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DEDICATIONS

This thesis is dedicated to my dear father, Ebrahim, my grandmother, Mirzadeh, and to the memory of my beloved mother, Iran. Their everlasting support, love, and encouragement have made my studies possible.

این رساله به پدر ارجمندم ابراهیم، مادر بزرگ عزیزم میرزاده، و به خاطرهٔ مادر گرامیم ایران، تقدیم میشود. پشتیبانی و عشق پیوستهٔ ایشان، تحصیلات مرا ممکن ساخته است.

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ABSTRACT

Biosynthesis and compartmentalization of Avena globulin polypeptides were investigated by in vitro translation, in vivo labelling, subcellular fractionation, and electron microscopic techniques. The 12S globulin α and β polypeptides were shown to be synthesized as common precursors with relative molecular weight of 58,000-62,000 both in vitro and in vivo.

These putative ~~precursors~~ were shown to be made primarily on polysomes attached to endoplasmic reticulum (rough endoplasmic reticulum). Upon synthesis, the globulin polypeptides were segregated in the endoplasmic reticulum cisternae rendering them resistant to proteolytic digestion. The association of these polypeptides with the endoplasmic reticulum was, however, transient. The globulin polypeptides disappeared from the endoplasmic reticulum shortly after synthesis and subsequently appeared in protein bodies. Pulse-chase labelling studies showed that the 58,000-62,000 globulin polypeptides arrived in protein bodies unprocessed. The site of proteolytic cleavage of these large polypeptides to globulin α and β subunits was therefore found to be the protein bodies. This processing event was gradual and not immediate. Sometimes during or after cleavage, the globulin polypeptides were assembled into 12S oligomers consisting of the processed α and β subunits. Such a 12S oligomer was absent in the endoplasmic reticulum and seemed to be formed only after deposition of globulin in protein bodies.

Electron micrographs obtained from developing oat endosperm active in protein synthesis (16-20 days after flowering) illustrated the

channelling of reserve proteins to intracellular vacuoles after their initial synthesis on the rough endoplasmic reticulum. Protein bodies which appeared to be formed at the interface between the rough endoplasmic reticulum and tonoplast, were subsequently secreted into the vacuoles. The newly- developed protein bodies fused to form larger bodies filling the entire vacuole.

Based on the above-mentioned observations, a hypothesis was proposed stating that globulin polypeptides are synthesized as larger precursors on the rough endoplasmic reticulum, sequestered within it, probably with the aid of a signal peptide, and channelled into intracellular vacuoles. Reserve globulins are packaged into membrane-bound vesicles or protein bodies and are secreted into the vacuole. During, or after, protein body formation, the globulin precursors are processed into the α and β subunits which are then assembled into 12S oligomers.

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ABBREVIATIONS

A_{260}	absorbance taken at 260 nm
A_{280}	absorbance taken at 280 nm
ATP	adenosine triphosphate
BIS	N,N'-methylene-bis-acrylamide
cDNA	complementary deoxyribonucleic acid
CNBr	cyanogen bromide
CPM	counts per minute
daf	days after flowering
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol-bis-(β-amino-ethyl ether) N,N'-tetra acetic acid
EM	electron microscopy
ER	endoplasmic reticulum
FITC	fluorescein isothiocyanate
X g	centrifugation force
GERL	Golgi endoplasmic reticulum lysosomes
GTP	guanosine triphosphate
h	hour
Hepes	2-(N-2-hydroxyethylpiperazine-N) ethane sulphonic acid
IEF	isoelectrofocusing
IgG	immunoglobulin G
K	one thousand
kDa	kilodaltons
Me	2-mercaptoethanol

M_r	relative molecular mass
min	minute(s)
mol wt	molecular weight
NADH	reduced nicotinamide adenine dinucleotide
O.D.	optical density
PB	protein bodies
PBS	phosphate buffered saline
PMSF	phenyl methyl sulfonylfluoride
Poly A^+ RNA	polyadenylated ribonucleic acid
RER	rough endoplasmic reticulum
RNase	ribonuclease
S	Svedberg unit
SDS	sodium dodecyl sulfate
SER	smooth endoplasmic reticulum
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	trichloroacetic acid
Tris	2-amino-2-hydroxymethylpropane-1,3-diol
TEM	transmission electron microscopy
waf	weeks after flowering

CHAPTER I

INTRODUCTION

1. BACKGROUND

One of the main nutritional deficiencies affecting populations in developing countries is 'protein-calorie deficiency' (1). This occurs in humans because the intake of energy and protein is lower than minimal requirements (2). Accordingly, protein quantity, and more specifically protein quality, defined as the content of essential amino acids, play an important role in human nutrition. Improving protein quality in food crops is of practical significance in terms of nutrition and health. Cereals provide more than 50% of world protein consumption. The successful development of opaque-2 maize (3) and new high protein oat cultivars (4) have demonstrated the genetic potential for improving the quality of cereal grains. Opaque-2 maize has a much higher lysine content than common maize. Also new oat cultivars such as Hinoat developed by Ottawa Research Station (Agriculture Canada) contain considerably higher amounts of protein with good quality, compared to existing economically beneficial cultivars, such as Elgin or Harmon. Such high quality crops were introduced through traditional plant breeding techniques, which are painstakingly long processes. The nutritional needs of the growing world population clearly demand much more efficient and rapid avenues for improving the quality and quantity of food crops. Modern genetic techniques may provide quicker tools for achieving these objectives. Therefore, to provide the breeder and geneticist with appropriate molecular information, researchers have been investigating the biology and biochemistry of seed reserve proteins. The last decade has been witness to great advances in understanding the mechanisms involved in seed reserve protein topogenesis (cellular biosynthesis, cytoplasmic transport, post-translational processing and organellar storage).

Several unique features have created interest in studying the mechanisms of storage protein synthesis in oat endosperm. Developing oat endosperms are very active protein synthesizing systems. More than 70 percent of the total protein synthesized is a storage fraction, globulin (5). Such a high rate of biosynthesis of a single class of storage proteins makes developing oat seeds a useful cereal system for studying genome expression in higher plants especially cereals. Such research has been conducted extensively in other cereals and legumes. Recent studies on zein in maize (6), phaseolin in french bean (7), hordein in barley (8), and glycinin in soybean (9) have substantially increased our knowledge of the biosynthesis and deposition of seed storage proteins as well as the structure, organization, and expression of their genes. More research on the molecular factors regulating storage protein biosynthesis may assist in improving the nutritional quality of cereal grains by manipulating the storage protein genes to alter their amino acid content and/or levels of expression.

2. PROTEIN BIOSYNTHESIS

The biosynthesis and packaging of cellular and viral proteins involve several distinct processes. These processes have been extensively studied for the last three decades. Palade (10) has outlined the basic steps of protein secretion as 1) synthesis, 2) segregation, 3) intracellular transport, 4) concentration, 5) intracellular storage; and in many cases 6) discharge into the outside environment.

The basic mechanisms underlying these steps are remarkably similar for organisms as diverse as bacteria, plants and animals. The relationships and interdependence of these various steps are becoming increasingly evident. The conceptual framework of these processes is now well established, but the specific details remain to be elucidated.

The pancreatic exocrine cell has been widely used as a model system to study the processes of protein biosynthesis and secretion (10,11). These cells synthesize and discharge several functionally important proteins and hormones (e.g. insulin). Based on available data, a protein to be secreted, for example, insulin, is first synthesized as a precursor (preproinsulin) on polysomes bound to rough endoplasmic reticulum (11). This precursor is segregated in the ER cisternae after the co-translational cleavage of the leader sequence. The proinsulin is then transported to the Golgi apparatus where it is packaged into zymogen granules. Post-translational cleavage of proinsulin occurs at this site to yield mature insulin. The mature insulin granules are finally secreted into the circulation (11). The model of insulin biosynthesis and secretion has been observed for numerous other cellular and viral secretory proteins (10,11,12). This mechanism is also closely imitated in the biosynthesis of integral and peripheral membrane proteins (12). In general, polypeptides which are destined for secretion into the environment or for deposition into intracellular organelles are thought to be synthesized and packaged along remarkably similar pathways and mechanisms.

Since the late 1950's, morphological and biochemical studies have strongly suggested that exportable proteins are synthesized on polysomes attached to the RER, while free polysomes in the cytosol are responsible for the synthesis of non-exportable proteins. The synthesis of secretory proteins is preceded by the proliferation of the ER membranes (13). The molecular basis for this discrimination was initially explained by Blobel and Dobberstein (14) who proposed the signal hypothesis. They postulated that a specific interaction of the NH_2-terminal portion of the growing polypeptide chain with the RER membrane results both in the association of polysomes with the RER and in the creation of a channel through which the growing polypeptide enters the RER cisternae (Figure 1). The signal hypothesis initially covered the secretory proteins, however, it is now known that this model may pertain to several other classes of proteins including polypeptides destined for transport to inner compartments of other cellular organelles, e.g., lysosomes, Golgi complex, peroxisomes, chloroplasts and mitochondria (12), and those which form the class of integral proteins tightly bound to membranes (12). Nevertheless, there are a few well- documented exceptions (12) where free polysomes appear to be responsible for the synthesis of secretory proteins and conversely, membrane-bound polysomes synthesize cytosolic proteins (e.g., tyrosine

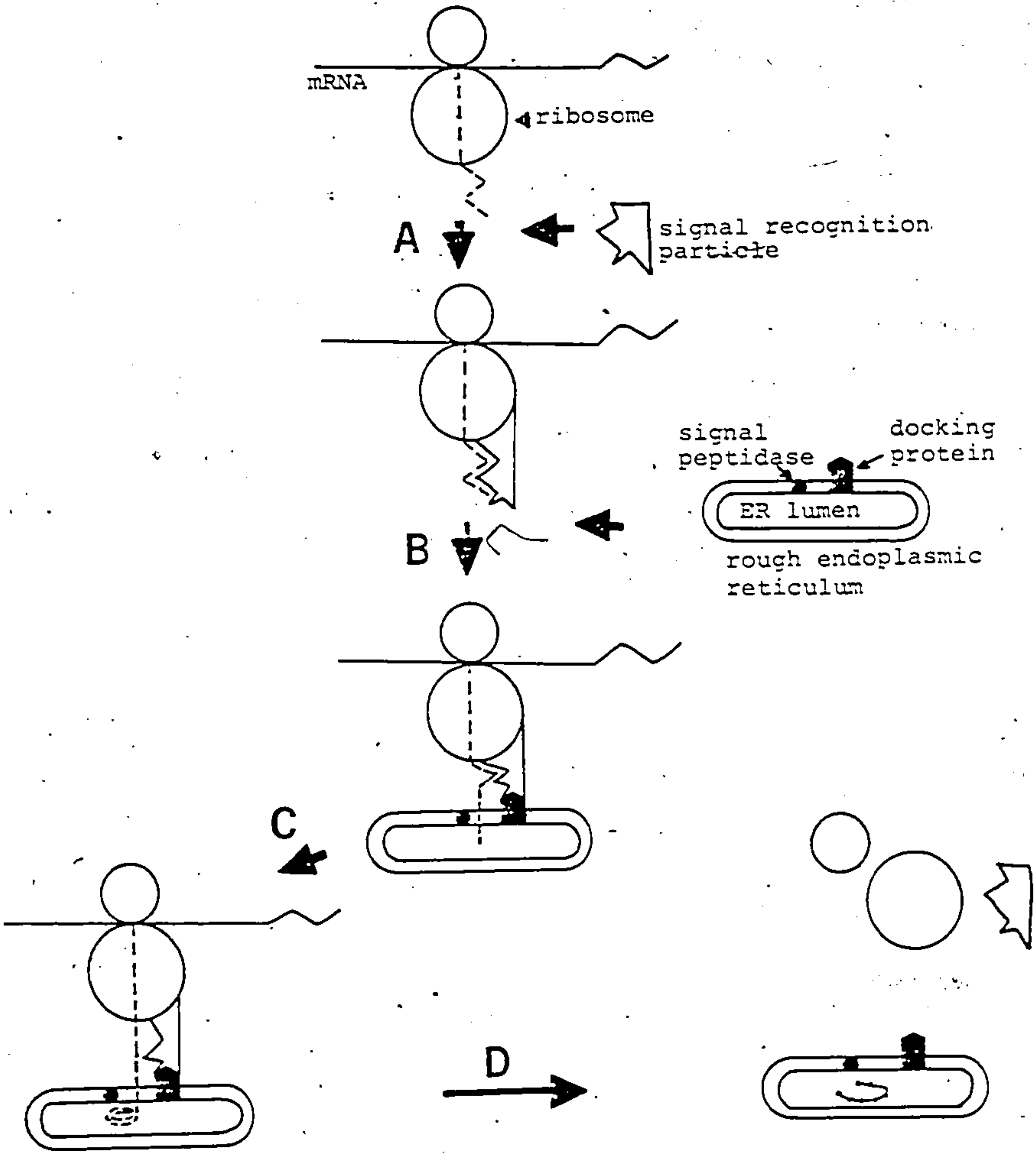


Fig. 1. Schematic details of the signal hypothesis.

(A) The specific binding of SRP to the signal sequence. This binding inhibits further translation.

(B) The inhibited ribosomal complex binds through the SRP to a membrane receptor, or docking protein. This binding allows translation to resume.

(C) The signal peptidase cleaves the signal sequence.

(D) After complete peptide transfer, the SRP and ribosomal subunits dissociate. The protein has been translocated into the lumen of the endoplasmic reticulum.

Adapted from Blobel and coworkers (17,18,19).

aminotransferase and serine dehydratase respectively). In some other cases, the controversy about the site of synthesis may be due to inadequate techniques presently available for the fractionation of membrane-bound and free polysomes.

The mechanisms involved in selecting specific mRNAs for translation by membrane-bound ribosomes have been studied extensively (12,15). Three alternative mechanisms have been described (12) suggesting how polyribosomes may interact with the ER membrane: 1) interactions between sites in the membrane and 60S ribosomal subunits, 2) interactions between nascent polypeptides and membrane receptors, and 3) binding of a region at the 3' end of the mRNA to a specific membrane site. The function of the N-terminal end of the nascent chain has been described in a well-known hypothesis called the signal hypothesis.

Signal Hypothesis

In accordance with the signal hypothesis, the primary translation products of most mRNAs coding for secretory proteins are usually larger than the final product secreted *in vivo* due to an extra N-terminal amino acid sequence. This signal peptide has been sequenced from several secretory protein precursors (16) and shown to have a high hydrophobic amino acid content. Recent studies by Blobel and coworkers (17,18,19) have resulted in a more detailed explanation of the mechanisms underlying the signal hypothesis.

Translation of the mRNA for a secretory protein begins on free ribosomes in the cytosol. Amino terminal amino acids are polymerized until the signal sequence has emerged from the large subunit of the ribosome. This is believed to occur after 60-70 amino acids have been polymerized (20). Around 40 of these amino acids, although polymerized, would reside in the large subunit of the ribosome and only 20-30 would be extruded out of the ribosomes (21).

The signal sequence then binds specifically to a signal recognizing particle (SRP) (19). The SRP is present as a soluble hexamer in the cytoplasm, but may also be bound to the large subunit of the ribosome (19). The binding of the SRP to the signal sequence inhibits the chain elongation process preventing further translation of the protein. This mechanism ensures that translation of secretory proteins is not completed in the cytoplasm, preventing their release into the cytoplasm. Translation can only resume when the inhibited complex binds to the appropriate receptor on the RER (20,21). This latter step provides the membrane specificity, ensuring that the proteins are translocated across the correct membrane.

With continued translation, the peptide is translocated across the membrane. The actual mechanism for this translocation event is not understood due to the lack of any experimental evidence. Blobel (14) suggested that the binding of the ribosome to the RER could cause a pore to appear, allowing the forming amino acid chain to pass through the membrane, however, in the absence of any evidence this remains speculative.

In order for the forming polypeptide chain to be vectorially discharged across the membrane, a driving force must be present. Several mechanisms for this process have been envisaged, including: (A) the energy of chain elongation pushes the peptide through the membrane (22), (B) the folding peptide on the luminal side of the RER membrane pulls the chain through (23), or (C) a specialized transport mechanism exists to transport the protein across the membrane (23,24).

Mechanism A would require that the binding of the ribosome to the membrane is fairly strong. The growing polypeptide chain can thus be pushed through the membrane without the ribosome being forced off the membrane. Situa-Mangano and Lane (25) attempted to demonstrate experimentally that mechanism A was not involved in the translocation of the secretory proteins. They found that puromycin, a translation terminator, did not interfere with the translocation of the incomplete secretory peptides. They concluded that once translation was terminated, the elongation force was removed. Since translocation of the peptides did occur, then the translocation process was not due to a chain elongation force, but to some other force. This conclusion is apparent in vivo when chain termination occurs naturally at the completion of the protein, and yet complete translocation still occurs. This experiment did not rule out the chain elongation force as a possible mechanism, but simply showed that it is not the only mechanism involved. Perhaps a better model for this translocation force, would be the combination of the elongation force and the force due to polypeptide folding on the luminal side of the RER membrane (mechanism B). In this model, the initial force would consist solely of the elongation force, but the final force required to complete the translocation would be entirely the force conveyed by the folding polypeptide chain (a pulling force).

The involvement of this latter source of energy was proposed by Smith et al. (23) as an alternative to the elongation force. This hypothesis was based on their finding that in E. coli, the bulk of ribosomes could be released from the membrane by treatment with puromycin. These results indicated that nascent peptide was the sole attachment of ribosomes to the membrane in bacteria and therefore the chain elongation energy on the ribosome could not be involved in forcing the growing chain through the membrane. However, they conceded that this might be the case only for prokaryotes (23). Finally, mechanism C envisages that certain protein translocators exist which could effect the unidirectional translocation of proteins across cellular membranes (24).

Another important feature of the signal hypothesis is the co-translational cleavage of the signal sequence (14) by a signal peptidase. The cleavage is thought to occur after the signal sequence has emerged from the luminal side of the RER membrane as the result of the action of a membrane-associated protease (26). Evidence for a signal peptidase gene on a bacterial chromosome has now been reported (27). Perhaps further research will uncover the existence of the genes coding for these important transport-related enzymes in higher organisms as well.

Segregation

The newly synthesized secretory proteins are segregated in the cisternal space of the RER after the vectorial transport of the nascent polypeptide from the large ribosomal subunit. Segregation appears to be an irreversible step; the nascent polypeptide is extruded in the cisternal space and once inside, can no longer get out (10). It is believed that upon such an entrapment, the polypeptides are converted into globular proteins too large (20 A diameter) to permeate the pores of the ER membrane (10 A diameter) (10). The modification of the protein's tertiary structure may include disulfide formation, hydroxylation of lysine and proline residues, glycosylation of polypeptide chains, and partial proteolytic cleavage (28), all of which can modify the secretory proteins so that they are unable to penetrate the ER membrane (10,29). The enzymes responsible for these reactions have been found to be associated with the ER vesicles (12,30). For example, glycotransferases were reported to be associated with the ER in rat liver (30).

3. BIOSYNTHESIS OF SEED RESERVE PROTEINS

All plant cells are secretory cells, since they synthesize and discharge the proteins of their cell wall (10). However, a variety of plant cells produce highly specialized secretory and reserve proteins. Much attention has been focused on the developing storage tissues of legume and cereal seeds which synthesize and store considerable amounts of proteins during embryogenesis (31,32). This has been largely due to the fundamental role of cereals and legumes as important sources of human dietary protein (2). The plant seeds are also suitable eukaryotic model systems for studying the regulation of seed protein gene expression (33,34). Electron microscopic investigations as well as in vitro synthesis and in vivo labelling studies have been employed to examine the depository processes in legume seeds (7,9,32,35,36,37,38). The results have revealed considerable homology with the processes involved in protein secretion in mammalian systems; although many specific details still remain to be explained.

Plant seed reserve proteins exhibit no enzymatic activity and have thus been classified on the basis of their physical properties into four solubility classes (Globulins, salt-soluble; albumins, water-soluble; glutelins, acid or base soluble; prolamins, alcohol-soluble) (39). The major storage protein fractions are globulins in legume seeds and prolamins in most cereals.

The biosynthesis of several well-studied reserve proteins is now discussed in some detail for the two major classes of legume globulins and cereal prolamins.

A. SYNTHESIS OF LEGUME RESERVE GLOBULINS

Among legumes, the reserve globulins of Pisum sativum (pea) and Glycine max (soybean) have been studied extensively.

Legume globulins are classified into two fractions, legumin-like and vicilin-like proteins, based on their solubility at various salt concentrations (40). To date, no evidence is available for the presence of alcohol-soluble prolamins in legumes.

Class a. LEGUMIN-LIKE PROTEINS: Pea legumin and soybean glycinin are well-studied examples of legumin-like proteins.

PEA LEGUMIN is a 12S oligomer consisting of two groups of acidic and basic subunits. The α and β subunits are linked together through disulfide bonds forming 58-60 kDa dimers (40). The in vitro and in vivo synthesis of pea legumin has been studied extensively (35,40,41,42,43). Legumin mRNA is translated in vitro into a 60 kDa precursor comprising of the α and β subunits in tandem (35). In vivo labelling experiments have demonstrated a precursor-product relationship (41). Legumin precursors are primarily synthesized on membrane-bound polysomes and segregated in the RER cisternae (35). Recent investigations by Chrispeels and coworkers have indicated that the newly-synthesized legumin polypeptides are transported into protein bodies after their synthesis on the RER (41). In protein bodies, the legumin precursors are cleaved into the α and β subunits (still disulfide-linked) which are then assembled into a 12S oligomer (41).

SOYBEAN GLYCININ: This globulin fraction is homologous in several physicochemical properties to pea legumin (42,44). It is found in soybean protein bodies as a 12S oligomer consisting of 40 kDa (acidic) and 20 kDa (basic) subunits (45). Interestingly, the mode of biosynthesis of soybean glycinin also resembles that discussed for pea legumin. Glycinin polypeptides are made primarily on membrane-bound polysomes as larger precursors (44). It has been shown that glycinin precursors carry a N-terminal leader sequence which is removed co-translationally (46). The 60 kDa glycinin precursor is also post-translationally cleaved into the mature acidic and basic subunits (44). However, a detailed in vivo labelling study has not been performed on soybean glycinin to clarify the mechanisms of its transport from the RER to protein bodies.

Class b. VICILIN-LIKE PROTEINS: Another group of globulins found in legumes have a smaller holoprotein size of 7S. These are referred to as vicilins.

PEA VICILIN: It is a 7S globulin complex found in mature pea seeds consisting of a heterogeneous group of polypeptides. The major vicilin species is a 50 kDa polypeptide (47) which is synthesized on the RER as a larger precursor (48). Recent cDNA sequencing studies by Lycett et al. (34) indicated its synthesis as a preprovicilin. The comparison of the vicilin cDNA sequence with its amino acid sequence data, suggested the removal of a N-terminal signal peptide and a C-terminal sequence (32). Partial post-translational proteolytic cleavage also occurs, yielding three α, β, and γ subunits (48). In vivo pulse-chase labelling studies have shown that pea vicilin is transported from the RER to protein bodies in a similar fashion as is legumin (41). The vicilin polypeptides are assembled in protein bodies into a 7S oligomer. Certain newly-synthesized vicilin polypeptides undergo glycosylation in the RER and possibly in the Golgi apparatus (49).

SOYBEAN CONGLYCININ: This fraction is found as a 7S oligomer consisting of a major 50 kDa polypeptide homologous to pea vicilin (44,47). The nucleotide sequences of the conglycinin and pea vicilin genes are homologous and only differ at the regions of internal proteolytic cleavage sites (34). Conglycinin is made on the RER as a larger precursor with a N-terminal signal peptide (44). Co-translational removal of the signal peptide and glycosylation of the conglycinin precursors have been demonstrated (44). The newly-synthesized conglycinin polypeptides are transported to protein bodies by yet unknown processes. Sengupta et al. (44) have outlined a detailed sequence of events that appear to be involved in the biosynthesis of the vicilin α subunit (Figure 2).

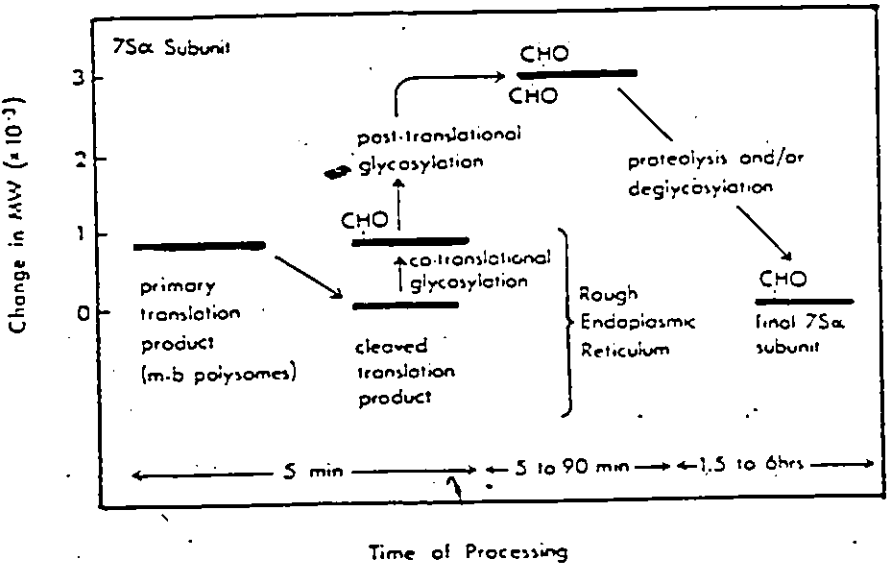


Fig. 2. Biosynthetic pathway of soybean 7S α subunit, a vicilin-like reserve protein. This process involves five consecutive steps.

1. Translation of 7S α subunit mRNA on the RER.
2. Co-translational cleavage of a signal sequence.
3. Core-glycosylation of the newly-synthesized polypeptide.
4. Further post-translational glycosylation.
5. Post-translational cleavage of the polypeptide in protein bodies.

Reproduced with permission from Sengupta et al. (44).

OTHER GLOBULINS Another vicilin-like protein, french bean phaseolin has also been the focus of extensive investigations (33,36,40,50). The biosynthetic and depository pathway of this reserve protein is similar to those described above for pea vicilin and soybean conglycinin and therefore is not discussed here.

The above discussion of legume reserve globulins indicates that these proteins share a common pathway for deposition in protein bodies. Globulins are probably synthesized on the RER with the aid of a N-terminal signal peptide, transported via secretory vesicles to protein bodies with the possible involvement of the Golgi apparatus. The role of the C-terminal extension found in some legume globulin precursors (pea vicilin) in their correct intracellular transport is not known.

In contrast, cereal reserve prolamins do not appear to be synthesized or deposited in the same way. This is illustrated by maize zein, barley hordeins, and wheat gliadin (51,52,53,54,55).

B. SYNTHESIS OF CEREAL RESERVE PROLAMINS

MAIZE PROLAMINS: Zeins are soluble in alcoholic solutions (e.g. 70% ethanol) and are characterized by a high content of glutamine, proline, leucine and alanine (these four residues account for about 70% of the total residues) and a low content of lysine and tryptophan (56). Zein polypeptides have been grouped into four classes of 22, 20, 14, 10 kDa. Approximately 100 copies of zein genes encoding zein polypeptides may exist per haploid genome (57), although it is believed that some of these genes are silent sequences which may be pseudogenes that are not expressed (56). Zein polypeptides are made primarily on membrane-bound polysomes and are processed by the removal of a signal peptide 10-20 amino acids long (58). The mature zeins are segregated within the lumen of the RER where they fuse together, forming granules (55,56,59). The signal peptidase activity has been detected in the RER membranes (59). The RER membranes completely surround the zein protein bodies (55). Zein polypeptides also undergo co-translational glycosylation by addition of a glucose unit to each nascent chain by a membrane-bound glucosyltransferase. Burr and Burr (59) have suggested that this glycosylation process may be essential for the correct compartmentalization of zein polypeptides. Dictyosomes do not seem to play any significant role in zein deposition (59).

BARLEY HORDEINS: Barley endosperm contains 11.5-14% protein consisting mainly of an alcohol-soluble hordein. The hordein fraction is comprised of two major groups of polypeptides called B and C hordeins (60,61). B hordeins have a mol wt range of 35-46 kDa whereas C hordeins are larger with a mol wt range of 55-75 kDa. Both B and C hordeins contain significant amounts of glutamate-glutamine and proline residues (55-70%). The particular pattern of B and C hordein polypeptides are under the control of two complex loci, Hor-1 and Hor-2, which respectively specify the C and B patterns (61). The pathway of hordein deposition closely resembles the process described for zein deposition with respect to its synthesis on the RER. Newly-synthesized hordein polypeptides also aggregate in the RER lumen into clumps (51). In contrast to zein, hordein granules, however, seem to break away from the RER and form irregular protein deposits with some ER membrane still attached (51).

4. GLOBULIN-PROLAMIN COMPARTMENTALIZATION

On the basis of the biochemical and electron microscopic evidence which was discussed above, two distinct systems for the synthesis and deposition of plant seed reserve proteins could be envisaged: a globulin-type system operating mostly in legumes and a prolamin-type system occuring in most cereals.

GLOBULIN SYSTEM - Several hypotheses have been proposed to explain the mechanisms involved in the synthesis and storage of reserve globulins in legume seeds. Recent studies suggest that reserve globulins follow a similar pathway for their synthesis and storage similar to that involved in the secretion of animal proteins. Boulter (62) has proposed a hypothesis which is now widely accepted. He postulated that reserve globulins are synthesized on the rough endoplasmic reticulum (RER), passed into the lumen where they are segregated and transported to the dictyosomes. The globulins are then carried into vacuoles in special vesicles which are cut off from the dictyosomes. This theory has also been adopted to explain the formation of protein bodies in oilseeds (63). The role of dictyosomes in this process is not supported by conclusive evidence. Some investigators have suggested that vesicles bud off directly from the RER and transport the proteins to the vacuoles (64). However, in a recent report, Chrispeels (36) presented evidence for the involvement of the Golgi apparatus in the transport and deposition of french bean globulins. Fig. 3 summarizes the hypothetical pathway of globulin synthesis and storage.

PROLAMIN SYSTEM - Reserve proteins in most cereals, e.g. wheat,

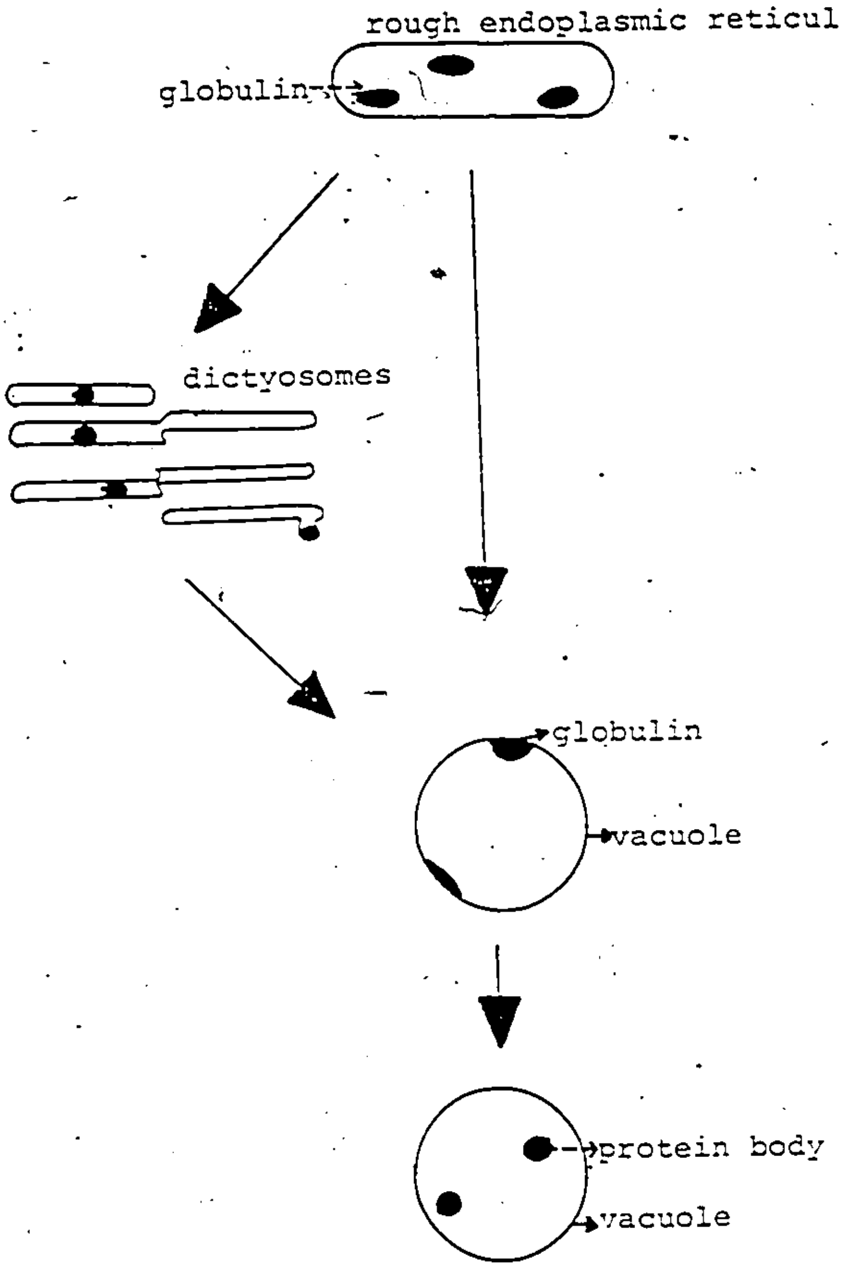


Fig. 3. Compartmentalization of reserve globulins.

Globulins are synthesized on the RER, transported either directly (a) or via dictyosomes (b) to intracellular vacuoles where they from protein bodies.

barley, maize, rye, and sorghum, consist mainly of alcohol soluble prolamins such as wheat gliadins, barley hordeins, and maize zein. Miflin et al. (51) have recently discussed the deposition of prolamins and the development of protein bodies in cereal seeds and proposed a common hypothesis. This hypothesis speculates that cereal reserve prolamins are synthesized on rough endoplasmic reticulum and segregated in the ER cisternae. The newly-synthesized reserve proteins aggregate into clumps within the ER cisternae. In barley and wheat, these aggregates form irregular deposits (protein bodies) after breakage from the RER. In maize, however, the reserve prolamins form spherical bodies which accumulate within the RER which completely surrounds them. Fig. 4 illustrates the development of protein bodies in the RER cisternae in corn endosperm cells (51,66).

AVENA GLOBULIN SYSTEM

Among cereals, oat (Avena sativa L.) seeds offer a unique cereal system due to the predominance of the salt-soluble globulins. This may indicate that oat seeds possess a different depository pathway from the prolamin system found in other cereals. Salt-soluble proteins are the major storage protein fraction in oat seeds accounting for up to 75% of total seed protein (5). This unique feature of oat seeds is responsible for its higher nutritional quality (salt-soluble globulins are richer in essential amino acids than the alcohol-soluble prolamins). Interestingly, oat globulin has been shown to be homologous to some legume globulin fractions such as pea legumin and soybean glycinin in several respects (67,68,69,70).

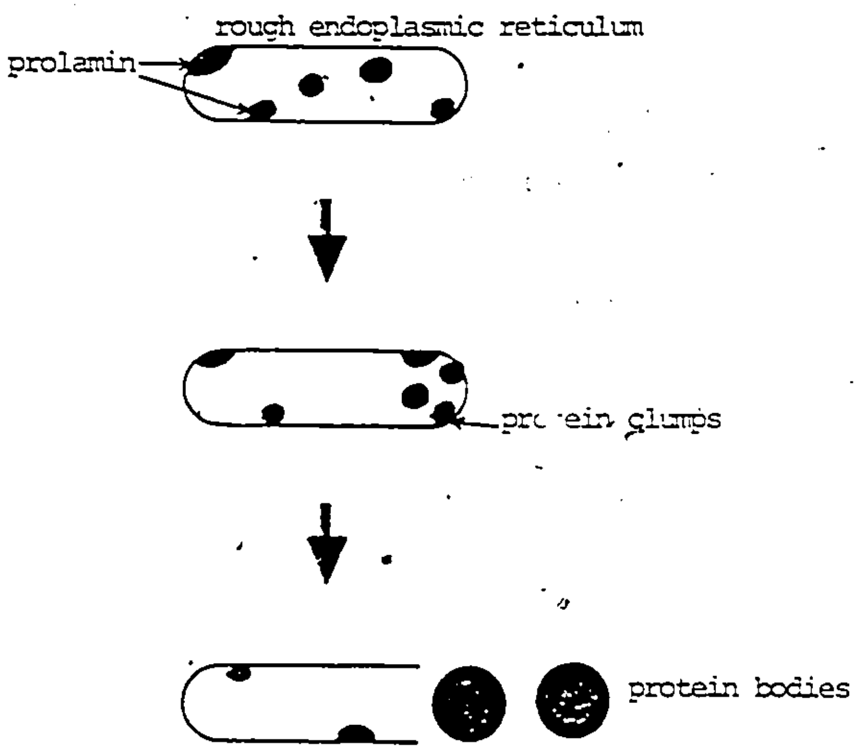


Fig. 4. Compartmentalization of reserve prolamins.

Prolamins are synthesized on the RER, aggregate into clumps, and are released as protein bodies. In some cases, these protein bodies would still be surrounded by ER membranes (maize protein bodies, ref. 65).

This suggests that the pathway of oat globulin deposition might be similar to the globulin system in legume seeds, rather than to the prolamin system predominantly in the other cereals.

DISCRIMINATORY FACTORS IN PROTEIN DEPOSITION

The regulatory factors which determine whether a certain gene product should remain in the RER or be transported into protein bodies are unknown. The differences observed between the two above depository systems may be due to several phenomena. One apparent factor may be the *in vivo* solubility characteristics of the particular storage protein sequestered in the RER. There is an obvious difference between the *in vitro* solubility of globulins (salt-soluble) and prolamins (alcohol-soluble) extracted from legume and cereal seeds respectively. There is no direct evidence, however, to support this theory. The observation that some cereal seed proteins such as thionins which are salt-soluble in nature remain in the RER cisternae after their synthesis on membrane-bound polysomes argues against the above assumption (71). Thionins, however, are minor constituents and are not considered as reserve proteins (not found in protein bodies) (71). Therefore, there is some indirect evidence for the role of protein solubility in deposition of reserve proteins. Further investigations are needed before assuming a direct role for solubility.

The nature of the signal peptides may also be an important factor. Differences in the N-terminal sequences of the nascent chains may contribute to the differential compartmentalization of prolamin and globulin polypeptides. It is generally believed that the mRNA molecule contains the entire information for the final destination of a protein which may be coded by signal sequences or other topogenic amino acid sequences (72,73,74). Blobel (73) has proposed a hypothesis which predicts that certain discrete segments of a polypeptide chain may determine its intracellular fate or topogenesis. These topogenic sequenc~~es would either~~ be permanent or transient (post-translationally removed) features of the protein. This phenomenon may be recognized by the presence of such sequences in many structurally different proteins which share a common translocation pathway (73).

The signal peptide is one type of topogenic sequence which has been observed in many secretory proteins (26,75). In most cases this signal peptide is present in the N-terminal end, but internal signal sequences have also been reported (76). The erythrocyte anion transport protein, Band III, was recently shown to contain a sequence near the middle of the protein that facilitates its segregation into the ER membrane (76). In some instances, the carboxyl end of the protein has been reported to play a role in the proper transport of the newly-synthesized protein across membranes. Koshland and Botstein (77) showed that the C-terminal amino acid sequence of β-lactamase from *Salmonella typhimurium* infected with P22 phage, is essential for correct transport across the cytoplasmic membrane. These investigators suggested that the N-terminal signal sequence of a protein does not suffice to cause its secretion and other sequences may be involved. The post-translational cleavage of cryptic C-terminal sequences has also been reported for the processing of procollagen and calcitonin (78,79).

In plant systems, Lycett *et al.* (34) recently showed by cDNA sequencing techniques that pea vicilin is synthesized as a preprovicilin with subsequent removal of a N-terminal signal peptide and a C-terminal peptide. The significance of this C-terminal sequence was not known. Another plant protein, thaumatin (sweet-tasting protein isolated from fruits of *Thaumatococcus daniellii*) has also been found to contain a C-terminal sequence which is removed post-translationally after the co-translational cleavage of a N-terminal signal peptide (80). This acidic C-terminal sequence (six amino acids long) was suggested to play a role as a topogenic signal for compartmentalization of thaumatin (80).

Recently, Crouch et al. (81) used cDNA sequencing techniques to demonstrate extensive post-translational cleavage of reserve napin precursors from rapeseed (Figure 5): in addition to the removal of a N-terminal leader peptide, two internal sequences which precede the two napin subunits are also post-translationally processed and deleted (81). The significance of these internal sequences may be in effecting charge neutrality for the napin precursors (each internal sequence possesses an opposite charge to the napin subunit immediately following it). A short peptide (three residues) was also detected at the C-terminal end of the precursors (81). The significance of this sequence was unknown.

Based on our current knowledge of the topogenic sequences, we may assume that the discrimination observed between the globulin and prolamin systems could reside in their corresponding signal and sorting sequences. This theory is supported by recent investigations on the secretion of plant storage proteins by Xenopus oocytes (52,72,53). As mentioned above, the oocytes apparently retained the prolamins and secreted the globulins (52,72,53). These studies revealed that the globulin mRNAs contain certain signals similar to those of the animal secretory proteins which can be decoded by the Xenopus oocyte secretory system; the prolamin mRNAs apparently lack such signals.

Discriminating Sequences in Globulin and Prolamin Topogenesis

According to the available data, many legume globulins and cereal

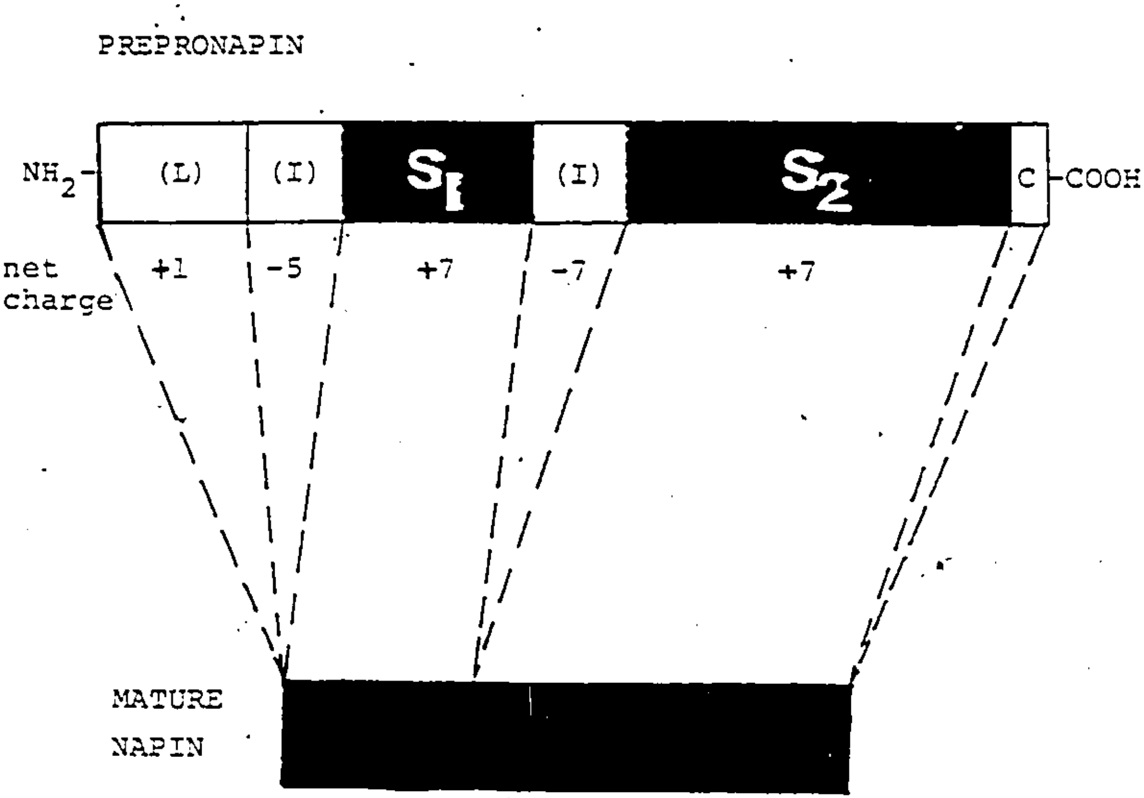


Fig. 5. Co-translational and post-translational processing of rapeseed napin precursor.

