Assembly of Storage Protein Oligomers in the Endoplasmic Reticulum and Processing of the Polypeptides in the Protein Bodies of Developing Pea Cotyledons

MAARTEN J. CHRISPEELS, THOMAS J. V. HIGGINS, and DONALD SPENCER
CSIRO, Division of Plant Industry, Canberra City, A.C.T. 2601, Australia. Dr. Chrispeels' present address is the Department of Biology, University of California/San Diego, La Jolla, California 92093.

ABSTRACT Cotyledons of developing pea seeds (*Pisum sativum* L.) were labeled with radioactive amino acids and glucosamine, and extracts were prepared and separated into fractions rich in endoplasmic reticulum (ER) or protein bodies. The time-course of synthesis of the polypeptides of legumin and vicilin and the site of their assembly into protein oligomers were studied using immunoaffinity gels and sucrose density gradients. When cotyledons were pulse-labeled (1–2 h), newly synthesized legumin was present in polypeptides with M_r 60,000–65,000, and newly synthesized vicilin was present as a series of polypeptides with M_r 75,000, 70,000, 50,000, and 49,000. These radioactive polypeptides were found primarily in the ER (Chrispeels et al., 1982, *J. Cell Biol.*, 93:5–14). During a subsequent chase period, newly synthesized reserve proteins were initially present in the protein bodies in the above-named polypeptides. Between 1 and 20 h later, radioactive legumin subunits (M_r 40,000 and 19,000) and smaller vicilin polypeptides (M_r 34,000, 30,000, 25,000, 18,000, 14,000, 13,000, and 12,000) appeared in the protein bodies. The appearance of these labeled polypeptides in the protein bodies was not the result of a slow transport from the ER (or cytoplasm).

Newly synthesized legumin and vicilin polypeptides were assembled into oligomers of 8S and 7S, respectively, in the ER. They appeared in the protein bodies in these oligomeric forms before the appearance of the smaller polypeptides (M_r <49,000). These results indicate that the smaller vicilin polypeptides (M_r <49,000) arise by delayed posttranslational processing of some or all of the larger vicilin polypeptides. The precursors of legumin are completely processed in the protein bodies 2–3 h after their synthesis. The processing of the vicilin precursors is much slower (6–20 h) and only a fraction of the precursor molecules are processed. As a result both large (M_r >49,000) and small polypeptides of vicilin accumulate in the protein bodies, whereas legumin accumulates only as polypeptides of M_r 40,000 and 19,000.

The large parenchymal cells of the cotyledons of pea (*Pisum sativum* L.) seeds contain 20–30% protein on a dry weight basis. The reserve proteins vicilin and legumin make up 70% of this protein. These reserve proteins are localized in protein bodies, which are spherical organelles measuring 1–3 μm in diameter consisting of an amorphous protein matrix surrounded by a limiting membrane. In mature seeds, legumin is a 12S protein (M_r 360,000) that consists of six acidic (M_r 40,000) and six basic (M_r 20,000) subunits, linked together in pairs by disulfide bridges (11). When legumin is fractionated by SDS PAGE, heterogeneity is found in both the acidic and basic subunits with three to five polypeptides in each molecular weight class. The vicilin fraction, which has a sedimentation coefficient of 7–8S, can be subdivided into at least five distinct oligomeric proteins on the basis of differential solubility and isoelectric precipitation. These proteins contain at least 13 different polypeptides in various proportions (24). The abundant polypeptides in the vicilin fraction have molecular weights of 75,000, 50,000 (doublet), 30,000, and 18,000, whereas the less abundant polypeptides have molecular weights of 70,000, 49,000, 34,000, 25,000, 14,000 (doublet), 13,000, and 12,000 (25).

When cotyledons are pulse-labeled with ^{14}C-amino acids, radioactive reserve proteins are associated with the rough endoplasmic reticulum (ER) before their arrival in the protein

THE JOURNAL OF CELL BIOLOGY · VOLUME 93 MAY 1982 306–313

bodies. Legumin is present in the ER as a family of polypeptides of M_r 60,000–65,000, whereas vicilin is represented only by polypeptides of M_r 75,000, 70,000, 50,000, and 49,000 (4). The smaller polypeptides of legumin and vicilin do not become labeled under these conditions. In this paper we have investigated both the kinetics of labeling of these smaller polypeptides of vicilin and legumin and the site of assembly of the polypeptides into oligomers. We have found that legumin is transported to the protein bodies as an 8S oligomer containing only the precursor polypeptides (M_r 60,000–65,000). Proteolytic processing of these precursors and assembly of 12S legumin occur in the protein bodies. Vicilin arrives in the protein bodies already assembled into 7–8S protein(s) containing polypeptides with M_r >49,000. The smaller vicilin polypeptides are detected only in the protein bodies, and we conclude that they result from the posttranslational processing of some of the high molecular weight polypeptides in the protein bodies.

MATERIALS AND METHODS

Details concerning plant material, radioactive labeling procedures, the preparation of cotyledon extracts and their fractionation on discontinuous sucrose density gradients to yield an ER-rich fraction, the isolation of protein bodies, the isolation of reserve proteins using immunoaffinity chromatography, and the fractionation of polypeptides by SDS-PAGE and detection by fluorography have all been described earlier (4). Immunoaffinity gels containing immunoglobin G against protein body extract (PBE), legumin, or vicilin covalently linked to Sepharose 4B (Pharmacia, Uppsala, Sweden) are referred to, respectively, as IgG-PBE-Sepharose, IgG-legumin-Sepharose, and IgG-vicilin-Sepharose. The developmental stage of pea cotyledons is defined as days after flowering (DAF). The ER-rich fraction obtained from sucrose gradients contained not only ER (as shown by the presence of NADH–cytochrome *c* reductase) but also some of the thylakoids (as shown by the faintly green color) and probably most of the Golgi-derived dictyosomes which band at a density of 1.13 $g \cdot cm^{-3}$ (19). The radioactive storage protein polypeptides in this fraction have been shown to be associated with the ER-derived membranes (4), and we refer to this microsomal fraction as ER. The M_r values shown on the figures are derived from a standard reserve protein (legumin plus vicilin) sample fractionated on the same gel.

Separation of Oligomers on Rate-zonal Sucrose Gradients

Isolated ER and protein bodies were dissolved in 0.25 M NaCl in 0.1 M Tris-HCl, pH 8.0, containing 1% Tween, and 0.5–1.0 ml of the protein solution was loaded on a linear 8–24% (wt/vol) sucrose gradient. The gradient solutions contained 0.25 M NaCl and 0.1 M Tris-HCl, pH 8.0. Gradients were centrifuged in a Beckman SW41 rotor at 5°C at 40,000 rpm (196,000 g_{av}) for 22 h for the separation of legumin oligomers, and for 34 h for the separation of vicilin oligomers. Purified protein body extracts containing 7S vicilin and 12S legumin, bovine serum albumin (4.4S), and sheep immunoglobulin G (7.0S) were run on parallel gradients and provided size markers. The gradients were monitored at 280 nm while they were being collected, and fractions (0.5 ml) were analyzed for radioactivity in protein either directly or after immunoaffinity chromatography with IgG-legumin-Sepharose or IgG-vicilin-Sepharose.

