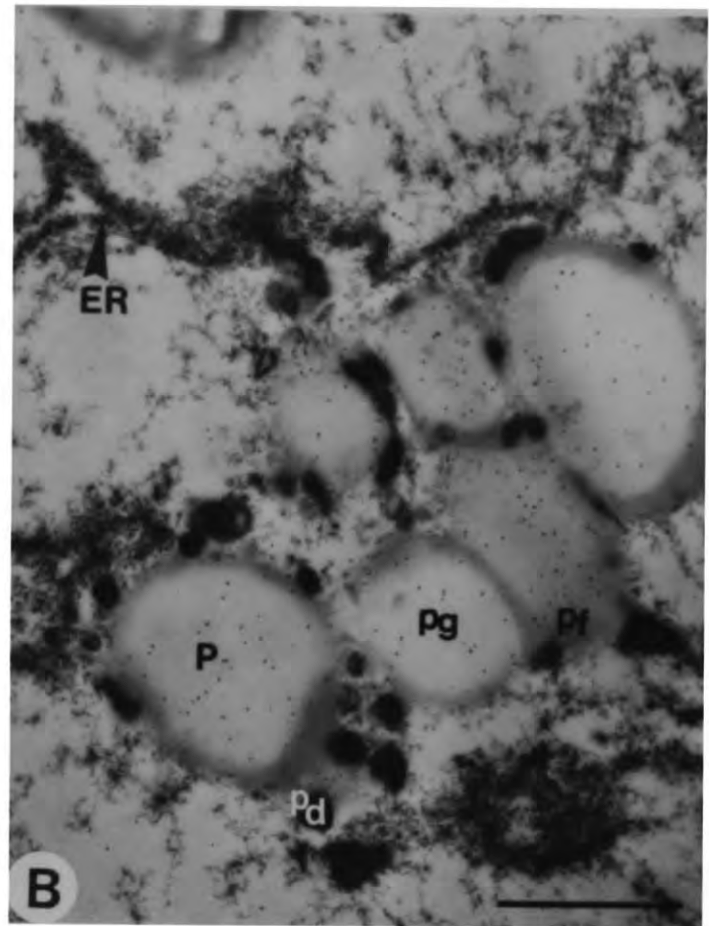
A : PAG_{10} labelling of C hordein in the aleurone shows a low level of non-specific binding over section. Bar = 1 µm.

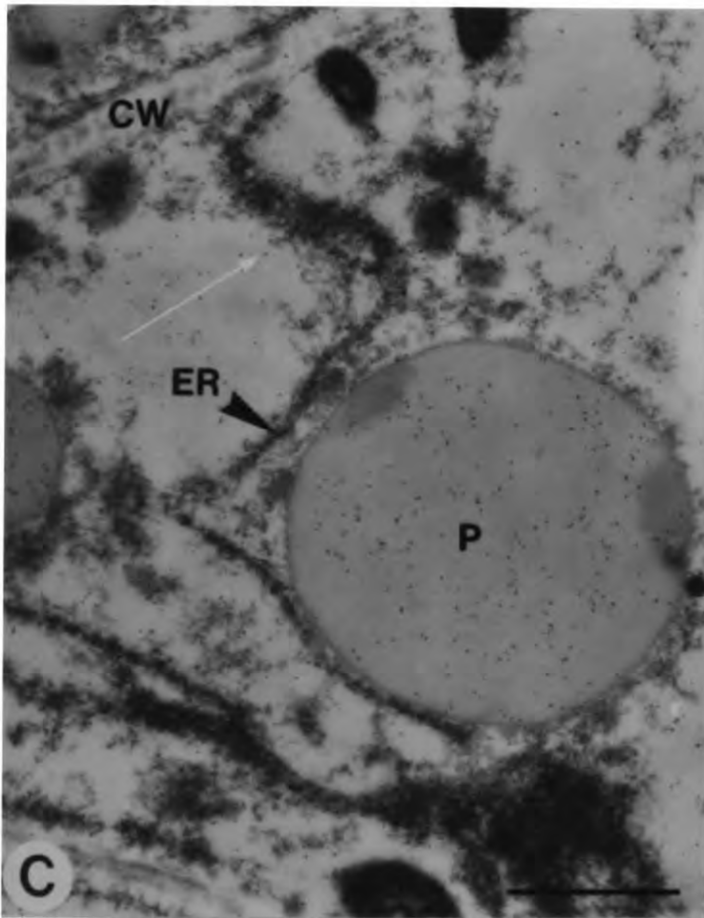
B : PAG_{10} labelling of C hordein in the sub-aleurone shows a high density of label associated with the protein body. Bar = 0.5 µm.

C : PAG_{10} labelling of C hordein is associated with the granular region of sub-aleurone protein bodies; there is no labelling of the endoplasmic reticulum. Bar = 0.5 µm.

D : PAG_{10} labelling of C hordein in the sub-aleurone protein body; there is no label in the lumen of the ER. Bar = 0.3 µm.







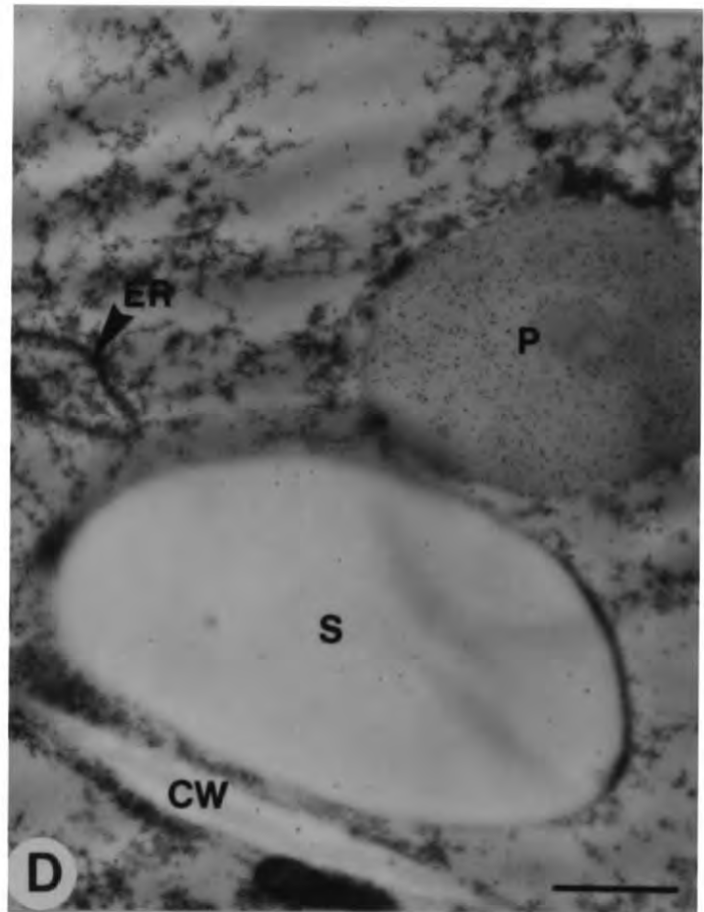


PLATE 42. Immunocytochemical localization of A, B, and C hordein in barley caryopsis, 14 DAA, embedded in Lowicryl K4M low-temperature embedding resin. Tissue was non-osmicated.

A : C hordein in a sub-aleurone protein body is labelled with PAG_{10}; there is a low level of non-specific binding. Section was not post-stained. Bar = 1 μm.

B : PAG_{10} labelling of C hordein in sub-aleurone; section post-stained with uranyl acetate and lead citrate. Bar = 1 μm.

C : PAG_{10} labelling of A hordein in sub-aleurone; section was post-stained. Bar = 1 μm.

D : PAG_{10} labelling of B hordein in sub-aleurone; section was post-stained. Bar = 1 μm.

3.4.4 Comparison of Fixative Buffers

A comparison was made of phosphate-buffered fixatives and PIPES-buffered fixatives using Bomi barley endosperm (14 DAA), embedded in LR white resin. B hordein was localized immunocytochemically in the sub-aleurone with protein A-gold (Plate 43).

For both phosphate-buffered fixed tissue (micrographs 43A, B) and PIPES-buffered fixed tissue, immunogold-labelling of B hordein was associated with the granular areas of the protein body. There is almost no label associated with the endoplasmic reticulum.

In PIPES-buffered fixed tissue, immunolabelling is associated with the granular region of the vacuolar protein complex (micrograph 43C). Rough microsomal vesicles, also present in the vacuole, are not labelled. In micrograph 43D, an increase in the lumen of the endoplasmic reticulum is a feature of PIPES-buffered fixed tissues. As with phosphate-buffered fixed tissues, there is no immunolabelling associated with the endoplasmic reticulum. Immunolabelling is not increased in PIPES-buffered fixed tissue.

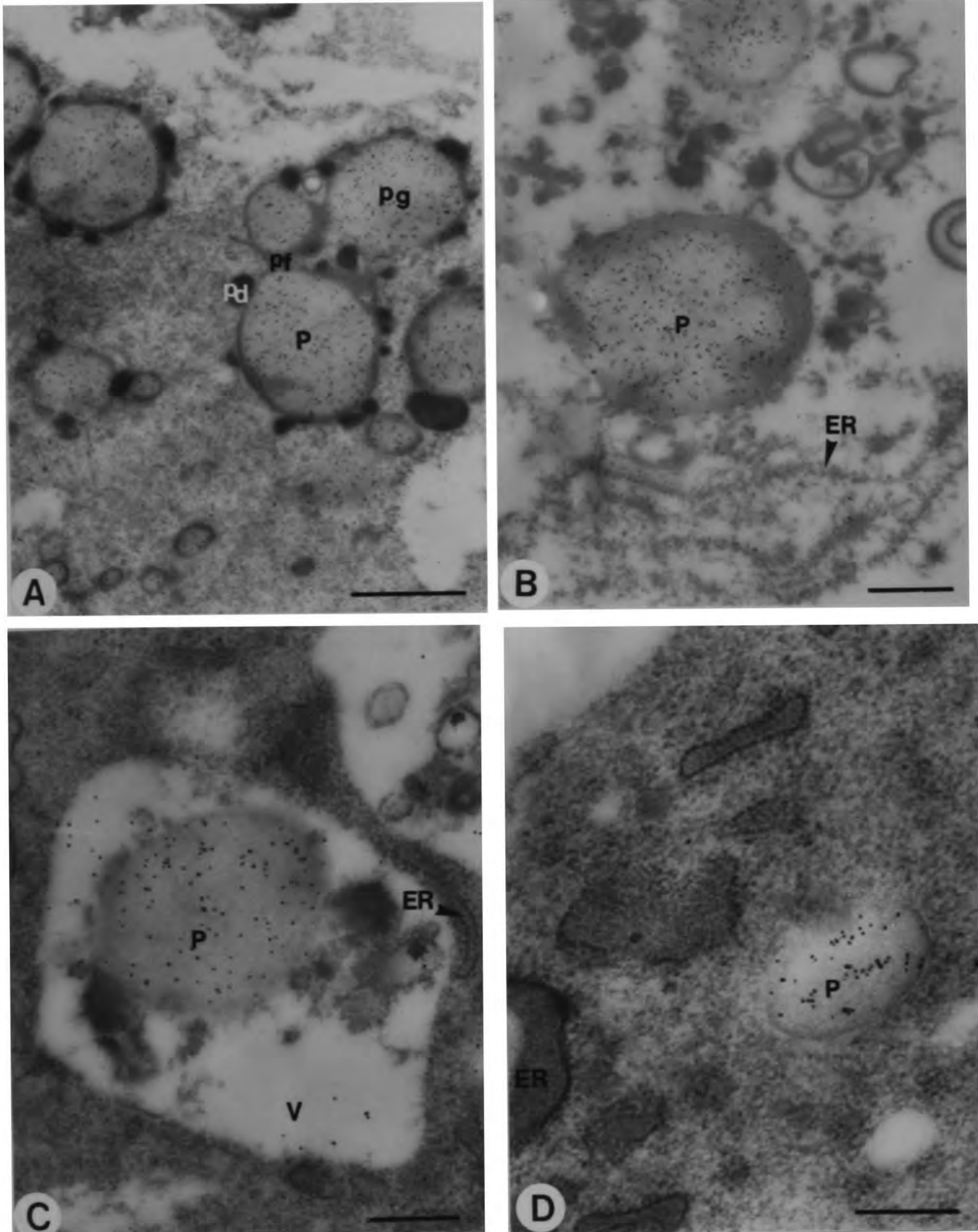


PLATE 43. Comparison of fixative buffers using LR white embedded tissue, 14 DAA. Immunocytochemical localization of B hordein in sub-aleurone with PAG_{10}.

A : Phosphate-buffered fixative: gold markers associated with granular protein bodies. Bar = 1 µm.

B : Labelling of protein body in phosphate-buffer-fixed tissue. Bar = 0.5 µm.

C : PIPES-buffered fixative: labelling of vacuolar protein complex. Rough microsomal vesicles are also present in the vacuole, but are not labelled. Bar = 0.3 µm.

D : Gold marker is associated with a small cytoplasmic protein deposit. The increase in ER lumen contents is a feature of PIPES-buffered fixatives; immunolabelling is not increased. Bar = 0.3 µm.

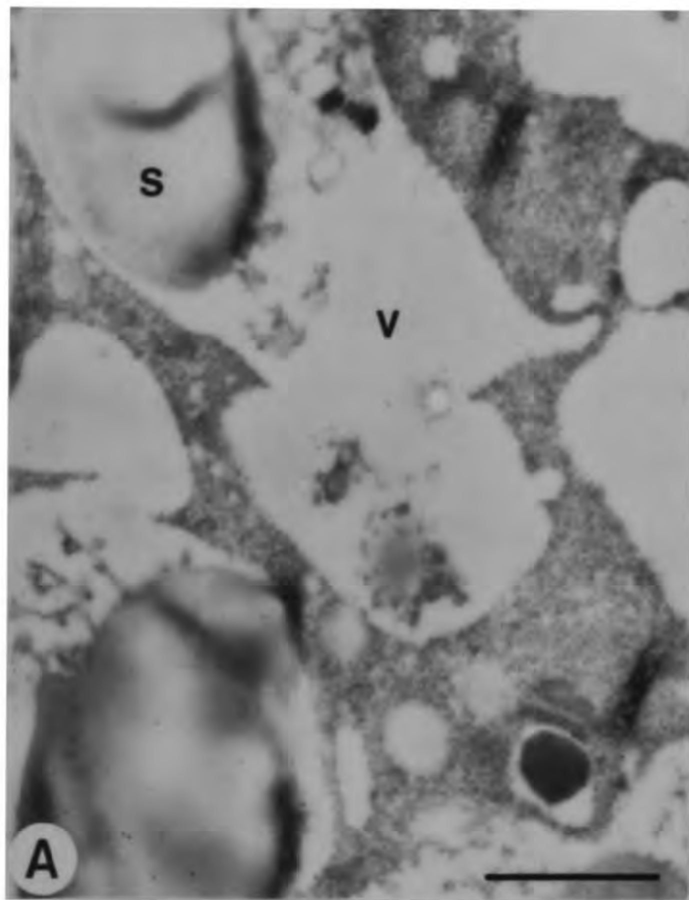
3.4.5 Immunocytochemical Localization of the Chymotrypsin Inhibitors, CI-1 and CI-2, in Bomi Barley

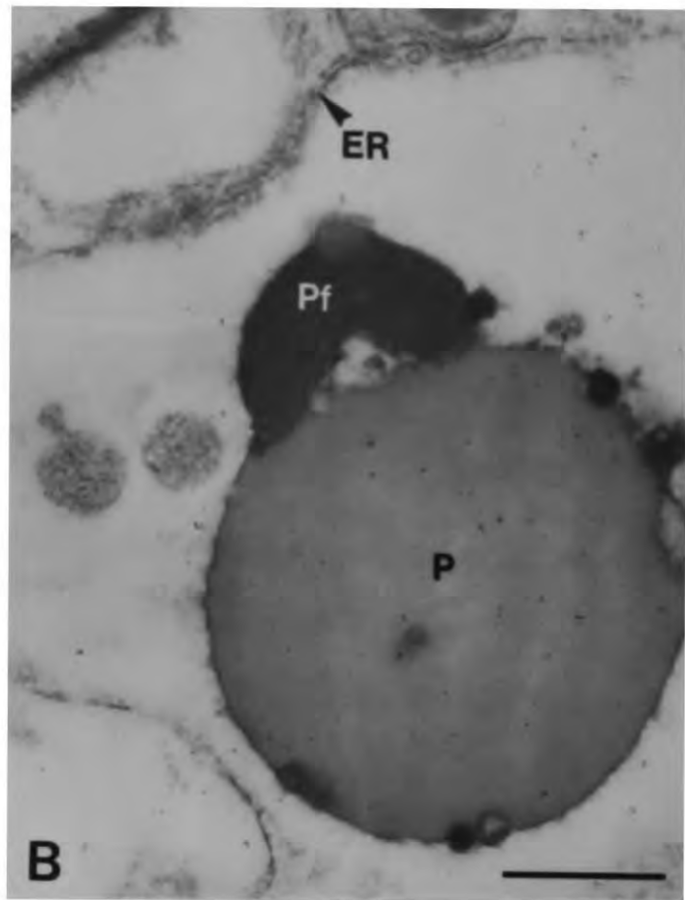
Plate 44 illustrates immunocytochemical labelling of the chymotrypsin inhibitors, CI-1 and CI-2, in Bomi barley at 16 DAA. The levels of these inhibitors are very low in the low-lysine, wild-type barley.

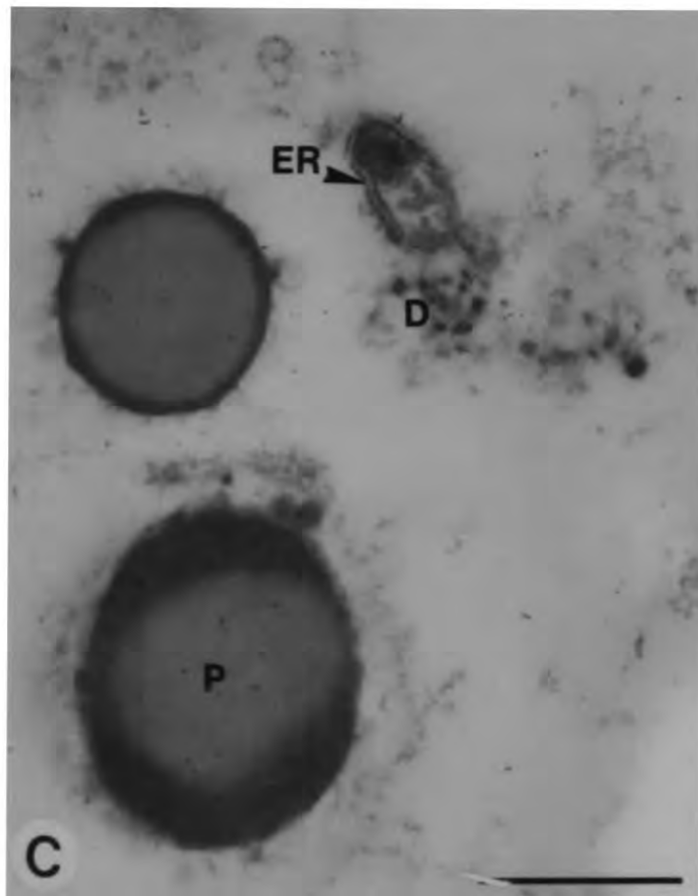
In micrograph 44A, gold-labelling of CI-2 in the aleurone shows a low-level of non-specific binding over the section. In micrograph 44B immunogold-labelling of CI-2 is associated with the protein body and cytoplasm.

Immunocytochemical localization of CI-1 in micrograph 44C shows labelling in sub-aleurone protein bodies, and a low level of cytoplasmic labelling. In micrograph 44D, labelling of CI-1 in the endosperm shows a similar distribution to CI-1 in the sub-aleurone.

For both CI-1 and CI-2, immunogold-labelling, although at a very low level, appears to be predominantly associated with protein bodies.







