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The Rough Endoplasmic Reticulum Is the Site of Reserve-Protein Synthesis in Developing *Phaseolus vulgaris* Cotyledons

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Abstract. Cotyledons of the common bean, *Phaseolus vulgaris* L., were incubated with radioactive amino acids at different stages of seed development. The proteins were fractionated by ion-exchange chromatography, sucrose gradients, and sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. From 16 to 28 d after flowering about 40% of the incorporated radioactivity was associated with the polypeptides of vicilin and 10% with those of phytohemagglutinin.

Polysomes were isolated from developing cotyledons 20–25 d after flowering and free polysomes were separated from membrane-bound polysomes. Aurintricarboxylic acid, an inhibitor of initiation in cell-free translation systems, did not inhibit the incorporation of amino acids into in-vitro synthesized proteins, indicating that synthesis was limited to the completion of already initiated polypeptides. Autofluorography of SDS-polyacrylamide gels showed that the two classes of polysomes made two different sets of polypeptides and that there was little overlap between these two sets.

Four polypeptides similar in size to the 4 polypeptides of vicilin were made by membrane-bound polysomes and not by free polysomes. Antibodies specific for vicilin bound to those 4 polypeptides. Free polysomes made only polypeptides which did not bind to antibodies specific for vicilin. Antibodies against phytohemagglutinin did not bind to any of the in-vitro synthesized polypeptides.

The membranes to which the polysomes were bound were characterized on sucrose gradients and by electron microscopy. Polysomes recovered from membranes which banded on top of 35 and 50% sucrose synthesized the vicilin polypeptides most rapidly. These membrane fractions were rich in vesicles of rough endoplasmic reticulum (ER). The ER marker-enzyme NADH-cytochrome-c reductase banded with an average density of 1.18 g/cm^3 (40% w/w sucrose) on continuous gradients. These experiments demonstrate that the ER is the site of vicilin synthesis in developing bean cotyledons. Quantitative determinations of several ER parameters (RNA and lipid-phosphate content, NADH-cytochrome-c-reductase activity) show that expansion of the cotyledons is accompanied by a 4–6-fold increase in ER.

Key words: Cotyledons (reserve proteins) – Endoplasmic reticulum – *Phaseolus* – Phytohemagglutinin – Protein (reserve) – Reserve protein vicilin.

Introduction

The seeds of the common bean (*Phaseolus vulgaris* L.) and many other legumes contain abundant supplies of reserve proteins stored in the parenchyma cells of the cotyledons. These cells are characterized by the presence of many large protein bodies measuring 3–10 μm in diameter and consisting of an amorphous protein matrix surrounded by a limiting membrane. In *Phaseolus vulgaris* the major reserve proteins are vicilin and phytohemagglutinin. They account respectively for 50 and 10% of the total protein of the cotyledons at seed maturity. Vicilin (also called glycoprotein II or globulin G1) is a 6.9 S protein which aggregates to form an 18-S tetramer at pH 4.5. The protein contains 3 or 4 non-identical polypeptides[1] which are glycoxylated. Phytohemagglutinin

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[1] The number of polypeptides seen in sodium dodecylsulfate-polyacrylamide gels is variable; the same preparation of vicilin can give 3 or 4 bands under apparently identical conditions (Pusztai and Watt, 1970; McLeester et al., 1973; Sun et al., 1974; Barker et al., 1976; Bollini and Chrispeels, 1978; Dale and Sussex, personal communication)

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(PHA) is a 6.4 S protein with a leucoagglutinating subunit (MW = 34,000) and an erythroagglutinating subunit (MW = 36,000) (Leavitt et al., 1977). Both vicilin and PHA have been shown to occur in the protein bodies (Barker et al., 1976; Bollini and Chrispeels, 1978). The reserve proteins are biosynthesized during seed development and maturation. Two lines of indirect evidence indicate that the rough endoplasmic reticulum (ER) may be the site of reserve protein biosynthesis. First, cotyledon development is accompanied by the proliferation of rough ER (Öpik, 1968; Briarty, 1973) and this rough ER is a major site of protein synthesis in the storage-parenchyma cells (Bailey et al., 1970). Second, glycosyl transferases involved in the biosynthesis of the carbohydrate moieties of reserve proteins appear to be associated with the ER (Nagahashi et al., 1978). In spite of these two lines of evidence identifying the rough ER as the likely site of reserve-protein synthesis in legume cotyledons, there is as yet no direct evidence that the polysomes which synthesize the polypeptides of the reserve proteins are associated with the ER. Sun et al. (1975, 1978) working with *Phaseolus vulgaris*, and Beachy et al. (1978) working with soybean, separated free and membrane-bound polysomes but found that they make similar polypeptides and that the polypeptides of the reserve proteins are made by both sets of polysomes. In contrast, research with animal cells has shown that proteins which are secreted or become incorporated into membranes or cytoplasmic organelles are usually associated with the ER (see reviews by Palade, 1975; Sabatini and Kreibich, 1976).

Recent demonstrations that polysomes active in in-vitro protein synthesis can readily be isolated from developing legume cotyledons (Sun et al., 1975, 1978; Higgins and Spencer, 1977; and Beachy et al., 1978) encouraged us to investigate the site of synthesis of vicilin in developing *P. vulgaris* cotyledons. We now present evidence that the polypeptides of vicilin are made exclusively by membrane-bound polysomes and that these polysomes can be recovered from rough ER fractionated on sucrose gradients. In addition, data ara presented which show that the rough ER proliferates at the time of synthesis and deposition of reserve proteins in the cotyledons.

Materials and Methods

Plants and Chemicals

Seeds of *Phaseolus vulgaris* L. cv. Greensleeves were purchased from W. Atlee Burpee, Co., Riverside, Cal., USA and planted in a mixture of sand, peatmoss and horticultural Perlite (Aztec Perlite, Escondido, Cal., USA) containing the controlled release fertilizer Osmocote (Sierra Chemical, Milpitas, Cal., USA). The plants were grown in a greenhouse under natural light in La Jolla. The flowers were tagged when they changed color from white to yellow (flower fading). The pods were harvested at different times after this color change and this time period is referred to as days after flowering.

Diethylpyrocarbonate, Triton X-100, tris (hydroxymethyl)-aminomethane (Tris), adenosinetriphosphate (ATP), guanosinetriphosphate (GTP), dithiothreitol, chloramphenicol, creatine phosphate, HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid), L-amino acids, thyroglobulin, creatine phosphokinase and ribonuclease were purchased from Sigma Chemical Co., St. Louis, Mo., USA. All radiochemicals were purchased from New England Nuclear Co., Boston, Mass., USA.

Extraction and Fractionation of Proteins

The proteins of the cotyledons were extracted and fractionated as previously described (Bollini and Chrispeels, 1978). Albumins and phytohemagglutinin were first separated from vicilin and minor salt-soluble proteins by chromatography on diethylaminoethyl cellulose (DEAE) and the polypeptides in these fractions separated by sodiumdodecylsulfate(SDS)-polyacrylamide gel electrophoresis using the method of Laemmli (1970). The best results were obtained with the following modifications: the stacking gel was only 2 or 3 mm high, and the ratio of acrylamide to bisacrylamide was 200:1. Autofluorography of the gels to locate the radioactive polypeptides was done according to the procedure of Bonner and Laskey (1974). Protein was measured with the method of Lowry et al. (1951) using bovine serum albumin as a standard.

In Vivo Protein Synthesis

Two beans were removed from the pods, and the cotyledons excised and incubated for 4 h at room temperature in 1 ml of sterile water containing 100 μCi of L-[^{3}H]amino-acid mixture (Lot No. 990–116) and 50 μg of chloramphenicol.

Extraction of Free and Membrane-Bound Polyribosomes

The extraction of polyribosomes was done using the method of Larkins and Davies (1975) in which the tissue is homogenized at high ionic strength and high pH to reduce ribonuclease activity. All solutions were sterilized by autoclaving and all glassware was rinsed with a boiling solution of 1% diethylpyrocarbonate and then heated overnight at 180° C.

The seeds were removed from the pods, usually 17–25 d after flowering; the cotyledons were removed from the seeds and frozen in liquid nitrogen. They were then used immediately or stored at −70° C. The frozen cotyledons were crushed and the powder transferred to a cold motar and extracted with 5 volumes of buffer A (0.2 M Tris-HCl at pH 8.6, 60 mM KCl, 30 mM $MgCl_2$, 0.2 M sucrose). The extract was centrifuged at 30000 × *g* for 20 min in a Sorvall (Norwalk, Conn., USA) RC2-B centrifuge. The sediment, containing most of the cellular organelles, was resuspended in 3 volumes of buffer A containing 1% v/v Triton X-100, and centrifuged again at 30000 × *g* for 20 min. The supernatant from the first centrifugation, containing the free polysomes, and from the Triton-X-100-solubilized material were then layered on a 2-ml cushion of 1.5 M sucrose in buffer B (40 mM Tris-HCl at pH 8.6, 20 mM KCl, 10 mM $MgCl_2$). The polyribosomes were sedimented by centrifugation at 134000 × *g* for 150 min in a Spinco ultracentrifuge (Beckman Instruments, Palo Alto, Cal., USA) and resuspended in 250 μl of 40 mM Tris-HCl at pH 8.6, 20 mM

$MgCl_2$. Profiles of polyribosomes were obtained by rate zonal centrifugation of the polyribosomal preparations on linear 15–50 % sucrose gradients (in buffer B). Centrifugation was for 2 h at 30000 rpm in the SW-41 rotor of a Spinco ultracentrifuge. The polysomal material had an A_{260}/A_{280} ratio around 1.5 for both the free and the membrane-bound polysomes.

