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The Golgi apparatus mediates the transport of phytohemagglutinin to the protein bodies in bean cotyledons

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Abstract. When developing cotyledons of *Phaseolus vulgaris* L. were labeled with [^{3}H]fucose, fucose-labeled phytohemagglutinin (PHA) was found in organelles with average densities of 1.13 g cm^{-3} and 1.22 g cm^{-3}. The position of these organelles on isopycnic sucrose gradients was independent of the presence of $MgCl_2$ and ethylenediaminetetraacetate in the media, indicating that the fucose-labeled PHA was not associated with the rough endoplasmic reticulum (ER). The organelles with a density of 1.13 g cm^{-3} were identified as membranes of the Golgi apparatus on the basis of the similarity of their sedimentation properties and those of the Golgi marker enzyme, inosine diphosphatase, in both isopycnic and rate-zonal sucrose gradients. The organelles with a density of 1.22 g cm^{-3} were identified as small (0.1–0.4 μm), electron-dense vesicles with a protein content similar to that of the protein bodies. Pulse-chase experiments with [^{3}H]fucose indicated that fucose-labeled PHA first appeared in the Golgi-apparatus-derived membranes and later in the dense vesicles. Fucose-labeled PHA chased out of the Golgi apparatus first, then out of the dense vesicles, and accumulated in the soluble portion of the homogenate which contained the contents of the broken protein bodies. Fucose-labeled PHA chased out of the two types of organelles with a $t_{1/2}$ of 20–30 min, a rate three to four times faster than newly synthesized PHA chases out of the bulk of the ER (Chrispeels, M.J., Bollini, R., 1982, Plant Physiol. **70**, 1425–1428). This result indicates that the Golgi apparatus is a much smaller compartment than the ER in the storage parenchyma cells. The sodium ionophore, monensin, which interferes with the function of the Golgi apparatus of animal cells, blocks the biosynthesis and–or transport of fucose- and galactose-labeled macromolecules to the cotyledon cell walls. Monensin also blocks the transport of labeled PHA out of the Golgi apparatus and into the protein bodies. These results provide the first biochemical evidence that a specific storage protein which accumulates in seeds is modified in, and passes through, the Golgi apparatus on its way to the protein bodies.

Key words: Endoplasmic reticulum – Golgi apparatus – Lectin – *Phaseolus* (transport of protein) – Phytohemagglutinin – Protein body – Storage protein – Transport (protein).

Abbreviations: endo-H = endo-β-N-acetylglucosaminidase; EDTA = ethylenediaminetetraacetate; ER = endoplasmic reticulum; IDPase = inosinediphosphatase; PHA = phytohemagglutinin; SDS-PAGE = sodium dodecylsulfate polyacrylamide-gel electrophoresis

Introduction

Bean seeds synthesise and accumulate large amounts of storage protein in the course of their development. In the mature seed these proteins accumulate within the cotyledon parenchyma cells in membrane-bounded organelles called protein bodies, which measure 1–5 μm in diameter. The major reserve proteins of the bean *Phaseolus vulgaris* are phaseolin and phytohemagglutinin (Barker et al. 1976; Bollini and Chrispeels 1978; Sun et al. 1978) which together make up 50–60% of the total protein of the mature seed. Phaseolin and phytohemagglutinin (PHA) are both glycoproteins containing mannose and glucosamine. The oligosaccharide side-chains of phaseolin are of the high-mannose type and are sensitive to digestion by endo-β-N-acetylglucosaminidase (endo-H) (Davies and Delmer 1981; Bollini et al. 1982), while the side-chains of PHA are only partially sensitive to digestion by endo-H (Chrispeels 1983)

and contain several other neutral sugars including fucose (Jaffé et al. 1974; Mialonier et al. 1973).

There is now a considerable amount of evidence that the storage proteins of leguminous seeds are synthesized exclusively on polysomes attached to the endoplasmic reticulum (ER) (Bollini and Chrispeels 1979), are inserted into the lumen of the ER (Higgins and Spencer 1981; Hurkman and Beevers 1982) and pass through the ER on their way to the protein bodies (Bollini et al. 1982; Chrispeels et al. 1982; Chrispeels and Bollini 1982). Biosynthesis of the glycosylated reserve proteins includes the cotranslational attachment of a high-mannose oligosaccharide via a dolicholphosphate intermediate (Sengupta et al. 1981; Bollini et al. 1983), a step which appears to be analogous in plant cells and animal cells (Foresee and Elbein 1975; Beevers and Mense 1977; Delmer et al. 1978; Staneloni et al. 1980). There is esentially no information on the transport of reserve proteins from the ER to the protein bodies. Some authors have postulated that the proteins pass through the Golgi apparatus, but arguments for and against this proposal are based entirely on ultrastructural observations (Bailey et al. 1970; Dieckert and Dieckert 1976; Harris 1979).

The recent finding that incubation of developing cotyledons of *Phaseolus vulgaris* in the presence of [^{3}H]fucose leads to the rapid incorporation of this sugar into the carbohydrate moiety of PHA and that this carbohydrate moiety becomes partially resistant to digestion by endo-H (Chrispeels 1983) has opened the way for a direct test of the hypothesis that the Golgi apparatus is involved in the modification of the carbohydrate moiety of PHA and the transport of reserve proteins to the protein bodies. Indeed, in animal cells, the attachment of fucose and the modification of oligosaccharide side-chains to make them endo-H resistant occurs in the Golgi apparatus (for review see Hubbard and Ivatt 1981).

In this paper we present evidence that the attachment of fucose to PHA occurs in the Golgi apparatus of the storage parenchyma cells. Pulse-chase experiments indicate that fucosylated PHA passes through the Golgi apparatus on its way to the protein bodies. The transport of PHA and that of phaseolin in inhibited by the ionophore monensin, a drug with interferes with the normal functioning of the Golgi apparatus of animal cells.

Materials and methods

Materials. Plants of *Phaseolus vulgaris* L. cv. Greensleeves (seeds from Burpee Seed Co., Riverside, Cal., USA) were grown as described in Bollini and Chrispeels (1979). Experiments were carried out with developing cotyledons weighing 175–225 mg, when the accumulation of PHA and phaseolin is rapid. Organic chemicals including monensin were purchased from Sigma Chemical Co. (St. Louis, Mo., USA) unless otherwise indicated. Radiochemicals were purchased from Amersham Co. (Arlington Heights, Ill., USA): L-[5,6-^{3}H]fucose (950 GBq mmol^{-1}), D-[6-^{3}H]galactose (999 GBq mmol^{-1}), D-[6-^{3}H]glucosamine hydrochloride (918 GBq mmol^{-1}), ^{3}H-amino acids (average specific activity 900 GBq mmol^{-1}) and ^{14}C-amino acids (6–20 GBq mmol^{-1}).