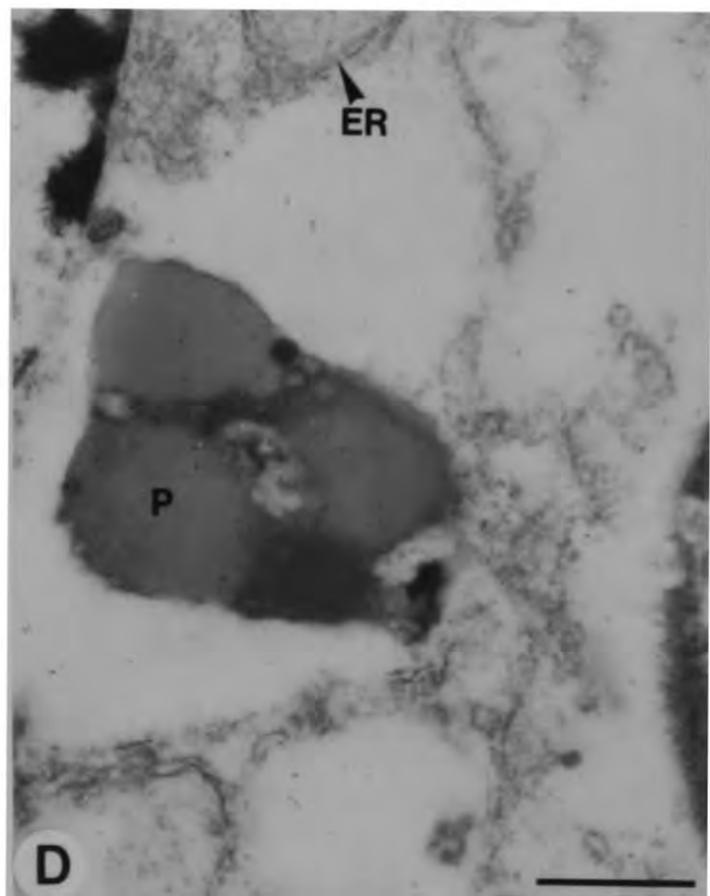


PLATE 44. Immunocytochemical localization of the chymotryptic inhibitors, CI-1 and CI-2, in Bomi barley, 16 DAA. Tissue was aldehyde fixed, embedded in LR White resin and sections post-stained after anti-body labelling.

A : PAG_{10} labelling of CI-2 in the aleurone shows a low level of non-specific binding. Bar = 1 μm.

B : PAG_{10} labelling of CI-2 in the endosperm. Bar = 1 μm.

C : PAG_{10} labelling of CI-1 in the sub-aleurone protein bodies and cytoplasm. Bar = 1 μm.

D : PAG_{10} labelling of CI-1 in the endosperm; shows similar label distribution to (C). Bar = 1 μm.

3.4.6 Immunocytochemical Localization of the Hordeins in the High-Lysine Barley Endosperms

High-lysine mutant barley lines were investigated using immunocytochemical localization to compare with the wild-type barley for the distribution of hordeins and chymotryptic inhibitors. The ultrastructural studies described earlier have shown an altered protein body morphology in the developing high-lysine barley mutants.

In the high-lysine mutant Hiproly at 14 DAA, immunocytochemical localization of C hordein with protein A-gold shows labelling to be associated with the granular protein bodies (micrograph 45A). In the endosperm immunogold-labelling is associated with the granular regions of protein bodies (micrograph 45B). At 21 DAA gold-labelling with goat anti-rabbit - secondary antibody of C hordein is localized in sub-aleurone vacuolar protein deposits (micrograph 46A), and in endosperm protein bodies (micrograph 46B). At a higher magnification (micrograph 46C), immunogold-labelling of C hordein is associated with the granular area of the protein body.

Protein A-gold immunocytochemical localization of B hordein in Hiproly endosperm at 21 DAA, shows B hordein to have a similar distribution to that of C hordein. In Plate 47 immunogold-labelling of B hordein is associated with sub-aleurone vacuolar protein

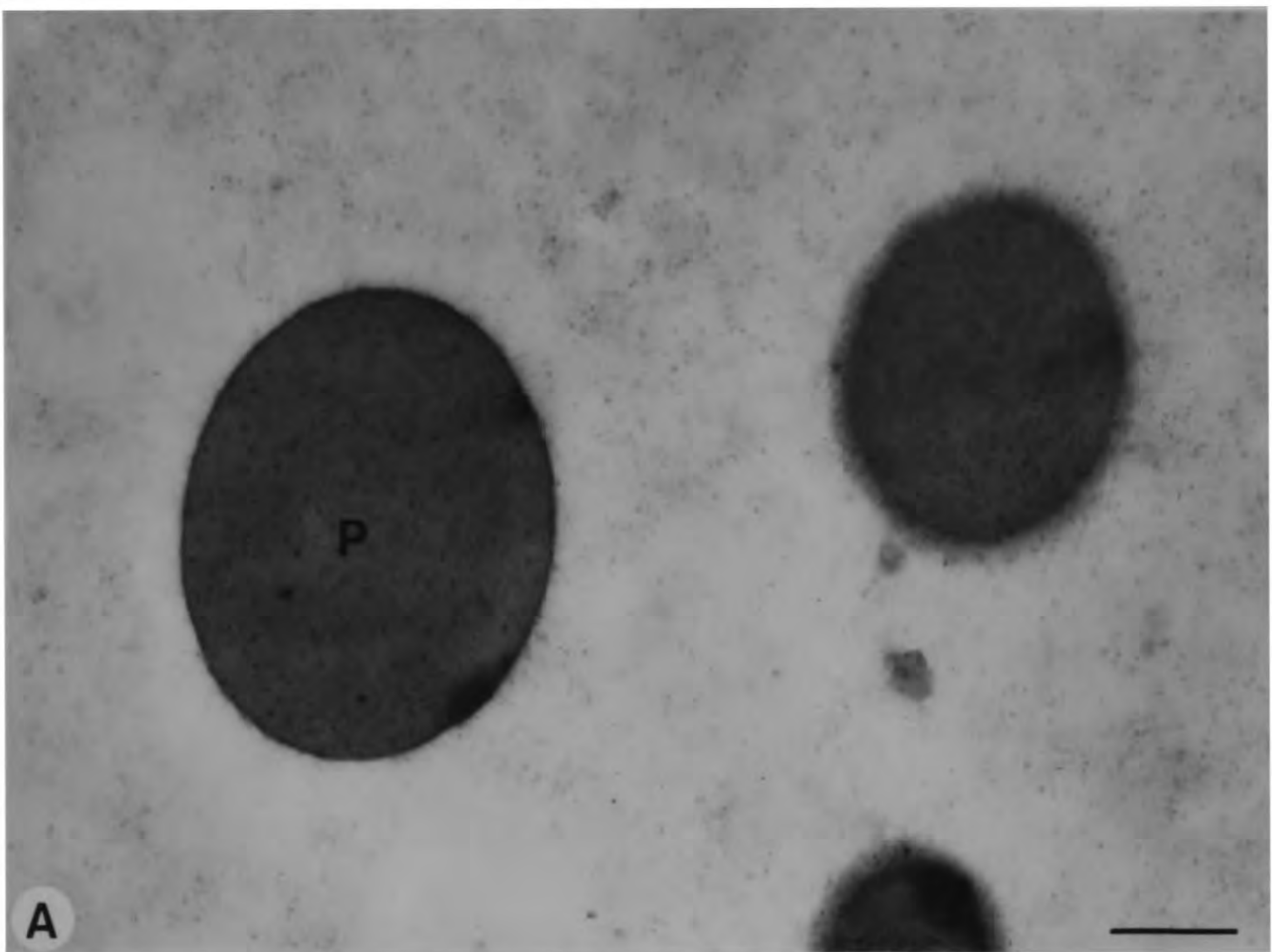
deposits (micrograph A). Micrograph 47B shows a higher magnification of micrograph 47A; gold particles are localized over the vacuolar, granular protein deposits. No gold particles are associated within the vacuolar vesicle. Micrograph 47C shows labelling of the distribution of B hordein in the endosperm protein bodies. At a higher magnification (micrograph 47D) these gold particles are shown to be associated with the granular areas of the protein body.

Plate 48 illustrates immunocytochemical localization of B and C hordein with protein A-gold in the high-lysine mutant Risø 56 at 17 DAA. In micrograph 48A, immunogold-labelling of the sub-aleurone protein complex shows C hordein is associated with the granular area. In the endosperm, immunogold particles are also localized over the granular areas of the protein complex (micrograph 48B). Protein A-gold-labelling of B hordein shows B hordein to have a similar distribution to C hordein; immunogold-labelling of B hordein is associated with the granular sub-aleurone protein complex in micrograph 48C. In micrograph 48D, immunogold particles are associated with the granular area of the endosperm protein complex, showing a distribution of B hordein similar to that of C hordein (micrograph 48B). In micrograph 48D however, labelling is also associated

with the protein-body-vesicle.

Immunocytochemical localization of B and C hordein in sub-aleurone protein bodies in Risø 1508, 17 DAA, is shown in Plate 49. In micrograph 49A, immunogold-labelling of C hordein shows labelling associated with the fibrillar area of the protein body. This fibrillar area is a more loose association compared with the fibrillar areas of the protein bodies of Risø 56 and Hiproly, and has a fibrillar/granular appearance. Similarly, in micrograph 49B, immunolabelling of B hordein is associated with the semi-fibrillar area of the protein body, and with the granular regions.

With regard to the ultrastructure of the protein body, the high-lysine mutants differ from the wild-type Bomi barley. In Bomi barley the protein bodies are almost entirely granular; in the high-lysine mutants there is a large increase in the fibrillar and electron-dense regions of the protein body with a diminished granular area. Immunocytochemical localization of the hordeins with protein A-gold shows immunogold-labelling to be associated with the granular regions of the protein body in Hiproly and Risø 56; in Risø 1508, comprising predominantly of a semi-fibrillar matrix, label is associated with this region.



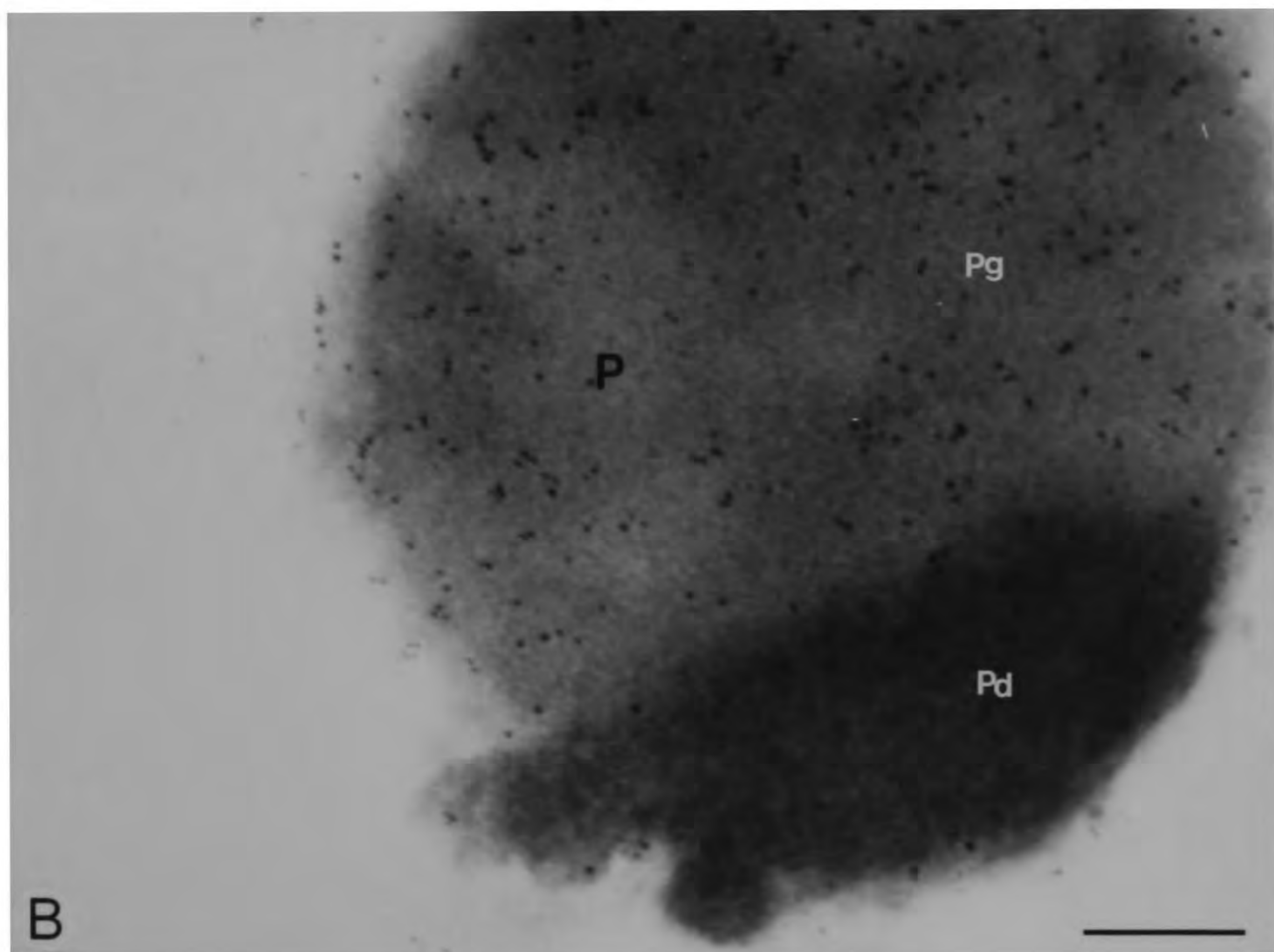
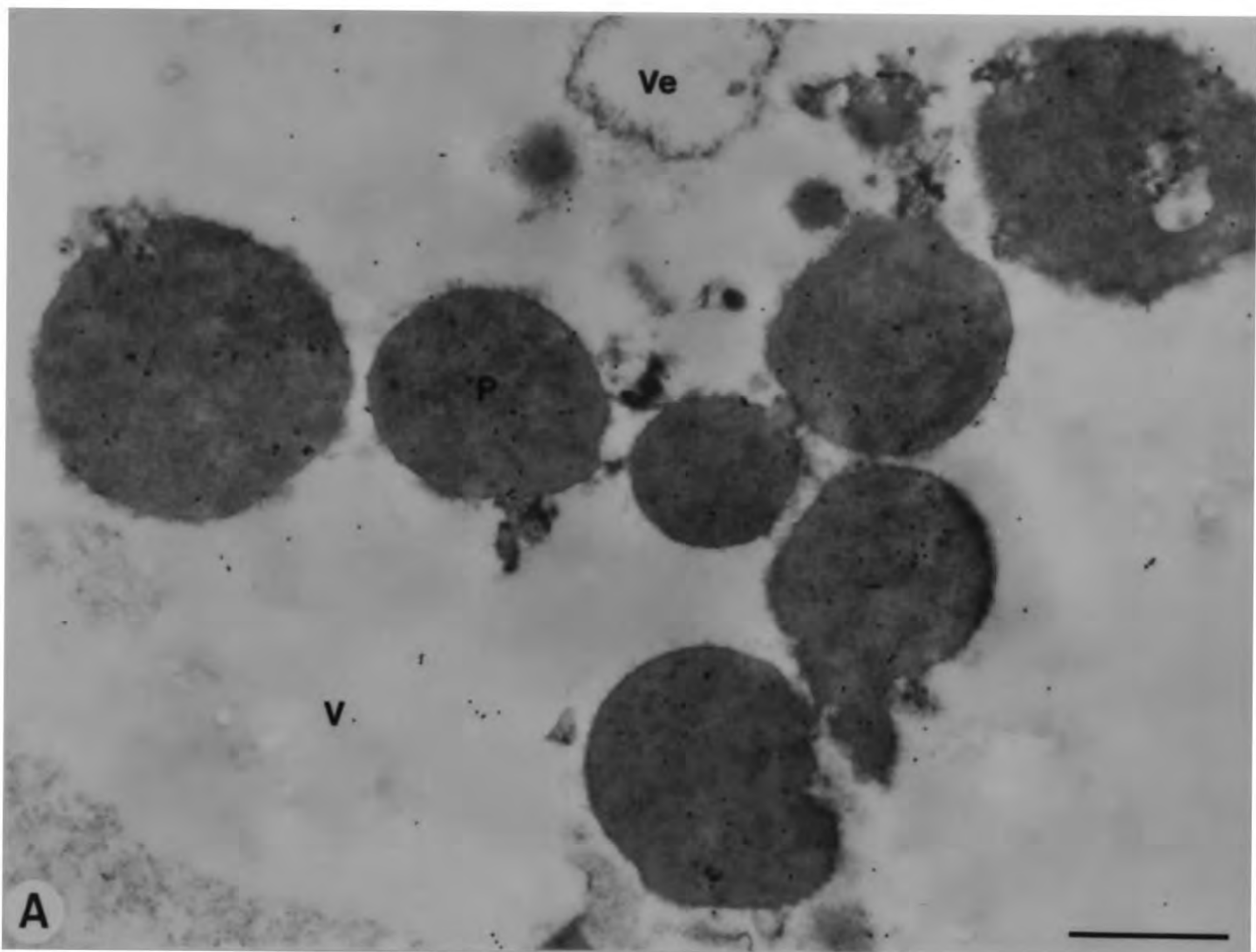
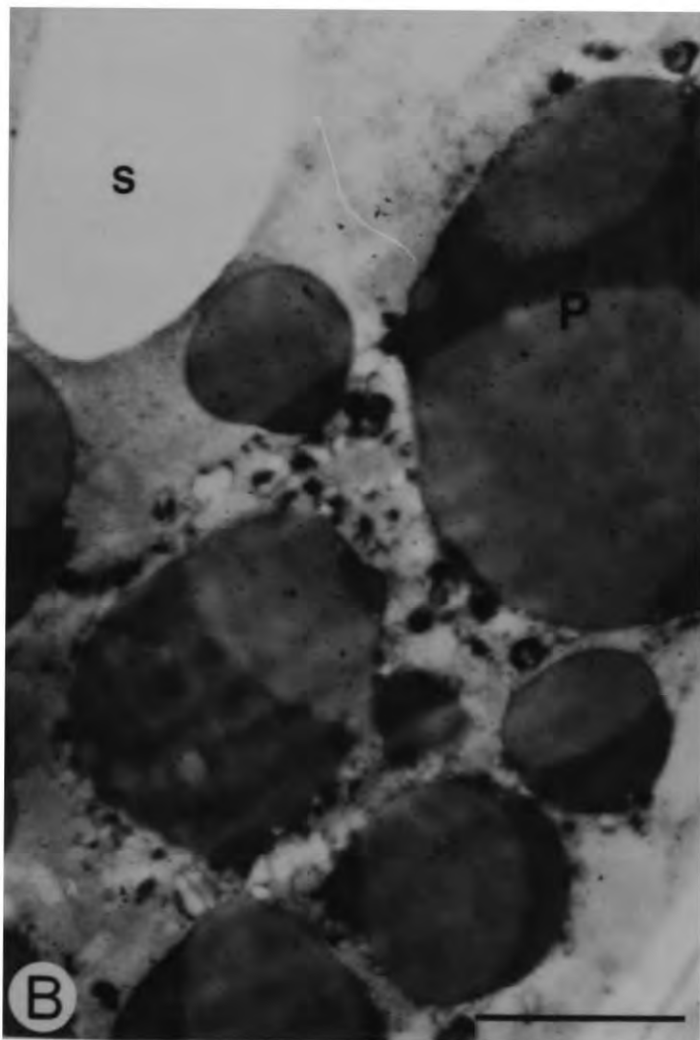


PLATE 45. Immunocytochemical localization of C hordein in the high-lysine mutant Hiproly, 14 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.
A : PAG_5 label associated with sub-aleurone granular protein bodies. Bar = 0.5 µm.
B : PAG_5 label associated with granular area of the endosperm protein body.
Bar = 0.15 µm.