Fractionation of ER on Sucrose Gradients

Cotyledons (0.5 g) were homogenized in 6 volumes of extraction medium (200 mM Tris-HCl pH 8.6, 0.2 M sucrose, 20 mM KCl, 0.1 mM $MgCl_2$, and 1 mM ethylendiaminotetraacetic acid (EDTA); or 200 mM Tris-HCl pH 8.6, 0.2 M sucrose, 30 mM KCl and 1 mM $MgCl_2$). The homogenate was centrifuged at $500 \times g$ for 3 min and loaded on linear or step 20–50% (w/w) sucrose gradient on a 60% sucrose cushion. The gradients contained 40 mM Tris-HCl pH 8.6, 20 mM KCl, and either 1 mM EDTA with 0.1 mM $MgCl_2$, or 1 mM $MgCl_2$. In some experiments 3 and 10 mM $MgCl_2$ was used in the homogenization medium and the gradients. The gradients were centrifuged at 25,000 rpm in the SW-27 rotor of a Spinco ultracentrifuge. Centrifugation times ranged from 30 to 120 min. For some experiments step gradients were used with steps of 20, 35 and 50% (w/v) sucrose.

In Vitro Protein Synthesis

In vitro protein synthesis was accomplished by the addition of a wheat germ S23 extract to the isolated polysomes in the presence of other chemicals necessary for translation. The wheat germ (obtained from General Mills, Los Angeles, Cal., USA) was fractionated according to Roman et al., (1976) to obtain the S23 extract; this was stored at −70° C. The in-vitro translation mixture (100 µl) had the following composition: 20 mM HEPES-KOH buffer at pH 7.6, 1 mM ATP, 20 µM GTP, 2 mM dithiothreitol, 8 mM creatine phosphate, 30 µM of each of 18 amino acids (without leucine or methionine), 50 µg of creatine phosphokinase (100 units/mg), and 50 µl of S23 wheat-germ extract. The concentrations of $MgCl_2$, KCl, L-[^{3}H]leucine (specific activity 60 Ci/mmol), L-[^{35}S]methionine (specific activity 564 Ci/nmol), the amount of polysomes used, and the incubation time and temperature are indicated in the "Results". The radioactively labeled polypeptides were separated from the free amino acids by filtration on nitrocellulose filters (Type HA, Millipore Co., Bedford, Mass., USA).

Immunological Techniques

Vicilin and PHA purified as described in Bollini and Chrispeels (1978) were injected subcutaneously into New Zealand white rabbits obtained from a local breeder. The antigens (1 mg) were emulsified with 1 ml of Freund's complete adjuvant (Calbiochem, San Diego, Cal., USA). Four weeks later, each rabbit received a second injection of 1 mg of protein in Freund's incomplete adjuvant. Seven and 10 d later blood was removed from the rabbit and the serum was collected after blood clotting. The serum was kept frozen, and thawed before use.

Two-dimensional double-diffusion tests (Ouchterlony) were carried out in 1.25% agar (Difco-Nobel, Difco Labs., Detroit, Mich., USA), 0.15 M NaCl and 1 mM EDTA. Diffusion was allowed to proceed for 24 h. Immunoelectrophoresis was performed according to the procedures of Grabar and Williams (1955). The antigens were electrophoresed in 1% agarose with 20 mM barbital buffer, pH 8.6, for 6 h at 8–10 V/cm. Antiserum was added to a slit in the agarose and diffusion was allowed to proceed for 24 h. The gel was washed to remove the salt, dried, stained with Coomassie Brilliant Blue (Imperial Chemical Industries, United States, Wilmington, Del., USA) and de-stained. Monospecific antibodies against vicilin were prepared from the vicilin antiserum by affinity chromatography on a column consisting of vicilin linked to polacrylamide beads with methods identical to those used to purify antibodies against vicilin-peptidohydrolase (Baumgartner et al., 1978). The concentration of anti-vicilin immunoglobulin G (IgG) in the preparation of purified monospecific antibodies was ca. 100 times greater than in the crude antiserum.

Precipitation of In Vitro Synthesized Polypeptides with Monospecific IgG against Vicilin

Polypeptides synthesized in vitro were precipitated with IgG using the method of Kessler (1975). This method depends on the presence of the A-protein on *Staphylococcus aureus* cells. The A-protein attached to the cell walls of the heat-killed bacteria binds IgG molecules or IgG-antigen complexes.

The translation mixture was centrifuged at $150000 \times g$ for 150 min and 50 µl of the supernatant was incubated overnight at 4° C with 10 µl of vicilin IgG (monospecific) or 5 µl of PHA antiserum, and 250 µl of TNT buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 0.1% Triton X-100). Heat-killed *Staphylococcus aureus* cells (40 µl of a 10% suspension in 50 mM Tris-HCl, pH 7.6, from Enzyme Center, Boston, Mass., USA) were added to the mixture which was incubated at room temperature for 30 min. The bacterial cells were sedimented by centrifugation and the pellet washed 5 times with 0.5 ml of TNT buffer and then resuspended in 40 µl of TNT buffer containing 2 M urea with 10 µl of a 10% SDS solution. The mixture was heated for 2 min in boiling water to break up any protein-protein interaction. β-Mercaptoethanol (2.5 λ) and bromophenol blue were added to the sample; the mixture was boiled briefly and loaded on the polyacrylamide gel.

Preparation of a Thyroglobulin Affinity Column and Purification of PHA

Thyroglobulin was linked to Sepharose 4B (Pharmacia, Uppsala, Sweden) using the cyanogen bromide method as described by Felsted et al. (1975) and Manen und Miège (1977). Cotyledon extract, purified PHA or in-vitro synthesized polypeptides were loaded on a column in 1 mM potassium phosphate, pH 7.0, containing 4% NaCl. The column was washed with the same buffer until the A_{280} was negligible. The PHA which remained bound to the column was then eluted with 50 mM glycine-HCl, pH 3.0, containing 0.5 M NaCl. The eluate contained all the agglutinating activity present in a crude extract of cotyledons.

Analytical Methods

To measure the lipid-phosphate content of the cotyledons, homogenates were made in media containing 1 mM EDTA and the membranes which passed through 20% (w/w) sucrose, but not through 33% (w/w) sucrose were collected on a step gradient. This procedure yielded most of the NADH-cytochrome-c-reductase-containing membranes, but eliminated the broken plastids which passed through the 33% sucrose. The lipid-phosphate content of the membranes was determined as described in Gilkes et al. (1979).

NADH-cytochrome-c reductase and RNA in membrane-associated polysomes were measured on membrane fractions obtained by differential centrifugation of homogenates ($30000 \times g$ for 30 min in a Sorvall RC2-B centrifuge). Enzyme activity was determined

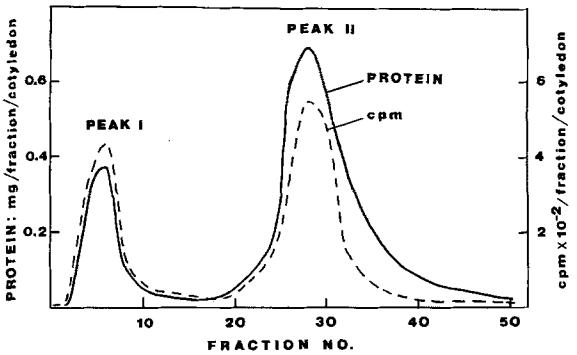

Fig. 1. Fractionation of proteins of bean cotyledons on DEAE-cellulose. Cotyledons were removed from the plants 25 d after flowering, and incubated for 4 h in small flasks at room temperature with 100 μCi of [^{3}H]amino acids. The extracted proteins were separated from the free amino acids by gel filtration, then loaded on the DEAE-cellulose column; this was eluted with a 0–0.5 M NaCl gradient. Protein was measured according to Lowry et al. (1951) and radioactivity determined by collecting the proteins precipitated with trichloroacetic acid on a filter

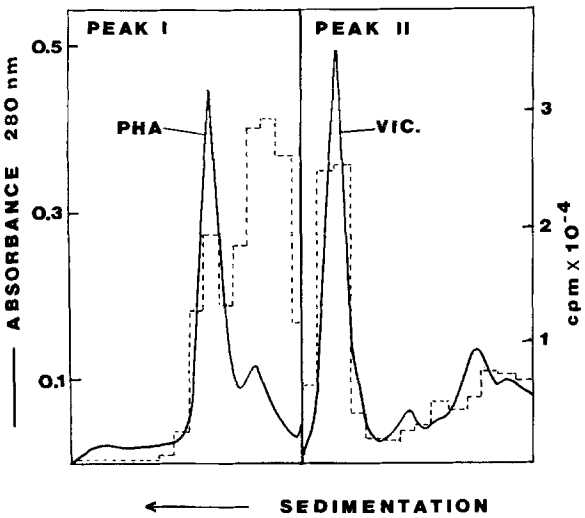

Fig. 2. Fractionation of proteins from bean cotyledons eluted from the DEAE-cellulose column on sucrose gradients. Proteins present in peak I and II (see Fig. 1) were dialyzed, lyophylized, and dissolved in 25 mM potassium phosphate, pH 4.5, containing 0.3 M NaCl. The dissolved proteins were fractionated on linear 5–25% sucrose gradients by centrifugation for 20 h at 38000 rpm in the SW41 rotor of a Spinco ultracentrifuge

by following the change in A_{550} of cytochrome c. The reaction mixture (1 ml) contained 0.80 mg of cytochrome c, 0.5 mg of NADH, 0.2 μmol of KCN and 80 μmol of potassium-phosphate buffer, pH 7.5. Cytochrome oxidase was measured by following the change in A_{550} of reduced cytochrome c. A 1-ml reaction mixture contained 0.65 mg of cytochrome c, 20 μmol of Tris buffer, pH 7.4, and 1 μmol of EDTA. RNA in polysomal preparations was estimated by measuring the absorbance at 260 nm and using an extinction coefficient of 20 ml mg^{-1} cm^{-1} for RNA.