Comparison of the nucleotide sequence and the amino acid sequence of a N-terminal signal peptide, a C-terminal extension, and two internal sequences on the napin precursor. Each internal sequence has a net charge opposite to the subunit immediately following it.

Abbreviations: L, leader sequence
I, internal sequence
S_1, small subunit
S_2, large subunit
C, C-terminal sequence

Adapted from ref. 81.

prolamins possess N-terminal signal peptides (32,44,54,82). This is to be expected since both globulins and prolamins are synthesized on membrane-bound polysomes, (35,44,55). Weber et al. (83) reported the reconstitution of functional hybrid microsomes from *Vicia faba* stripped microsomes (smooth ER) and barley endosperm polysomes. They also demonstrated the transfer of *in vitro* synthesized barley hordeins across the *Vicia faba* microsomal membranes (83). Based on these results, they suggested that an evolutionarily conserved apparatus for the initial steps in the transport mechanisms may exist for legume globulins and cereal prolamins (83). Therefore, the difference in the intracellular compartmentalization of these two types of reserve proteins may reside in their hypothetical sorting sequences. The C-terminal peptides found in pea vicilin (32) and rapeseed napin (81) as well as the internal sequences reported for napin precursors, may represent such sorting sequences. However, no data are available to support this assumption. It may be worth noting that so far among seed reserve proteins, C-terminal sequences have only been reported for globulins, vicilin and napin. The cDNA sequence analysis of a typical prolamin (maize zein) failed to demonstrate the presence of similar extra C-terminal peptides (84). Further search in other globulins and prolamins is essential before perceiving a role for these C-terminal sequences in differentiating between globulin and prolamin compartmentalization. A list of some putative topogenic sequences in plant proteins and in an animal protein is presented in Table I.

Finally the differential glycosylation of the newly-synthesized polypeptides may also play a role in compartmentalization of seed reserve

Table I

Some Putative Topogenic Sequences in Plant and Animal Proteins

N-Terminal	Internal	C-Terminal
Napin (<u>Brassica</u> <u>napus</u>) (81)		
^{1}MANKLFVSATLAFFFLLTNA-^{21}	^{74}-SIYRTVVEFDEDDATNS-^{93}	^{176}-PSY-^{198}
Vicilin (<u>Pisum</u> <u>sativum</u>) (34)		
^{1}MLLAIAFLASVSS-^{13}		^{439}-IKEHLYSILGAF^{450}
Thaumatin (<u>Thaumatococcus</u> <u>danielli</u>) (80)		
^{1}MAATTCFFFLFPFLLLLTLSRA-^{22}		^{208}-LELEDE^{213}
Zein (<u>zea</u> <u>mays</u>) (84)		
^{1}MLLGLSASAATA-^{12}		
Calcitonin (79)		
^{1}VLLAALVQDVVQMKASEQEREGSSLDSPRSKR-^{36}		^{69}-GKKRDMSSDLERDHRPHVSMPQNAN^{93}

Amino acid symbols:
A - Alanine
R - Arginine
N - Asparagine
D - Aspartic acid
C - Cysteine
Q - Glutamine
E - Glutamic acid
G - Glycine
H - Histidine
I - Isoleucine
L - Leucine
K - Lysine
M - Methionine
F - Phenylalanine
P - Proline
S - Serine
T - Threonine
W - Tryptophan
Y - Tyrosine
V - Valine

proteins. In animal cells, the glycosylation of lysosomal proteins can result in their secretion, instead of their segregation into the intracellular lysosomes (85). Several subunits of the legume 7S holoproteins (40) as well as maize zein (86) are known to be glycosylated. Reserve protein glycosylation could be a co-translational (86) or a post-translational event (34). The evidence, however, provided by Badenoch-Jones et al. (49) argues against the role of glycosylation in reserve protein deposition. The inhibition of in vivo protein glycosylation by tunicamycin did not alter the correct synthesis, cleavage and assembly of pea vicilin polypeptides (49).

The reserve protein which is the subject of this thesis (oat globulin), is known to be homologous to the legume reserve globulins in many physical and chemical properties as described above. This homology may also extend to include the depository pathways of the oat and legume reserve globulins. If so, the sorting sequences on the oat globulin polypeptides could be similar or identical to the legume globulin sorting peptides. These assumptions could be tested by performing several experiments designed to elucidate the pathway of oat globulin deposition and examine the presumed signal and sorting sequences present on the oat globulin polypeptides. The comparison between the topogenic sequences of the oat globulin and legume globulins might provide further information regarding the elements involved in the correct compartmentalization of reserve proteins in cereal and legume seeds. But before such comparison could be done, a detailed study of the mechanisms of oat globulin deposition is essential.

5. DEVELOPMENT OF AVENA STORAGE TISSUE AND CHEMISTRY OF ITS RESERVE PROTEINS

A) DEVELOPMENT

Aveneae is a tribe in the Graminae (recently renamed Poaceae) family. It is subdivided into approximately four genera. Avena sativa L. (common oat) is the widely cultivated species.

Oat grain is the product of a developmental process. This process starts with a double fertilization of nuclei in the ovary of the flower. Approximately 6 weeks later, a large and dry seed with several protective layers is developed. In the double-fertilization event, the stigma (feathery receptor structure) receives a pollen grain on the upper part of the ovary. Two (male) copies of nuclei are released into the ovary through a pollen tube. An embryo is produced by the combination of a male nucleus with the female egg cell. Endosperm is developed by genetic combination of the other male nucleus and a double complement of genetic material (the two polar nuclei). Multiple division of these two new genetic combinations results in the development of distinct embryo and endosperm organs. The pericarp layers which surround endosperm and embryo originate initially from part of the maternal ovary. In summary, the mature oat groat (caryopsis) is comprised of three different organs which originate from three different genetic origins; the embryo, endosperm, and pericarp layers (87a). These are described below.

1. Aleurone Layer

The aleurone layer is an organized array of cells which surround

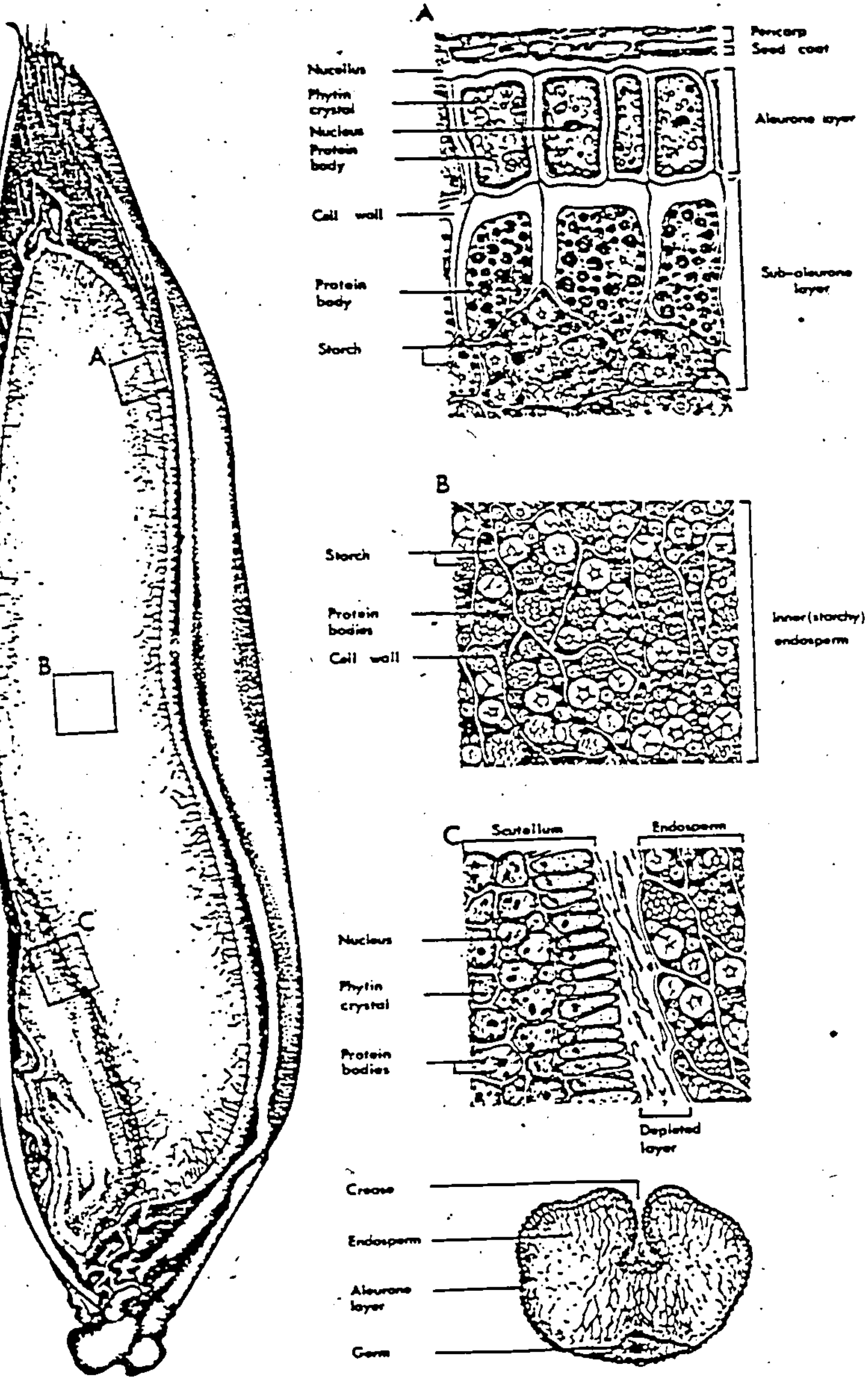


Fig. 6. Major structural features of the oat kernel.

A. Bran B. Starchy endosperm C. Embryo

Reproduced with permission from Fulcher and Wong (87b) the starchy endosperm (Fig. 6A). It contains large amounts of autofluorescent phenolic compounds (mainly ferulic acid). Inside each aleurone cell, several hundred protein bodies are packed. Two different types of structures have been found in protein bodies; globoids, spherical particles containing large amounts of a calcium-magnesium salt of myo-inositol phosphate (phytin), and so-called 'protein-carbohydrate bodies', also present in aleurone cells, which are apparently composed of periodate-sensitive carbohydrates. It appears that the chemical composition of the proteins present in the aleurone layer protein bodies is different from that of protein bodies in starchy endosperm (119). The level of lysine seems lower in the former (87a).

The aleurone layer has some enzymatic and/or hydrolytic functions necessary for germination. It contains many enzymes such as lipases, proteases, and β-glucanases (88).

2. Starchy Endosperm

The starchy endosperm is a metabolically inactive tissue which comprises up to 80% of the oat groat. Only one type of cell exists in which a huge amount of starch, protein, lipid, and gums are deposited. These cells are contained by particular cell walls which are rich in β-glucans (gums). Endosperm proteins contain relatively high amounts of lysine and other essential amino acids. Endosperm proteins are accumulated in the cells in spherical protein bodies (0.2 to 6 µm in diameter) (87a) (Fig. 6B).

In high protein cultivars, the sub-aleurone cells are particularly active in protein biosynthesis and store much more protein with very little starch. In low protein varieties, the starch-protein ratio is much higher. In general, the outer-endosperm cells contain more protein than the inner-cells. This gradation exists in all oat cultivars (87).

Proteins, lipids, and gums represent a total of 20 to 30% of the grain's dry weight. Starch is the largest component (43-60%). It exists as starch granules in endosperm cells. It is somewhat similar to wheat and barley starch (89).

3. Embryo

This germ tissue is metabolically and enzymatically active. A major part of the germ is called 'scutellum' which consists of two distinct tissues, the parenchyma and the epithelium. The parenchymal tissue is made of roughly spherical cells which are used for nutrient storage. It constitutes the major part of the scutellum (80%). The epithelial tissue is constructed of a row of elongated cells which are located on the surface of the parenchymal tissue. It functions during germination by absorbing nutrients derived from endosperm cells adjacent to scutellum which contain a large number of protein bodies (87).

4. Protein Bodies

In all plants, protein bodies are cellular organelles containing storage proteins. In monocots (e.g. cereal grains), protein bodies are mainly found in a triploid tissue, endosperm (starchy endosperm and aleurone layers) as well as in scutellum. In dicots (e.g. legumes) protein bodies are found within cotyledons (part of the embryo). In some seeds, two internal inclusions, the globoid and the crystaloid, are located within protein bodies. A crystaloid is a storage protein deposit but a globoid is not a proteinaceous inclusion (90,91).

Protein bodies can be isolated from cereals and legumes by aqueous solvents. Density gradient centrifugation is also used successfully for their separation. Average size of protein bodies ranges from 0.1 to 25 µm. In most cases, they are surrounded by a single membrane.

B) Storage Protein Classification

Seed proteins were classified in 1895 by Osborne on the basis of their solubility in water and various aqueous solutions. Four classes were defined:

Albumins	- Proteins soluble in water
Globulins	- Proteins insoluble in water but soluble in dilute salt
Glutelins	- Proteins insoluble in water and dilute salt but soluble in weak acid and base
Prolamins	- Proteins insoluble in the above solutions but soluble in 70-80% ethanol.

Although a number of modifications have since been introduced, this classification is still valid and widely used.

The proportions of these protein fractions vary considerably among legume and cereal seeds. In legume seeds, the major reserve proteins are the salt-soluble globulins.

In most cereals (maize, wheat, barley, rye) the alcohol-soluble prolamins comprise up to 60% of total seed proteins. The predominance of this fraction contributes to the low nutritional quality of the proteins of these cereal grains. Prolamins are very low in essential amino acids such as lysine, methionine, and threonine. Among cereal grains, Avena sativa L. (oat) and Oryza sativa L. (rice) are exceptions. Prolamins are only a minor fraction of the total seed proteins in these two grains. Oryza sativa L. contains glutelins as the major reserve protein (about 80%) (92). The structure and physicochemical properties of oat globulins are described below in more detail.

C) Avena Globulins

The chemistry and biosynthesis of oat globulin have been studied by several research groups (5,67,68,69,70,93). Recent investigations by Burgess et al. (94) and the author (95) have revealed the presence of three distinct protein oligomers in the salt-soluble protein fraction of oat endosperm. These have sedimentation coefficients of 12S, 7S, and 3S. The 12S holoprotein is the major component (93%) and has been characterized extensively (5,67,69,70). This fraction is soluble at an optimum NaCl concentration of 0.8 - 1.0 molar at pH 8.5 (93). The 12S holoprotein is a 332 kDa oligomer consisting of two groups of subunits, α(36-42 kDa) and β(18-22 kDa). It is assumed that the 12S complex contains 12 such components ($6\alpha + 6\beta$) (93). The α and β subunits are held together through disulfide linkages forming 58-60 kDa dimer molecules.

Isoelectric focusing and 2-dimensional electrophoretic analyses have indicated a high degree of heterogeneity in the subunits of the 12S globulin. The amino acid composition of this fraction shows a high proportion of aspartate/asparagine and glutamate/glutamine residues which is typical of seed reserve proteins (Table II).

Several lines of evidence (67,68,69,70) have suggested a close homology between the oat 12S globulin and the 11-12S globulins from various legumes (pea and soybean). Pea 12S legumin and oat 12S globulin are immunologically related (68), have homology at the DNA level (68),

TABLE II

The Amino Acid Composition[a] of Oat Globulin Fraction and its Major Components

Amino Acid	Globulin	β-Subunit	α-Subunit
Asp/asn	9.9	12.8	9.1
Thr	3.5	4.0	3.3
Ser	6.2	6.3	7.9
Glu/Gln	21.4	15.3	21.4
Pro	5.3	5.2	4.5
Gly	8.2	7.0	12.1
Ala	6.4	7.2	5.8
Val	5.8	6.2	5.6
Ile	4.4	5.3	3.9
Leu	7.7	7.9	7.7
Tyr	3.5	3.6	3.0
Phe	5.6	4.9	5.7
His	2.0	2.2	1.6
Lys	2.6	3.3	2.1
Arg	6.4	7.2	5.9

a. Half cystine and methionine were not reported because of the partial destruction by HCl hydrolysis, and tryptophan because of complete destruction.

(Reproduced with permission from Peterson D.M. (93))

and have similar N-terminal amino acid sequences (69), and similar physicochemical properties (68,69).

EXPERIMENTAL DESIGN AND RESEARCH OBJECTIVES

The discussion of the compartmentalization of seed reserve proteins earlier in this chapter revealed that oat 12S globulin might be deposited via pathways similar to those described for legume 11-12S reserve globulins. This assumption was based on some homology at the protein level although no concrete data were available on the actual depository mechanisms. The investigations of several laboratories (67,68,69,70) and a previous graduate student in our laboratory (Matlashewski G.J., Ph.D. Thesis, 1983, University of Ottawa) had suggested that there was immunological and physicochemical homology among oat globulins and the 11-12S globulins in legume seeds (legumins). These investigators also showed the *in vitro* synthesis of oat globulin as 60 kDa putative precursors (67,69,70), similar to the precursors of legumins (35).

Based on the existing data, a number of experiments were designed by this author in order to further investigate the mechanisms of oat globulin deposition and compare these with the pathway of legumin deposition.

These experiments were original, designed to answer several questions regarding the synthesis, transport, processing, and deposition of oat 12S globulin polypeptides. This clarification was needed before comparing the pathway of oat 12S globulin deposition with that already described for 11-12S legumins. The mechanisms of legumin deposition were previously discussed in detail and could be summarized into the following steps: 1) Synthesis as a 60 kDa precursor on membrane- bound polysomes (RER); 2) Post-translational transport from the RER into protein bodies; 3) Cleavage of the 60 kDa precursor into α-polypeptides and β-polypeptides in protein bodies; 4) Assembly of the processed polypeptides into a 12S oligomer.

The available data at the time suggested homology between oat globulin and legumins in respect to the first step of deposition (synthesis as larger precursors). No evidence was however available regarding the other steps of oat 12S globulin deposition and therefore a thorough comparison could not be made between the depository pathways of oat 12S globulin and legumins. Several experiments were thus performed to answer the following questions regarding the mechanisms of oat 12S globulin deposition.

(1) Were the 12S globulin precursors synthesized on the RER or by free polysomes?

(2) Were these precursors segregated within the RER? If so, was this association permanent or transient?

(3) Were the newly-synthesized precursors transported to protein bodies or remained in the RER?

(4) Did this intracellular transport involve a direct channelling of protein or occur via organelles such as the Golgi apparatus?

(5) How and where did the cleavage of the precursors into smaller subunits occur?

(6) How and where did the globulin polypeptides assemble into the 12S oligomer (the authentic form of globulin)?

CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Plant Tissues

Oat cultivars (Avena sativa L.) were grown at the Ottawa Research Station, Agriculture Canada. Samples (seeds or panicles) were collected at various stages of development. The high protein cultivar, Hinoat, was chosen for the purpose of protein and RNA extractions. Groats were obtained in large quantities by mechanical dehulling of frozen developing spikelets. This procedure was developed in summer 1982 and was published in Analytical Biochemistry (96). To have a constant access to fresh developing oat endosperm throughout the winter, oat plants (Hinoat) were grown in a glasshouse under defined conditions (18 h. day, 24°C day, 16°C night) in the Department of Biology greenhouse, University of Ottawa.

2. Chemicals

Sigma Chemical Co.:	Lauryl sulfate SDS, Hepes buffer, Magnesium acetate, EDTA, EGTA, FITC-conjugated goat IgG,
Baker Analyzed Reagents:	ammonium persulfate, lithium chloride, phenol, formamide, formaldehyde, 2-mercaptoethanol, potassium acetate, Tris base, ethanol, potassium sulfate, mercuric oxide, sulfuric acid, sodium hydroxide, hydrochloric acid, trichloroacetic acid, 2-propanol, urea, chloroform, glycerol, ether.

Bio-Rad Laboratories: chemicals for SDS-polyacrylamide gel electrophoresis, acrylamide, TEMED, Coomassie Brilliant Blue.

Fisher Scientific Company: ammonium sulfate, potassium chloride, paraformaldehyde, lysine-HCl, sodium m-periodate.

Bethesda Research Labs: oligo-d(T) cellulose, RNase-free sucrose, rabbit reticulocyte lysate, wheat germ extract.

Boehringer Corporation Ltd.: RNaseA, Proteinase K.

BDH Lab. Reagent: sodium azide.

Difco Laboratories: sodium deoxycholate.

New England Nuclear: rabbit reticulocyte lysate translation kit, [3,4,5,-^{3}H(N)]leucine (140 Ci/mmol), [^{14}C(U)]leucine (300 mCi/mmol), (^{35}S)methionine (800 Ci/mmol), (^{35}S)sulfate (900 mCi/mmol)

B. METHODS

1. Protein Extractions:

a) Extraction of salt-soluble globulins

Developing or mature oat kernels (Hinoat) were dehulled (and frozen in case of developing seeds) and ground finely using a mortar and pestle. The flour (3 g) was mixed with 200 ml of I M NaCl, 0.05 M Tris (pH 8.5) for 3 h at room temperature. The homogenate was centrifuged for 20 min at 3,000 X g. The supernatant was removed (the pellet was kept for the prolamin extraction) and dialyzed against distilled water for 48 hours. After centrifugation at 13,000 X g for 20 min the precipitate was suspended in distilled water (the supernatant was preserved for albumin "extraction") and freeze-dried overnight. This procedure is a modification of that of Peterson and Smith (97).

b) Extraction of water-soluble albumins

The supernatant obtained after dialysis and centrifugation during globulin extraction was used for albumin recovery. The albumin fraction was precipitated with 0.25 volume of 50% (w/v) TCA, centrifuged, and suspended in water for lyophilization (97). Alternatively, the supernatant was directly lyophilized.

c) Extraction of alcohol-soluble prolamins

The flour pellet obtained after the isolation of globulins was suspended in 60 ml 55% 2-propanol, 43% H_2O, 2% 2-mercaptoethanol and stirred for 2 h at 60°C. The mixture was centrifuged for 25 min at

13,000 X g. The supernatant was made 4% NaCl to precipitate prolamins and left overnight. After centrifuging at 20,000 X g for 25 min, the pellet (prolamin) was suspended in 100 ml of H_2O and dialyzed against distilled water for 24 h. The dialyzed solution was then centrifuged and freeze-dried. The above procedure was a modification of that of Shewry et al. (98).

d) Extraction of base-soluble glutelins

The pellet from prolamin extraction was extracted with 0.1 N NaOH for 2 h at room temperature. After centrifugation for 30 min at 13,000 X g, the supernatant was dialyzed for 24 h and centrifuged again. The precipitate was suspended in distilled water and freeze-dried (97).

e) Extraction and fractionation of globulin components

The procedure for the separation of minor components of salt-soluble globulins was based on classical isoelectric point precipitation (IEP) of proteins frequently applied to leguminous seeds. Mature oat seeds (20 g) were ground to flour, defatted with petroleum ether and dried. The flour was stirred in extraction buffer (10 ml/g meal) (0.1M Na_2HPO_4-NaH_2PO_4, pH 7.5, 1 M NaCl, 0.02% sodium azide, 1 mM PMSF) for 1 h at 22°C. The supernatant was then dialyzed for 48 h at room temperature against 4 changes of 0.1 M NaH_2PO_4, pH 4.8, 0.2 M NaCl. The precipitate (major globulin component) was recovered by centrifugation and the supernatant was dialyzed against 4 changes of dH_2O for a further 48 h period at 4°C. The second precipitate (minor components) was then recovered by centrifugation at 4°C as above. Both pellets were lyophilized and stored at -20°C. The final supernatant (albumins) was also lyophilized for SDS-PAGE (Sodium dodecylsulfate-polyacrylamide gel electrophoresis) and IEF (Isoelectrofocusing) analyses.

Linear sucrose density gradients of 5-20% were used to fractionate the various globulin components. Gradients were formed in 50 mM Tris-HCl, pH 8.0, 1 M NaCl in 38 ml cellulose nitrate tubes. Protein preparations (20 mg) were dissolved in the gradient buffer and layered on the gradients.

Centrifugation was for 24 h at 20°C at 142,000 x g. Pea 7S and 11S oligomers were isolated by the IEP procedure and run on parallel gradients as standards. After the spin, the gradients were fractionated and analyzed using an ISCO-UA5 absorbance monitor and Type 6 optical unit. The peak fractions were dialyzed against dH_2O overnight and the precipitates analyzed by SDS-PAGE (see below).

2. Isolation of free and membrane-bound polyribosomes

Polyribosomes were isolated from developing oat groats by a modification of the method of Matthews and Miflin (8). Oat groats were frozen in liquid N_2 and ground in a coffee grinder. The flour was mixed with extraction buffer containing 0.2M Tris (pH 7.5), 100 mM KCl, 10 mM Mg acetate, 0.25 M sucrose, 10 mM DTT; and homogenized in a blender for 2 min. After 2 cycles of centrifugation at 6,000 X g for 5 min, the supernatant (containing free polysomes) was layered on 4 ml of 1.5M sucrose in the same buffer: The pellet was re-extracted by the same extraction buffer containing 1% Triton X-100. The mixture was centrifuged once at 6,000 X g for 5 min and then at 17,000 X g for 15 min. The supernatant (containing membrane-bound polysomes) was also layered on an identical sucrose cushion. All solutions were centrifuged in a Beckman Ti-60 rotor at 130,000 X g for 4 h.

Polysome pellets were carefully washed twice with sterile-distilled water (s-d), suspended in s-d H_2O and stored at -80°C.

3. Isolation of Polysomal RNA and Poly A^+ RNA

Isolated polysomes were dissociated with SDS and polysomal RNA was extracted with phenol-chloroform as described (99). Polysomes were suspended in dissociation buffer (0.2 M Tris, pH 9.0, 0.1 M NaCl, 10 mM EDTA, 0.5% SDS) mixed with one volume of phenol-chloroform (v/v-1:1) and centrifuged at 3,000 X g for 10 min. The aqueous phase was removed and the organic phase was re-extracted with one volume of buffer. The aqueous phases were pooled and extracted with half-volume of phenol-chloroform. The aqueous phase was removed and the organic phase was extracted with half-volume of buffer. All aqueous phases were again pooled, made 0.2 M ammonium acetate and mixed with 2.5 volumes of ethanol and left at -20°C overnight. Polysomal RNA was recovered by centrifuging at 12,000 X g for 30 min.

Oligo d(T)-Cellulose Chromatography:

The column was run as described by Aviv and Leder (100).

Oligo-d(T)cellulose (1 gram) was suspended in high-salt binding buffer (20 mM Tris/HCl, pH 7.4, 0.4 M NaCl, 0.2% SDS), and poured into a column (1cm X 6cm). The column was then washed extensively with high-salt binding buffer and stored at 4°C in 0.02% sodium azide. The chromatography was performed as described below.

The column was first washed with s-d H_2O and then with high-salt buffer. Total RNA in dH_2O was heated to 60°C for 10 min and mixed with one volume of high-salt buffer and applied to the column. The first column eluate was passed through the column a second time to maximize poly A^+ RNA adsorption. Then, the column was washed with 10 volumes of high salt buffer and 5 volumes of low salt buffer (20 mM Tris/HCl, pH 7.4, 0.2 M NaCl) respectively. Poly A^+ RNA was eluted with 1 volume of salt-free buffer (20 mM Tris/HCl, pH 7.4). The eluant was made 0.3 M NaCl, mixed with 2.5 volumes of 99% ethanol and left at -20°C for 24 h. The poly A^+ RNA was recovered by centrifugation and stored at -80°C.

The column was monitored by a Dual path (254, 280 nm) UV-2 monitor (Pharmacia Fine Chemicals).

4. In vivo labelling and Pulse-chase labelling of developing seeds

Spikelets were detached from developing oat plants and were immediately placed in a radioactive solution so that the pedicels of the spikelets were submerged. In pulse-labelling experiments, spikelets were incubated for several hours and certain number of groats were detached at specific times. In pulse-chase labelling experiments, spikelets were incubated for 1 h and subsequently transferred into a chase medium containing cold amino acids (leucine or methionine). In some in vivo labelling experiments, after the initial pulse, the spikelets were incubated with 0.5 mM cycloheximide. [^{3}H]leucine (140 Ci/mmol), [^{14}C]leucine (300 mCi/mmol), [^{35}S]methionine (900 Ci/mmol) and [^{35}S]sulfate were used as radioactive precursors.

Incorporation of radioactivity into proteins was determined by spotting an aliquot of labelled fractions on Whatman No. 1 filter paper. These filters were placed in 10% TCA for 15 min, boiled in 5% TCA in presence of cold amino acids, washed with cold 5% TCA, dried with ethanol and ether, and counted in 10 ml of scintillation solution.

5. Electrophoretic Analyses

a) Sodium dodecyl sulfate - polyacrylamide gel electrophoresis

SDS-PAGE was performed based on the method of Laemmli (101). The resolving gel contained 10%, 12% or 14% acrylamide, 0.17% N, N'-methylene bisacrylamide (bis), and 0.1% SDS, in 0.375 M Tris/HCl buffer, pH 8.8. The stacking gel was made of 5% acrylamide, 0.63% bis and 0.1% SDS in 0.125 M Tris/HCl, pH 6.8. The running buffer was 3.0% Tris, 14.4% glycine and 0.1% SDS solution. Protein samples were dissolved in the sample buffer (0.054 M Tris, pH 6.8, 1.0% SDS, 1.0% 2-mercaptoethanol, 20% glycerol), boiled for 2 min, and analyzed. The samples were electrophoresed at a constant voltage of 80V for 16 h. After electrophoresis, the gels were stained with 0.2% Coomassie Brilliant Blue (CBBR-250) in 10% acetic acid, 40% methanol and destained with the acetic acid-methanol-water solution. The standard molecular weight markers used were: Phosphorylase B (94,000 daltons), albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), trypsininhibitor (20,100 daltons), and α-lactalbumin (14,400 daltons).

b) Isoelectro focusing (IEF) IEF gels were composed of 5% acrylamide, 0.2% bis, 6.0 M deionized urea and 2% pH 3.5-10 Ampholine (LKB). Samples (10 mg/ml) were solubilized in a sample buffer containing 10mM glycine, 6.0 M urea, 1% Triton X-100 and 1% 2-mercaptoethanol (pH 8.0 adjusted with Tris). Gels were prefocused at 9W for 150 Vh and then focused at 12W for 2,500 Vh. Following protein fixation with 10% TCA for 1 h, the gels were washed overnight with running water, then stained (0.05% CBBR-250) and destained as described above.

c) Two-dimensional analysis (IEF and SDS-PAGE) For two-dimensional analysis, samples were first resolved according to charge by IEF as described above. Separate lanes were cut and equilibrated in O'Farrell et al. (102) Buffer O (without Me or glycerol) for 30 min. A lane was then placed on top of a second gel and proteins were separated by SDS-PAGE as above. The IEF lane was held in place within an additional layer of stacking gel. After electrophoresis, the gels were stained for 1 h and destained as above.

d) Agarose gel electrophoretic analysis of RNA. Total and poly A$^+$ RNA samples were electrophoresed on a 1.4% agarose gel after denaturation with glyoxal and dimethylsulfoxide. RNA (2-10 μg) was mixed with 6M glyoxal, dimethylsulfoxide, and 0.1 M NaH_2PO_4, pH 7.0 buffer. The RNA solution was incubated at 50°C for 60 min. After cooling to 20°C, sterile loading buffer (50% glycerol, 0.01 M NaH_2PO_4, pH 7.0, 0.4% bromophenol blue) was added and the sample was loaded on the gel.

The gel was poured and run in 0.01 M NaH_2PO_4, pH 7.0. The gel was run submerged in buffer at 20 V for 16 h. The buffer was recirculated during electrophoresis. At the end of the run, the gel was stained with 0.5 µg/ml ethidium bromide in 50 mM NaOH for 1 h. The gel was neutralized with 200 mM NaAc, pH 4.0 for 30 min, and photographed with a type 55 Poloroid film.

6. Fluorography

Polyacrylamide gels which had been stained and destained were incubated with Enhance (New England Nuclear) for 1 h, and with distilled water for 30 min respectively. The gels were then laid on a sheet of chromatography paper (Whatman no. 1), covered with Saran wrap, and dried using a commercial gel dryer. X-ray films were placed on the dried gels inside a X-ray Exposure holder and left at -70°C. After an appropriate exposure period, the films were developed and fixed using commercial methods (Kodak reagents). Kodak X-Omat AR films were used.

7. Immunological techniques

a) Antibody production:

The native 12S globulin was purified by isoelectric point precipitation and sucrose density gradient centrifugation. The purified globulin was then emulsified in Freund's Adjuvant (complete) either in native form or after denaturation with SDS. The emulsion was injected into rabbits at four weekly intervals. The pre-immune serum was collected before injections. The immune-serum was obtained at the end of the four week period and tested against the antigen by Ouchterlony double-diffusion analysis (103). A booster injection was given with incomplete Freund's

Adjuvant at the end of the four week period. Two weeks later, the rabbits were subjected to heart puncture and the blood was collected. Total IgG population was then isolated from the immune-serum by affinity chromatography on Protein A-Sepharose column (67). Globulin antibodies were further purified by immunoaffinity chromatography on CNBr-activated Sepharose covalently attached to purified globulin using the method of Casey (105). The characterization of the anti-12S globulin IgG is presented separately in Appendix II.

b) Immunoprecipitation of Translation Products:

In vitro translation mixtures were diluted with 1 volume of 10 mM Tris-HCl, pH 7.5, 1% NP40, 2 mM EDTA, 150 mM NaCl, and incubated with preimmune-serum for 30 min to eliminate any non-specific binding. After 30 min, 50 µl of Protein A-Sepharose (100 µg/ml) was added and incubation continued for another 30 min. The mixtures were centrifuged for 2 min in a microfuge and the pellet was discarded. The supernatant was then incubated with anti-globulin IgG (10 µl of a 1 mg/ml IgG solution) for 2 h. Protein A-Sepharose (50 µl) was added to the mixtures and incubated for 1 h. After centrifugation for 2 min, the supernatant was discarded and the pellet was washed 3 times with 10 mM Tris-HCl, pH 7.5, 0.2% NP40, 2 mM EDTA, 500 mM NaCl, and twice with 10 mM Tris-HCl, pH 7.5. The sepharose beads were finally suspended in SDS-PAGE sample buffer (see section 5a) and boiled for 5 min. The amount of immunoprecipitate was determined by spotting 5 µl of the buffer on Whatman No. 1 filter paper and precipitating with TCA (see Section 4). Appropriate amounts of immunoprecipitates were then analyzed by SDS-PAGE.

c) Immunoaffinity chromatography:

Anti-12S globulin IgG was first coupled with CNBr-activated Sepharose using a method recommended by the manufacturer (Pharmacia) a modification of the method of Casey (104). The efficiency of the protein coupling was normally greater than 90%. The globulin IgG-Sepharose gel was then divided into several aliquots and used as required.

Samples (e.g. ER, PB) were suspended in buffer containing 20 mM NaH_2PO_4, 0.14 M NaCl, pH 7.4, 1% Triton X-100, and applied to the globulin IgG-Sepharose column. The non-bound material was washed out with the same buffer. The bound polypeptides were eluted with 0.2 M glycine, pH 2.8 and the eluant was immediately adjusted to pH 7.4. The bound polypeptides were then analyzed by SDS-PAGE.

8. Sucrose Density Gradient Fractionations

This technique was used in many cases including ER extraction, PB isolation, globulin size determination, and polysome size determination.

a) Subcellular Isolations

Linear sucrose density gradients were used for subcellular fractionation. Labelled developing seeds (0.5 g) were homogenized in 12% sucrose in a medium containing 100 mM Tris-HCl, pH 8.6, 30 mM KCl, 5 mM EDTA (medium A). The homogenate was centrifuged at 500 X g for 10 min. and the supernatant applied to a Sepharose 4B column to separate organelles from soluble proteins and small molecules. The organelles were eluted with 12% sucrose in medium A containing only 1 mM EDTA in the void volume of the column. The most light-scattering fractions were pooled and layered on a linear 16-50% sucrose gradient made up in medium A (1 mM EDTA). Centrifugation was for 2 h at 150,000 X g in a SW 41 rotor in a Beckman ultracentrifuge. The gradients were fractionated and monitored using an ISCO flow cell in a UV5 optical unit. Fractions were tested for enzyme activities and examined for radioactivity as described above.

Fractions rich in ER were isolated by 16% and 35% (w/v) discontinuous sucrose gradients. In this procedure, most of the NADH-cytochrome c reductase activity could be recovered at the 16/35 interface. The labelled seeds were homogenized and prepared for sucrose gradient centrifugation as described above. The gradient contained 3 ml of 35% (w/v) and 8 ml of 16% (w/v) sucrose made up in medium A. After centrifugation at 150,000 X g for 90 min, the organelles visible at the 16/35 interface were collected and analyzed by SDS-PAGE and fluorography either before or after immunochromatography on globulin-IgG-Sepharose as explained later.

Linear sucrose gradients were used for the isolation of rough endoplasmic reticulum (RER). Linear gradients of 16- 50% (w/v) sucrose were formed in a buffer containing 40 mM Tris/HCl, pH 8.6, 30 mM KCl, and 10 mM MgAc. Developing seeds were homogenized in 100 mM Tris/HCl pH 8.6, 50 mM KCl, 10 mM MgAc. Magnesium salt was added to retain the ribosomes on ER (105). After a 10 min spin at 1,000 X g, the supernatants were layered on the gradients. Centrifugation was for 90 min at 32,000 X g in a SW 41 Ti rotor of a Beckman ultracentrifuge. Gradients were fractionated and the fractions were tested for NADH-cytochrome c reductase activity. The fractions with the highest enzyme activity (density of 1.15 g/cm^3) were collected, treated with 1% Triton X- 100, and used for polysome isolation as described above.

b) Isolation of Protein Bodies (PB).

Developing seeds were homogenized with a razor blade and the homogenate was filtered by Miracloth. The filtrate was layered on a discontinuous sucrose gradient of 16% and 65%, and centrifuged at 1000 X g for 10 min. The material banding at the 16/65 interface was collected and referred to as protein bodies. This was later confirmed using an electron microscope (see below).

c) Size determination

Linear sucrose gradients of 5-20% (w/w) were normally used for protein size determinations. Protein samples were dissolved in 50 mM Tris/HCl, 1 M NaCl, pH 8.0 and applied to 5-20% NaCl gradients made in the same buffer. Centrifugation was for 24 h at 20°C in a Beckman SW 28.1 rotor at 27,000 rpm. The gradients were fractionated and monitored at 280 nm. Molecular weight markers (pea 7S and 11S protein oligomers) were run on parallel gradients to estimate the size of globulin samples.

9. In vitro Translations

a) Wheat Germ Translations

Oat globulin poly A^+ RNA, polyribosomes, and RER were used as templates for protein synthesis in a cell-free translation system, wheat germ extract, basically as described by Roberts and Paterson (106). Translation mixture (10 μl wheat germ S30 extract, 20 μCi [^{35}S]methionine or 5 μCi of [^{3}H]leucine, 50 mM of each unlabelled amino acid, 1 mM GTP, 8 mM creatine phosphate, 300 mM spermidine, 50 mM ATP, 2 mM DTT, 50 mg/ml creatine phosphokinase, and varying amounts of RNA, polysomes or RER. Incubation was for 60 min at 30°C. Incorporation of radioactivity into protein was determined by spotting 2 μl aliquots onto strips of Whatman No. 1 filter paper. These were placed in cold 10% TCA, boiled in 5% TCA, followed by washing in cold 5% TCA. They were then washed in ethanol and diethylether for 2 min each, placed in non-aqueous scintillation fluid and their radioactivity determined by scintillation spectrometry. In some cases, translation products were treated with RNase A before counting.

b) Reticulocyte Lysate Translations.

Translations were also carried out in reticulocyte lysate. A 30 µl translation contained 80% lysate (Amersham), appropriate amount of RNA or polysome, and 100 µCi of [^{3}H]leucine (Amersham, 120 Ci/mmol). Potassium and magenesium concentrations were 118 mM and 2.5 mM respectively. Incubation was for 60 min at 30°C. Incorporation of radioactivity into protein was determined as described above (see Wheat Germ Translations).

10. Concanavalin A Sepharose Chromatography

Con A Sepharose (Sigma) was washed with 10 volumes of 0.05 M Tris/HCl, pH 7.4, 0.5 M NaCl and incubated with protein samples (4 mg/5 ml above buffer) for 30 min at room temperature by shaking with an end-over-end mixer. The suspension was poured into a column and the non-bound material was removed with 20 ml of the buffer. The bound proteins were then eluted with 20 ml of 0.2 M α-methyl-D-glucoside in the same buffer and 2 ml fractions were collected. The column was monitored at A_{280} using an ISCO-UA5 absorbance monitor and a Type 6 optical unit. The non-bound and bound fractions were made to 10% TCA and the precipitated proteins were analyzed by SDS-PAGE as described above.

11. Assay of NADH-Cytochrome C Reductase Activity

NADH-Cytochrome c reductase was assayed as described by Bollini and Chrispeels (107). Enzyme activity was measured by following the change in A_{550} of cytochrome c. A 1 ml reaction mixture contained 0.8 mg of cytochrome c, 0.5 mg of NADH, 0.2 μmol of KCN and 80 μmol of potassium-phosphate buffer, pH 7.5. Enzyme activity was expressed as ΔA_{550} min^{-1} ml^{-1}.

12. Proteinase K Digestion of Protein Bodies

Linear 16-65% or discontinuous 16% and 65% sucrose gradients were used to isolate protein bodies. The protein body preparations were incubated with proteinase K at a concentration of 500 μg/ml in the presence of 50 μg/ml lauric acid at 10°C for 45 min. Proteinase K was omitted in control samples. After treatment, the suspension was made 2 mM PMSF and centrifuged over a 16%-65% linear sucrose gradient in a SW 28.1 rotor at 25,000 rpm in a Beckman ultracentrifuge for 2.5 h. The gradients were then monitored at 280 nm and fractionated.

13. Extraction of Globulin from Organelles

One volume of 100 mM Tris HCl, pH 8.0, 2 M NaCl was added to ER and protein body preparations and the samples were then homogenized manually. After centrifugation in a Fisher Microfuge, the supernatant was removed and either used directly (in the case of ER) or was dialyzed against dH_2O at 4°C (in the case of PB) to precipitate globulins.

14. Immunofluorescent Techniques

Tissue fixation method was based on that described by McLean and Nakane (108). Mature oat groats were cut into small pieces (2 mm) and placed in a fixative solution (0.05 M phosphate, 0.1 M lysine-HCl, pH 7.4, 0.1 M sodium periodate, 10% paraformaldehyde) and fixed for 18 h at 4°C. Seed blocks were washed with 0.05 M phosphate buffer pH 7.4 and dehydrated through an alcohol series (methyl alcohol, 99% ethanol, n-propanol, and n-butanol). Dehydrated blocks were incubated in a glycol methacrylate (GMA) mixture (95 ml GMA, 5 ml Carbowax 400, 0.5% benzoyl peroxide) for 4-5 days at room temperature in the dark and for 2-3 days at 60°C in an oven. Sections were cut using a microtome and placed on glass slides. For immunofluorescent experiments, slides were left in acetone overnight before use. In a few cases, seed blocks were used to prepare frozen sections immediately after fixation.

For immunolabelling, plastic sections were washed extensively with PBS buffer (0.1 M NaH_2PO_4-Na_2HPO_4, 0.15 M NaCl, pH 7.4) and incubated with antibodies or pre-immume serum for various periods of time (15-60 min). The sections were then washed extensively with PBS buffer and incubated with FITC-conjugated anti-rabbit goat immunoglobulin (IgG) for 30-60 min. After a 10 min wash with PBS buffer, the sections were viewed under a Zeiss fluorescent microscope. For normal viewing, sections were stained with acid fuchsin for 2 min. The microscope was equipped with both a conventional bright-field illuminating system and a III RS epi-illuminating condenser combined with an HBO 200 W mercury-arc illuminator for fluorescence analysis. The III RS condenser contained three fluorescence filter combinations each with a dichromatic beam splitter and an exciter/barrier filter set with maximum transmission at 365nm/418nm (FC I), 450-490nm/520nm (FC II) and 546nm/ 590nm (FC III). Photomicrographs were obtained using Ektachrome 400 Daylight film.

