Role of the Endoplasmic Reticulum in the Synthesis of Reserve Proteins and the Kinetics of Their Transport to Protein Bodies in Developing Pea Cotyledons

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ABSTRACT Developing pea (Pisum sativum L.) cotyledons were labeled with radioactive amino acids, glucosamine, and mannose in pulse and pulse-chase experiments to study the synthesis, glycosylation, and transport of the reserve proteins vicilin and legumin to the protein bodies. Tissue extracts were fractionated on sucrose gradients to isolate either the endoplasmic reticulum (ER) or the protein bodies. Immunoaffinity gels were used to determine radioactivity in the reserve proteins (legumin and vicilin).

After pulse-labeling for 45 min with amino acids, about half the total incorporated radioactivity coincided closely with the position of the ER marker enzyme NADH-cytochrome c reductase at a density of 1.13 $g \cdot cm^{-3}$ on the sucrose gradient. Both radioactivity and enzyme activity shifted to a density of 1.18 $g \cdot cm^{-3}$ in the presence of 3 mM $MgCl_2$ indicating that the radioactive proteins were associated with the rough ER. Approximately half of the incorporated radioactivity associated with the rough ER was in newly synthesized reserve protein and this accounted for 80% of the reserve protein synthesized in 45 min. Trypsin digestion experiments indicated that these proteins were sequestered within the ER.

In pulse-chase experiments, the reserve proteins in the ER became radioactive without appreciable lag and radioactivity chased out of the ER with a half-life of 90 min. Radioactive reserve proteins became associated with a protein body-rich fraction 20-30 min after their synthesis and sequestration by the ER. Pulse-chase experiments with radioactive glucosamine and mannose in the presence and absence of tunicamycin indicated that glycosylation of vicilin occurs in the ER. However, glycosylation is not a prerequisite for transport of vicilin from ER to protein bodies. Examination of the reserve protein polypeptides by SDS PAGE followed by fluorography showed that isolated ER contained legumin precursors (M_r 60,000-65,000) but not the polypeptides present in mature legumin (M_r 40,000 and 19,000) as well as the higher molecular weight polypeptides of vicilin (M_r 75,000, 70,000, 50,000, and 49,000). The smaller polypeptides of vicilin present in vicilin extracted from protein bodies (M_r 12,000-34,000) were absent from the ER. The results show that newly synthesized reserve proteins are preferentially and transiently sequestered within the ER before they move to the protein bodies, and that the ER is the site of storage protein glycosylation.

Pea seeds synthesize and accumulate large amounts of reserve proteins in the course of their development. In the mature seed, these proteins are accumulated within the cotyledon parenchyma cells in small (1-3 μm), membrane-bounded organelles known as protein bodies (10, 18, 41). The major reserve proteins of the pea are legumin and vicilin (14, 31) which together make up ~70% of the total protein of the mature seed.

Legumin of mature seeds is a 12S protein (M_r 360,000) consisting of six acidic (M_r 40,000) and six basic (M_r 20,000) subunits (15). It is initially synthesized as a group of polypep-

tides of M_r 60,000–65,000 (11, 34), which are then processed by proteolytic cleavage to yield the acidic and basic subunits. This processing step is not cotranslational but occurs 1–2 h after synthesis (34).

The vicilin fraction (7S) of mature pea seeds can be subdivided into at least five distinct protein oligomers on the basis of differential solubility and isoelectric precipitation. These oligomers contain at least 13 different polypeptides in various proportions (38). The most abundant vicilin polypeptides have molecular weights of 75,000, 50,000 (doublet), 30,000, and 18,000. The vicilin fraction also contains less abundant polypeptides of M_r 70,000, 49,000, 34,000, 25,000, 14,000 (doublet), 13,000, and 12,000 (39). Recent evidence indicates the presence of an additional distinct oligomer called convicilin, which contains only the M_r 75,000 polypeptide (13). The vicilin polypeptides of M_r 70,000, 50,000, and 14,000 have been shown to be glycosylated (1).

The synthesis of vicilin is not as well understood as that of legumin. Only the larger polypeptides with an M_r >49,000 can be detected after short-term pulse labeling (see Fig. 1A in reference 34) or after in vitro translation of isolated RNA (12, 20, 21). The smaller polypeptides of vicilin, present in immature and mature seeds, are not found among the radioactive products under these conditions.

It is known from in vitro studies that the polypeptides of both legumin and vicilin are synthesized only on membrane-bound polysomes and that, at least in the case of the vicilin polypeptides, these are cotranslationally modified in the presence of microsomal membranes (21, 34). However, little further information is available about the involvement of the endoplasmic reticulum in the synthesis of reserve proteins and in the sequence of events before their accumulation in the protein bodies. In this paper we present evidence that the polypeptides of both legumin and vicilin are transiently sequestered within the ER, and also kinetic data on the movement of these proteins out of the ER and into the protein bodies. Vicilin polypeptides are glycosylated while associated with the ER but glycosylation is not a prerequisite for subsequent transport to the protein bodies. Legumin is associated with the ER in its precursor form (M_r 65,000–60,000), and vicilin is represented only by polypeptides of M_r 49,000 and greater.

MATERIALS AND METHODS

Plant Material

A selection from *Pisum sativum* cv. Greenfeast (line PI/G 086, stock collection, CSIRO, Canberra) was grown in growth chambers as described (28) except that the temperature was 20° rather than 25°C. Developing seeds were harvested at specified times (expressed as days after flowering [DAF]), the seedcoats and embryonic axes removed, and the intact cotyledons used immediately for pulse-labeling studies. Under these growing conditions the major period of vicilin accumulation occurred between 12 and 32 DAF and that of legumin between 19 and 32 DAF (37).

Radioactive Labeling

The cotyledons were labeled with ^{14}C-amino acids, [^{3}H]2-mannose, or [^{3}H]-glucosamine as described (35). In experiments where the radioactivity was chased, the cotyledons were rinsed three times with water, blotted dry, and transferred to a 20-μl drop containing 4% (wt/vol) sucrose, 10.7 g/liter of asparagine, and the major mineral nutrients (29). Radioactive precursors were obtained from Amersham Australia (Sydney, Australia) and had the following specific activities: ^{14}C-amino acids (6–20 GBq/mmol), [^{3}H]2-mannose (590 GBq/mmol), and [^{3}H]-glucosamine (836 GBq/mmol). The ^{14}C-amino acids and the [^{3}H]glucosamine were used as supplied; to remove the ethanol, the [^{3}H]mannose was reduced to one-quarter of the original volume under a stream of N_2, and H_2O was added to

replace the liquid lost by evaporation. Incorporation of radioactivity was measured after precipitation in 10% (wt/vol) TCA (27).