Radioactive labeling. Radioactive labeling was carried out with excised cotyledons as described (Spencer et al. 1980). In some experiments a nutrient medium containing 4% (w/v) sucrose, 10.7 g l^{-1} of asparagine and the major mineral nutrients (Millerd et al. 1975) was used. The labeled tissue was collected by cutting a thin slice from the cotyledon with a razor blade. The remainder of the cotyledon was discarded. The tissue was homogenized in 100 mM 2-amino-2(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 7.8, containing either 1 mM sodium ethylenediaminetetraacetate (EDTA) (medium A) or 2 mM $MgCl_2$ (medium B) and 12% (w/w) sucrose. The cell walls and debris were removed by centrifugation at 1000 *g* for 5 min and the supernatants used for fractionation of subcellular organelles.

Fractionation of subcellular organelles. In many experiments the organelles were first separated from the soluble molecules by means of Sepharose 4B (Pharmacia, Uppsala, Sweden) columns as described (Van der Wilden et al. 1980). Subsequent fractionations were on continuous or discontinuous sucrose gradients. The discontinuous gradients consisted of a layer (8 ml) of 16% (w/w) sucrose over a layer (1 ml) of 35% and – or 54% (w/w) sucrose always in the same medium (A or B). These gradients were centrifuged for 90 min at 35,000 rpm (150,000 *g*) in the SW41 rotor of a Spinco-Beckman (Palo Alto, Cal., USA) model L3–50 centrifuge. After centrifugation the Golgi-apparatus-rich fraction is on top of the 35% sucrose layer, and the dense-vesicle-rich fraction on top of the 54% sucrose. If only 54% (w/w) sucrose is used, all the organelles will be at the 16–54 interface. The continuous gradients were either 22% to 54% (w/w) sucrose, or 16% to 40% (w/w) sucrose on a 1-ml cushion containing 54% (w/w) sucrose. All sucrose solutions were made in either medium A or B. These gradients were centrifuged for 2 h at 150,000 *g*.

Isolation of PHA. The affinity procedure of Felsted et al. (1975) was used to separate PHA from all other cellular proteins. This procedure allows PHA to be purified from a crude homogenate in one step. The affinity gel consisted of porcine thyroglobulin covalently linked to Sepharose 4B. The affinity gel and the cellular fractions containing PHA were mixed together for 1 h in the presence of 1% Tween-20 (polyoxyethylene sorbitan monolaurate; Sigma) and 2 mM $MgCl_2$ in a small plastic column fitted with a fritted disk. The affinity gel (0.1–0.5 ml depending on the amount of material to be extracted) was then washed five times with 5 ml of phosphate-buffered saline (PBS) (10 mM KH_2PO_4-KOH, pH 7.4, and 15 mM NaCl) and five times with 5 ml of PBS containing 1 M NaCl. The PHA was eluted with 2 or 3 ml of 0.1 M glycine-HCl pH 3.0 containing 0.5 M NaCl. The eluted material contained the two polypeptides of PHA. Fluorographs of radioactive PHA recovered in this manner have been published (Chrispeels and Bollini 1982; Chrispeels 1983).

Determination of radioactivity. Aliquots of cellular fractions were mixed with an equal volume of 10% (w/w) trichloroacetic acid (TCA) and 3 ml of 5% (w/w) TCA. The mixtures were allowed to stand for 15 min at room temperature and the protein was collected by filtration on membrane filters (type HAWP 02500; Millipore Corp., Bedford, Mass., USA). The filters were washed with 5% TCA, dried, and the radioactivity was determined after immersion in a toluene-based liquid scintillation mixture.

Radioactivity in the cell walls was determined in the same way after washing the walls six times by resuspension and sedimentation (1000 *g* for 10 min) in NaH_2PO_4-NaOH buffer (10 mM, pH 7.4) containing 0.15 M NaCl and 1% (w/w) (Tween 20). Radioactivity taken up by the cells was determined by mixing a small aliquot of the homogenate with a water-miscible scintillant (3a70B; Research Products International, Elk Grove, Ill., USA).

Enzyme assays. Inosinediphosphatase (IDPase) was determined by mixing 100 µl of the gradient fractions with 400 µl of inosinediphosphate (1 mg ml^{-1}) dissolved in 100 mM Tris-HCl (pH 7.4) containing 3 mM $MgCl_2$. After a 2-h incubation at 37° C inorganic phosphate was determined by the method of Ohnishi et al. (1975). Under these conditions 0.7 µmol of inorganic phosphate gives an A_{600} of 1.0, and the release of 1 µmol h^{-1} is defined as one unit. NADH-cytochrome c reductase was assayed as described by Sottocasa et al. (1967).

Sodium-dodecylsulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and fluorography. Aliquots of protein fractions were precipitated with 1.5 volumes of methanol and 0.006 volumes of glacial acetic acid, and kept at −20° C for 12 h. The protein was washed with 2 ml of 90% (v/v) aqueous acetone, and subjected to analysis by SDS-PAGE as described in Bollini and Chrispeels (1978). Fluorographs were made as described by Bonner and Laskey (1974) using XAR-5 film (Eastman-Kodak, Rochester, N.Y. USA).

Electron microscopy. Cell organelles were recovered from sucrose gradients and mixed with an equal volume of a sucrose solution containing 4% (v/v) glutaraldehyde. The sucrose concentration in the fixative was 30%, 40% or 48% (w/w) for, in this order, the class-I particles, ER, and class-II particles. After allowing them to stand 30 min at 0° C the organelle fractions were further diluted with 12% (w/w) sucrose containing 2% (v/v) glutaraldehyde, and filtered under pressure on a membrane filter (GSWP 01300, pore size 0.22 µm; Millipore Corp.). The organelles and filters were post-fixed in 2% (w/v) osmium tetroxide in 25 mM NaH_2PO_4-NaOH buffer (pH 7.2) for 1 h at 0° C. The samples were dehydrated through a graded series of ethanol and propylene oxide and embedded in Spurr's resin (1969). The thin sections were stained with lead tartrate and uranyl acetate for 5 min each and examined in an electron microscope.