RESULTS

Newly Synthesized Polypeptides in the ER

Our previous work showed that there is a major discrepancy in the number and size of the storage protein polypeptides sequestered by the ER before transport to the protein bodies, on the one hand, and of those present in the protein bodies on the other (4). Protein bodies contain reserve protein polypeptides ranging in size from M_r 12,000 to 75,000, whereas the ER of cotyledons labeled with ^{14}C-amino acids only had radioactive storage protein polypeptides with M_r 49,000 or greater. To eliminate the possibility that the smaller polypeptides were synthesized in the ER at a stage of cotyledon development not examined in the previous study, we examined newly synthesized reserve protein polypeptides in the ER over almost the entire period of reserve protein synthesis. Cotyledons (from 12 to 27 DAF) were labeled with ^{14}C-amino acids for 1 h and the ER fraction was isolated on discontinuous sucrose gradients. The reserve protein polypeptides in this fraction were isolated with IgG-PBE-Sepharose, fractionated by SDS PAGE, and a fluorograph was prepared (Fig. 1). A Coomassie Blue–stained sample of an extract of protein bodies isolated from cotyledons at 24 DAF is shown for comparison (Fig. 1, track *PB*). By comparing the fluorograph of the newly synthesized polypeptides associated with the ER with the Coomassie-stained polypeptides in the protein bodies, it can be concluded that storage protein polypeptides with M_r <49,000 are not associated with the ER as newly synthesized polypeptides at any time during cotyledon development. We have previously (4) identified these high molecular weight reserve protein polypeptides associated with the ER. Legumin is present as a group of precursors with M_r 60,000–65,000. The synthesis of legumin begins around 15 DAF but its synthesis relative to that of other reserve proteins is most pronounced from 21 to 30 DAF. Vicilin synthesis occurred throughout the developmental period examined, but there was a change in the polypeptides synthesized. The synthesis of polypeptides with M_r 50,000 and 49,000 dominated

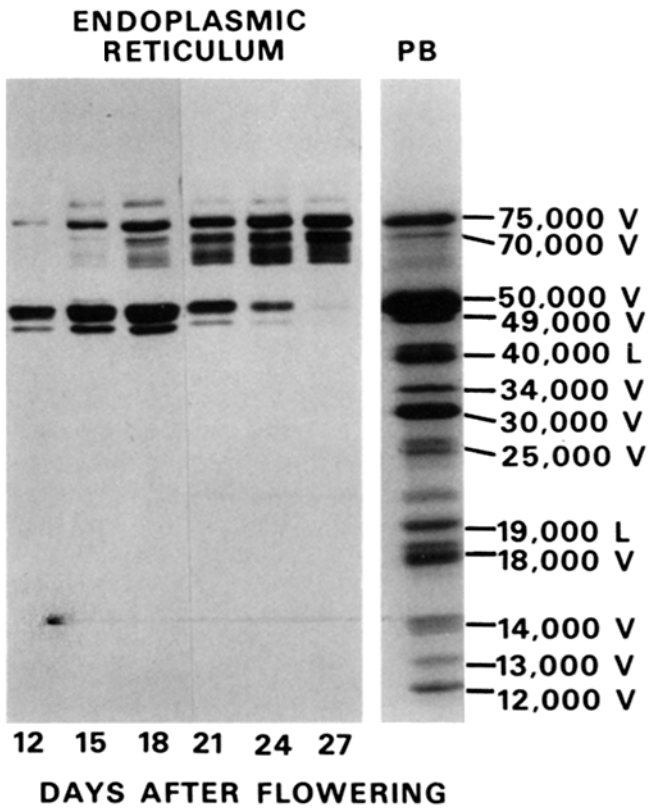

FIGURE 1 Fractionation by SDS PAGE of newly synthesized reserve protein polypeptides associated with the ER at different stages of seed development. Cotyledons (four per treatment) were labeled with ^{14}C-amino acids (21 kBq each) for 1 h and the ER was isolated on a discontinuous sucrose gradient (16% over 35%, wt/wt). Reserve protein polypeptides were isolated with IgG-PBE-Sepharose, fractionated by SDS PAGE, and a fluorograph was prepared. The numbers at the bottom of each lane indicate the stage of development as days after flowering. The lane on the right is a Coomassie Blue-stained track of reserve proteins present in protein bodies isolated from cotyledons at 24 DAF. The M_r of the vicilin (*V*) and legumin (*L*) polypeptides are shown.

the early stages of protein accumulation (12–21 DAF), whereas the synthesis of the larger vicilin polypeptides (M_r 75,000 and 70,000) was most pronounced at the end of the developmental period.

Synthesis of Small Reserve Protein Polypeptides

To find out when the smaller polypeptides (M_r <49,000) of the reserve proteins become radioactively labeled, cotyledons (20 DAF) were labeled with ^{14}C-amino acids for 1.5 h and then transferred to a nonradioactive nutrient solution. Cotyledons cultured on this medium continue to synthesize and accumulate protein for several days (18). This medium also provides effective chase conditions, since total incorporation of ^{14}C-amino acids ceases 10–15 min after transfer. We found that the radioactivity of the reserve proteins in the ER reaches a maximum 15 min after transfer and that chase-out of the proteins proceeds with a half-life of 90 min (4). The cotyledons were collected at different times after transfer (0, 2, 4, 7, 11, and 22 h) and a portion of the total tissue extracts was challenged with IgG-PBE to isolate the reserve proteins, which were then fractionated by SDS PAGE. The lanes were loaded with equal amounts of radioactive reserve protein, but all samples had nearly the same specific radioactivity (275 cpm/μg of protein). A number of radioactive polypeptides that were either absent or only barely detectable at the end of the 1.5-h pulse (0-h chase) gradually became more heavily labeled with time after transfer (Fig. 2). This increase in radioactivity was particularly noticeable in polypeptides of M_r 40,000, 34,000, 30,000, 25,000, 19,000, 18,000, 14,000, 13,000, and 12,000. These polypeptides correspond in size to the smaller polypeptides of vicilin and legumin.