Results

In Vivo Synthesis of Proteins

Cotyledons, excised from seeds at different stages of maturation, were incubated in the presence of [^{3}H]-amino acids to label the proteins and to determine when during development the major seed proteins are synthesized. The radioactive proteins present in an extract of the cotyledons were fractionated first by DEAE-cellulose chromatography and then by sucrose gradients or SDS-polyacrylamide gel electrophoresis as described by Bollini and Chrispeels (1978). Chromatography on DEAE cellulose resulted in the separation of the proteins in two fractions; radioactivity was associated with each fraction (Fig. 1). The proteins in each peak were concentrated and fractionated on sucrose gradients (Fig. 2). Peak I from the DEAE-cellulose column was separated into PHA (6.3 S) and a peak of smaller proteins. Peak II from the DEAE-cellulose column was separated into vicilin (18 S at pH 4.5) and smaller proteins. Radioactivity was associated with each of these protein peaks.

The data shown in Figs. 1 and 2 were obtained by labeling cotyledons 25 d after flowering. Similiar experiments were conducted at 5 other stages of cotyledon development and the data are summarized in Table 1. The experiments allowed us to calculate the proportion of radioactivity which accumulates in PHA and vicilin at different times during cotyledon development. The capacity to incorporate amino acids increased about 3-fold between 19 and 28–31 d after flowering, then declined again during the final phase of seed maturation. The biosynthesis of PHA and vicilin declined more rapidly than total protein synthesis in the cotyledons. By 31 d after flowering little or no radioactivity was incorporated into the two reserve proteins.

The polypeptides present in the two peaks obtained from the DEAE-cellulose column were separated by SDS-polyacrylamide gel electrophoresis on slab gels. Proteins extracted from cotyledons obtained on different days after flower fading were added to the different wells and an equal amount of radioactivity was added to each well. The gels were stained with Coomassie brilliant blue and a single lane of the stained gel is shown on the right-hand side of Fig. 3A and B. An autofluorograph of the same gel with 7 stages of bean development is shown in Fig. 3A and B. The most abundant polypeptides in the first peak of the DEAE-cellulose column were those of PHA (Fig. 3A, right-hand lane). These polypeptides were actively synthesized between 16 and 28 d after flower fading and synthesis slowed down

Table 1. Incorporation and accumulation of ^{3}H-amino acids in PHA and vicilin at different development stages of bean cotyledons.
Calculations were made after separation of PHA and vicilin by DEAE-cellulose chromatography and sucrose gradients as shown in Figs. 1 and 2

Days after flowering	Peak I		Peak II		PHA as % of total	Vicilin as % of total
	cpm $\times 10^{-3}$	% in PHA	cpm $\times 10^{-3}$	% in vicilin		
16	147	38	675	44	6.8	36
19	197	42	725	48	9.0	38
22	420	37	1,170	42	9.7	31
25	775	29	2,100	54	7.8	39
28	1,075	24	1,900	30	8.7	19
31	650	5	2,670	13	1.0	10
34	546	< 1	1,200	< 1	–	–

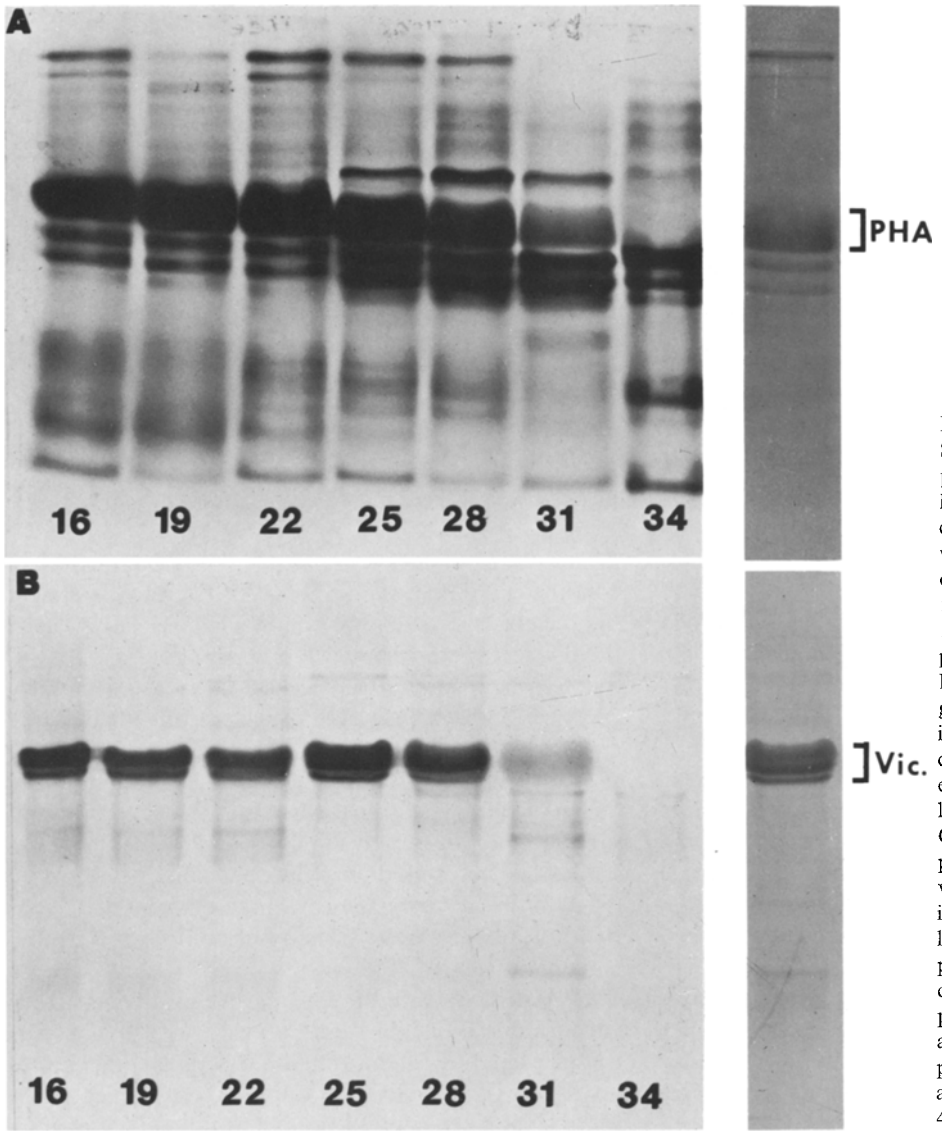

Fig. 3A and B. Autofluorograph of SDS-polyacrylamide gel of polypeptides of bean cotyledons labeled in vivo at different stages of cotyledon development. Cotyledons were obtained at different stages of development, the numbers indicating days after flowering. **A** (upper gel) represents all the polypeptides in Peak I from the DEAE-cellulose column. **B** (lower gel) represents all the polypeptides in Peak II from the DEAE-cellulose column. The right-hand lanes in each case are photographs of one lane of the gel after staining with Coomassie Brilliant Blue. The positions of the polypeptides of vicilin and phytohemagglutinin are indicated on the side. The numbered lanes show the radioactively-labeled polypeptides at different stages of development. **A** 18% polyacrylamide gel, 28000 cpm added to each lane; **B** 15% polyacrylamide gel, 10000 cpm added to the first 4 lanes and 4000 cpm to the last 2 lanes

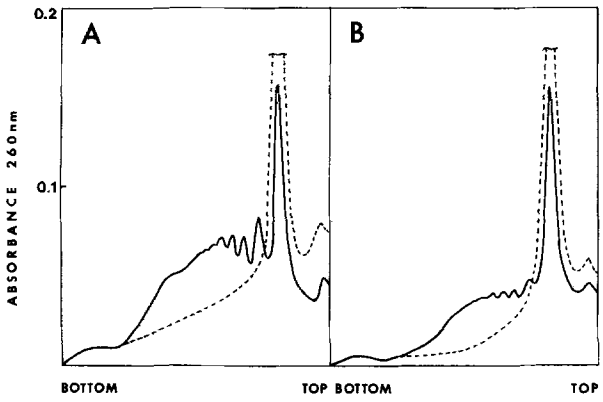

Fig. 4A and B. Fractionation of free and membrane-bound polysomes from bean cotyledons on sucrose gradients. Centrifugation for 2 h at 30,000 rpm on a linear 15–50% (w/v) gradient in a SW41 rotor of a Spinco ultracentrifuge. **A** membrane-bound polysomes, **B** free polysomes; solid curves = control, broken curves = +1 μg RNase

later on. The most abundant polypeptides in the second peak of the DEAE-cellulose column are those of vicilin (Fig. 3B, right-hand lane). These polypeptides were also synthesized primarily between 16 and 28 d after flower-fading, with synthesis slowing down after 28 d.

These results indicate that vicilin and PHA, two proteins which have been shown to be sequestered in subcellular organelles, were major products of protein synthesis 16–28 d after flower fading. Cotyledons in this stage of development were used as a source of free and membrane-bound polysomes to study in vitro protein synthesis.