Results

Fucosylated PHA is associated with two classes of subcellular organelles. Earlier experiments showed that when intact bean cotyledons were exposed to a small amount of [^{3}H]fucose (185 kBq per cotyledon), the radioactivity was rapidly taken up by the tissue and incorporated into a variety of macromolecules associated with the cell walls, membranous organelles and the cytosol. Analysis of the proteins by SDS-PAGE followed by fluorography showed that PHA was the major fucosylated protein synthesized in the cotyledons. Phytohemagglutinin containing [^{3}H]fucose was present in the membranous organelles before it appeared in the soluble portion of a tissue homogenate. The soluble fraction of the homogenate contained not only the cytosolic proteins, but also the contents of most of the protein bodies. Labeled PHA did not appear in the soluble fraction until 20–30 min after the start of the incubation with [^{3}H]fucose (Chrispeels 1983). Isopycnic sucrose gradients were used to find out with which organelles the [^{3}H]fucose-labeled PHA was associated. The cotyledons were labeled simultaneously with ^{14}C-amino acids and [^{3}H]fucose for 1 h, the radioactive tissue was homogenized in a medium containing 1 mM EDTA (medium A) or 2 mM $MgCl_2$ (medium B), and the organelles were separated from the soluble molecules with Sepharose 4B columns. The organelles were then fractionated on linear 16 to 54% (w/w) sucrose gradients and the gradient fractions were assayed for IDPase, a Golgi marker (Dauwalder et al. 1969; Ray et al. 1969; Gardiner and Chrispeels 1975), for NADH-cytochrome c reductase, an ER marker, for total incorporated radioactivity, and for radioactivity incorporated into PHA (Fig. 1). The latter was determined after the affinity purification of PHA from every gradient fraction by means of thyroglobulin-Sepharose affinity gel. The results (Fig. 1 A, B) show that in the presence of EDTA or $MgCl_2$, IDPase banded at a density of 1.13 g cm^{-3}. The NADH-cytochrome c reductase, on the other hand, banded at a density of 1.125 g cm^{-3} when EDTA was present, and at 1.18 g cm^{-3} when $MgCl_2$ was present. This density shift of the ER marker enzyme has been shown to be the consequence of the presence or absence of ribosomes on the microsomal vesicles (Ray et al. 1976; Lord et al. 1973). Incorporation of ^{14}C-amino acids was most pronounced in organelles having a density of 1.135 g cm^{-3} in the presence of EDTA and 1.18 g cm^{-3} in the presence of $MgCl_2$ (Fig. 1 C, D). Thus most of the newly synthesized proteins were associated with organelles that behaved in the gradients in the same way as the ER marker enzyme. The slight discrepancy between the average density of the NADH-cytochrome c reductase and the newly synthesized proteins was also observed in pea cotyledons (Chrispeels et al. 1982) and may be the result of the presence of newly synthesized protein in a subset of all the ER-derived vesicles. The distribution of organelles containing [^{3}H]fucose-labeled macromolecules was quite different from that of the ^{14}C-

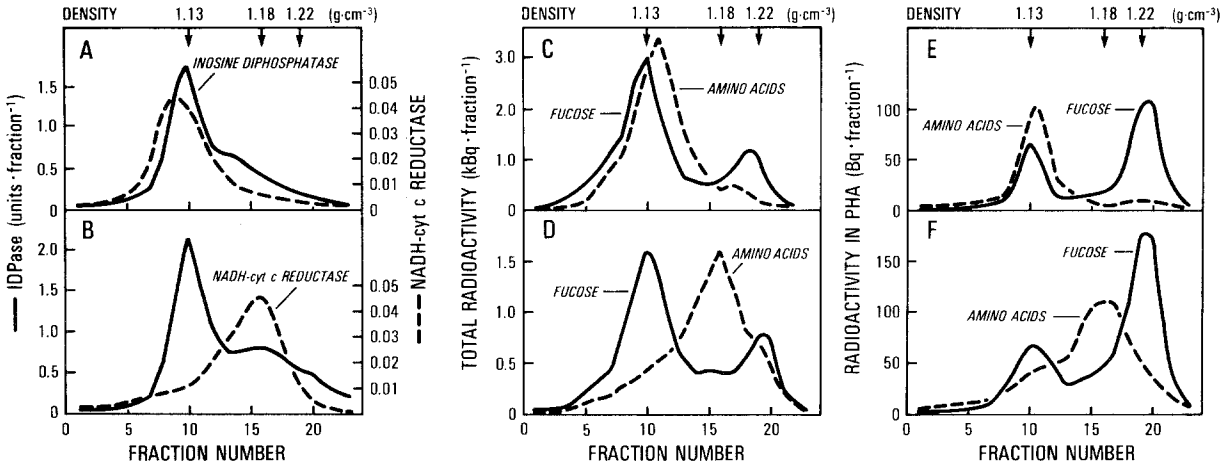

Fig. 1A–F. Fractionation of organelles containing newly synthesized PHA on isopycnic sucrose gradients after labeling the cotyledons of *P. vulgaris* with [^{3}H]fucose and ^{14}C-amino acids. Cotyledons (7 per treatment; 200 mg each) were labeled simultaneously with 185 kBq of [^{3}H]fucose and ^{14}C-amino acids for 1 h. The radioactive tissue was homogenised in medium A (EDTA) or B (2 mM $MgCl_2$) and all subsequent manipulations (Sepharose 4B chromatography, isopycnic sucrose gradients) done in the same media. Sucrose gradients (16 to 54% w/w, sucrose) were centrifuged at 150,000 *g* for 2 h. Fractions were assayed for IDPase and NADH-cyt c reductase activities (**A** = EDTA; **B** = $MgCl_2$) for incorporation into all macromolecules (**C** = EDTA; **D** = $MgCl_2$) and for incorporation into PHA purified by affinity chromatography (**E** = EDTA; **F** = $MgCl_2$)

amino-acid-labeled macromolecules and was the same whether $MgCl_2$ or EDTA was present in the media (Fig. 1C, D). There was a large peak of [^{3}H]fucose-labeled macromolecules at 1.13 g cm^{-3}, the location of the Golgi marker, and a smaller one at 1.22 g cm^{-3}. This finding is in agreement with the observation that fucosyltransferase activity is associated with the Golgi apparatus, rather than with the ER (Munro et al. 1975).

Incorporation of ^{14}C-amino acids and [^{3}H]fucose into PHA (Fig. 1E, F) followed a pattern similar to that found for incorporation into total macromolecules. Phytohemagglutinin labeled with ^{14}C-amino acids was found primarily in the rough ER as shown by the shift in density from 1.18 g cm^{-3} to 1.135 g cm^{-3} when $MgCl_2$ was omitted and EDTA was added to the medium. The [^{3}H]fucose-labeled PHA on the other hand was present in organelles with a density of 1.13 g cm^{-3} (hereafter called class-I particles) and 1.22 g cm^{-3} (class-II particles) in both media. Like other reserve proteins, PHA is synthesized on membrane-bound polysomes and is transiently associated with the rough ER (Chrispeels and Bollini 1982); the observations described above are consistent with the interpretation that fucose becomes attached to PHA after it leaves the rough ER and passes through some other compartment of the endo-membrane system.