Tissue Homogenization and Isolation of ER on Sucrose Gradients

After incubation with isotope, the cotyledons were rinsed four times with cold water and kept on ice. A thin (1-mm) slice of tissue previously in contact with the radioactive precursor was removed and the rest of the cotyledon was discarded. Data were calculated back to a value for a single cotyledon slice that for convenience is referred to as "per cotyledon." Two media were used for homogenization and the isolation of ER. Medium A contained 100 mM Tris-HCl, pH 7.8, and 1 mM EDTA; medium B contained in addition 3 mM MgCl$_2$. Linear sucrose gradients were formed with solutions of 16% (wt/wt) and 48% (wt/wt) sucrose in medium A or B. In some experiments, organelles were first separated from soluble proteins and small molecules with a Sepharose 4B (Pharmacia, Uppsala, Sweden) column as described (40). The columns were equilibrated and eluted with 12% (wt/wt) sucrose in medium A or B. The same medium (A or B) was used throughout for homogenization, Sepharose 4B chromatography, and sucrose gradients. The cotyledon slices were homogenized with a cold mortar and pestle with 12% (wt/wt) sucrose dissolved in medium A or B, and the homogenate was centrifuged at 1,000 g_{avg} for 10 min to remove starch, nuclei, and cell wall debris. The gradients were centrifuged for 2 h at 35,000 rpm (150,000 g_{avg}) in the SW41 rotor of a Spinco/Beckman (Palo Alto, CA) ultracentrifuge. Fractions of 0.55 ml were collected with a density gradient fractionator (ISCO, Lincoln, NB), and the absorbance at 280 nm was simultaneously recorded.

For many experiments, the ER was recovered by fractionating the homogenate on a discontinuous sucrose gradient. The homogenate in 12% (wt/wt) sucrose in medium A was centrifuged at 1,000 g_{avg} for 10 min and then layered on a discontinuous gradient consisting of 2.5 ml of 35% (wt/wt) sucrose and 7.5 ml of 16% (wt/wt) sucrose in medium A. After centrifugation for 2 h at 35,000 rpm (150,000 g_{avg}) in the SW41 rotor of a Spinco ultracentrifuge the sample volume of the gradient, together with the top 1.0 ml of the layer of 16% (wt/wt) sucrose, was collected, as well as the organelles at the interface of the 16% (wt/wt) and 35% (wt/wt) sucrose layers. This organelle fraction, referred to as ER, contained not only ER (as shown by the presence of NADH-cytochrome c reductase) but also some of the thylakoids (as shown by the faintly green color) and probably most of the Golgi-derived dictyosomes which band at a density of 1.13 g·cm^{-3} (30).

Treatment of Isolated ER with Trypsin

ER isolated as described above was diluted with medium A. Aliquots (0.5 ml) were mixed with 50 μl of trypsin (300 μg/ml of trypsin TPCK, Worthington Biochemical Corp., Freehold, NJ) and 50 μl of Triton X-100 (5% vol/vol) and incubated at 0°C for 1 h; H_2O was substituted for the reagents in the controls. After incubation, 100 μl of soybean trypsin inhibitor (5 mg/ml) was added and the samples were incubated for a further 30 min. Reserve protein was then isolated with IgG-vicilin-Sepharose (see below).

Isolation of Protein Bodies

Homogenization procedure and medium were optimized to provide a maximum yield of intact protein bodies. Slices from three to five cotyledons were chopped by hand with a razor blade in a cold plastic petri dish with a total of 2.0 ml of 12% (wt/wt) sucrose with 2 mM MgCl$_2$. The homogenate was then filtered through one layer of Miracloth (Chicopee Mills, Milltown, NJ), layered over 2.0 ml of 16% (wt/wt) sucrose containing 2 mM MgCl$_2$, and centrifuged in a rotor with swinging buckets at 1,000 g_{avg} for 10 min. The supernatant was decanted and the pellet, containing starch, broken chloroplasts, and a large proportion of protein bodies, was resuspended in 4 ml of medium A.

SDS PAGE and Fluorography

Aliquots of sucrose gradient fractions were treated as described (34) to precipitate the proteins, and the polypeptides fractionated on slab polyacrylamide gels (either 18% or a gradient from 12.5 to 25%) by a modification (34) of the Laemmli and Favre (24) procedure. Fluorographs were made by exposing the dried gels to preflashed Kodak x-ray film RP 54 (5). All gels included standards consisting of an extract of protein bodies isolated from mature pea cotyledons that had been labeled with [^{3}H]sodium borohydride (23). These extracts contained principally legumin and vicilin.

Antibodies and Immunochromatography

Protein body extracts (PBE), legumin, and vicilin were all prepared from

mature seeds, antibodies were raised in sheep, immunoglobulins were prepared and covalently attached to cyanogen bromide-Sepharose (Pharmacia, Uppsala, Sweden), and immunoaffinity chromatography was carried out as described elsewhere (1). The affinity gels are referred to as IgG-PBE-Sepharose, IgG-legumin-Sepharose, or IgG-vicilin-Sepharose.

Enzyme Assays

NADH-cytochrome c reductase and cytochrome oxidase were assayed as described, respectively, by Bowles and Kauss (6) and Sottocasa et al. (33).

Electron Microscopy

Isolated protein bodies were sedimented through 3% (vol/vol) glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.2) and fixed for 1 h at 22°C. For in situ work, cotyledon tissue was cut and fixed in the above fixative but kept at 22°C for 2 h. Both the isolated protein bodies and the tissue pieces were postfixed in 2% (wt/vol) osmium tetroxide in the same buffer, dehydrated through a graded series of ethanol, and embedded in Spurr's resin (36). Sections were counter-stained with lead (42) and uranyl acetate (a saturated solution in 50% ethanol). All these operations were carried out at room temperature. The sections were examined in a JEOL JEM 100 CX electron microscope.

It should be noted that the seeds in this report were obtained from plants grown at 20°C, while the seeds used in previous anatomical studies (8–10) were from plants grown at 25°C. The lower temperature results in a slower growth rate. The developmental stages illustrated here (16 and 24 DAF) are similar in ultrastructure to 11–12 DAF and 17–18 DAF, respectively, in our earlier reports.

RESULTS

Newly Synthesized Reserve Proteins Are Associated with the Rough ER

When intact pea cotyledons are exposed to a small amount of ^{14}C-amino acids (18.5 kBq in 10 µl per cotyledon), the radioactivity is rapidly taken up by the tissue in contact with the isotope and incorporated into protein. Incorporation proceeds nearly linearly with time and without detectable lag (Spencer and Higgins, unpublished observation). When a homogenate of cotyledons (15 DAF), which had been labeled with ^{14}C-amino acids for 45 min, was fractionated on a sucrose gradient (medium A), radioactivity was detected in proteins in the sample volume at the top of the gradient and in a broad band with a density of 1.13 g·cm^{-3} (Fig. 1a). The position of

this band coincided approximately with that of the ER marker enzyme, NADH-cytochrome c reductase. The proteins present in the sample volume of the gradient and in the ER region were fractionated by SDS PAGE and a fluorograph was prepared. The stained gel (Fig. 2, lanes a and b) showed that most of the protein remained in the sample volume of the gradient. The most abundant polypeptides in this fraction were the M_r 75,000, 50,000, and 30,000 of vicilin. (The cotyledons contain very little legumin at 15 DAF.) When the tissue is homogenized in medium A, nearly all the protein bodies are ruptured, and >95% of the reserve protein remains in the sample volume at the top of the gradient (Chrispeels, unpublished observation). The fluorograph of the same gel (Fig. 2, lanes c and d) showed that many radioactive proteins were present both in the sample volume and associated with the ER. However, the most heavily labeled polypeptides were associated with the ER, and their position in the gel coincided with that of the vicilin polypeptides of M_r 50,000 and 49,000. Much less radioactivity was associated with the large amount of vicilin polypeptides in the sample volume of the gradient (Fig. 2, cf. lanes a and c). Confirmatory evidence that these comigrating polypeptides are indeed vicilin is given below.