15. Electron Microscopy

Oat developing seeds (7-16 days after flowering) were fixed in 3% glutaraldehyde in 50 mM KH_2PO_4-K_2HPO_4, pH 7.2 for 3-6 days at

20°C. Protein body and ER pellets were fixed similarly for 2 h at 20°C. All samples were washed extensively in the above buffer minus glutaraldehyde. The samples were post-fixed in 1% OsO_4 in the same buffer for 18 h at 20°C, washed extensively, dehydrated with an ethanol series (20%, 40%, 60%, 80%, 100%, 100%, propylene oxide), stained *en bloc* with 1.5% uranyl acetate for 1 h at 20°C and embedded in Spurr's resin (109). The seed samples were subjected to an *en bloc* stain of 1.5% uranyl acetate (aqueous) for 1 h at 20°C before embedding. Gold sections (90 nm) were cut on a Reichert OmU_2 Ultra-microtome and were examined with a Philips 300 transmission electron microscope operating at an accelerating voltage of 60 kV. Micrographs were recorded on 35 mm Kodak fine grain release positive electron microscope film.

CHAPTER III

RESULTS

PART I

GLOBULIN SYNTHESIS

A number of experiments were performed in order to investigate the pattern and site of globulin synthesis during oat seed development. These experiments included: A) extraction and *in vitro* translation of polysomes and poly A$^+$ RNA from developing seeds, B) isolation and translation of rough endoplasmic reticulum, C) *in vivo* labelling and analysis of cell membranes, as well as D) electron microscopic investigations.

A. Extraction and in vitro Translation of Seed Polysomes and Poly A$^+$ RNA.

Two classes of free and membrane-bound polysomes were extracted by conventional differential centrifugation techniques. These polysomes were then used to extract total polysomal RNA by phenol-chloroform extraction procedure. Poly A$^+$ RNA fractions were selected by oligo (dT)-cellulose chromatography. Table III describes the yield and translational capability of polysomes, RNA and poly A$^+$ RNA fractions in two classes of free and membrane-bound.

Generally, free polysomes gave a higher yield than membrane-bound polysomes. The A_{260}/A_{280} ratios for both polysomal fractions were within an acceptable range (1.5-1.85) indicating the purity of these polysomes. Total RNA extracted from free polysomes was also higher than that extracted from membrane-bound polysomes. However, the yield of membrane-bound poly A$^+$ RNA (35 μg) was about 30% higher than that of free poly A$^+$ RNA (25 μg).

TABLE III. Yield and Translational Capability of Oat Polysomes and RNA.

	YIELD A_{260} units/25g	TRANSLATIONAL EFFICIENCY cpm/ul. (per ug RNA)
FREE		
POLYSOMES	253 units	1,500
TOTAL RNA	116 units(4.6 mg)	1,100
POLY A^+ RNA	0.62 units(25 ug)	2,500
MEMBRANE-BOUND		
POLYSOMES	237 units	2,950
TOTAL RNA	96 units(3.84 mg)	990
POLY A^+ RNA	0.88 units(35 ug)	2,300

All RNA fractions (total, poly A$^-$, and poly A$^+$ RNAs) were further analyzed by denaturing agarose gels (Fig. 1). The total RNAs from free and membrane-bound polysomes consisted of two major RNA species. These species corresponded to the plant 25S and 18S ribosomal RNAs. Other minor species were present between 18S and 25S and below 18S bands. Poly A$^-$ RNA samples from both free and membrane-bound classes showed similar profiles to those of total RNAs (not shown). Some ribosomal 25S and 18S RNA were present in both poly A$^+$ RNA preparations despite oligo (dT) cellulose chromatography. Fig. 1, lanes 2 and 4 show the patterns of membrane-bound and free poly A$^+$ RNAs after one round of oligo (dT)-cellulose chromatography respectively. Some ribosomal RNAs (25S and 18S) were still present in the poly A$^+$ RNA samples. Both samples seemed to be enriched in RNA molecules smaller than 18S, after oligo (dT)-cellulose chromatography. Analysis of membrane-bound poly A$^+$ RNA samples isolated after two cycles of oligo (dT)-cellulose chromatography normally shows the presence of three major RNA species with approximate sizes of 18S, 15S, and 12S (data not shown). Based on the behaviour of free and membrane-bound RNA samples on agarose gels, it could be assumed that both these RNA preparations were intact and not degraded.

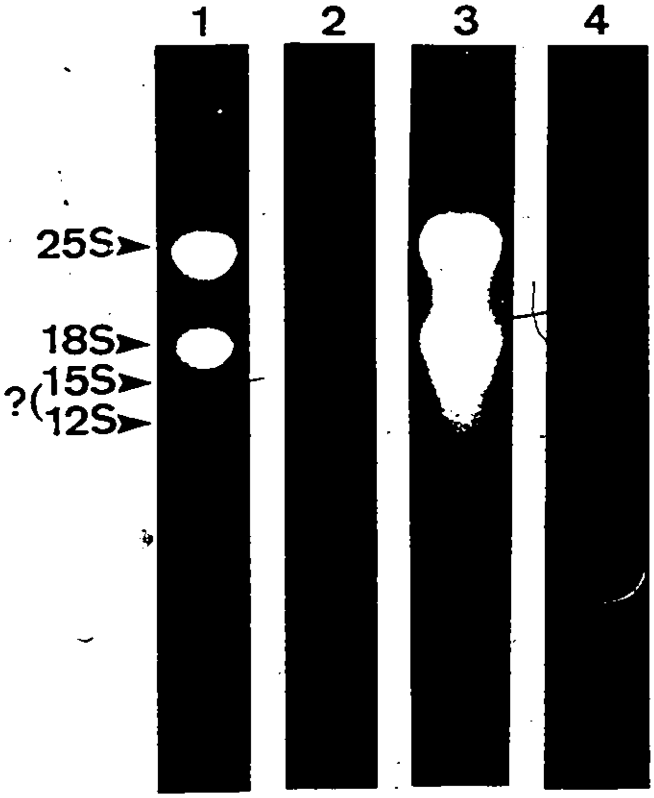
Free and membrane-bound polysomes and poly A$^+$ RNA were translated *in vitro* in a rabbit reticulocyte lysate system in order to compare their translational capability and products. The incorporation of [^{3}H]leucine into TCA-precipitable products was higher by membrane-bound polysomes than free polysomes (2,950 cpm/µl and 1,500 cpm/µl respectively). Free and membrane-bound poly A$^+$ RNA preparations directed the synthesis of approximately equal amounts of protein (2,300 cpm/µl and 2,500 cpm/µl respectively). Poly A$^+$ RNA samples translated more efficiently than

Fig. 1 Agarose gel electrophoresis of free and membrane-bound RNA preparations.

Total RNA was isolated from free and membrane-bound polysomes. The total RNA samples were applied to one cycle of oligo (dT)-cellulose chromatography to separate the poly A^+ RNA fractions.

Lane 1, total membrane-bound RNA.
Lane 2, membrane-bound poly A^+ RNA.
Lane 3, total free RNA.
Lane 4, free poly A^+ RNA.

FIG.1



polysomes did in the rabbit reticulocyte system.

Equal number of counts from the translation products of polysomes and poly A$^+$ RNA were analyzed by SDS-PAGE under reducing conditions. The translation products were visualized by fluorography (Fig. 2). A number of differences existed between the translation products of free and membrane-bound templates. One major difference could be observed in polypeptides with molecular weight of 58-62 kDa. These polypeptides were major translation products of membrane-bound polysomes and poly A$^+$ RNA. The 58-62 kDa polypeptides corresponded in size to unreducible globulin polypeptides. These polypeptides were also synthesized by free polysomes and poly A$^+$ RNA. This became more clear once a similar gel was run and exposed for a longer period of time (Fig. 2a). However, these 58-62 kDa polypeptides were minor relative to the total translation products of free templates. Scanning of lanes FA$^+$ and MA$^+$ in Fig. 2 quantitated the difference in the level of synthesis of the 58-62 kDa polypeptides by free and membrane-bound templates (Fig. 2b). The 58-62 kDa polypeptides accounted for about 8.3% of the total products made by membrane-bound poly A$^+$ RNA and about 0.85% of total products made by free poly A$^+$ RNA (calculated by cutting and weighing the peak areas). These results suggested that the globulin putative precursors may be primarily synthesized by membrane-bound templates.

The translation products of free and membrane-bound poly A$^+$ RNAs were immunoprecipitated by globulin antibodies to reveal whether the 58-62 kDa polypeptides were indeed related to globulin polypeptides (Fig. 3). Globulin antibodies immunoprecipitated a higher amount of counts from the translation products of membrane-bound poly A$^+$ RNA (14,800 cpm, from a total of 65,000 cpm = 22.7%) than from those of free poly A$^+$ RNA

Fig. 2 *In vitro* translation of free and membrane-bound templates.

Developing groats (18 daf) were used to isolate free and membrane-bound polysomes by differential centrifugation. The polysomes were used to isolate poly A$^+$ RNA by two cycles of oligo (dT)-cellulose chromatography. The templates were then translated in a rabbit reticulocyte lysate system in presence of a RNase inhibitor (RNasin, Amersham). The radioactive label was [^{3}H]leucine (190 Ci/mmol). The products were analyzed by SDS-PAGE (13%, +Me) and fluorography. Lane C is the control (no RNA added). Lanes FP and FA$^+$ represent translation products of free polysomes (0.7 O.D. units) and free poly A$^+$ RNA (2.5 µg). Lanes MP and MA$^+$ represent translation products of membrane-bound polysomes (0.7 O.D. units) and membrane-bound poly A$^+$ RNA (2.5 µg). Arrowheads indicate the position of putative globulin precursors. Equal number of counts (20,000 cpm) were loaded on each lane (except for the control lane) and the fluorograph was exposed for 8 days.

Molecular weight markers: [^{14}C]methylated bovine serum albumin (69,000), [^{14}C]methylated ovalbumin (46,000), [^{14}C]methylated carbonic anhydrase (30,000), and [^{14}C]methylated lysozyme (14,300).

Fig. 2a Translation products of free and membrane-bound templates from Fig. 2 were reanalyzed by SDS-PAGE and fluorography. Equal number of counts were loaded on each lane (except for the control lane). The exposure time was longer (12 days). Arrowheads indicate the position of putative globulin precursors. The arrow indicates the position of a 40 kDa polypeptide preferentially made by free templates.

FIG. 2

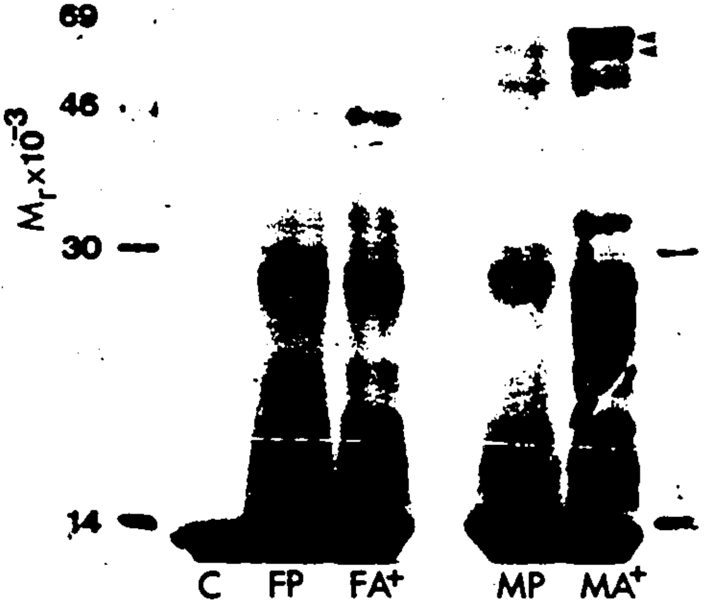

69
46
30
14
$M_r \times 10^{-3}$
C
FP
FA+
MP
MA+

FIG. 2a

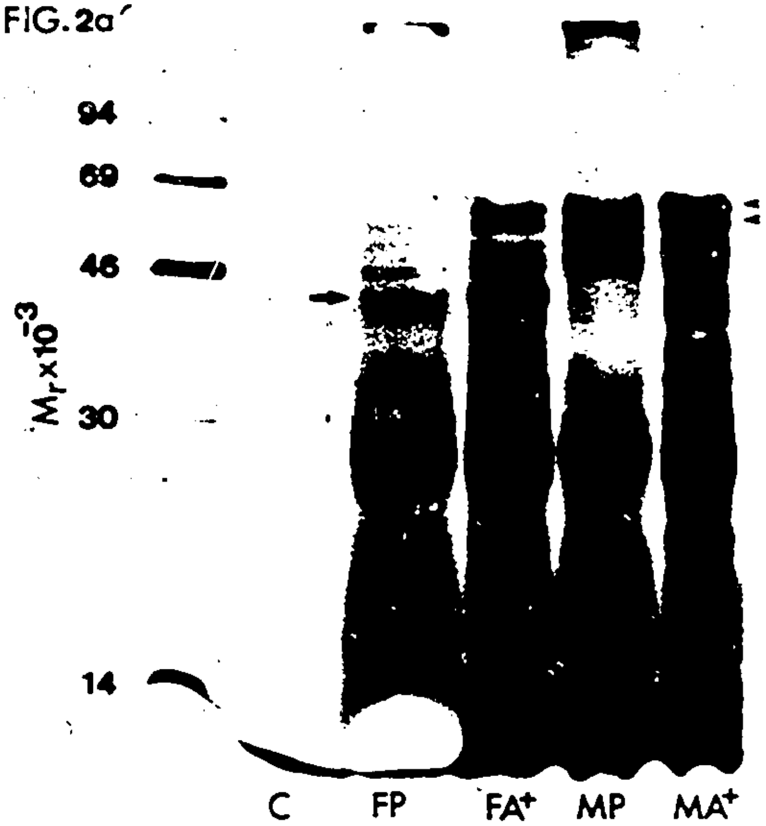

94
69
46
30
14
$M_r \times 10^{-3}$
C
FP
FA+
MP
MA+

Fig. 2b Scanning of translation profiles.-

Lanes FA^+ and MA^+ corresponding to translation profiles of free and membrane-bound poly A^+ RNA were scanned using a densitometer in order to estimate quantitatively the differences between the translation products of the two templates. Free A^+ is the profile of translation products of free poly A^+ RNA. M.B.A^+ is the profile of translation products of membrane-bound poly A^+ RNA.

FIG. 2b

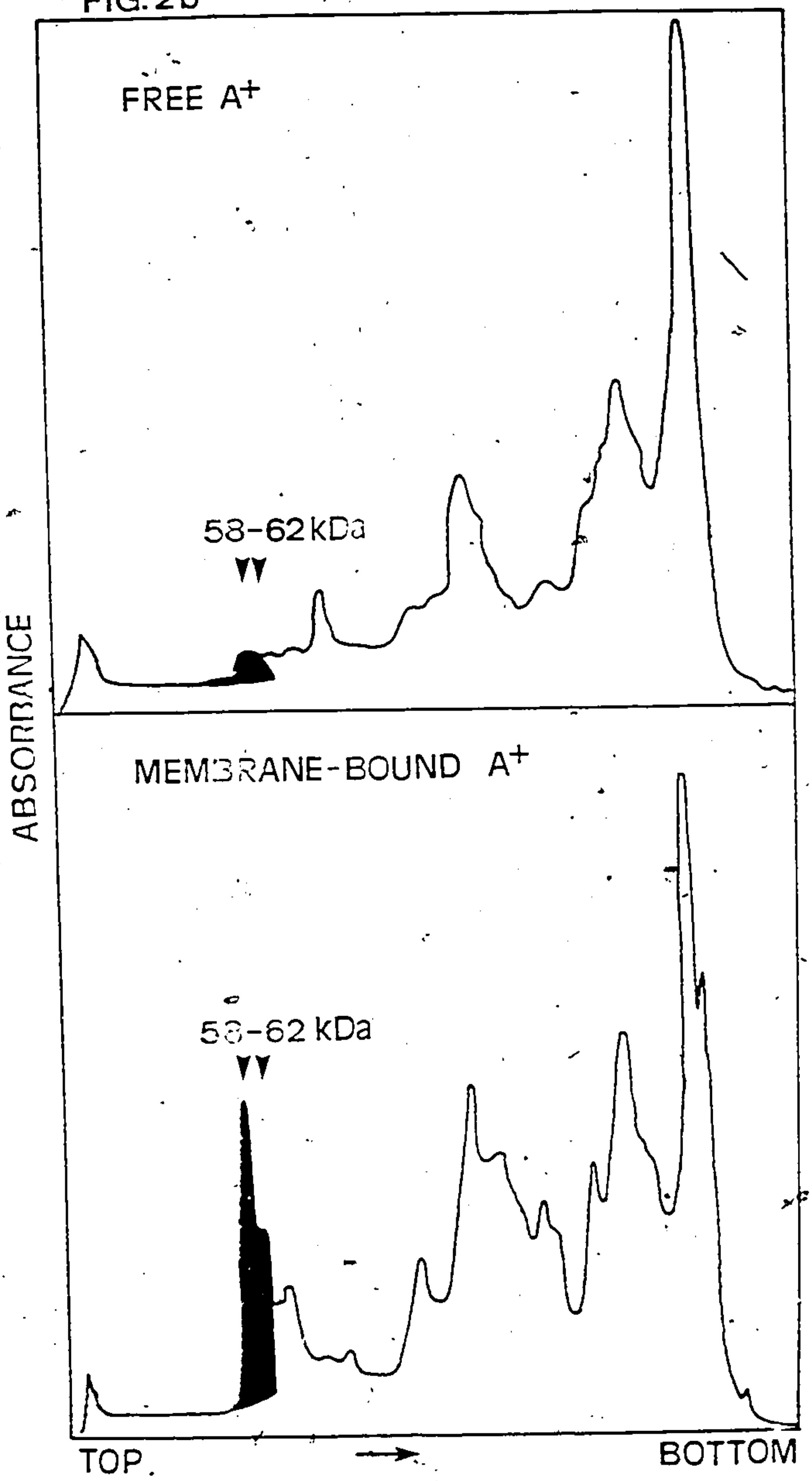

FREE A+
58-62 kDa
MEMBRANE-BOUND A+
58-62 kDa
ABSORBANCE
TOP
BOTTOM

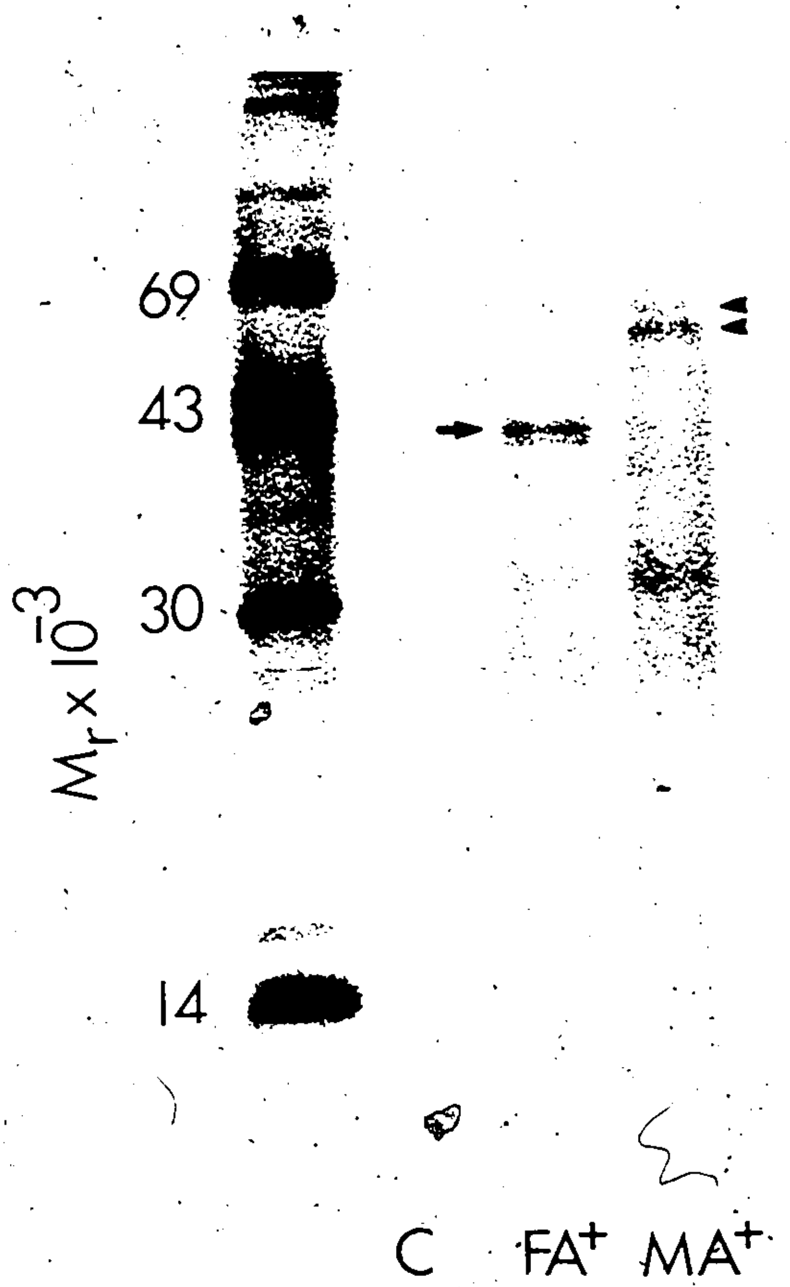
Fig. 3 Immunoprecipitation of translation products.

Free and membrane-bound poly A^+ RNA preparations were translated in rabbit reticulocyte lysate with [^{3}H]leucine. Equal number of counts of translation products (65,000 cpm) were immunoprecipitated with 12S globulin antibodies and Protein A-Sepharose. The immunoprecipitates were dissolved in SDS-PAGE sample buffer and boiled for 5 min. Equal number of counts (4000 cpm) were analyzed by SDS-PAGE (13%, +Me) and fluorography. Lane C is the control (immunoprecipitate of control translation with no RNA added). Lanes FA^+ and MA^+ represent immunoprecipitates of the translation products of free and membrane-bound poly A^+ RNA respectively. The fluorograph was exposed for 15 days. Arrowheads indicate the position of globulin 58-62 kDa polypeptides. The arrow indicates the position of a 40 kDa polypeptide immunoprecipitated from translation products of free polysomes.

(7,900 cpm from a total of 65,000 cpm = 12.1%). However, this comparisonmay not be totally reliable since variations may occur in the efficiency of immunoprecipitation between the two samples. The percentage of radioactivity immunoprecipitated by antibodies was higher than those estimated from densitometery (about 60% higher). This excess radioactivity may be a result of contamination by free label trapped within antibody-antigen aggregates. It may also be a result of non-specific precipitation of non-globulin polypeptides. Finally, it could represent some smaller globulin-related polypeptides (incomplete translation products).

The SDS-PAGE analysis of equal number of counts from the two immunoprecipitates (Fig. 3) indicated that most of the counts precipitated from the products of membrane-bound poly A$^+$ RNA corresponded to the 58-62 kDa polypeptides. Most of the radioactivity immunoprecipitated from the translation products of membrane-bound poly A$^+$ RNA were represented on the fluorograph by two 58-62 kDa polypeptides. Antibodies seemed to have also precipitated such 58-62 kDa polypeptides antigenically related to globulin from translation products of free poly A$^+$ RNA. However, the signals at this region of the gel were minor relative to the total signals on the lane. This indicated that most of the radioactivity immunoprecipitated from the translation products of free poly A$^+$ RNA either represented other seed proteins antigenically related to globlulin or, these signals were the result of non-specific precipitation. According to Fig. 2b and Fig. 3, free poly A$^+$ RNA synthesized the globulin 58-62 kDa polypeptides but the level of this synthesis was about 10 fold lower than the level of synthesis of these polypeptides by membrane-bound poly A$^+$ RNA. The major polypeptide

FIG. 3



immunoprecipitated from the translation products of free poly A$^+$ RNA was a 40 kDa molecule. The relation of this polypeptide with globulins is not clear. It may be a distinct globulin polypeptide synthesized by free templates. It may also be related to another group of seed proteins having homologous antigenic domains to globulin polypeptides. Finally, it may be the result of non-specific precipitation. The synthesis of some globulin by free polysomes was theoretically expected even if membrane-bound polysomes were the exclusive site of globulin synthesis. Prior to formation of membrane-bound polysomes in the cell, the synthesis of a secretory protein begins on free polysomes. After the synthesis of a nascent chain, such free polysomes bind to rough endoplasmic reticulum creating membrane-bound polysomes (17,18,19). When developing seeds were harvested and frozen to be used for polysome extraction, some of the poly A$^+$ RNA molecules coding for globulin polypeptides might have been at an early stage of globulin synthesis and thus were present as free polysomes. However, despite this expectation, the presence of globulin poly A$^+$ RNA molecules in the free polysomes may be partially the result of incomplete fractionation and contamination from the membrane-bound polysomes. Finally, synthesis of globulin on free polysomes may be a true phenomenon occurring <u>in vivo</u>. It may be that some globulin species are synthesized via an alternative pathway (synthesis on free polysomes and deposition in the cytosol).

Nevertheless, the bulk of the data indicated that membrane-bound polysomes contained a major portion of poly A$^+$ RNA molecules coding for globulin precursors. Membrane-bound polysomes could therefore be regarded as the major site of globulin synthesis in developing seeds.

B. Isolation and In Vitro Translation of Rough Endoplasmic Reticulum.

If the globulin poly A^+ RNA molecules were abundant in the membrane-bound polysome fraction, they should also be detectable in the rough endoplasmic reticulum (RER) fraction of developing seeds. An experiment was conducted to extract and translate a RER preparation. RER was isolated by linear sucrose gradient centrifugation. Developing oat groats were homogenized in a buffer containing 10 mM MgAc and the extract fractionated on a linear sucrose gradient (16-50% w/v). Under these conditions, the ER marker enzyme (NADH-cytochrome c reductase) formed a broad band with an average density of 1.15 g/cm^3 (Fig. 4). ER membranes should retain their ribosomes in these conditions (105). The fractions with the highest NADH-cytochrome c reductase activity were therefore referred to as RER, and used to isolate polysomes. The fractions 10-14 were pooled and treated with 1% Triton X-100 to dissociate the bound polysomes which were then sedimented through 1.5 M sucrose as described in Materials and Methods. The polysomes were suspended in translation buffer and translated in a wheat germ extract (Fig. 5). As standards, a membrane-bound polysome preparation and a poly A^+ RNA preparation were also translated in order to compare their in vitro translation products (lanes B and C) with the products of polysomes isolated from the RER fraction (lane D). The ER-associated polysomes (lane D) directed the synthesis of two polypeptides (arrows) with molecular weights of 58-62 kDa which corresponded in size to some of the polypeptides made by membrane-bound polysomes (lane B) and poly A^+ RNA (lane C).

The data indicated that polysomes associated with the RER could direct in an in vitro cell-free system the synthesis of polypeptides corresponding in size to the unreducible globulin polypeptides.

Fig. 4 Isolation of RER membranes on a sucrose gradient. Developing groats (18 daf) were homogenized in a buffer containing Mg^{+2} ions and the homogenate was filtered and layered on a linear 16–50% (w/v) sucrose gradient made in the same buffer. The gradients were centrifuged at 30,000 rpm in a Beckman SW 41 rotor for 90 min at 4°C. After centrifugation, the gradient was monitored for protein at 280 nm (------) and fractionated into 0.5 ml fractions. Each fraction was then tested for NADH-cytochrome c reductase activity (●——●). Enzyme activity is expressed as ΔA_{550} min^{-1} ml^{-1}.

FIG.4

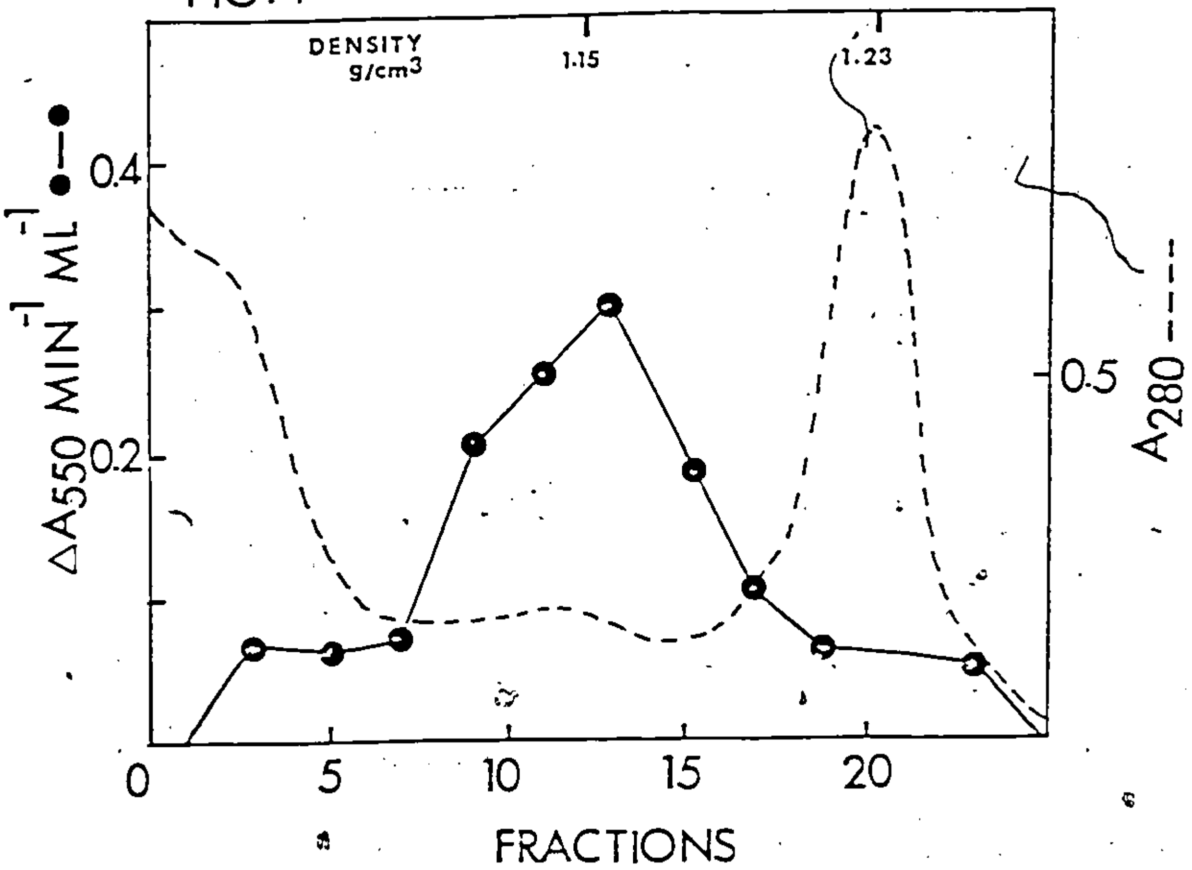
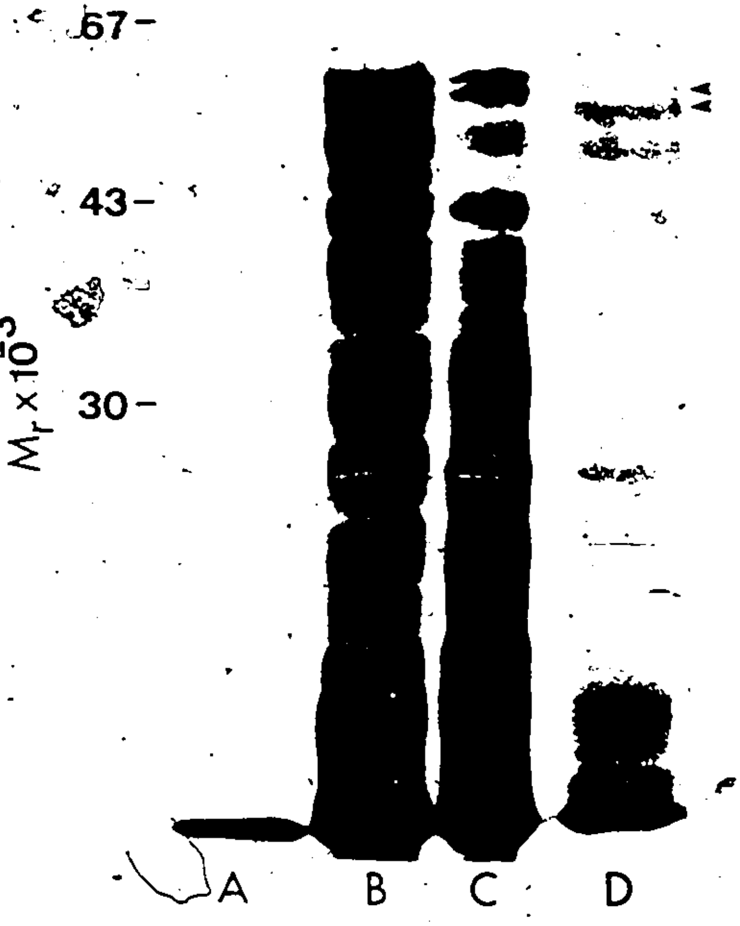

DENSITY g/cm3
1.15
1.23
ΔA550 MIN-1 ML-1 ●—●
0.4
0.2
0.5
A280 - - - -
0
5
10
15
20
FRACTIONS

Fig. 5 Isolation and cell-free translation of RER.

Developing groats (18 daf) were homogenized in 100 mM Tris-HCl pH 8.3, 10 mM MgAc, and the extract was fractionated on a 16-50% linear sucrose gradient. The organelles at the density of 1.15 g/cm^3 with the highest NADH-cytochrome c reductase activity were collected and treated with Triton X-100. The polysomes associated with this RER fraction were isolated by centrifugation through a 1.5 M sucrose solution. The polysome samples were translated in a wheat germ extract in the presence of [^{35}S]methionine (800 Ci/mmol) and the products were analyzed by SDS-PAGE (12%, +Me) and fluorography. A, blank (no polysomes added); B, translation products of membrane-bound polysomes (0.4 A_{260} units) isolated as described in Materials and Methods; C, translation products of poly A$^+$ RNA (3 μg); D, translation products of polysomes isolated from RER fractions (0.25 A_{260} units). Lanes B and C contained 200,000 cpm. Lane D contained 100,000 cpm. Arrows indicated the two polypeptides corresponding in size to globulin dimers.

FIG. 5



C. In Vivo Labelling and Analysis of Globulin Polypeptides Associated with the Endoplasmic Reticulum.

A final approach to study the mode of globulin synthesis was an *in vivo* labelling experiment. This experiment was designed so that labelled proteins associated with ER membranes could be analyzed.

Developing oat groats were labelled with [^{14}C]leucine for 50 min, homogenized, and the homogenate was fractionated on a sucrose gradient. Under these conditions, TCA-precipitable radioactivity could be detected both in the supernatant at the top of the gradient and in a band at the density of 1.10 g/cm^3. This finding indicated that the sample volume of the gradient (supernatant) contained high levels of radioactivity which could be due to unincorporated label and labelled proteins soluble in homogenization buffer. Since the removal of this soluble radioactivity could clarify the pattern of radioactivity distributed in the gradient, a simple column chromatography technique was used to separate the soluble fraction from organelles. Therefore, organelles were separated by Sepharose 4B chromatography from soluble proteins prior to sucrose gradient centrifugation. Under these conditions, the radioactivity peaked at the density of 1.10 g/cm^3 coinciding with the position of NADH-cytochrome c reductase, the ER marker enzyme (Fig. 6A). The density of the peaks of NADH-cytochrome c reductase activity and TCA-precipitable radioactivity increased to 1.15 g/cm^3 when the labelled seeds were homogenized in a medium containing 5 mM MgAc instead of EDTA (Fig. 6B). This was probably due to the retention of ribosomes on the ER membranes (105). The effect of magnesium ions on the density of ER membranes is known to be a good marker for the detection of these membranes (105). The simultaneous shift of the peak of TCA-precipitable

Fig. 6 A & B Distribution of incorporated radioactivity and NADH-cytochrome c reductase activity from organelle extracts on linear sucrose gradients. Developing oat groats (18 daf) were labelled for 50 min with 2 μCi of [^{3}H]leucine (800 Ci/mmol) each. The labelled groats were homogenized in 50 mM Tris-HCl pH 8.0, 100 mM KCl, 5 mM EDTA or 3 mM MgAc, 12% sucrose and spun for 10 min at 2000 X g. The supernatant was applied to a Sepharose 4B column in the same media. The organelles eluted in the void volume of the column and were layered on a 16-50% sucrose gradient in 50 mM Tris-HCl pH 8.0, 100 mM KCl, 1 mM EDTA. After centrifugation at 150,000 X g for 2 h, the gradients were fractionated into 1 ml fractions. TCA precipitable radioactivity was determined and expressed as cpm x 10^{-4}. NADH-cytochrome c reductase activity was expressed as ΔA_{550} min^{-1} ml^{-1}. A, the profile obtained in absence of Mg^{+2} ions. B, the profile in presence of Mg^{+2}.

FIG. 6 A

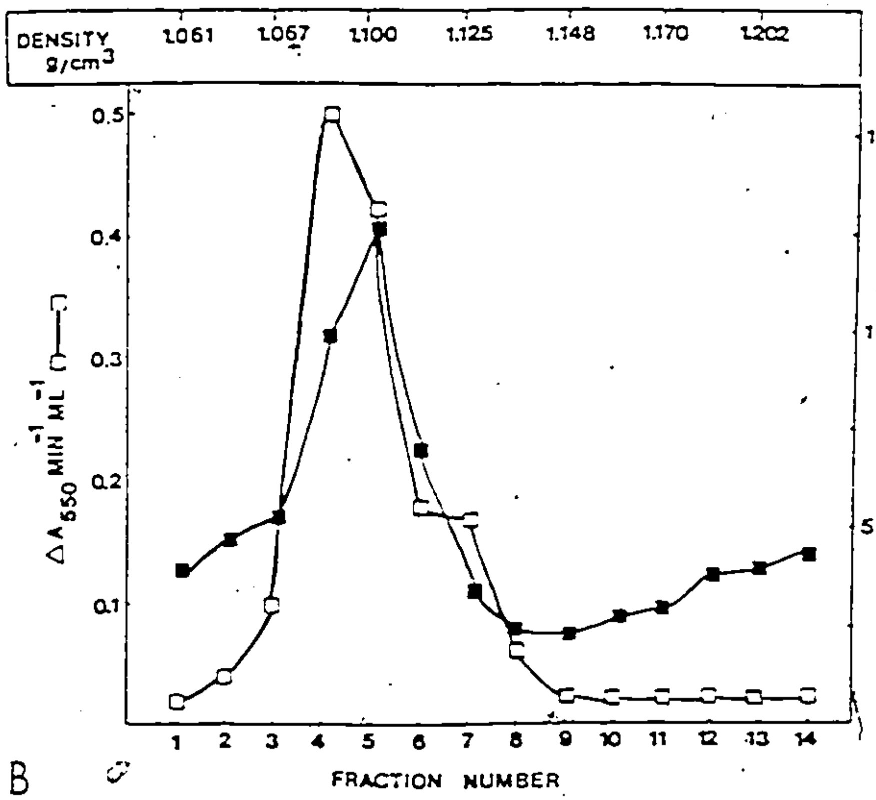
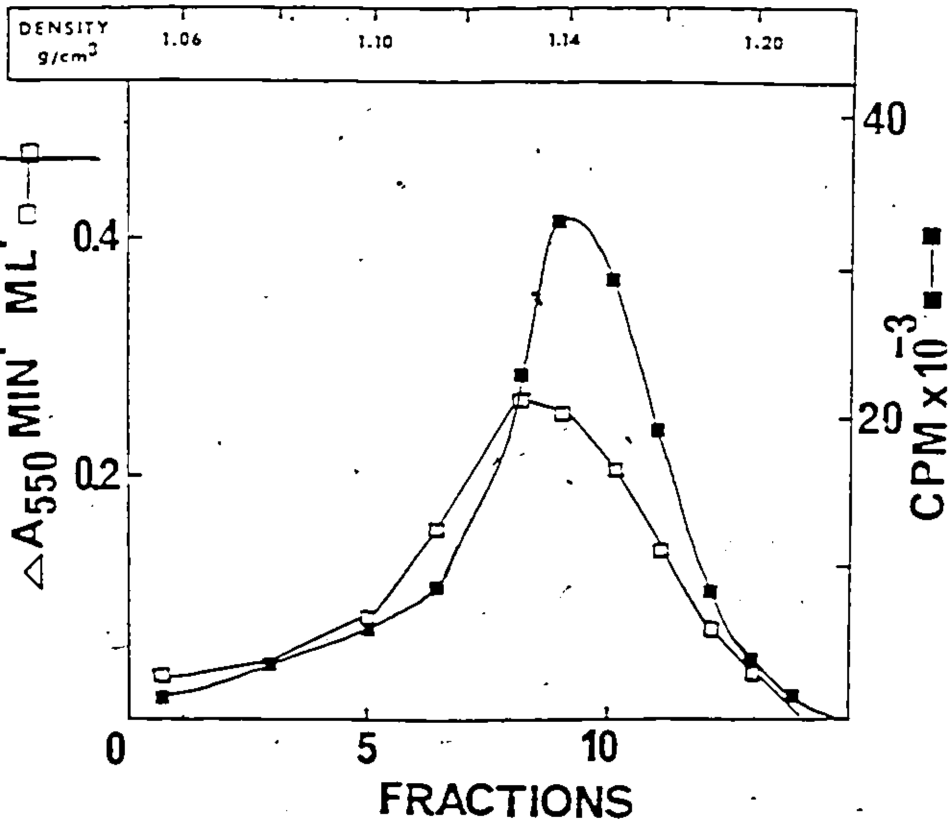

DENSITY g/cm³
1.061
1.067
1.100
1.125
1.148
1.170
1.202
0.5
0.4
0.3
0.2
0.1
ΔA₅₅₀ MIN⁻¹ ML⁻¹ □—□
15
10
5
CPM x 10⁻⁴ ■—■
1
2
3
4
5
6
7
8
9
10
11
12
13
14
FRACTION NUMBER

FIG. 6 B


DENSITY g/cm³
1.06
1.10
1.14
1.20
0.4
0.2
ΔA₅₅₀ MIN⁻¹ ML⁻¹ □—□
40
20
CPM x 10⁻³ ■—■
0
5
10
FRACTIONS

radioactivity with that of the ER marker enzyme suggested that these two parameters were associated with each other. This might in turn indicate that newly-synthesized radioactive polypeptides were associated with ER membrane.

To examine the nature of the newly-synthesized polypeptides associated with ER, [^{14}C]leucine-labelled groats were used to isolate the ER fraction. Groats were labelled for 50 min, extracted, and the extract was fractionated on a 16% and 35% discontinuous sucrose gradient. This perparative procedure allowed the isolation of sufficient quantities of ER membranes for the detection of labelled polypeptides. The membrane fraction at the 16/35 interface had the highest NADH-cytochrome c reductase activity among the four fractions of the gradient (supernatant, 16% sucrose, 16/35 interface, and 35% sucrose). The ER membranes sedimented to the 16/35 interface because of their lower density under the conditions of this experiment (no magnesium salt present). The purpose of this experiment was to demonstrate whether or not the newly-synthesized globulin polypeptides were associated with the ER directly and not through ribosomes. The use of a 16-35% discontinuous sucrose gradient in a buffer containing EDTA and free of magnesium would ensure the isolation of EDTA-stripped ER membranes at the 16/35 interface. The analysis of the labelled polypeptides associated with ER by SDS-PAGE (+Me) and fluorography revealed the presence of many radioactive polypeptides (Fig.7, lane A). Two polypeptides with molecular weight of 58-62 kDa corresponded in size to unreducible globulin dimer polypeptides (arrows). The same ER fraction was challenged with an affinity gel specific for globulin (globulin-IgG Sepharose) and the bound polypeptides were analyzed by SDS-PAGE (+Me) and

Fig. 7 Analysis of radioactive polypeptides associated with ER.