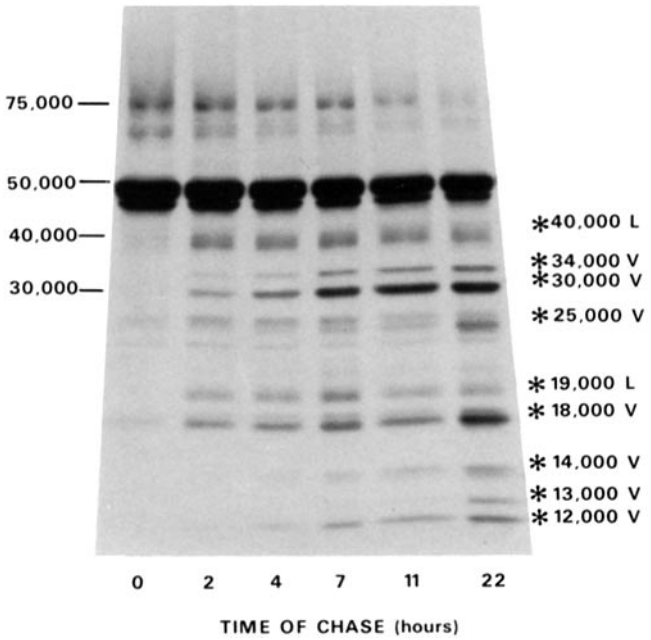

FIGURE 2 Change in the labeling pattern of polypeptides during a long-term chase of incorporated ^{14}C-amino acids. Cotyledons (20 DAF) were labeled for 90 min (0-h chase) and then transferred to nutrient medium for 2, 4, 7, 11, or 22 h. The tissue was extracted, and the extract was challenged with IgG-PBE-Sepharose. The bound polypeptides were fractionated on SDS PAGE and detected by fluorography. An asterisk denotes those legumin (*L*) and vicilin (*V*) polypeptides that gradually increased in radioactivity with time after transfer. Total incorporation did not increase after transfer. Gel slots were loaded with equal amounts of radioactive protein. The numbers show the M_r of the legumin and vicilin polypeptides.

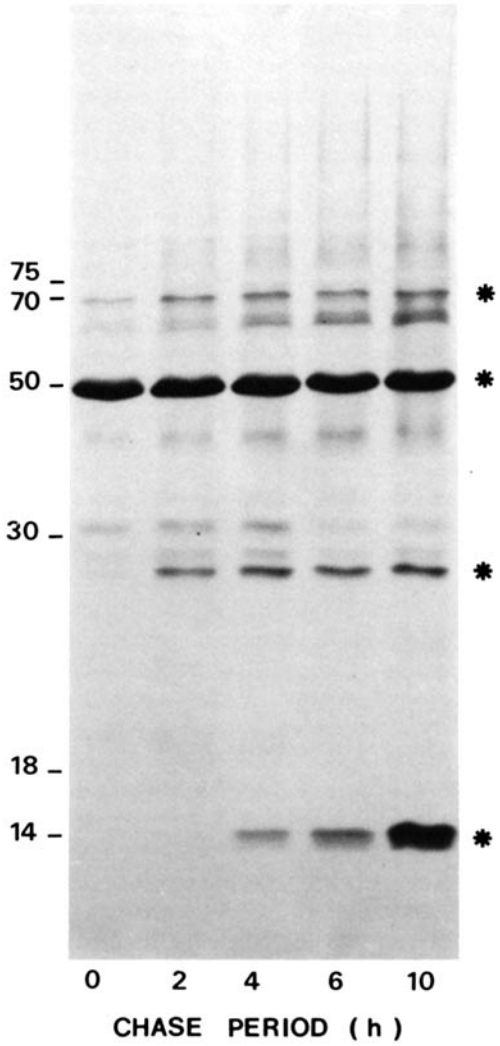

FIGURE 3 Change in the labeling pattern of polypeptides during a long-term incorporation of [^{14}C]glucosamine. Cotyledons (18 DAF, four cotyledons per time-point) were labeled for 2 h with 37 kBq each, then transferred to a medium containing 50 mM glucosamine and collected after a chase period of 2, 4, 6, or 10 h. The tissue was homogenized, the supernatant fractionated by SDS PAGE, and a fluorograph prepared. The polypeptides marked with asterisks correspond in size to the major glycopolypeptides of vicilin (1). Gel slots were loaded with equal amounts of radioactive protein. The numbers show the $M_r \times 10^{-3}$ of vicilin polypeptides.

The delayed appearance of smaller vicilin polypeptides (M_r <49,000) was confirmed by studying the known glycosylated vicilin polypeptides. The most abundant glycosylated reserve protein polypeptide is the M_r 14,000 polypeptide of vicilin (9), although, when cotyledons are incubated with [^{14}C]glucosamine for 12 h, radioactivity is also incorporated into vicilin polypeptides of M_r 70,000, 50,000 (doublet), and 26,000 (1). The sequence of labeling of these glycosylated polypeptides was examined in a pulse-chase experiment in which cotyledons (16 DAF) were incubated with [^{14}C]glucosamine for 2 h and transferred to a nutrient medium containing 50 mM glucosamine for various times up to 10 h. Total protein extracts of the cotyledons were fractionated on SDS PAGE, and newly synthesized, glycosylated proteins were detected by fluorography (Fig. 3). At the end of the 2-h pulse-labeling, the M_r 50,000

polypeptide of vicilin was most heavily labeled and incorporation was readily detected in the M_r 70,000 polypeptide. During the chase period, radioactivity increased first in the M_r 26,000 and subsequently in the M_r 14,000 polypeptide with a big increase in the latter between 6 and 10 h after the chase commenced. The M_r 14,000 component frequently resolves into two polypeptides, as seen in Fig. 3. The delayed appearance of radioactivity in the M_r 14,000 polypeptide is particularly striking in view of the fact that it is the most abundant glycosylated polypeptide in mature seeds.

Appearance of Smaller Polypeptides of Legumin and Vicilin in the Protein Bodies

The above results indicate that the smaller polypeptides of vicilin are not synthesized in appreciable amounts until at least several hours after the larger polypeptides and that they continue to accumulate for up to 22 h into the chase period. Previous work (4) showed that the half-life of storage proteins in the ER is ~1.5 h. This raises the possibility that the smaller polypeptide arise by processing of the larger polypeptides in the protein bodies. To examine this possibility further, we isolated protein bodies from cotyledons during a pulse-chase experiment and determined the time-course for the appearance of the smaller polypeptides in the protein bodies. Total incorporation of ^{14}C-amino acids into protein in the tissue extracts was approximately the same at different sampling times, indicating that there was little incorporation after transfer to nutrient medium. Radioactivity in the reserve proteins present in the protein body fraction continued to increase for 3–5 h after the transfer because of continued transport of labeled polypeptides from the ER to the protein bodies (4). Legumin and vicilin were isolated from the protein body fraction and the supernatant with IgG-legumin-Sepharose or IgG-vicilin-Sepharose and fractionated by SDS PAGE, and fluorographs were prepared. The gels were loaded with aliquots of protein body extract representing equal amounts of tissue. Thus total radioactivity in the gel lanes increased for up to 3–5 h after the beginning of the chase.