Characterization of class-I and -II particles. A combination of rate-zonal and isopycnic gradients was used to examine further the sedimentation properties of the two classes of particles. Cotyledons were labeled for 1 h with [^{3}H]fucose and the radioactive tissue homogenized in medium A (EDTA). The cleared homogenate was passed through a Sepharose 4B column to isolate the organelles. The organelles were layered on a sucrose gradient consisting of an 8 ml continuous 20 to 30% (w/w) layer over a 1-ml layer of 54% (w/w) sucrose. The gradients were centrifuged at 25,000 rpm (75,000 *g*) for 30 min. Fractions were collected and assayed for IDPase and incorporation of [^{3}H]fucose into PHA (Fig. 2A). The results indicate the presence of IDPase and [^{3}H]fucose-labeled PHA in slowly sedimenting organelles (peak I) near the top of the gradient, and the presence of [^{3}H]fucose-labeled PHA in rapidly sedimenting organelles which were at the bottom of the gradient. These two organelle fractions were recovered, diluted to 20% sucrose, and layered on linear 22 to 54% (w/w) sucrose gradients which were centrifuged for 2 h at 35,000 rpm (150,000 *g*). These gradients were fractionated and the fractions assayed again for IDPase activity and [^{3}H]fucose-labeled PHA. The results (Fig. 2B) show that peak I contained organelles which banded at 1.13 g cm^{-3} at the same density as the IDPase, while peak II contained organelles which banded primarily at 1.22 g cm^{-3}. The nature of the shoulder on the main peak is not known. Together these results indicate that [^{3}H]fucose-labeled PHA was present in small and–or light (1.13 g cm^{-3}) organelles which comigrated

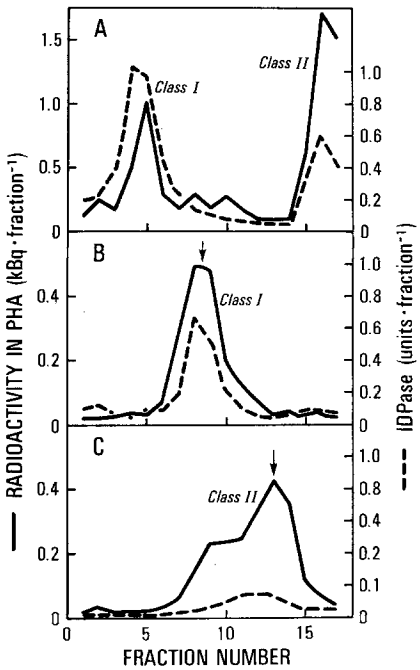

Fig. 2A–C. Fractionation of organelles isolated from cotyledons of *P. vulgaris* containing [^{3}H]fucose-labeled PHA and IDPase on rate-zonal and isopycnic sucrose gradients. Cotyledons (6) were labeled with [^{3}H]fucose (185 kBq each) for 1 h, and the radioactive tissue homogenized in medium A (EDTA). The homogenate was layered on a continuous 20 to 30% (w/w) sucrose gradient sitting on a 1 ml cushion of 54% (w/w) sucrose. The gradient was centrifuged for 30 min at 75,000 *g* and the fractions assayed for IDPase and radioactive PHA (**A**). Peaks I and II were collected, diluted to 20% (w/w) sucrose and loaded on continuous sucrose gradients (22 to 54%, w/w) which were centrifuged at 150,000 *g* for 2 h. The fractions were collected and again assayed for IDPase activity and radioactive PHA (**B** and **C** for peaks I and II, respectively)

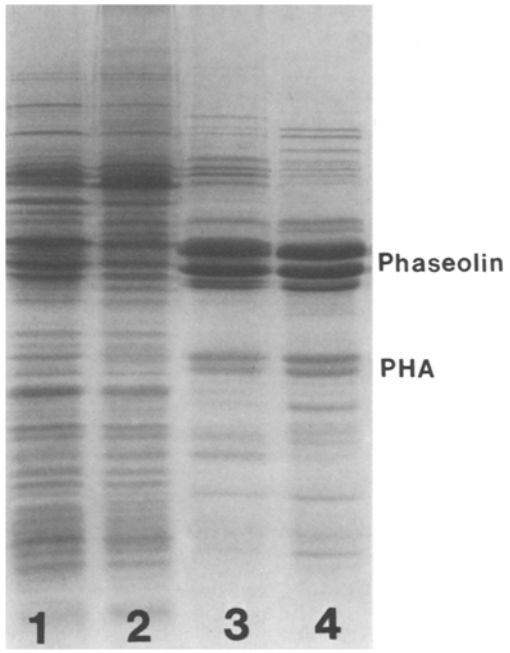

Fig. 3. Analysis by SDS-PAGE of the polypeptides present in the different subcellular fractions isolated from cotyledons of *P. vulgaris*. Cotyledons (2) were homogenized in medium B and the homogenate fractionated on a 16 to 54% (w/w) isopycnic sucrose gradient. Fractions corresponding to the soluble portion, class-I particles, ER and class-II particles were collected by hand. The polypeptides were fractionated by SDS-PAGE. Lane 1: ER corresponding to 0.25 of a cotyledon; lane 2: class-I particles corresponding to 0.66 of a cotyledon; lane 3: class-II particles corresponding to 0.66 of a cotyledon; lane 4: soluble corresponding to 0.012 of a cotyledon

with the Golgi marker enzyme IDPase, and in large and–or dense organelles.

The polypeptides present in different subcellular fractions were analyzed by SDS-PAGE. Cotyledons were homogenized in medium B (with Mg^{2+}) and the homogenate fractionated on a linear 16 to 54% (w/w) sucrose gradient. The soluble fraction, and the organelles corresponding to sucrose densities of 28 to 32% w/w) (class-I particles), 38 to 42% (w/w) (ER and mitochondria) and 46 to 50% (w/w) (class-II particles) were recovered. The results (Fig. 3) show that the polypeptides pattern of the class-I particles was rather similar to that of the ER (compare lanes 1 and 2). The polypeptides of phaseolin can be clearly distinguished in the ER and class-I particles and are among the most abundant polypeptides in these membranous organelles. This is also the case in pea cotyledons where the polypeptides of vicilin can be readily identified in a stained gel of the ER (Chrispeels et al. 1982). The polypeptides of PHA cannot be easily identified in lanes 1 and 2 because of the presence of many polypeptides with similar M_r in this region of the gel. The uncertainty is compounded by the observation that the polypeptides of PHA in the ER have a slightly lower mobility than the same polypeptides in the protein bodies (Chrispeels and Bollini 1982). In the soluble fraction (lane 4) the polypeptides of phaseolin and PHA are very abundant and readily identified. Their presence in the soluble fraction is the result of the breakage of the protein bodies during homogenization. It should be noted that the protein in lane 1 represent the ER from 0.25 of a cotyledon, while the protein in lane 4 represents the protein in 0.012 of a cotyledon. Thus the ER contains only a few percent of the storage proteins in the cotyledons. Phaseolin and PHA are very abundant in, and can be readily identified among the polypeptides present in class-II particles. With the excep-