Five developing groats (18 daf) were labelled with 2 μCi [^{14}C]leucine (300 mCi/mmol) for 50 min. After homogenization, the extract was fractionated on a step gradient of 16% and 35% (w/v) sucrose. The organelles at the 16/35 interface were collected and analyzed by SDS-PAGE (12%, +Me), and a fluorograph was prepared (Lane A). The ER fraction was also challenged with globulin-IgG Sepharose. Lane B shows the labelled polypeptides which bound to globulin-IgG Sepharose. Lanes A and B contained approximately 24,000 cpm each.

Fig. 7a Transient association of globulin polypeptides with ER.

As in Fig. 7, developing groats were labelled for 50 min. In this experiment, at the end of labelling, groats were transferred into a label-free medium for a further 3 h incubation. ER was isolated as in Fig. 7 and applied to globulin-IgG Sepharose. The bound fraction was analyzed by SDS-PAGE (12%, +Me) and fluorography. Lane 0, is labelled globulin polypeptides immunoselected from the ER fraction of 50 min-labelled groats. Lane 3 is the bound fraction of the ER from groats which had been incubated for 3 h. Lane 0 contained 24,000 cpm. Lane 3 contained about 900 cpm (total amount of counts immunoselected).

Fig. 8 A similar experiment to that described in Fig. 7 was conducted but instead of using [^{14}C]leucine, [^{35}S]sulfate (900 mCi/mmol) was used for labelling developing groats for 50 min. ER was isolated and applied to a globulin-IgG Sepharose and the bound fraction was analyzed by SDS-PAGE (12%, +Me) and fluorography. Arrows indicate two labelled globulin polypeptides associated with the ER. Total of 15,000 cpm was loaded on the gel.

FIG. 7

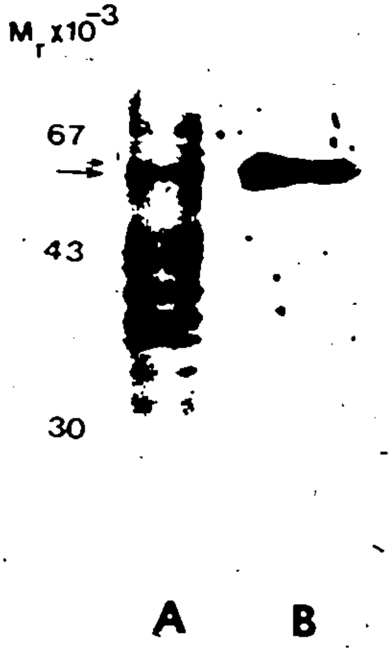


FIG. 8

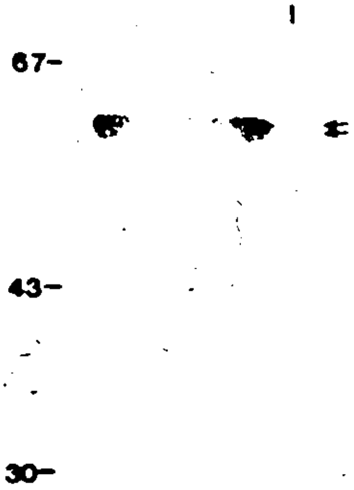
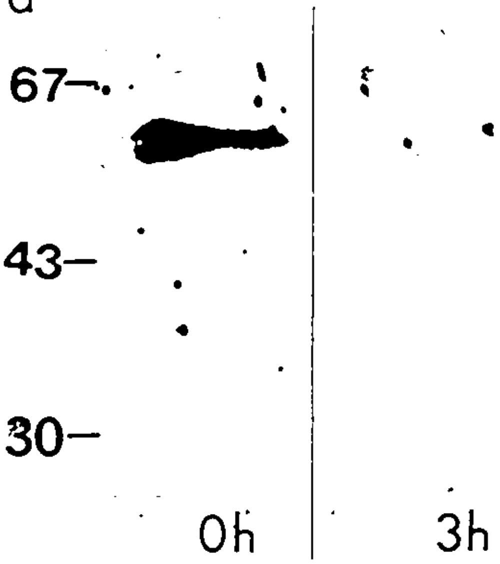


FIG. 7a



detected by fluorography (Fig. 7, lane B). Labelled globulin polypeptides with molecular weight of 58-62 kDa were detectable in the ER.

In a second part of this experiment, the 50 min labelled groats were incubated for 3 h in dH_2O, homogenized and used to isolate ER membranes. These membranes were then challenged with globulin-IgG Sepharose to detect the presence of any globulin polypeptides. The analysis of the material bound to the globulin antibodies by SDS-PAGE and fluorography showed the absence of any globulin 58-62 kDa polypeptides (Fig. 7a, lane 3). It should be mentioned that the ER fraction (from seeds labelled for 50 min and incubated for 3 h) contained TCA-precipitable radioactivity (about 900 cpm) before chromatography on globulin-IgG Sepharose. This indicated that no polypeptide in this ER fraction was capable of binding to the globulin-IgG Sepharose.

This disappearance of globulin polypeptides from the ER membranes may represent some sort of intracellular transport of these polypeptides from the ER to another site. Further investigations on such a transport mechanism will be presented in part II of the Results.

Because of the low quality of the fluorograph in Fig. 7, the above labelling experiment was repeated. Developing seeds were labelled this time with [^{35}S]sulfate for 50 min and then used for ER extraction. The ER fraction was applied to a globulin-IgG Sepharose column in order to select any globulin polypeptides present. The bound fraction was analyzed by SDS-PAGE (+Me) and fluorography. Fig. 8 is the fluorograph of the labelled globulin polypeptides selected by globulin-IgG Sepharose. The globulin 58-62 kDa polypeptides were again found in ER membranes.

In summary, Figs. 7 and 8 indicated that newly-synthesized globulin polypeptides could be found associated with the ER membranes when developing groats were labelled for a short period of time (50 min). These polypeptides seemed to be transiently and not permanently associated with the ER membranes.

D. Electron Microscopic Investigations

The role of RER in biosynthesis of reserve globulin polypeptides was suggested by the above biochemical evidence. Nevertheless, it was desirable to examine these results by electron microscopy. Therefore, in the course of the isolation, in vivo labelling, and in vitro translation of RER, various samples were subjected to eletron microscopy. Throughout this thesis multiple micrographs have been presented for each examination since this is a convention in electron microscopy.

1. Examination of the RER in intact developing oat groats.

To examine the state of the rough and smooth ER membranes in endosperm cells of groats at this period of oat seed development, fresh groats were fixed in 3% glutaraldehyde, post-fixed in 1% OsO_4 and dehydrated through an ethanol series. Gold sections (90 nm) were prepared and examined at 60 kV. Figs. 9 A-D illustrate the transmission electron micrographs of 16 daf oat groats. Membranes (ER) were abundant in developing cells. Fig. 9A shows a number of protein bodies varying considerably in size. These protein bodies seemed to be connected with one another through a network of membranes. These interconnections may however be artifactual and could be due to structural damage during fixation of seed sections. In Fig. 9A, stacks of ER membranes were visible stretching and surrounding the protein bodies. In the same figure (upright position) four protein bodies were present with no detectable association with the ER membranes. Fig. 9B displayed three circular vacuoles consisting of many protein bodies. A large starch granule was also present which seemed to be intact. Starch granules in other figures showed certain cracks which could be a result of sectioning

Fig. 9 Transmission electron micrographs of developing (16 daf) oat starchy endosperm cells.

Fig. 9A. A partial image of an endosperm cell containing protein bodies and stacks of membranes resembling the ER. Magnification (M) x 14,000.

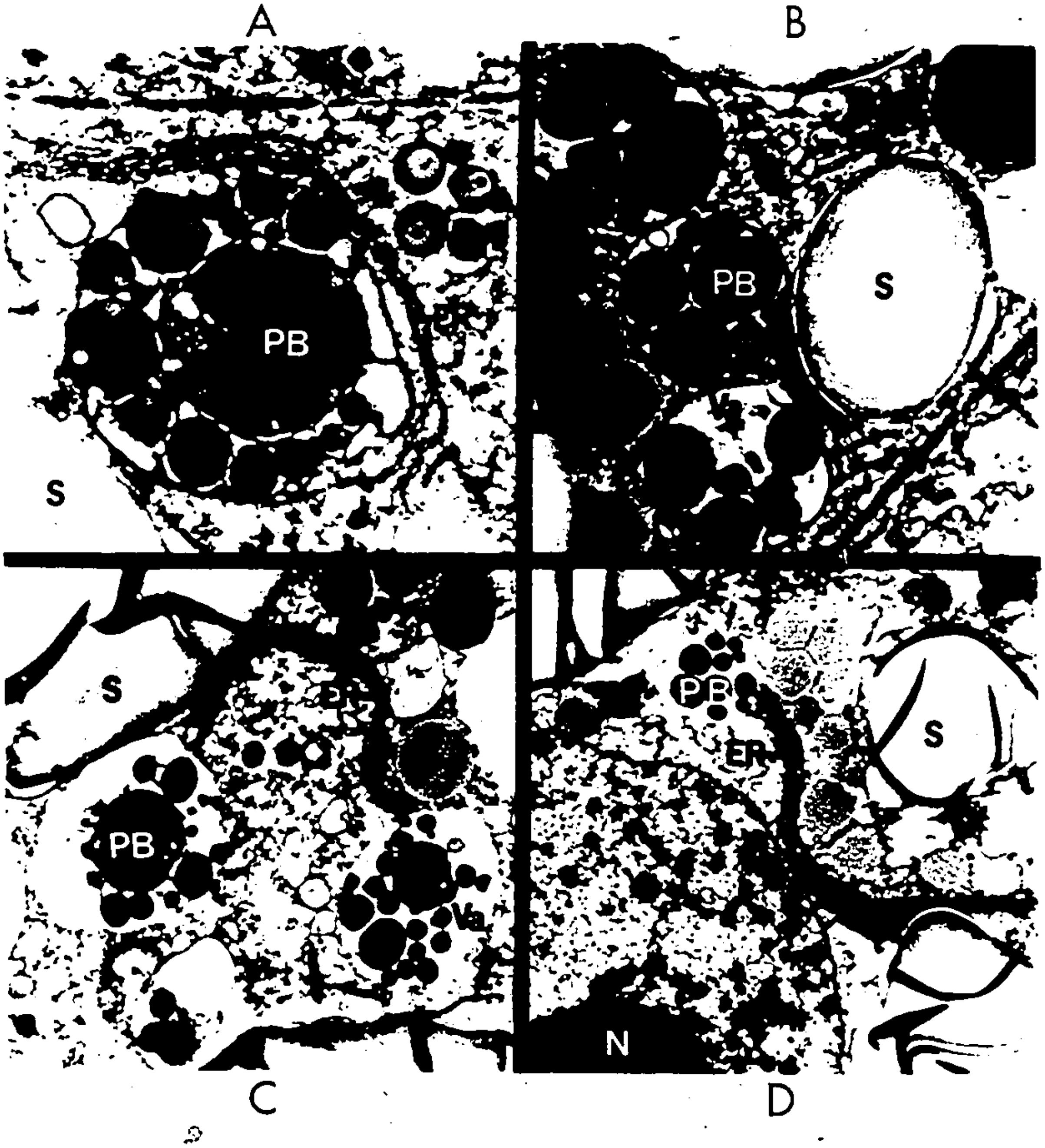
Fig. 9B. Numerous protein bodies are found within intracellular vacuoles demonstrating an organized pattern of deposition. The size of these protein bodies varies considerably within the same vacuole. M x 11,000.

Fig. 9C. Stacks of membranes are observed stretching from one vacuole to another. Each vacuole contains protein bodies of various size. M x 9,000.

Fig. 9D. This micrograph also shows the connections between ER-like membranes and tonoplast (vacuolar membrane). The ER strands seem to be intensely stained which may suggest them to contain electron-dense material. M x 6,000.

Abbreviations: ER, endoplasmic reticulum; L, lipid body; N, nucleus; PB, protein body; S, starch; Va, vacuole; W, cell wall.

FIG. 9



and fixing the seeds. As in Fig. 9A, there were two large vacuoles loaded with protein bodies in Fig. 9C. These two vacuoles seemed to be connected via stacks of ER membranes. In Fig. 9C, numerous small vesicles could be observed in the cytosol and within the vacuoles; these may be artifactual resulting from the fixation of the cells. Some of these vesicles may however represent true structures involved in protein body development. Interconnections between the ER membranes and intracellular vacuoles could also be detected in Fig. 9D. This electron micrograph also showed the association of the ER with nuclear membrane.

The apparent presence of significant amounts of ER membranes and their association with electron-dense protein bodies tended to support the notion that 16 daf seeds were actively involved in protein synthesis.

2. Isolation and examination of ER membranes

For experiments concerning <u>in vivo</u> labelling and <u>in vitro</u> translation of the RER, sucrose gradient fractionation techniques were used to isolate EDTA-stripped ER membranes and RER membranes (see Materials and Methods). The identity and purity of these membrane preparations were checked biochemically by testing for ER marker enzyme activity. This was not, however, adequate to confirm sample integrity or purity. Therefore, elecron microscopy was employed to further examine these membranes. Membrane fractions were pelleted and the pellets were fixed in 3% glutaraldehyde. The fixed pellets were post-fixed in 1% OsO_4, dehydrated, and embedded in plastic (Spurr's resin). Gold sections (90 nm) were prepared and examined with a Philips electron microscope.

Figs. 10 A-C show the TEM images of the ER membranes isolated by a 16%/35% step sucrose gradient. This membrane fraction was assumed to be rich in ribosome-free ER under the conditions used in this extraction method. Figs. 10A and 10B illustrate stacks of ribosome-free ER membranes; but few RER membranes were actually detectable. This was due to the absence of magnesium ions in the extraction buffer which caused the stripping off and loss of ribosomes. ER membranes also formed spherical vesicles (ERv). Vesicle-formation is a common phenomenon in the isolation of ER membranes. ER cannot be isolated in an intact form because it forms an interconnecting network of membranes throughout the cell. Homogenization of the tissue results in the vesiculation of the membranes. Generally the membrane preparations were not contaminated with protein bodies or other major membrane fractions.

The RER samples which were isolated by a linear 15-50% and a step 20%/35% sucrose gradients were also examined. These preparations should theoretically be rich in RER because of the presence of magnesium during sample preparation (see Materials and Methods). Figs. 11 A-C illustrate the TEM images of the RER pellets (linear 15-50% sucrose gradient). The RER sample isolated from the 20/35 interface looked identical to that isolated by linear gradient (not shown). Membrane vesicles covered with numerous ribosomes were visible. Ribosomes were well stained with OsO_4, but the membranes were less visible. Alternatively, when the RER preparations were post-fixed with K_2MnO_4 instead of OsO_4, membranes were preferably stained over ribosmes (Fig. 11D). As a result of centrifugation, some of the membranes appeared to have lost their ribosomes. Similar to ribosome-free ER membranes, the RER preparations were also found in the form of vesicles.

Fig. 10 TEM examination of ribosome-free ER extracts (isolated by 16-35% step sucrose gradient).

Fig. 10A. This ER extract contains primarily long strands of membranes. Ribosomes are present but at low frequency. M x 88,000.
Fig. 10B. A second image of the same extract showing the presence of some membranes in the form of vesicles. M x 34,000.
Fig. 10C. This extract contains mainly vesicles with occasional ribosomes attached. M x 34,000.

Abbreviations: ER, endoplasmic reticulum;
ER_v, endoplasmic reticulum vesicle.

FIG.10

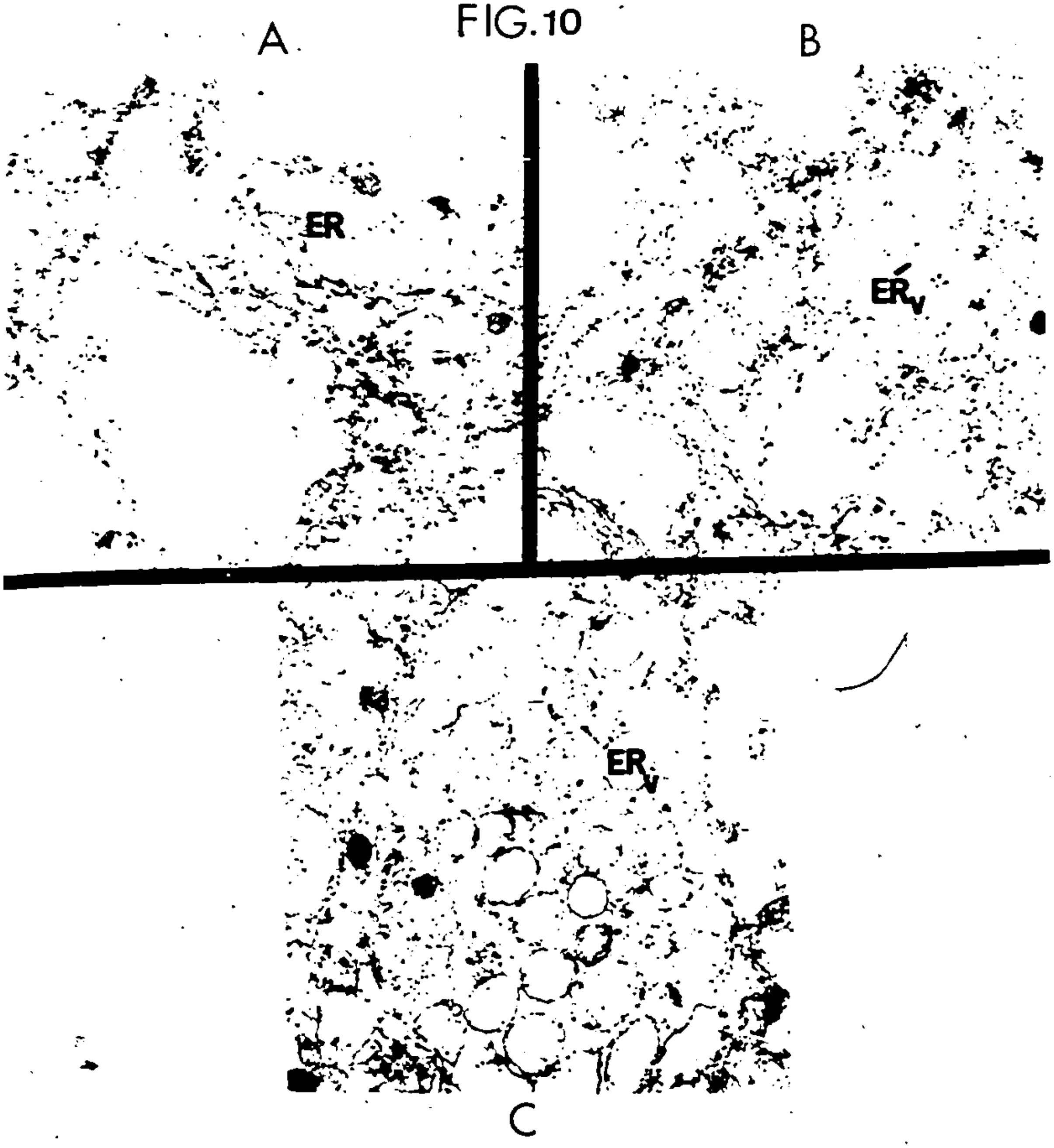
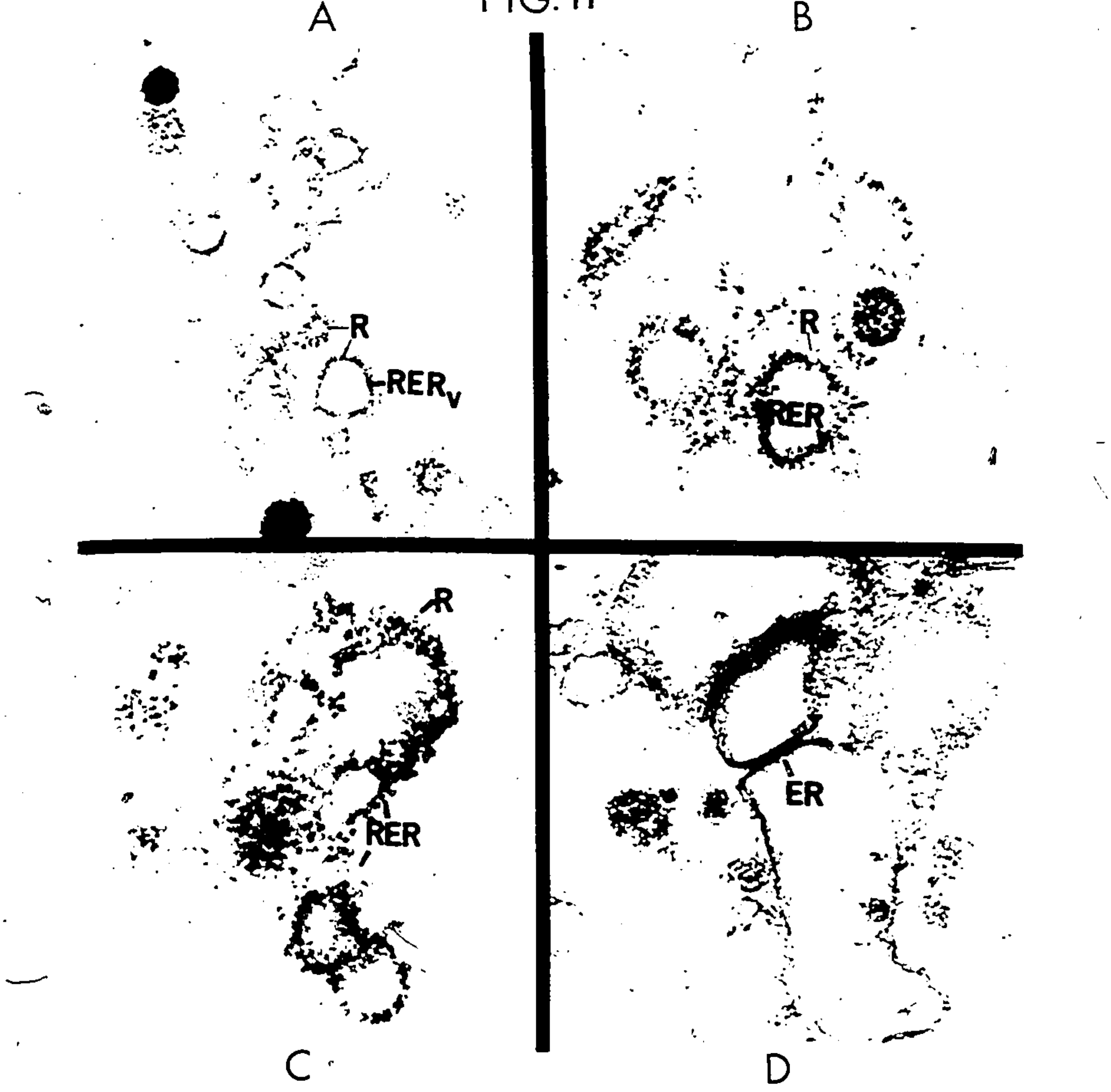


Fig. 11 TEM examination of rough ER extracts (isolated by 15-50% linear sucrose gradient) of developing oat groats.

Fig. 11A-C. An extract of RER membranes which was post-fixed with OsO_4. Membranes in the form of vesicles are observed with numerous ribosomes attached. Post-fixation with OsO_4 preferably stained ribosomes. M x 27,000, M x 52,000, and M x 52,000 respectively.
Fig. 11D. The same extract as in Figs. 11A-C which was post-fixed with K_2MnO_4 instead of OsO_4 in order to stain membranes. Large and small vesicles are observed. Ribosomes are not stained. M x 52,000.

Abbreviations: ER, endoplasmic reticulum; R, ribosomes; RER, rough endoplasmic reticulum. RER_v, rough endoplasmic reticulum vesicle.

FIG. 11



These observations confirmed the pureness of the RER samples analyzed in the above-mentioned _in vivo_ and _in vitro_ experiments and indicated the reliability of the procedures used to isolate these membranes.

PART II - GLOBULIN TRANSPORT

It was previously demonstrated that oat globulin 58-62 kDa polypeptides associated with ER membranes after a short labelling period (see Fig. 7). These associated proteins were also shown to be transiently associated with the ER membranes. The disappearance of the globulin polypeptides from the ER posed a question as to the eventual destination of these polypeptides. An experiment was designed to analyze various intracellular organelles simultaneously during an in vivo labelling experiment. Fractionation of a homogenate of developing groats on a 16-65% sucrose gradient provided a system where all major organelles such as ribosome-free ER, RER, microbodies, mitochondria, chloroplasts, and protein bodies could be separated on a single gradient. Based on this system, the following labelling experiment was carried out.

A. In vivo labelling of developing seeds and analysis of proteins associated with various intracellular organelles.

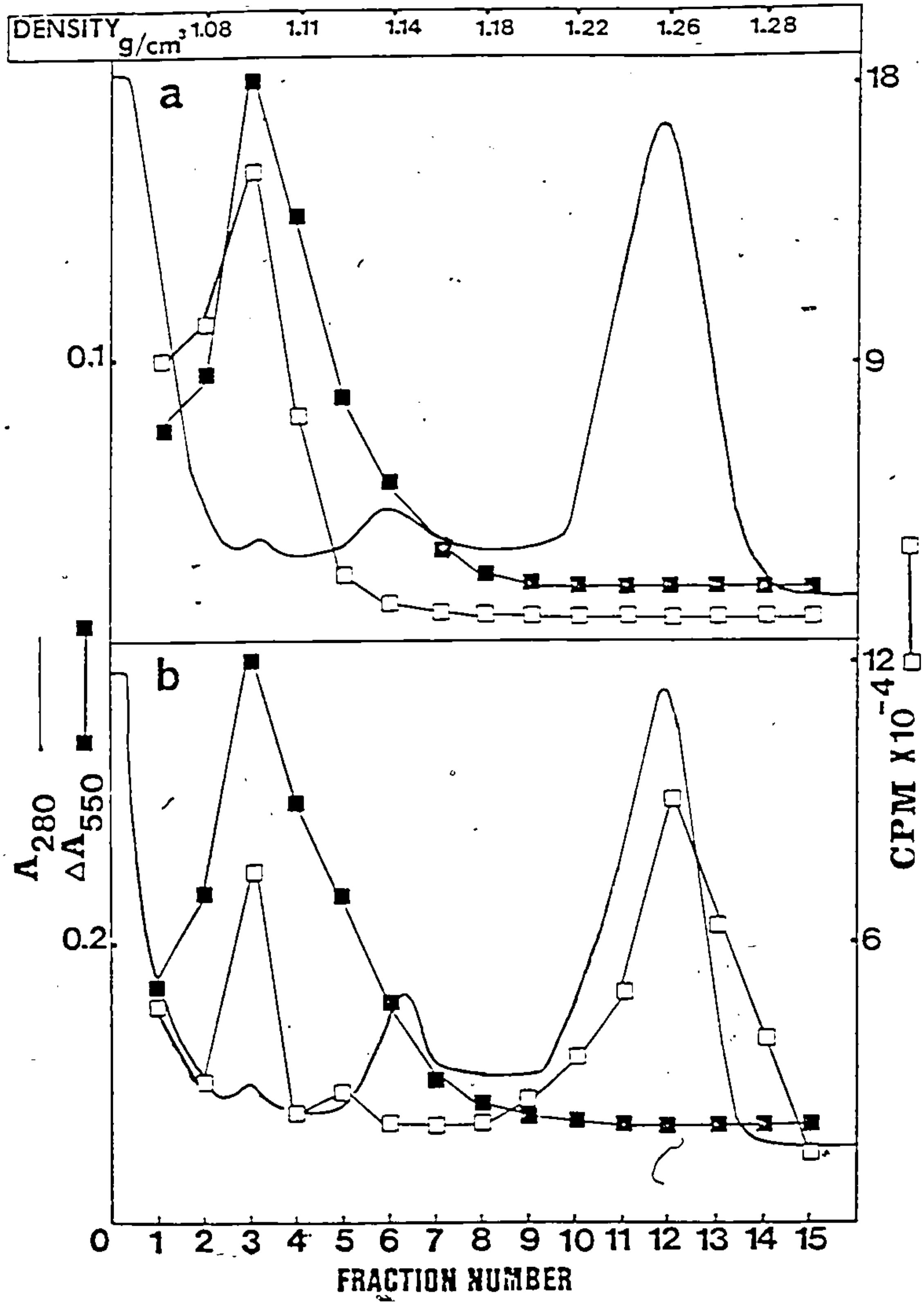
Developing spikelets were labelled with [^{35}S]sulfate for 45 min, a number of seeds were harvested (0 h) and the remainder were incubated for 20 h in the presence of cycloheximide (20 h). The labelled seeds were homogenized and the homogenates fractionated on a linear 16-65% sucrose density gradient. The gradient fractions were monitored at 280 nm for protein, and analyzed for NADH-cytochrome c reductase activity and for TCA-precipitable radioactivity (Fig. 12a an b). At 0 h, most of the radioactivity was located at the density of 1.10 g/cm^3 corresponding to the position of the ER marker enzyme in the gradient (Fig. 12a). No significant proportion of the radioactivity was detected in the protein

Fig. 12 Distribution of incorporated radioactivity in ER and protein bodies upon *in vivo* labelling of developing groats.

Developing spikelets (18 daf) were labelled with $[^{35}S]$sulfate (900 mCi/mmol) for 1 h and transferred to a label-free medium. Groats were detached at 0 and 20 h after transfer, homogenized, and fractionated on linear 15-65% sucrose gradients. The gradients were monitored for absorbance at 280 nm and fractionated into 1 ml fractions. TCA-precipitable radioactivity was determined and expressed as CPM x 10^{-4}, (□—□). NADH-cytochrome c reductase activity was expressed as A_{550} min^{-1} ml^{-1} (■—■).

(a). The gradient profile of the extract from seeds with a 0 h incubation period.
(b). The gradient profile of the extract from seeds with a 20 h incubation period.

FIG. 12


DENSITY g/cm³
1.08
1.11
1.14
1.18
1.22
1.26
1.28
a
b
0.1
0.2
18
9
12
6
A280
ΔA550
CPM X10⁻⁴
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
FRACTION NUMBER

body fraction (density of 1.26 g/cm^3). No appreciable radioactivity was found in the mitochondria and chloroplast fractions from the gradient (1.16-1.20 g/cm^3) (105). These observations indicated that most of the newly-synthesized reserve proteins were associated with ER after 1 h pulse and compared to the previous results (see Fig. 6). However after 20 h, a large proportion of the incorporated radioactivity (about 50%) was found associated with the protein body fractions (density of 1.26 g/cm^3, Fig. 12b). The ER fractions contained a much lower proportion of total radioactivity (about 25%). The radioactivity associated with the ER may represent membrane proteins or reserve proteins which were not transported out of the ER. It may also represent newly-synthesized proteins yet to be transported out of the ER. Some radioactivity was present in other organelles but was insignificant compared to that of protein bodies. Such a shift of labelled proteins from ER to the protein bodies after a long incubation period might indicate the intracellular transport of the newly-synthesized reserve proteins from the ER to protein bodies. Further discussion of deposition pathways will be provided in the Discussion Chapter.

During the 20 h incubation of spikelets, cycloheximide at a concentration of 0.5 mM was included in the incubation medium. The application of cycloheximide in this and a few other <u>in vivo</u> labelling experiments was to inhibit continued incorporation of radioactivity into proteins after termination of labelling. However, since cycloheximide is a potent inhibitor of protein synthesis, possible drawbacks of its use should therefore be considered. While inhibiting the synthesis of reserve proteins, cycloheximide may also inhibit the synthesis of membrane proteins and thus prevent the formation of new membrane structures. However, in the relatively short period of time in which these experiments were carrried out, synthesis of new membrane structures may not be required. This assumption was supported by the fact that results were similar whether or not cycloheximide was included in labelling experiments. For example, in the case of the above transport experiment, the shift of radioactivity from the ER to protein bodies was observed in presence or absence of cycloheximide. The only detectable effect of cycloheximide was partial inhibition of continued incorporation of radioactivity into proteins associated with the ER.

B. Electron Microscopic Investigations.

The transmission electron microscopic observations, discussed in part I.D. of this chapter, indicated the close association of ER with protien bodies (Figs. 9 A-D). The intracellular transport of reserve globulin was also briefly discussed based on those TEM investigations. A more comprehensive search of seed sections revealed further evidence for the involvement of the ER membranes in the development of protein bodies. Oat developing seeds (16 daf) were fixed, dehydrated, embedded in plastic (Spurr's resin), and sectioned. The sections were viewed under an electron microscope. Figs. 13 A-D illustrate the TEM images of developing endosperm cells. In Fig. 13A, a large vacuole was seen with close association with two stacks of ER membranes. Electron-dense protein bodies could be seen at the sites of connections among the vacuole and the ER membranes. Higher magnification of the upper section of Fig. 13A reveals this phenomenon more clearly (Fig. 13B). A protein deposit was found within the stacks of ER in the vicinity of the vacuole. Other protein bodies were found in the same vacuole. Figs. 13C and 13D also demonstrate the development of a protein deposit within the ER membranes. In Fig. 13C, stacks of ER membranes were seen stretching to the surface of a vacuole. At the site of connection between the ER membranes and the tonoplast, a protein deposit could be seen. This area of interest in Fig. 13C was magnified to show the interconnections more clearly (Fig. 13D).

The electron microscopic images discussed above could be interpreted as showing a seqeence of events leading to the development of protein bodies in developing seeds. This sequence may have occurred as follows: proteins were synthesized and accumulated in the ER lumen; the

Fig. 13 TEM investigations on intracellular transport of reserve proteins in developing oat endosperm (16 daf).

Fig. 13A. A large vacuole is observed with protein bodies of various size. At two corners of this vacuole, connections exist with stacks of membranes resembling the ER. M x 13,600. The arrows indicate the protein bodies which are assumed to be at a developing stage.

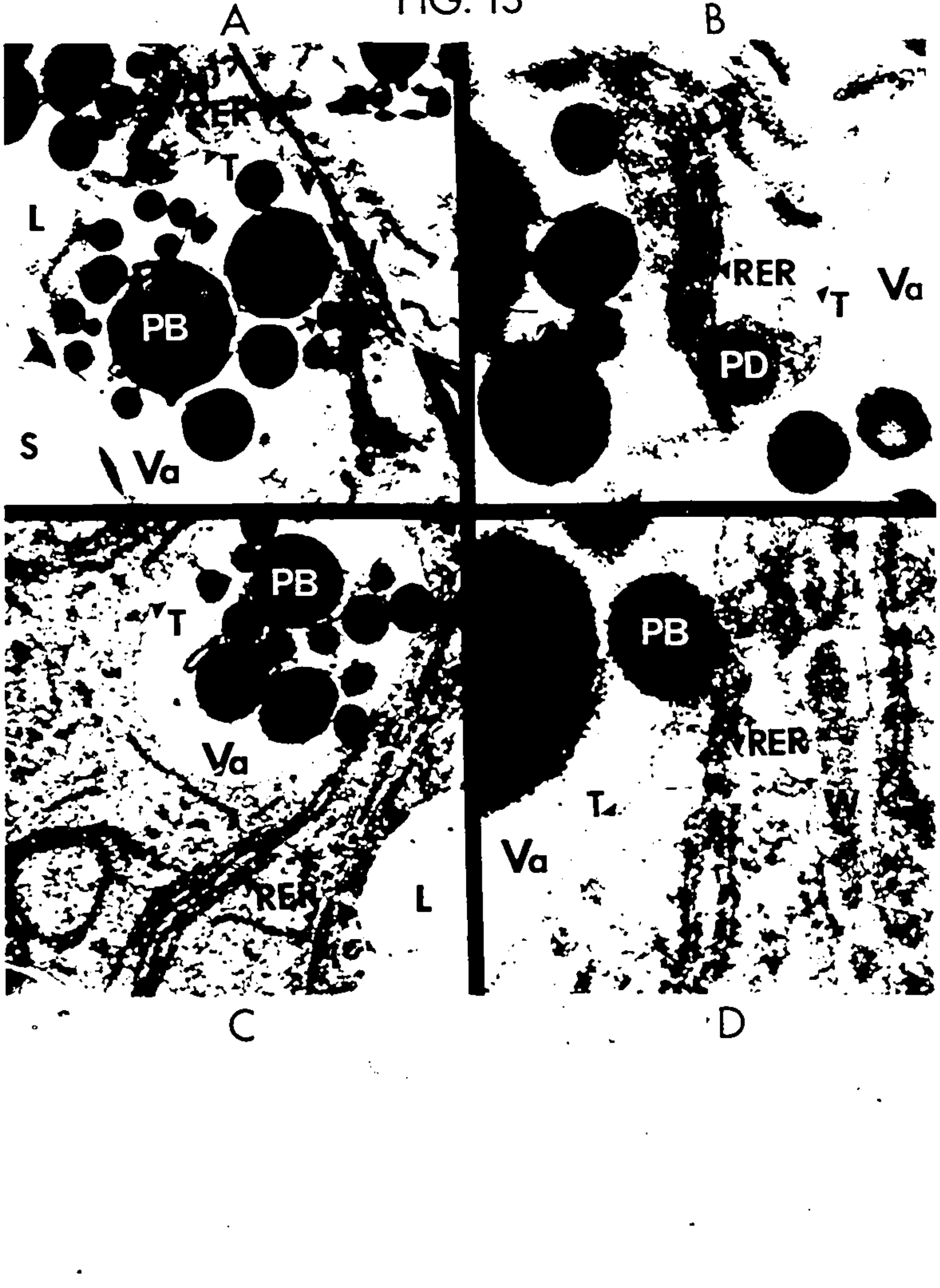
Fig. 13B. A higher magnification image illustrates more clearly the development of a protein deposit at the interface between the ER and the vacuole. A few other protein bodies in the vacuole may have just been developed. M x 34,000.

Fig. 13C. ER strands appear to be connected to tonoplast of a large vacuole. At the site of connection a protein body is observed. This electron-dense vesicle is inside the vacuole but appears to be still attached to the ER. M x 11,000. The arrow indicates a protein body assumed to be developing.

Fig. 13D. A higher magnification of Fig. 13c reveals the interconnections more clearly. M x 44,000. The arrow indicates the site of interconnections among a protein body, endoplasmic reticulum, and tonoplast.

Abbreviations: RER, rough endoplasmic reticulum; L, lipid droplet; PB, protein body; PD, protein deposit; S, starch; T, tonoplast, Va, vacuole; W, cell wall.

FIG. 13



newly-synthesized proteins were chanelled via the ER lumen to form a protein deposit at the surface of the tonoplast (vacuolar membrane); finally the protein deposit was released into the vacuole as a protein body possibly through the fusion of the ER membranes with the tonoplast. This mechanism may be regarded as the major pathway of globulin synthesis and deposition since such electron microscopic images could be found frequently within the seed sections examined. However, there may be other possibly minor pathways of globulin deposition.

PART III - GLOBULIN PROCESSING

A. In Vivo Labelling Studies on Cleavage of Globulin Precursors.

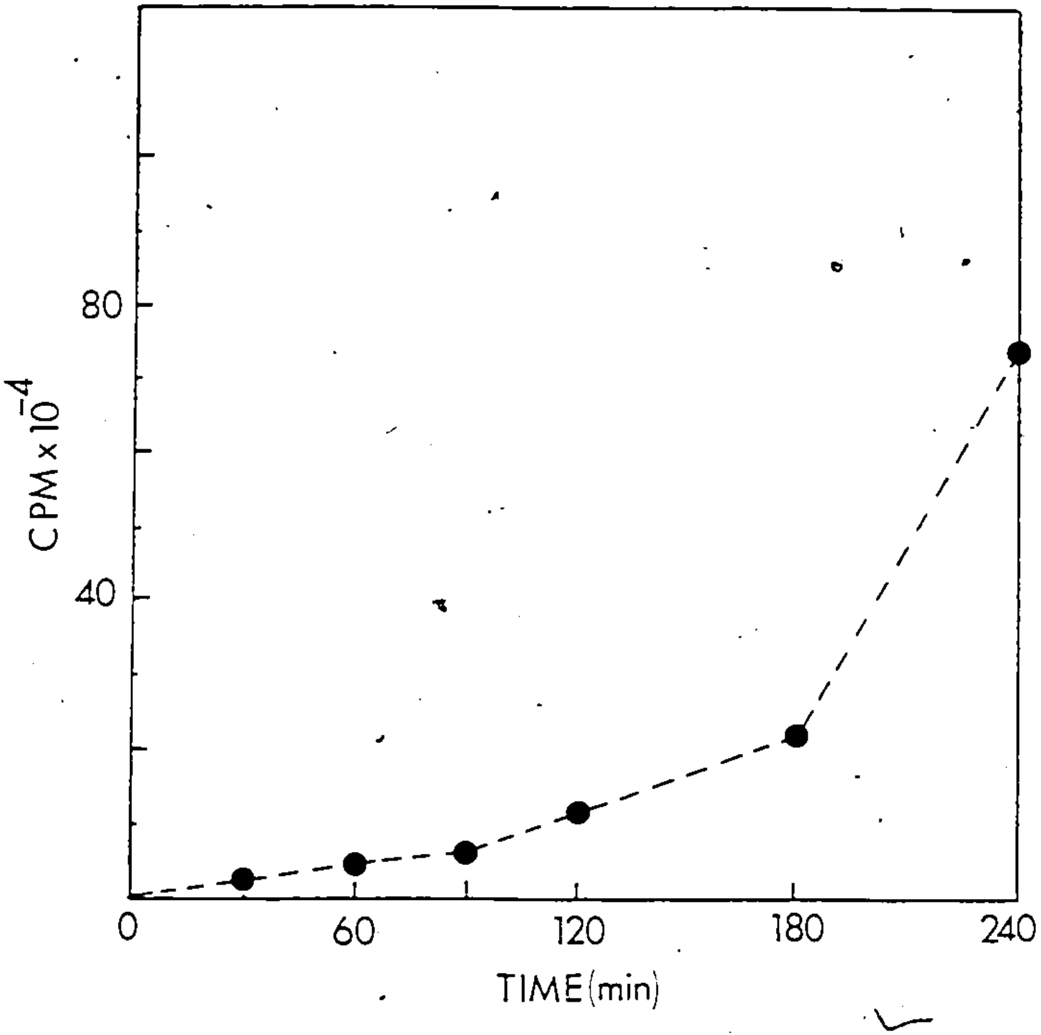
In vitro translations and preliminary in vivo labelling studies of the ER (See PART I) suggested that globulin polypeptides were synthesized as larger 58-62 kDa precursors. This hypothesis which has also been suggested by other investigators in the field (67,69,70) was tested by a series of in vivo labelling and pulse-chase labelling experiments.

First, developing oat spikelets were labelled continuously with [^{35}S]methionine to determine the pattern of incorporation into globulin polypeptides. Spikelets were labelled for 30, 60, 90, 120, 180, and 240 min. Groats (total of about 100 mg) were detached at each time period and were extracted for globulin. The globulin extracts were counted after precipitation in 10% TCA and boiling in 5% TCA. Fig. 14 demonstrates the total amount of radioactivity incorporated into the globulin fraction. The rate of incorporation was slow at the beginning of labelling and gradually increased. The most pronounced increase in incorporation was observed between 180 min and 240 min. This may indicate a slow rate of uptake of radioactivity by the spikelets. However, a qualitative (using a radioactive monitor) monitoring of the whole spikelets during the labelling period showed that radioactivity was taken up by the plant after only 5 min. At this time the hulls (covering the seeds) had already taken up a significant amount of radioactivity but the seeds were relatively less radioactive. These observations indicated a slow rate of transport of radioactive amino acids from the hulls to the seeds. It seemed likely that a major portion of the label was metabolized in other tissues such as hulls before entering the seeds.

Fig. 14 In vivo incorporation of [^{35}S]methionine into globulin polypeptides.

Developing spikelets (16 daf) were labelled with [^{35}S]methionine for 30, 60, 90, 120, 180, and 240 min. Groats were detached and used to extract globulins. The globulin extracts were counted after TCA precipitation in 10% TCA for a minimum of 15 min, boiling in 5% TCA in presence of cold methionine for 5 min, and washing in cold 5% TCA. Incorporation into TCA-precipitable radioactivity is expressed as CPM x 10^{-4}.