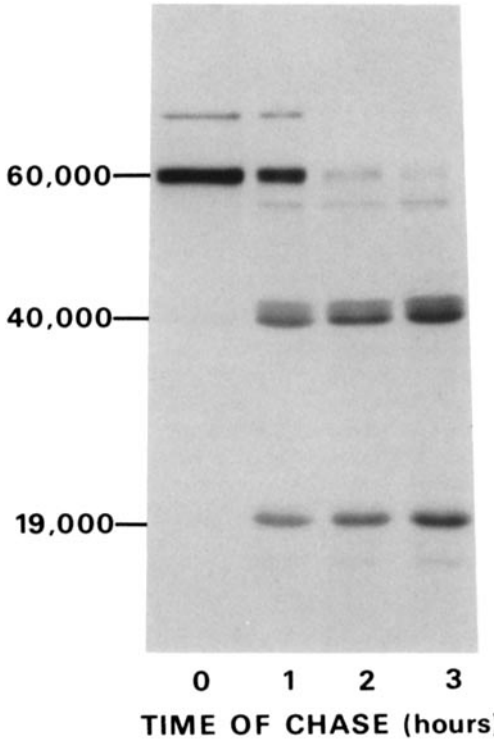

FIGURE 4 Change in the labeling pattern of polypeptides of legumin in the protein bodies during a chase of incorporated radioactivity. Cotyledons (21 DAF, two per time-point) were labeled with ^{14}C-amino acid (21 kBq each) for 1 h and subsequently transferred to nutrient medium to chase the radioactivity. Cotyledons were harvested at the time of transfer as well as 1, 2, and 3 h later, and protein bodies were isolated. Legumin was separated from other labeled proteins with IgG-legumin-Sepharose, fractionated by SDS PAGE, and a fluorograph was made.

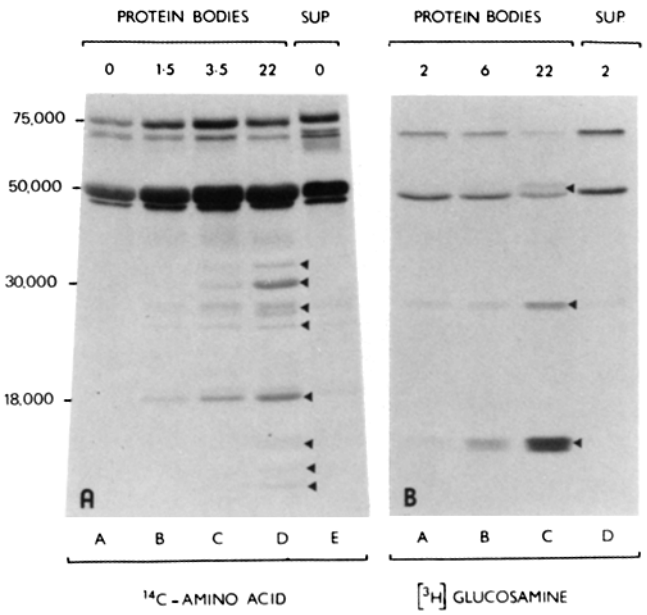

FIGURE 5 Change in the labeling pattern of polypeptides of vicilin in the protein bodies during a chase of incorporated radioactivity. (*A*) Cotyledons (19 DAF, three per time-point) were labeled with ^{14}C-amino acids (21 kBq each) for 1.5 h and subsequently transferred to nutrient medium. Cotyledons were harvested 0, 1.5, 3.5, and 22 h after the transfer, and protein bodies were isolated. Vicilin was separated from other labeled proteins with IgG-vicilin-Sepharose and fractionated by SDS PAGE, and a fluorograph was made. Slots were loaded with an equal proportion of protein body extract, or supernatant representing the same amount of tissue. (*B*) Cotyledons (20 DAF, three per time point) were labeled with [^{3}H]glucosamine (740 kBq each), and the radioactivity was chased by transfer to nutrient medium. Cotyledons were harvested 2, 6, and 22 h after transfer, and protein bodies were isolated. Reserve proteins were isolated and fractionated as in *A*. See text for explanation of the arrowheads.

To study the processing of legumin, cotyledons (21 DAF) were pulse-labeled for 1 h with ^{14}C-amino acids and chased for either 0, 1, 2, or 3 h (Fig. 4). Legumin first arrived in the protein bodies (Fig. 4, 0-h chase) as precursor polypeptides (M_r ~60,000) and was gradually processed to polypeptides with M_r 40,000 and 19,000. 3 h after the transfer, nearly all the precursor had been processed. Because the half-life of storage protein in the ER is ~90 min (4), legumin appears to be processed soon after it reaches the protein bodies. The relationship of the minor radioactive polypeptide (M_r ~80,000) to legumin is not understood (see Discussion).

To investigate the appearance of vicilin polypeptides in the protein bodies, cotyledons (19 DAF) were pulse-labeled for 1.5 h with ^{14}C-amino acids and chased for either 0, 1.5, 3.5, or 22 h. A fluorograph of the vicilin polypeptides fractionated by

SDS PAGE after isolation from the protein bodies with IgG-vicilin-Sepharose is shown in Fig. 5*A*. There was a general increase in the total radioactivity in the protein bodies during the chase (from 17,500 cpm/cotyledon at the beginning of the chase to 44,000 cpm/cotyledon after 3.5 h of chase), and this is reflected in an increase in intensity of the polypeptides with M_r 75,000, 50,000, and 49,000 in Fig. 5*A*, lanes *A*, *B*, and *C*. The labeling pattern of the vicilin polypeptides became increasingly complex with time after transfer. Some polypeptides became labeled within 3.5 h (e.g., M_r 25,000 and 18,000), whereas others were not labeled until some time between 3.5 and 22 h (e.g., M_r 30,000, 14,000, 13,000, and 12,000; marked with arrowheads in lane *D*). Lane *E* of Fig. 5*A* shows the radioactive vicilin polypeptides in the supernatant of the tissue homogenate after sedimentation of the protein bodies shown in lane *A*. In other words, lanes *A* + *E* show the vicilin polypeptides in the whole tissue extract at the beginning of the chase. The smaller radioactive polypeptides, which become more heavily labeled in the protein bodies after the beginning of the chase, were either absent or present in very small amounts in the supernatant fraction. Their gradual appearance in the protein bodies was therefore not the result of a slow transport out of the ER or the cytosol. Furthermore, we were unable to detect polypeptides of M_r <49,000 in the ER fraction when it was isolated after a 12-h labeling period (data not shown).

In a similar pulse-chase experiment, using [^{14}C]glucosamine instead of ^{14}C-amino acids, the only radioactive reserve protein polypeptides that were detectable in the protein bodies after a 2-h pulse were the M_r 70,000 and 50,000 polypeptides of vicilin (Fig. 5*B*). The much-delayed appearance of radioactivity on the M_r 26,000 and especially the M_r 14,000 polypeptides of vicilin (cf. Fig. 3) was detected only in the protein bodies and with increasing intensity during a lengthy (up to 22 h) chase period.