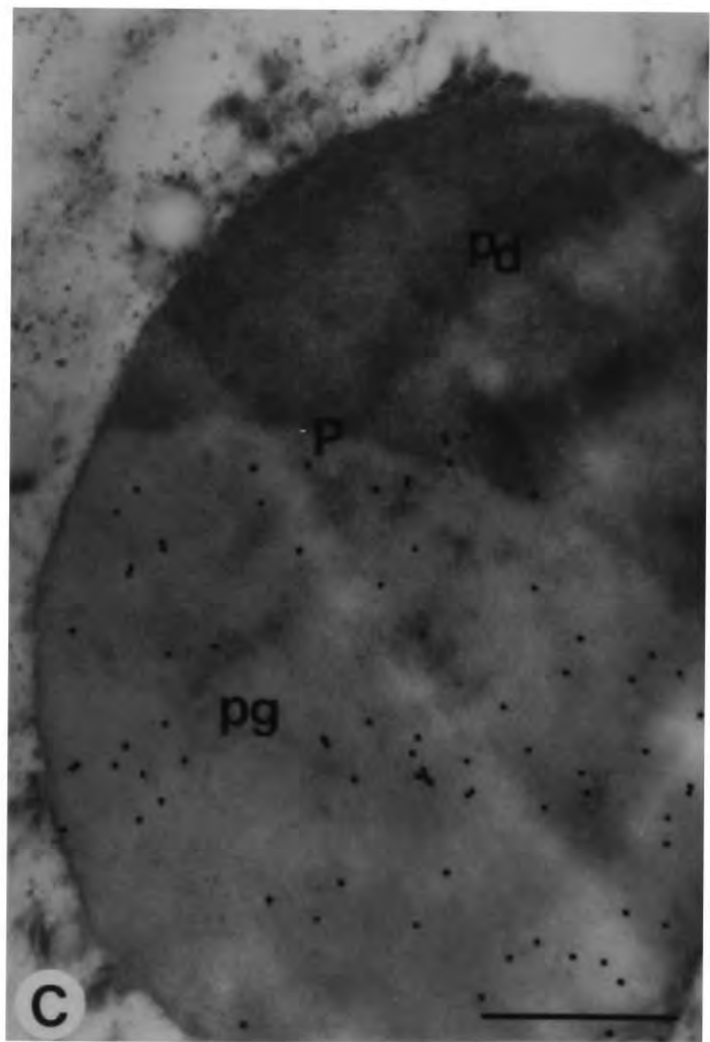
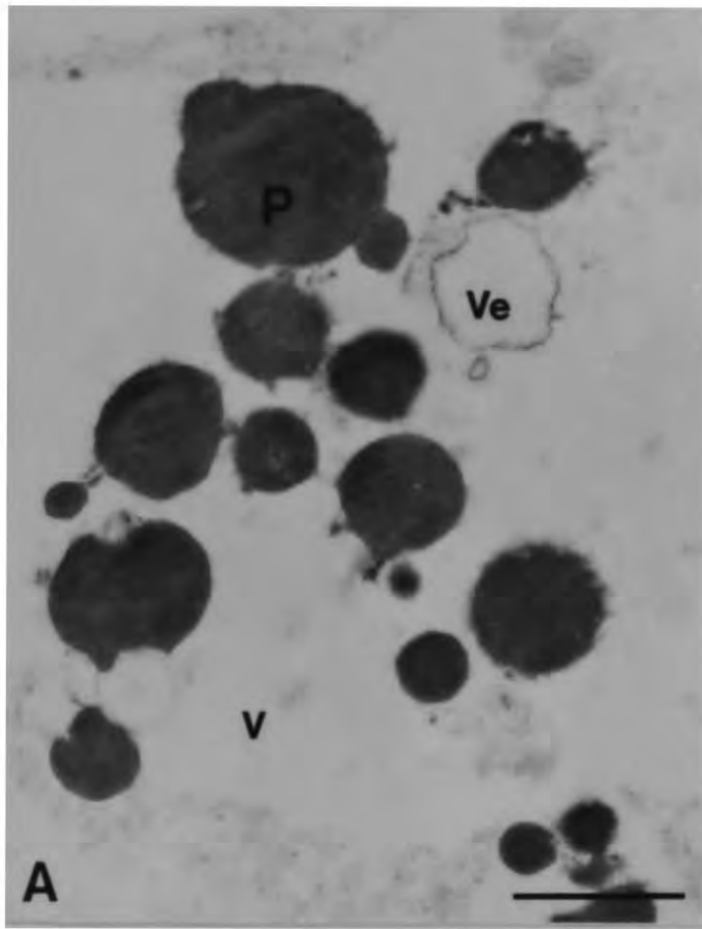
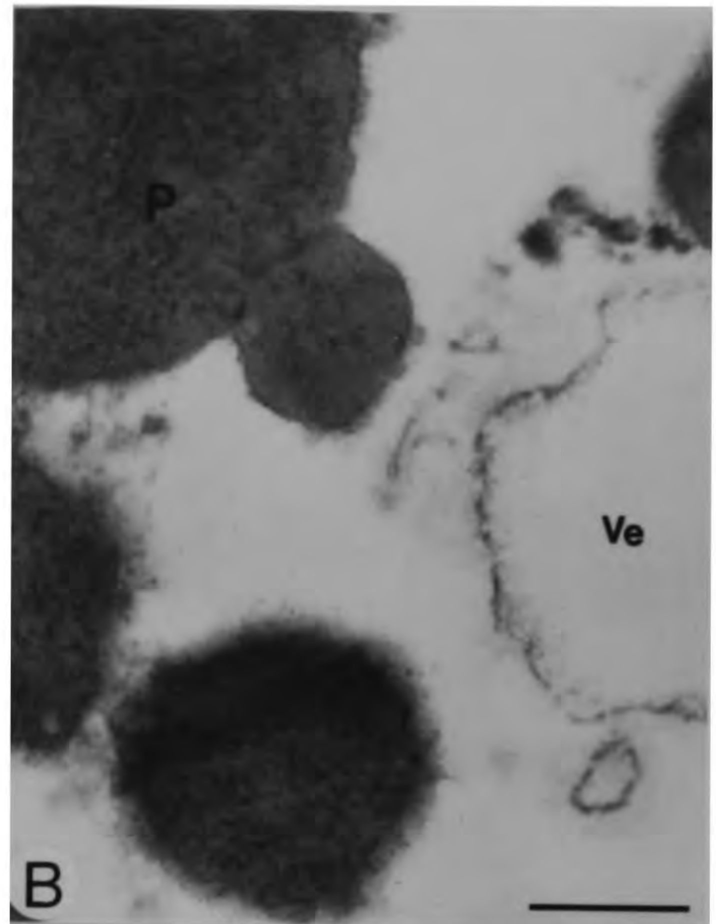
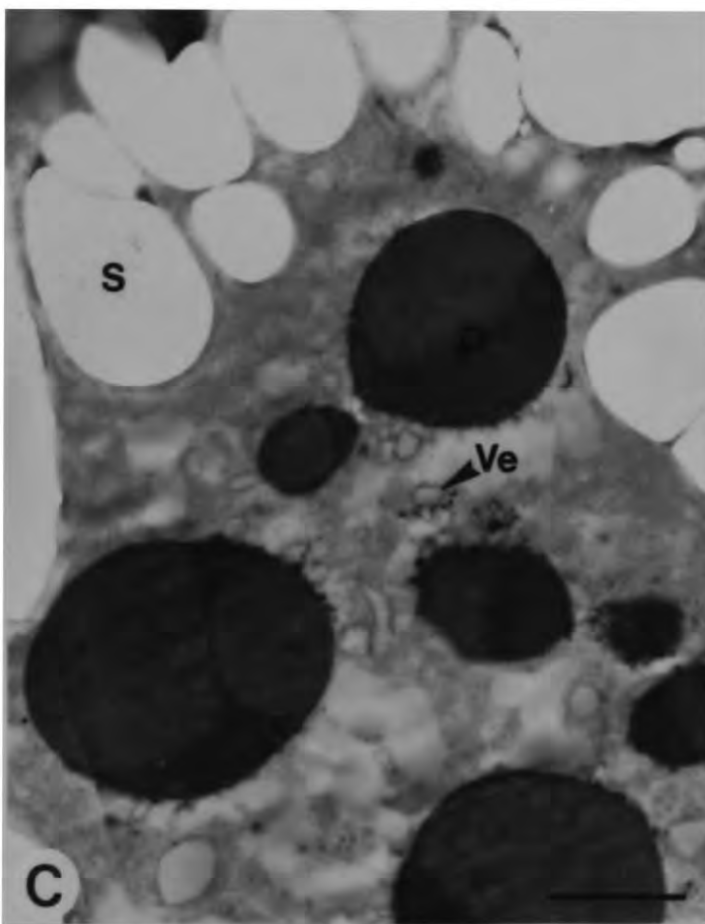


PLATE 46. Immunocytochemical localization of C hordein in the high-lysine mutant Hiproly, 21 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.
A : GAR_{15} label associated with vacuolar protein deposits in the sub-aleurone. Bar = 1 µm.
B : GAR_{15} label associated with endosperm protein bodies. Bar = 2 µm.
C : GAR_{15} label associated with granular area of protein body. Bar = 0.5 µm.







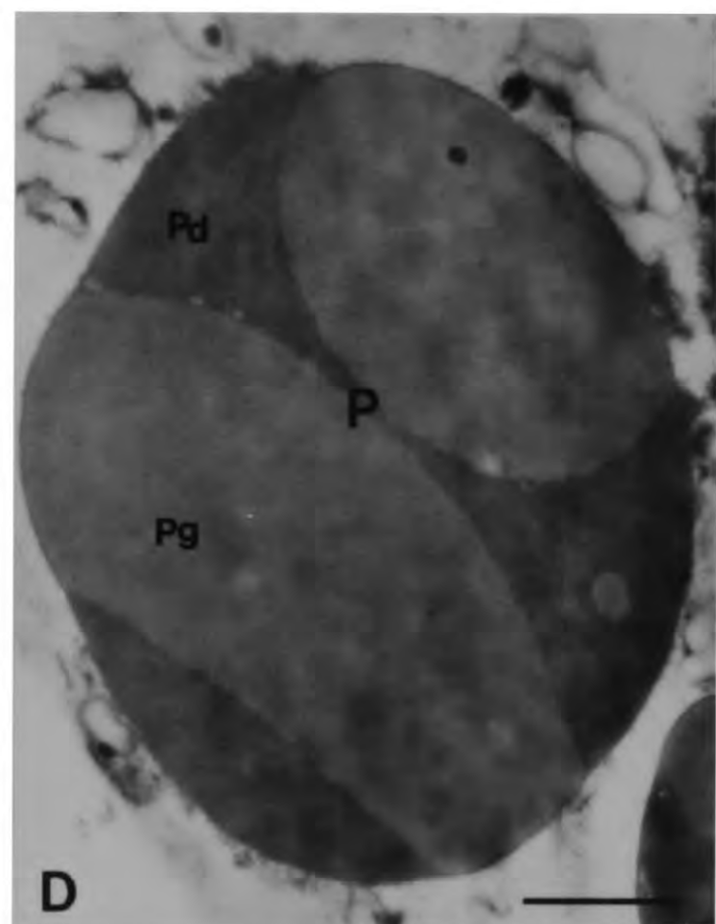
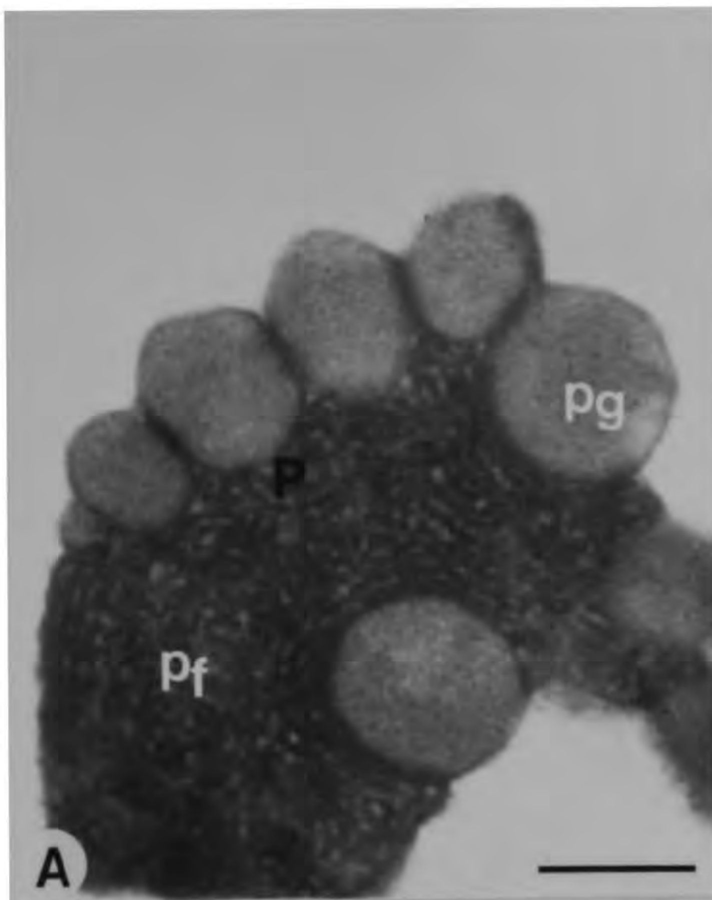
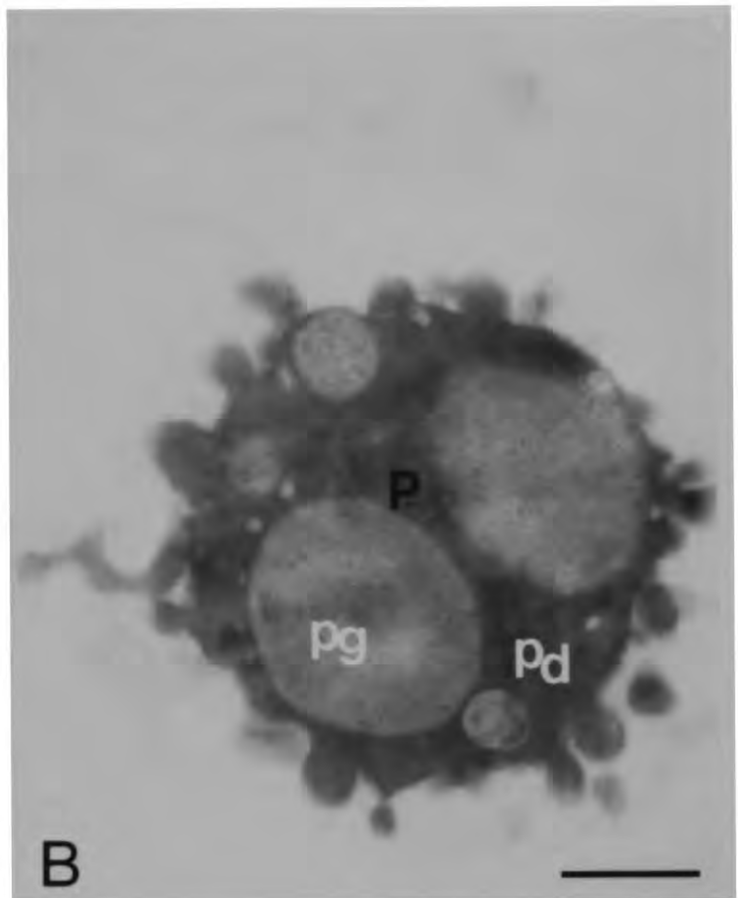
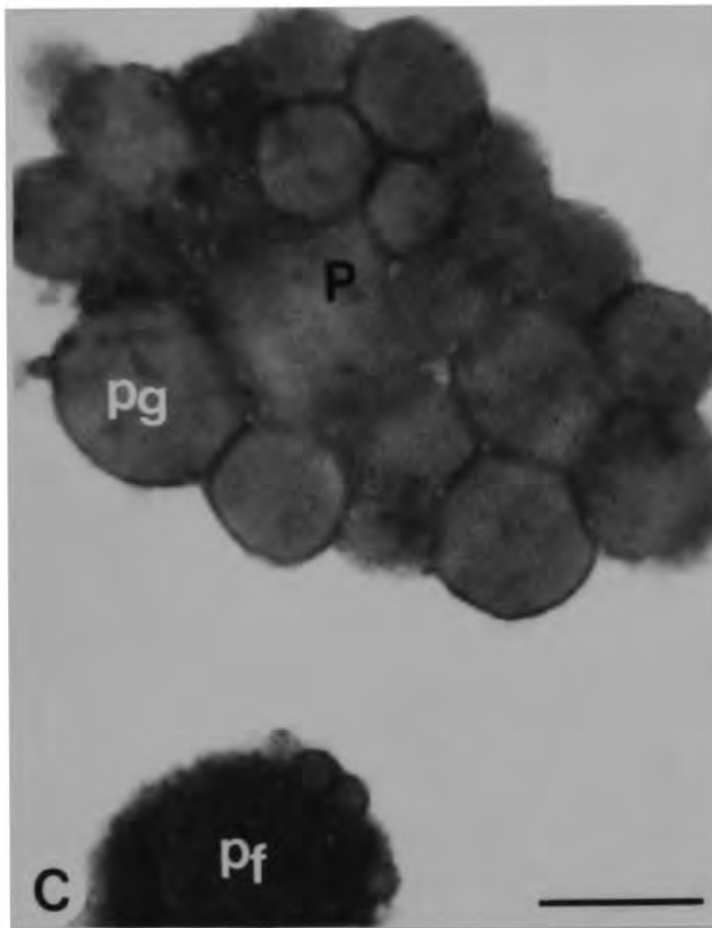


PLATE 47. Immunocytochemical localization of B hordein in the high-lysine mutant Hiproly, 21 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.
A : PAG_5 labelling of vacuolar protein deposits in the sub-aleurone. Bar = 2 μm.
B : Detail of A; gold label associated with protein deposits. Bar = 0.5 μm.
C : PAG_5 labelling of endosperm protein bodies. Bar = 3 μm.
D : PAG_5 label associated with granular region of the protein body. Bar = 1 μm.