Isolation of Free and Membrane-Bound Polysomes

Free and membrane-bound polysomes were separated by differential centrifugation of a cotyledon homogenate at $30000 \times g$ for 20 min. Such a centrifugation sedimented 95% of the activity of NADH-cytochrome-c reductase, a marker enzyme for the ER. The polysomes were freed from the membranes by resuspending the pellet in buffer containing 1% Triton X-100. The polysomes in the detergent-treated membrane pellet and in the supernatant of the first centrifugation were sedimented (2.5 h at $134000 \times g$), resuspended, and fractionated on continuous sucrose gradients. The polysome profiles obtained in such an experiment are shown in Fig. 4. Both fractions apparently contained single ribosomes as well as polysomes of various sizes. Incubation of the unfractionated polysomes with ribonuclease (10 min at 0° C) followed by fractionation of the material on sucrose gradients resulted in decreased absorbance at 260 nm in the polysome region of the gradient, and increased absorbance in the single ribosome peak.

Translation Activity of Free and Bound Polyribosomes

The translation activity of the polyribosome preparations was determined by incubating the polyribosomes in the presence of an S23 wheat germ fraction and radioactive amino acids. First we determined the concentrations of potassium (KCl) and magnesium ($MgCl_2$) which resulted in the highest rates of incorporation. The presence of 100 mM KCl and 2.5 mM $MgCl_2$ in the incubation mixture resulted in maximum rates of incorporation for both the free and the membrane-bound polysomes (data not shown). These conditions were used for all further experiments.

Next we determined the extent to which incorporation was consequence of the completion of already initiated polypeptides or of the initiation of new polypeptides. This was done by following the time-course of incorporation in the absence and in the presence of 100 μM aurintricarboxylic acid. The results show that this inhibitor of polypeptide-chain initiation did not inhibit the translation activity of either set of polysomes, indicating that the incorporation of amino acids resulted almost exclusively from the completion of already initiated peptide chains (data not shown).

Estimation of the Amount of Free and Membrane-Bound Polysomes

The amount of RNA present in the preparations of free and bound polysomes was estimated by determining the absorbance at 260 nm. While the results varied from one experiment to the next we found approximately equal amounts of RNA in each of the two preparations. Analysis of the polysome profiles showed that only 40–45% of the A_{260} in the free-polysome preparation was accounted for by polysomes and 55–60% by single ribosomes. In the preparation of membrane-bound polysomes 63–68% of the A_{260} could be accounted for by polysomes and only 32–37% by free ribosomes. These data indicate that the ratio of membrane-bound polysomes to free polysomes was nearly 2:1.

When the isolated polysomes were allowed to incorporate radioactive amino acids in vitro it was found that the membrane-bound polysomes were nearly twice as active as the free polysomes when the results were expressed as cpm/unit of A_{260} (Ta-

Table 2. Activity of free and membrane-bound polysomes from bean cotyledons in a cell-free incorporation system. The polyribosomes were translated in 50 µl of reaction mixture containing 125 mM KCl, 2.5 mM $MgCl_2$ and 5 µCi L-[^{3}H]leucine. Reaction products were treated with hot (10 min at 90° C) trichloroacetic acid prior to collection on membrane filters. Incorporation in the absence of added polyribosomes was 3000 cpm; this value has been subtracted

Free polysomes		Membrane-bound polysomes	
A_{260}	cpm	A_{260}	cpm
0.147	116000	0.156	215000
0.368	158000	0.390	309000
0.735	272000	0.780	571000

ble 2). The preparation of free polysomes incorporated 370,000 cpm of [^{3}H]leucine/unit of A_{260} compared to 730,000 cpm of [^{3}H]leucine/A_{260} for the membrane-bound polysomes. These data are consistent with a ratio of membrane-bound to free polysomes of 2:1.

Comparison of Polypeptides Synthesized In Vitro by Free and Membrane-Bound Polysomes

The radioactive polypeptides completed in vitro by the free and membrane-bound polysomes were fractionated on SDS-polyacrylamide gels and visualized by autofluorography of the gels. Centrifugation of the in-vitro protein-synthesis reaction mixture at the end of incorporation period indicated that 80–90% of the incorporated radioactivity could be sedimented at $180000 \times g$ for 150 min. This centrifugation separated the polypeptides which had been released from the polysomes from those which remained attached. A comparison of these two classes of polypeptides by SDS-polyacrylamide gel electrophoresis indicated few differences in the high molecular-weight range (data not shown). This was true for both the free and the membrane-bound polysomes. A direct comparison of the polypeptides completed and released by free and membrane-bound polysomes showed that both sets of polysomes were engaged in the synthesis of different sets of polypeptides.

A clear example that the free and membrane-bound polypeptides make different sets of polypeptides is shown in Fig. 5 where the lanes marked F and B contain the radioactive polypeptides made by free and membrane-bound polysomes, respectively. The lane marked V contains a sample of radioactively labeled vicilin synthesized in vivo. Since the three polypeptides were only barely visible on the original autofluorogram their position has been marked on the side with black dots.

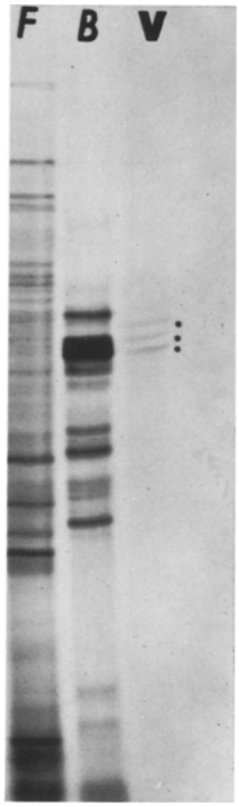

Fig. 5. Autofluorograph of an SDS-polyacrylamide gel used to separate the soluble polypeptides made by free and membrane-bound polysomes. F = free polysomes. B = membrane-bound polysomes. V = in-vivo labeled vicilin; the three polypeptides were clearly visible on the original and their position is marked by black dots

Characterization of ER

The ER was localized on isopycnic sucrose gradients by measuring the distribution of the marker enzyme NADH-cytochrome c reductase. When the cotyledons were homogenized in a medium containing 0.1 mM $MgCl_2$ and 1 mM EDTA this marker enzyme banded with an average density of 1.125 g/cm^3 (Fig. 6A). The curve which is shown represents NADH-dependent cytochrome c reductase activity. The four fractions at the top of the gradient contained NADH-independent cytochrome-c-reductase activity. The NADH-independent cytochrome-c reductase appeared to be a soluble enzyme and did not move into the gradient during the 2-h centrifugation. When the cotyledons were homogenized in a medium containing no EDTA and 1, 3 or 10 mM $MgCl_2$ the NADH-cytochrome-c reductase had a density range of 1.15 g/cm^3 to 1.23 g/cm^3 and formed a broad band in the gradient. The position of the mitochondria was determined by assaying for cytochrome-c-oxidase activity. This enzyme

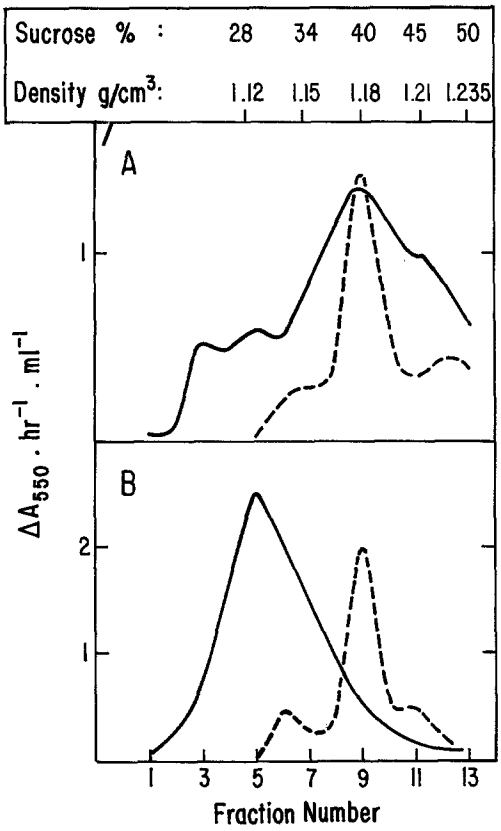

Fig. 6A and B. Distribution of NADH-cytochrome-c reductase and cytochrome oxidase on isopycnic sucrose gradients of proteins from bean cotyledons. **A** Homogenization medium and gradients contained 1 mM $MgCl_2$. **B** Homogenization medium and gradients contained 0.1 mM $MgCl_2$ and 1 mM EDTA. Gradients (20 to 50% w/w) were centrifuged for 2 h at $82000 \times g$. Each gradient contained the extract of 0.5 g of cotyledons obtained 25 d after flowering. Solid curves = NADH-cytochrome-c reductase (ER marker); broken curves = cytochrome-c oxidase (mitochondrial marker)

banded with a density of 1.18 g/cm^3 in both types of gradients (Fig. 6B).

Some aggregation of ER, mitochondria and protein bodies was apparent at 10 mM $MgCl_2$, but most of the NADH-cyt c reductase activity still had the same broad distribution with an average density of 1.18 g/cm^3 (data not shown). This concentration of $MgCl_2$ was used to isolate ER for electron microscopic examination and to test the translational activity of polysomes isolated from membranous organelles.