After 1 h of labeling, ~30% of the total incorporated radioactivity present in a cotyledon homogenate bound to the IgG-PBE-Sepharose, indicating that about one third of the total protein synthesis was directed toward storage proteins. Of the newly synthesized storage protein, ~80% was associated with the ER fraction, whereas >95% of the (nonradioactive) storage protein was in the soluble fraction.

To determine whether newly synthesized vicilin is associated with the ER or with some other organelle that also bands at a density of 1.13 g·cm^{-3}, organelles were fractionated on sucrose gradients either with or without 3 mM MgCl₂ (mediums A and B; see Materials and Methods). It has been shown that this results in ER membranes with differing densities, owing to the retention of ribosomes in the presence of MgCl₂ (22, 26, 32). Cotyledons (18 DAF) were labeled with ^{14}C-amino acids for 45 min, and the homogenates were fractionated on a Sepharose 4B column to separate the organelles from the soluble proteins
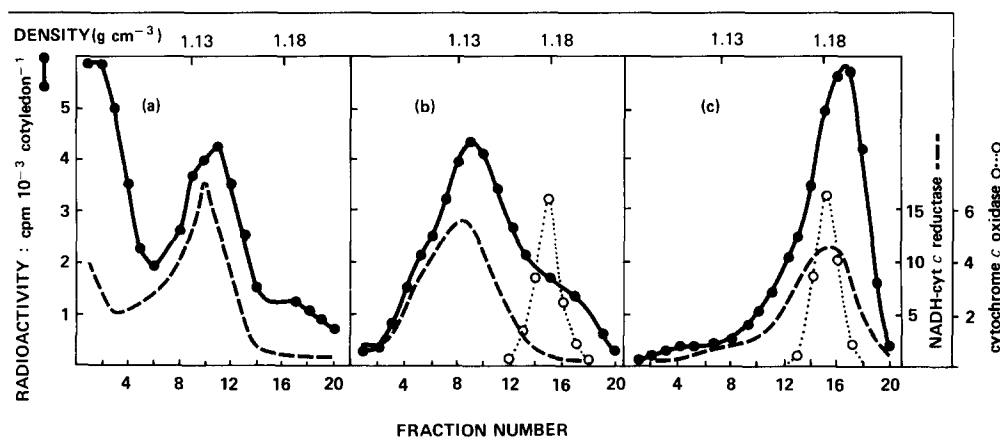

FIGURE 1 Distribution of NADH-cytochrome c reductase and incorporated radioactivity on an isopycnic sucrose gradient. (a) Four cotyledons (15 DAF) were labeled for 45 min with 13 kBq of ^{14}C-amino acids each. Extracts were prepared and, after a clearing spin (1,000 g_{avg} for 10 min), were fractionated on a 16–48% (wt/wt) linear sucrose gradient. All media contained 1 mM EDTA and no MgCl₂. (b and c) Eight cotyledons (18 DAF) were labeled for 45 min with 18.5 kBq of ^{14}C-amino acids each and four cotyledons were homogenized in 100 mM Tris, pH 7.8, 1 mM EDTA, 12% sucrose, either without (b) or with (c) 3 mM MgCl₂. Organelles were separated from soluble proteins on Sepharose 4B in the same media, and organelles were fractionated on a 16–48% (wt/wt) sucrose gradient. Sucrose gradients were centrifuged at 150,000 g_{avg} for 2 h, fractions were collected, and the TCA-insoluble radioactivity in each fraction was determined. Radioactivity is expressed as cpm·10^{-3}·cotyledon^{-1}, NADH-cytochrome c reductase and cytochrome c oxidase as ΔA_{550}·h^{-1}·cotyledon^{-1}.

STAIN   FLUOR

75,000 —

50,000 — ⟵

30,000 —

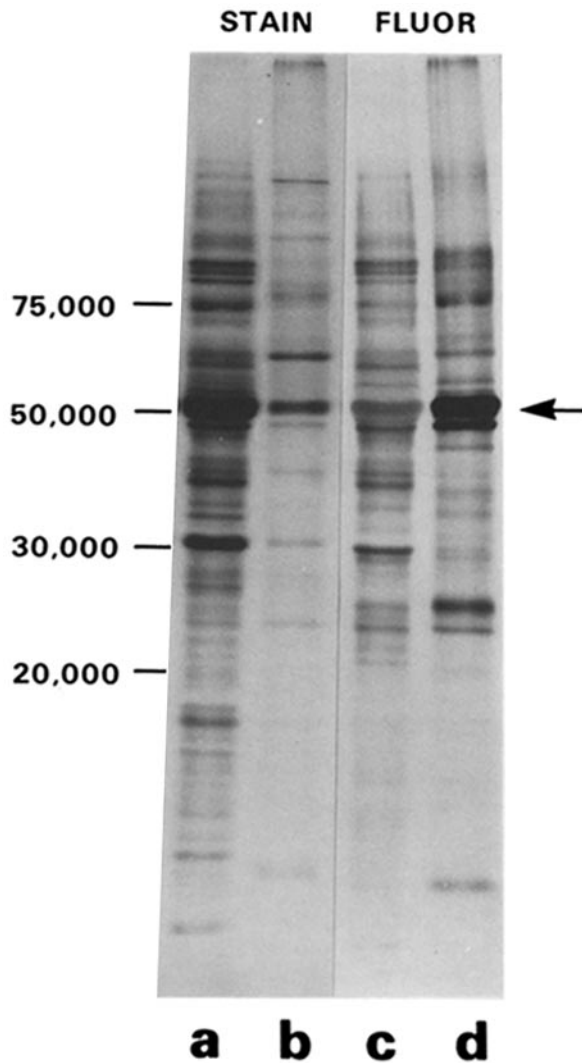20,000 —

a   b   c   d

FIGURE 2   Fractionation by SDS PAGE of the polypeptides present in the sample volume and the ER region of the sucrose gradient shown in Fig. 1 a. Lanes a and c are, respectively, the stained gel and the fluorograph of the proteins in the sample volume of the gradient. Note the abundance of protein (especially vicilin, M_r = 75,000, 50,000 and 30,000) in the sample volume of the gradient due to the solubilization of the reserve protein in the protein bodies. Lanes b and d are, respectively, the stained gel and the fluorograph of the proteins in the ER. The heavily labeled polypeptides in the ER (arrow to the right) correspond in size to vicilin polypeptides (M_r = 50,000 and 49,000).