Formation of Oligomers

The data presented so far are consistent with the conclusion that proteolytic processing of storage protein polypeptides may occur in the protein bodies. This prompted us to ask when the assembly of the storage protein polypeptides into oligomers occurred, relative to the appearance of the smaller polypeptides. In the mature seed, vicilin and legumin occur as oligomers of 7S and 12S, respectively (11). In the immature seed, legumin is first assembled into an oligomer of 8S before conversion to the 12S form (22). The sedimentation coefficients of newly synthesized vicilin and legumin in the ER and the protein bodies were determined in sucrose gradients. Cotyledons (22 DAF) were labeled with ^{14}C-amino acids or [^{35}S]methionine, either for 1 h, after which the ER was isolated on a discontinuous gradient, or for 3 h, after which the protein bodies were isolated. The proteins in the ER and the protein bodies were fractionated on sucrose gradients, gradient fractions were challenged with IgG-vicilin-Sepharose and IgG-legumin-Sepharose, and radioactivity in legumin and vicilin was determined for each fraction. The profiles in the sucrose gradients of vicilin (labeled with ^{14}C-amino acids) and legumin (labeled with [^{35}S] methionine) in the ER and protein bodies are shown in Fig. 6. In the protein bodies of cotyledons labeled for 3 h, all the radioactive vicilin was present as 7.5S oligomers, whereas ~70% of the radioactivity in legumin was in an 8.1S oligomer and the remaining 30% in the 12S form. In the ER, on the other hand, ~70% of the vicilin was present as 7.5S oligomers and the remainder in a 3–4S form (probably single polypeptide chains). Thus vicilin oligomers appear to be assembled in the ER. Legumin in the ER was present in a 4–5S form (60–70% of the radioactivity) and in an 8S form. There was no peak of 12S legumin in the ER after 1 h of labeling. It is noteworthy that a much smaller proportion of legumin polypeptides than of vicilin polypeptides were assembled into oligomers in the ER. These results indicate that legumin is assembled in an 8S form in the ER (with chains of M_r 60,000–65,000) and transferred to the protein bodies in this form. The conversion of 8S legumin into 12S legumin was studied in an experiment in which 4.0 MBq of [^{35}S]methionine were injected into the pedicel of a pea pod at 21 DAF. The cotyledons were harvested 1 wk later and protein bodies were isolated. After separation of the reserve proteins on a rate-zonal sucrose gradient, it was found that 68%

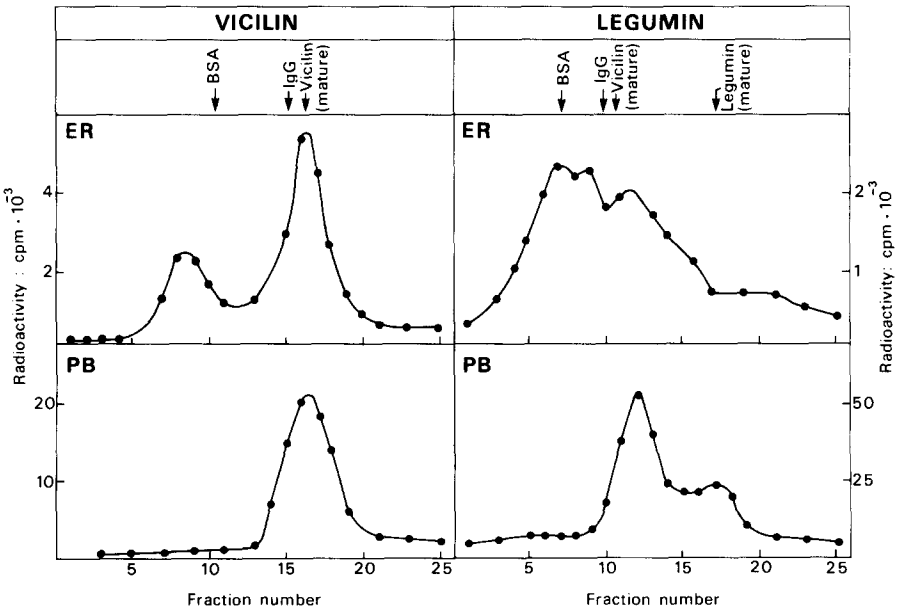

FIGURE 6 Fractionation on rate-zonal sucrose gradients of newly synthesized vicilin (*left*) and legumin (*right*) isolated from ER (*top*) and protein bodies (*PB, bottom*). Cotyledons (22 DAF, six per sample) were labeled with 34 kBq of ^{14}C-amino acids (for vicilin labeling) or 3 kBq of [^{35}S]methionine (for legumin labeling), either for 1 h (for the isolation of ER) or for 3 h (for the isolation of protein bodies). ER and protein bodies were isolated as described and dissolved in 100 mM Tris-HCl pH 8.0, 250 mM NaCl containing 1% Tween. A 1-ml portion was layered over an 8–24% (wt/wt) sucrose gradient and centrifuged at 200,000 g_{av} at 5°C for 36 h (for the fractionation of vicilin) or for 23 h (for the fractionation of legumin). The gradients were fractionated (25 fractions of 0.5 ml), and each fraction was challenged with IgG-vicilin-Sepharose or IgG-legumin-Sepharose, and the radioactive polypeptides were recovered. The positions of marker proteins (bovine serum albumin, M_r 68,000, and rabbit IgG, M_r 150,000) and 7S vicilin and 12S legumin present in protein bodies isolated from mature seeds are indicated by vertical arrows.

of the radioactivity in legumin was in the 12S form and 32% in the 8S form (data not shown).

The polypeptides present in 3–4S vicilin and 7S vicilin in the ER and the protein bodies and in 8S legumin and 12S legumin in the protein bodies were fractionated by SDS PAGE, and a fluorograph was made (Fig. 7). The results showed that the large vicilin polypeptides (M_r 75,000, 70,000, 50,000, and 49,000) were present in both forms of vicilin in the ER as well as in the protein bodies. There is very little processing of vicilin during a 3-h labeling period (cf. Fig. 5 *A*, lane *B*). Legumin polypeptides were present in the ER entirely as precursors of M_r 65,000–60,000 (data not shown, but see Fig. 5 *B* for example), whereas in the protein bodies legumin was present in the processed form (M_r 40,000 and 19,000) in both the 8S and 12S oligomers with some precursor still apparent in the 8S form. These results indicate that assembly of both vicilin and legumin polypeptides into oligomeric forms can occur before the processing steps that result in the formation of the M_r 40,000 and 19,000 polypeptides of legumin and the M_r <49,000 of vicilin. Results in Figs. 4 and 5 indicate that processing occurs in the protein bodies.