The membranous organelles present at the 20/38% (w/w) interface of a discontinuous sucrose gradient were examined with the electron microscope and a representative view is shown in Fig. 7. The isolation medium and the gradients contained 10 mM $MgCl_2$ to insure the integrity of the ribosomes. The particulate fraction consisted almost entirely of membranous vesicles with attached ribosomes. Tangential sections through these vesicles show clusters of ribosomes against a grey background (arrows). The preparation contained clusters of ribosomes which appear to be free. To what extent these clusters represent aggregated free polysomes or different views of membrane-bound polysomes was not determined. The preparation was free of intact mitochondria (which band at 40% w/w sucrose) but contained some double-membrane vesicles, possible derived from broken plastids (which band at 35% w/w sucrose).

Polypeptides Synthesized by ER-Associated Polysomes

Rough ER membranes of different densities were obtained by fractionating a cotyledon homogenate (no EDTA, 10 mM $MgCl_2$) on a step gradient using steps of 20, 35 and 50% (w/v) sucrose. The gradients were centrifuged for 30 min at $32000 \times g$, and density equilibrium may not have been reached during this short centrifugation. The short centrifugation time was chosen to avoid sedimenting free polysomes into the sucrose gradient. The membranes present at each interface were recovered and treated with Triton X-100. Polysomes were isolated from these detergent-treated membrane fractions. The upper part of the gradient (labeled supernatant) was also recovered, treated with Triton X-100, and used for polysome isolation.

The isolated polysomes were used in an in-vitro protein synthesis system with [^{35}S]methionine. The products of translation were fractionated on an SDS-polyacrylamide gel and visualized by autofluorography (Fig. 8). The data show that the polysomes present in the different fractions did not all make the same polypeptides. Certain polypeptides were made primarily by the polysomes which remained at the top of the gradient or were present on top of the 20% sucrose layer. The polypeptides which correspond in size to the four polypeptides of vicilin were made by polysomes attached to the membranes which were collected at the 20/35 and the 35/50% sucrose interfaces. The lane labeled "V" in Fig. 8 contained radioactive vicilin but the radioactivity was so low that the four polypeptides could only be seen on the original autofluorograph, and their position is marked by the four horizontal black lines. The right-hand lane is a photograph of the lane marked V after staining with Coomassie Brilliant Blue. It is clear that there is little size difference between the in-vivo synthesized polypeptides and the in-vitro synthesized polypeptides. The polypeptides made in vivo appear slightly larger than those made in vitro.

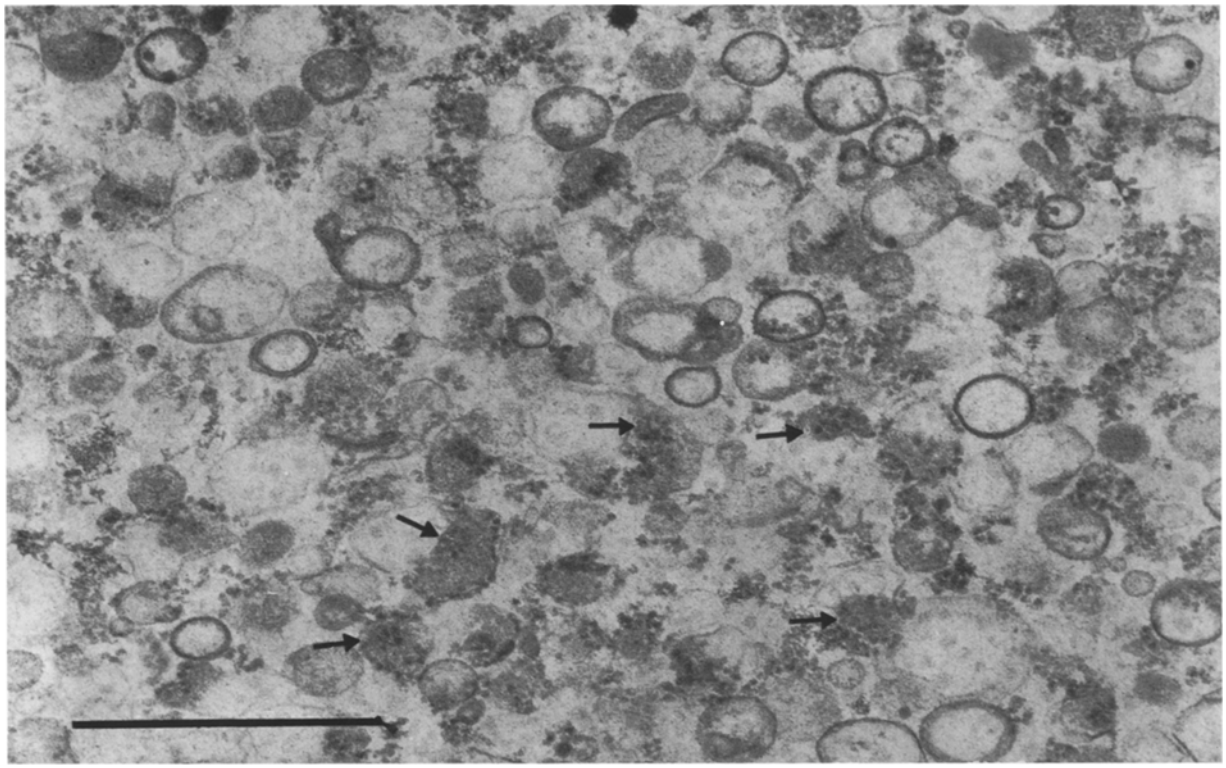

Fig. 7. Electron micrograph of a section through a pellet of ER membranes from bean cotyledons. The cotyledons were homogenized in a medium containing 0.2 M sucrose, 200 mM Tris-HCl, pH 8.6, 30 mM KCl and 10 mM $MgCl_2$. The extract was loaded on a step sucrose gradient and the membranes present at the 20/38% (w/w) interface were collected. Arrows indicate tangential sections through rough-ER vesicles showing clusters of ribosomes against a grey background. Bar represents 1 μm

Specificity of Vicilin Antiserum

An antiserum against purified vicilin was used to determine if any of the in-vitro made polypeptides would bind to anti-vicilin immunoglobulins. The specificity of this antiserum was tested in three ways. Two-dimensional double-diffusion tests (Ouchterlony) of the antiserum against purified vicilin showed the presence of a single precipitin arc between the wells containing vicilin and antiserum. No pricipitin lines were formed between PHA and vicilin antiserum or between vicilin and a pre-immune serum (data not shown).

Secondly, immunoelectrophoresis was used to test for the presence of contaminating antigens. The results of such an electrophoretic fractionation of the antigen followed by diffusion of antiserum from the rectangular trough showed the presence of a single precipitin line (Fig. 9A). Finally, the specificity of the antiserum was tested by using it to precipitate radioactive polypeptides from a homogenate made from cotyledons which had been labeled in-vivo with ^{35}S]-methionine. The precipitated polypeptides were then fractionated on an SDS polyacrylamide gel and visualized by autofluorography (Fig. 9B). Lane 1 shows all the radioactive proteins present in the homogenate. Lane 2 the polypeptides precipitated by the *Staphylococcus* IgG complex. Lane 3 is a photograph of Lane 2 after staining with Coomassie Brilliant Blue. The 4 polypeptides of vicilin are clearly visible. The additional polypeptide is the heavy chain of the IgG molecule.

Precipitation of Vicilin Polypeptides Synthesized In Vitro

The antiserum was then used to check whether free or membrane-bound polysomes carried out the in-vitro synthesis of polypeptides which could bind to the anti-vicilin IgGs. In this experiment membrane-bound polysomes were separated from free polysomes by differential centrifugation. The polypeptides made in vitro which bound to the IgG corresponded in size to the polypeptides of vicilin, and were made exclusively by membrane-bound polysomes but not by free polysomes (Fig. 10, lanes 2 and 5). In some experiments the anti-vicilin IgG precipitated addi-

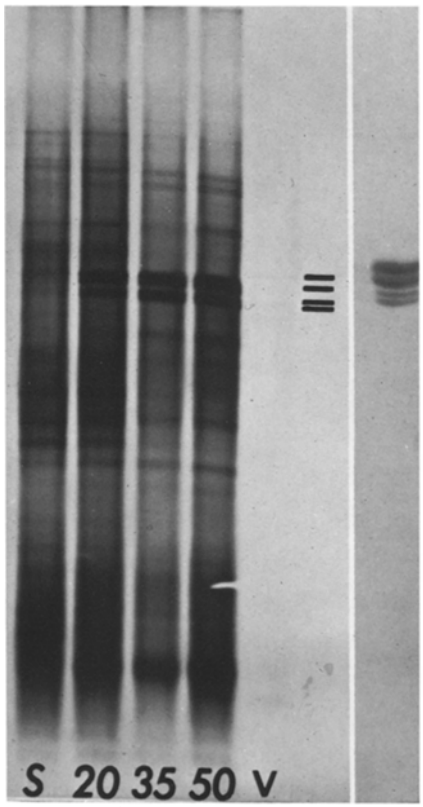

Fig. 8. Autofluorograph of the fractionated translation products of bean-cotyledon polysomes obtained from different regions of a sucrose-gradient. Cotyledons were homogenized in a medium containing 0.2 M sucrose, 200 mM Tris-HCl pH 8.6, 30 mM KCl, 10 mM $MgCl_2$, and the homogenate fractionated on a step gradient with layers of 20, 35 and 50% (w/v) sucrose in 40 mM Tris-HCl, pH 8.6, 20 mM KCl and 10 mM $MgCl_2$. The gradient was centrifuged for 30 min at $32000 \times g$. The different membrane fractions at the interfaces, and the supernatant were recovered, treated with Triton X-100, and the polysomes isolated and translated with [^{35}S]methionine. The translation products were fractionated on an SDS polyacrylamide gel and visualized. The lanes marked S, 20, 35 and 50 represent the polysomes obtained from the supernatant, the supernatant/20, 20/35 and 35/50% sucrose interfaces of the gradient. The fifth lane (marked V) contained a sample of cotyledon extract labeled in-vivo with [^{3}H]amino acids; the position of vicilin (visible on the original autofluorograph) has been marked by 4 horizontal lines. A photograph of a lane of the gel containing purified vicilin is shown at the extreme right