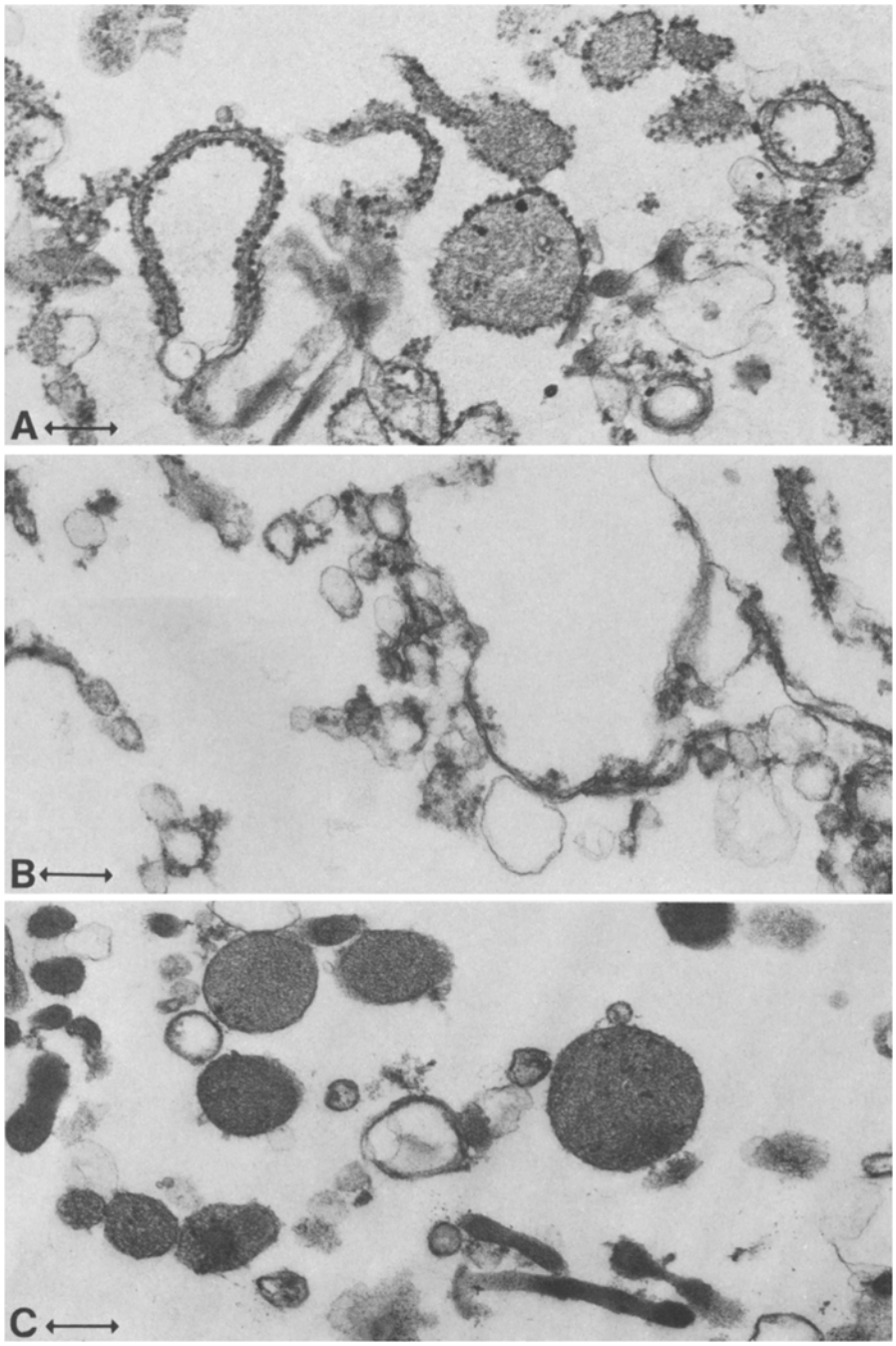

Fig. 4A–C. Electron micrographs of subcellular fractions isolated from *P. vulgaris* cotyledons. Cotyledons (4) were homogenized in medium B and the homogenates fractionated on a 16 to 54% (w/w) sucrose gradient. Organelle fractions corresponding in average sucrose densities of 1.13 (**B**), 1.19 (**A**) and 1.22 (**C**) were recovered, fixed with glutaraldehyde, collected on membrane filters and processed for transmission electron microscopy. **A** Rough-ER-rich fraction; **B** class-I-rich fraction; **C** class-II-rich fraction. Size marker = 0.2 μm

tion of the polypeptides of phaseolin and PHA, the polypeptide pattern of the class-II particles is not similar to that of the ER of the class-I particles.

Electron microscopy. Further characterization of the subcellular organelles was carried out by electron microscopy. The colyledons were homogenized, and the organelles separated on sucrose gradients using medium B (with $MgCl_2$) as described above for the analysis of polypeptide patterns. The organelles were fixed in glutaraldehyde, collected on membrane filters and further processed for transmission electron microscopy. Fractions A, B and C (Fig. 4) were recovered at average densities of 1.19, 1.13 and 1.22 g cm^{-3}, in the order named.

Fraction A (average density of 1.19 g cm^{-3}) contains mostly rough ER vesicles with an electron-dense matrix (Fig. 4A). The abundance of reserve protein in this fraction (see Fig. 3, lane 1) indicates that the electron-dense material is prob-

ably reserve protein. Since the phaseolin in this fraction has a different mobility from the phaseolin in the protein bodies (Bollini et al. 1982) the electron-dense material does not represent storage protein entrapped in these vesicles during homogenization, but represents storage protein on its way to the protein bodies.

Fraction B (average density of 1.13 g cm^{-3}) represents the class-I particles. It contains a mixture of rough-ER vesicles generally devoid of an electron-dense matrix, and aggregates of small and large membrane vesicles without attached ribosomes (Fig. 4B). While it is not possible to determine the identity of these membranes, it is likely that they are derived from the Golgi apparatus and represent inflated cisternae with their associated vesicles. The Golgi apparatus bands at the density at which these organelles were recovered, as indicated by the peak of IDP-ase in this region of the gradient (Fig. 1).

Fraction C (average density 1.22 g cm^{-3}) represents the class-II particles. It consists predominantly of vesicles with an electron-dense matrix (Fig. 4C). Some empty vesicles are also present. The particles measure 0.1–0.4 μm in diameter. Many are spherical, but some appear to be tubular. The larger vesicles resemble isolated protein bodies, but are much smaller (see, for example, Fig. 7B in Chrispeels et al. 1982). The abundance of reserve proteins in this fraction (Fig. 3, lane 3) indicates that the electron-dense material is probably storage protein. It is not clear whether the [^{3}H]fucose-labeled PHA is associated with all these vesicles or only with a subset.

Pulse-chase experiments. Pulse-chase experiments were carried out to find out if the [^{3}H]fucose-labeled PHA was transiently associated with the membranous organelles in which it was found. Cotyledons (eight batches of four cotyledons weighing 175–195 mg each) were labeled with 148 kBq of [^{3}H]fucose each for up to 60 min. Some were collected after 15, 30 and 45 min of labeling. After 60 min of labeling the remaining cotyledons were rinsed and transferred to a drop of nutrient medium, and the radioactivity was chased for 30, 60, 90 or 120 min. At all these timepoints, cotyledons were collected and the radioactive tissue homogenized in 12% sucrose in medium A. After a clearing spin (1000 *g* for 5 min) each homogenate was layered on sucrose gradients consisting of a 1-ml layer of 54% (w/w) sucrose and an 8-ml continuous gradient of 16 to 40% (w/w) sucrose, all in medium A. The gradients were centrifuged for 2 h at 150,000 *g* and divided in three portions: the load