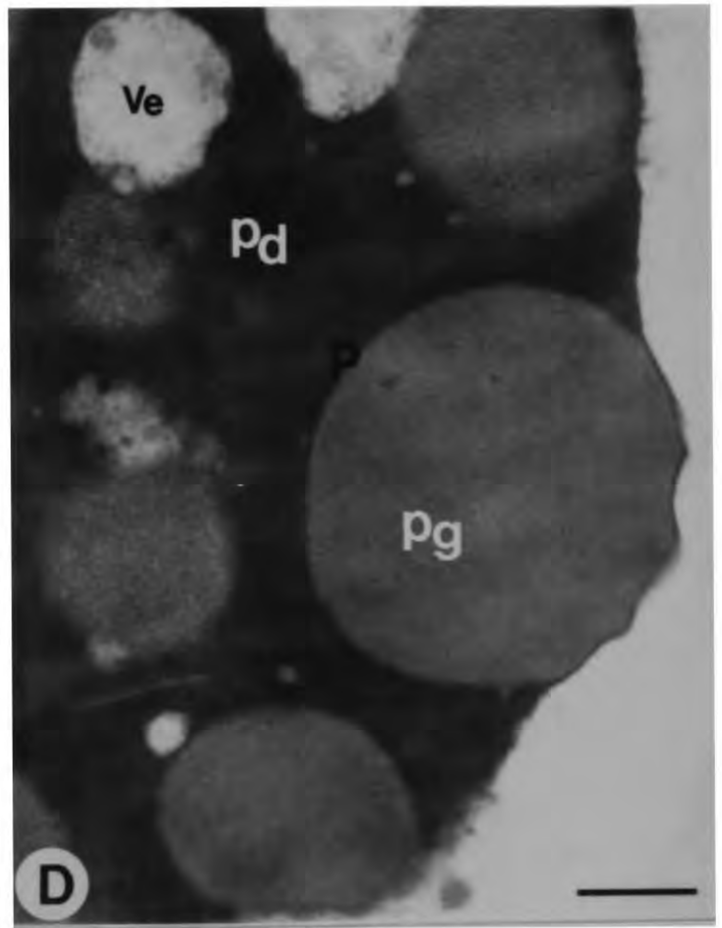


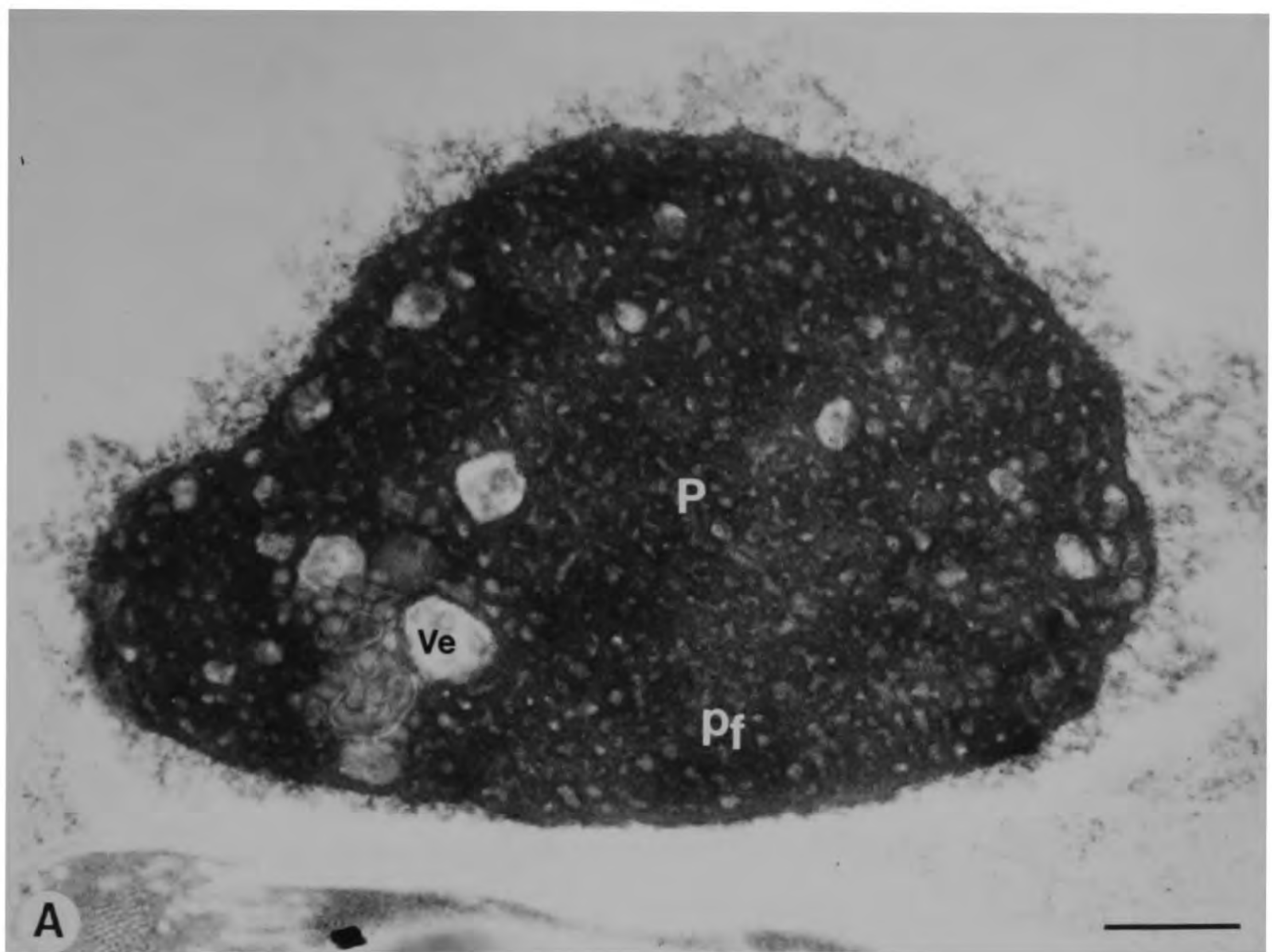
PLATE 48. Immunocytochemical localization of B and C hordein in the high lysine mutant, Risø 56, 17 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.

A : PAG_5 labelling of C hordein associated with the granular area of the sub-aleurone protein complex. Bar = 0.3 µm.

B : PAG_5 labelling of C hordein is associated with the granular area of the endosperm protein complex. Bar = 0.5 µm.

C : PAG_5 labelling of B hordein in granular sub-aleurone protein complex. Bar = 0.5 µm.

D : PAG_5 labelling of B hordein is associated with the granular area of the endosperm protein complex and over Ve. Bar = 0.5 µm.



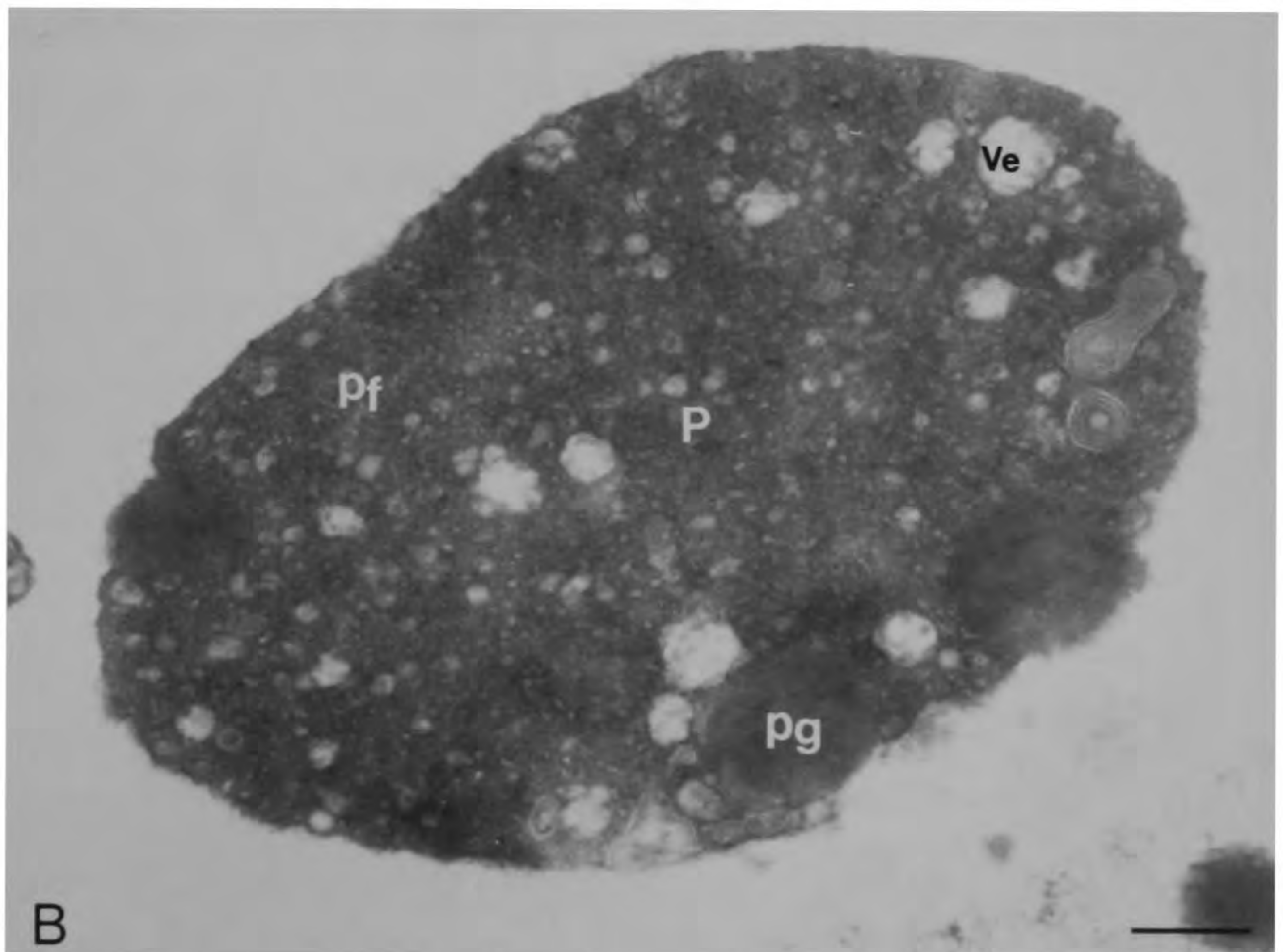


PLATE 49. Immunocytochemical localization of B and C hordein in sub-aleurone protein bodies of the high-lysine mutant, Risø 1508, 17 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.

A : PAG_5 labelling of C hordein shows label associated with the fibrillar region of the protein body. Bar = 0.3 µm.

B : PAG_5 labelling of B hordein shows B hordein has a similar distribution to C hordein.

Bar = 0.3 µm.

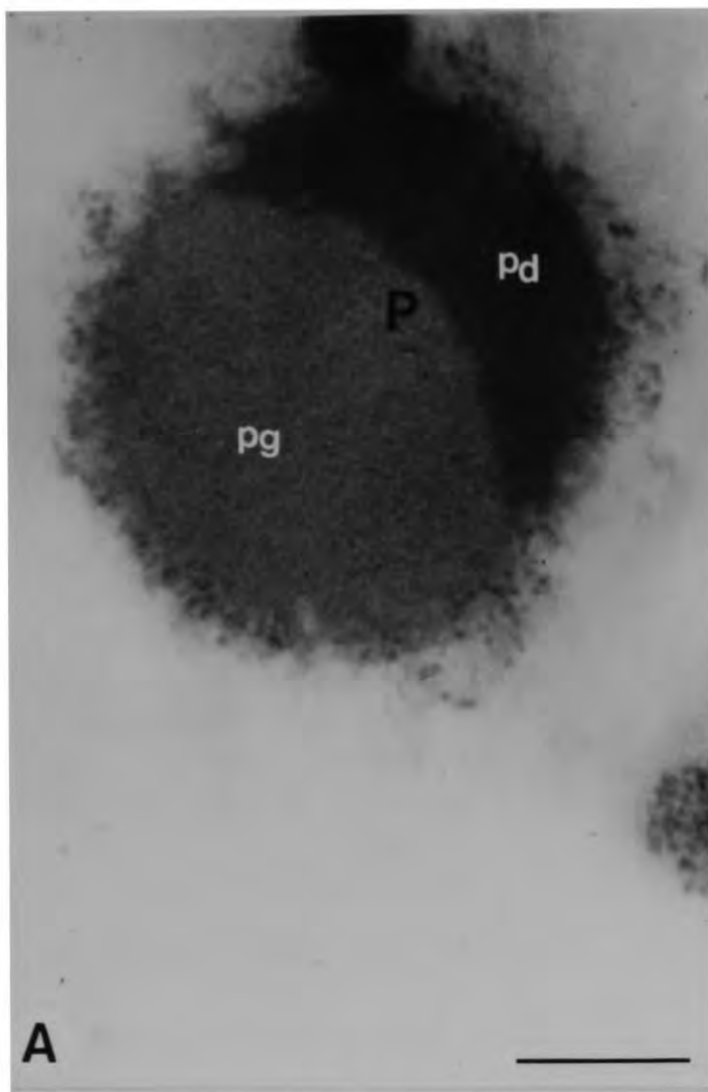
3.4.7 Immunocytochemical Localization of the Chymotrypsin Inhibitors, CI-1 and CI-2, in the High-Lysine Mutant Hiproly

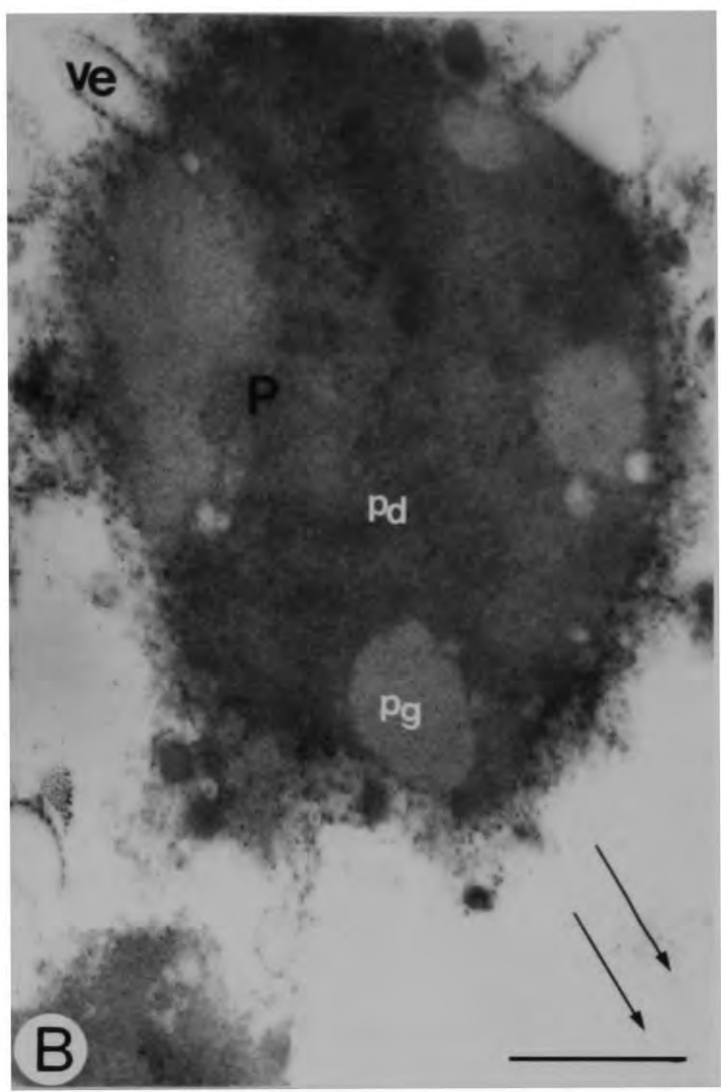
Plate 50 illustrates immunocytochemical localization of the chymotrypsin inhibitors CI-1 and CI-2 in the high-lysine mutant Hiproly at 21 DAA.

Localization of chymotrypsin inhibitor-2 with protein A-gold shows a distribution of antigenic sites over the protein bodies and surrounding cytoplasm in the sub-aleurone and endosperm. This is shown in the endosperm in micrograph 50A.

Immunocytochemical localization of chymotrypsin inhibitor-1 shows this inhibitor to be primarily associated with the protein body in the sub-aleurone and endosperm. In micrograph 50B, immunogold-labelling is associated with the electron-dense region of the protein body. There is a lower level of labelling in the cytoplasm, of which there is a higher level around one side of the protein body. This is probably a smearing effect produced during tissue processing (arrows show direction of smearing).

At a higher magnification, micrograph 50C, immunogold-labelling of CI-1 is predominantly associated with the granular area of the endosperm protein body. A smearing effect is seen (shown by arrows) close to the protein body. This may account for immunogold-labelling over the fibrillar area of the protein body.





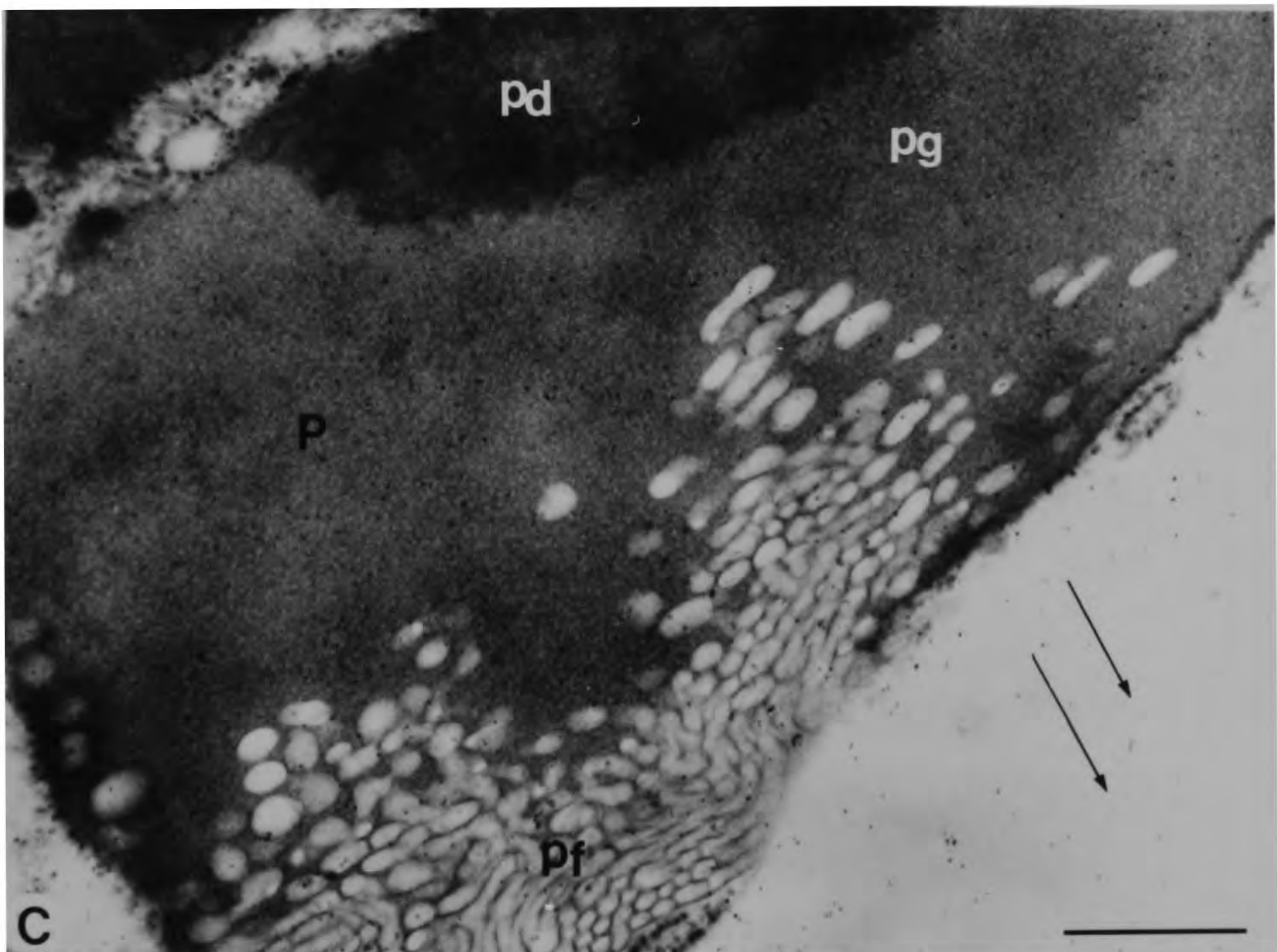


PLATE 50. Immunocytochemical localization of the chymotryptic inhibitors, CI-1 and CI-2, in the endosperm of the high-lysine mutant Hiproly, 21 DAA. Tissue was fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.

A : PAG_5 labelling of CI-2 shows a random distribution of gold particles. Bar = 0.25 µm.

B : PAG_5 labelling of CI-1 is associated with the electron-dense region of the protein body. Bar = 0.5 µm.

C : PAG_5 labelling of CI-1 shows high density of gold located over the protein body. Bar = 0.3 µm.