FIG. 14



An equal amount of protein from each time period was analyzed by SDS-PAGE and fluorography to visualize the labelled globulins. Fig. 15 demonstrates the pattern of labelled globulins upon continuous labelling of seeds. This pattern compared well with the results in Fig. 14 and demonstrated a pronounced increase in the amount of labelled globulin between 180 min and 240 min. At the early time periods, the labelled globulin was represented by two polypeptides with molecular weight of 58-62 kDa. However, after 120 min of labelling, other polypeptides with a molecular weight range of 12-40 kDa also appeared on the fluorograph. Two groups of polypeptides with average molecular weights of 20 and 38 kDa corresponded in size to the cold globulin α-polypeptides and β-polypeptides (visualized by staining the gel with Coommassie Brilliant Blue dye). The globulin large subunits were not labelled as well as the smaller subunits. The reason behind this particular labelling pattern will be discussed later in this section.

Pulse-chase labelling with [^{35}S]methionine:

The data in Figs. 14 and 15 indicated that a 60 min or a 90 min labelling period was sufficient to label the globulin 58-62 kDa putative precursors. This period was short enough not to allow the labelling of the other smaller globulin polypeptides. Therefore for pulse-chase labelling, developing spikelets were labelled for 60 min with [^{35}S]methionine and then were transferred into a chase medium containing cold methionine. Groats were detached after 1 h of labelling and at 1, 2, 3, 5, and 10 h of chase. Globulin was extracted and the total amount of incorporated radioactivity was determined. Fig. 16 shows the total

Fig. 15 Pattern of $[^{35}S]$methionine-labelled globulins.

The globulin samples from Fig. 14 were analyzed by SDS-PAGE (13%, +Me) and fluorography. An equal amount of protein was loaded on each lane. Fluorograph was exposed for 5 days. Numbers represent times of labelling in minutes. Arrowheads indicate the 58-62 kDa polypeptides. Molecular weight markers are $[^{14}C]$methylated bovine serum albumin (69,000), $[^{14}C]$methylated ovalbumin (46,000), $[^{14}C]$methylated carbonic anhydrase (30,000), and $[^{14}C]$methylated lysozyme (14,300).

FIG.15

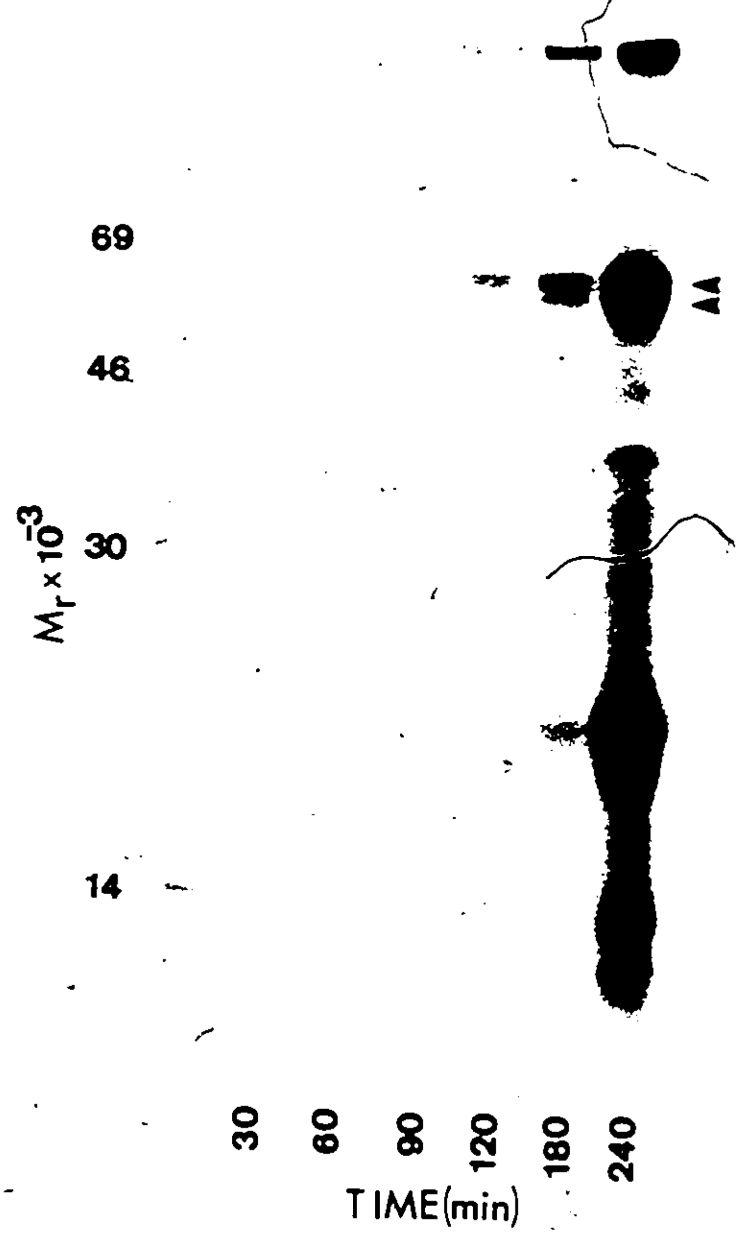
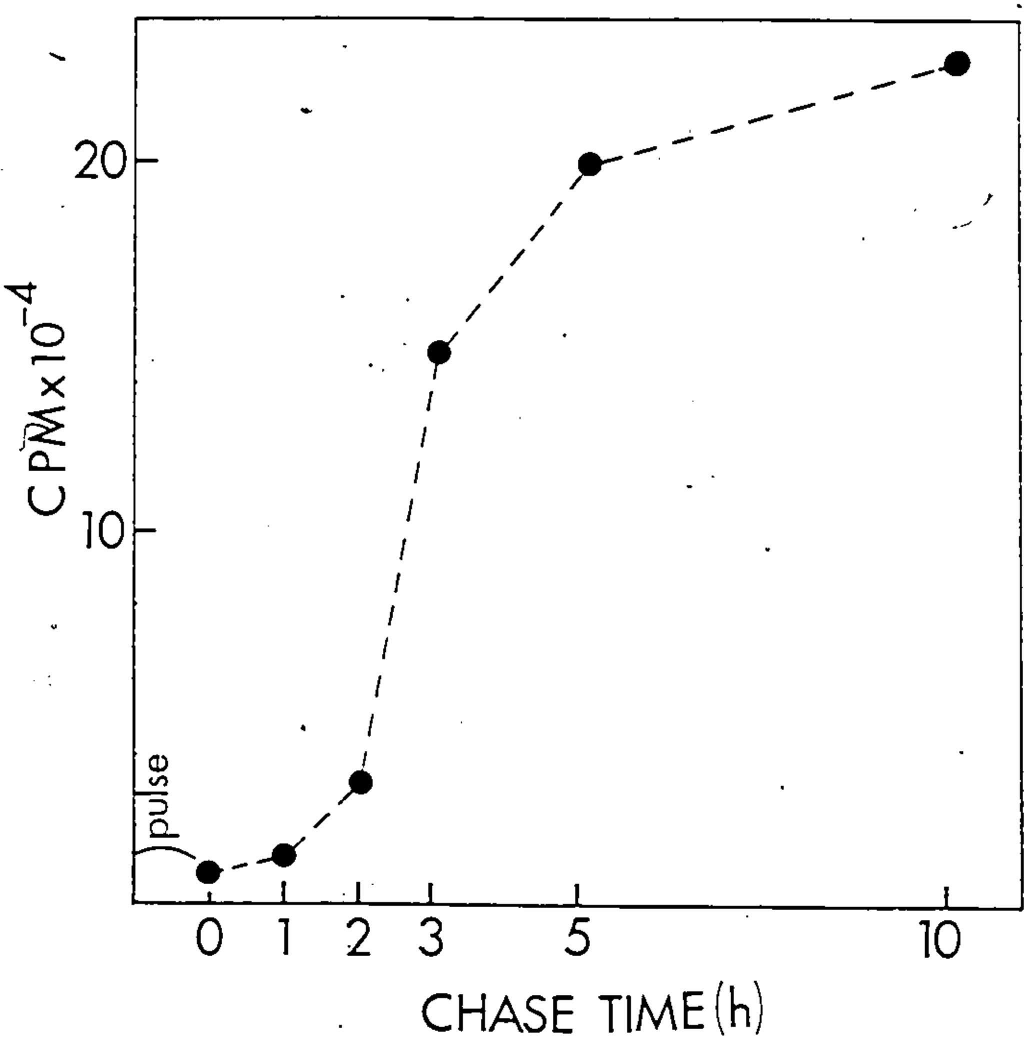


Fig. 16 Pulse-chase labelling of globulin polypeptides.

Developing spikelets were labelled for 1 h with [^{35}S]methionine and transferred into a chase medium containing cold methionine. Groats were detached at various times (0, 1, 2, 3, 5, and 10 h) and used to extract globulins. The globulin extracts were counted by TCA-precipitation. Incorporation into TCA precipitable radioactivity is expressed as CPM x 10^{-4}.

FIG. 16



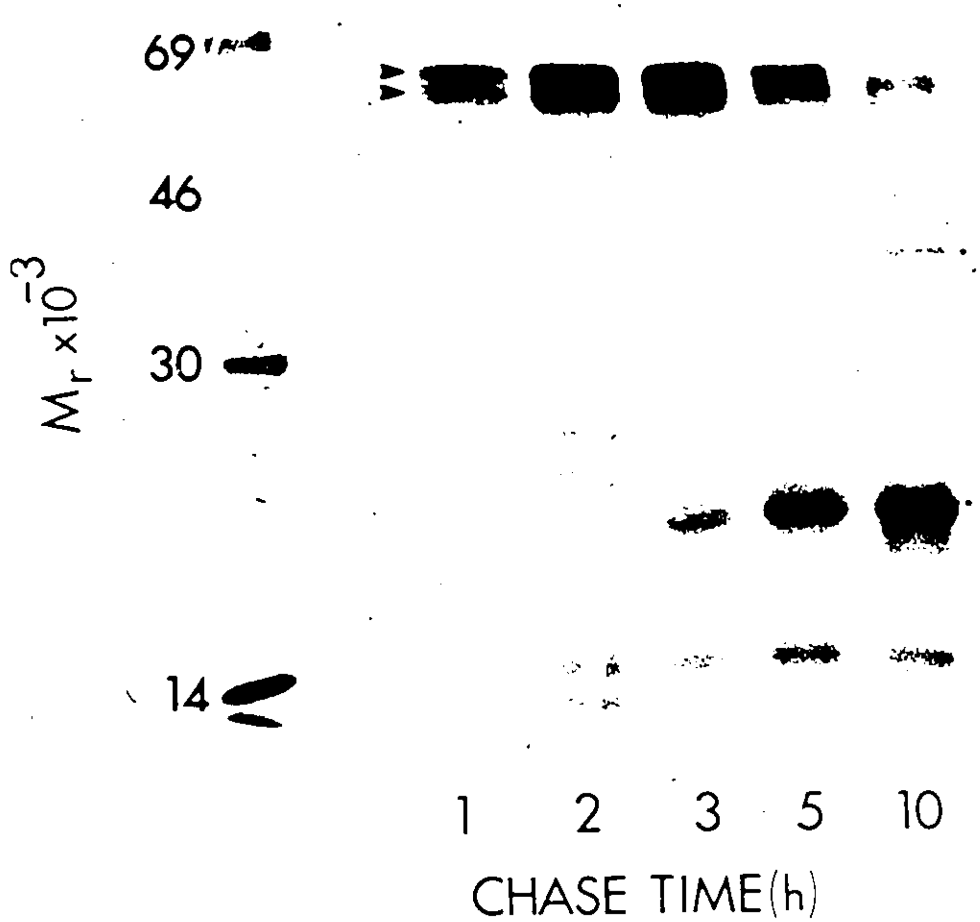
amount of incorporation of $[^{35}S]$methionine into the globulin fraction in pulse-chased seeds. A significant increase in the incorporated radioactivity occurred after 2 h chase. This increase in incorporation continued even after 10 h chase although the rate of increase was lower than that observed between 2 and 3 h chase. Interestingly, the pattern of incorporation was similar to the pattern previously seen in the continuous labelling experiment (Fig. 14). The most pronounced increase in incorporation occurred after 3 h in both cases (3 h of labelling in Fig. 14; 1 h of labelling and 2 h of chase in Fig. 16). Therefore, it appeared that the transfer to chase medium had very little effect at the early hours of chase. However, the chase began to take effect ~~at later~~ times since the peak of radioactivity appeared to be reaching a plateau at 10 h chase.

To investigate the pattern of labelled globulins in the above pulse-chased seeds, equal number of counts (5000 cpm) were analyzed by SDS-PAGE and fluorography (Fig. 17). After 1 h of chase, two 58-62 kDa globulin polypeptides were labelled. These polypeptides persisted during the chase period although their relative intensity decreased between 3 and 10 h of chase. Presumed α-polypeptides and β-polypeptides appeared after 2 h of chase and their intensity increased gradually afterwards. A correlation seemed to exist between the decrease in the amount of the 58-62 kDa polypeptides and the increase in the amount of the lower molecular weight polypeptides. There are two possible explanations for this labelling pattern. First, the 58-62 kDa and α and β polypeptides were labelled at different times independently of each other. Secondly, the 58-62 kDa polypeptides were first labelled and later converted to the lower molecular weight polypeptides. If the first possibility were

Fig. 17 Pattern of pulse-chased globulin polypeptides.

The globulin samples from Fig. 16 were analyzed by SDS-PAGE (13%, +Me) and fluorography. Equal numbers of counts (5000 cpm) were loaded on the gel. The fluorograph was exposed for 10 days. Numbers represent the chase times in hours. Arrowheads indicate putative globulin precursors. Dots indicate the position of globulin α and β-polypeptides (corresponding to cold globulins present in the gel and visualized by Coommassie Brilliant Blue staining).

FIG. 17



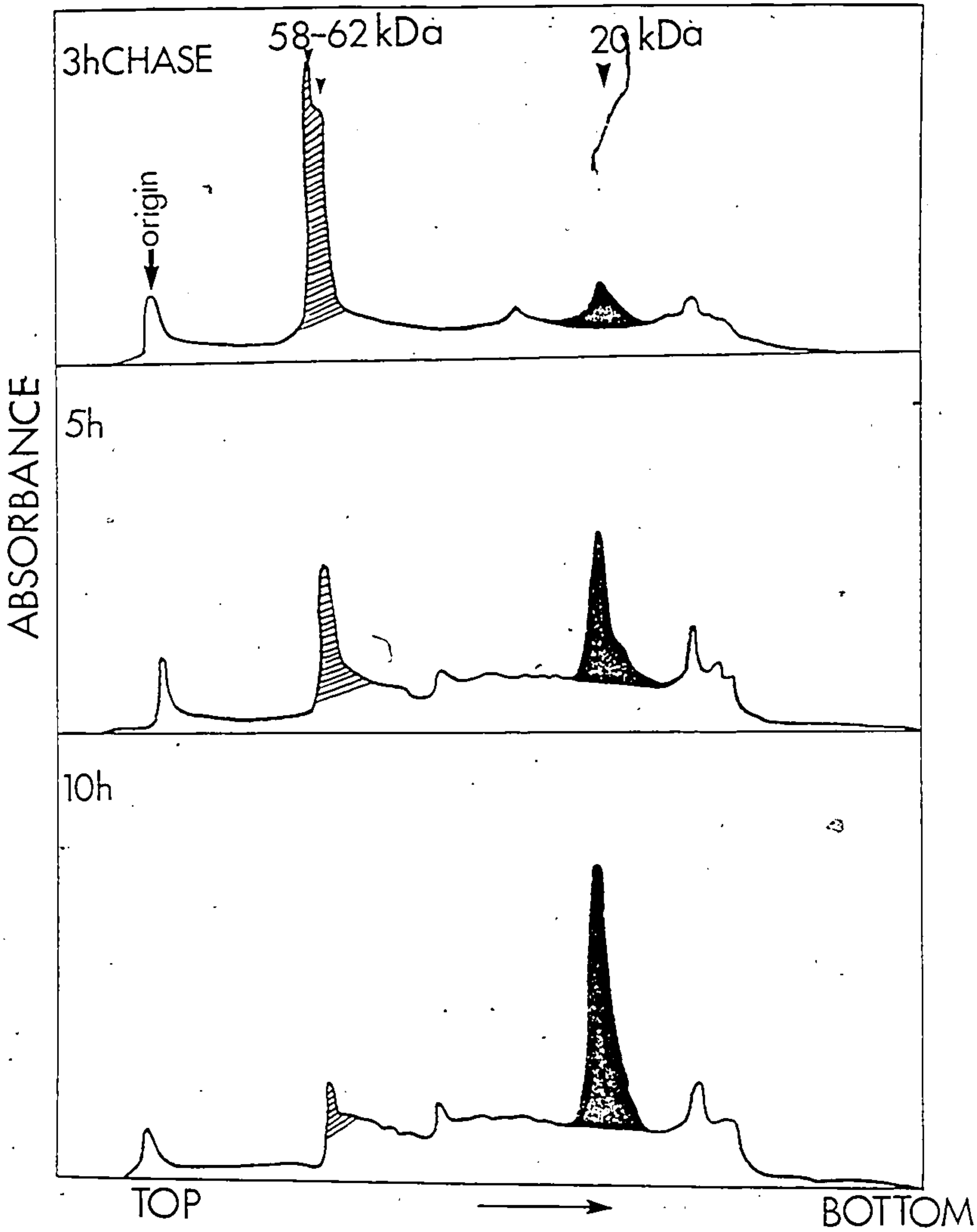
true, the decrease in the intensity of the 58-62 kDa polypeptides could not be logically explained. Evidence seemed to point to the second theory of precursor-product relationship. In order to quantitate the changes in the intensities of globulin polypeptides, lanes 3, 5, and 10 were scanned using a densitometer. Fig. 18 shows the quantitative relationships among the 58-62 kDa and the smaller 20 kDa polypeptides. There was a 4 fold reduction (calculated by measuring peak weights) in the amount of 58-62 kDa polypeptides from 3 h to 10 h chase. At the same period of time, the β-polypeptides increased in intensity by about 4 fold. Only the β-polypeptides were quantitated because they were labelled preferentially over the α-polypeptides (38 kDa). The plot of peak weights revealed a direct relationship between the reduction in the amount of precursors and the increase in the amount of the β-globulins (data not shown). The total signal on each lane was roughly equal. These data provided some quantitative evidence for the precursor-product relationship.

There were two major problems encountered in the above pulse-chase labelling experiment. First, incorporation of label into proteins continued despite the chase. Secondly, [^{35}S]methionine did not label both globulin subunits equally. Two new approaches were undertaken to attempt to solve the above problems. In one approach, spikelets were labelled with [^{35}S]sulfate in the presence of cycloheximide. In another approach, spikelets were labelled with [^{14}C]leucine and chased with cold leucine. These experiments are described below.

Fig. 18 Scanning of *in vivo* labelling patterns.

Lanes 3, 5, and 10 from Fig. 17 were scanned using a densitometer in order to quantitate the changes in the signal intensities of globulin polypeptides during these chase periods. Crossed peak areas correspond to the 58-62 kDa globulin precursors. Filled-in peaks represent the globulin β-polypeptides.

FIG.18



In vivo labelling with [^{35}S]sulfate in presence of cycloheximide:

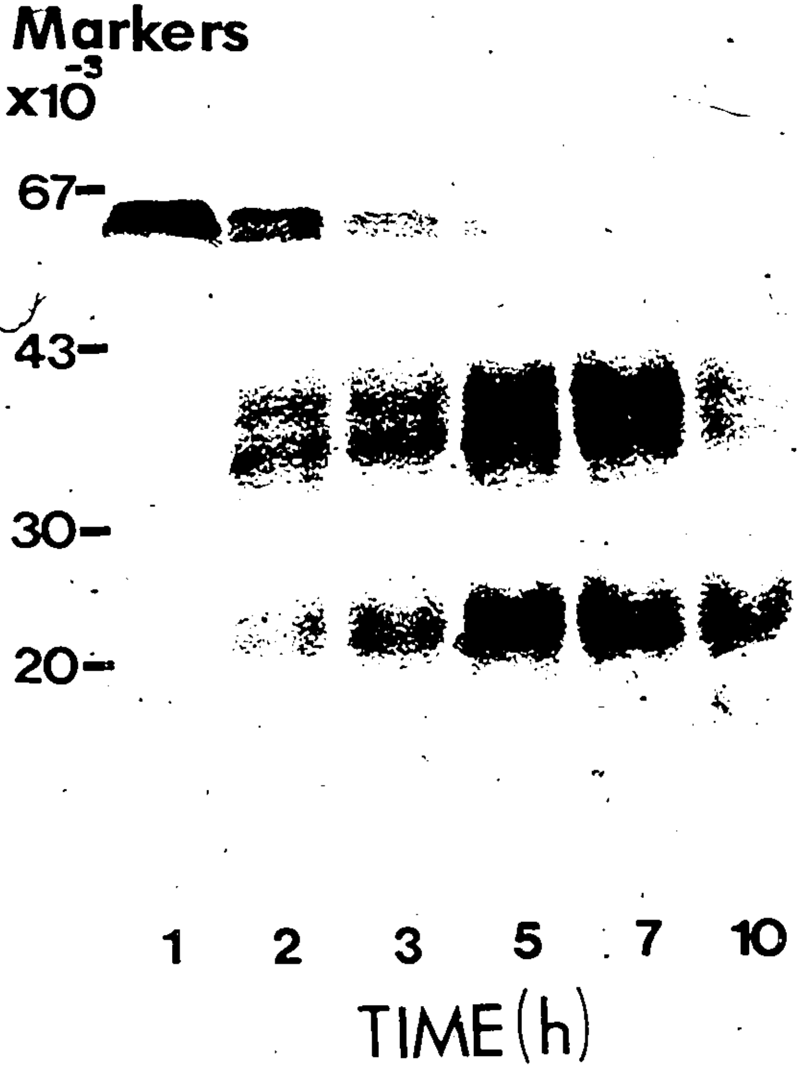
It appeared that an efficient and successful chase of radioactivity could not be achieved at the time periods used in the above experiments (Figs. 16 and 17). It might have been possible to completely chase out the radioactivity if the chase period was prolonged for several days. However, this was not practical since the plants do not survive in these conditions for long periods. They start to dry out quickly resulting in the shrinkage of the seeds making their analyses unreliable. A possible solution to the difficulties of chasing the radioactivity in such pulse-chase labelling experiments was the use of a protein synthesis inhibitor at the beginning of the chase. Although the addition of the inhibitor could negate the chase portion of the experiment it could solve the problem of continuous radioactive incorporation during the chase. In this experiment, spikelets were labelled for 1 h with [^{35}S]sulfate, transferred into a medium containing 0.5 mM cycloheximide and incubated for 1, 2, 3, 5, 7, and 10 h. Globulin was extracted from detached seeds and equal number of counts were analyzed by SDS-PAGE and fluorography (Fig. 19). Globulin 58-62 kDa polypeptides were labelled after 1 h incubation and disappeared from the fluorograph gradually. After 2 h incubation, α-polypeptides and β-polypeptides were labelled. The intensities of these polypeptides increased during the incubation period. The data could be interpreted as a conversion of the 58-62 kDa polypeptides into the smaller globulin polypeptides. This approach resulted in equal labelling of both subunits and the disappearance of the 58-62 kDa precursors, therefore, achieving its objectives.

The possible drawbacks of using cycloheximide were discussed earlier

Fig. 19 *In vivo* labelling of globulin polypeptides in developing oat endosperm (18 daf) in presence of cycloheximide.

Oat spikelets were labelled with [^{35}S]sulfate for 1 h and subsequently transferred to a medium containing cycloheximide. Groats were harvested at times indicated after transfer and the globulin fraction was isolated. The globulin extracts were analyzed by SDS-PAGE (12%, +Me) and fluorography. Approximately equal number of counts (14,000 cpm) were loaded on each lane.

FIG.19



in PART II of the results. The comparison of the data in Fig. 19 with those in Fig. 17 argues against any interference of cycloheximide in correct processing and deposition of globulin polypeptides. In presence or absence of the inhibitor, the 58-62 kDa globulin polypeptides were synthesized and processed similarly.

Overall, the results of this experiment compared with the data obtained from the earlier pulse-chase labelling experiment described in Figs. 16-18.

There was an interesting observation from the above labelling experiments with [^{35}S]. When spikelets were labelled with [^{35}S]methionine, the label was found incorporated mainly into the smaller β-polypeptides. Members of α-polypeptides were not all labelled. Only a 40 kDa component of this group of polypeptides was labelled. This pattern of labelling reflects the number of methionine residues present in the two groups of globulin subunits. It was interesting to note that when [^{35}S]sulfate was used as the radioactive precursor both subunits were labelled to the same extent. The discrepancy between the two experiments was probably in labelling the cysteine residues of the globulin polypeptides. Apparently, [^{35}S]sulfate labelled both the methionine and cysteine residues. However, [^{35}S]methionine seemed to have labelled only the methionine residues of the globulin polypeptides. Although theoretically, [^{35}S]methionine should also be converted by the plant to [^{35}S]cysteine and thus label both sulfur-containing amino acid residues of globulin polypeptides. One may conclude that in the short period of time that the above labelling experiment was carried out, the metabolic conversion of labelled methionine to cysteine had not taken place. It is conceivable to assume that both subunits would have been labelled if the [^{35}S]methionine labelling was continued for longer periods allowing for the labelling of cysteine residues.

Pulse-chase labelling with [^{14}C]leucine:

A pulse-chase labelling experiment was performed in which developing spikelets were labelled with [^{14}C]leucine for 1 h and chased for 1, 2, 3, 4, and 5 h with cold leucine. Labelled seeds were detached and used to extract globulin. Equal number of counts from various chase periods were analyzed by SDS-PAGE and fluorography (Fig. 20a). At early chase times, globulin 58-62 kDa polypeptides were labelled as well as a number of smaller polypeptides with molecular weights of 26-32 kDa. After 3 h of chase, globulin large and small subunits were labelled with increasing intensities. A gradual decrease in the intensities of the 58-62 kDa globulin polypeptides was noted from 3 h to 5 h of chase. This indicated that the chase might have started to take effect after 5 h. Therefore, an identical pulse-chase labelling was performed with longer chase periods of 10 h and 15 h. Fig. 20b shows the pattern of labelled globulins in this experiment. Apparently, longer chase times led to further reduction in the intensity of the globulin 58-62 kDa polypeptides (see Fig. 20b, lanes 10 and 15). A clear relationship among the 58-62 kDa polypeptides and the labelled globulin subunits could now be assumed. To quantitate this relationship, lanes 1 and 15 of the fluorograph were scanned using a gel scanner. The results (Fig. 21) revealed that the reduction in the intensity of the 58-62 kDa polypeptides (5 fold) equalled the increase in the intensity of labelled α and β-polypeptides (5 fold). However, the total signals on these two lanes were not equal.

Fig. 20 a and b Pulse-chase labelling of globulin polypeptides with $[^{14}\mathrm{C}]$leucine.

(a) Developing spikelets were labelled with $[^{14}\mathrm{C}]$leucine for 1 h and transferred into a chase medium containing cold leucine. Groats were detached at various times (0, 1, 2, 3, 4, 5) and used to extract globulins. The globulin extracts were counted and equal number of counts (10,000 cpm) were analyzed by SDS-PAGE (13%, +Me) and fluorography. The fluorograph was exposed for 7 days. Numbers represent chase times. Arrowheads indicate the position of the globulin 58-62 kDa precursors.

(b) Developing spikelets were labelled with $[^{14}\mathrm{C}]$leucine for 1 h and transferred into a chase medium containing cold leucine. Groats were detached at various times (1, 2, 3, 5, 10, 15 h) and used to extract globulins. The globulin extracts were counted and equal number of counts (5,000 cpm) were analyzed by SDS-PAGE (13%, +Me) and fluorography. The fluorograph was exposed for 12 days. Arrowheads indicate the globulin precursors. L and S represent the large and small globulin polypeptides respectively (corresponding to stained cold globulin polypeptides).

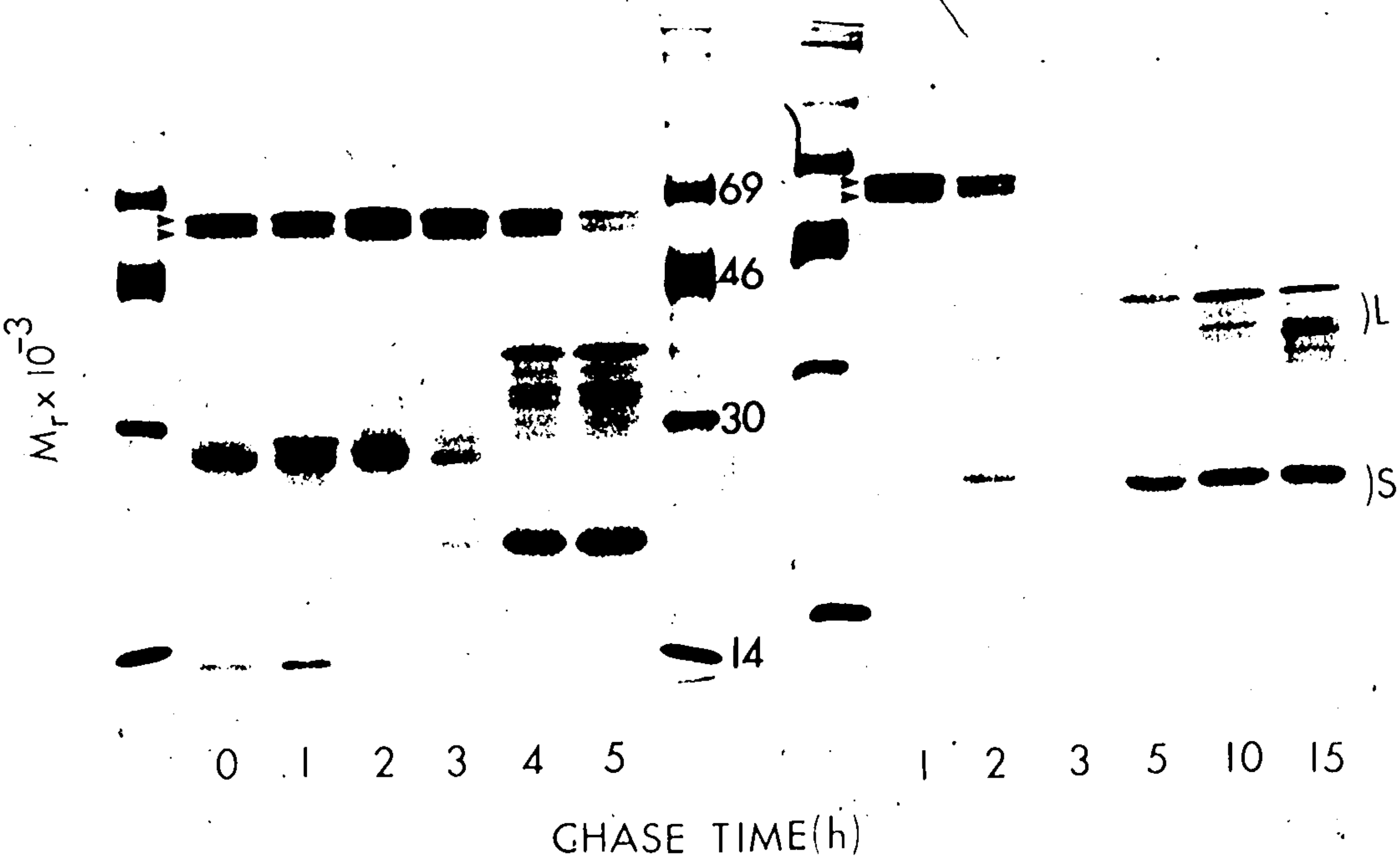


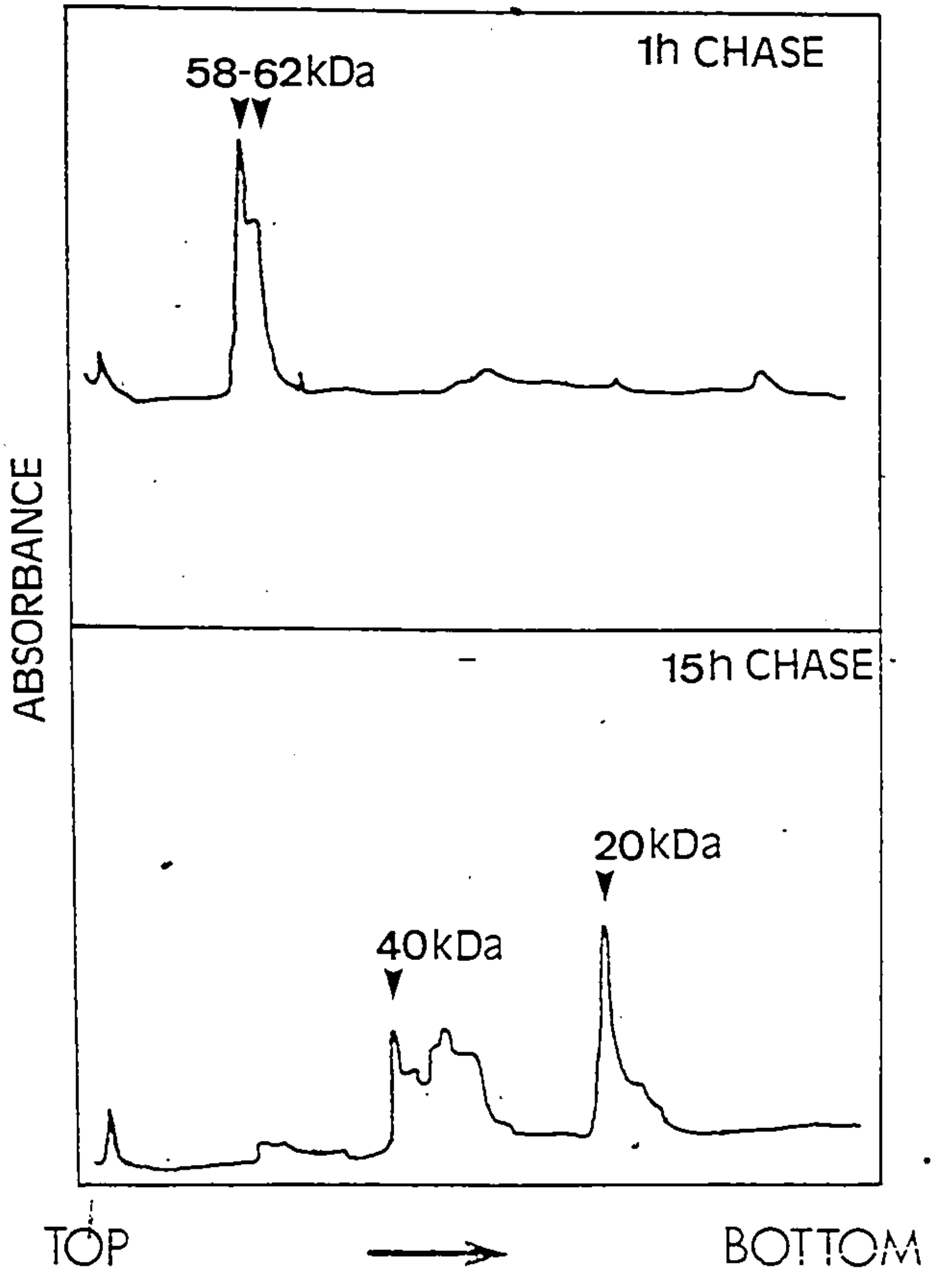
FIG. 20a

FIG. 20b

Fig. 21 Scanning of pulse-chase labelling patterns of globulin polypeptides.

Lanes 1 and 15 from Fig. 20b were scanned using a densitometer in order to quantitate the changes in the globulin 58-62 kDa, α-polypeptides, and β-polypeptides in the beginning and at the end of the chase.

FIG. 21



58-62kDa
1h CHASE
ABSORBANCE
15h CHASE
20kDa
40kDa
TOP
BOTTOM

There was a 30% discrepancy which cannot be explained logically since equal number of counts were loaded on each lane. This difference may have originated from the contamination of protein samples with free label despite the fact that samples were counted by precipitating and boiling in TCA in presence of cold leucine.

The identity of the labelled 26-32 kDa polypeptides was not clear. These polypeptides were not present in labelling experiments with [^{35}S]. They might belong to the class of minor globulins (see appendix I). They may also be prolamins contaminating the globulins extract. However, only labelling with [^{14}C]leucine seemed to lead to labelling of these 26-32 kDa polypeptides.

As a whole, the data supported the hypothesis that globulin 58-62 kDa polypeptides were precursors of the globulin α and β-polypeptides. This processing was probably due to proteolytic cleavage of globulin precursors by specific enzyme.

PROCESSING SITE OF GLOBULIN PRECURSORS:

The detection of globulin for long periods in the above *in vivo* labelling experiments indicated that these precursors were relatively stable. The proteolytic cleavage seemed to occur hours after synthesis. This suggested that such a processing event was post-translational. This cleavage could occur during: transport of precursors from the RER to protein bodies; or during the formation of protein bodies on the protein body membrane; or after deposition in protein bodies. An *in vivo* labelling experiment was carried out to determine whether globulin polypeptides arrived in protein bodies as unprocessed precursors or as mature subunits.

Developing spikelets were labelled with [^{35}S]sulfate for 1.5 h. The spikelets were removed from the radioactive solution and were incubated with cold sulfate in presence of cycloheximide for various periods of time (2, 4, 6, 8, and 10 h). The seeds were detached and used to isolate protein bodies. Globulin was extracted from the protein bodies and was analyzed by SDS-PAGE and fluorography (Fig. 22). Polypeptides with molecular weights of 58-62 kDa appeared in protein bodies after 2 h incubation (lane 2). The intensity of these polypeptides decreased when the spikelets were incubated for longer periods of time. After 4 h incubation, the two groups of α-polypeptides and β-polypeptides appeared on the fluorograph and gradually increased in intensity.

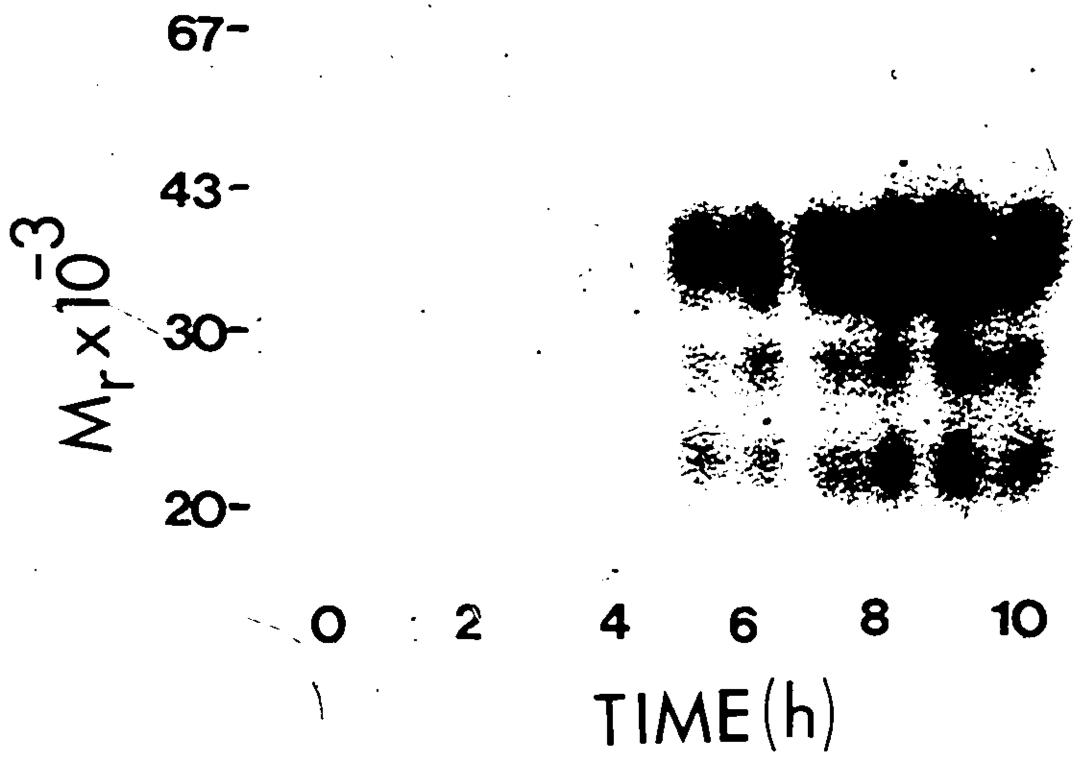
The appearance of the putative 58-62 kDa globulin precursors in protein bodies in the above experiment suggested that these precursors were not processed before their arrival into protein bodies. The processing event apparently occurred only after their entry into protein bodies suggesting these vesicles to be the site of cleavage.

The use of a protein synthesis inhibitor during the incubation period was required to assist in blocking a continuous incorporation of [^{35}S]sulfate into globulin precursors. Complications in the use of cycloheximide was discussed earlier. For this particular experiment with protein bodies no evidence was obtained for any interference of cycloheximide with the pathway of globulin deposition. Other _in vivo_ labelling experiments, which used cold sulfate without the use of cycloheximide showed similar results. Labelled globulins arrived into the protein bodies in the form of the larger 58-62 kDa polypeptides in presence or absence of cycloheximide. Processing seemed to occur afterwards. The difference with the experiment in Fig. 22 was that the

Fig. 22 _In vivo_ labelling of globulin polypeptides in protein bodies in presence of cycloheximide.

Oat spikelets (18 daf) were labelled with [^{35}S]sulfate (900 mCi/mmol) for 1.5 h, transferred to a medium containing cold sulfate and cycloheximide. Groats were harvested at various times indicated (0, 2, 4, 6, 8, 10 h) and used to isolate protein bodies. Each protein body preparation was then extracted for globulin and analyzed by SDS-PAGE (12%, +Me) and fluorography. Lanes 2, 4, 6, 8, and 10 contained 4000 cpm.

FIG. 22



58-62 kDa globulins persisted on the fluorographs after long periods of chase. Therefore, it appeared that cycloheximide only caused the inhibition of further synthesis of the 58-62 kDa polypeptides.

B. In Vivo Labelling Studies on Assembly of 12S Globulin.

The deposition of globulin polypeptides as a 12S oligomer in protein bodies suggests that at a certain point during synthesis, segregation, transport or deposition, globulin polypeptides are assembled into a 12S complex. To examine the site of globulin assembly, an in vivo labelling experiment was conducted and the labelled polypeptides in two organelles (ER and protein bodies) were size-fractionated on a sucrose gradient.

The first part of the experiment was designed to demonstrate whether or not a 12S oligomer was present in association with the ER membranes. Developing spikelets were labelled for 1 h with [^{35}S]sulfate, homogenized and the homogenate fractionated on a 16-35% step sucrose gradient. The ER fraction (EDTA-stripped ER) at the 16/35 interface was collected and dialyzed against a 1 M NaCl buffer. The ER suspension was then applied to a 5-20% sucrose gradient made in 1 M NaCl buffer. Fig. 23 shows the gradient profile of the labelled ER fraction. A major and a minor radioactive peak were detectable in the gradient. Relative to the marker proteins (oat 3S, 7S, and 12S), the major and minor peaks had sedimentation coefficients of 3S and 8-9S respectively (Fig. 23). Interestingly, no 12S peak was evident in the ER fraction. These results suggested that the globulin precursors were not assembled into a 12S oligomer in the ER. The 3S and 8-9S protein peaks may represent either large protein molecules or protein oligomers. In the later case, this may indicate that globulin polypeptides were assembled in the ER as 3S or 8-9S oligomers. Unfortunately, this could not be examined by SDS-PAGE and fluorography due to the low yield of radioactive polypeptides recovered from the two peaks.

Fig. 23 Incorporation of radioactivity into globulin molecules in the ER.

Oat spikelets (18 daf) were labelled with [^{35}S]sulfate (900 mCi/mmol) for 50 min. Labelled groats were detached and used for ER extraction on a step gradient of 16%/35% sucrose. The ER fraction was collected, and extracted with 1 M NaCl, 50mM Tris-HCl, pH 8.0 buffer. The extract was fractionated on a 5-20% sucrose gradient made in the same buffer. The gradients were divided into 1 ml fractions and TCA-precipitable radioactivity of each fraction was determined (expressed as CPM x 10^{-2}).

Fig. 24 Incorporation of radioactivity into globulin molecules in protein bodies.