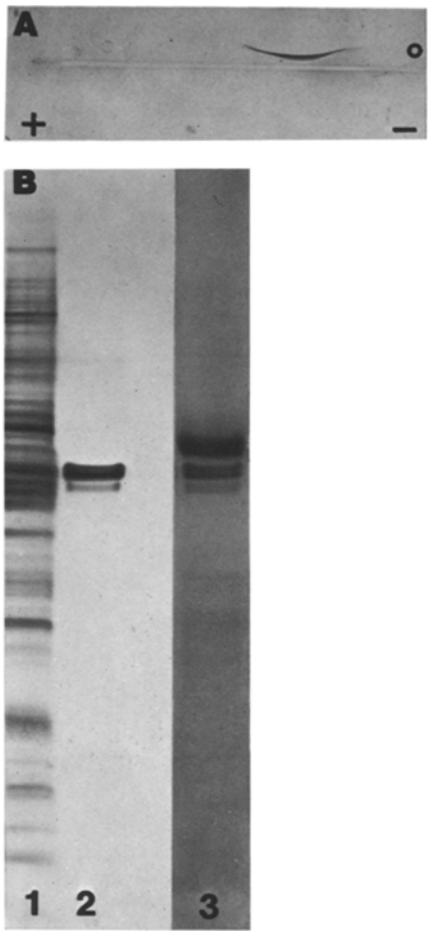

Fig. 9. A Immunoelectrophoresis of vicilin and vicilin antiserum. A sample of vicilin was put in the circular well and subjected to electrophoresis for 6 h. Antiserum was placed in the long slit and diffusion allowed to proceed overnight. A single precipitin line is visible. **B** Precipitation of vicilin polypeptides from a homogenate of cotyledons labeled in-vivo with [^{35}S]methionine. Cotyledons (8) were obtained 14 d after flowering and incubated with 125 μCi of [^{35}S]methionine for 6 h at room temperature. The tissue was homogenized in 20 mM sodium borate pH 9.0, and a cleared homogenate used for precipitation of vicilin with IgG and heat-killed *Staphylococcus aureus* cells. The polypeptides present in the total homogenate and in the precipitate were separated by SDS-polyacrylamide gel electrophoresis and visualized by autofluorography. Lane 1: total homogenate; note that vicilin is not heavily labeled 14 d after flowering. Lane 2: immunoprecipitate of the total homogenate treated with vicilin IgG and *Staphylococcus aureus* cells. Lane 3: photograph of lane 2 after staining with Coomassie Brilliant Blue. Note the prominent band caused by the presence of the heavy chain of IgG

tional smaller polypeptides made by membrane-bound polysomes, but never did we get precipitation of polypeptides made by free polysomes.

In Vitro Synthesis of Phytohemagglutinin

The same techniques which were employed to demonstrate that the polypeptides of vicilin made by membrane-bound polysomes were used to find out if these polysomes also synthesized PHA. An antiserum was prepared against PHA and the antiserum gave a strong precipitin line when tested against PHA in a two-dimensional double-diffusion test (data not shown). When this antiserum was used to precipitate in-vitro synthesized polypeptides, no radioactive polypeptides bound to the heat-killed *Staphylococus au-*

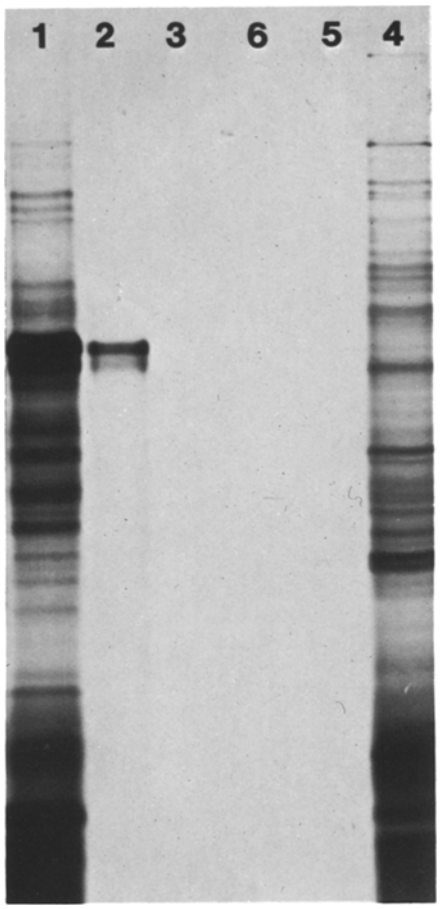

Fig. 10. Immunoprecipitation of in-vitro synthesized polypeptides with vicilin and PHA antisera and autofluorography of these polypeptides fractionated on SDS-polyacrylamide gels. The polypeptides released by free and membrane-bound polysomes were treated with antiserum and the antibody-antigen complex separated from the other radioactive polypeptides by precipitation with heat-killed *Staphylococcus aureus* cells as described in methods. Lane 1: polypeptides made by membrane-bound polysomes and released in the medium. Lane 2: polypeptides precipitated with vicilin IgG and heat-killed *Staphylococcus aureus* cells from polypeptides released by membrane-bound polysomes. Lane 3: polypeptides precipitated with PHA antiserum and heat-killed *Staphylococcus aureus* cells from polypeptides released by membrane-bound polysomes. Lanes 4, 5 and 6: same as Lanes 3, 2 and 1, respectively, but representing polypeptides made by free polysomes

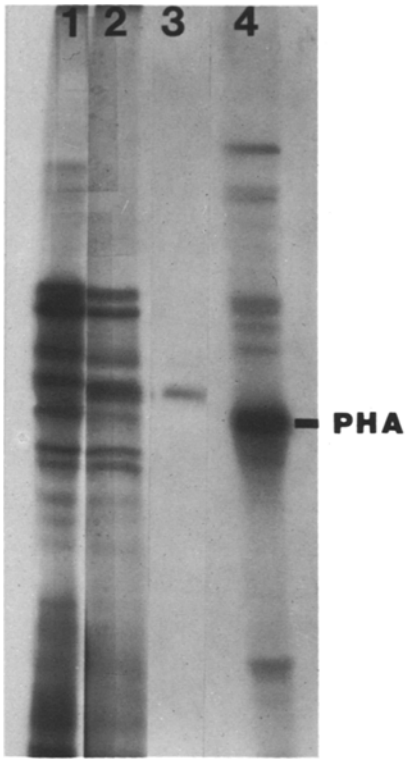

Fig. 11. Autofluorography of polypeptides fractionated on SDS-polacrylamide gels after elution from a thyroglobulin affinity column. Lane 1: total polypeptides made by membrane-bound polyribosomes. Lane 2: polypeptides which did not bind to the column. Lane 3: polypeptides which bound to the column and were eluted at pH 3.0. Lane 4: partially purified PHA (peak 1 from DEAE cellulose column) from in-vivo labeled cotyledons

reus cells (see lanes 3 and 6, Fig. 10). A different method was therefore used to separate the in vitro synthesized PHA from all other polypeptides. Polypeptides made in vitro were fractionated on a thyroglobulin affinity column which was eluted at pH 3.0 to remove the bound PHA or PHA-like polypeptides. The eluted polypeptides were concentrated by freeze-drying, separated by SDS-polyacrylamied gel electrophoresis, and visualized by autofluorography (Fig. 11). A small proportion (5%) of the total radioactivity was bound to the column when the products of free polysomes were loaded on the affinity column, but no distinct band was formed when the eluted material was subjected to electrophoresis. A similar proportion of the total radioactivity was bound to the column when the products of membrane-bound polysomes were loaded. When the bound polypeptides were eluted and subjected to electrophoresis one major radioactive polypeptide was found (Fig. 11, lane 3) which was slightly larger than the polypeptides of PHA (Fig. 11, lane 3) which was slightly larger than the polypeptides of PHA (Fig. 11, lane 4; the two polypeptides of PHA were not separated).

Changes in the Endoplasmic Reticulum during Cotyledon Expansion

A number of ER-parameters were measured at different stages of cotyledon development to find out how the abundance of the ER changed during bean maturation. The effect of cotyledon age on NADH-dependent cytochrome-c reductase, lipid phosphate, membrane-associated RNA and translational activity of

Table 3. Changes in ER-associated parameters during development of bean cotyledons. Seeds were harvested at different times, weighed and homogenized in extraction buffer (with or without EDTA and $MgCl_2$). Lipid-phosphate was determined on the membranes which sediment through 20% sucrose, but not through 33% (w/w) in a medium containing EDTA. NADH-cytochrome c reductase activity was determined on a total membrane fraction sedimented at $30000 \times g$ for 20 min, and RNA and translational activity were measured on the polysomes isolated from such a total membrane fraction

Age (d after flowering)	Fresh weight (mg/cot[a])	NADH-Cyt-c reductase (ΔA550/min/cot)	Lipid-PO_4 (μmol/cot)	RNA (μg/cot)	Translational activity (cpm $\times 10^{-3}$/cot)
16	75	0.60	0.127	10.1	57
19	104	0.87	0.254	22.0	129
23	169	2.32	0.387	33.8	245
31	205	3.92	0.682	22.5	143

[a] cot = cotyledon

membrane-bound polysomes, all expressed per cotyledon, are shown in Table 3. The activity of the ER-marker enzyme NADH-cytochrome-c reductase increased 6-fold between the 16th and the 31st days after flowering. Lipid phosphate in the microsomal fraction on top of the 33% sucrose layer increased 5-fold during the same period.