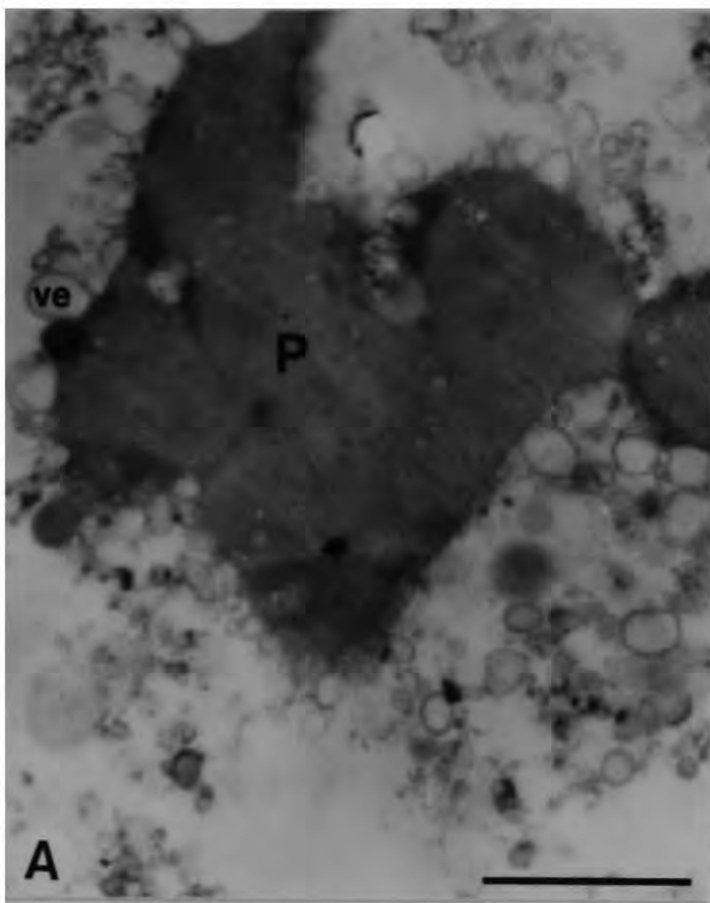
3.4.8 Immunocytochemical Localization of C Hordein on Subcellular Isolations from Sucrose Density Gradient Separation Homogenates of Barley (cv. Bomi) Endosperms

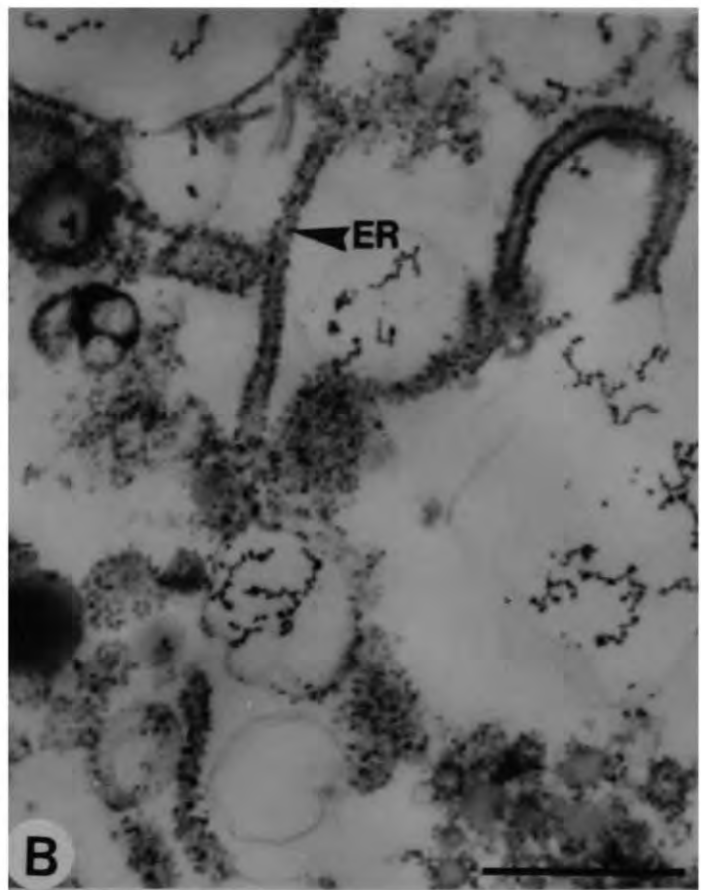
Subcellular fractionation techniques were used to isolate protein body fractions, and endoplasmic reticulum fractions, from a sucrose density gradient separation of a homogenate of Bomi barley endosperms. This occurred in the presence of magnesium (Plate 51A, B), and in the absence of magnesium (Plate 51C, D). Fractions were fixed with aldelyde and osmium tetroxide and embedded in Spurr resin. 15nm protein A-gold was used in immunolabelling of sections to localize C hordein.

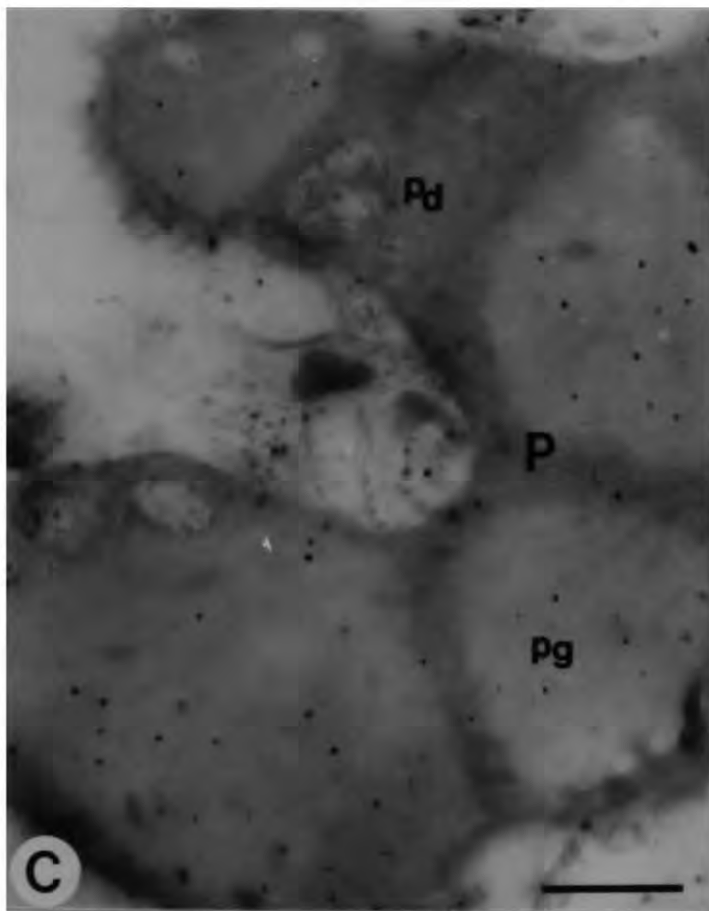
In micrograph 51A, immunogold-labelling of C hordein is associated entirely with the granular areas of the protein body. The presence of magnesium in the subcellular fractionation stabilizes the ribosomes on the endoplasmic reticulum. Vesicles surrounding the protein body are studded with microsomes. In micrograph 51B, no immunogold-labelling is seen over the endoplasmic reticulum.

The absence of magnesium during subcellular fractionation of an homogenate of barley endosperms, induces the loss of ribosomes from the endoplasmic reticulum. In micrograph 51C immunogold-labelling of C hordein is associated with the granular

regions of the protein body. Smooth vesicles are seen around the protein body periphery. These are not immunolabelled with gold. The smooth endoplasmic reticulum fraction in micrograph 51D has also no labelling associated with it.







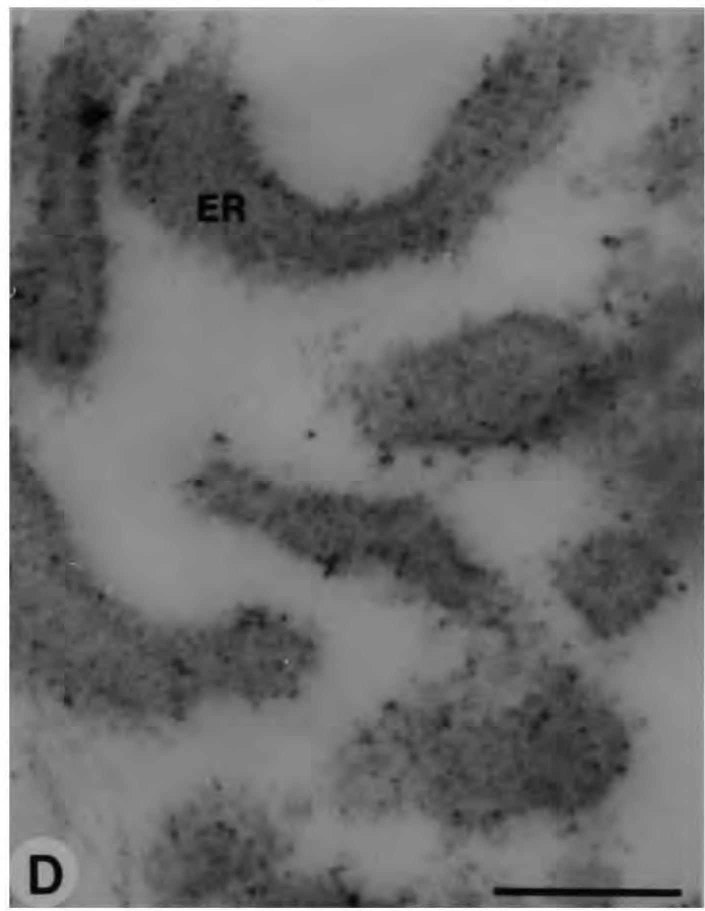


PLATE 51. Immunocytochemical localization of C hordein on sucrose density gradients separation homogenates of barley endosperms. Fractions were fixed with aldehyde and osmium tetroxide, and embedded in Spurr resin. Sections were post-stained.

A : Protein body fraction (Mg^+ gradient) shows PAG_{15} labelling of C hordein associated with protein body. Bar = 2 µm.

B : (Mg^+ gradient). PAG_{15} labelling of C hordein in endoplasmic reticulum fraction. Bar = 0.5 µm.

C : (Mg^- gradient). PAG_{15}- labelling of C hordein in protein body fraction. Bar = 0.3 µm.

D : (Mg^- gradient). PAG_{15} labelling of C hordein in endoplasmic reticulum fraction. Bar = 0.25 µm.

3.5 *In situ* Hybridization using a Biotinylated cDNA Probe

In situ hybridization was used to locate mRNA for the chymotrypsin inhibitors, CI-1 and CI-2, in the endosperm of the high-lysine barley mutant, Riso 1508.

Endosperms, with a caryopsis fresh weight of 40 mg, were harvested from Risø 1508 grown in the greenhouse, as previously described (Section 2.1.1.1). At this stage storage proteins are being actively synthesized. The barley grain was immersed in fixative immediately upon harvesting, and subsequently a 3mm slice was cut transversely across the middle region of the grain. Tissue was then further processed for *in situ* hybridization, as described in Section 2.11.

3.5.1 Localization of Chymotrypsin Inhibitor (CI-1 and CI-2) mRNAs in the Endosperm of the Barley Mutant Risø 1508

cDNAs for chymotrypsin inhibitors (CI-1 and CI-2) were obtained from Rothamsted (Williamson and Kreis, unpublished; and Williamson et al., 1986a, b, manuscripts in preparation.) These cDNAs related to CI-1 and CI-2, were prepared using synthetic oligonucleotides as probes. "Northern hybridization" experiments show that the abundances of CI-1 and CI-2 mRNAs correlate with changes in the amounts of the proteins *in vivo* , in normal and high-lysine barley lines.

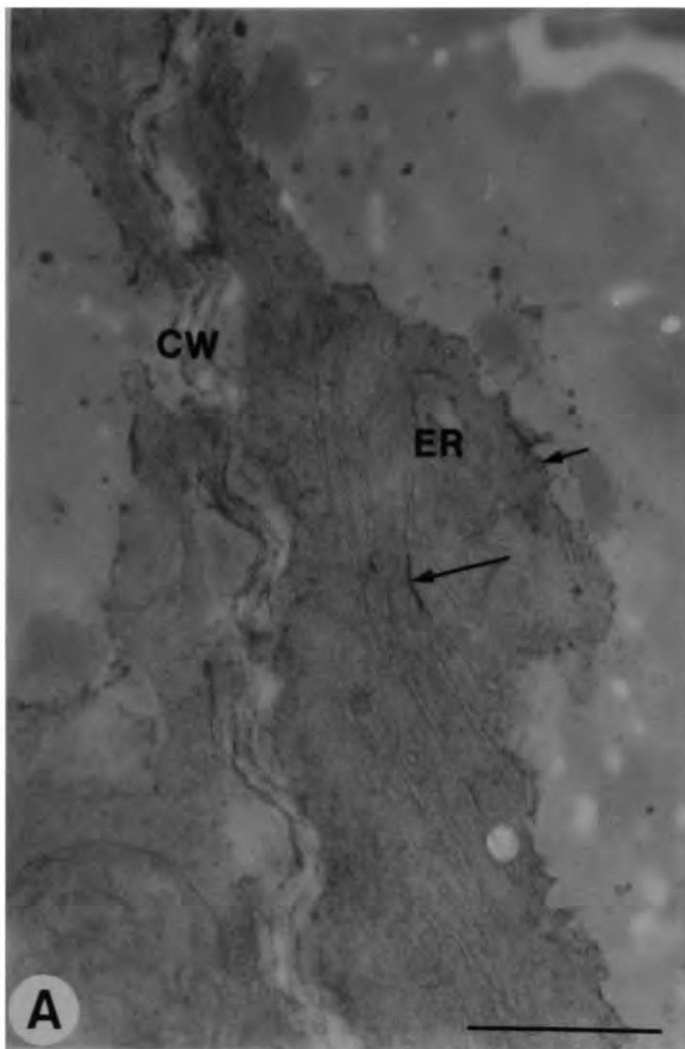
Chymotrypsin inhibitor (CI-1 and CI-2) mRNAs were localized in the endosperm of the high-lysine barley mutant Risø 1508 by hybridization with biotinylated cDNA, and subsequent avidin-peroxidase/DAB reactions. Peroxidase/DAB staining, after avidin-peroxidase binding to the biotin, is shown in Plate 52 for CI-1, where sections have been post-stained with uranyl acetate and lead citrate. In micrograph 52A, stain deposits are located in the endoplasmic reticulum of the sub-aleurone. At a higher magnification (micrograph 52B), peroxidase/DAB staining is seen to be deposited in the lumen of the endoplasmic reticulum. Micrograph 52C shows a dictyosome in the aleurone of Riso 1508. The mRNA for chymotrypsin inhibitor-1 is located in the cytosol. In the endosperm however, micrograph 52D, peroxidase-DAB staining is associated with the tubular (fibrillar) area of the protein body.

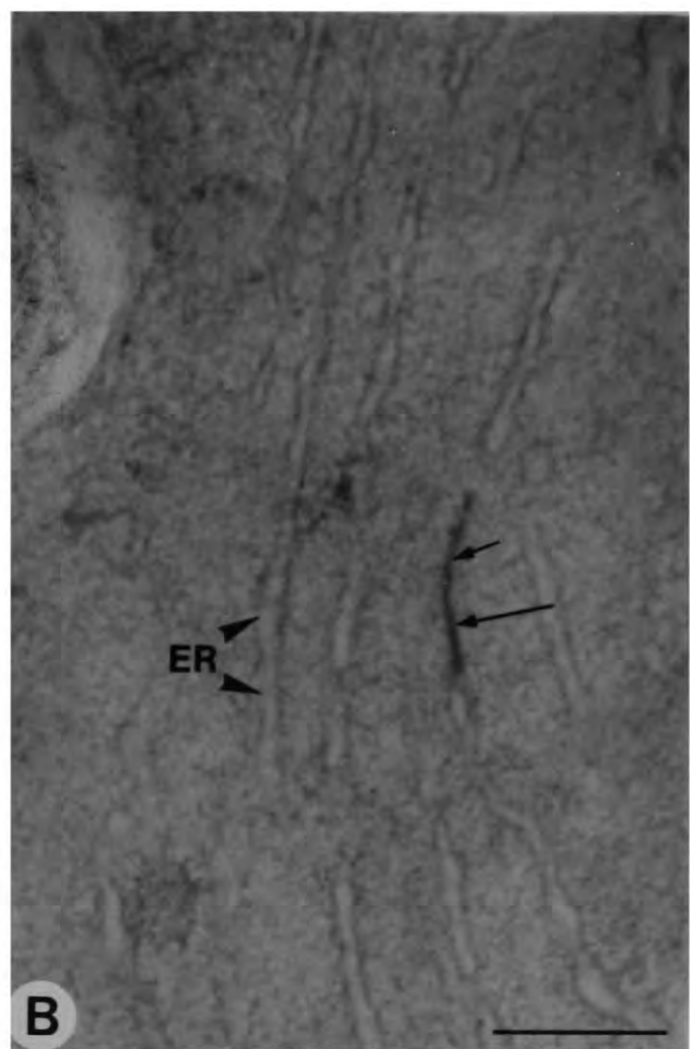
Plate 53 illustrates the localization of mRNA for chymotrypsin inhibitor-2 in the endosperm of Risø 1508. Hybridization with biotinylated cDNA was followed by avidin-peroxidase binding, and subsequent DAB staining reactions. Grids were post-stained with uranyl acetate and lead citrate before viewing in the transmission electron microscope. In micrograph 53A, stain deposits are seen in the lumen of the endoplasmic reticulum and

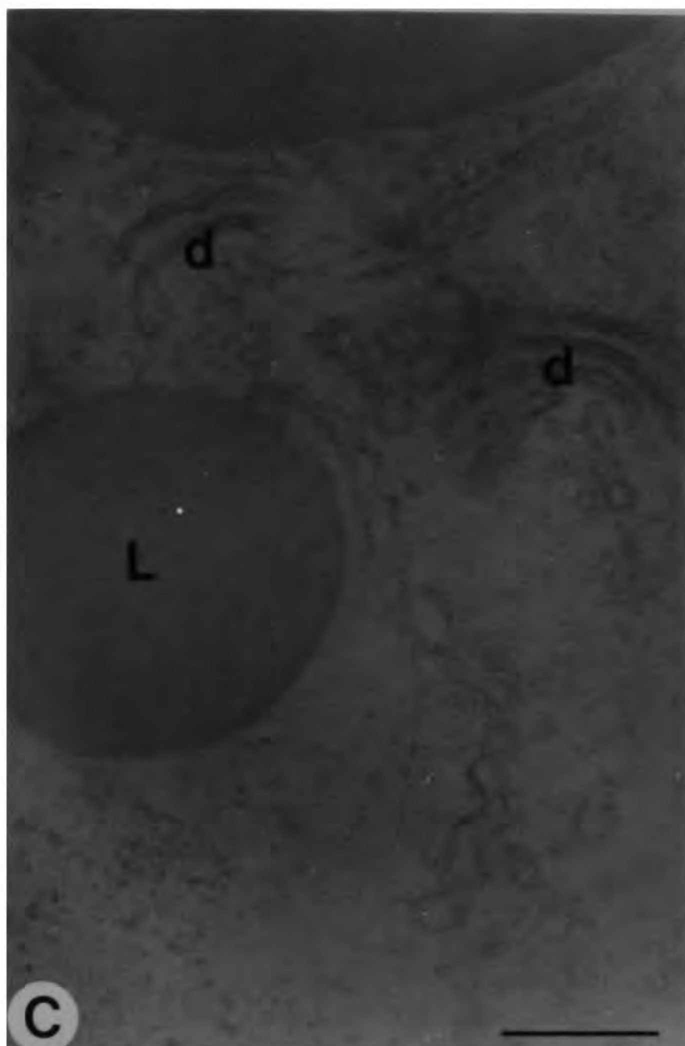
are also located in the protein body (indicated by arrows) of the sub-aleurone. In the starchy endosperm (micrograph 53B), stain deposits are located only in the lumen of the endoplasmic reticulum.

The results show that the mRNAs for both CI-1 and CI-2, are associated primarily with the endoplasmic reticulum, and some staining deposits are located in protein bodies.

The control tissues for CI-1 and CI-2 using a biotinylated plasmid without a cDNA insert, show no peroxidase/DAB staining deposits in comparison with the cDNA probed tissue, and there are no stain deposits in the lumen of the endoplasmic reticulum of the control tissues.