and smaller molecules. This additional purification step results in an organelle fraction that is free of cytosolic proteins, and subsequent fractionation of the organelles results in more reproducible profiles of NADH-cytochrome c reductase activity (22). The organelle fraction was then fractionated on a linear 16–48% (wt/wt) sucrose gradient containing Tris and EDTA. The same medium, either without MgCl₂ (medium A) or with 3 mM MgCl₂ (medium B), was used for all operations (homogenization, Sepharose 4B column, sucrose gradient). The results of two parallel gradients showed that without MgCl₂ the NADH-cytochrome c reductase activity banded at a density of 1.13 g·cm⁻³ (Fig. 1 b), while in the presence of 3 mM MgCl₂ the activity banded at 1.18 g·cm⁻³ (Fig. 1 c). As in Fig. 1 a, there was not an exact coincidence between the position of the radioactive protein and the NADH-cytochrome c reductase on

the gradient. The position of the mitochondrial marker, cytochrome c oxidase, was the same in both gradients. The position of inosine diphosphatase, a Golgi apparatus marker enzyme (17), also remains the same under these conditions (30), as do the positions of other membranous structures without attached ribosomes. Fractions of the ER peaks of both gradients were combined separately and challenged with IgG-PBE-Sepharose to determine the proportion of radioactivity in reserve protein. Values of 52 and 49% were obtained, respectively, for the gradients without and with MgCl₂. In the presence of MgCl₂, a small amount of radioactive protein remained in the region of the gradient corresponding to a density of 1.13 g/cm³ (Fig. 1 c), and 38% of this radioactivity was in reserve protein. The presence of radioactive reserve protein at a density of 1.13 g·cm⁻³ in the gradient containing 3 mM MgCl₂ can probably be accounted for by the incomplete shift of the ER, as shown by the NADH-cytochrome c reductase profile. Taken together, these data show that the newly synthesized reserve protein polypeptides were associated with the rough ER.

Reserve Protein Is Glycosylated in the ER

Certain polypeptides of the vicilin fraction (M_r = 70,000, 50,000, 26,000 and the 14,000 doublet) have been shown to become radioactive when cotyledons are incubated with [¹⁴C]-glucosamine, and this incorporation is inhibited by tunicamycin (1). Cotyledons (18 DAF) were labeled for 60 min with [³H]glucosamine or with [³H]mannose and the homogenates fractionated on Sepharose 4B. The organelle fraction was collected and further fractionated on a linear sucrose gradient. The experiments were carried out either with 3 mM MgCl₂ or without MgCl₂ in all media. The results (Fig. 3) showed that both radioactive precursors were incorporated primarily into a membrane fraction with density 1.13 g·cm⁻³ and that this membrane fraction shifted to a higher density (1.18 g·cm⁻³) in the presence of MgCl₂. However, there was a significant difference in the radioactive profiles of incorporated glucosamine and mannose when the gradients had 3 mM MgCl₂. The shift of incorporated [¹⁴C]glucosamine to a higher density in the presence of MgCl₂ was fairly complete and comparable to that seen after ¹⁴C-amino acid incorporation (Fig. 1 c), but with [³H]mannose a major residual peak of incorporated radioactivity remained at 1.13 g·cm⁻³. This latter peak is in the region where the Golgi apparatus bands (30).

Pooled fractions from the peaks in Fig. 3 were challenged with IgG-PBE-Sepharose to determine the proportion of radioactivity in reserve proteins. After labeling with [¹⁴C]glucosamine, 18–20% of the incorporated counts in the peaks at both 1.13 g·cm⁻³ (no MgCl₂, Fig. 3 a) and 1.18 g·cm⁻³ (3 mM MgCl₂, Fig. 3 b) were in reserve proteins. With [³H]mannose labeling, the rough ER peak at 1.18 g·cm⁻³ (3 mM Cl₂, Fig. 3 d) had 19% of its radioactivity in reserve proteins but the residual peak of radioactivity at 1.13 g·cm⁻³ contained only 4.5% reserve proteins. The lighter membranes were relatively richer in other mannose-containing compounds (such as proteins, lipids or carbohydrates) than the rough ER.

Identity of Reserve Protein Polypeptides within the ER

To find out which newly synthesized reserve protein polypeptides were associated with the ER, the ER was isolated on discontinuous sucrose gradients from cotyledons (18 DAF) that had been labeled with ¹⁴C-amino acids, [³H]glucosamine, and
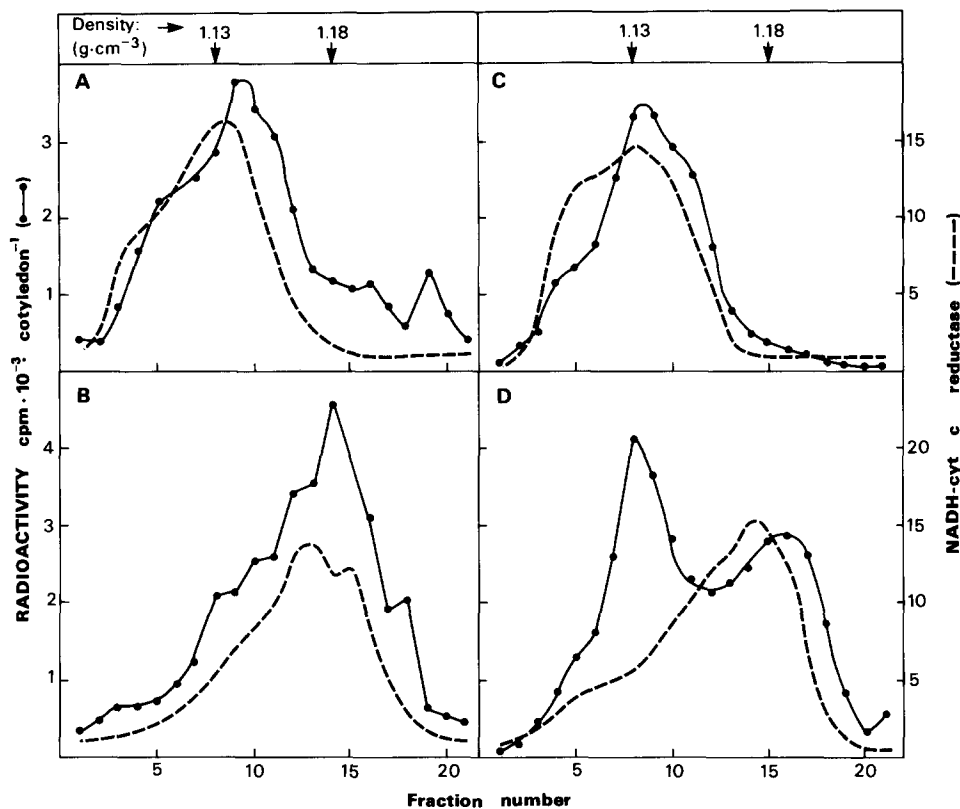
FIGURE 3 Distribution of NADH-cytochrome c reductase and incorporated [³H]glucosamine and [³H]-mannose on isopycnic sucrose gradients in the presence (B and D) or absence (A and C) of 3 mM MgCl₂. Four cotyledons (18 DAF) were labeled with 370 kBq of [³H]glucosamine or 370 kBq of [³H]mannose for 1 h, homogenized and after a clearing spin (1,000 g for 10 min) the homogenates were fractionated on a Sepharose 4B column. The organelles were further fractionated on linear 16–48% (wt/wt) sucrose gradients, and the gradient fractions were assayed for NADH-cytochrome c reductase and radioactivity. NADH-cytochrome c reductase is expressed as A_{550} h⁻¹·cotyledon⁻¹, and incorporated radioactivity as cpm·10⁻³·cotyledon⁻¹.