RNA content and translational activity of ER-associated polysomes were determined after separating membrane-bound polysomes. The RNA present in the pellet of polysomes, sedimented after dissolving the membranes with Triton X-100, increased 3.35-fold between 16 and 23 d after flowering, and then declined again. Similarly, the translational activity associated with these polysomes increased 4.2-fold during the same time period and then declined again. Taken together these results indicate that the rough ER proliferates at the time of reserve-protein deposition.

Discussion

In Vivo Synthesis of Vicilin and PHA

This study confirms and extends a recently published study by Sun et al. (1978) who showed that the accumulation of vicilin occurs primarily during the 3rd and 4th weeks of seed development. While we did not attempt to pinpoint the onset of vicilin synthesis, we observed that the polypeptides of vicilin became labeled as early as 12 d after flowering. The biosynthetic period of PHA coincides with that of vicilin.

During the 4-h labeling period of the excised cotyledons 40% of the radioactivity accumulated in vicilin and 10% in PHA. When these figures are compared with the known levels of these proteins in the cotyledons-50% for vicilin and 10% for PHA (Bollini and Chrispeels, 1978) – it appears that the processes of protein synthesis were not unduly disturbed during the labeling period.

Properties of Free and Membrane-Bound Polysomes

The free and membrane-bound polysomes appeared to have similar properties. The optimal Mg^{2+} and K^+ concentrations for protein synthesis were identical, synthesis was limited to completion of already initiated polypeptides, and most of the polypeptides were not released from the polysomes. Such observations indicate that the two classes of polysomes cannot be differentiated on the basis of their "functional" properties.

We observed a ratio of 1:2 for free polysomes to membrane-bound polysomes. The same ratio was observed whether we used A_{260} or incorporation activity of the isolated polysomes. Evans et al. (1979) observed the same ratio in developing pea cotyledons. Sun et al. (1978) reported a ratio of 18:1 in developing bean (*P vulgaris*) cotyledons.

Characterization of the Polypeptides Synthesized In Vitro

In the last few years polysomes active in the biosynthesis of polypeptides have been isolated from the reserve tissues of the seeds of many species including the common bean (Sun et al., 1975, 1978), soybean (Beachy et al., 1978), pea (Higgins, and Spencer, 1977; Evans et al., 1979), corn (Larkins and Dalby, 1975; Burr and Burr, 1976), oats (Luthe and Peterson, 1977) and barley (Fox et al., 1976). Different methods have been used to characterize the products of in vitro synthesis and to determine that reserve polypeptides had been synthesized including similarity in size, immunological precipitation and peptide mapping.

Our characterization of vicilin as one of the products of in vitro protein synthesis is based on the size of the polypeptides in SDS polyacrylamide gels and the use of a vicilin antiserum. The polypeptides

synthesized in vitro were usually slightly smaller, but similar in number, when compared with in vivo synthesized polypeptides. The smaller size can readily be accounted for by the lack of the carbohydrate moiety, the molecular weight of which is around 2000 (Ericson and Delmer, 1978). We found no evidence for the existence of precursors which might indicate the presence of a leader sequence at the N-terminal end of the vicilin polypeptides. Our results are in agreement with those of Sun et al. (1978) who also observed that the in-vitro made polypeptides of vicilin are slightly smaller than those made in vivo.

The specificity of the vicilin antiserum was checked in two ways: by immunoelectrophoresis and by precipitation of radioactive vicilin from an extract of cotyledons labeled in vivo with [^{35}-S]methionine. The results showed that the antigen was pure and that the antiserum specifically interacted with the polypeptides of vicilin. The antiserum and the monospecific IgG molecules purified by affinity chromatography precipitated radioactive in vitro synthesized polypeptides similar in size to the polypeptides of vicilin. Additional, smaller polypeptides were precipitated in some experiments when the precipitation had gone further towards completion. These smaller polypeptides may represent early termination products of the vicilin polypeptides which nevertheless bear the same antigenic sites as the large molecules. Higgins and Spencer (1977) also found that the polysomes isolated from pea cotyledons made numerous smaller polypeptides, the peptide-maps of which were similar to the peptide maps of the reserve proteins.

None of the heavily labeled, in-vitro made polypeptides was similar in size to the polypeptides of PHA. An antiserum against PHA did not precipitate any in-vitro synthesized polypeptides although it gave a strong precipitation line when tested against PHA in an Ouchterlony test. Others have also observed that antibodies against glycoproteins do not always precipitate the in-vitro synthesized polypeptides (Higgins and Spencer, 1978). This would be expected if the polysaccharide moiety was the major antigenic site of the protein. We, therefore, relied on the binding of PHA to thyroglobulin to find out if the polysomes synthesized PHA polypeptides. Using a thyroglobulin affinity column we found that a single polypeptide, slightly larger in size than the two polypeptides of PHA, was synthesized by the membrane-bound polysomes. Whether this polypeptide is related to one or both of the PHA polypeptides remains to be demonstrated.

Characteristics of the Endoplasmic Reticulum

ER has been isolated from a number of plant tissues and its density, as determined by sedimentation on sucrose gradients, has been shown to depend on the isolation medium. The presence of EDTA in the medium removes the ribosomes and decreases the density of the ER (Lord et al., 1973). Whether this shift is entirely a consequence of the removal of ribosomes or also of the removal of peripheral membrane proteins remains to be demonstrated. The observed density of the ER membranes varies depending on the tissue and on ribosome density (for references see Philipp et al., 1976).

The data presented here show that several ER parameters increased 4- to 6-fold when the fresh weight of the cotyledons increased from 75 to 250 mg. It is of interest to note that NADH-cytochrome-c reductase and lipid phosphate continued to increase up to the end while ER-associated polysomes reached a maximum and declined later on. This decline in membrane-bound polysomes may be related to the decrease in vicilin synthesis which accompanies the final stages of bean maturation. Beevers and Poulson (1972) demonstrated that the final stage of cotyledon development is characterized by the limited availability of mRNA for polysome formation and protein synthesis.

The observed increase in the rough ER during cotyledon expansion and reserve protein deposition confirms the early qualitative observations of Öpik (1968) and the morphometric measurements of Briarty (1973). Briarty found that the volume occupied by the rough ER in the cotyledons of *Vicia faba* increased from 1.5 to 18% of the cytoplasm during the time period when the fresh weight of the cotyledons increased from 40 to 120 mg.

Rough ER as the Site of Reserve-Protein Synthesis

Several lines of evidence indicate that the rough ER is the most likely site of reserve protein synthesis in cotyledons of legumes. Using autoradiography Bailey et al. (1970) demonstrated that the rough ER is a major site of amino-acid incorporation and that proteins made by ER-bound polysomes can be chased to the protein bodies. While they did not positively identify the proteins which were being synthesized on the rough ER as reserve proteins, they suggested that the rough ER is the site of reserve-protein synthesis.

The second line of evidence derives from the biosynthesis of the carbohydrate moiety of the reserve proteins. Reserve proteins are glycoproteins with a small polysaccharide moiety consisting of N-acetyl glucosamine and mannose residues (Ericson and Chrispeels, 1976; Ericson and Delmer, 1978). The biosynthesis of the carbohydrate moiety is carried out by

glycosyl transferases and the attachment of this moiety to the polypeptide is mediated by a lipid carrier molecule (Ericson and Delmer, 1977; 1978). Recent experiments by Nagahashi and Beevers (1978) show that the glycosyl transferases for mannose and N-acetyl glucosamine are associated with the membranes of the ER.

Attempts to demonstrate directly that polysomes which synthesize polypeptides of reserve proteins are uniquely associated with the ER have not been unequivocally successful. In the endosperm of developing corn and barley seeds most of the prolamine synthesis is carried out by membrane-bound polysomes (Fox et al., 1976; Burr and Burr, 1976; Larkins and Dalby, 1975) although there is considerable controversy about the nature of the membranes which bind the polysomes. Larkins and Hurkman (1978) claim that the ER is the site of zein synthesis in corn endosperm while Burr and Burr (1976) contend that the polysomes are directly attached to the protein body membranes. ER and protein-body membranes are known to be continuous in corn endosperm (Khoo and Wolf, 1970; Larkins and Hurkman, 1978).

The experiments with developing legume cotyledons were much less clear-cut and indicated a lack of qualitative difference between the polypeptides synthesized by free and membrane-bound polysomes (Sun et al., 1978; Beachy et al., 1978; Evans et al., 1979). Experiments by Sun et al. (1978) indicating that 95% of the polysomes were not attached to membranes, but did synthesize vicilin polypeptides led to the conclusion that most of the reserve proteins were synthesized on free polysomes. The results presented in this paper are in agreement with results obtained with developing corn endosperm and support the conclusion that membrane-bound polysomes are the exclusive site of reserve-protein synthesis. In legume cotyledons there is not continuity between the ER and the protein-body membrane and the latter does not appear to have polysomes associated with it. The sucrose-density gradient experiments presented here support the conclusion that the membranes which bind the polysomes are part of the ER. The demonstration that the biosynthesis of polypeptides of a protein which is sequestered in a membrane-limited organelle is carried out by polysomes bound to the ER confirms the generally accepted ideas concerning the spatial differentiation of protein synthesis in eukaryotic cells (Palade, 1975; Sabatini and Kreibich, 1976).