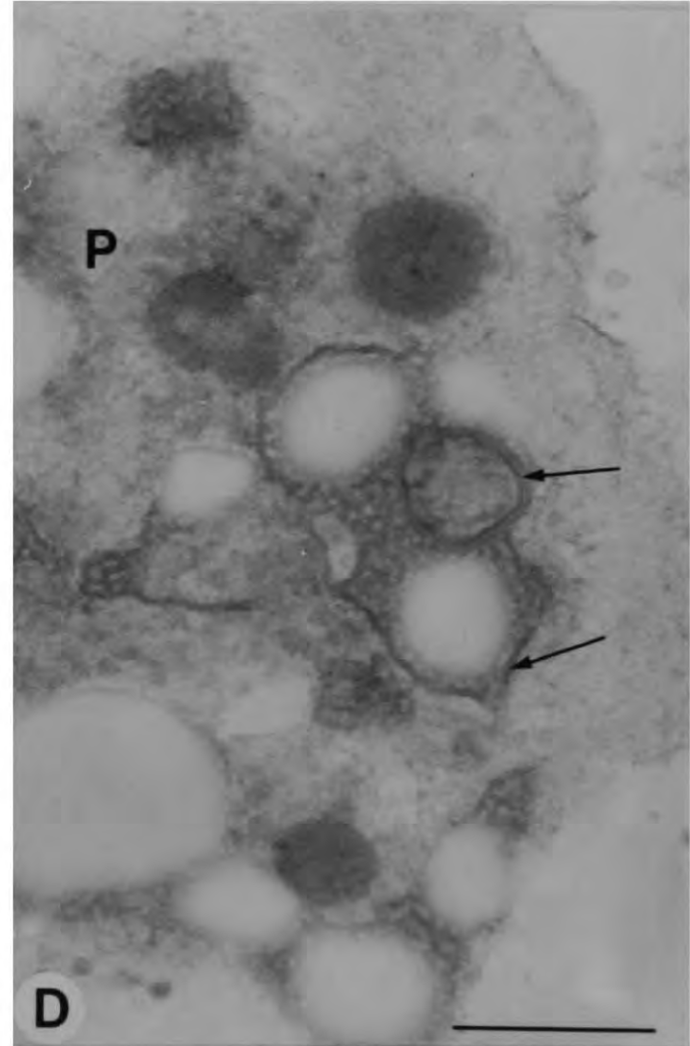


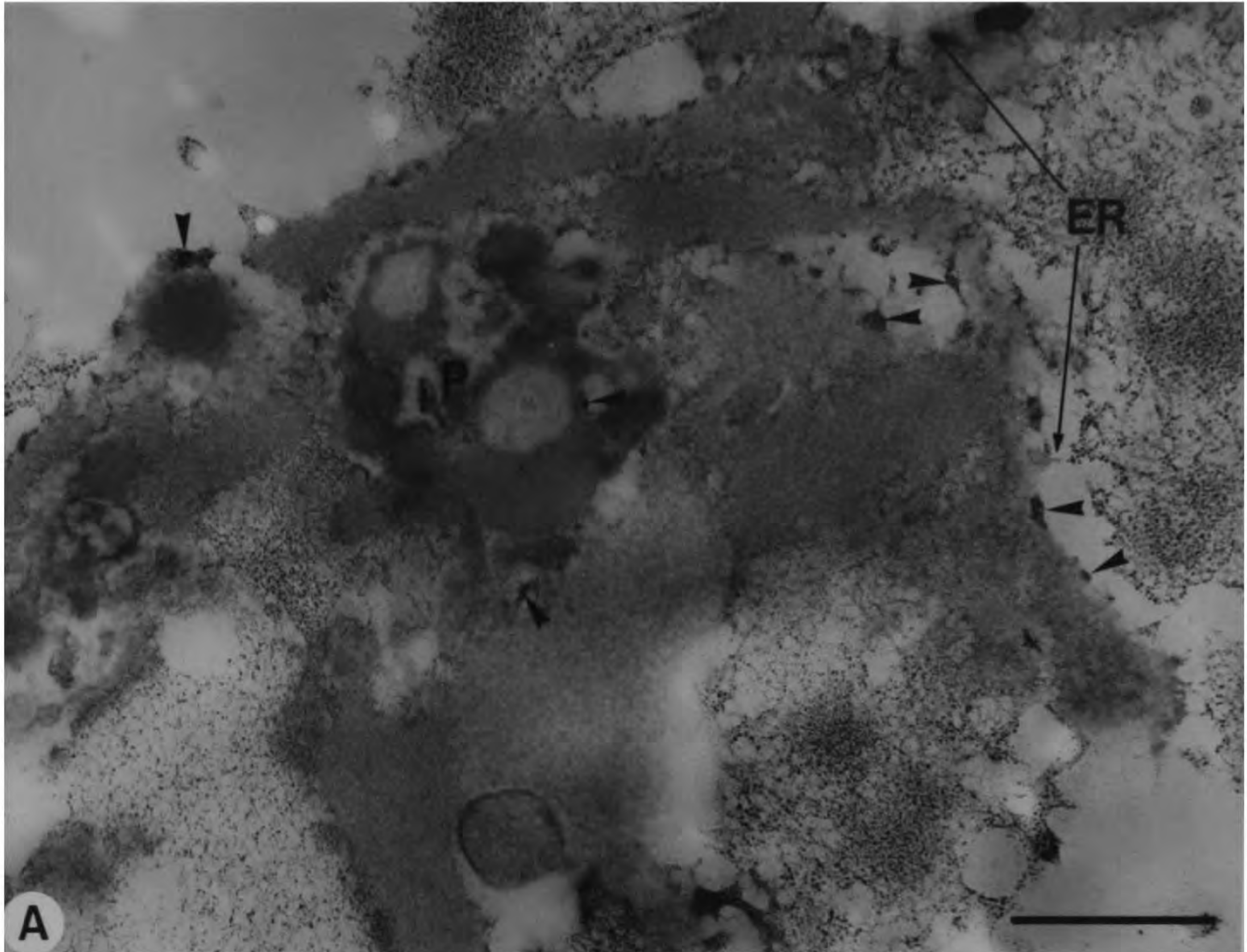
PLATE 52. Localization of CI-1 mRNA by hybridization with a biotinylated cDNA probe and subsequent avidin peroxidase/DAB reactions in the mutant 1508, 21 DAA (42 mg).

A : Peroxidase-DAB reaction product associated with the ER (arrows) in the sub-aleurone.
Bar = 1 µm.

B : Detail of (A) ; arrows indicate stain deposits within the lumen of the ER. Bar = 0.25 µm.

C : No stain deposits are seen in the aleurone. Bar = 0.25 µm.

D : Stain deposits are associated with the fibrillar areas of the endosperm protein complex. Bar = 0.5 µm.



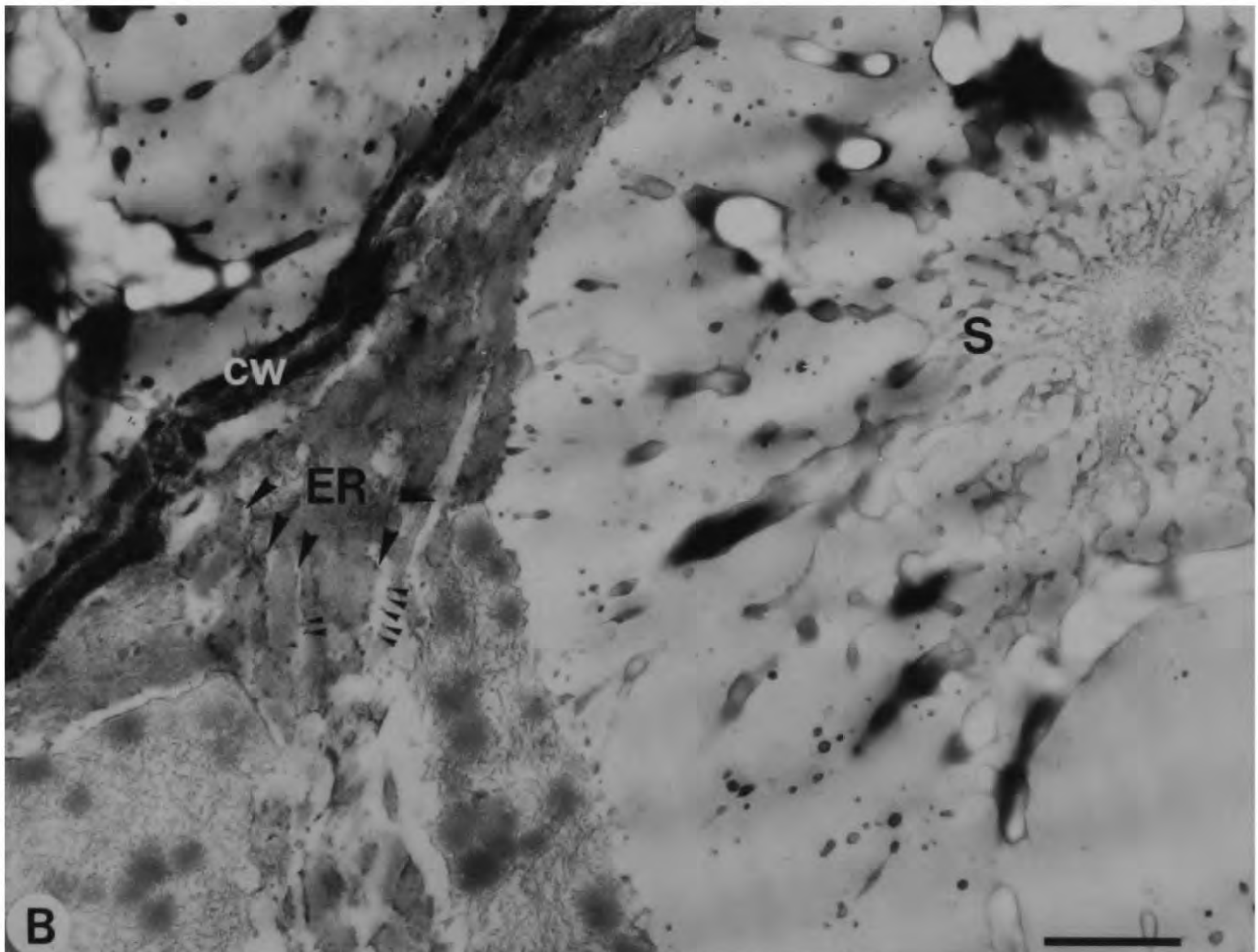


PLATE 53. Localization of CI-2 mRNA by hybridization with a biotinylated cDNA probe and subsequent avidin-peroxidase/DAB reactions, in the mutant, Riso 1508, 21 DAA (42 mg).

A : In the sub-aleurone, stain deposits are associated with the protein deposit and in the lumen of the ER (arrows). Bar = 0.5 µm.

B : In the endosperm, stain deposits are located in the lumen of the ER (arrows). Bar = 1 µm.

CHAPTER FOUR

DISCUSSION

DISCUSSION

The cereal storage proteins are the primary source of harvested plant proteins. The peculiar properties of these proteins in part determine the nutritional quality (for non-ruminants) of the grain, and its technological uses (Miflin and Shewry, 1983). As cereal grains are the major source of protein consumed by man, but are nutritionally unbalanced, the need for better nutritionally balanced cereal proteins is urgent. The endosperm storage proteins are a major component of the nutritionally important proteins, but the subcellular locations of many of the storage proteins are undetermined. Some are known to be located in protein bodies, but there is some controversy over the way in which these deposits are formed.

The cereal endosperm is ideal experimental material for the molecular biologist, being highly specialized tissue primarily engaged in the synthesis and accumulation of starch and storage proteins. Whereas there is a large body of literature on molecular composition of storage proteins and their primary structure and gene expression (Kreis et al., 1985; Shewry and Miflin, 1985), controversy exists surrounding the synthesis and deposition of these storage proteins. Briefly, for barley, one theory suggests that all

protein bodies are vacuolar in origin, whilst others argue that the prolamin containing bodies are derived from and associated with, the endoplasmic reticulum.

The characterization of seed proteins, and the improvement in both their quality and yield, has been investigated for several decades. Outstanding advances have been made in the past few years, in particular in the molecular biology and regulation of synthesis, in the deposition and the degradation of seed proteins, and the regulation of enzymic activities.

One approach to the improvement of the nutritional qualities of barley has been to look for, and characterize, the high-lysine barley lines. The first high-lysine barley recognized, was found by screening the world barley collection using the dye-binding capacity technique (Munck et al., 1970). The variety Hiproly was found to have relatively more lysine in the seed than other varieties and also higher protein content, but the grain weight was lower.

The same screening procedure was used at the Danish Atomic Energy Research Station at Risø to examine seeds treated with either chemical or physical mutagens. Although Risø 56 and 1508 mutant lines have a higher lysine content than their parent varieties, making them of better nutritional quality, the grain yield is reduced to 80% of that of the parents. Attempts to

incorporate the high-lysine characteristic of Risø 1508 into well-filled grain have so far been unsuccessful (Rhodes and Jenkins, 1978). The lower grain yields have so far rendered these lines commercially non-viable, but the production of these lines has stimulated the biochemical investigation of the development of both the mutant and normal lines. A reduced and modified synthesis of storage proteins may be associated with an altered protein body morphology; such information may provide a more precise description of the protein deposition in normal lines.

The lack of knowledge concerning the ontogeny of protein deposition is creating a handicap in the understanding of seed development, particularly as the molecular biological techniques are progressing quickly. Hence, the purpose of the research described in this thesis was to investigate the deposition of storage proteins in the barley endosperm.

Firstly, barley endosperm development was investigated by thin-section transmission electron microscopy. The results were compared with existing evidence used to support the vacuolar or endoplasmic reticulum derived theories for storage protein origination. Thick section studies of zinc iodide-osmium tetroxide impregnated barley endosperm provided new information on subcellular inter-

relationships. Storage proteins were, for the first time, localized in developing endosperm cells by immunocytochemistry.

The control of gene expression during seed development is of major importance. So far, studies have shown that the expression of various genes within the endosperm is regulated by organ specific, temporal, nutritional and transacting mutations (Miflin et al., 1983; Kreis et al., 1983c; Rahman et al., 1982; Rahman et al., 1984). However, further studies are hampered by the lack of a precise description of endosperm development and protein deposition. In particular, although endosperm is known to be a complex organ consisting partly of terminally differentiated cells, which will die on maturity (the starchy endosperm), and partly of cells which will survive dormancy and function during germination (the aleurone), almost all studies have been done on homogenized total endosperm or total caryopsis (Rahman et al., 1984). Furthermore, it is apparent that the stage of cellular development differs within different parts of the same tissue. Hence *in situ* hybridization was applied to study the developmental regulation of gene expression in barley endosperm.

Fresh and dry weights of the developing Bomi barley endosperm were followed from anthesis to maturity (Figure 3.1). The results have shown that the fresh

weight of grains harvested at Rothamsted in the summer of 1984, increased with time until 32 days after anthesis and decreased after that. The increase in fresh weight of the caryopsis to 32 DAA was largely due to an increase in growth of the endosperm. The dry weight constituted an increasing proportion of the fresh weight all through development of the caryopsis; at 45 DAA this was about 80% of the fresh weight.

Del Rosario et al. (1968) have shown that, for the greater part of grain filling in rice endosperm, considerable amounts of water loss occur from the caryopsis, which undergoes a progressive reduction in moisture content. Jenner (1980) has drawn attention to the fact that about half of the final starch content of cereal grains is deposited with no change in water content of the endosperm. Oparka and Gates (1981) suggest that in rice, as in other cereals, water leaves the caryopsis during grain filling as assimilates continue to enter. This was found for barley endosperm (Figure 3.1) where the dry weight constituted an increasing proportion of the fresh weight in grain filling.

The developmental pattern of Rothamsted field-grown barley was similar in 1985; although anthesis occurred later, fresh and dry weights correspond closely to those obtained at the same developmental stages in 1984

(results from 1985 are not included). Both 1984 and 1985 fresh and dry weights of caryopsis and endosperm development of the wild-type Bomi barley agree with the results presented by Rahman (1982) from anthesis to maturity for the barley grain, cv. Sundance.

Bishop (1930) was probably the first person to study the developmental sequence in which different protein fractions accumulate in barley grain grown at Rothamsted. His results differ little from the more recent results studying barley grain grown in the field at Rothamsted (Rahman et al., 1982). Rahman et al. (1982) found that the hordeins appear in the endosperm during the phase of rapid growth, and they continue to accumulate linearly until maturity. Controversy existed in the literature as to whether there are changes (Briedert and Schön, 1974) or not (Brandt, 1976) in the rate of accumulation of different hordein polypeptides. Rahman et al. (1982) have indicated that there are differences in the composition of the hordein fraction in the barley grain cv. Sundance, at different stages of development. C hordein polypeptides form a greater proportion of the total in the early stages of development, but during the latter stages, there is an increase in the relative amount of the B hordein and in one specific group of polypeptides (B1 hordein) within this fraction.

Storage proteins have been defined (Boulter, 1977)

as those proteins laid down in one phase of development but which function at a later metabolic phase to supply intermediary nitrogen compounds for biosynthesis. Seed protein reserves are usually deposited in membrane-bound organelles now commonly termed 'protein bodies'. In cereals these protein reserves occur mainly in the triploid tissues of the endosperm, and are found predominantly in the sub-aleurone and outer starchy endosperm (Briggs, 1978). They are hydrolysed during germination from non-living tissues in a relatively uncontrolled manner prior to peptide uptake at the embryo scutellum. By contrast, dicotyledons such as the legume seeds, contain substantial reserves of protein in the protein bodies within the cotyledon storage parenchyma tissues, from which mobilization within living tissues is regulated (review: Chrispeels, 1985).

The results in this thesis show that two types of protein body can be recognized in the endosperm of the developing barley grain. In the sub-aleurone, protein bodies are smaller in size and are granular in appearance under the electron microscope, whereas in the cells of the starchy endosperm the protein bodies are larger and more heterogeneous. The latter consist of complex granular clusters with smaller fibrillar and electron dense components. Results confirm some of the work of earlier studies (Munck and von Wettstein, 1976;

von Wettstein, 1979; Cameron-Mills and von Wettstein, 1980).

Two schools of thought exist as to the nature and origin of the protein bodies in developing barley endosperm. The first suggests that, in common with protein bodies of other seeds, they are derived from vacuoles into which the protein is transported and deposited (discussed by Matile, 1976; Ashton, 1976). This theory has been most fully supported by von Wettstein and colleagues (Munck and von Wettstein, 1976; Cameron-Mills and von Wettstein, 1980) and they have produced micrographs to show that the storage proteins of barley are accumulated within vacuoles. Numerous small vesicles aggregate around the growing protein body inside the vacuole, which might imply the involvement of vesicles in the transport of hordein from the rough endoplasmic reticulum to the vacuoles (Cameron-Mills and von Wettstein, 1980).

Miflin and Shewry (1979) and Miflin et al. (1983) have, however, proposed a contrasting theory. In this they have suggested that protein bodies of barley endosperm are derived from the rough ER. Such a mechanism has been reported for maize (Khoo and Wolf, 1970; Larkins and Hurkman, 1978; Miflin et al., 1981). The hypothesis put forward by Miflin et al. suggests that the hordeins are deposited inside the ER where they

aggregate. As the aggregate increases in size so it breaks away from the endoplasmic reticulum and is deposited in the cytoplasm, and is not considered to be enclosed within a membrane. Micrographic evidence is presented in support of this (Miflin et al., 1981; Miflin and Shewry, 1979).

Miflin et al. (1981) have presented electron micrographs of isolated protein bodies and propose from these that the protein bodies are not enclosed within a limiting membrane. To test this hypothesis, they incubated protein bodies with proteinase K and showed that they were not protected from digestion. In parallel experiments, Miflin and Burgess (1982), found that other subcellular organelles of barley and also protein bodies from pea cotyledons were protected from digestion.

Miflin and coworkers (including Shewry and Miflin, 1983) derived a working hypothesis suggesting that the hordein is not deposited in protein bodies of vacuolar origin, but is present as aggregates of insoluble protein associated with fragments of the endoplasmic reticulum.

The ultrastructure of developing Bomi barley grains reported here, was first studied by thin-section transmission electron microscopy (Section 3.1.3). The endosperm differentiates to form the sub-aleurone, underlying the aleurone, and the outer and inner starchy

endosperm. By 10 DAA the protein bodies in the sub-aleurone are both cytoplasmic and vacuolar. Many of these protein bodies are encircled by endoplasmic reticulum. As reported by Cameron-Mills and von Wettstein (1980) the cells of the sub-aleurone are the smallest and last to be formed. Although starch is seen in the deeper endosperm cells, no granules are seen in the sub-aleurone; conversely no protein bodies are evident in the starchy endosperm at 10 DAA, indicating differential regulation of the deposition of the different types of storage compounds in the different tissues of the endosperm.

Endoplasmic reticulum was also seen to surround and enclose areas of cytoplasm. At the same stage in the development of the barley endosperm, some dictyosomes were evident in the cytoplasm. Although dictyosomes at 10 DAA could be shown from zinc iodide-osmium tetroxide staining to be numerous in cells of the sub-aleurone, with conventional thin sectioning they were seldom seen, and then only in slightly thicker sections which had been subject to osmium tetroxide for longer periods than in normal fixation regimes. The fact that dictyosomes are seldom seen in thin sections of endosperm has been documented by Parker for wheat (Parker, 1981; 1982).

Rapid protein deposition occurred 14 DAA when many of the protein deposits in the sub-aleurone were

vacuolar. Starch granules began to accumulate in cells of the sub-aleurone which also showed a marked increase in endoplasmic reticulum. Endoplasmic reticulum encircled vacuoles containing protein deposits. The protein complex was mainly granular but had a smaller fibrillar component (e.g. Plate 13). In the endosperm, the protein deposits were both vacuolar and cytoplasmic, although both types were in close proximity to the endoplasmic reticulum.

Cameron-Mills and von Wettstein (1980) reported barley endosperm protein bodies developing within a vacuolar membrane. These protein bodies were seen to consist predominantly of a granular component. At a later stage in development, the protein complex has also a smaller fibrillar component and an electron-dense component.