Oat spikelets (18 daf) were labelled with [^{35}S]sulfate (900 mCi/mmol) for 1 h and subsequently transferred into a medium containing cold sulfate and cycloheximide for 6 h. Groats were detached at 1 and 6 h after transfer and used to prepare protein bodies. The protein body samples were used to extract globulin. The globulin extracts were then dissolved in 1 M NaCl and fractionated on a 5-20% sucrose gradient. The gradients were monitored at A_{280} and divided into 1 ml fractions. TCA-precipitable radioactivity was determined and expressed as CPM x 10^{-3}; 1 h incubation (○————○), 6 h incubation (▲————▲). Fractions 15, 16, and 17 from the 6 h gradient were pooled, dialyzed, and analyzed by SDS-PAGE (10%, +Me) and fluorography (Lane a)

FIG.23

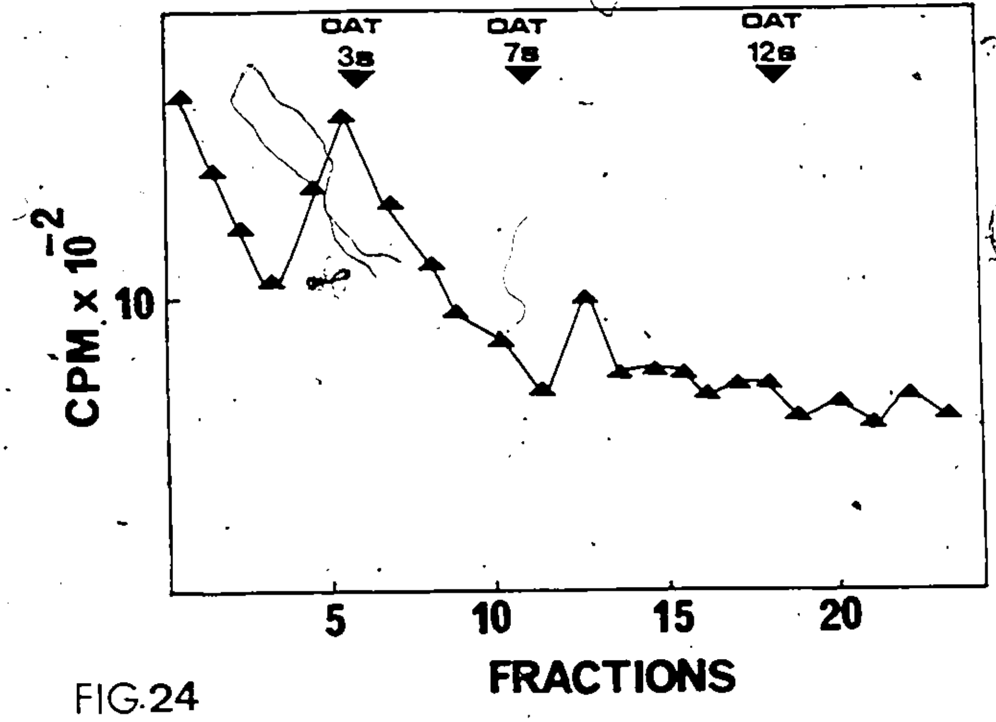
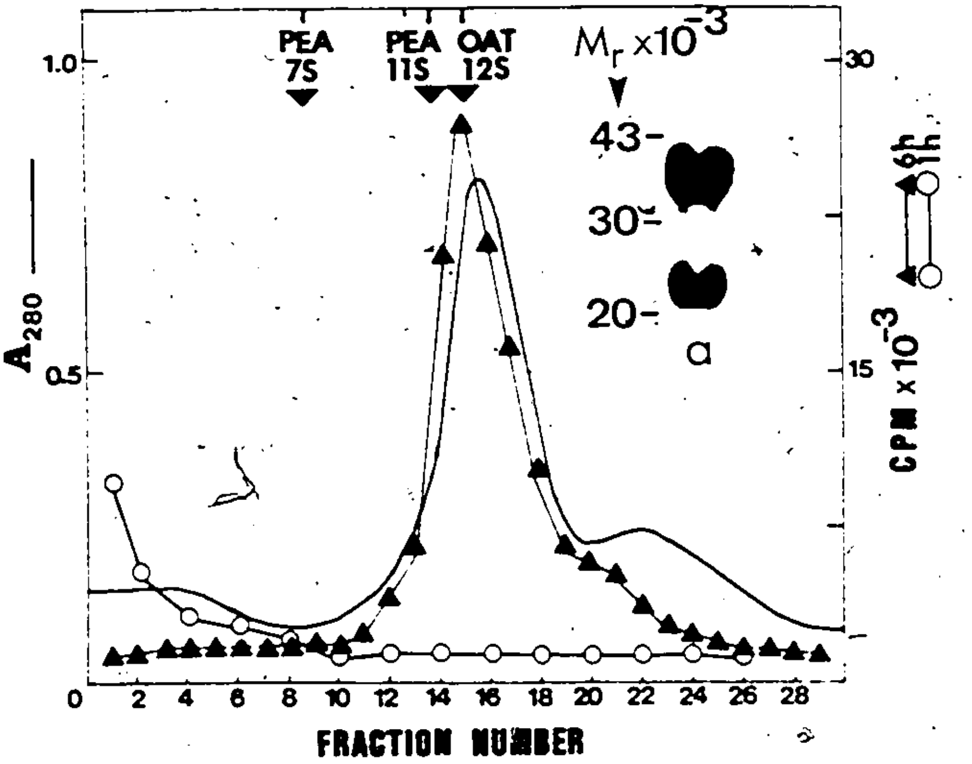

OAT
3s
OAT
7s
OAT
12s
CPM x 10^{-2}
10
5
10
15
20
FRACTIONS

FIG.24


PEA
7S
PEA
11S
OAT
12S
M$_r$ x10^{-3}
43-
30-
20-
a
1.0
0.5
0
A$_{280}$
30
15
CPM x10^{-3}
6h
1h
2
4
6
8
10
12
14
16
18
20
22
24
26
28
FRACTION NUMBER

To investigate oligomer formation in protein bodies, a similar labelling experiment was carried out in which spikelets were labelled for 1 h and then transferred into a non-radioactive solution containing cycloheximide and incubated for 6 h. The labelled seeds were used to prepare protein bodies which were then extracted for globulin. The globulin extract was finally fractionated on a 5–20% sucrose gradient. No radioactive oligomer was found in protein bodies after 1 h pulse (Fig. 24). However, a major radioactive peak with an approximate sedimentation coefficient of 12S was detected in the gradient after 6 h of incubation (Fig. 24). This radioactive peak corresponded approximately with the unlabelled 12S peak (present in the gradient and detected by monitoring the gradient at A_{280}). These observations suggested that globulin polypeptides were assembled in protein bodies as a 12S oligomer several hours after synthesis. The nature of the assembled polypeptides in the radioactive 12S peak was investigated by SDS-PAGE (+Me) and fluorography (Fig. 24, lane a). Labelled globulin α-polypeptides and β-polypeptides were detected on the fluorograph. This confirmed the identity of the 12S radioactive peak and its homology to the cold 12S peak. It might also indicate that the globulin precursors were processed before or during assembly into the 12S oligomer. Nevertheless, further studies are essential to clarify the kinetic relationship between cleavage and assembly.

The identification of the 12S radioactive peak and the labelled globulin subunits was convenient because of the presence of cold globulins in the samples acting as internal markers.

PART IV - GLOBULIN DEPOSITION

In vivo pulse-chase labelling and electron microscopic investigations suggested that reserve globulin polypeptides were translocated into protein bodies after their synthesis on the RER. In this section, oat endosperm protein bodies have been isolated and analyzed. Deposition of globulin polypeptides in seed protein bodies has also been examined by immunocytochemical techniques.

A. Biochemical and Electron Microscopic Analyses of Protein Bodies

Protein bodies were isolated in earlier studies by the method of Chrispeels et al. (35). This procedure involved homogenizing developing or mature groats and centrifuging the filtered extract through 16% (w/w) sucrose. The pellet contained protein bodies but was heavily contaminated by starch as shown by electron microscopic analysis (Figs. 25 A and B). Protein bodies only constituted a minor portion of the sample and appeared broken and damaged. Consequently, the above method was modified by including a second sucrose solution of 65% (w/w) to separate the starch granules. When the seed extract was sedimented through a 16%/65% discontinuous sucrose gradient, the majority of protein bodies banded at the interface (16/65). The starch granules were heavier and pelleted. Such preparations consisted almost exclusively of protein bodies as shown by electron microscopic examination (Figs. 26 A-C).

The morphology of protein bodies was also examined in intact seeds by electron microscopy (Figs. 27 A-D). Membranes appeared to be surrounding each protein body (Fig. 27A). A more magnified image showed this phenomenon more clearly (Fig. 27B). Interconnections among the

Fig. 25 TEM examination of a protein body extract isolated using a single 16% sucrose solution.

Fig. 25A. Developing oat groats (16 daf) were used to extract protein bodies. The protein bodies were obtained by filtering a seed extract by Miracloth and sedimenting the filtrate through 16% sucrose. TEM analysis of this pellet revealed the presence of large chunks of starch and a lot of broken protein bodies. M x 4000.

Fig. 25B. A higher magnification image of the same pellet. M x 7000.

Abbreviations: PB, Protein body; S, starch.

FIG.25

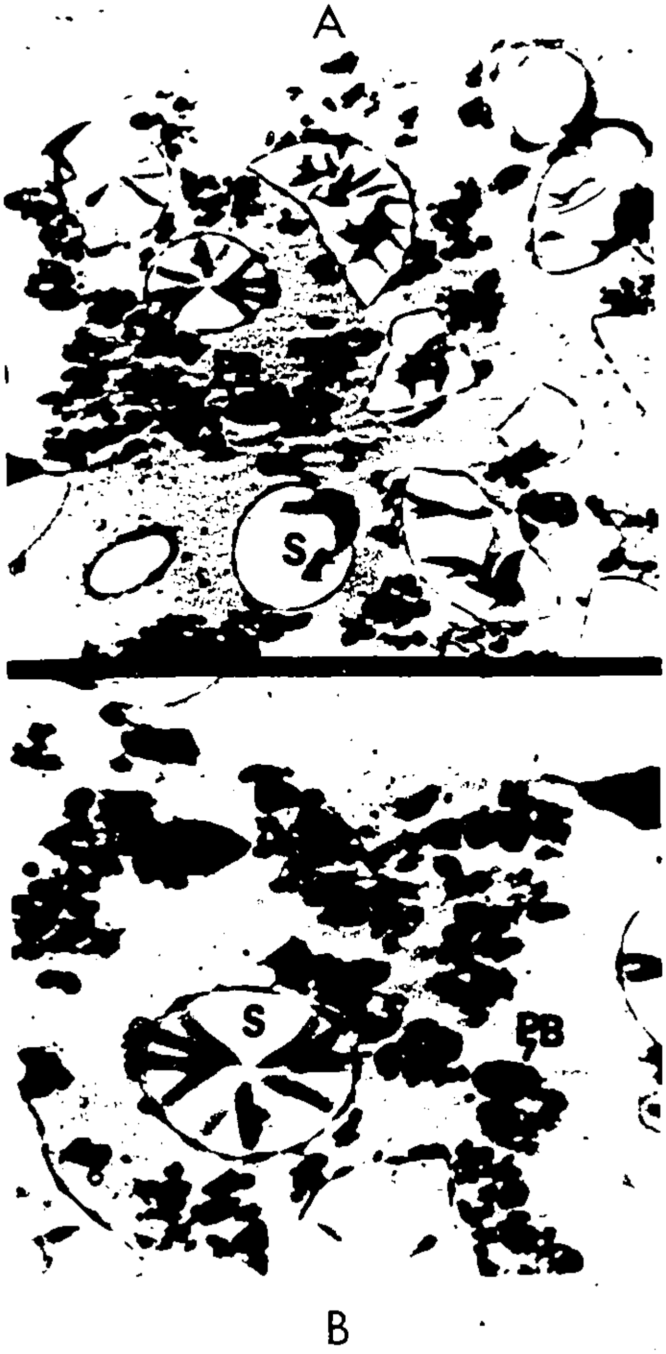


Fig. 26 TEM examination of a protein body extract isolated using a step gradient.

Fig. 26A. Oat groats (16 daf) were homogenized, and the homogenate filtered through Miracloth. The extract was layered on a step gradient of 16% and 65% sucrose and centrifuged for 10 min at 2,000 X g. The material at 16/65 interface was analyzed by TEM. This pellet is almost comprised exclusively of protein bodies. M x 14,000.
Fig. 26B. Another image of the same pellet. M x 14,000.
Fig. 26C. An image of a single protein body indicating it to be intact. M x 14,000.

Abbreviations: PB, protein body.

FIG.26

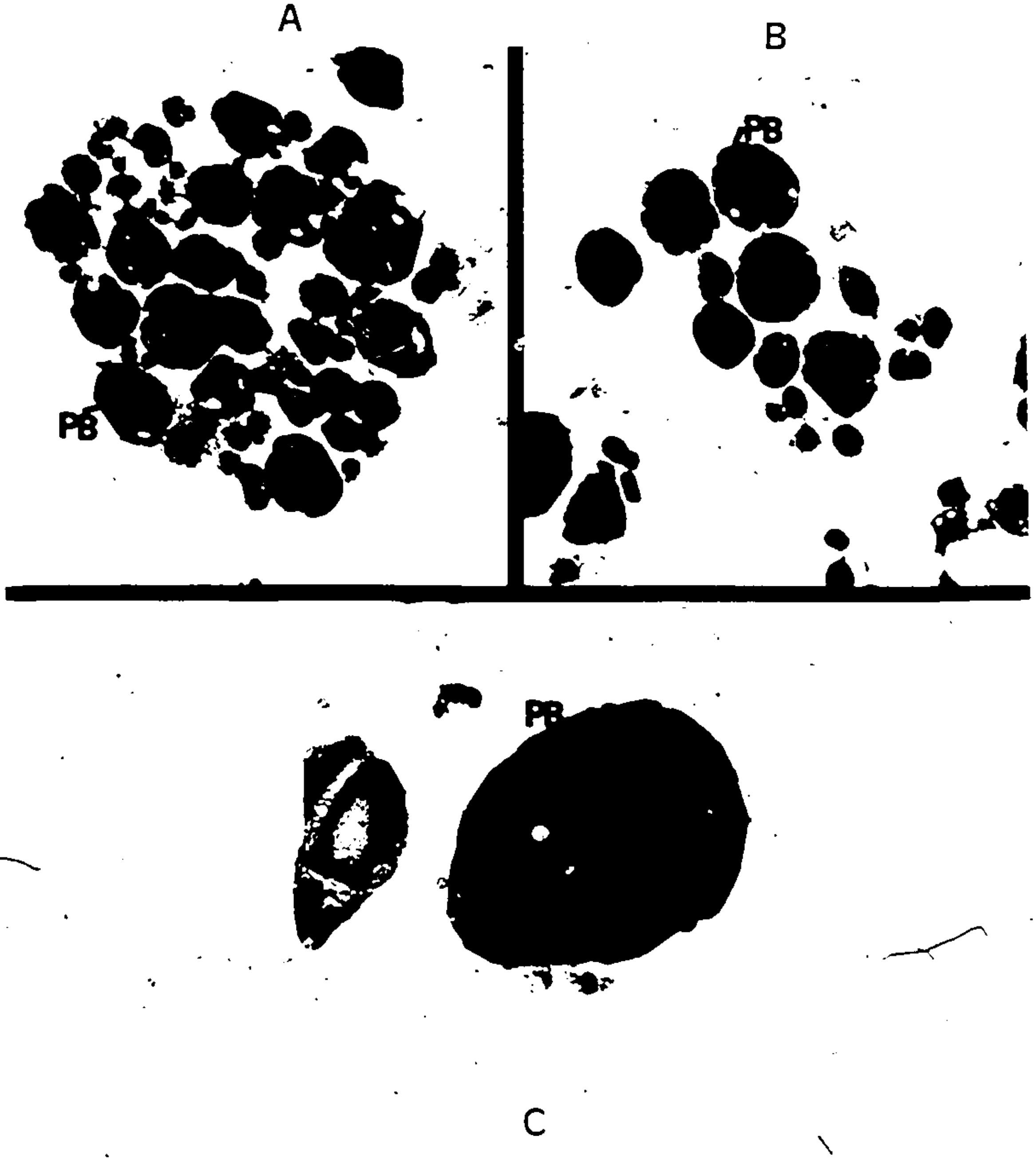
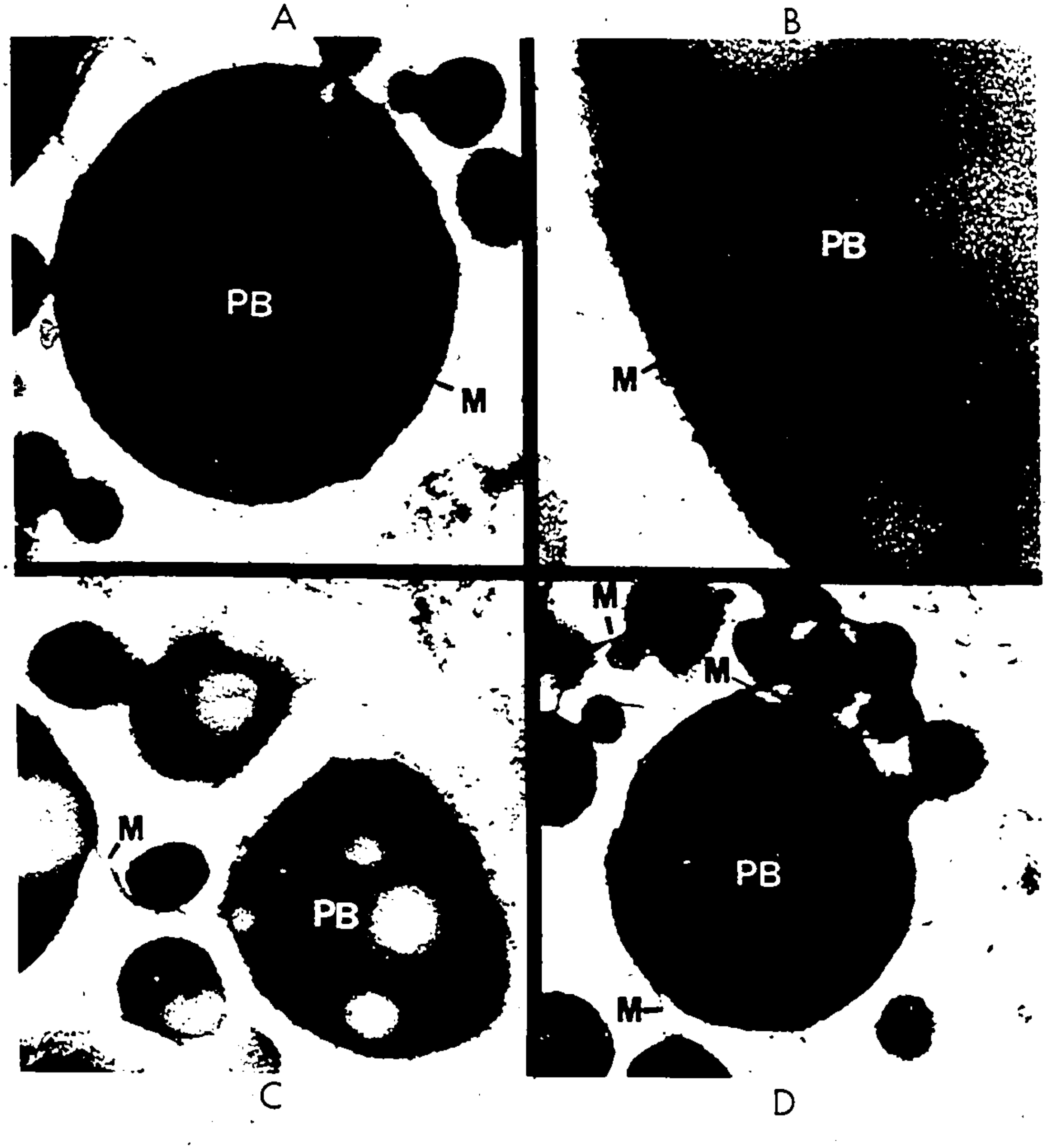


Fig. 27 TEM examination of membranes surrounding protein bodies.

Fig. 27A. A protein body in developing (18 daf) oat groats. Membrane surrounding the protein body is not clearly observed. M x 34,000.
Fig. 27B. A higher magnification image does illustrate the presence of possibly a double membrane surrounding the protein body. M x 14,000.
Fig. 27C & 27D. The interconnections among protein bodies via membranes are illustrated. It seems that all protein bodies are connected to one another. M x 44,000 and M x 34,000 respectively.

Abbreviations: M, membrane; PB, protein body.

FIG. 27



membranes of all protein bodies could also be found, but significance of this continuity was not clear (Figs. 27C and 27D). These loose membranes may be artifacts of the fixation of seed sections by glutaraldehyde.

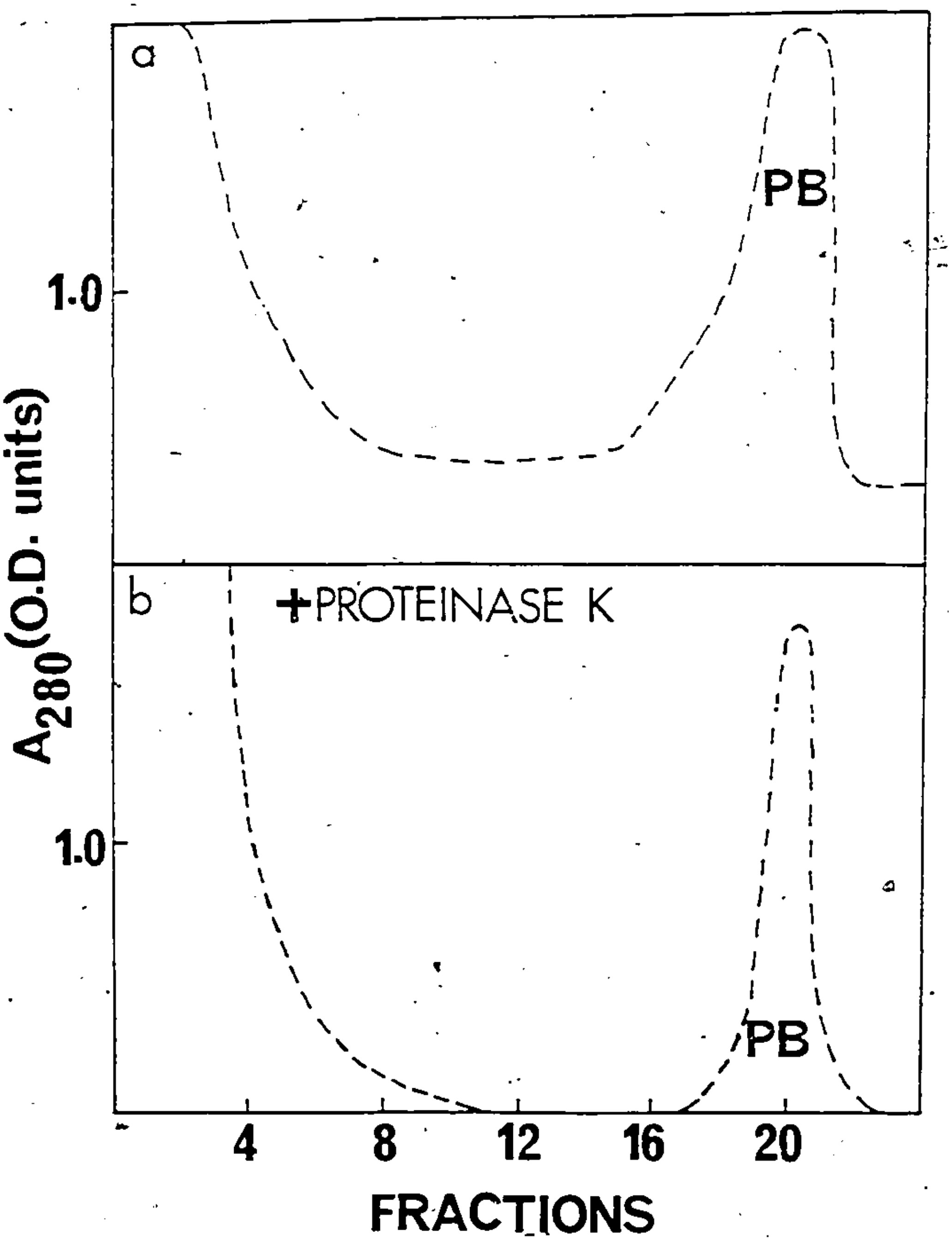
Comparison of the morphology of the protein bodies in the *in vitro* samples (Figs. 26 A-C) with those from the *in vivo* samples (Figs. 27 A-D) revealed considerable variations. Extracted protein bodies had lost their *in vivo* spherical structure. These observations were not surprising since the *in vivo* protein bodies were subjected to harsh manipulations (e.g. homogenization, centrifugation).

Proteinase K Treatment of Protein Bodies:

Fractionation of protein body extracts on linear 16-65% sucrose gradients showed the presence of a major peak at an approximate density of 1.23 g/cm^3 (Fig. 28a). Incubation of the material in this peak with proteinase K did not result in appreciable digestion (Fig. 28b). The proteinase K treatment, however, resulted in the sharpening of the protein body peak (Fig. 28b). One possible cause of this resistance against proteolytic digestion might be the presence of a membrane surrounding each protein body. However, the electron micrographs of protein bodies isolated by sucrose gradient fractionation in Figs. 26 A-C did not clearly show the presence of such membranes. Although membranes might have been present but not stained well enough to become visible. Alternatively, protein bodies might have lost their membranes during centrifugation. If this were the case, the resistance of the protein bodies against proteolysis must have been conferred by another factor. For instance, globulin deposits in protein bodies could have a tertiary structure making the polypeptide chains inaccessible to proteolytic

Fig. 28 a & b Proteinase K digestion of oat endosperm protein bodies. Mature and/or developing groats were used to prepare protein bodies (PB) with linear or step 16-65% sucrose gradients. The PB extracts were treated with proteinase K and after addition of PMSF (as a protease inhibitor), the treated extracts were fractionated on linear 16-65% sucrose gradients. After centrifugation, the gradients were monitored at 280 nm and fractionated. (a), the profile of the gradient of the PB extract without proteinase K digestion. (b), the profile of the proteinase K-treated PB extract.

FIG. 28



digestion. This resistance may also be partly due to the presence of significant amounts of phytic acid (a phenolic compound) in protein bodies which could form insoluble complexes with globulin 12S molecules.

The protein composition of oat protein bodies was also investigated. The protein body fractions from sucrose gradients were pooled and extracted with 1M NaCl. This salt extract was fractionated on a 5-20% sucrose gradient to determine the size of any protein oligomer contained therein. The gradient's A_{280} profile is shown in Fig. 29a. This profile was identical to the pattern normally obtained from the total seed globulin extract (see Appendix I). Therefore, it appeared that a 12S oligomer was the predominant protein component of the protein bodies. Electrophoretic analysis of this protein peak under reducing conditions indicated the abundance of the two groups of globulin α-polypeptides and β-polypeptides (Fig. 29b).

In summary, a method was developed to isolate pure protein body preparations free of starch. These protein bodies were resistant to proteolytic digestion. Electron microscopic investigations suggested that these protein bodies might be surrounded by membranes. The major protein component of these protein bodies was found to be the 12S globulin composed of the globulin 20 kDa and 38 kDa subunits.

FIG. 29

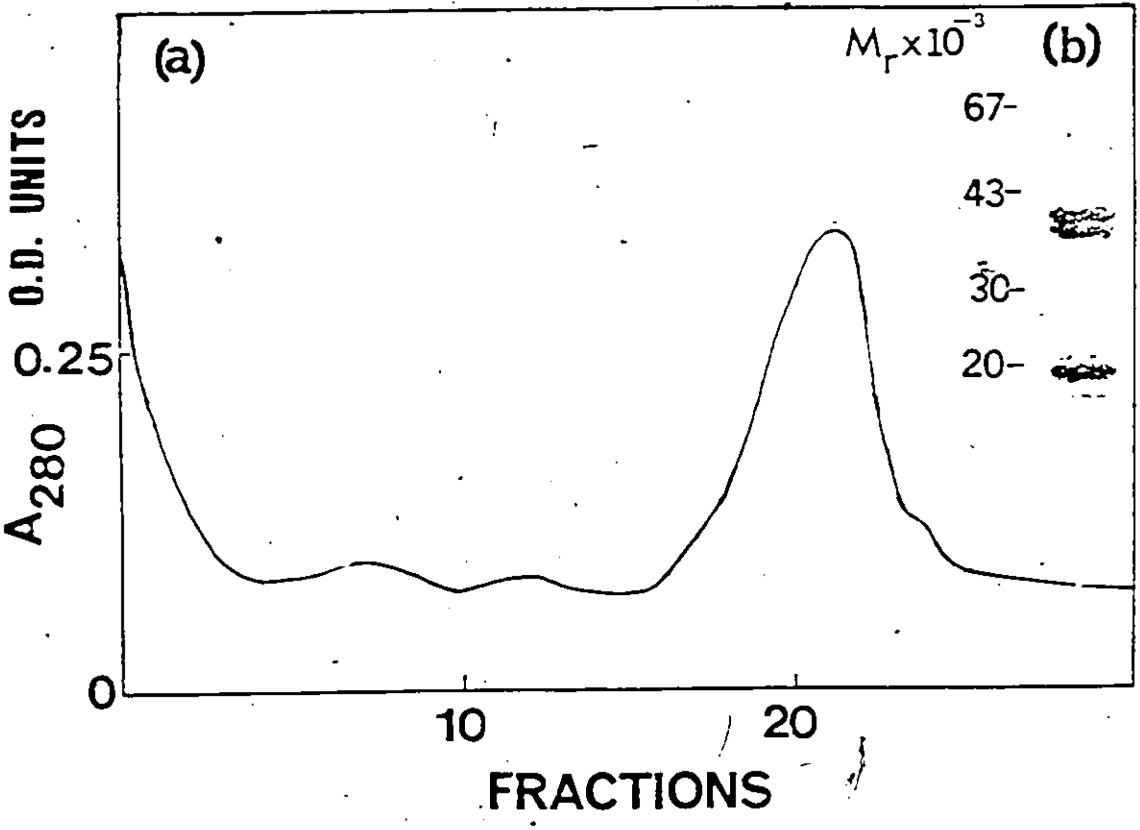


Fig. 29 Size fractionation and electrophoretic analyses of globulin from oat endosperm protein bodies. (a), globulin was extracted from protein bodies isolated from mature seeds. The globulin extract was fractionated on a 5-20% sucrose gradient. After centrifugation, the gradient was monitored at 280 nm and fractionated into 1.2 ml fractions. (b), the same globulin extract from protein bodies was analyzed by SDS-PAGE (12%, +Me).

B. Immunocytochemical Localization of the 12S Globulin in Protein Bodies.

Although the electrophoretic analysis of the protein body preparations indicated the occurrence of the 12S globulin in protein bodies, evidence was needed to show specific localization of globulins in the oat endosperm. The previous data were inadequate to demonstrate any differences in the localization of the 12S globulin in the aleurone layer, subaleurone layer, or starchy endosperm protein bodies. Immunocytochemical techniques were employed to achieve this objective due to the availability of the 12S globulin antibodies. In this approach, mature oat seeds were cut and fixed in 10% paraformaldehyde. This specific fixative was used because of minimum alterations to protein structure and antigenic properties (110).

Plastic (glycol methacrylate) sections (2 μm) were prepared and incubated with anti-12S globulin rabbit IgG and fluorescein conjugated anti- rabbit goat IgG (FITC-goat IgG, Sigma). Finally after extensive washing, the sections were viewed under a Zeiss fluorescent microscope.

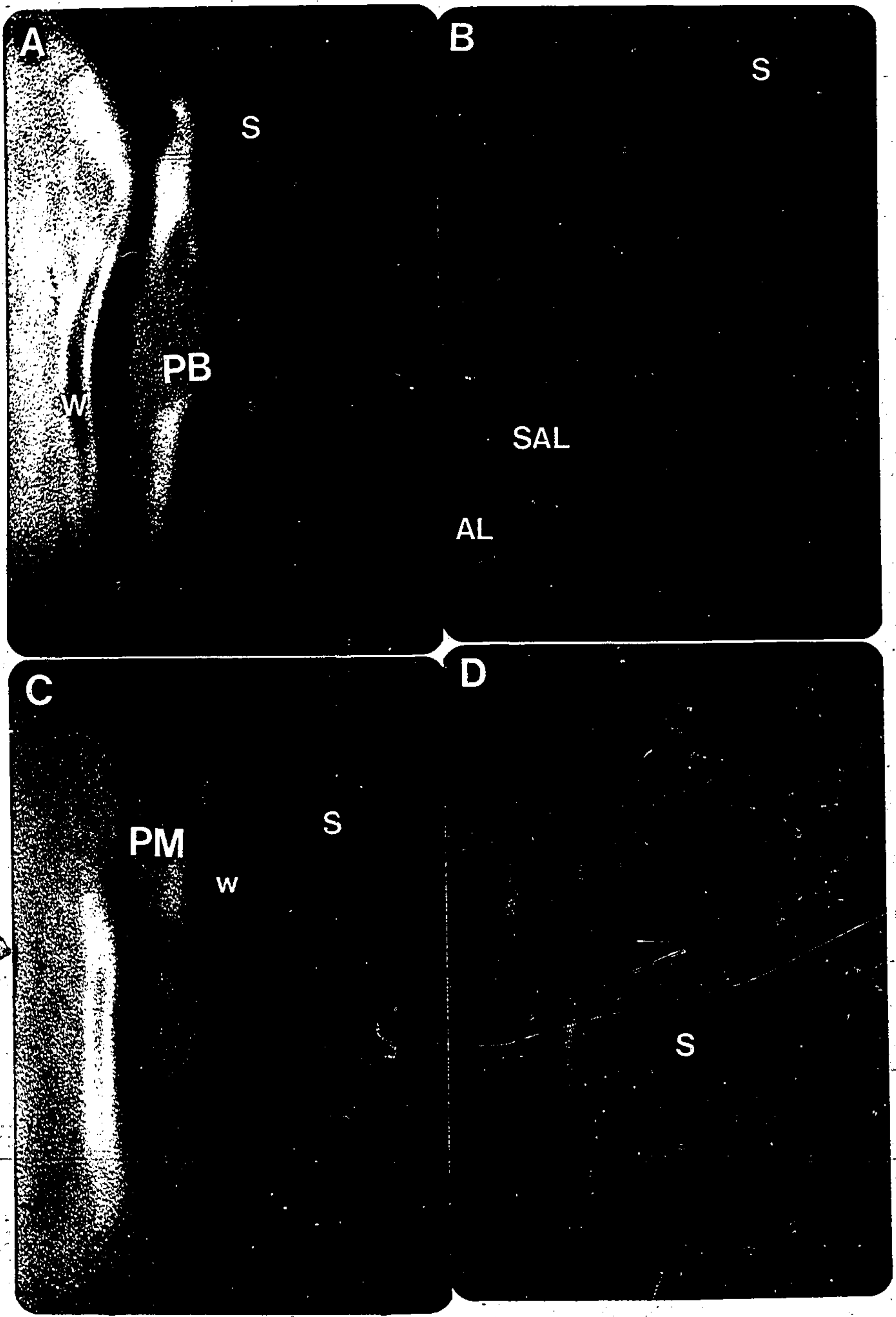
Before presenting the results of the immunofluorescence experiments, a few fluorescence micrographs of mature oat endosperm are first illustrated (Figs. 30 A-D). Figs. 30 A-C show fluorescence images of plastic sections which were stained for protein with acid fuchsin. Individual protein bodies were stained (Fig. 30A). However, in most cases, staining occurred over the entire cytosol except for starch granules (Figs. 30 B and C). This was probably due to the presence of tremendous numbers of protein bodies in the cell matrix. Therefore the

Fig. 30 Structural analyses of mature oat endosperm by fluorescence microscopy.

Fig. 30A. A plastic section stained with acid fuchsin. Protein bodies have been stained within subaleurone layer cells. (FC III). M x 600.
Fig. 30B. Aleurone and subaleurone layer cells treated with acid fuchsin. Protein inclusions are abundant. (FC III). M x 600.
Fig. 30C. Starchy endosperm cells of mature oat seeds containing numerous starch granules together with protein bodies seen in the form of protein matrix (stained with acid fuchsin). (FC III). M x 600.
Fig. 30D. Iodine-potassium iodide staining of a frozen oat section. Starch granules are stained heavily in the inner endosperm cells. Photographed using a plain broad field. M x 1500.

Abbreviations: AL, aleurone layer cell; PB, protein bodies; PM, protein matrix; SAL, subaleurone layer cells; S, starch; W, cell wall.

FIG.30



term "protein matrix" (PM) was used to label the fluoromicrographs. Acid fuchsin staining was less intense in the aleurone layer (AL) compared to the subaleurone layer cells (SAL) (Fig. 30B). This might be because of the occurrence of large amounts of phenolic compound in the aleurone layer protein bodies which prevent proper staining with acid fuchsin. When a frozen oat section was stained with iodine, starch granules were heavily stained (Fig. 30D). Protein bodies were also visible (Fig. 30D, arrow).

Starchy endosperm cell constituents possessed no appreciable autofluorescence when a frozen oat section was viewed under a plain broad field (Fig. 31A). Instead, the seed coat and outer aleurone layer showed significant autofluorescence (Fig. 31A).

In the immunofluorescent experiments, two types of controls were incorporated. In the first control, sections were treated with FITC-goat IgG (Fig. 31B). No appreciable staining was visible after a 60 second exposure (Fig. 31B). However, slight fluorescence was detectable after a very long exposure time (5 min), (Fig. 31C). In the second control, sections were first treated with pre-immune serum for 60 min and then stained with FITC-goat IgG for 30 min. Identical observations to that of the first control were made and therefore the micrographs are not shown. In a normal exposure time (60 seconds), no staining of the section was observed.

When oat sections embedded in plastic were incubated with 12S globulin antibodies for 60 min and were then treated with FITC-goat IgG for 30 min, considerable fluorescein staining of the protein bodies occurred (Figs. 32 A-C). Exposure time for these micrographs was 60

Fig. 31 Various controls incorporated in the immunofluorescence localization experiment.

Fig. 31A. Examination of a frozen section of the mature oat endosperm reveals fluorescence material in the seed coat and aleurone layer. No visible fluorescence is found in the subaleurone and starchy endosperm cells. Photographed using PBF. M x 60.

Fig. 31B. FITC-goat IgG staining (30 min) of oat plastic sections. Occasional fluorescent spots (arrow) are due to nonspecific FITC-goat IgG precipitates (60 second exposure). (FC II). M x 600.

Fig. 31C. FITC-goat IgG staining (30 min) of oat plastic sections. Slight fluorescence is detectable after a long exposure time (5 min). (FC II). M x 600.

Abbreviations: AL, Aleurone layer; C, seed coat; S, starch; SAL, subaleurone layer: SE, starchy endosperm; W, cell wall. PBF, plain broad field.

FIG. 31

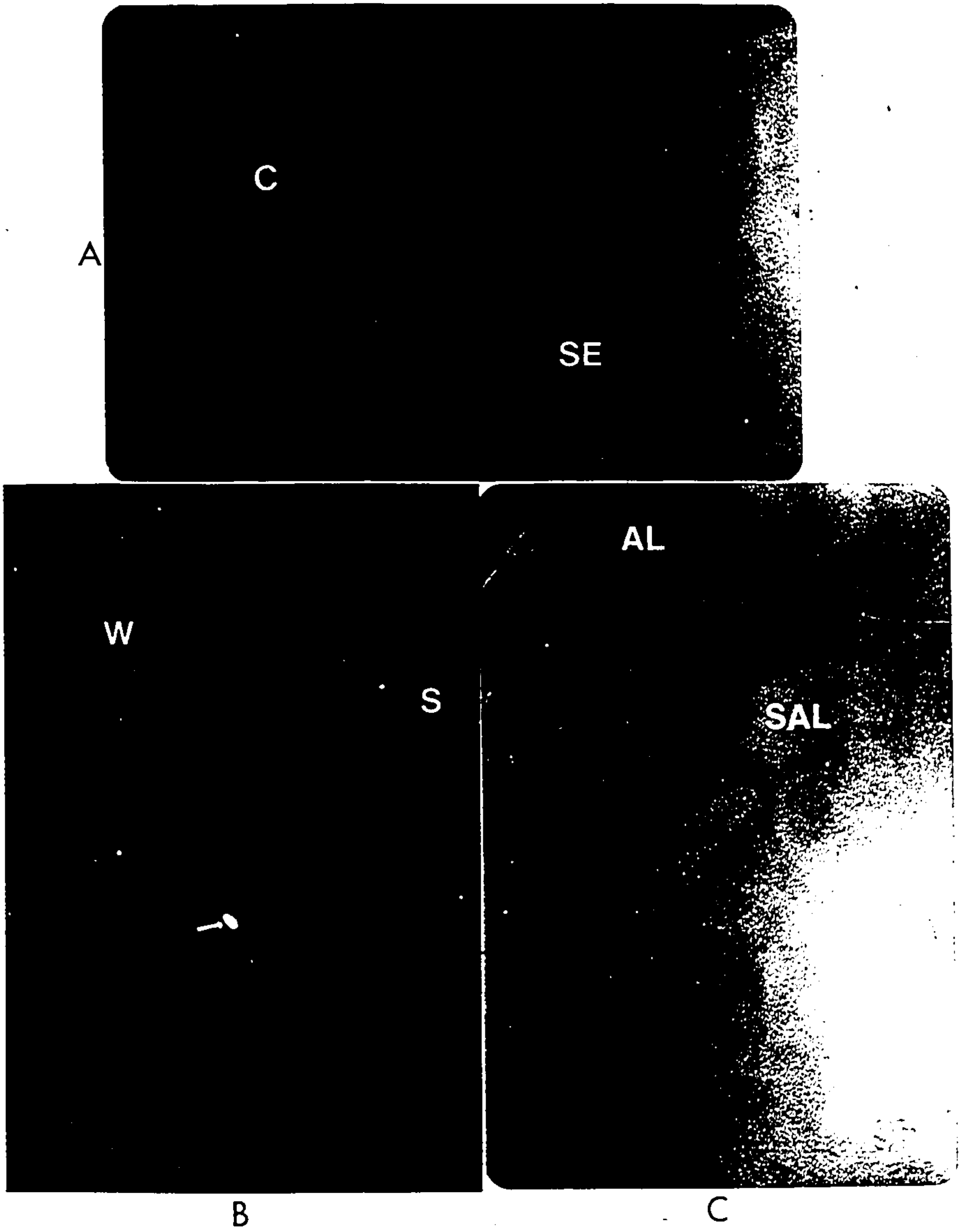


Fig. 32 Immunofluorescence localization of 12S globulin using 12S antibodies.

Fig. 32A. Subaleurone cells treated with 12S globulin antibodies (60 min) and FITC-goat IgG (30 min). Fluorescein staining of protein bodies is observed throughout (60 second exposure). M x 600.