[³H]mannose. After treatment with 1% Tween-20, the ER was challenged with three separate affinity gels specific for protein body extract, vicilin, and legumin, and the polypeptides that bound to the affinity gel were fractionated by SDS PAGE and detected by fluorography (see below). While mature protein bodies contain polypeptides ranging in M_r from 75,000 to 12,000 (Fig. 4, lane a), the ER does not contain reserve protein polypeptides with M_r <49,000 (Fig. 4, lane b). (The M_r 23,000 polypeptide visible in lane b binds to IgG specific for pea lectin; Chrispeels and Higgins, unpublished.) Legumin is represented in the ER by a minor polypeptide of M_r 80,000 and a major group of polypeptides of M_r 60,000–65,000 (these are not resolved in Fig. 4, lane e). Vicilin is represented in the ER by polypeptides of M_r 75,000, 70,000, 50,000, and 49,000. When cotyledons were labeled with [³H]glucosamine or [³H]mannose, the isolated ER contained only two labeled reserve protein polypeptides (Fig. 4, lanes c and d). These polypeptides, with M_r 70,000 and 50,000, are part of the vicilin complex (1).

Treatment of isolated ER with trypsin in the presence and absence of detergent as described in Materials and Methods was used to determine whether the ER-associated reserve protein was membrane-protected. Radioactivity in vicilin in control samples (no trypsin) was 4,550 and 4,150 cpm in the absence and presence of 0.4% Triton X-100, respectively. Radioactivity in vicilin in trypsin-treated samples was 5,130 and 1,450 cpm in the absence and presence of 0.4% Triton X-100, respectively, showing that digestion occurred only in the presence of both trypsin and detergent. This was confirmed by fractionation of the polypeptides by SDS PAGE followed by fluorography. The radioactivity remaining in the sample containing both trypsin and detergent was present in a series of polypeptides of M_r <22,000 (data not shown).

Kinetics of Transport of Reserve Proteins out of the ER

Pulse and pulse-chase experiments were carried out to determine whether the newly synthesized reserve proteins were transiently associated with the ER. In these experiments, the ER was isolated on discontinuous sucrose gradients (see Materials and Methods) and aliquots of the ER and the sample volume of the gradients were challenged with IgG-PBE-Sepharose to determine the proportion of radioactivity in reserve protein, and the total incorporation in reserve protein was calculated. Under pulse-labeling conditions with cotyledons at 15 DAF, the reserve proteins in the ER initially became labeled with ¹⁴C-amino acids six to seven times faster than the reserve proteins in the sample volume (soluble) portion of the gradient. Radioactivity in the reserve proteins in the ER reached a maximum after 90 min, while radioactivity in the soluble reserve proteins at the top of the gradient (derived from protein bodies) continued to accumulate for at least 2 h (Fig. 5 a).

A parallel experiment was carried out in which the cotyledons were transferred to a nonradioactive culture medium (see Materials and Methods) after labeling for 45 min. Transfer results in the cessation of incorporation within 10–15 min (Spencer and Higgins, unpublished observations). The radioactivity in the reserve protein associated with the ER reached a maximum 15 min after the transfer to chase medium, then declined with a half-life of ~90 min (Fig. 5). The radioactivity in the solubilized reserve proteins at the top of the gradient continued to increase for the entire period of the chase (3 h), leveling off towards the end.

Pulse and pulse-chase experiments were also carried out with cotyledons obtained at 23 DAF when both vicilin and legumin

syntheses are in progress and similar results were obtained (data not shown). In these experiments, we also fractionated the reserve proteins by SDS PAGE to find out whether the individual radioactive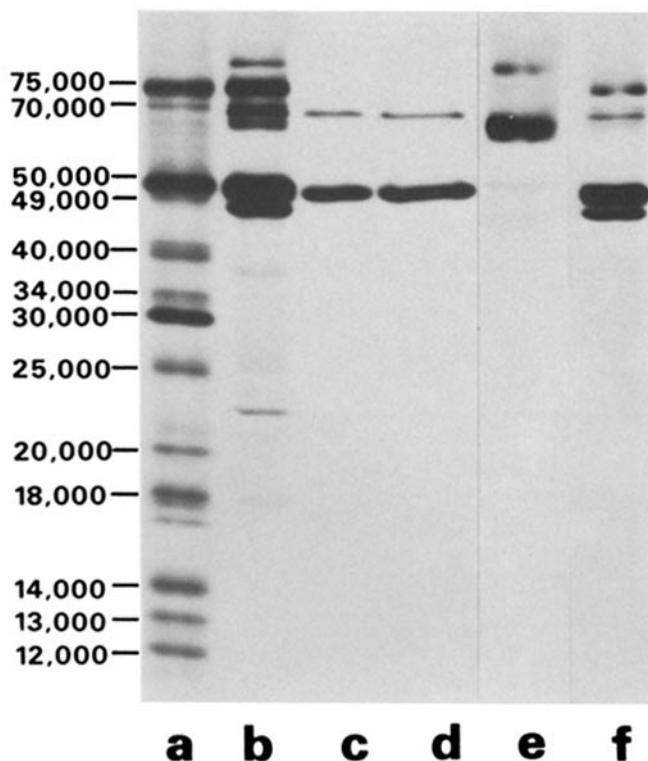 polypeptides chased out of the ER at approximately similar rates. One such fluorograph, showing only the point of maximum incorporation into the ER (45-min pulse followed by 15-min chase) and the long chase (2 h) for both the ER and the soluble proteins, is shown in Fig. 6. The polypeptides of vicilin (M_r 75,000, 50,000, and 48,000) and legumin (M_r 65,000–60,000) that accumulate in the ER during the 45-min pulse are largely lost during the next 2-h chase period (Fig. 6a and b). The same polypeptides of vicilin (M_r 49,000 and greater) show a corresponding increase in the soluble fraction during the chase. Legumin, which also increases in the soluble fraction, is present not as the precursor with M_r 65,000–60,000 but in its processed form as polypeptides of M_r 40,000 and 19,000. During this chase period, the movement of legumin polypeptides appears more complete than that of vicilin polypeptides. These results suggest a precursor-product relationship between the reserve protein in the ER and in the soluble fraction. It should be kept in mind that the reserve protein in the sample volume of the gradient comes from broken and solubilized protein bodies.