By 18 DAA (e.g. Plate 16B), the endosperm protein complex in Rothamsted Bomi barley exhibited the three components described by Cameron-Mills and von Wettstein (1980). The complex consisted of large granular spheres embedded in a fibrillar component with small electron dense spheres. Many vesicles were seen at the periphery of the protein complex; Cameron-Mills and von Wettstein (1980) have suggested the involvement of these vesicles in the transport of the protein synthesized on the endoplasmic reticulum to the site of storage in the

protein bodies.

As amplified above in the results section, the term "fibrillar" does not seem entirely appropriate for a structure that is predominantly tubular. However, to avoid the confusion of introducing an alternative terminology, the term "fibrillar" has been retained.

By 18 DAA the aleurone cells of barley were highly vacuolate; these vacuoles contained a dense deposit, with a transparent central core, within a peripheral matrix, inside the vacuole. This is similar to the developing maize aleurone grain (Kyle and Styles, 1977). Aleurone grains are organelles of protein and phytin storage. Oleosomes (lipid bodies) were seen packed tightly around the aleurone grains. Lipid bodies were occasionally observed in the cytoplasm of the sub-aleurone; Cameron-Mills and von Wettstein (1980) have also reported a similar finding. Whereas only occasional lipid bodies are found in the sub-aleurone, their numbers are much higher in the cells of the aleurone. Hydrolysis of these lipid reserves by lipases from aleurone grains is probably an important source of fatty acids during germination.

By 20 DAA (e.g. Plate 17), large protein aggregates were evident in vacuoles of the sub-aleurone. In the endosperm, protein bodies became compressed into angular shapes between the large and small starch grains; only

large starch grains were seen in cells of the sub-aleurone. At a later stage in development (45 DAA, results not reported) starch granules were embedded in the proteinaceous matrix.

The application of electron microscopic techniques to developing endosperms is fraught with difficulties of fixation and sectioning (see Campbell et al., 1981). Miflin et al. (1983) suggested that this can produce artefacts and that fixation can influence the interpretation of results. They showed micrographs of protein deposits in close association with endoplasmic reticulum from their best fixation régimes. Other micrographs show protein deposits within vacuoles (comparable to Cameron-Mills and von Wettstein, 1980), but Miflin et al. interpret these results as the consequence of poor fixation. Conversely, Cameron-Mills and von Wettstein (1980) question the results of Miflin and Shewry (1979) which suggest that protein bodies are formed in the endoplasmic reticulum and subsequently released into the cytoplasm. Cameron-Mills and von Wettstein suggest that a tonoplast can be seen around these supposedly free protein bodies in the cytoplasm.

The results of barley protein deposition in endosperm cells reported in this thesis have been derived from initial work which assessed the best fixation régime which conferred the most accurate

interpretation of cell ultrastructure. This was based on the following criteria; lack of plasmolysis, distribution of organelles, no dilation of the endoplasmic reticulum etc. The use of different fixatives and buffers has been described above. Aldehyde fixation in phosphate buffer produced the best fixation; this fixation regime produced the most reliable and consistent ultrastructural preservation. From such fixations it could be concluded that most protein deposits were vacuolar although some were free in the cytoplasm. This will be discussed later with respect to the mode of protein deposition in barley endosperm.

Descriptions of the ultrastructure of Bomi barley have defined three components, namely, the granular deposits (1) embedded in a fibrillar component (2) and frequently associated with electron dense spheres (3). The ultrastructural investigation of the mutant barley lines was carried out to provide an additional insight into protein body formation as affected by a reduced and altered protein content.

The condensation of the storage proteins is markedly altered in the barley mutants Risø 56 and Risø 1508 (Munck and von Wettstein, 1976; Cameron-Mills, 1980; Cameron-Mills and von Wettstein, 1980.) Cameron-Mills and von Wettstein (1980) have shown that at 20 DAA protein bodies in the normal barley line

comprise clusters of granular components with an associated fibrillar matrix and electron dense spheres. In the high-lysine mutants the proportion of fibrillar matrix to homogeneous component is considerably increased. The electron dense component is also increased with deposits located at the periphery, and embedded within, the fibrillar matrix. Membrane vesicles, commonly found at the periphery of normal barley protein bodies, are more numerous and located also within the protein complex in the high-lysine mutants. Cameron-Mills and von Wettstein (1980) suggest that the fibrillar matrix appears to be a transient stage in the condensation of the storage proteins into the homogeneous structure. Reduction in hordein synthesis increases the proportion of the fibrillar matrix, apparently retarding the condensation process.

The results presented above show that in the high-lysine mutant Hiproly at 14 DAA, single protein bodies are found both in the cytoplasm and in vacuoles in the sub-aleurone. However at the same stage in the development the cells of the sub-aleurone of Bomi, many more protein deposits were observed. Protein deposits of the sub-aleurone and starchy endosperm of Hiproly had many electron dense spheres at their periphery. By 21 DAA, protein deposits increased in number in the vacuoles of the sub-aleurone, but still did not equal

the amount at 14 DAA in Bomi barley. There was however, an increase in vesicles and vesicular material within the sub-aleurone protein vacuoles in Hiproly. Hiproly starchy endosperm at 21 DAA contained cytoplasmic protein bodies comprising granular components with large electron dense areas. In Bomi these electron dense components were very small and only visible from 18 DAA.

Among the various mutants examined, the greatest depression in hordein synthesis occurs in Risø 1508, derived from Bomi barley. This depression was found to be accompanied by an altered protein body structure. Single protein bodies were observable in vacuoles of the sub-aleurone at 17 DAA; these consisted of a predominant fibrillar region with some granular structure. Cameron-Mills and von Wettstein (1980) reported similar structures, and interpreted these as indicating the failure of storage protein to condense into a granular component. The tightly packed fibrillar structure is discernable throughout the protein body of the mutant. The electron dense component is more prolific than in the wild type and the accumulation of vesicles within the vacuoles is also observed. Cameron-Mills and von Wettstein (1980) again reported similar results.

In the mutant Risø 56, derived from Carlsberg II, the hordein content is reduced from 60% to 45% of total

seed nitrogen (Shewry et al., 1980c). Protein bodies were discernable 17 DAA in Risø 56, and in the sub-aleurone they consisted of granular components embedded in large fibrillar areas. The fibrillar area is more prolific than in the wild type Bomi barley that were examined. Cameron-Mills and von Wettstein (1980) report an increase in the fibrillar matrix in Risø 56 in comparison to Carlsberg II, the wild type it was derived from.

Electron dense spheres were present in Riso 56 protein bodies both at the periphery and embedded within the fibrillar matrix. These results confirm those reported by Cameron-Mills and von Wettstein (1980) that there is an increase in membrane vesicles associated with Risø 56 protein bodies, and that, unlike the wild type, these are also found embedded within the protein body cluster.

Investigations using a zinc iodide-osmium tetroxide staining complex in conjunction with conventional transmission electron microscopy have been reported for some seed endosperms such as wheat (Parker and Hawes, 1982) and rice (Oparka and Harris, 1982; Bechtel and Juliano, 1980). The results for the thick section study reported in this thesis are however, probably the first reported for barley endosperm.

By 10 DAA, cells of the sub-aleurone were highly vacuolate. These vacuoles were commonly encircled by

one or more sheets of endoplasmic reticulum (e.g. Plate 19). Dictyosomes with prominent cisternae and densely staining vesicles, were abundant during the early endosperm development and may function in producing membrane for the rapidly developing cells. Protein bodies were found in close association with cisternal and tubular ER; the latter having close associations with dictyosomes.

By 14 DAA, when protein deposition was at a maximum, there were fewer dictyosomes than seen at 10 DAA. Cells of the sub-aleurone contained fewer dictyosomes with dense vesicles around a single cisternal plate. Both cytoplasmic and vacuolar protein deposits had similar densely staining vesicles at the periphery. From thin-section studies, Cameron-Mills and von Wettstein (1980) have suggested the involvement of these vesicles in the transport of the protein synthesized on the endoplasmic reticulum to the protein bodies.

By 20 DAA dictyosomes were only occasionally evident (e.g. Plate 26). The dictyosome distribution throughout development in barley shows a similar trend for that described for wheat (Parker, 1981, 1982; Parker and Hawes, 1982). Parker and Hawes (1982) have implicated the involvement of the Golgi apparatus in the transport of protein synthesized on the ER to protein

bodies. However, although micrographs (e.g. Plates 22 and 25) show interrelationships between the dictyosome, protein body and endoplasmic reticulum, similar to that described by Parker and Hawes (1982) for wheat, the reduction in dictyosome population during the period of maximum rates of protein deposition suggests that the dictyosomes do not play a major role in protein deposition in barley. This suggestion is further strengthened by the failure of attempts to confirm a dictyosomal role by immunocytochemical localizations.

High-lysine barley mutants were also investigated by the thick sectioning of zinc iodide-osmium tetroxide impregnated caryopsis. In Hiproly, Risø 56 and Risø 1508 protein was found to be deposited in vacuoles surrounded by endoplasmic reticulum, as in the wild type Bomi. Like Bomi these protein deposits had electron-dense vesicles at their periphery, although more numerous than in the wild type. An obvious divergence from the wild type is the abundance of the tubular ER in the cells of the sub-aleurone of the high-lysine mutants.

High-lysine mutants are characterized by a reduction in grain size which is mainly due to reduced starch accumulation (Køie et al., 1976; Kreis, 1978; Doll and Køie, 1978). This is accompanied by an increased sucrose concentration. In the sub-aleurone

cells of Bomi there is an abundance of rough cisternal ER. This is characteristic of cells exhibiting high rates of protein synthesis for either intracellular accumulation within vacuolar protein bodies or for external secretion. The cisternae are interconnected by smooth tubular ER (e.g. Plate 22a). Cells involved in the transport or secretion of lipids or sugars have an extensive network of tubular ER; such structural features are the case in the sub-aleurone cells of high-lysine mutants which exhibit an increased sucrose accumulation.

High-lysine barley mutants are characterized by an overall increase in the lysine content of the mature endosperm, compared with parental varieties. In most cases the high-lysine character involves a reduction in the synthesis of the lysine-poor prolamin fraction. While the mutant Risø 1508 (lys 3A) (Brandt, 1976; Shewry <u>et al</u>., 1979) synthesises relatively less of all of the prolamin polypeptides when compared with the wild type, the mutation Risø 56 reduces specifically the synthesis of the B hordein polypeptides (Doll, 1980). The high-lysine mutant Hiproly (lys) displays none or only a small reduction in prolamin synthesis, the elevated lysine content resulting from a change in the composition of the albumin and globulin fraction. The increased deposition of one polypeptide, SPII albumin,

accounts for 37% of the overall lysine increase in the Hiproly endosperm when compared with a normal variety (Jonassen, 1980a, 1980b; Svendsen et al., 1980b). The grain weight of the Riso high-lysine mutants is reduced by about 20% and the endosperm is shrunken (Doll, 1976); the reduction in grain size is mainly due to reduced starch accumulation (Køie et al., 1976; Kreis, 1978; Doll and Køie, 1980) which is associated with an increased sucrose concentration. The latter is the precursor of starch synthesis. Kreis and Doll (1980) suggest from their results based on studies of the individual high-lysine genes in the double mutants, that the influences of these genes on prolamin and starch synthesis are independent.

As part of a cellular investigation of the molecular aspects of gene expression in storage protein synthesis, mRNA for barley chymotrypsin inhibitors was localized using the *in situ* hybridization technique. Hybridization sites were located using a biotinylated cDNA probe which was visualized under the electron microscope using a labelled avidin complex which binds strongly to biotin.

Classical and conventional methodology allows classification of cells within functionally complex and cellularly heterogeneous tissues. These parameters provide information on ultrastructure (from thin

sections), subcellular interrelationships (from thick sections), and the presence of proteins in the cell (from immunocytochemical localization). Whereas immunocytochemical localization is a useful tool for detecting the presence of a protein, it is not definitive in regard to function of the particular gene concerned. At any one time insufficient protein may be available for positive detection. Protein detected in cells may have arrived there other than from *de novo* synthesis e.g. special transport systems, entrapment etc. Furthermore, non-specific immunological cross-reactions may lead to erroneous conclusions. Hence, *in situ* hybridization techniques were used to determine where in the cells transcription rather than translation products are present.

The distribution of the peroxidase/DAB reaction product indicated that in Risø 1508, both CI-1 and CI-2 mRNAs were associated with the cisternal endosplasmic reticulum and with the protein body (Plate 52 and 53). Much of the stain deposit was associated with the endoplasmic reticulum; especially within the lumen of the endoplasmic reticulum. Stain deposition apparently within the endoplasmic reticulum lumen may be explained as a consequence of localized changes within the section; the stain deposits are smaller than the section thickness. Cell preservation after hybridization and

peroxidase reaction was not as good as that revealed by conventional fixation, however the general form of the major organelles and their distribution was sufficiently good to give confident identification. The control tissues show no peroxidase/DAB staining deposits in comparison with the cDNA probed tissue, and there are no stain deposits in the lumen of the endoplasmic reticulum or in protein bodies.

In situ hybridization and immunocytochemistry provides information on the timing and position of specific events during cell and tissue differentiation. This could not be obtained by either biochemical or microscopical techniques alone. Both transcription and translation products of CI-1 and CI-2 were localized in Risø 1508 when the grain had a fresh weight of about 40 mg. At this time, CI-1 and CI-2 proteins are present in the endosperm. CI-1 and CI-2 mRNAs were associated with the fibrillar region of the protein complex. Conventional thin sectioning, in which tissue preservation is better than following *in situ* hybridization, has shown that many of the Risø 1508 protein complexes are vacuolar, with a granular region and prominent fibrillar and vesicular components. The association of mRNAs with the vesicular and fibrillar regions of these protein deposits, as well as with the cisternal endoplasmic reticulum, suggests that the

vesicles/fibrillar material of the vacuolar protein complex may be derived from the endoplasmic reticulum.

The marking of cellular components for microscopical observation is an extremely important technique for studying molecular organization and cell function. Indeed biochemical analysis alone would be incomplete since it would give little information regarding the distribution of cellular components. Immunocytochemical techniques were employed to identify and localize specific proteins with the intention of gaining an understanding of the dynamics of cell components at the ultrastructural level, in particular, protein body ontogeny.

Controls are of enormous importance in immunocytochemistry to establish the validity of observations. These controls were described in section 3.4.1; there was no non-specific binding of protein A-gold after 'gold-only' incubation, and none after labelling of pre-immune serum incubations. Controls were employed on sections from the <u>same</u> tissue block as that used for labelling of the antigens.

The storage proteins are difficult to study immunologically as they are extracted by alcoholic solvents; they can be redissolved in denaturing solvents such as SDS and 6M urea, but these may interfere with antigen-antibody reactions. Festenstein and Hay (1982)

have reported that the non-specific reaction of SDS with rabbit serum in double diffusion tests can be minimized by reducing the SDS concentration; a quantitative measure of antigen-antibody reaction following SDS precipitation of non-specific reacting material can be provided by a nephelometric assay.

Festenstein and Hay (1982) have found reported common antigenic determinants for A and B hordeins and also for B and C hordeins. B hordein contains extra determinants not present on A hordein and also not present on C hordein. From immunocytochemical localization, A, B and C hordein were located in protein bodies of barley endosperm, specifically within the granular component. Although all antisera were polyclonal raised in rabbits and do not allow the identification and comparison of single antigenic determinants, it does allow an assessment of the site of deposition and temporal accumulation of storage proteins.

Rahman et al. (1982) illustrated the characteristic temporal differences in accumulation of the component polypeptides of the hordein fraction from analysis by gel electrophoresis. These results showed that C hordeins made up a relatively higher percentage of total hordein at the early stages, decreasing from 20% of the total at 33% final dry weight to 15% at maturity,

although the absolute amount increased linearly. The relative amount of the lowest molecular weight B hordein band (B1) increased throughout development from 30% of the total at 33% final dry weight, to about 45% at maturity.

The temporal accumulation of C hordein in developing Bomi barley endosperm has been demonstrated by immunocytochemical labelling with protein A-gold. At 10 DAA, there is a low density of gold label over small cytoplasmic protein deposits. However, at 14 DAA, during the phase of rapid protein deposition, protein deposits are heavily labelled with protein A-gold. Protein has formed a matrix in the interstices between starch grains by 32 DAA, and is heavily labelled with protein A-gold after previous incubation with the primary antibody. At 14 DAA, immunocytochemical localization has shown A, B and C hordein to have a similar distribution in Bomi endosperm.

Many antigens do not tolerate post-fixation with osmium tetroxide. The reduced antigenicity resulting from osmium tetroxide post-fixation was observed with C hordein in Bomi Spurr resin-embedded tissue. Bendayan and Zollinger (1983) showed that the antigenicity of certain proteins can be restored after post-osmification by treatment of thin sections with sodium metaperiodate. Binding sites were "unmasked" by

"etching" with periodate prior to incubation with antibodies. Such treatment resulted in an intensified labelling of C hordein over protein bodies. The effect of omitting osmium post-fixation increases the density of labelling after antibody incubation, however contrast is poor although this can be slightly improved by post-staining the grid with uranyl acetate and lead citrate. Hearn et al. (1985) found that periodate "etching" of tissue fixed in glutaraldehyde, post-fixed in osmium tetroxide and embedded in epoxy resin permitted multiple antigen labelling in the same tissue, and labelling of antigens in tissues that had been stored in epoxy resins for many years.

In recent years a number of new resin embedding media have appeared, of which at least two have enabled distinct improvements to be made in post-embedding technology. The development of the "Lowicryl" range has made possible the embedding of tissue at temperatures of $-30\,^{o}C$ or below, where the denaturation and extraction of protein during dehydration is minimized (Carlemalm et al., 1982). The recently formulated "LR White" acrylic resin has a number of advantageous characteristics and is simpler to use. It is not used at low temperatures, but like "Lowicryl K4M", "LR White" is hydrophilic, so that ultrathin sections permit improved penetration of aqueous solutions. The lipid solvency of these two

resins appears to be significantly lower than that of the epoxides and polyesters and therefore membranes and organelles remain for observation even when the tissue is not post-fixed in osmium. Importantly, the plastic itself does not have an avidity for antibodies nor does it prevent them from binding to tissue antigens.

A comparison was made of labelling of A, B and C hordein in Bomi barley endosperm embedded in Spurr resin, "LR White" resin or "Lowicryl K4M". Generally, in comparison with Spurr resin, there is a high degree of labelling over the protein bodies with the acrylic resins. Contrast and preservation in "LR White"-embedded tissue is superior to "Lowicryl K4M"-embedded tissue, however, in both cases a unidirectional-smearing effect is apparent. This is probably due to smearing of protein by the glass-knife during sectioning. With both types of resin, immunocytochemical labelling of hordein antibodies is confined to the granular areas of protein bodies, once smearing effects have been taken into consideration.

This smearing effect is not evident in Spurr resin-embedded tissue, and ultrastructural preservation and contrast is best in tissue post-fixed in osmium tetroxide and embedded in Spurr resin. Although osmium tetroxide post-fixation does lead to a reduction in antigenicity, for the hordein antigens this is not too

important as they do tolerate a substantial degree of further fixation with osmium. Furthermore, "etching" did not reveal any further sites "masked" by osmium tetroxide; neither the endoplasmic reeticulum or dictyosomes were labelled.

From a comparison of fixative buffers (described in "Materials and Methods" and section 3.4.4) it was found that phosphate-buffered aldehyde fixative produced the most consistent results throughout the whole developmental sequence of Bomi barley from anthesis to maturity. From immunocytochemical labelling, PIPES-buffered fixed tissue did not reveal any different antigenic sites from those revealed in phosphate-buffered fixed tissue. An increase in the lumen of the endoplasmic reticulum is a feature of PIPES-buffered fixatives; no gold labelling was associated with the endoplasmic reticulum.

Ultrastructural investigation revealed that the high-lysine barley lines, namely Risø 56, Risø 1508 and Hiproly, have a modified protein body structure in comparison with the normal wild type barley. This modification comprises of a reduced granular protein body component when compared with the wild type, but a more prolific fibrillar component and an increased amount of electron-dense spheres. Cameron-Mills and von Wettstein (1980) interpreted the fibrillar matrix as a

transient phase in the condensation of storage proteins into a granular component, and stated that the polypeptide composition of protein bodies determines their ultrastructure. They found that the reduction in the synthesis of B type hordein in mutant Risø 56 increases the proportion of the fibrillar matrix, while a more drastic alteration in storage protein condensation was observed in mutant Risø 1508, which is highly deficient in both B and C type polypeptides.