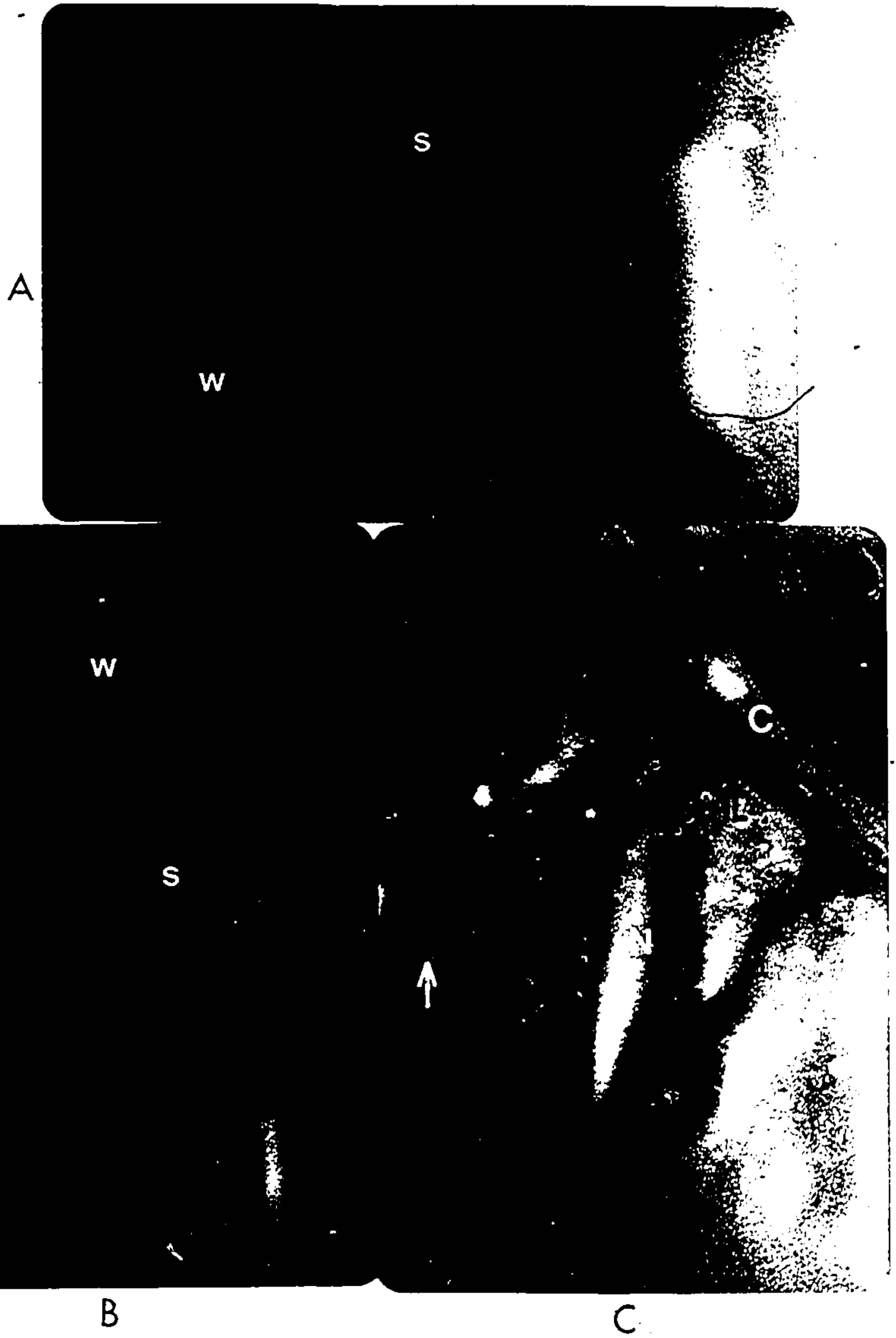
Fig. 32B. Starchy endosperm cells treated with 12S globulin antibodies (60 min) and FITC-goat IgG (30 min). Even staining of protein bodies is observed with no binding to cell walls and/or starch granules (60 second exposure). M x 1500.

Fig. 32C. Aleurone layer cells treated with 12S globulin antibodies (60 min) and FITC-goat IgG (30 min). Fluorescein staining of protein bodies is observed but many intense autofluorescence spots (arrow) are present which mask the positively stained regions. This autofluorescence was previously reported to exist in aleurone layer cells (see Fig. 31B).

All three figures were photographed using FC II.

Abbreviations: PB, protein bodies; S, starch granule; W, cell wall; C, seed coat; AL, aleurone layer cell; SAL, subaleurone layer cell.

FIG. 32



seconds, comparable with those used for the controls (see above). The fluorescein binding seemed to be specific for protein bodies; cell walls and starch granules were not stained (Figs. 32 A-C). Subaleurone layer cells (Figs. 32 A and B) were evenly stained indicating that all protein bodies apparently contained 12S globulin molecules. In aleurone layer cells (Fig. 32C), in addition to fluorescein staining of the protein bodies, considerable autofluorescence was also observed. This could be due to the presence of high amounts of ferulic acid and other phenolic compounds in the aleurone layer cells. The autofluorescence intensity was apparently amplified after staining with FITC-goat IgG and globulin antibodies. The control for aleurone autfluorescence was shown in Fig. 31C (AL).

In summary, 12S globulin molecules were found localized within oat endosperm protein bodies. No preferential staining of the protein bodies in all endosperm cell types was detectable indicating the even distribution of 12S globulin molecules in all protein bodies.

CHAPTER IV

DISCUSSION

The synthesis of oat globulin polypeptides in the form of larger molecular weight precursors had been previously suggested by several research groups (67,69,70,111, and Matlashewski G.J., 1983, Ph.D. Thesis, University of Ottawa). The basis of this assumption was the repeated observations that globulin polypeptides were synthesized as 60 kDa polypeptides in *in vitro* translation systems. These 60 kDa polypeptides were unreducible under reducing conditions (+ 2-mercaptoethanol). In mature seeds, globulin polypeptides exist as 60 kDa polypeptides which are reducible into two groups of subunits with average molecular weights of 38 and 20 kDa. Therefore, *in vitro* translation of globulin mRNA molecules into unreducible 60 kDa polypeptides tended to indicate that globulin polypeptides were first synthesized as larger precursors. Although the *in vitro* synthesis of globulin polypeptides as 60 kDa polypeptides was widely accepted, sufficient evidence to establish a clear precursor-product relationship was lacking. There was also little information regarding the site of globulin synthesis, the mode of its deposition in developing endosperm, and, the pattern and site of possible processing events.

The experiments in this thesis were designed to answer some of the above questions. The results of these experiments, reported earlier in the Results Chapter, will be discussed here in an attempt to clarify whether any of the above questions have been answered.

Pattern and Site of Globulin Synthesis:

Several publications have reported that polysomes (69,70), total poly A$^+$ RNA (69,70), and membrane-bound polysomes (67,111) are capable of directing the *in vitro* synthesis of 60 kDa polypeptides immunoprecipitable with globulin antibodies. In addition, two reports had suggested the preferential synthesis of oat globulin by membrane-bound polysomes (112,116). There was little biochemical evidence to support such a preferential synthesis by membrane-bound polysomes. No biochemical evidence was also available to indicate the involvement of ER membranes in globulin synthesis.

A number of experiments were designed to address the role of ER membranes in globulin synthesis. Three approaches were undertaken: A, Comparison between polypeptides synthesized by free and membrane-bound polysomes and poly A$^+$ RNA. B, Isolation and cell-free translation of RER membranes to reveal the presence of any mRNA molecules coding for globulin polypeptides. C, *in vivo* labelling and analysis of ER membranes for the presence of any globulin polypeptides.

A. Comparison of polypeptides synthesized by free and membrane-bound polysomes and poly A$^+$ RNAs.

Developing oat groats appeared to contain approximately equal proportions of free and membrane-bound polysomes. The yield of total polysomal RNA extracted from free polysomes was however higher than from membrane-bound polysomes. In contrast, membrane-bound polysomes appeared to contain a higher amount of poly A$^+$ RNA transcripts than free polysomes. Membrane-bound poly A$^+$ RNA preparations were rich in RNA molecules corresponding in size to 18S, 15S, and 12S based on agarose gel electrophoresis. The 18S RNA molecule which has been suggested to code for the globulin 60 kDa polypeptides (111) was also observed in free poly A^+ RNA preparations. Although this 18S band might have been ribosomal RNA, it was not possible to draw any conclusions on the site of RNA molecules coding for globulin polypeptides based on the agarose gel electrophoresis data. Therefore, the free and membrane-bound templates were translated in an in vitro cell-free protein synthesizing system.

The analysis of the in vitro translation products revealed a number of differences among the products of free templates and membrane-bound templates. One major discrepancy was in the synthesis of two 58-62 kDa polypeptides. These two polypeptides comprised a significant portion of the translation products of membrane-bound poly A^+ RNA and only a minor part of the translation products of free poly A^+ RNA. Scanning of the translation products showed that membrane-bound poly A^+ RNA directed the synthesis of globulin 58-62 kDa polypeptides 10 fold higher than free poly A^+ RNA could. Immunoprecipitation of the translation products with globulin antibodies also showed that these 58-62 kDa polypeptides could be immunoprecipitated from the translation products of membrane-bound poly A^+ RNA. These results indicated that the 58-62 kDa polypeptides were related to globulin polypeptides and that they were synthesized primarily by membrane-bound templates. There were some synthesis of these globulin polypeptides by free templates since the 58-62 kDa polypeptides were also immunoprecipitated from the translation products of free poly A^+ RNA although the signals were relatively weak.

As a whole, the results supported the assumption that membrane-bound polysomes of developing seeds were enriched in mRNA molecules coding for globulin polypeptides.

B. Isolation and analysis of RER membranes for the presence of any mRNA molecules coding for globulin polypeptides.

Sucrose gradient centrifugation was used to isolate RER membranes from developing oat groats. These membranes sedimented to a density of 1.15 g/cm^3 when a seed extract was fractionated on a linear 16-65% sucrose gradient. The integrity of these membranes were examined by electron microscopy. Such preparations appeared to be enriched in ER membranes with attached ribosomes. When polysomes were isolated from these RER membranes and translated in vitro in a wheat germ extract, they were translationally active and could direct the synthesis of many polypeptides. Among these translation products, two polypeptides with molecular weights of 58-62 kDa corresponded in size with the globulin polypeptides previously shown to be primarily synthesized by membrane-bound templates. This might thus indicate that mRNA molecules coding for globulin polypeptides were present in RER membranes isolated from developing groats. This indication compared well with the earlier findings that globulin polypeptides were synthesized preferentially by membrane-bound templates. One may thus refer to the RER as the major site of globulin synthesis.

C. In vivo labelling and analysis of ER membranes for the presence of any globulin polypeptides.

Since evidence was obtained for the presence of mRNA molecules coding for globulin polypeptides in the RER fraction of developing groats, globulin polypeptides were expected to be bound to the ER membranes. To examine this assumption, developing seeds were labelled in vivo and ER membranes were isolated in order to analyze for the presence of labelled globulin polypeptides. Fractionation of an extract of labelled groats on a linear sucrose gradient showed that incorporated radioactivity was largely associated with the ER marker enzyme activity; suggesting that most of the labelled polypeptides were present in the ER fraction of the groats. This assumption was supported by the important observation that the peak of incorporated radioactivity shifted together with the peak of ER marker enzyme activity to 1.15 g/cm^3 when magnesium ions were present.

The nature of the radioactive polypeptides associated with ER membranes were then examined. Globulin antibodies were used to challenge any globulin polypeptides present in the ER. Two polypeptides with molecular weights of 58-62 kDa were selected by the antibodies. Therefore, globulin putative precursors were found associated with the ER membranes suggesting that the 58-62 kDa polypeptides might be segregated within these membranes after or during translation.

The segregation of the globulin precursors within the ER membranes may be assisted by a N-terminal signal sequence. The presence of such a sequence was previously suggested by Brinegar and Peterson (70). These investigators reported that <u>in vitro</u> synthesized precursors were slightly larger than the <u>in vivo</u> synthesized precursors (70). Further clarification of the existance of signal sequences awaits complete sequencing of the globulin gene(s).

Transmission electron microscopic examinations of developing seeds at 16 daf appeared to confirm the above biochemical data by showing an abundance of ER membranes at this stage of seed development. Interconnections of the ER with protein bodies also tended to support the notion that ER membranes were actively involved in protein synthesis and formation of protein bodies.

Finally, the presence of mRNA molecules coding for globulin polypeptides in the ER as well as the association of globulin polypeptides with the ER confirmed that globulin polypeptides are synthesized and probably segregated within ER membranes in developing seeds.

Previously, Luthe and Peterson (112) had isolated and translated both free and membrane-bound polysomes from developing oat groats. They reported no qualitative difference in the products made by membrane-bound and free polysomes. However, they did not challenge the translation products with globulin antibodies.

Membrane-bound polysomes have been shown to be the primary site of synthesis of many legume and cereal reserve proteins (8,35,43,44,50,54,59,113). For example, pea legumin, maize zein, soybean glycinin, and french bean phaseolin are synthesized on polysomes associated with the ER membranes. Using in vivo labelling techniques, Chrispeels et al. (35) and Bollini et al. (50) have also shown transient association of legumin and vicilin polypeptides and phaseolin polypeptides with ER membranes in pea and french bean cotyledons respectively. Bollini and Chrispeels (107) also isolated RER membranes from french bean cotyledons and demonstrated that these membranes were enriched in polysomes capable of directing the in vitro synthesis of phaseolin. Synthesis on membrane-bound polysomes appears to be an essential step for these reserve proteins which are bound for deposition in protein body vesicles. In other words, reserve globulins which are destined for vacuoles must first be channelled through the RER lumen. This could therefore be regarded as a sort of intracellular secretion.

Intracellular Translocation of Globulin Precursors:

In vivo labelling experiments were conducted which showed transient association of globulin 58-62 kDa precursors with ER membranes. Globulin polypeptides disappeared from the ER shortly after their synthesis and did not remain there permanently. This suggested that ER membranes (ER lumen) were not the final site of globulin deposition. These observations agreed well with the data on the transport of legume reserve globulins (35,50). This translocation of newly-synthesized globulin polypeptides differs however with the mode of synthesis and deposition of maize zein (55). Zein polypeptides remain associated with the ER after their synthesis and the resulting protein bodies possess significant amount of ER-marker enzyme activity (55,59). It therefore appears that oat 12S globulin polypeptides follow a deposition pathway similar to that observed for legume globulins and different from that described for zein polypeptides. The factors influencing the discrepancy between globulin and prolamin compartmentalization were discussed in detail in the introduction.

Further *in vivo* labelling and subcellular fractionation experiments indicated a gradual translocation of proteins from the ER to protein bodies. *In vivo* labelling of developing spikelets and subsequent incubation with cycloheximide showed that at first radioactive proteins accummulated in the ER fraction of the cells. However, as the incubation was prolonged, radioactive protein began to accummulate largely in the protein body fraction. This apparent translocation of proteins from the ER to protein bodies suggested that protein bodies may be the final site of reserve protein deposition during seed development.

Electron micrographs presented in this thesis and those reported by

Saigo et al. (116) concur with one another in suggesting that RER is the site of globulin synthesis. Electron microscopic investigations complemented the biochemical data in confirming the abundance of RER membranes during reserve protein synthesis in developing oat seeds. Such membranes were found continuous with the vacuolar membranes surrounding protein bodies. ER membranes were abundant at 16-20 daf concurrent with the time of maximum reserve protein synthesis. The examination of the ER fractions isolated from developing (16 daf) seeds indicated the association of numerous ribosomes with these membranes suggesting them to be active in reserve protein deposition. Similarly, Saigo et al. (116) reported the proliferation of ER membranes in developing oat seeds (16 mg groats) concurrent with reserve protein deposition. Such a direct correlation between the appearance of ER membranes and the synthesis of some enzymes and reserve proteins has been reported in other systems (114,115).

Together the data suggest that ER initiates the synthesis of reserve proteins (specifically reserve globulins) and translocates them to vacuoles. A direct continuity between the ER and the tonoplast membranes was shown by Saigo et al. (116) and the electron micrographs in this thesis. The newly-synthesized proteins aggregate at the surface of tonoplast to form protein deposits. The membranes needed for this process may originate from the ER, tonoplast, and/or dictyosomes. Membrane vesicles observed in the vicinity and within vacuoles may participate in this process. However, as suggested previously, some of these vesicles may be artifacts of tissue fixation and cannot be assumed as true structures by certainty. Protein deposits formed at the surface of tonoplast appear then to be secreted into the vacuole. These relatively small protein bodies may then fuse together to form larger protein bodies eventually filling the entire vacuole.

Post-translational Processing of Globulin Precursors:

Some sort of processing was previously predicted based on the finding that globulin polypeptides (which exist in mature seeds as two groups of subunits) were synthesized _in vitro_ as larger 58-62 kDa precursors. Such a processing event could be considered post-translational due to the stability of the globulin precursors, for a relatively long time after synthesis. To examine the pattern and the site of globulin processing, a number of _in vivo_ labelling experiments were conducted. A series of observations were made which are discussed here in two sections: A. Precursor-Product Relationship.

B. Site of Globulin Processing.

A. Precursor-Product Relationship:

Preliminary _in vivo_ labelling experiments using developing spikelets showed that radioactivity was taken up very quickly and distributed promptly into the leaves and the hulls. The seeds also took up the radioactivity but at a slower rate. Incorporation of the radioactivity by the seeds was detectable after 30 min of labelling and increased dramatically afterward. The analysis of the incorporated radioactivity indicated the labelling of two 58-62 kDa globulin polypeptides after 1 h of labelling. These results compared with the earlier _in vitro_ synthesis studies which showed the translation of membrane-bound poly A^+ RNA into two 58-62 kDa globulin polypeptides. Two other research groups have also reported the synthesis of such globulin polypeptides upon _in vivo_ labelling of developing seeds (69,70).

In order to clarify the relationship among the 58-62 kDa polypeptides and the mature globulin 38 kDa and 20 kDa subunit groups, pulse-chase labelling experiments were carried out. In one such experiment using [^{35}S]methionine, the 58-62 kDa polypeptides were shown to be labelled when developing seeds were incubated for 1 h. The removal of the spikelets and subsequent chase with cold methionine appeared to result in appearance of smaller molecular weight polypeptides corresponding in size to the two globulin subunit groups. This also resulted in some reduction in the amount of 58-62 kDa polypeptides as the chase was prolonged. Overall, there was a correlation between the appearance of the 38 kDa and 20 kDa globulins and the reduction in the amount of labelled 58-62 kDa globulins.

In a second attempt, developing spikelets were labelled with [^{35}S]sulfate and subsequently incubated with cycloheximide. This experiment showed similar results to the above pulse-chase labelling experiments with the exception that the reduction in the intensity of globulin 58-62 kDa polypeptides was more drastic. This indicated that cycloheximide was inhibiting the continued incorporation of free label into the 58-62 kDa polypeptides allowing for a better correlation to be drawn among the precursors and the products.

In a third approach, developing spikelets were labelled with [^{14}C]leucine and chased with cold leucine. The 58-62 kDa globulins were first labelled followed by the labelling of the 38 kDa and the 20 kDa globulins. A correlation was again observed between a gradual reduction in the amount of labelled 58-62 kDa polypeptides and a gradual increase in the amount of the labelled 38 kDa and 20 kDa globulin polypeptides. This pulse-chase labelling pattern compared well with the patterns obtained with [^{35}S]methionine and [^{35}S]sulfate. However, [^{14}C]leucine labelled both groups of globulin subunits in contrast to [^{35}S]methionine which preferentially labelled the 20 kDa globulin subunits. These labelling patterns were indicative of the absence of methionine residues in most species of the larger 38 kDa globulin subunits.

Overall, a clear relationship was found between the globulin precursors and the mature globulin subunits. Developing seeds appeared to synthesize two 58-62 kDa polypeptides and later process them into smaller 38 kDa and 20 kDa subunit groups. Such a processing event is common among many seed storage proteins. Pulse-chase labelling experiments have been used to show post-translational processing of pea legumin (41), rice glutenin (113), and soybean glycinin (44). It appears that all seed reserve globulins are synthesized as larger precursors during their deposition in developing seeds. Interestingly, the biosynthesis of plant seed lectins appears to follow similar pathways. Higgins et al. (117) have demonstrated the synthesis of pea lectin as a 23,000 Da precursor which is transported from the ER to protein bodies where it is processed into its mature form (polypeptides with molecular weight of 17,000 and 6,000). Similarly, Lord (118) has shown post-translational processing in protein bodies of castor been lectin precursors (64,000-68,000 Da) into two chains with molecular weight of 32,000 and 34,000. In this case, the synthesis as larger precursors is vital to the cells since the mature form of this lectin (32,000 Da chain known as ricin) is highly toxic and its release in cytoplasm will inactivate 60S ribosomal subunits (118).

The significance of synthesis of reserve proteins as larger precursors is not clear. Crouch et al. (81) have suggested that the net charge of the newly-synthesized napin precursors might facilitate their topogenesis in the cell.

B, Site of Globulin Processing:

Mature globulin polypeptides could be found in protein bodies of developing seeds. The site of maturation of these polypeptides was not known. Globulin precursors may be processed before or after their arrival into protein bodies.

An *in vivo* labelling experiment showed that when developing seeds were labelled for 1.5 h and incubated for 2 h, labelled globulin 58-62 kDa precursors were detectable in protein bodies. This observation therefore suggested that globulin precursors entered the protein bodies intact; processing must have occurred afterwards. In the same *in vivo* labelling experiment, globulin 38 kDa and 20 kDa polypeptides were detected in protein bodies only after the appearance of the 58-62 kDa precursors. One may therefore conclude that protein bodies are the site of globulin processing. The enzyme(s) responsible for cleavage of globulin precursors in protein bodies are not known. Numerous acid hydrolases including proteases have been localized in plant seed protein bodies (119,120,121). Donhowe and Peterson (119) recently demonstrated the proteolytic activity of oat aleurone cell protein bodies. A specific protease or a combination of a number of proteolytic enzymes may be involved in cleaving the globulin precursors. It has been shown, for example, that a combination of two enzymes, trypsin and carboxypeptidase B could correctly process the proinsulin precursors into mature insulin molecules in an *in vitro* system (122). It is thought that in proinsulin processing an endopeptidase is required to cleave on the carboxyl side of the cleavage site (basic amino acids), and an exopeptidase is then needed to trim off the remaining basic residues from the newly-exposed C-terminus (122). Little data are available about the mechanisms involved in the proteolytic cleavage of seed reserve proteins. Recent sequence analyses of seed globulin genes have, however, provided some interesting information regarding the processing sites. A Asn-Gly sequence seems to be conserved at the site of proteolytic cleavage for all globulin sequences that have been examined (42,123,124,125,126,127). Partial nucleotide sequencing of a cDNA clone for a oat 12S globulin precursor has also revealed the presence of this Asn-Gly sequence at the site of proteolytic cleavage (128). Interestingly, processing of the soybean globulin precursor includes the removal of a peptide of four amino acids between the acidic and basic polypeptides (125). However, in pea the C-terminal amino acid of the acidic polypeptide immediately precedes the N-terminal of the basic polypeptide (123). The precise processing pattern of oat globulin precursors is not yet clear since the amino acid sequence of the acidic (larger) subunits is not known.

Assembly of Globulin Polypeptides in Protein Bodies:

Since globulin molecules are normally found as 12S oligomers in oat seeds, a mechanism must therefore exist which results in formation of these oligomers by assembly of the two 38 kDa and 20 kDa globulin subunits. The timing of this assembly was unknown. Globulin 12S oligomer might be assembled during any one of the various steps of globulin deposition. Globulin precursors synthesized on the RER may be immediately assembled into such 12S molecules. Alternatively, this assembly might occur during transport or deposition of globulin polypeptides in protein bodies.

A combination of <u>in vivo</u> labelling techniques and density gradient fractionation methods were used to determine which of the above mechanisms was actually occurring <u>in vivo</u>. These experiments showed the appearance of protein molecules with sedimentation coefficients of 3S and 8-9S in the ER after a short pulse but no oligomer with sedimentation coefficient of 12S was detected in the ER. The identity of the 3S and 8-9S globulin molecules was not clear. They could represent large globulin protein molecules or might be protein oligomers assembled from globulin polypeptides in the ER. It was previously demonstrated that under these conditions (labelling for 50 min) only the globulin precursors with molecular weights of 58-62 kDa were detectable in the ER (see Results, Part Ic., Figs. 7 and 8). It could thus be suggested that the 3S and/or the 8-9S molecules were assembled forms of the globulin precursor polypeptides. Further studies are however needed to examine this assumption.

Interestingly, in their studies on pea legumin deposition, Chrispeels et al. (35) reported the formation of 4-5S and 8S oligomers in ER membranes of developing pea cotyledons.

In vivo labelling and density gradient fractionation experiments also showed the formation of a predominant 12S holoprotein in protein bodies after a short pulse (50 min) and a long incubation period (6 h). This newly-assembled 12S oligomer was not detected in protein bodies after 50 min of labelling and 1 h of incubation. These results therefore suggested that the 12S oligomer was assembled probably after the arrival of the newly-synthesized precursors into protein bodies. Electrophoretic and fluorographic analyses of the 12S oligomer demonstrated that this oligomer consisted of mature globulin 38 kDa and 20 kDa subunits. This finding suggested that the cleavage either preceeded or accompanied the oligomer formation. In the former case, the cleavage of globulin precursors may then be a prerequisite for the assembly of the 12S oligomer suggesting its role in proper packaging of reserve globulin in protein bodies. Further studies are essential, however, to clarify the kinetic relationship between cleavage and assembly.

The accumulation of globulin polypeptides in protein bodies only as 12S molecules may indicate that the protein's three-dimensional structure plays an important role in the proper packaging of globulin subunits. In other seeds, Bollini et al. (50) provided evidence for assembly of french bean phaseolin into an 18S molecule after arrival into protein bodies. Pea 11-12S legumin was also reported to be assembled in protein bodies (41). Although, final assembly of reserve globulins appears to take place in protein bodies, there is evidence for partial assembly of globulin precursors in the ER. In the case of pea legumin, newly-synthesized precursors were shown to be assembled into oligomers in the ER which appeared to be half the size of mature legumin (41). Such a pattern of assembly has also been reported for soybean glycinin

(129). One experiment in this thesis (Fig. 23, Results Chapter) suggested the presence of large globulin molecules in the ER but the evidence was not adequate to indicate a partial assembly of oat globulin precursors in the ER.

Protein Bodies as the Site of Globulin Deposition

Protein bodies, which are the final site of oat globulin deposition, were characterized by several biochemical and electron microscopic techniques. The conditions for isolation of intact protein bodies needed to be optimized. This was critical for the validity of the results of those experiments dealing with protein bodies. A discontinuous sucrose gradient of 15% and 65% was found to be ideal for the separation of protein bodies from starch granules which sedimented in the bottom of the tube. Centrifugation time was also important. A short spin (10 min) at low speed (1000 X g) in a swinging bucket rotor was sufficient to separate the protein bodies banding at the 15/65 interface. Magnesium ions were included in the extraction buffer in order to stabilize various organelles. The use of a 16-65% linear sucrose gradient also resulted in the isolation of intact protein bodies, but the yield of protein bodies was much lower and the technique is less rapid. This was mainly due to the high ionic strength of the buffer used for homogenization which causes disruption of protein bodies.

Once a reliable protein body preparation was obtained, various electrophoretic and microscopic analyses could be carried out. Oat protein bodies had an average density of 1.23 g/cm^3. This value compared well with that reported for oat starchy endosperm protein bodies (119). When compared to other cereals, oat protein bodies had a density equal to maize protein bodies (1.23 g/cm^3) but lower than those reported for wheat (1.27 g/cm^3) and barley (1.26 g/cm^3) protein bodies (51).

Electron microscopic examination of protein bodies in intact seeds showed the presence of membranes surrounding each protein body. These surrounding membranes were not visible once the protein bodies were extracted from intact seeds. It was previously argued that the process of protein body preparation might have resulted in the loss of membranes. Alternatively, it was suggested that membranes might have been present in such protein body preparations but were not stained well enough to become visible.

Proteinase K treatment of extracted protein bodies did not result in any significant digestion. This resistance of protein bodies against proteolysis may be conferred by membranes surrounding the protein bodies, although such membranes were not easily visible from the electron micrographs. Therefore, there may be another factor contributing to the resistance of these protein bodies against proteinase K digestion. One possible factor suggested previously was a special three-dimensional structure of globulin deposits which could make them resistant to proteolysis. Such a structure may actually be an essential feature of the protein deposits making them resistant to digestion by vacuolar proteases which have been reported to exist in protein bodies (117,118). Miflin and Burgess (130) used the proteinase K treatment approach to show that among maize, barley, wheat and pea, only maize and pea protein bodies were surrounded by membranes. The results presented in this thesis were comparable with that reported for maize protein bodies. Interestingly, legume seed protein bodies were also resistant to proteolytic digestion indicating them to be similar to oat and maize protein bodies in this respect (130). It should be mentioned that the membranes surrounding the maize protein bodies must have originated from the RER membranes since these protein bodies possessed considerable NADH-cytochrome c reductase activity (55). This was not the case with oat protein bodies (no ER- marker enzyme activity was found in oat protein body preparations). This discrepancy may be indicative of the separate pathways by which oat and maize protein bodies are developed.

In terms of protein composition, oat protein bodies had sucrose gradient and SDS-PAGE profiles similar to those obtained from the whole endosperm. The 12S globulin polypeptides were the major protein components of protein bodies, therefore suggesting that most of the developing seed's protein was deposited in these vesicles, and little, if any, may be left "loose" in the cytosol.

During the course of this project, other investigators (119) had also been characterizing oat starchy endosperm and aleurone layer protein bodies. Their results demonstrated the similarity of these two classes of protein bodies in terms of amino acid composition and SDS-PAGE profile (119). They concluded that globulins and prolamins might be equally distributed in all protein bodies.

In the present study, no attempts were made to separate starchy endosperm from aleurone layer. It was not practically feasible to separate these two compartments from developing oat groats during the _in vivo_ labelling studies. Based on the available data, it was assumed that globulin polypeptides were synthesized in both the aleurone and subaleurone cells. A subsequent immunofluorescent study (see below) supported this assumption.

Localization of 12S globulin using a fluorescent probe was made possible by the availability of specific 12S globulin antibodies. A commonly used fluorescent dye (fluorescein) was found suitable and preferable over rhodamine. Positive fluorescence staining of all protein bodies in the oat endosperm cells (including aleurone layer, subaleurone and starchy endosperm) indicated an even distribution of 12S globulin within various parts of oat endosperm. An obvious limitation of this technique was poor resolution of individual protein bodies. This problem could be overcome by an immunocytochemical localization study employing protein A-gold as the probe. Unavailability of EM facilities has delayed the initiation of this project.

In conclusion, the results of the immmunofluorescent experiment supported the notion that globulin was evenly distributed in oat endosperm protein bodies. This is in contrast to rice protein bodies which are classified into two types based on their protein composition (131). Other cereals, however, are like oat and seem to have only one type of protein body (132).

GENERAL DISCUSSION

Hypothesis

Based on the data presented in this dissertation and the published reports, a general hypothesis on the mechanisms of synthesis, processing, transport and deposition of oat 12S globulin can now be proposed. Many parts of this hypothesis are supported by biochemical and microscopic evidence but some sections are suggested solely based on indirect evidence or indications from other seed depository systems.

1. During development of oat endosperm, 12S globulin polypeptides are synthesized primarily on membrane-bound polysomes as larger precursors.

2. The newly-synthesized precursors are segregated into the ER lumen possibly with the aid of signal sequences.

3. After segregation, the precursors are channelled via the ER cisternae to the vacuolar membranes (tonoplast).

4. The globulin polypeptides are condensed and compartmentalized with either part of the tonoplast membrane or vesicles derived from the Golgi bodies, to form protein bodies.

5. The newly-assembled protein bodies are then fused into larger ones filling the entire vacuole.

6. During or after the process of protein body assembly, proteolytic cleavage of globulin precursors occurs yielding the α and β subunits.

7. The globulin α and β species are then put together to form a 12S oligomer. This assembly occurs during or after the cleavage of the precursors.

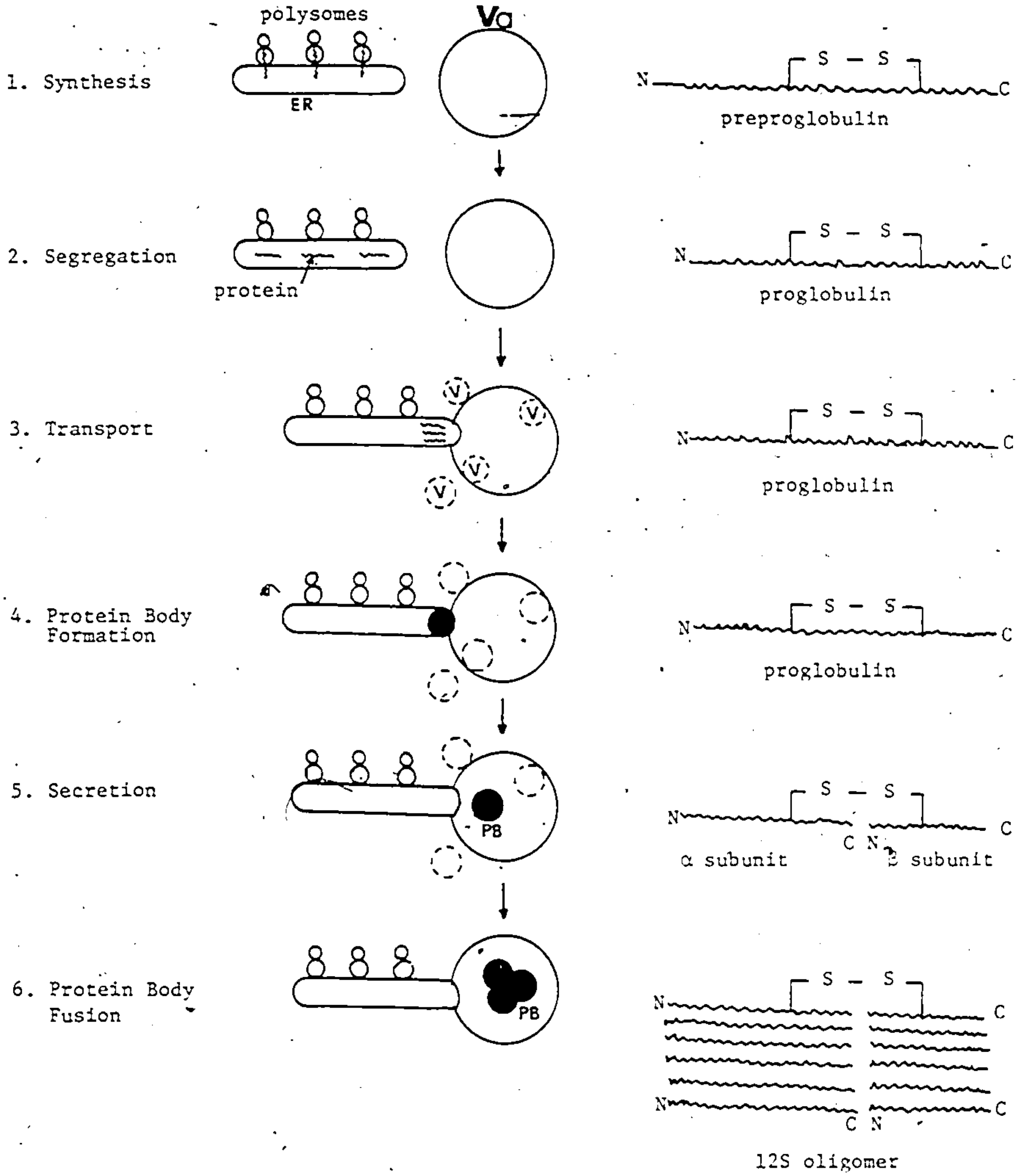
Fig. 1 illustrates a schematic view of the above hypothesis.

The synthesis of globulin 58–62 kDa precursors on the RER is supported by in vitro translation, in vivo labelling, subcellular fractionation and electron microscopic data presented in this thesis and other published reports (67,69,70). Co-translational segregation of globulin polypeptides was indicated by the proteinase K digestion of mRNA translation products (see Results, B_4).

Transmission electron micrographs presented in this thesis and in a published article (116) both illustrated the transport of reserve proteins through the ER cisternae into vacuoles. Micrographs in this thesis also showed the assembly of protein bodies at the interface between the ER and tonoplast. It may be assumed that protein channelled in the ER is encircled by membranes provided by tonoplast or vesicles derived from other sources such as dictyosomes. Such vesicles could be clearly observed in the cytoplasm and occassionally inside vacuoles or in association with tonoplast. However, it is not clear whether the membrane is directly provided by tonoplast and replaced by extra vesicles or the small vesicles are filled with protein without the involvement of tonoplast. In any case, the assembled protein bodies seem to be released (secreted) into the vacuole and later fused forming larger protein bodies. It should be emphasized that this process of protein body development was not probably the only one operating in the cell but it seemed to be the predominant pathway. Occassionally, individual protein bodies were observed in the cytoplasm which were not within vacuoles and might have thus been developed via separate pathway(s).

Fig. 1. Synthesis and compartmentalization of oat globulin. Based on the results of the present thesis and other published reports a general hypothesis on globulin deposition is proposed. Oat 12S globulin is synthesized as precursor molecules on the RER, segregated, and transported to tonoplast. At this site, protein deposits are formed which are secreted into the vacuole. The smaller protein bodies fuse together to form larger bodies. In the meantime, globulin molecules go through several structural changes including disulfide bond formation, proteolytic cleavage, and oligomer formation

Abbreviations: ER, endoplasmic reticulum; PB, protein body; V, vesicle; Va, vacuole.


1. Synthesis
polysomes
ER
Va
N
S – S
C
preproglobulin
2. Segregation
protein
proglobulin
3. Transport
V
proglobulin
4. Protein Body Formation
proglobulin
5. Secretion
PB
α subunit
β subunit
6. Protein Body Fusion
PB
12S oligomer

The formation of large protein bodies from the fusion of smaller ones may occur due to the gradual dehydration of endosperm. Membrane interconnections among protein bodies may assist in the fusion process. Such membrane continuity was clearly observed in the micrographs presented here.

The proglobulin molecules packaged into protein bodies were cleaved at this site into the corresponding α and β subunits. <u>In vivo</u> labelling studies reported here in this thesis demonstrated this post-translational cleavage of globulin precursors occurring only after arrival in protein bodies. In the course of or after globulin processing, protein molecules were assembled into a 12S oligomer. This part of the hypothesis was supported by <u>in vivo</u> labelling and gradient fractionation experiments reported in the Results chapter.

In summary, most portions of the above hypothesis are backed by some sort of evidence, but some details still remain to be worked out. Two major points that specially need to be explained are first, the role of a hypothetical signal sequence in the segregation process and secondly, the source of membranes (vesicles) required for protein body assembly.

The pathway of oat 12S globulin synthesis and deposition is not unique and has indeed considerable homologies to the mechanisms described for other seed reserve globulins. The synthesis of reserve proteins as larger molecular weight precursors and post-translational processing of these precursors has been reported for rice glutelin (113), pea legumin and vicilin (41), soybean glycinin (44), rapeseed napin (81) and various types of seed lectins (133,134). It is thought that oligomeric proteins present in protein bodies which contain significantly different subunits are synthesized as a single large precursor chain which is subsequently cleaved to generate the different subunits (133). In contrast, oligomeric proteins with identical or nearly identical subunits appear not to be synthesized via such precursors (133). Oat globulin which possesses two distinct subunits fits into the first category of oligomeric proteins being synthesized as a single precursor. The synthesis of a precursor form of two subunits with significant differences in charge may neutralize the protein molecule and thus facilitate its intracellular transport into protein bodies. As mentioned previously in the Introduction, rapeseed napin subunits with opposite charges form a precursor molecule with a pH close to neutrality (81).

It is now well accepted that during seed development the origin of many types of reserve proteins is the RER. Oparka and Harris (38) employed electron microscopy to demonstrate that all three types of rice protein bodies are initiated by dilation of the endoplasmic reticulum. Miflin <u>et al</u>..(51) used subcellular fractionation techniques to show the synthesis of maize, barley and wheat reserve proteins on the RER. In legume seeds, the RER is also the primary site of reserve protein synthesis. Therefore, regardless of the cell depository pathway, development of protein bodies are all initiated in the RER. This is one piece of evidence which supports the notion that seed protein deposition is a type of intracellular secretion.

Many other secretory and reserve proteins are translocated from the RER into protein bodies (10,41,44) in a manner similar to oat globulin. In animal cells, transport of secretory proteins from the RER into condensing vacuoles has been shown to require energy (dependent on ATP production) (10). In the absence of ATP production, secretory proteins remain in the RER (10). The mechanisms of this intracellular transport, however, are still unknown and several different hypotheses have been proposed. Organelles such as tubular ER, GERL (Golgi ER lysosomes), Golgi complex and transporting vesicles could be involved. The overall interpretation of the available data is that reserve proteins which are glycosylated normally pass through the Golgi complex on their way from the RER to protein bodies. Non-glycosylated proteins such as oat 12S globulin may follow an alternative route. It should, however, be mentioned that dictyosomes may be involved in some other way for example in providing vesicles which participate in development of protein bodies. Chrispeels (36), however, argued that all reserve globulins pass through the Golgi on their way to protein bodies. Evidence was recently provided for the Golgi-mediated transport of vicilin in pea cotyledons (135). Upon application of ionophores, monensin and nigericin, vicilin was transported to plasmalemma instead of normal transport to the vacuole (135). Interestingly, in presence of these drugs, vicilin was released from the cotyledon cells to accummulate between the plasmalemma and the cell wall. This study indicates the significance of ionic equilibria within the cell in proper compartmentalization of reserve globulins.

Oat 12S globulin appears to be deposited in protein bodies with a vacuolar origin. This aspect of oat globulin deposition resembles what has so far been proposed for oilseed and legume reserve protein accumulation. Oilseed protein bodies develop by the secretion of newly-synthesized proteins into vacuoles via dictyosome-derived vesicles (63). Legumin and vicilin, the main storage globulins of peas and field beans are also deposited in vacuolar protein bodies. The vacuoles may be transformed into protein bodies by fragmentation (37). Adler and Müntz (37) studied the development of <u>Vicia faba</u> protein bodies and suggested initial formation of protein bodies by the transformation of large vacuoles into protein bodies; but in later stages of maturation, swollen ER strands appeared which generated ER-produced protein bodies. Both mechanisms seemed to suggest a vacuolar origin for protein bodies (37). Among cereals, wheat protein bodies may be incorporated into vacuolar-like structures (136). However, Miflin et al. (51) reported no vacuolar enzyme activity associated with wheat protein bodies. These investigators have suggested an alternative pathway for wheat protein body development. They proposed that the storage proteins of the wheat and barley starchy endosperms are synthesized on the RER and aggregate into clumps within it. These clumps then separate from the RER and form irregular protein bodies. A similar pathway was also shown to operate for zein deposition in maize (51), the difference being that maize protein bodies are completely surrounded by RER (51,86).

The mechanisms of oat globulin deposition appear to be similar in some aspects to that observed for other seed reserve globulins. This could be due to the remarkable homology which exists between oat globulin and globulins in legumes. The following paragraphs will examine in detail the homologous aspects of oat 12S globulin and pea legumin (a typical legume globulin) deposition.

Homologous Aspects of Oat Globulin and Pea Legumin Topogenesis

To further explore the degree of homology between the depository mechanisms of oat 12S globulin and 11-12S legumins, a detailed comparison is now made between the hypothesis proposed for oat 12S globulin deposition and the data available in the literature (35,41,43,62) on pea legumin compartmentalization.

a) Synthesis as 58-62 kDa (oat globulin) and 60 kDa (pea legumin) precursors was shown by *in vitro* translation, *in vivo* labelling and pulse-chase labelling techniques.

b) Synthesis of the above precursors was shown to occur on the RER by subcellular fractionation and *in vitro* translation techniques.

c) The association of the globulin and legumin precursors with the RER was shown to be transient and not permanent.

d) Intracellular transport of the newly-synthesized precursors from the RER to protein bodies was demonstrated by *in vivo* labelling for both globulin and legumin. The transport mechanisms seem to be rather different. Electron microscopic investigations have suggested direct channelling of oat globulin from the RER into the vacuoles. Legumins seem, however, to be transported via dictyosomes (62). This aspect of depository process needs to be further explored, for a more comprehensive comparison of the transport pathways.

e) Cleavage of the precursors of oat 12S globulin and pea legumin was shown to occur after their arrival into protein bodies. The processed products are two groups of polypeptides with similar average molecular weights (20 kDa and 40 kDa for pea legumin, and 20 kDa and 38 kDa for oat globulin).

f) Assembly of the mature polypeptides into 12S oligomers was demonstrated for both reserve globulin and legumin. The 12S oligomer was not found at any step prior to the arrival of the precursors into protein bodies. The site of assembly was protein bodies in both cases.

Consequently, the homology between oat 12S globulin and pea legumin seems to extend to most if not all the steps of deposition. Except for the pathway of transport from the RER to protein bodies which might differ between the two systems, all other steps seem to be identical. This extensive homology in depository mechanisms may indicate that oat 12S globulin precursors possess certain topogenic sequences homologous to those of pea legumin, which govern their topogenesis in developing seeds. The hypothetical role of topogenic sequences was discussed extensively in the introduction. Such sequences may aid the newly-synthesized globulins to be correctly transported, processed, and assembled. Further investigations are required before comparing oat globulin and pea legumin in respect to their signal and sorting sequences. The sequence of pea legumin signal peptide was recently determined by cDNA sequencing (123).