Role of Glycosylation in Transport of Reserve Proteins out of the ER

A pulse-chase experiment similar to the one shown in Fig. 5, but with [^{3}H]glucosamine as precursor, showed that glucosamine-labeled polypeptides are also chased out of the ER with a half-life of ~90 min (data not shown). The incorporation of glucosamine into the reserve proteins is inhibited by >80% when the cotyledons are pretreated with tunicamycin (50 µg/ml; 20 µl/cotyledon) (1). This observation makes it possible to find out whether glycosylation is necessary for transport out of the ER. Cotyledons (18 DAF) were pretreated for 2 h with tunicamycin (50 µg/ml) and then, still in the presence of tunicamycin, labeled with ^{14}C-amino acids for 1 h. Some cotyledons were then harvested, while others were transferred to chase medium for 2 h. The homogenates were fractionated on discontinuous gradients, the ER was recovered, and the reserve proteins were isolated with IgG-PBE-Sepharose. After 1 h of labeling, radioactivity in reserve proteins associated with the ER was 15,300 cpm per cotyledon for the control sample and 13,050 cpm per cotyledon for the tunicamycin-treated sample. After 2 h of chase the figures were 3,170 and 3,080 cpm, respectively. The control and tunicamycin-treated samples had similar amounts of radioactivity in the soluble reserve protein fraction. These results indicate that glycosylation is not a prerequisite for the movement of reserve proteins into or out of the ER.
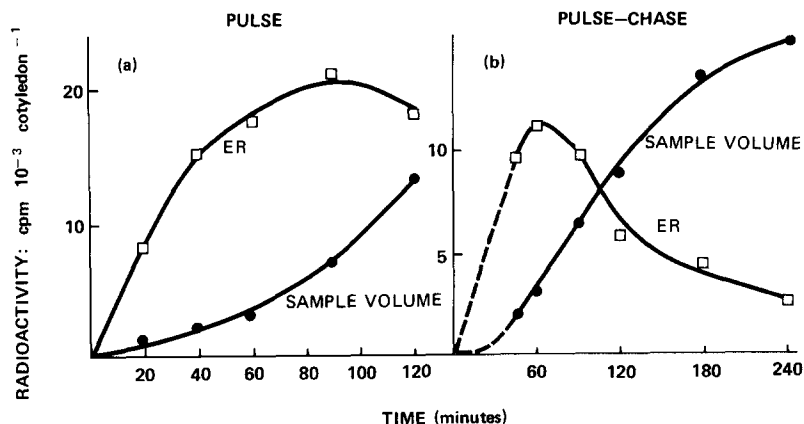

FIGURE 4 Fluorograph of radioactive storage protein polypeptides isolated from the ER. Three cotyledons (18 DAF) were labeled with ^{14}C-amino acids (b), [^{3}H]glucosamine (c), or [^{3}H]mannose (d) for 1 h using 27.7, 370, and 370 Bq, respectively, of each radioactive precursor. The cotyledons were homogenized, and the ER was isolated on a discontinuous sucrose gradient consisting of a layer of 16% (wt/wt) sucrose over a cushion of 35% (wt/wt) sucrose, both in 100 mM Tris, pH 7.8, containing 1 mM EDTA. After centrifugation at 150,000 g_{avg} for 2 h, the ER was collected and challenged with IgG-PBE-Sepharose. Lanes e and f show the ^{14}C-amino-acid-labeled proteins in the ER which bound, respectively, to IgG-legumin-Sepharose or IgG-vicilin-Sepharose. The reserve protein polypeptides were recovered and fractionated by SDS PAGE. Lane a shows chemically labeled reserve proteins present in an extract of protein bodies, and numbers indicate the M_r of reserve protein polypeptides.

FIGURE 5 Pulse and pulse-chase labeling of reserve proteins in the ER of pea cotyledons (15 DAF). (a) 20 cotyledons were labeled with 27.7 kBq of ^{14}C-amino acids each, and four cotyledons were harvested at times indicated. Extracts were prepared and fractionated on a sucrose gradient as described in Fig. 4. The ER and sample volume were collected, the reserve proteins were isolated with IgG-PBE-Sepharose, and their radioactivity was determined. (b) 24 cotyledons (15 DAF) were each labeled for 45 min with 27.7 kBq of ^{14}C-amino acids, then transferred to nonradioactive nutrient solution, and four cotyledons were harvested at the times indicated. The cotyledons were extracted and fractionated, and the radioactivity in reserve proteins in the ER and sample volume of the gradient was determined as in a.

ER   SOLUBLE

75,000 —
Legumin —
precursors

50,000 —

40,000 —

Legumin
(processed)
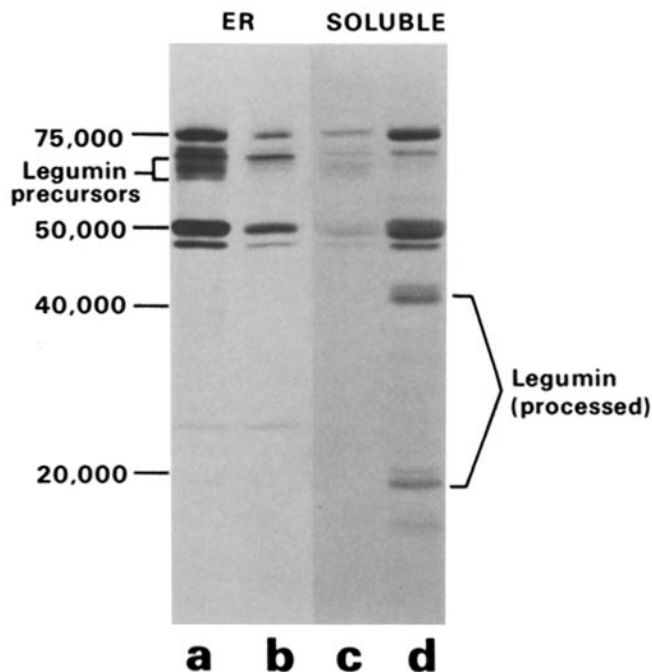
20,000 —

a   b   c   d

FIGURE 6   Fluorograph showing the disappearance of radioactive storage proteins from the ER and their appearance in the soluble fraction in a pulse-chase experiment. Cotyledons (23 DAF) were each labeled with 28 kBq of ^{14}C-amino acids, and the cotyledons were transferred to chase medium after 45 min. Cotyledons were harvested after another 15 min (lanes *a* and *c*) or 135 min (lanes *b* and *d*). Extracts were prepared and, after centrifugation, the reserve proteins in the ER and sample volume of the gradients were isolated with IgG-PBE-Sepharose affinity gel fractionated by SDS PAGE and a fluorograph was prepared. Lanes *a* and *b*: radioactive reserve proteins in the ER after 15 and 135 min of chase, respectively. Lanes *c* and *d*: radioactive reserve proteins in the soluble fraction after 15 and 135 min of chase, respectively. Lines on the left indicate the position of molecular weight markers with M_r = 75,000, 50,000, 40,000, and 20,000. Lines on the right indicate the polypeptides of legumin (M_r = 40,000 and 19,000) which are not present in the ER but are formed as a result of the processing of the legumin precursors (M_r = 60,000–65,000).