Despite this, the granular areas of protein bodies of Risø 56 were labelled with B1 hordein antibodies. It was hoped at the beginning of this project that antisera available against the hordeins would be relatively more specific and would indicate the distribution of the most prominent antigen. The high level of apparent labelling of B hordein in Risø 56 indicates however, that this is not the case with B/C cross determinancy, since the level of B labelling would have arisen only by the presence of C hordein.

Immunocytochemical localization of B and C type hordeins in the high-lysine barley mutants illustrated, by protein A-gold labelling, that both hordeins were located in the granular component of protein bodies. The large fibrillar matrices were unlabelled in Risø 56. In Risø 1508 where a small granular component is present around the prominent tubular/fibrillar matrix, labelling

shows storage protein deposition to be restricted to the granular component.

The chymotrypsin inhibitors CI-1 and CI-2 have been the focus of much attention due to their unusually high contents of lysine which contribute towards the increased lysine content of some barley lines. In Hiproly, CI-1 and CI-2 contain 9.5% and 11.5% lysine respectively (Hejgaard and Boisen, 1980). The increased lysine content leads to an improved nutritional quality of the grain.

Immunocytochemical localization has shown CI-1 and CI-2 associated predominantly with the protein body. Although these proteins primarily have a specific metabolic function, evidence from immunocytochemical localization indicates that as they can be present in sufficiently large amounts in some genotypes, they probably have a secondary role as storage proteins. The localization of CI-2 in membrane-bound compartments is consistent with observations for the chymotrypsin inhibitors in tomato and potato (Graham et al., 1985; Walker-Simmons and Ryan, 1977). Also, it has been shown by Jonassen et al. (1981) and Williamson et al. (1986) that CI-2 is probably synthesized on membrane-bound polysomes.

The results described in this thesis do not show a close fit to either of the existing, but contradictory,

models proposed by Cameron-Mills and von Wettstein (1980), and by Miflin *et al.* (1983), for the development of barley storage protein deposition.

Since neither of the barley models seems appropriate the results of this research are compared with other existing models of plant storage protein deposition. In legume seeds the storage proteins are synthesized on the rough endoplasmic reticulum, pass into the lumen and then to the dictyosomes where they are packaged into vesicles which move to vacuoles into which they are deposited (Boulter, 1979; Bailey *et al.*, 1970; Harris and Boulter, 1976). Immunocytochemical studies have confirmed the role of the Golgi apparatus in the transport of some storage proteins (Craig and Goodchild, 1984; Greenwood and Chrispeels, 1985); and the central role of the Golgi apparatus in post-translational modification and transport has been reviewed by Chrispeels (1985).

However, with respect to protein deposition in barley, the "legume model" does not seem to be a viable proposition. No evidence has been found to document the pathway from protein synthesis on the rough endoplasmic reticulum into the lumen, then the pathway from the dictyosome to vesicles. Only one micrograph illustrates immunogold labelling of C hordein over a possible dictyosome (Plate 39B). However, whilst this structure

has some resemblance to a dictyosome with its peripheral vesicles, it may also represent a tangential section through the periphery of a protein complex; the latter is more probably the case. The only evidence in partial support of this theory is that protein deposits are mainly in vacuoles.

In maize, protein deposition has been explained based on the work of Khoo and Wolf (1970). They found that maize storage proteins are synthesized on, and deposited within, the rough endoplasmic reticulum. In maize, there is ultrastructural and biochemical evidence to suggest that (a) polyribosomes are directly associated with the protein body membrane (Burr and Burr, 1976) and that (b), rough microsomal preparations are capable of synthesizing the storage protein zein (Burr and Burr, 1981) with cleavage of the preprotein occurring co-translationally. In barley however, polyribosomes are not seen associated with the vacuolar protein body membrane, rather electron-dense vesicles (enhanced by selective staining with zinc iodide-osmium tetroxide) are evident at the periphery of cytoplasmic protein bodies and at the periphery of protein deposits within vacuoles. There are no micrographs, either ultrastructural or from immunocytochemistry, to support a "maize-type" deposition model for barley storage protein deposition.

Cameron-Mills and Ingversen (1978) have shown from an *in vitro* reconstitution of rough microsomes that hordeins were discharged vectorially into the lumen of the endoplasmic reticulum. However, contrary to maize, Cameron-Mills and von Wettstein (1980) have not shown any continuity between protein bodies and the endoplasmic reticulum. They have however, observed small vesicles implicated in the transport of storage proteins from the endoplasmic reticulum to the protein bodies. Small vesicles are also a distinctive feature at the periphery of protein bodies, illustrated in this thesis.

In the "oat model", evidence from Adeli and Altosaar (1983) suggest that although oat globulins are also synthesized by endoplasmic reticulum-associated polyribosomes, the high molecular weight proteins are probably transported to the protein bodies and cleaved into the smaller α and β subunits. The marked dissimilarity in types of storage protein suggests, that an "oat-type" model would not be applicable to barley storage proteins.

Rice has the additional feature of having various distinguishable types of protein bodies, each thought to contain predominantly different storage proteins (Bechtel and Juliano, 1980). The "rice model" proposes that all types of rice protein bodies are derived from

the endoplasmic reticulum, although transport from the Golgi apparatus may be involved in the accumulation of some types of protein deposits (Oparka and Harris, 1982).

The results presented for barley storage protein deposition fail to fit the "rice model". From immunocytochemical localization there is no evidence of different types of protein bodies for different storage proteins. There is no evidence to support protein body derivation from endoplasmic reticulum, nor to implicate the Golgi apparatus in the accumulation of protein deposits.

In wheat, ultrastructural examination of the development of protein body formation in the endosperm (Bechtel *et al.*, 1982a; Parker, 1982) indicates that storage proteins are initially deposited within a membrane-bound compartment and are subsequently released into the cytosol where they may continue to aggregate additional protein and membranous material before forming a matrix during seed desiccation. In conventionally prepared material, it has been shown that storage protein may accumulate directly within the dilated lumen of the cisternal endoplasmic reticulum (Briarty, 1978; Campbell *et al.*, 1981; Parker, 1982). Thick section studies of wheat endosperm indicate the presence of numerous dictyosomes closely associated with

both cisternal endoplasmic reticulum and the forming protein bodies (Parker and Hawes, 1982). Barley protein deposits are found in membrane-bound compartments, although some are also seen at the same developmental stage, free in the cytosol. At maturity, protein forms a matrix in the interstices of starch grains; this agrees with the "wheat model". However, in contrast to wheat, there is no micrographic evidence that storage proteins of barley accumulate in the dilated lumen of the endoplasmic reticulum.

Dictyosomes are numerous at 10 DAA, during the early endosperm development in barley. During the period of rapid protein deposition (14 DAA), dictyosomes are still evident although not so numerous, and have a more condensed appearance with vesicles located around a central cisternal plate. In the maturing barley endosperm (20 DAA) dictyosomes are still found, although less frequently. This shows a similar trend to the dictyosomal distribution throughout development described in wheat (Parker and Hawes, 1982; Parker 1981, 1982; Buttrose, 1963a; Campbell et al., 1981). Parker and Hawes (1982) have implicated the involvement of the Golgi in storage protein deposition; they have suggested from their results that endosperm storage proteins are transported from their site of synthesis on the endoplasmic reticulum to their site of storage in

protein bodies, via the Golgi.

Although thick-section micrographs have illustrated close interrelationships between the dictyosomes, endoplasmic reticulum and protein bodies in barley, similar to that found by Parker and Hawes (1982) in wheat, it cannot be implied that the Golgi is necessarily involved in the transport of synthesized protein. The numerous dictyosomes present in barley endosperm 10 DAA may function in synthesizing membranes and/or wall material in the rapidly developing sub-aleurone cells.

The established model systems for wheat, rice, and maize cannot be entirely satisfactorily applied to the results found in this thesis. A new model (Figure 4.1) is thus proposed for protein deposition in developing barley endosperm. This model is not only consistent with the results of the research above, but is corroborated by the results of other workers.

By 10 DAA, many vacuoles are seen in the sub-aleurone. This is the initial stage seen from the results, and is represented at the top of figure 4.1. The highly vacuolated sub-aleurone is a feature of thin section-transmission-electron-micrographs. In these and thicker sections, impregnated with zinc iodide-osmium tetroxide, these vacuoles were seen surrounded by endoplasmic reticulum. The number of surrounding

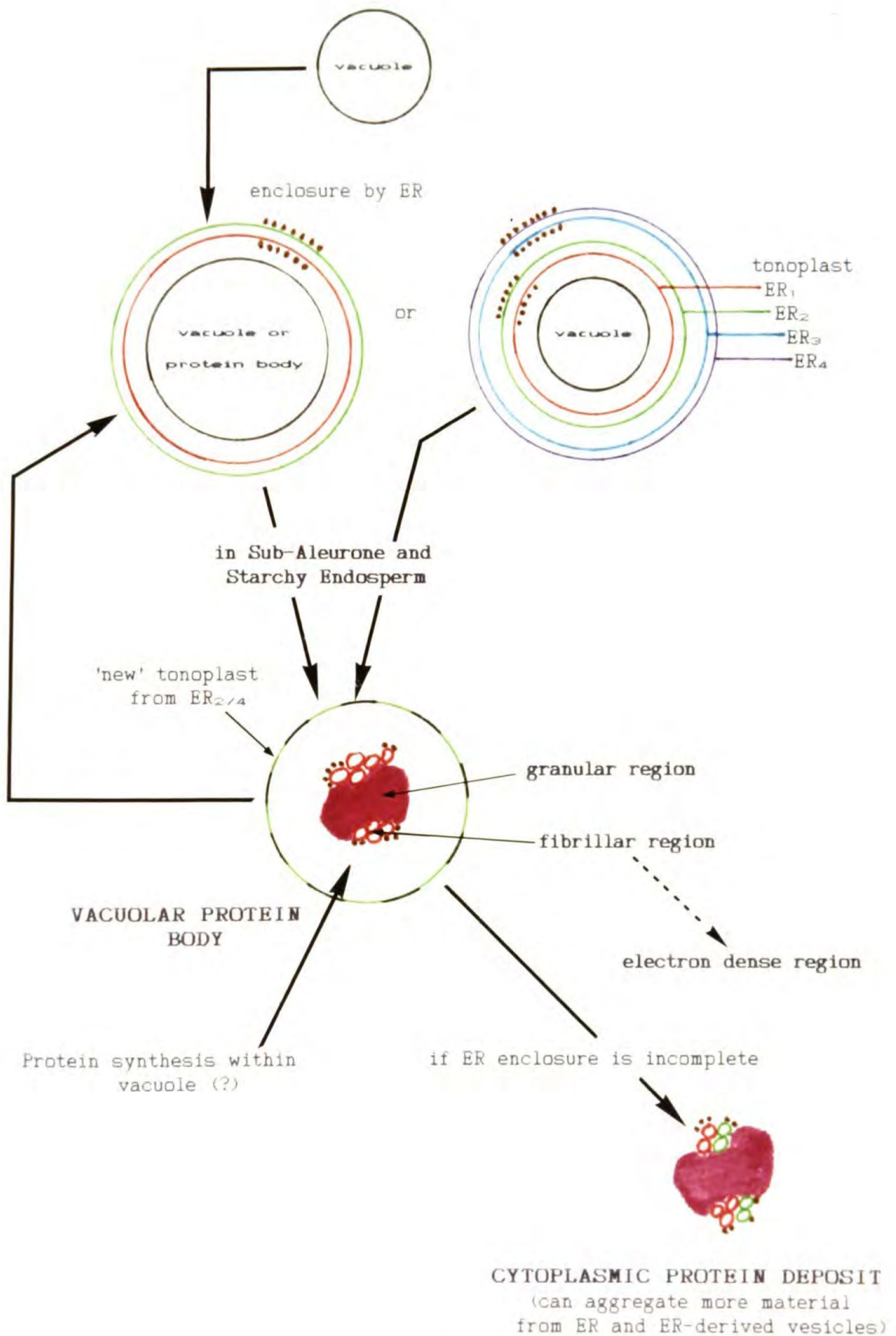


FIGURE 4.1 Model for storage protein deposition in developing barley endosperm.

cisternae can vary, but in figure 4.1 two are represented; these are labelled ER(1) to ER(4), representing the effective layers of membrane.

The morphology of vacuolation and an evaluation of its functional significance in cell metabolism has been documented by Matile and Moor (1968); Matile (1966); Marty (1973a); Matile (1968). Matile and Moor (1968) proposed that the genesis of vacuoles involved firstly, the formation of small endoplasmic-reticulum-derived vesicles (provacuoles). The fusion of these provacuoles results in the formation of small vacuoles, and this is followed by fusion and expansion of vacuoles. Large dictyosome-derived vesicles are incorporated into vacuoles by invagination of the tonoplast. This invagination of the tonoplast results in the incorporation of cytoplasmic material into the vacuoles. The vacuoles have been shown to contain a number of hydrolytic enzymes which characterize these vacuoles as lysosomes (Matile, 1966; Matile and Wiemken, 1967).

Matile and Moor (1968) suggest that provacuoles (ER-derived vesicles) are the primary lysosomes; their hydrolases arise from the endoplasmic reticulum. Vacuoles represent the secondary lysosomes (digestive vacuoles) of the higher-plant cell. Hence, Matile (1966) suggests that the vacuoles of the higher plant cell represent organelles in which the processes of

intracellular digestion takes place. Marty (Marty, 1973b, c; Marty, 1978; Marty et al., 1980) considers that the endoplasmic reticulum and the vacuoles are integrated in a membranous continuum in line with the "Endomembrane Concept" of Morré and Mollenhauer (1974). Plant vacuoles may thus serve as a multifunctional compartment, including storage protein sequestration.

Authors who consider that storage proteins are deposited in vacuoles also include Ashton (1976) and Pernollet (1978). As vacuoles in general originate from the endoplasmic reticulum in plant cells (Matile, 1976), Adler and Muntz (1983) have proposed a uniform theory of generation of vacuolar protein bodies formed either by vesicles generated from the endoplasmic reticulum or from vesicles derived from dilated endoplasmic reticulum.

It is proposed that storage protein deposition in developing barley endosperm is initially vacuolar, with deposition in endoplasmic reticulum-generated vacuoles. There is however, no micrographic evidence to support the latter view of Adler and Muntz (1983) that protein bodies arise as vacuolar-type products of swollen endoplasmic reticulum cisternae.

Figure 4.1 illustrates the ontogeny of a vacuolar protein body. ER(1/3) and the original tonoplast break-down into vesicles and move into the vacuolar

space. ER (2/4) forms the "new" vacuolar tonoplast. Protein associated with the endoplasmic reticulum, at low levels in the lumen, will be incorporated into the new vacuole, condensing to become a deposit as a consequence of its hydrophobicity. This process may be repeated as successive encirclings of endoplasmic reticulum are incorporated into the vacuole, which thus contain increasing amounts of protein deposits. The incorporation of ER-microsomal membrane and ribosomes, as seen by conventional thin-sectioning, and messenger RNA as shown by *in situ* hybridization, suggests the possibility of protein synthesis within the vacuole were amino acids to be transported across the tonoplast.

Both cytoplasmic and vacuolar protein bodies are seen in tissue sections. It is suggested that the cytoplasmic deposits form if the endoplasmic reticulum enclosure is incomplete, the protein complex thus losing its surrounding membrane and giving rise to such protein deposits. The form of the cytoplasmic complexes indicates that the hydrophobic protein bodies would continue to aggregate more material from the endoplasmic reticulum and the derived vesicles.

Numerous small vesicles are found at the periphery of the protein deposit within the vacuole (e.g. Plate 13). Such vesicles are more numerous in the high-lysine mutants, where they can often be found embedded within

the protein deposit (e.g. Plate 31). These vesicles are electron-dense when impregnated with zinc iodide-osmium tetroxide which is selective for the endomembrane system. Cameron-Mills and von Wettstein (1980) have also reported numerous small vesicles around the growing protein body inside the vacuole, have suggested they may be involved in the transport of hordein from the rough endoplasmic reticulum to the vacuoles, however the presence of transport vesicles actually within the vacuole suggests something other than simply deposition into the vacuole from ER-derived vesicles as proposed by Cameron-Mills and von Wettstein (1980); it seems unlikely that vesicles would pass through the tonoplast.

In the sub-aleurone, the protein deposits consist of a granular component. In the wild type barley, sub-aleurone protein bodies are almost entirely granular throughout development, although they increase in size with maturity. At 14 DAA, the starchy endosperm protein bodies, however, have small fibrillar regions both in the cytoplasmic and vacuolar deposits. These regions do not label in immunocytochemical localizations for the storage proteins and the scheme in Figure 4.1 proposes that fibrillar regions, which actually have a tubular appearance, are derived from vesicles. The electron-dense spheres seen later in development are compacted residues of the vesicle membranes.

The fibrillar and electron-dense regions are more prominent in the high-lysine mutants, although the granular component is reduced in comparison to the wild type. By 23 DAA, sub-aleurone protein bodies are still semi-fibrillar, but starchy endosperm protein bodies consist of a loose network of tubules interspersed with granular matrix with associated electron-dense spheres. Risø 56 follows a similar pattern; sub-aleurone protein bodies at 17 DAA and 24 DAA comprise granular components embedded in a fibrillar matrix. In the starchy endosperm, protein complexes consist of granular spheres embedded in an electron-dense matrix.

From *in situ* hybridization investigations localizing the chymotryptic inhibitors in the endosperm of Risø 1508 (secction 3.5.2), the mRNA was found to be associated with the endoplasmic reticulum, and fibrillar areas of the protein complex. This supports the model in Figure 4.1. Some of the vesicles surrounding the protein body are ER-derived. These vesicles give rise to the fibrillar matrix around which synthesized protein is sequestered.

The model proposed in Figure 4.1 fits well with the published data used by Cameron-Mills and von Wettstein (1980) to defend their earlier model. Miflin et al. have disagreed with Cameron-Mills and von Wettstein's conclusion and, on the basis of subcellular isolation

work, suggested an alternative. Miflin and Burgess (1982), from an investigation of the effect of proteinase-K on isolated protein bodies, proposed that barley endosperm protein bodies are not surrounded by a complete membrane. However, they do not provide substantial evidence against the likelihood that intact membranes present in the original endosperm were damaged during isolation. Part of this evidence came from Miflin et al. (1981) where protein bodies were shown to be not vacuolar; this was based on the absence of vacuolar marker enzymes in association with protein bodies, but instead there was a large peak of NADH-cytochrome *c* reductase activity associated with the protein body peak. Miflin and Burgess (1982) also criticised other work (e.g. Cameron-Mills and von Wettstein, 1980; Parker, 1980; and Campbell et al., 1981). Where electron micrographs show protein deposited within vacuoles; Miflin and Burgess (1982) suggested that as cereal endosperm is difficult to fix and section, published work does not convincingly show the presence of vacuolar membranes surrounding protein bodies *in vivo* . Hence, Miflin et al. propose storage protein deposition occurs by synthesis of protein on the RER which subsequently passes into the lumen where it aggregates. The results presented in this thesis do not, however, support the model proposed by Miflin et

al., although their results can be explained by the model in Figure 4.1.

Further evidence in support of the proposed model 4.1 for protein deposition in developing barley endosperm comes from sucrose density gradient separation of homogenates of barley endosperms in the presence and absence of Mg^{2+} (Figures 3.2 and 3.4). In the presence of Mg^{2+}, most NADH cytochrome *c* reductase activity is associated with the protein body fraction. (Figure 3.2). In the absence of Mg^{2+}, most enzyme activity is associated with the endoplasmic reticulum fraction (Figure 3.4); peak at 1.21 g/ml in the presence of Mg^{2+} becomes peak 1.14 g/ml in the absence of Mg^{2+} indicating endoplasmic reticulum-association with the protein body fraction. This fits the proposed model 4.1 where ER (1/3) breaks down and forms vesicles within the protein body vacuole.

In conclusion, the work described in this thesis proposes that storage protein deposition in developing barley endosperm is initially vacuolar, and the endoplasmic reticulum is involved not only in protein synthesis but is also involved in the aggregation of storage proteins into complex protein bodies. A combined approach using the techniques of thin-

and thick-section-conventional-transmission-electron-microscopy, and the combination of electron microscopy in association with the molecular techniques of immunocytochemistry and *in situ* hybridization, yielded information that could not be obtained from either biochemistry or microscopy alone.

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