Precursor-Product Relationships

The precursor nature of the legumin polypeptides of M_r 60,000–65,000 has been documented previously (7, 22, 23). The current experiments show that the processing of the precursor occurs in the protein bodies after oligomer formation has occurred. In addition, they strongly indicate that the smaller polypeptides (M_r <49,000) of vicilin also arise by posttranslational processing of the larger polypeptides. This conclusion is based on the delayed appearance of the smaller polypeptides during the chase period, on the fact that they are only detected in the protein bodies, and on the finding that they are not detected until several hours after oligomer formation has occurred. These observations make it unlikely that the smaller polypeptides are the products of individual mRNAs that are present in low concentration. It seems highly unlikely that such products would be incorporated into preformed vicilin oligomers.

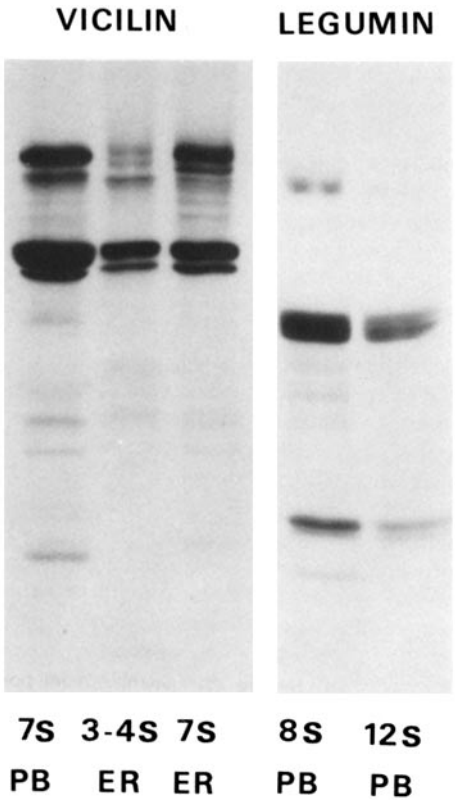

FIGURE 7 Fractionation by SDS PAGE of the polypeptides of vicilin and legumin recovered from the sucrose gradients described in Fig. 6. Three fractions of each peak were combined, to provide the material for each lane of the gel. From left to right: vicilin 7S from protein bodies, vicilin 3–4S from ER, vicilin 7S from ER, legumin 8S from protein bodies, and legumin 12S from protein bodies.

The complexity of vicilin polypeptides with M_r >49,000 which are potential precursors, and polypeptides with M_r <49,000 which are the processing products, makes it difficult to deduce precise precursor-product relationships. Prolonged incubation did not result in the total disappearance of any of these polypeptides (Fig. 5 *A*) and indeed they are present in significant amounts in mature seeds. To shed further light on possible specific precursor-product relationships of the vicilin polypeptides, we made use of the following two observations: firstly, at 13 DAF the cotyledons make relatively much more vicilin polypeptides with M_r 50,000 and 49,000 than vicilin polypeptides with M_r 75,000 and 70,000; and, secondly, when cotyledons at 27 DAF are labeled with [^{35}S]methionine for 2 h, vicilin polypeptides with M_r 75,000 and 70,000 become radioactive, but those with M_r 50,000 and 49,000 remain unlabeled (Higgins and Spencer, unpublished observations). Cotyledons (13 and 27 DAF) were labeled for 1.5 h with ^{14}C-amino acids or [^{35}S]methionine (27 DAF only) and then transferred to nonradioactive medium for a 20-h chase period. Total extracts were prepared, treated with IgG-legumin-Sepharose to remove all legumin and then with IgG-vicilin-Sepharose to isolate the vicilin fraction, which was further analyzed by SDS PAGE and fluorography. Equal amounts of protein were analyzed in all treatments. After pulse-labeling cotyledons at 13 DAF with ^{14}C-amino acids, radioactivity was found almost exclusively in the polypeptides of M_r 50,000 and 49,000 (Fig. 8, lane *a*). After a 20-h chase period, radioactivity was readily detected in all smaller vicilin polypeptides (M_r 34,000, 30,000, 25,000 [a complex of bands], 18,000, 14,000 [a doublet], 13,000, and 12,000) but not in the M_r 75,000 or 70,000 polypeptides (Fig. 8, lane *b*). This indicates strongly that the vicilin polypeptides of M_r <49,000 are derived from the processing of either or both the M_r 50,000 and 49,000 polypeptides.

After pulse-labeling 27 DAF cotyledons with ^{14}C-amino acids, the polypeptides of M_r 75,000 and 70,000 were the only radioactive vicilin products detected. After a 20-h chase, a very small amount of radioactivity was detected in the M_r 50,000 polypeptides (Fig. 8, lanes *c* and *d*). This sequence was seen more clearly when cotyledons (27 DAF) were pulse-labeled with [^{35}S]methionine. Incorporation was detected mainly into vicilin polypeptides with M_r 75,000 and 70,000 (two components). During the following 20-h chase period, the M_r 70,000 components decreased markedly and a major radioactive component appeared of M_r 50,000 (Fig. 8, lanes *e* and *f*). These results suggest that processing of the polypeptides of M_r 70,000 can give rise to smaller products, particularly of M_r 50,000. It should be emphasized that this experiment reflects changes that are quantitatively minor. The M_r 50,000 region of vicilin is complex and the major components of this complex are primary translation products that are made early in seed development (see Fig. 8, lane *a* and *b*). At 27 DAF, little total