This work was carried out while R.B. was on leave from the Laboratorio Biosintesi Vegatali nelle Piante di Interesse Agrario. Financial support for this work came from the Cosiglio Nazionale della Ricerche of Italy in the form of a fellowship to R.B., and from the National Science Foundation in the form of research grants to M.J.C. The isolation of the monospecific antibodies from the vicilin antiserum was done by G. Hahn in our laboratory. S.H. Howell gave us advice on many aspects of in-vitro protein synthesis. Bobbie Bradford provided technical assistance, and E. Herman made the electron micrograph of the rough ER.

References

Bailey, C.J., Cobb, A., Boulter, D.: A cotyledon slice system for the electron autoradiographic study of the synthesis and intracellular transport of the seed storage protein of *Vicia faba*. Planta **95**, 103–118 (1970)

Barker, R.D.J., Derbyshire, E., Yarwood, A., Boulter, D.: Purification and characterization of the major storage proteins of *Phaseolus vulgaris* seeds, and their intracellular and cotyledonary distribution. Phytochemistry **15**, 751–757 (1976)

Baumgartner, B., Tokuyasu, K.T., Chrispeels, M.J.: Localization of vicilin peptidohydrolase in the cotyledons of mung bean seedlings of immunofluorescence microscopy. J. Cell. Biol. **79**, 10–19 (1978)

Beachy, R.N., Thompson, J.F., Madison, J.T.: Isolation of polyribosomes and messenger RNA active in in-vitro synthesis of soybean seed proteins. Plant Physiol. **61**, 139–144 (1978)

Beevers, L., Poulson, R.: Protein synthesis in cotyledons of *Pisum sativum* L.I. Changes in cell-free amino acid incorporation capacity during seed development and maturation. Plant Physiol. **49**, 476–491 (1972)

Bollini, R., Chrispeels, M.J.: Characterization and subcellular localization of vicilin and phytohemagglutinin, the two major reserve proteins of *Phaseolus vulgaris* L. Planta **142**, 291–298 (1978)

Bonner, W.M., Laskey, R.A.: A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**, 83–88 (1974)

Briarty, L.G.: Stereology in seed development studies: some preliminary work. Caryologia **25**, Suppl., 289–301 (1973)

Burr, B., Burr, F.A.: Zein synthesis in maize endosperm by polyribosomes attached to protein bodies. Proc. Natl. Acad. Sci. USA **73**, 515–519 (1976)

Ericson, M.C., Chrispeels, M.J.: The carbohydrate moiety of mung bean vicilin. Aust. J. Plant. Physiol. **3**, 763–769 (1976)

Ericson, M.C., Delmer, D.P.: Glycoprotein synthesis in plants. I. Role of lipid intermediates. Plant Physiol. **59**, 341–347 (1977)

Ericson, M.E., Delmer, D.P.: Glycoprotein synthesis in plants. III. Interaction between UDP-N-acetylglucosamine and GDP-mannose as substrates. Plant Physiol. **61**, 819–823 (1978)

Evans, M., Croy, R.R.D., Hutchinson, P., Boulter, D., Payne, P.I., Gordon, M.E.: Cell free synthesis of some storage protein subunits by polyribosomes and RNA isolated from developing seeds of pea (*Pisum sativum* L.). Planta **144**, 455–462 (1979)

Felsted, R.L., Leavitt, R.D., Bachur, N.R.: Purification of the phytohemagglutinin family of proteins from red kidney beans (*Phaseolus vulgaris*) by affinity chromatography. Biochim. Biophys. Acta **405**, 72–81 (1975)

Fox, J.E., Pratt, H.M., Shewry, P.R., Miflin, B.J.: The in-vitro synthesis of hordeins with polysomes from normal and high lysine varieties of barley. In: Acides Nucléiques et Synthèse des Protéines chez les Végétaux, pp. 501–509, Bogorad, L., Weil, J.H., eds. Colloq. Intern. CNRS No. 261, Paris 1976

Gilkes, N.R., Herman, E.M., Chrispeels, M.J.: Rapid degradation and limited synthesis of phospholipids in the cotyledons of mung bean seedlings. Plant Physiol. (in press)

Grabar, P., Williams, C.A.: Methode permettant l'étude conjugée des propriétés électrophoretiques et immunochimiques d'un me-

lange de proteines; application au sérum sanguin. Biochim. Biophys. Acta **10**, 193–194 (1955)
Higgins, T.J.V., Spencer, D.: Cell-free synthesis of pea seed proteins. Plant Physiol. **60**, 655–661 (1977)
Kessler, S.W.: Rapid isolation of antigens from cells with a staphylococcal protein-A-antibody absorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. **115**, 1617–1624 (1975)
Khoo, V., Wolf, J.J.: Origin and development of protein granules in maize endosperm. Am. J. Bot. **57**, 1042–1050 (1970)
Laemmli, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**, 680–685 (1970)
Larkins, B.A., Dalby, A.: In-vitro synthesis of zein-like protein by maize polyribosomes. Biochem. Biophys. Res. Commun. **66**, 1048–1054 (1975)
Larkins, B.A., Davies, E.: Polysomes from peas. V. An attempt to characterize the total free and membrane-bound polysomal population. Plant Physiol. **55**, 749–756 (1975)
Larkins, B.A., Hurkman, W.J.: Synthesis and deposition of zein in protein bodies of maize endosperm. Plant Physiol. **62**, 256–263 (1978)
Leavitt, R.D., Felsted, R.D., Bachur, N.R.: Biological and biochemical properties of *Phaseolus vulgaris* isolectins. J. Biol. Chem. **252**, 2961–2966 (1977)
Lord, J.M., Kagawa, T., Moore, T.S., Beevers, H.: Endoplasmic reticulum as the site of lecithin formation in castor bean endosperm. J. Cell Biol. **68**, 11–29 (1973)
Lowry, D.M., Rosebrough, M.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275 (1951)
Luthe, D.S., Peterson, D.M.: Cell free synthesis of globulin by developing oat (*Avena sativa* L.) seeds. Plant Physiol. **59**, 836–841 (1977)
Manen, J.F., Miège, M.N.: Purification et caractèrisation des lectines isolées dans les albumines et les globulines de *Phaseolus vulgaris*. Physiol. Vég. **15**, 163–173 (1977)
McLeester, R.C., Hall, T.C., Sun, S.M., Bliss, F.A.: Comparison of globulin proteins from *Phaseolus vulgaris* with those from Vicia faba. Phytochemistry **12**, 85–93 (1973)
Nagahashi, J., Beevers, L.: Subcellular localization of glycosyltransferases involved in glycoprotein biosynthesis in the cotyledons of *Pisum sativum* L. Plant Physiol. **61**, 451–459 (1978)
Öpik, H.: Development of cotyledon cell structure in ripening *Phaseolus vulgaris* seeds. J. Exp. Bot. **19**, 64–76 (1968)
Palade, G.E.: Intracellular aspects of the process of protein synthesis. Science **189**, 347–358 (1975)
Phillipp, E.-I., Franke, W.W., Keenan, T.W., Stadler, J., Jarasch, E.-D.: Characterization of nuclear membranes and endoplasmic reticulum isolated from plant tissue. J. Cell Biol. **68**, 11–29 (1976)
Pusztai, A., Watt, W.B.: Glycoprotein II. The isolation and characterization of a major antigenic and non-haemaglutinating glycoprotein from *Phaseolus vulgaris*. Biochim. Biophys. Acta **207**, 413–431 (1970)
Roman, R., Brooker, J.D., Seal, S.N., Marcus, A.: Inhibition of the transition of a 40S ribosome-Met-tRNA$_i^{met}$ complex to an 80S ribosome-Met-tRNA$_i^{met}$ complex by 7-methylguanosine-5′-phosphate. Nature **260**, 359–360 (1976)
Sabatini, D.D., Kreibich, G.L.: Functional specialization of membrane-bound ribosomes in eukaryotic cells. In: The Enzymes of Biological Membranes, vol. 2, pp. 531–579, Martonosi, A., ed., New York: Plenum Publ. Corp. 1976
Sun, S.M., Buchbinder, B.U., Hall, T.C.: Cell-free synthesis of the major storage protein of the bean, *Phaseolus vulgaris* L. Plant Physiol. **56**, 780–785 (1975)
Sun, S.M., McLeester, R.C., Bliss, F.A., Hall, T.C.: Reversible and irreversible dissociation of globulins from *Phaseolus vulgaris* seeds. J. Biol. Chem. **249**, 2218–2221 (1974)
Sun, S.M., Mutschler, M.A., Bliss, F.A., Hall, T.C.: Protein synthesis and accumulation in bean cotyledons during growth. Plant Physiol. **61**, 918–923 (1978)

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Note added in proof: While this paper was in press Püchel et al. (1979) presented evidence that in developing cotyledons of the field bean (*Vicia faba* L.) synthesis of the reserve proteins vicilin and legumin is carried out preferentially by membrane-bound polysomes. [Püchel, M., Müntz, K., Parthier, B., Aurich, O., Bassüner, R., Manteufel, R., Schmidt, P.: RNA metabolism and membrane-bound polysomes in relation to globin biosynthesis in cotyledons of developing field beans (*Vicia faba* L.). Eur. J. Biochem. **96**, 321–329 (1979)].