Kinetics of Transport of Reserve Proteins into Protein Bodies

Because most of the reserve proteins remained at the top of the sucrose-gradients (as in Fig. 2) we have assumed that the sample volume of the gradients contained not only the cytosolic proteins but also the protein body proteins. We have therefore equated labeling of the reserve proteins in the sample volume with labeling of the reserve proteins in the protein bodies. Experimental confirmation of this assumption depended on the development of a method to isolate protein bodies from immature cotyledons quickly, in high yield and relatively free of ER. The simple procedure described in Materials and Methods was applied to cotyledons 16, 20, 24, and 28 DAF. This period spans the transition from the vacuolar stage to the protein body stage of cotyledon development (9, 10). The fractions rich in protein bodies contained only 5–7% of the NADH-cytochrome *c* reductase present in the homogenate, indicating that contamination by ER was minimal. The yield of protein bodies was assessed by comparing the amount of reserve protein in the protein body pellet with that in the supernatant. At 16 DAF, approximately half the reserve protein

was recovered in the protein body pellet. By 24 DAF, and especially at 28 DAF, ~80% of the reserve protein was present in the protein body pellet.

The integrity of the protein body preparations from several developmental stages was checked by electron microscopy and compared with the equivalent structures in corresponding intact tissue (Fig. 7). At 16 DAF the cotyledon cells still contain several large vacuoles within which are the electron-dense reserve protein deposits (Fig. 7 *c*, arrows). Vacuoles with similar electron-dense material are present in the protein body–rich pellets (Fig. 7 *a*). However, most of the vacuoles appear to have ruptured and the pellets contain separate membranes and electron-dense masses. At 24 DAF the cells contain numerous small protein bodies with an electron-dense matrix (Fig. 7 *d*). Similar protein bodies were found in the pellets (Fig. 7 *b*). These results indicate that this relatively simple method of isolating protein bodies can be used most effectively at the later stages of cotyledon development (24 DAF and older).

Cotyledons (24 DAF) were incubated with ^{14}C-amino acids for different times and a protein body–rich fraction was isolated from a thin slice of the tissue which had been in contact with the label. Aliquots of the total homogenate and the protein body fraction were challenged with IgG-PBE-Sepharose, the storage protein was isolated, and the radioactivity in the storage protein was determined (Fig. 8). Incorporation into storage protein in the total homogenate was linear for ~2 h, leveled off thereafter, and showed no appreciable lag at the start. Incorporation into the protein body fraction was linear but showed a lag of ~20–30 min. Labeling of the reserve proteins in the protein body fraction leveled off after 3 h, reaching a level ~70% of that in the total homogenate. The remaining 30% of the labeled reserve protein was in the supernatant probably as a result of the breakage of some protein bodies during the isolation procedure. These results support our assumption that when tissue homogenates are prepared in medium A or B the protein bodies are disrupted and the reserve proteins are solubilized and retained in the sample volume of the sucrose gradient.

DISCUSSION

Role of the Rough ER

The results presented here show that newly synthesized reserve proteins are preferentially but only transiently associated with the rough ER and later accumulate in the protein bodies. Our conclusion that the radioactive reserve proteins are associated with the rough ER is based on the known sedimentation properties of ER in the presence or absence of $MgCl_2$ (22, 26, 32). The peak of incorporated radioactivity was always slightly more dense than the peak of NADH-cytochrome *c* reductase activity (Fig. 1). This could be interpreted to mean that only a particular fraction of the ER cisternae is engaged in reserve protein synthesis.

Our experiments provide confirmation of the autoradiographic experiments of Bailey et al. (2) carried out with *Vicia faba* cotyledons. The authors concluded that the rough ER is an important site of protein synthesis, and that proteins made on the rough ER accumulate in the protein bodies after a lag of 20–30 min. Our conclusion complements other findings showing that reserve proteins are made by polysomes bound to the ER, but not by free polysomes (4, 21, 25), and that reserve proteins can be visualized within the ER cisternae with ferritin-coupled antibodies (3). There is evidence for the cotranslational
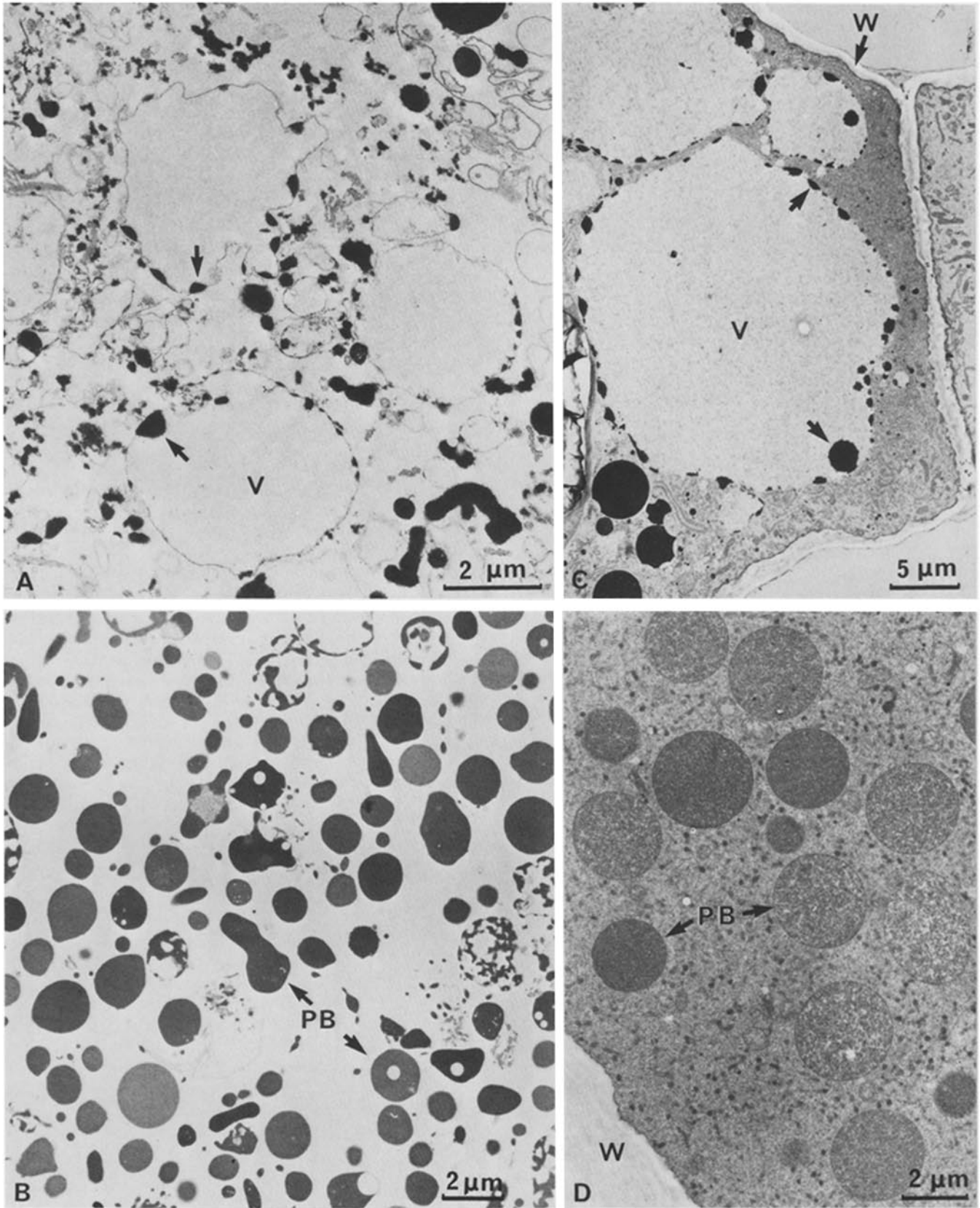
FIGURE 7 Electron micrograph of isolated protein bodies and storage parenchyma cells of pea cotyledons at the same stage of development. (*a* and *b*) Isolated protein bodies 16 and 24 DAF, respectively; (*c* and *d*) intact tissue 16 and 24 DAF, respectively. *v*, Vacuole; *PB*, protein body; arrow indicates electron-dense deposits in the vacuole; *W*, cell wall.
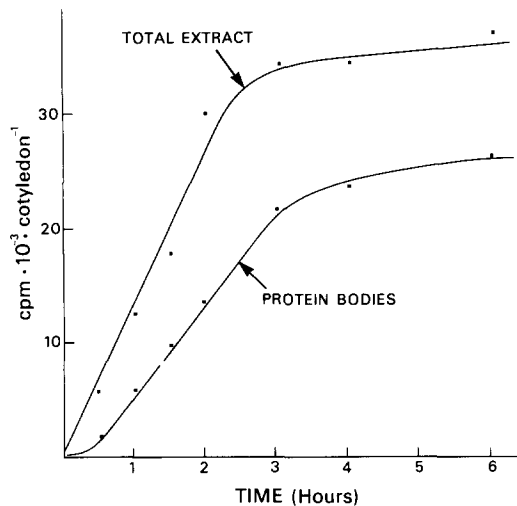
FIGURE 8 Arrival of radioactive reserve protein in the protein bodies. 21 cotyledons (23 DAF) were each labeled with 27.7 kBq of ^{14}C-amino acid, three cotyledons were collected at the times indicated, and protein bodies were isolated from a thin slice of the tissue which had been in contact with the label. The protein bodies were isolated as described in Materials and Methods. The total extract (before sedimentation of the protein bodies) and the resuspended protein bodies were challenged with IgG-PBE-Sepharose, the reserve protein was isolated and radioactivity was determined. Radioactivity is expressed as $cpm \cdot 10^{-3} \cdot cotyledon^{-1}$.