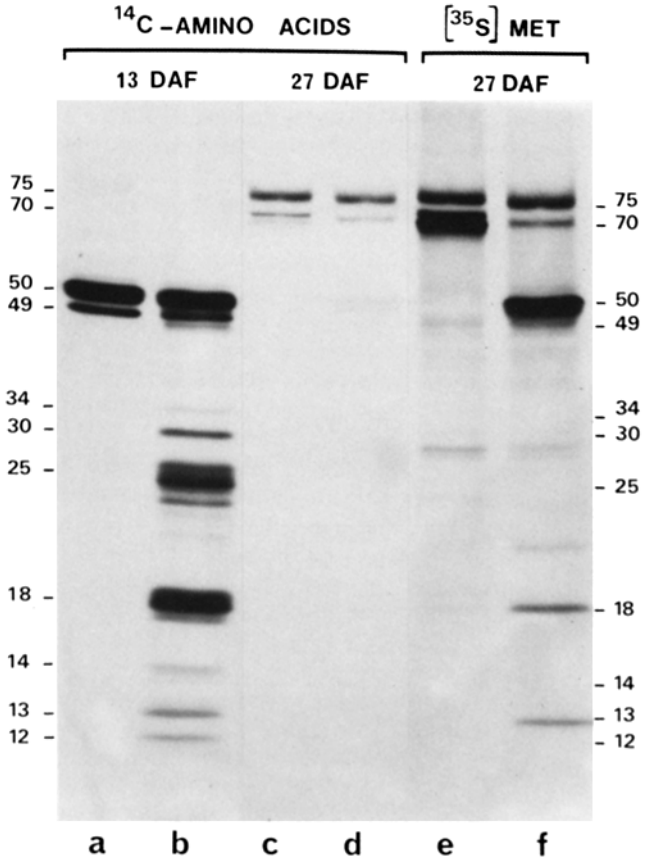

FIGURE 8 Distribution of vicilin polypeptides in cotyledon extract under pulse-chase conditions. Cotyledons (13 and 27 DAF) were labeled for 1.5 h with ^{14}C-amino acids or [^{35}S]methionine (27 DAF only), transferred to nutrient medium for an additional 20 h, and a total homogenate was prepared. Vicilin was isolated with IgG-vicilin-Sepharose and fractionated by SDS PAGE, and a fluorograph was made. Each lane was loaded with equal amounts of protein. Lanes *a*, *c*, and *e* show radioactive vicilin polypeptides at the end of 1.5-h labeling period and lanes *b*, *d*, and *f* are those after a 20-h chase period. Lanes *a–d* show extracts labeled with ^{14}C-amino acids and lanes *e* and *f* those labeled with [^{35}S]methionine. Numbers indicate the $M_r \times 10^{-3}$ of vicilin polypeptides.

vicilin synthesis is occurring, as evidenced by the extent of ^{14}C-amino acid incorporation (Fig. 8, lanes *c* and *d*). Labeling with [^{35}S]methionine exaggerates the contribution, to the M_r 50,000 complex, of minor components that appear to arise from processing of the M_r 70,000 polypeptides. Of the radioactive vicilin polypeptides of M_r <49,000 detected after the chase period (Fig. 8, lane *f*), only one (of M_r 18,000) comigrated with recognized vicilin polypeptides.

DISCUSSION

The protein bodies of pea cotyledons contain a wide spectrum of legumin and vicilin polypeptides ranging in molecular size from 75,000 to 12,000. The results presented in this paper support the conclusion that legumin and vicilin are synthesized as large M_r precursors, and that the smaller polypeptides (M_r 40,000 and 19,000 for legumin, and M_r 12,000–34,000 for vicilin) are formed by proteolytic processing of the larger polypeptides in the protein bodies. This conclusion helps to explain several apparently anomalous earlier observations. In vitro translations of mRNA extracted from developing cotyledons showed that only three major groups of storage protein–related polypeptides were formed, namely, polypeptides with M_r 75,000 (vicilin), 60,000–65,000 (legumin) and 50,000 (vicilin) (7, 8, 15, 22). These polypeptides were synthesized by membrane-bound polysomes, and some of them were made as preproteins that could be processed to a slightly smaller size by membranes isolated from dog pancreas or pea cotyledons (15). Chrispeels et al. (4) carried out in vivo labeling experiments and observed that newly synthesized storage protein polypeptides are sequestered by the ER before being transported to the protein bodies. They found, however, that polypeptides with M_r <49,000 were not associated with the ER. The results presented here show that such polypeptides are formed after the reserve proteins arrive in the protein bodies.

In addition, the results indicate that the assembly of polypeptide chains into oligomers occurs in the ER and that reserve proteins are probably transported as oligomers (7S for vicilin and 8S for legumin) to the protein bodies. Thus the formation of oligomers precedes the processing of the polypeptides. The assembly of legumin into its mature 12S form is a process that occurs slowly in the protein bodies, after the legumin precursors have been processed. The processing of legumin precursors was shown earlier to be quite rapid (22). Here we have shown that most of the legumin precursors (M_r 60,000–65,000) synthesized in a 1-h pulse are transported to the protein bodies and processed there during a 3-h chase. The processing of vicilin molecules appears to be much slower, with most of the increase in the radioactivity of some polypeptides occurring more than 6 h after the beginning of the chase (e.g., polypeptide with M_r 14,000 in Fig. 4). Thus considerable time may elapse between the arrival of the polypeptides in the protein bodies and the processing step giving rise to the smaller polypeptides. Similar cases of slow, posttranslational processing have been observed for several lysosomal or vacuolar enzymes: β-galactosidase in rat macrophages (21), carboxypeptidase Y in yeast (13), β-hexosaminidase, cathepsin D, and α-glucosidase in fibroblasts (12). It is worth noting in this context that protein bodies are part of the vacuolar/lysosomal compartment of plant cells. Recent evidence shows that protein bodies are derived from vacuoles during seed maturation (5, 6, 27) and that they contain numerous acid hydrolases (14, 17, 20, 26). Protein bodies therefore have an enzymic complement and function similar to that of the central vacuole of plant cells or the lysosomes of animal cells. It is likely, although there is as yet no supporting evidence, that protein bodies of developing cotyledons contain proteases, as part of their battery of acid hydrolases. These enzymes might be responsible for endoproteolytic cleavage of the reserve protein polypeptides after their arrival in the protein bodies. This nicking or processing may be the consequence of existing in a lysosomal compartment and could represent the first stages in the remobilization of this protein which mainly occurs during seed germination.

Precursor-Product Relationships

The precursor-product relationship of the major legumin polypeptides has been established previously (7, 22). The minor legumin polypeptide in the ER (M_r 80,000) can be seen only when the fluorographs are exposed for a long time (Fig. 4). This polypeptide is also processed and may give rise to a globulin similar to the legumin/like globulins found in small amounts in cowpea, kidney bean, and mungbean (10, 16).

These globulins have two disulfide-bonded chains with a total molecular weight of 80,000.

The precursor-product relationships for the polypeptides of vicilin are much less clear. The experimental evidence indicates that several of the large molecular weight polypeptides are broken down: the M_r 50,000–49,000 complex gives rise to all polypeptides of M_r <49,000, and polypeptides of M_r ~70,000 give rise to a minor component of the M_r 50,000 complex (Fig. 8). This finding, that components of the M_r 50,000 complex are both primary and secondary products of translation, compounds the difficulty in showing precursor-product relationships. In addition, some polypeptides are probably intermediates (for example, the M_r 26,000 glycosylated polypeptide), while others are clearly end-products (M_r 14,000 in Fig. 5 *B*, lane *c*). It is clear that not all the large polypeptide molecules are processed. Some large polypeptides are broken down, while others with the same M_r are not. Indeed, mature seeds contain a considerable amount of vicilin polypeptides with M_r 75,000 and 50,000 and smaller amounts with M_r 70,000 and 49,000. It is not clear whether the polypeptides that are not processed have a primary structure different from that of products that are. Experiments with phaseolin (the vicilinlike reserve protein of *Phaseolus vulgaris* with polypeptides of M_r 50,000) show that there is considerable charge heterogeneity among the polypeptides in the same molecular weight class. This heterogeneity can be detected only by using a combination of isoelectrofocusing and SDS gels (2, 3). The existence of a similar heterogeneity in the various vicilin polypeptides of peas could explain the apparent failure to process all vicilin polypeptides in the M_r 70,000 and 50,000 size class. This processing of only a fraction of the larger polypeptides could account for an anomaly that has been recognized for a long time (25), namely that the sum of the molecular weights of the component vicilin polypeptides (M_r ~440,000) far exceeds the observed size of the oligomer (M_r 180,000–200,000).