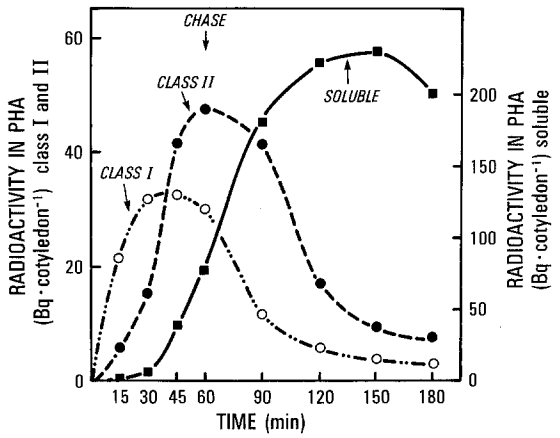

Fig. 5. Pulse and pulse-chase labeling of PHA with [^{3}H]fucose in different subcellular fractions of *P. vulgaris* cotyledons. Cotyledons were labeled with 148 kBq of [^{3}H]fucose each and collected after 15, 30, 45 or 60 min. Some cotyledons were chased after 1 h of labeling and collected 30, 60, 90 and 120 min later. The radioactive tissue was homogenized in medium B ($MgCl_2$) and the homogenates fractionated on isopycnic sucrose gradients. Fractions corresponding to the soluble, the class-I and the class-II particles were collected by hand, and PHA isolated with affinity gel

fraction at the top (soluble), the middle region corresponding to 22 to 35% (w/w) sucrose (class-I particles), and the band of organelles on top of the 54% (w/w) sucrose (class-II particles). Phytohemagglutinin was extracted from each fraction and the radioactivity in PHA determined. The results of this experiment show (Fig. 5) that [^{3}H]fucose was incorporated sequentially into the PHA present in class-I particles, class-II particles, and the soluble fraction. Chase-out of radioactivity in PHA followed the same pattern: radioactivity disappeared first from class-I particles then from class-II particles, but accumulated in the soluble fraction. Careful examination of the chase-out kinetics shows that radioactivity in the class-I particles had already started to decrease at the time the chase was applied. This is the consequence of an internal chase. The amount of radioactive fucose used (148 kBq per cotyledon) was not quite enough to have linear incorporation for the entire 60 min of the pulse. Together these results are consistent with the interpretation that [^{3}H]fucose becomes attached to PHA in the class-I particles, and that fucosylated PHA then moves to the class-II particles and subsequently to the soluble fraction. The soluble fraction contains, as stated before, the contents of the protein bodies as well as the cytosolic proteins.

It is noteworthy that the half-time of chase-out ($t_{1/2}$) of the fucosylated PHA from both class-I and class-II particles was 20–30 min. The half-time of

Table 1. Effect of monensin on the incorporation of ^{3}H-amino acids, [^{3}H]galactose and [^{3}H]fucose into cytoplasmic and cell-wall macromolecules of developing cotyledons of P. vulgaris. Cotyledons (four per sample) were pre-incubated with a 25-µl drop of 50 µM monensin in 1% ethylalcohol and half-strength nutrient solution. Controls were incubated in 1% ethylalcohol in half-strength nutrient solution. After 2 h, 5 µl of radioactive precursor was added (185 kBq per cotyledon), and incorporation allowed for another 2 h. The tissue was then homogenized and fractionated into cytoplasm and cell wall. The numbers indicate kBq cotyledon^{-1}

Precursor	Treatment	Uptake	Incorporation into cytoplasm	Incorporation into cell wall
Amino acids	Control	5.72	2.20	0.15
	Monensin	4.48	1.48	0.11
Fucose	Control	12.21	2.91	2.08
	Monensin	14.43	2.22	0.53
Galactose	Control	5.31	0:85	14.13
	Monensin	5.42	1.08	3.16

chase-out of ^{3}H-amino acid labeled PHA from the ER was found to be 90 min (Chrispeels and Bollini 1982).

Monensin blocks transport. Monensin is a low-molecular-weight monovalent ionophore which interferes with the secretory pathways of various animal cell types. The available evidence implicates the Golgi complex as a primary site of action of monensin. As an indirect test of the role of the Golgi apparatus in the transport of protein-body proteins, the effect of monensin on PHA transport was examined. How monensin affects the uptake and incorporation of ^{3}H-amino acids, [^{3}H]fucose and [^{3}H]galactose into different cellular fractions of the cotyledons was determined in a preliminary experiment. The results (Table 1) indicate that a 2-h pre-treatment with monensin had relatively little effect on the uptake of radioactive precursors or their incorporation into cytoplasmic macromolecules. The ionophore greatly inhibited the incorporation of fucose and galactose into the cell wall. Fucose- and galactose-containing macromolecules in the cell wall are part of the hemicellulose-pectic fraction whose synthesis and secretion is mediated by the Golgi apparatus (Ray et al. 1969, 1976; Ray, 1980). The inhibitory effect of monensin on the labeling of the cell wall indicates that it interferes with the normal functioning of the Golgi apparatus in these cells.

The effect of monensin on the transport of PHA trough the endomembrane system was examined in a pulse-chase experiment with control and

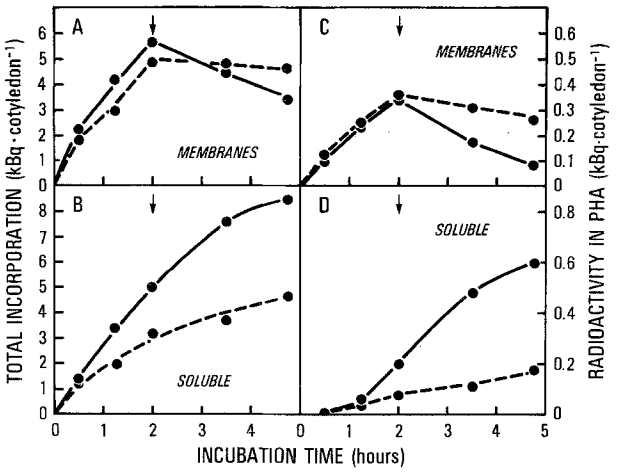

Fig. 6A–D. Effect of monensin on the synthesis and transport of PHA in *P. vulgaris* cotyledons. Cotyledons were given a 2-h pre-treatment without or with 50 µM monensin and then labeled with ^{3}H-amino acids for 30, 75 or 120 min. After 2 h (arrow) the label was removed and the cotyledons chased for 90 or 180 min. The radioactive tissue was homogenized (medium A) and the homogenates fractionated into a membranous-organelle fraction and a soluble fraction; this fractionation was done with discontinuous gradients each consisting of a layer of 16% (w/w) sucrose over 54% (w/w) sucrose. These fractions were analysed for total incorporation and incorporation into affinity purified PHA. **A, B** Total incorporation into membranous organelles and soluble macromolecules, respectively; **C, D** [^{3}H] amino acids in PHA in membranous organelles and soluble fraction. —— Control; ----- monensin. Note that monensin slows down the chase-out of PHA from the membranous-organelle fraction and prevents its accumulation in the soluble fraction

monensin-treated cotyledons. The cotyledons were pretreated for 2 h with 20 µl of half-strength nutrient medium or with the same medium containing 50 µM monensin, and 5 µl of ^{3}H-amino acids containing 185 kBq were then added to each cotyledon. The cotyledons were collected at 30, 75 and 120 min, and at this time (120 min) the remaining cotyledons were rinsed to remove the radioactive precursor and further incubated with or without monensin. These cotyledons were harvested after 1.5 h and 3 h of chase. The homogenates were fractionated into soluble and membrane fractions by means of discontinuous gradients consisting of a layer of 16% (w/w) sucrose over 54% (w/w) sucrose in medium A. The organelles were collected from the top of the 54% sucrose layer, while the soluble fraction represents the load portion of the gradients. Aliquots were used to determine incorporation into trichloroacetic-acid (TCA) precipitable macromolecules (Fig. 6A, B) while the remainder was used to extract PHA. Incorporation of ^{3}H-amino acids into PHA is shown in Fig. 6C, D. The results of this experiment show that treatment with monensin did not inhibit the biosynthe-