removal, by the ER, of a leader sequence on vicilin polypeptides of M_r 75,000 and 50,000 (13, 22). Isolated ER contains so much reserve protein that some of the individual polypeptides (especially the M_r 50,000 of vicilin) can be readily detected by staining SDS polyacrylamide gels with Coomassie Blue.

The transient association of reserve proteins with the ER indicates that this is the first compartment that sequesters the reserve protein on its way to the protein bodies. The half-time of the reserve proteins in the ER is ~90 min (Fig. 5) and reserve proteins in the protein bodies were labeled with a lag of 30 min, while there was no lag in the labeling of ER-associated reserve proteins (Fig. 8).

The Path of Transport

Little is known about the path of transport between the ER and the protein bodies in legume seeds. Transport could be mediated by ER-derived vesicles or could involve the Golgi apparatus; a less likely alternative is that reserve protein molecules might be released into the cytosol and then taken up by the protein bodies.

Suggestions for or against the involvement of the Golgi apparatus in reserve protein transport have been based entirely on morphological evidence (7, 8, 16, 19). It is clear from the experiments in which 3 mM $MgCl_2$ was present in the media that only a small fraction of the radioactivity and of the reserve protein was present at the density where the Golgi-derived dictyosomes band (1.13 $g \cdot cm^{-3}$ [30]). The radioactivity profile generally followed the NADH-cytochrome c reductase profile, indicating that the radioactivity in reserve proteins in this region of the gradient (1.13 $g \cdot cm^{-3}$) could be accounted for by the presence of ER that had not shifted to a higher density. When mannose was used as a label there was a definite peak of incorporation at 1.13 $g \cdot cm^{-3}$ in the presence of $MgCl_2$, while the ER banded at 1.18 $g \cdot cm^{-3}$. However, the peak at 1.13 $g \cdot$

cm^{-3} had much less reserve protein associated with it (4.5% of incorporated [^{3}H]mannose) than did the rough ER at 1.18 $g \cdot cm^{-3}$ (19%). The results do not rule out the possibility that the Golgi apparatus is involved in reserve protein transport, but neither do they lend support to that suggestion.

The similarity in lag times (20–30 min) between the arrival of reserve protein in the protein bodies (Fig. 8) and in the soluble fraction of a homogenate (Fig. 5 b) indicates that, if the cytosol is in the transport pathway, transit through the cytosol should be quite rapid.

Reserve Protein Polypeptides

The composition of the reserve protein polypeptides associated with the ER indicates that not all the polypeptides found in legumin and vicilin of mature peas are present in the ER. Legumin is represented only by its precursors, a group of polypeptides with M_r 65,000–60,000. These polypeptides are not present in mature legumin, which consists of small (M_r 20,000) and large (M_r 40,000) subunits. Our results show that the processing step, which takes 1–2 h, takes place after the legumin leaves the ER. Vicilin is represented only by polypeptides with M_r 75,000, 70,000, 50,000 (doublet), and 49,000. The smaller polypeptides that are present in vicilin of mature peas are absent from the ER. We suggest that the smaller polypeptides found in vicilin are formed by proteolytic cleavage of the larger vicilin polypeptides after these proteins leave the ER.

Glycosylation of Reserve Protein Polypeptides

The experiments with [^{14}C]glucosamine and [^{3}H]mannose provide the first proof that in situ glycosylation of the storage proteins occurs in the ER. Nagashi and Beevers (30) showed that the glycosyltransferases for glucosamine and mannose are associated primarily with the ER, although they are also present in other membrane fractions, in particular the Golgi apparatus. It appears that only two glycosylated polypeptides are made initially, namely, the M_r 50,000 and 70,000 of vicilin. We postulate that the other glycosylated polypeptides found in long-term labeling experiments, namely M_r 26,000 and 14,000 (1), are processing products of either or both of these. The experiments also show that legumin is not made as a glycosylated precursor, which is later deglycosylated. Since legumin is not glycosylated at any stage, a carbohydrate side-chain is probably not necessary for intracellular transport. That is confirmed by our finding that tunicamycin at a level that inhibits glycosylation by ~80% does not inhibit transport of either legumin or vicilin.

The principal questions raised by this work are: where and when are the smaller polypeptides of legumin and vicilin formed and when are the polypeptides assembled into oligomers? Work on these problems is now in progress and will be the subject of a subsequent publication.

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