It is a pleasure to acknowledge the expert technical assistance of Sue Button.

This work was supported by a grant of the U.S.–Australia Cooperative Science Project.

Reprint requests should be addressed to Dr. Chrispeels at his permanent address: Department of Biology, C-016, University of California/San Diego, La Jolla, CA 92093.

Received for publication 12 November 1981.

REFERENCES

1. Badenoch-Jones, J., D. Spencer, T. J. V. Higgins, and A. Millerd. 1981. The role of glycosylation in storage protein synthesis in developing pea seeds. *Planta (Berl.)*. In press.
2. Bollini, R., and A. Vitale. 1981. Genetic variability in change heterogeneity and polypeptide composition of phaseolin, the major storage protein of *Phaseolus vulgaris*, and peptide maps of its three subunits. *Physiol. Plant.* 52:96–100.
3. Brown, J. W. S., F. A. Bliss, and T. C. Hall. 1980. Microheterogeneity of globulin-1 storage protein from French bean with isoelectrofocusing. *Plant Physiol. (Bethesda)*. 66:838–840.
4. Chrispeels, M. J., T. J. V. Higgins, S. Craig, and D. Spencer. 1982. The role of the endoplasmic reticulum in the synthesis of reserve proteins and the kinetics of their transport to protein bodies in developing pea cotyledons. *J. Cell Biol.* 93:5–14.
5. Craig, S., D. J. Goodchild, and C. Miller. 1980. Structural aspects of protein accumulation in developing pea cotyledons. II. Three-dimensional reconstructions of vacuoles and protein bodies from serial sections. *Aust. J. Plant Physiol.* 7:329–337.
6. Craig, S., A. Millerd, and D. J. Goodchild. 1980. Structural aspects of protein accumulation in developing pea cotyledons. III. Immunocytochemical localization of legumin and vicilin using antibodies shown to be specific by the enzyme-linked immunosorbent assay (ELISA). *Aust. J. Plant Physiol.* 7:339–351.
7. Croy, R. R. D., J. A. Gatehouse, I. M. Evans, and D. Boulter. 1980. Characterization of the storage protein subunits synthesized *in vivo* by polyribosomes and RNA from developing pea (*Pisum sativum* L.). I. Legumin. *Planta (Berl.)* 148:49–56.
8. Croy, R. R. D., J. A. Gatehouse, I. M. Evans, and D. Boulter. 1980. Characterization of the storage protein subunits synthesized *in vitro* by polyribosomes and RNA from developing pea (*Pisum sativum* L.). II. Vicilin. *Plant (Berl.)*. 148:57–63.
9. Davey, R. A., and W. F. Dudman. 1979. The carbohydrate of storage glycoproteins from seeds of *Pisum arvense*: characterization and distribution on component polypeptides. *Aust. J. Plant Physiol.* 6:435–437.
10. Derbyshire, E., and D. Boulter. 1976. Isolation of legumin-like protein from *Phaseolus aureus* and *Phaseolus vulgaris*. *Phytochemistry (Oxf.)*. 15:411–414.
11. Derbyshire, E., D. J. Wright, and D. Boulter. 1976. Legumin and vicilin storage proteins of legume seeds. *Phytochemistry (Oxf.)*. 15:3–24.
12. Hasilik, A., and E. F. Neufeld. 1980. Biosynthesis of lysosomal enzymes in fibroblasts. Synthesis as precursors of higher molecular weight. *J. Biol. Chem.* 255:4937–4945.
13. Hasilik, A., and W. Tanner. 1978. Biosynthesis of the vacuolar yeast glycoprotein carboxypeptidase Y. *Eur. J. Biochem.* 85:599–608.
14. Herman, E. M., and M. J. Chrispeels. 1980. Characteristics and subcellular localization of phospholipase D and phosphatidic acid phosphatase in mung bean cotyledons. *Plant Physiol. (Bethesda)*. 66:1001–1007.
15. Higgins, T. J. V., and D. Spencer. 1981. Precursor forms of pea storage proteins: modification by microsomal membranes during cell-free translation. Plant Physiol. 67:205–211.
16. Khan, R. I., J. A. Gatehouse, and D. Boulter. 1980. The seed proteins of cowpea (*Vigna unguiculata* L. Walp.). *J. Exp. Bot.* 31:999–1011.
17. Matile, P. 1968. Aleurone vacuoles as lysosomes. *Z. Pflanzenphysiol.* 58:365–368.
18. Millerd, A., D. Spencer, W. F. Dudman, and M. Stiller. 1975. Growth of immature pea cotyledons in culture. *Aust. J. Plant Physiol.* 2:51–59.
19. Nagahashi, J., and L. Beevers. 1978. Subcellular localization of glycosyl transferase involved in glycoprotein biosynthesis in the cotyledons of *Pisum sativum* L. *Plant Physiol. (Bethesda.)* 61:451–438.
20. Nishimura, M., and H. Beevers. 1978. Hydrolases in vacuoles from castor bean endosperm. *Plant Physiol. (Bethesda)*. 62:44–48.
21. Skudlarek, M., and R. T. Swank. Biosynthesis of two lysosomal enzymes in macrophages. *J. Biol. Chem.* 254:9939–9942.
22. Spencer, D., and T. J. V. Higgins. 1980. The biosynthesis of legumin in maturing pea seeds. *Biochem. Int.* 1:502–509.
23. Spencer, D., T. J. V. Higgins, S. C. Button, and R. A. Davey. 1980. Pulse-labeling studies on protein synthesis in developing pea seeds, and evidence of a precursor form of legumin small subunit. *Plant Physiol. (Bethesda)*. 66:510–515.
24. Thomson, J. A., H. E. Schroeder, and W. F. Dudman. 1978. Cotyledonary storage proteins in *Pisum sativum*. I. Molecular heterogeneity. *Aust. J. Plant Physiol.* 5:263–279.
25. Thomson, J. A., H. E. Schroeder, and A. M. Tassie. 1980. Cotyledonary storage proteins in *Pisum sativum*. V. Further studies on molecular heterogeneity in the vicilin series of holoproteins. *Aust. J. Plant Physiol.* 7:271–282.
26. Van der Wilden, W., E. M. Herman, and M. J. Chrispeels. 1980. Protein bodies of mung bean cotyledons as autophagic organelles. *Proc. Natl. Acad. Sci. U. S. A.* 77:428–432.
27. Yoo, B. Y., and M. J. Chrispeels. 1980. The origin of protein bodies in developing soybean cotyledons: a proposal. *Protoplasma* 103:201–204.