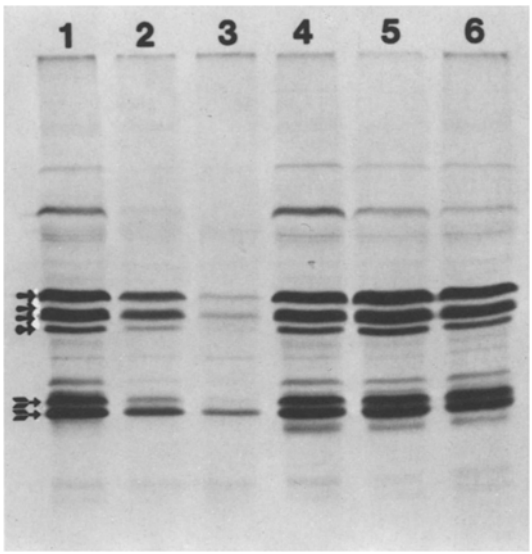

Fig. 7. Analysis by SDS-PAGE and fluorography of the membranous-organelle fractions shown in Fig. 6C. Lanes 1, 2 and 3: control; lanes 4, 5 and 6: monensin. Lanes 1 and 4: 2 h labeling, no chase; lanes 2 and 5: 2 h labeling and 1.5 h chase; lanes 3 and 6: 2 h labeling and 3 h chase. Phaseolin is shown by the set of four arrows, PHA by two arrows

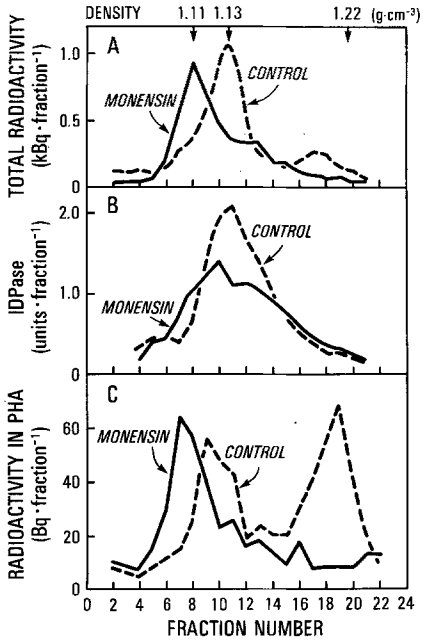

Fig. 8A–C. Effect of monensin on the distribution of [^{3}H]fucose-labeled PHA from on isopycnic sucrose gradients. Cotyledons were given a 2-h pre-treatment with or without 50 μM monensin, and then labeled for 1 h with 185 kBq of [^{3}H]fucose. The radioactive tissue was homogenized in medium A (EDTA), and the organelles fractionated as in Fig. 1. The fractions were assayed for total incorporation (**A**), IDPase and activity (**B**) and incorporation of [^{3}H]fucose into PHA (**C**)

sis of membrane-associated proteins in general or PHA in particular. However, labeling of proteins in the soluble fraction was inhibited by treatment with monensin. There was a particularly large inhibition of the labeling of PHA in the soluble fraction (Fig. 6D), presumably the consequence of the failure of radioactive PHA to the chased out of the membranous organelles. Since the soluble fraction represents the broken protein bodies as well as the cytosol, it appears that monensin prevented the movement of PHA from the membranous organelles to the protein bodies.

To find out if the movement of other proteins, in particular the storage protein phaseolin, was also inhibited by pretreatment with monensin, aliquots of the membranous-organelle fractions were analyzed by SDS-PAGE (Fig. 7). The results showed a rapid chase-out of radioactive PHA and phaseolin from the control samples (lanes 1, 2, 3) but no chase-out in the monensin-treated cotyledons (lanes 4, 5, 6). These results indicate that monensin interfered with the transport of phaseolin as well as with that of PHA.

To find out at which point monensin interfered with this transport the distribution of [^{3}H]fucose-labeled PHA on sucrose gradients was determined. Cotyledons were pretreated for 2-h with monensin as before and then labeled for 1 h with 185 kBq of [^{3}H]fucose per cotyledon. The radioactive tissue was homogenized, the debris removed by centrifugation at 1000 *g* for 10 min, and the supernatants fractionated on linear 16 to 54% (w/w) sucrose gradients (medium A). The gradients were fractionated and the fractions assayed for total incorporation into TCA-precipitable material, IDPase activity and incorporation of [^{3}H]fucose into PHA. The results (Fig. 8) showed that treatment with monensin caused a shift in the density from 1.13 to 1.11 g cm^{-3} of the organelles which contain most of the macromolecular [^{3}H]fucose (panel A) as well as the [^{3}H]fucose-labelled PHA. The pattern of IDPase distribution on the gradient was also affected by the monensin treatment (panel B). After treatment with monesin the organelles containing this enzyme formed a much broader band on the gradient. The most marked effect of monensin treatment was on the distribution of [^{3}H]fucose-labeled PHA. The treatment abolished the peak of [^{3}H]fucose-labeled PHA in the class-II particles at a density of 1.22 g cm^{-3}. The results are consistent with the interpretation that monensin alters the Golgi apparatus and prevents the transport of PHA from class-I to class-II particles.

Discussion

The role of the Golgi apparatus. The results presented in this paper show that newly synthesized

PHA is transiently associated with two classes of subcellular organelles distinct from the ER. These organelles termed class-I and -II particles have densities of 1.13 and 1.22 g cm^{-3}, respectively, and were identified tentatively as fragmented dictyosomes and small dense vesicles containing reserve protein. The results therefore provide the first biochemical evidence that the Golgi apparatus mediates the transport of PHA between the ER and the protein bodies.

Arguments in favor or against a role for the Golgi apparatus in the transport of protein-body proteins have been based entirely on ultrastructural observations. The presence of electron-dense deposits in Golgi-associated vesicles in developing cotton cotyledons led Dieckert and Dieckert (1976) to the conclusion that the Golgi apparatus is probably involved in reserve protein transport. Harris (1979) on the other hand carefully examined thick sections of cotyledon parenchyma cells of *Vicia faba* and found little evidence for or against a role of the Golgi apparatus in protein transport. It is unlikely that ultrastructural observations alone without supporting immunocytochemical data can resolve this issue (see Baumgartner et al. 1980).