Future Endeavours

The major objectives of this research project have been achieved. A more clear picture of oat globulin biosynthesis and deposition can now be envisaged. Despite a number of questions which remain to be answered, the general depository pathway of oat globulin is now better understood. Further research focuses on experiments designed to examine the remaining uncertain portions of the hypothesis proposed earlier. These experiments include peptide mapping and NH_2-terminal sequence analyses of oat globulin precursors, as well as immunocytochemical localization studies. Work is also underway in preparation of cDNA libraries complementary to oat poly A^+ RNA sequences, using PBR322 and λ phage as cloning vectors. These libraries are being screened with several probes including synthetic oligo-nucleotides coding for a partial sequence of oat globulin β subunit as well as pea legumin cDNA sequences. This approach may enable one to examine the globulin cDNA molecules for the presence of any possible topogenic sequences. Expression of globulin genes in hosts such as *E. coli* and *Xenopus* oocytes may also assist in clarifying the significance of any putative signal or sorting sequences. Such an approach was recently used to express the thaumatin gene in yeast demonstrating the necessity for NH_2- and C-terminal sequences of preprothaumatin (137). A storage protein was also recently expressed in *E. coli* using novel expression vectors (138). Bartels et al. (138) were able to show the expression of a subunit of wheat glutenin in *E. coli* demonstrating the stability of a plant storage protein in a foreign genetic background.

The long term objectives of this project are still far reaching. Improving seed protein quantity and quality by manipulating the genes coding for reserve proteins could only be achieved through acquiring a sound understanding of mechanisms underlying the expression of these genes. The results of the present project may assist in this direction.

APPENDICES

APPENDIX I. CHARACTERIZATION OF THE MAJOR AND MINOR COMPONENTS OF OAT GLOBULINS

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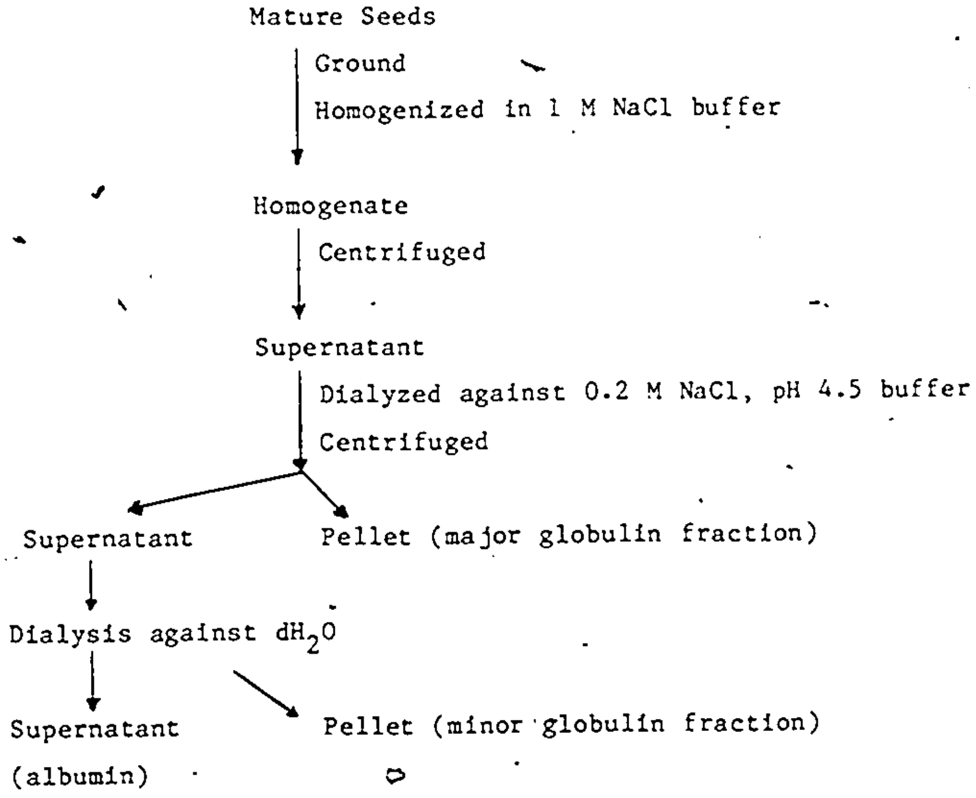
APPENDIX I

CHARACTERIZATION OF MAJOR AND MINOR COMPONENTS OF OAT GLOBULINS

The salt-soluble globulin extract from mature oat seeds consists of a complex mixture of polypeptides. The 12S globulin is the major fraction constituting about 93% of the total globulin extract. The remaining 7% of the extract contains a number of heterogeneous polypeptides. General characteristics of the major and minor globulins are presented in this appendix.

Protein Extraction.

Various components of oat globulin fraction were isolated by isoelectric precipitation as shown in the following diagram.



Observations

Three distinct protein components could be separated from the salt-soluble oat extract: fraction precipitated at pH 4.8 (major globulin), fraction soluble at pH 4.8-0.2 M NaCl (minor globulin), and proteins soluble in dH_2O (albumin). The minor globulin fraction comprised about 7.0% (w/w) of total seed globulins, or in other words, about 5% of total seed protein.

Fractions from isoelectric precipitation (IEP) were analyzed by SDS-PAGE (Fig. 1) under reducing and non-reducing conditions together with total salt-soluble globulin prepared according to Peterson (93). The SDS-PAGE profiles of the major globulin fraction (lane b and b') were compared with total salt-soluble globulin extracted with 50 mM Tris, pH 8.5, 1 M NaCl (lanes a and a'). Reduction of the 60 kDa globulin dimers (Fig. 1, lanes a and b) into the α(~38 kDa) and β(~20 kDa) polypeptides could be observed under reducing conditions (lane a' and b'). The pattern of the minor globulin fraction did not change significantly in the presence or absence of 2-mercaptoethanol (lanes c and c'). However, a few minor bands (e.g. 18 kDa) were affected by the reducing agent. These observations suggested that the major components of the minor globulin fraction might not be linked together through disulfide bonds. Similarly, upon reduction, the SDS-PAGE pattern of the albumin fraction (lanes d and d') did not vary noticeably. Under reducing conditions, several polypeptides in the minor globulin and albumin fractions comigrated with the α subunit components of the 12S globulin which raised the possibility of cross-contamination. Antibodies raised against

Fig. 1 SDS-PAGE analysis of the salt-soluble protein fractions from mature oat flour under non-reducing and reducing conditions. Protein fractions were isolated from oat flour using isoelectric point precipitation. Samples were analyzed on a 12% acrylamide gel in the presence or absence of 2-mercaptoethanol. Lanes a, a' show total salt-soluble globulins extracted by 50 mM Tris-HCl, 1 M NaCl, pH 8.0. Lanes b, b' are the globulin fraction precipitated at pH 4.8. Lanes c, c' show the globulin fraction soluble at pH 4.8, 0.2 M NaCl. Lanes d, d' represent the albumin fraction soluble in dH_2O. Numbers indicate the protein standards: albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and α-lactalbumin (14,000).

Fig. 2 Isoelectric focusing pattern (pH 3.5-10) of reduced salt-soluble protein fractions from mature oat flour. Lane A shows the pattern of the pH 4.8 precipitated globulin. Lane B shows the globulin fraction soluble at pH 4.8, 0.2 M NaCl. Lane C is the pattern of the water-soluble albumin. pI determinations were done using pI standards: 1, amyloglucosidase pI 3.5; 2, bovine carbonic anhydrase pI 5.85; 3, human carbonic anhydrase pI 6.55; 4, horse myoglobin pI 7.35; and 5, lentil lectin pI 8.15.

FIG.1

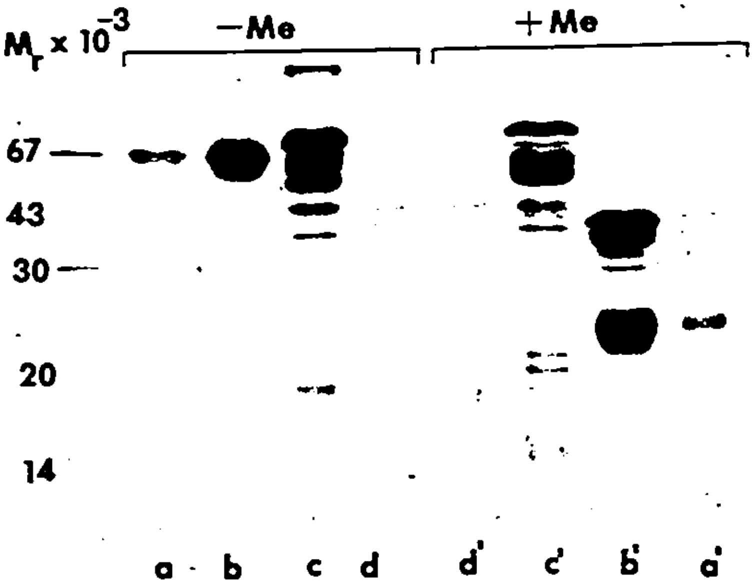
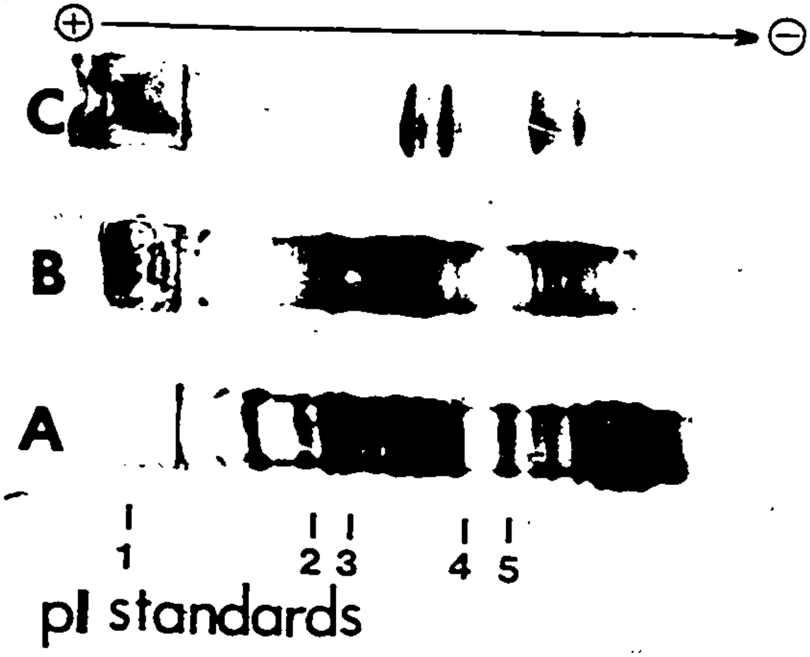

$M_r \times 10^{-3}$
−Me
+Me
67
43
30
20
14
a
b
c
d
d'
c'
b'
a'

FIG. 2


⊕
⊖
C
B
A
1
2
3
4
5
pI standards

purified 12S globulin cross-reacted with the crude vicilin fraction.

However, the anti-12S IgG did not cross-react with individual 3S and 7S vicilins once they were further purified by sucrose density fractionation (see below). This suggested that the crude minor globulin fraction contained trace amounts of the 12S globulin. It is interesting to note that the antibody raised against total salt-soluble globulin (67) did cross-react with both of the major and minor globulin IEP fractions. Therefore, although minor globulin polypeptides cannot be observed in the SDS-PAGE pattern of total globulin (lanes a and a'), they must be present and should be detectable with either higher sample loading or more sensitive protein stains. The anti-12S IgG did not react with the albumins.

The complexity of the 12S globulin, minor globulin, and albumin fractions was investigated by IEF with a pH range of 3.5–10 (Fig. 2). The electrophoretic pattern of the 12S fraction (lane A) was similar to that reported earlier for total oat seed globulin (5,67,69,93,139), showing an acidic (α) and a basic (β) region. Two major regions were observed for the minor globulin fraction (lane B, one acidic, pI ≈5.0–7.0, and the other basic, pI ≈8.0–9.0). The albumin fraction (lane C) contained fewer bands than those of the other two fractions. Since anti-12S globulin IgG did not react with albumins, the albumin fraction was not just a small portion of the globulin fraction. The SDS-PAGE and IEF data indicated that during the dialysis step, the major and minor globulins were precipitated efficiently and therefore ready for further characterization.

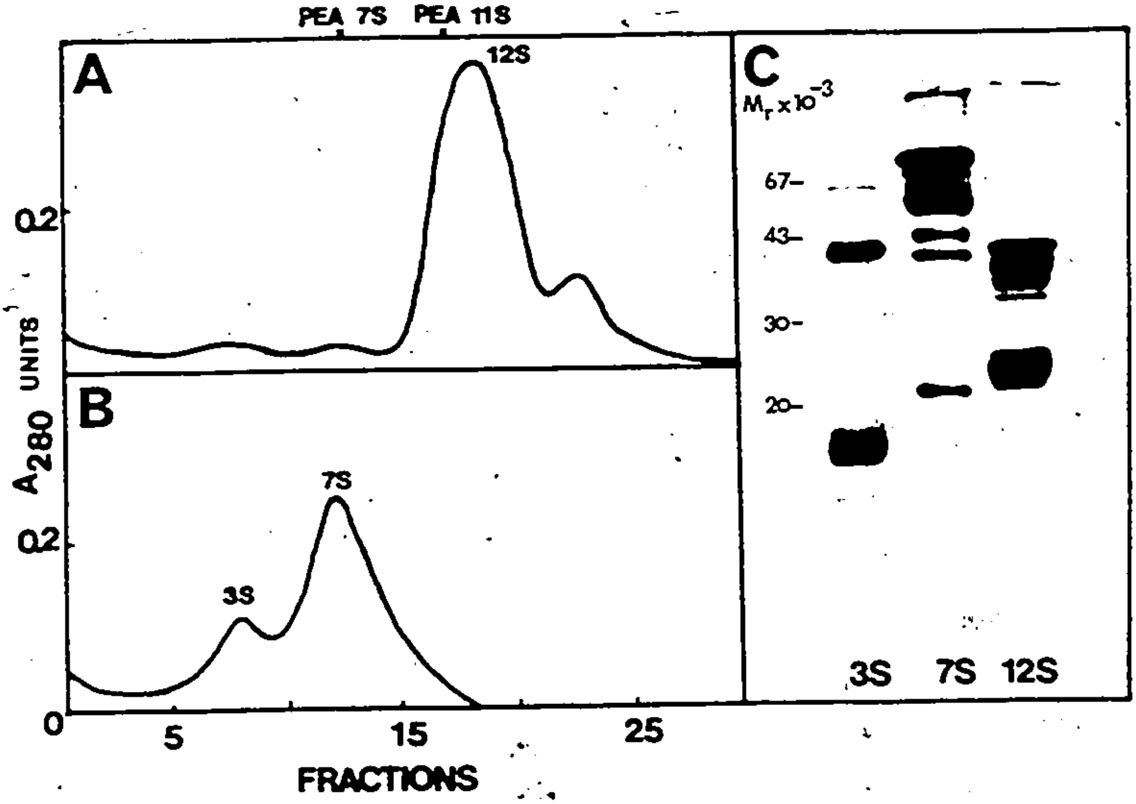
The crude preparations of the two globulin fractions were fractionated on 5–20% sucrose gradients (Fig. 3, A and B). The major globulin banded in a peak with a sedimentation coefficient of 12S (Fig. 3A) which corresponds to the S values reported for total salt-soluble globulin (93,94). A second minor peak with approximate S value of 18S was also observed. The analysis of this peak by SDS-PAGE indicated an identical profile to that of the 12S fraction. This may indicate the association of 12S globulin into a larger oligomer. Upon ultracentrifugation of the crude vicilin preparation two major peaks with approximate S values of 3S and 7S were observed (Fig. 3B). The sedimentation coefficients of 12S, 7S and 3S were estimated using pea 7S and 11S proteins as standards.

The analysis of the 3S, 7S, and 12S protein peaks by SDS-PAGE revealed that these holoproteins consist of distinct and complex groups of proteins (Fig. 3C). The 12S peak of the globulin fraction (lane 12S) was comprised of two groups of polypeptides with average mol wt of 38 and 20 kDa, the globulin α and β polypeptides respectively. The 7S and 3S species (see corresponding lanes) possessed different sets of polypeptides. The 3S holoprotein consisted of 3 major polypeptides with mol wt of 12, 16, and 40 kDa, while the 7S peak consisted of about 7 major polypeptides with approximate mol wt of 20, 39, 43, 50, 65, and 75 kDa.

The SDS-PAGE and IEF profiles indicated that many of the minor globulin polypeptides comigrated with components of the 12S globulin α and β subunit groups. However, two-dimensional analysis showed that the polypeptides with similar isoelectric points possessed different

Fig. 3 Sucrose density gradient fractionation of the globulin IEP fractions. Samples (15-20 mg) were dissolved in 50 mM Tris-HCl, pH 8.0, 1 M NaCl and layered on 5-20% (w/w) sucrose gradients made in the same buffer. The gradients were centrifuged, monitored at 280 nm and fractionated into 1.2 ml fractions. A, shows the profile of the major globulin fraction. B, illustrates the gradient profile of the minor globulin. C, shows the SDS-PAGE (12%, +Me) patterns of the major peaks from the two gradients (3S, 7S, 12S).

FIG. 3


PEA 7S
PEA 11S
A
12S
0.2
A280 UNITS
B
7S
3S
0.2
0
5
15
25
FRACTIONS
C
$M_r \times 10^{-3}$
67–
43–
30–
20–
3S
7S
12S

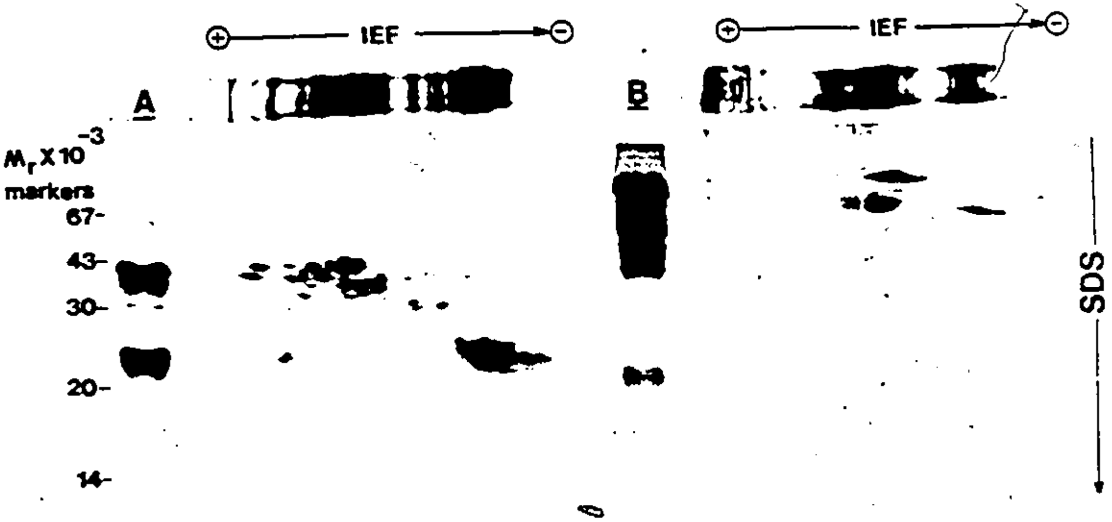
molecular sizes (Fig. 4). Fig. 4A shows the two-dimensional pattern of the 12S globulin. The two major IEF regions of the 7S + 3S fraction had mol wt ranges of 42-70 kDa and 48-52 kDa (Fig. 4B). In summary, the analysis of the 12S globulin and 7S + 3S fractions by SDS-PAGE, immunodiffusion, IEF, and two-dimensional electrophoresis showed the distinct nature of these two salt-soluble protein components and indicated the effectiveness of the IEP and ultracentrifugation procedure for their separation.

Repeatedly, salt-soluble vicilin polypeptides in leguminous seeds have been shown to contain a high carbohydrate content (49,140,141). The two globulin IEP fractions from oat seeds were analyzed separately by affinity chromatography on Concanavalin-A Sepharose columns to determine whether or not these reserve proteins were also glycosylated. No detectable quantity of the 12S globulin bound to the column indicating the absence or low content of carbohydrate in this fraction (Fig. 5, profile G). However, when the 7S + 3S fraction was applied to the Con-A Sepharose column, a high proportion of it did bind to the column (~60%), Fig. 5, profile V). When both unbound (U) and bound (B) eluants were analyzed by SDS-PAGE (Fig. 5, lanes U and B), the polypeptides corresponding to the 3S holoprotein (see Fig. 3C, Lane 3S) were primarily observed in the unbound portion (Fig. 5, Lane U). However, the 40 kDa polypeptide of the 3S could be detected in the bound portion (Fig. 5, Lane B). In contrast, the components of the 7S holoprotein (see Fig. 3C, Lane 7S) were mostly bound to the affinity column (Fig. 5, Lane B). Together, the data suggested that the major components of the 7S holoprotein from oat were glycosylated. This finding indicated

Fig. 4 Two-dimensional electrophoresis of 12S globulin and the 7S +3S fraction. The protein samples were separated by isoelectric focusing (first dimension) from acidic (left) to basic (right) in the presence of a reducing agent. The second dimension was SDS-PAGE (12%, +Me). A, the two-dimensional pattern of the 12S globulin; B, the pattern of the 7S + 3S fraction.

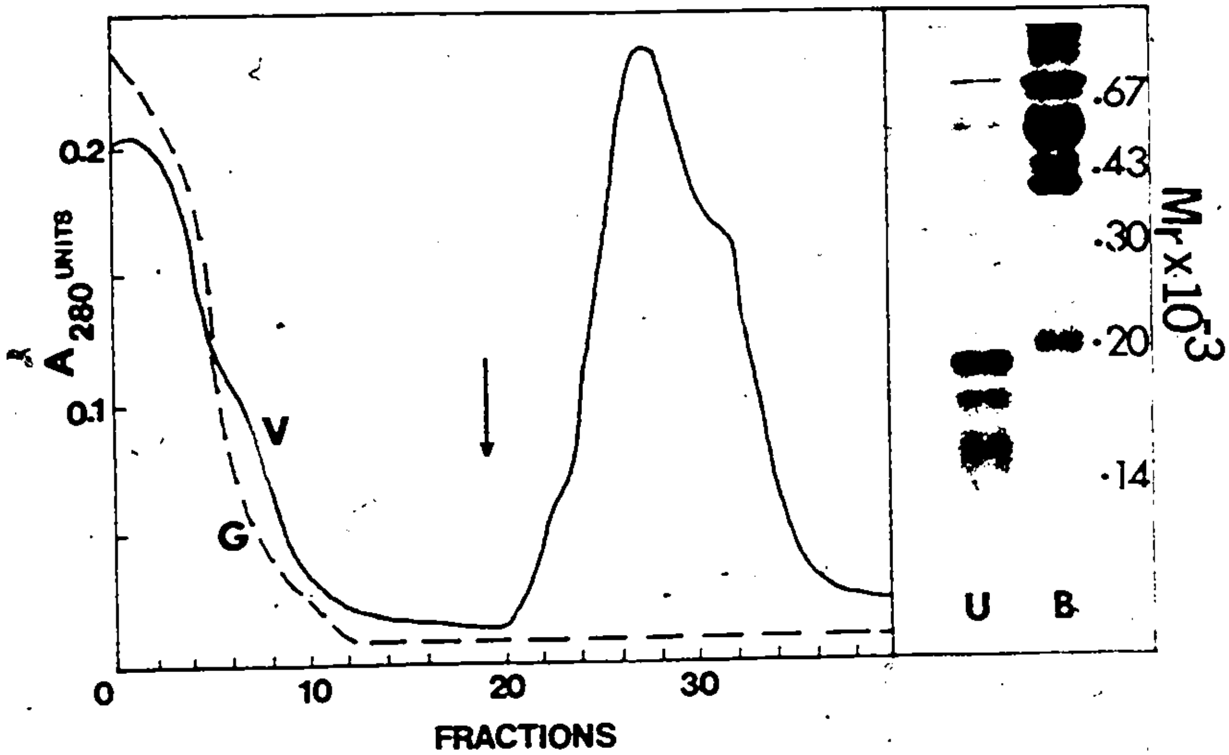
Fig. 5 Concanavalin A-Sepharose chromatography. 12S globulin and the vicilin fraction were incubated separately with Con-A Sepharose and the non-bound material was collected in each case. The bound polypeptides were eluted with α-methyl-D-glucoside. G is the profile obtained when 12S globulin was chromatographed. V shows the profile of the 7S +3S fraction. The unbound and bound fractions from 7S + 3S fraction were analyzed by SDS-PAGE (12%, +Me) (Lanes U and B respectively).

FIG. 4



IEF
IEF
A
B
$M_r \times 10^{-3}$ markers
67-
43-
30-
20-
14-
SDS

FIG. 5



A_{280} UNITS
0.2
0.1
V
G
0
10
20
30
FRACTIONS
U
B
67
43
30
20
14
$M_r \times 10^{-3}$

differential post-translational modifications of the 3S, 7S and 12S globulins and may thus suggest differential compartmentalization pathways. The glycosylated globulins (7S) could be deposited with a mechanism different from that involved in compartmentalization of non-glycosylated globulins (12S).

The 7S globulin may be homologous to the vicilin protein fractions from legume seeds, particularly pea and soybean (142,143) and therefore may be referred to as oat vicilin. The oligomeric size and the SDS-PAGE pattern of oat 7S vicilin resembles closely those reported for the vicilin fractions from pea and soybean. The molecular weight heterogeneity of oat vicilin polypeptides, 12 to 75 kDa, is also found in pea and soybean vicilins. However, the IEF pattern of the oat vicilin differs from that of pea 7S vicilin in the basic region (data not shown). These discrepancies may reflect evolutionary changes in the amino acid sequence of vicilin polypeptides.

Globulins with sedimentation coefficients of 2-3S and 7S have been identified in many dicotyledonous and a few monocotyledonous plant. In most legumes the vicilin fraction is a major reserve protein. However, in some non-leguminous dicots including Helianthus annuus, Beta vulgaris, and Telfairnea pedata, the vicilins are only minor constituents and the 12S globulin is major (40). Interestingly, a similar protein ratio seems to exist in the oat endosperm where the vicilin fraction comprises only about 5% of the total seed protein. The differential expression of genes coding for these heterogeneous polypeptides in oat and legumes may be the result of strict genetic control. The role of vicilin as a reserve protein is not yet certain, although this protein fraction is present in oat endosperm protein bodies (data not shown).

The analysis of the three oat salt-soluble protein fractions, the 12S, 7S and 3S globulins, by Con A-Sepharose chromatography indicated the glycoprotein nature of the 7S holoprotein. Reserve proteins of several legumes (dicots) including *Pisum sativum*, *Glycine max*, *Phaseolus vulgaris*, *Vigna radiata*, *Lupinus augustifolins* and *Lupinus luteus* L. have been shown to be glycosylated (49). In *Pisum sativum* and *Glycine max*, it has been shown that only the vicilin fractions are glycoproteins (44,49). Kitamura *et al*. (144) used Con A-Sepharose chromatography to show that the 7S component of *Glycine max* globulins is a glycoprotein and contains more carbohydrate than 11S globulin. The nature of carbohydrates bound to the 7S vicilin polypeptides from various legumes has been shown to be similar and to consist of neutral sugars and hexosamine (40). The absorption of the oat 7S holoprotein to Con A-Sepharose may indicate the presence of hexosamine and possibly glucosamine molecules. Among pea 7S vicilin subunits, carbohydrate has been shown to be associated mainly with the 14 kDa and 50 kDa polypeptides (145). Recent *in vivo* labelling experiments by Badenoch-Jones *et al*. (49) strongly indicated the glycosylation of five vicilin polypeptides at various stages of seed development. It has been suggested that the glycosylation of plant proteins involves the formation of lipid-sugar intermediates, N-acetylglucosamine and dolichol phosphate (146). Endoplasmic reticulum was shown to be the site of glycosyltransferases responsible for the glycosylation of pea proteins (147).

The mechanisms involved in the glycosylation of oat 7S vicilin polypeptides are unknown and may ~~be~~ similar to those studied in legumes. The data are consistent with the notion that the 7S fraction is commonly glycosylated and may indicate that such selective glycosylation of seed proteins al~~so~~ occurs in monocotyledonous plants.

APPENDIX II

PURIFICATION AND CHARACTERIZATION OF THE 12S GLOBULIN ANTIBODIES

In numerous occasions in this thesis, the 12S globulin antibodies have been used to specifically identify the globulin polypeptides. It is thus crucial that such antibody preparations are adequately pure and well-characterized.

In several previous reports on the synthesis of oat globulin, antibody preparations were used which had been raised against a total globulin extract (67,69,70). Once the presence of other minor globulins (3S and 7S) was reported in a total globulin (94,95), it was deemed necessary that a purified 12S globulin preparation should be used for antibody production. This appendix describes briefly the procedure for the purification of the 12S holoprotein, production of anti-12S IgG, and characterization of such a antibody preparation.

1. Purification of the 12S globulin

Crude 12S globulin was prepared by isoelectric precipitation as described in the Materials and Methods. The protein fraction precipitated at pH. 4.8, 0.2 M NaCl was layered on a 5-20% sucrose gradient to separate the major 12S holoprotein. This first 12S preparation was fractionated twice more on the same sucrose gradient so that a homogeneous and sharp peak was obtained with no shoulders present. The 12S fractions from the final gradient were dialyzed and the precipitated globulin was used for antibody production.

2. Production and purification of anti-12S IgG.

New Zealand rabbits were used to raise antibodies against the 12S globulin as described in the Materials and Methods. The immune-serum was collected by centrifuging the blood samples coagulated at 4°C for 16h. Total IgG was obtained by immunoaffinity chromatography of the immune-serum on a Protein A-Sepharose column. Anti-12S globulin IgG was then purified by applying the total IgG preparation to a 12S globulin-Sepharose column (the purified 12S globulin was covalently attached to CNBr-activated Sepharose). The bound IgG fraction was collected and referred to as the 12S globulin antibodies.

3. Characterization of the 12S globulin antibodies.

The purification scheme of the 12S globulin antibodies is presented in Table IV. The data indicated that the purified anti-12S globulin IgG had a much higher specific activity than the total IgG and the immune-serum. A purification factor of 480 was obtained after the two cycles of affinity chromatorgraphy (Protein A-Sepharose column and 12S globulin-Sepharose column).

Antigenic properties of the anti-12S globulin IgG were first examined by double-diffusion immunoprecipitation technique (104). Anti-12S globulin IgG was tested against several protein fractions including total salt-soluble globulin, purified 12S globulin, purified 7S + 3S globulin fraction, and albumin (all these proteins were obtained from mature oat groat, cv. Hinoat). Fig. 1 shows that the anti-12S globulin could only cross-react with the total globulin and the purified 12S globulin. No cross-reactivity with the minor globulins (7S + 3S) and albumins was observed (Fig. 1).

Table IV.

PURIFICATION OF GLOBULIN ANTIBODIES

	protein[1] concentration (mg/ml)	specific[2] activity	purification[3] factor
Serum	80	0.1	1
Total IgG[4]	2.4	4.2	42
Anti-globulin[5] IgG	0.25	48	480

1. Protein concentration was determined by the standard Bio-Rad method.

2. Specific activity was measured by dividing the maximum serial dilution factor (determined by immunodiffusion precipitation) by the protein concentration.

3. Purification factor was determined by dividing the specific activity of total IgG and purified anti-globulin IgG by the specific activity of the serum.

4. Total IgG was obtained by immunoaffinity chromatography of the serum on a Protein A-Sepharose column.

5. Anti-globulin IgG was purified by applying total IgG to a globulin-Sepharose column (12S globulin coupled to CNBr-activated Sepharose).

Fig. 1 Double-diffusion immunoprecipitation assay for 12S globulin antibodies.

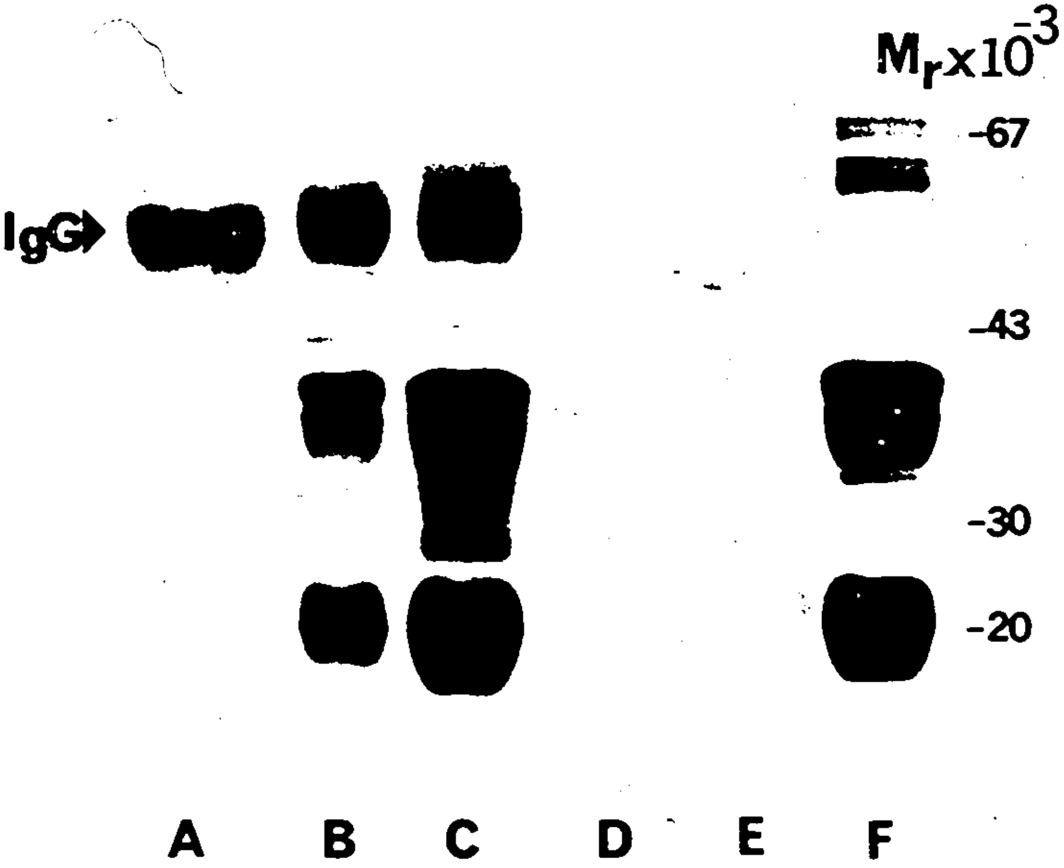
The plate contained 1.5% agarose in 20 mM Na_2HPO_2-NaH_2PO_4, pH 7.4, 140 mM NaCl. The center well contained 10 µl of the anti-12S globulin IgG. The outer wells contained 10 µl of 4.0 mg/ml total globulin (A), purified 12S globulin (B), purified (7S +3S) globulins (C), and albumin (D). The plate was incubated overnight at room temperature. A visible precipitation line indicates a positive reaction.

Fig. 2 Immnunoprecipitation of various oat protein fractions by 12S globulin antibodies.

Total globulin, purified 12S globulin, purified (7S +3S) globulins, and albumins were dissolved in 50 mM Tris, pH 8.0, 1 M NaCl with a concentration of 8 mg/ml. The protein solutions were centrifuged in a Fisher microfuge for 5 min to remove any undissolved protein. The clear supernatant from each protein solution was collected and a 100 µl aliquot was incubated with 100 µl of the anti-12S globulin IgG. After overnight incubation at room temperature, the mixtures were centrifuged in a microfuge for 5 min and the immunoprecipitates were dissolved in SDS-PAGE sample buffer containing 2-mercaptoethanol. The protein samples were then analyzed by SDS-PAGE (12% acrylamide) and the gel was stained with Coomassie Brilliant Blue. Lane A is the SDS-PAGE pattern of the antibody alone. Lanes B and C, proteins precipitated from the purified 12S globulin by anti-12S globulin IgG respectively. Lanes D and E, proteins precipitated from the purified (7S + 3S) globulins and albumins by anti-12S globulin igG respectively. Lane F is the pattern of total globulin run as a standard.

FIG.1

FIG.2

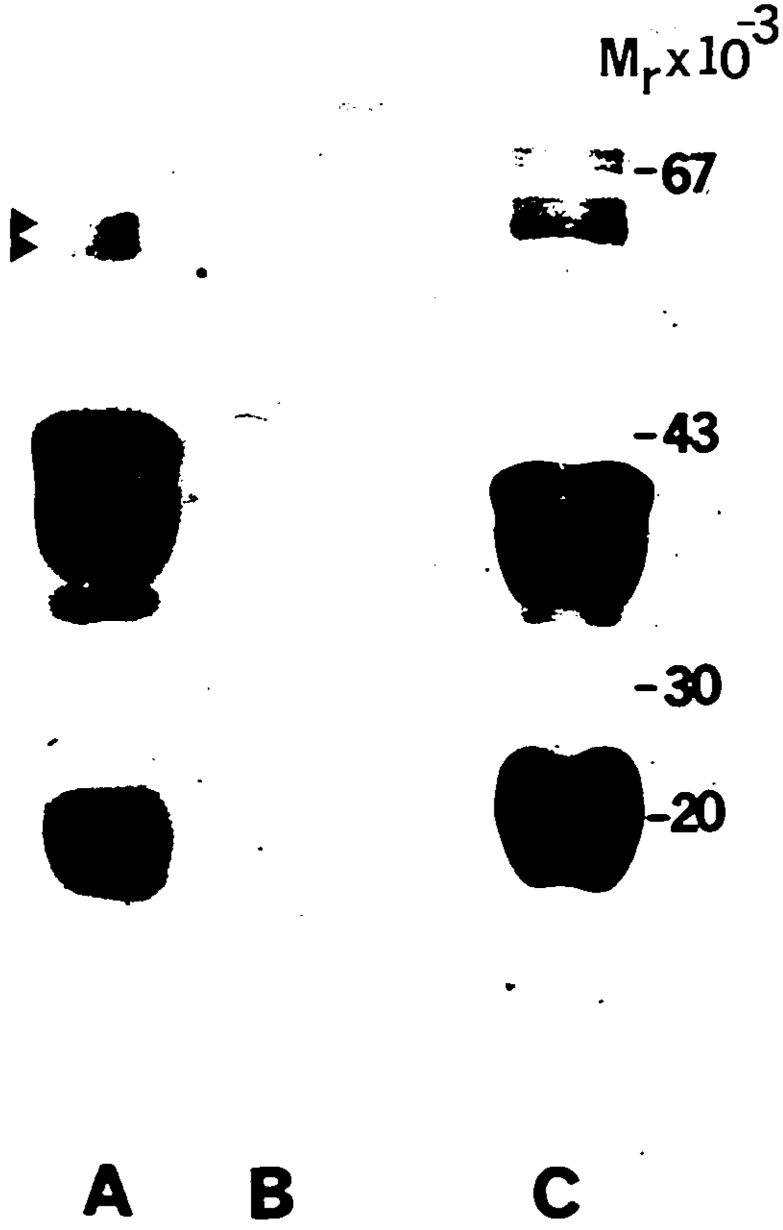


The antibodies were further characterized by immunoprecipitation of the above-mentioned oat protein fractions. These protein fractions were dissolved in 50mM Tris, pH 8.0 1 M NaCl and an aliquot of each protein sample was mixed with an equal volume of the anti-12S globulin IgG. After incubation overnight at room temperature, the mixtures were centrifuged for 5 min in a Fisher microfuge and the immunoprecipitates were dissolved in SDS-PAGE sample buffer. Fig. 2 depicts the SDS-PAGE pattern of the immunoprecipitates obtained by reacting the anti-12S globulin IgG with total globulin, purified 12S globulin, minor 7S + 3S globulins, and albumins. Lane A is the SDS-PAGE pattern of the anti-12S globulin IgG (run for comparison). The globulin antibodies could immunoprecipitate the α and β globulin polypeptides from both the total globulin and the purified 12S globulin (Lanes B and C). No detectable polypeptides were immunoprecipitated from the albumins (Lane D). The anti-12S globulin IgG immunoprecipitated a 70 kDa polypeptide from the minor globulin fraction (7S + 3S), (Lane E). Lane F is the SDS-PAGE pattern of the total oat globulin fraction which is run as a standard. Overall, these observations indicated that the 12S globulin antibodies were quite specific for the α and β globulin components of the 12S globulin except for the immunoprecipitation of a 70 kDa polypeptide from the minor globulin preparation. This may be due to non-specific precipitation of this polypeptide during the overnight incubation. It could also be explained by contamination of the 12S globulin preparation used for antibody preparation. Thirdly, sequence homology may exist between the 70 kDa polypeptide and the α and β globulin polypeptides. To clarify this problem, another experiment was carried out in which the 12S globulin antibodies were labelled with ^{125}I and tested against the total globulin and the minor 7S + 3S globulin fractions using the Western-blot technique (148). In this experiment, the total globulin and the minor globulin fractions were first run on a polyacrlamide gel in the presence of SDS. The separated proteins were transferred to nitrocellulose paper by blotting and the blots were incubated with ^{125}I-labelled anti-12S globulin IgG. After hybridization, the blots were exposed to X-ray films. Fig. 3 shows the cross-hybridization of the labelled 12S globulin IgG with the electrophoretically separated polypeptides of the total globulin IgG cross-reacted with the α and β globulin polypeptides as well as the unreduced globulin 60 kDa polypeptides (Lane A arrows). No cross-reactivity with any of the minor globulin polypeptides was observed (Lane B). These results demonstrated that the 70 kDa polypeptide precipitated in the previous experiment (Fig. 2) might have been a non-specific precipitate, and not due to impurities in the 12S globulin used for the production of antibodies.

In summary, the overall results of the above experiments indicate that a pure and specific antibody preparation was used in studying the biosynthesis of oat 12S globulin in developing seeds.

Fig. 3 Western-blot analysis using ^{125}I-labelled anti-12S globulin IgG against total globulin and purified (7S + 3S) globulins. Labelling of IgG with ^{125}I was performed according to the method of Greenwood et al. (149). The specific activity was approximately 30 μCi/μg of IgG. Western-blotting was carried out according to the method of Towbin et al. (148). Briefly, the protein samples were analyzed by SDS-PAGE, and the separated proteins were transferred electrophoretically to a nitrocellulose sheet. This sheet was incubated with ^{125}I-labelled anti-12S globulin IgG, washed in PBS buffer, dried, and exposed to Kodak X-Omat AR film at -70°C. Lane A represents the polypeptides in the total globulin extract which cross-reacted with anti-12S globulin IgG. Lane B shows the absence of any polypeptide from the purified (7S + 3S) globulin preparation which could cross-react with anti-12S globulin IgG. Lane C is the stained SDS-PAGE pattern of the total globulin (run as a standard).

FIG.3



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CLAIMS TO ORIGINAL CONTRIBUTIONS

The results concerning the synthesis of oat 12S globulin were obtained using purified 12S globulin antibodies raised against this purified globulin. These results confirmed the previous studies on the synthesis of oat globulin.

The results concerning globulin transport and processing were original findings and have contributed to the field of reserve globulin deposition in the following manner.

METHODOLOGY

Among the methods, two precedures were developed by this author which could be useful in studying the deposition of seed reserve proteins in any cereal system.

- Isolation of intact protein bodies.

A step sucrose gradient of 16% and 65% sucrose was used to isolate protein bodies from developing or mature seeds. This method yielded intact protein bodies.

- Simultaneous isolation of organelles for pulse-chase labelling studies.

A single sucrose gradient of 15-65% sucrose was employed to isolate ER and protein bodies from <u>in vivo</u> labelled developing oat groats. This technique was found to be simple and more reliable than other commonly used methods which separate these organelles from different batches of seeds with different procedures.

ORIGINAL FINDINGS

- Evidence was provided for the preferential synthesis of oat 12S globulin precursors on rough endoplasmic reticulum.

- Biochemical and microscopic evidence was provided for the post-translational transport of the newly-synthesized globulin precursors from the rough endoplasmic reticulum into protein bodies.

- The globulin precursors were shown to be processed into the smaller α and β globulin polypeptides. The site of this cleavage was found to be the protein bodies.

- Evidence was provided for the assembly of the globulin polypeptides into a 12S oligomer within protein bodies.

- Finally, two minor groups of globulin polypeptides (3S and 7S) were extracted from mature oat flour which resembled the major globulin fractions in legumes. These polypeptides were partially characterized.

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