The observation that the oligosaccharide moiety of PHA can be readily labeled when the cotyledons are incubated with [^{3}H]fucose (Chrispeels 1983) allowed a direct biochemical analysis of the role of the Golgi apparatus in PHA synthesis and transport. In animal cells, incorporation of fucose into glycoproteins occurs in the Golgi apparatus (see Hubbard and Ivatt 1981). Our identification of the class-I particles as Golgi-apparatus-derived membranes or fragmented dictyosome cisternae is based primarily on the following observations. [^{3}H]Fucose-labeled PHA bands with IDPase – a Golgi marker enzyme – in both rate-zonal sucrose gradients and isopycnic sucrose gradients, and its position in the isopycnic gradients was unchanged whether EDTA or $MgCl_2$ was present in the isolation media. This treatment changes the position of the rough ER, and changed the position of the major peak of ^{14}C-amino-acid-labeled PHA, indicating that most of the ^{14}C-amino-acid-labeled PHA was in the rough ER, while the fucose-labeled PHA was in a smooth-membrane system. Such a smooth-membrane system is present in this region of the gradients and consists of large vesicles with associated small vesicles. These structures probably represent disrupted Golgi apparatus.

An additional line of evidence for a role of the Golgi apparatus in the transport and biosynthesis of PHA comes from the observation that the oligosaccharide side-chains of PHA are modified and become in part resistant to endo-H degradation. In animal cells glycoproteins acquire resistance to endo-H as they pass through the Golgi apparatus (see Hubbard and Ivatt 1981), and acquisition of endo-H resistance is taken as evidence that glycoproteins have been modified in and passed through the Golgi apparatus.

The absence of a clear peak of ^{14}C-amino-acid-labeled PHA in the Golgi-region of the gradients (1.13 g cm^{-3}) when $MgCl_2$ is present allows one to infer that the Golgi apparatus must be a much smaller compartment than the ER. A smaller compartment which processes the same amount of protein as a larger one should have a shorter $t_{1/2}$ of chase-out for the radioactive protein. This is precisely what was observed. Amino-acid-labeled PHA chased out of the membrane system with a $t_{1/2}$ of 90 min (Chrispeels and Bollini 1982) while the $t_{1/2}$ for [^{3}H]fucose-labeled PHA was 20–30 min. The rapid turnover of PHA and other reserve proteins in the Golgi makes it difficult to detect the transport of reserve proteins through this compartment, unless new sugars such as fucose are specifically added.

We interpret these results to mean that the ^{3}H-amino-acid-labeled PHA moves from the ER to the Golgi apparatus where the oligosaccharide side-chain of PHA is modified by the incorporation of fucose (and possibly other sugars). Little is known about the transport of protein between the ER and the Golgi apparatus. The resent discovery by Harris and Oparka (1983) that in mungbean cotyledons there are numerous tubular connections between the cisternae of the ER and the Golgi apparatus indicates that transport between these two may be direct and need not involve vesicles.

The role of the Golgi apparatus in storage-protein transport is further confirmed by the results of the experiments with monensin. This drug has, to the best of my knowledge, not been used to probe macromolecular transport in plant cells, but has been used extensively in investigations of animal cells. In animal cells monensin blocks the secretion of macromolecules, the transport of membrane proteins to the plasma membrane, the recycling of low-density lipoprotein receptors, as well as the post-translational processing of proteoglycans (Tartakoff and Vassalli 1978; Uchida et al. 1980; Vladutiu and Ratazzi 1980; Nishimoto et al. 1982, and references therein). The available evidence implicates the Golgi apparatus as the primary site of action of this ionophore. Ultrastructural observations with plant and animal cells show that monensin blocks the formation of secretory vesicles at the trans face of the Golgi

apparatus and causes the cisternae to swell up and form large round vacuole-like structures (Grimes et al. 1982). The results described here indicate that monensin interferes with the synthesis and–or secretion of cell-wall hemicellulose, a known function of the Golgi apparatus. The ionophore also blocked the chase-out of labeled PHA and phaseolin from the class-I particles (Golgi apparatus-rich fractions) into the class-II particles. Treatment with monensin prevented the appearance of radioactive PHA, and probably also of radioactive phaseolin, into the class-II particles.

Transport to the protein bodies. Transport from the Golgi apparatus to the protein bodies appears to involve the class-II particles which were identified as small (0.1–0.4 μm), dense vesicles with an electron-dense matrix. The main proteins in these particles are PHA and phaseolin. Similar dense vesicles are seen in situ between the ER and the protein bodies (Baumgartner et al. 1980).

The rapid chase-out of the PHA from these dense vesicles compared with the chase-out from the ER again makes it difficult to locate the radioactive proteins in these vesicles on sucrose gradients if ^{3}H-amino acids are used as precursors. The vesicles, like the Golgi apparatus, constitute a much smaller compartment than the ER, and the pulse-chase of radioactive proteins through these vesicles can only be visualized if a radioactive precursor is used which is not incorporated into the PHA in the ER.

In this particular study I did not attempt to demonstrate the arrival of intact protein bodies from developing cotyledons in adequate yields. With the homogenization procedures used here most of the protein bodies break and most of the storage protein and protein-body hydrolases are released and present in the soluble fraction. The protein-body proteins are removed by the Sepharose 4B columns or remain at the top of the isopycnic sucrose gradients (see for example Fig. 3 in Bollini et al. 1982). When cotyledons are pulsed with radioactive precursors, storage proteins in the membranous organelles become labeled immediately (Chrispeels et al. 1982; Chrispeels and Bollini 1982) while there is a lag of 20–30 min in the labeling of the storage proteins in the soluble fraction (Fig. 5). A similar lag is found if one examines the arrival of radioactive storage protein in the protein bodies (Chrispeels et al. 1982). Thus we have shown already for pea cotyledons that the storage proteins in the soluble fraction of the homogenate become labeled at the same time as they arrive in the protein bodies in the cell.

The proteins stored in protein bodies include glycoproteins with high-mannose side-chains such as phaseolin and pea vicilin, glycoproteins with complex side-chains such as PHA, and proteins such as pea legumin which are totally devoid of carbohydrate (Badenoch-Jones et al. 1981). The evidence presented in this paper shows that proteins with fucosylated side-chains pass through the Golgi on their way to the protein bodies. The conclusion that phaseolin also passes through the Golgi is based entirely on the results obtained with the inhibitor monensin. I would predict that unglycosylated proteins such as pea legumin will also be found to be transported via the Golgi apparatus. This prediction is based on the role of the Golgi complex in animal cells. In animal cells the Golgi complex is involved in the transport and modification of glycoproteins as well as the transport of proteins which lack carbohydrates. Not all secreted proteins are glycoproteins, yet there is no documented example of a cell in which the secretory product bypasses the Golgi (Farquhar and Palade 1981).

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