Developmental Biochemistry of Cottonseed Embryogenesis and Germination

XIII. REGULATION OF BIOSYNTHESIS OF PRINCIPAL STORAGE PROTEINS

Leon Dure III and Glenn A. Galau
Department of Biochemistry, University of Georgia, Athens, Georgia 30602

ABSTRACT

The synthesis of the principal cottonseed storage proteins during embryogenesis has been followed by analyses of stained protein gels and of fluorographs of protein synthesized in vivo and from purified RNA in vitro in the wheat germ system. The kinetics of in vivo labeling as well as immunochemical cross-reactivity indicate that the 52- and 48-kilodalton mature storage protein sets are derived from 70- and 67-kilodalton precursor protein sets that are abundant proteins in embryonic cotyledons and disappear in late embryogenesis. Identification of the initial translation products of the storage protein mRNA has not been clearly established although proteins of apparent molecular weights of 69,000 and 60,000 are the likely storage protein precursors.

Storage protein synthesis falls off markedly in late embryogenesis simultaneously with the loss of a superabundant class of mRNAs (shown by cDNA:RNA reassociation) that are presumed to be those for the storage proteins. The synthesis of these proteins ceases abruptly when immature embryos are removed from the bolt and allowed to germinate precociously or when this precocious germination is prevented by incubation in abscisic acid. Thus, abscisic acid is not implicated in the expression of the storage protein genes.

A scheme involving co-translational processing into vesicles, glycosylation, and slow in situ cleavage to produce the mature storage proteins is proposed.

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We have attempted to follow during embryogenesis the synthesis of the principal storage proteins of cotton cotyledons which were identified and partially characterized in the preceding paper (8). The accumulation of these proteins (and their precursors) was followed by the 2D electrophoresis of proteins extracted at intervals during embryogenesis. To follow their synthesis, the incorporation of radioactive amino acids into them in intact cotyledons was assessed by the fluorography of 2D protein gels (in vivo synthesis) and estimates of the levels of their mRNAs were made by measuring the synthesis of their initial translation forms with mRNA isolated at stages in embryogenesis utilizing the wheat germ in vitro protein-synthesizing system.

The measurements reported here were made with cotyledons taken from embryos that were 50 and 100 mg in wet weight and from cotyledons from the mature seed. These sizes were chosen since they represent distinct phases of embryogenesis, i.e. 50-mg embryos are in the cell division phase of embryogenesis whereas 100-mg embryos are in the maturation phase characterized by a high endogenous level of AbA (17). The mature seed represents the termination of embryogenesis.

MATERIALS AND METHODS

Plant Material, Chemicals, Isotopes. Mature seeds and immature embryos of cotton (Gossypium hirsutum, var. Coker 201) were taken from greenhouse plants. Acrylamide, bisacrylamide, and SDS were obtained from Bio-Rad Laboratories, ampholines from LKB, agarose for immunoelectrophoresis (Seakem HGT) from Marine Colloids, and Coomassie Brilliant Blue R-250 and creatine kinase from Sigma Chemicals. S, nuclease (EC 3.1.4.-) used in the nucleic acid reassociation experiments was obtained from Miles Laboratories. For in vivo labeling of proteins, U-14C-labeled algal protein hydrolysate (>50 mCi/mg atom) from Amersham Corporation (CFB.25) was used. For in vitro protein synthesis, L-[3,4,5-3H]leucine (110 Ci/mmole) from New England Nuclear (NET-460) was used. Wheat germ was a gift from General Mills, Modesto, CA. Heat-killed, formalin-fixed Staphylococcus aureus was a gift of Dr. Judith Foster. Avian myeloblastosis virus reverse transcriptase, used for the synthesis of cDNA, was a gift from Dr. J. Beard.

Protein Extraction. The protein content of cotyledons was either solubilized en masse with 2% SDS, 1% mercaptoethanol, 50 mM Tris-HCl (pH 8.3) which yields the "total protein" fraction, or divided into two subfractions, the "soluble" fraction and the "pellet" fraction as described in reference 8. The soluble fraction was obtained by homogenizing cotyledons in 0.1 M NaCl, 1% mercaptoethanol, 50 mM Tris-HCl (pH 8.3), and taking the supernatant after extracting the tissue for 6 h at room temperature. The pellet fraction was obtained by re-extracting the insoluble, pelleted material obtained above with the 2% SDS, 1% mercaptoethanol, Tris-HCl (pH 8.3) for 6 h at room temperature and taking the supernatant after centrifugation. The extracted proteins were precipitated by making the extracts 95% in acetone. The precipitates were prepared for electrophoresis or isoelectric focusing as described in the preceding paper (8).

Measurement of in Vivo Protein Synthesis. Embryos were dissected from immature seeds, rinsed in distilled H2O, placed on moist filter paper in Petri dishes, and incubated in darkness for the periods indicated in the text. The filter paper was moistened with a solution containing the 14C-labeled algal hydrolysate (5 μCi ml-1), gramicidin D (10 μg ml-1) and, where indicated, AbA (5 μM). After the incubation period, the embryos were thoroughly rinsed in distilled H2O, axes removed and discarded, and the cotyledons extracted for protein as given above. In the pulse-chase experiment, the embryos were removed from the filter paper...
containing the 14C-labeled algal hydrolysate, rinsed well with distilled H2O, and placed on filter paper moistened with a solution containing gramicidin and, where indicated, AbA at the same concentrations as given. To measure synthesis after several days of incubation ± AbA, the dissected, rinsed embryos were incubated on filter paper wetted with the gramicidin solution ± AbA for 3.5 days in darkness, then transferred to filter paper wetted with the same medium containing the isolopes and incubated for 12 additional h.

**Measurement of Protein Synthesis with Wheat Germ System (in Vitro Synthesis).** Wheat germ supernatant was prepared according to Marcu and Dudock (23) with potassium acetate replacing potassium chloride. Translation reaction mixtures contained, in a final volume of 50 μl, 35 mM Hepes-KOH (pH 7.5), 1 mM ATP, 0.4 mM GTP, 8 mM creatine phosphate, 100 μg ml−1 creatine kinase, 2 mM magnesium acetate, 0.15 M potassium acetate, 8.5 mM DTT, 0.3 mM phenylmethylsulfonil fluoride, 25 μg each of unlabelled amino acids, 4.5 μM 13,4,5-3Hleucine (110 Ci/mmol), 18 μl wheat germ supernatant, and 20 to 30 μg total high mol wt cotton RNA. In some cases the reaction mixture was supplemented with 20 μg yeast tRNA. Incubation was for 90 min at 25 C after which the mixtures were treated with RNase A (100 μg ml−1) for 15 min at 37 C and precipitated with 10 volumes acetone.

In our hands, the wheat germ system with [3H]leucine at the specific activity and concentration used incorporates between 0.5 to 1 × 10−3 dpm into hot TCA-insoluble material in the 50 μl reaction mixture. This equates to 25 to 50 pmol leucine/25 μg total cotton RNA. If leucine is assumed to constitute about 7% of the amino acids in protein, then about 360 to 720 pmol total amino acids are incorporated in the 90-min period. The average mol wt of proteins synthesized in developing cotyledons is about 40,000 (derived from in vivo labeling), which equates to about 365 amino acids. Thus, 365 pmol amino acids equals about 1 pmol protein. From the above it is apparent that the system is synthesizing between 1 to 2 pmol protein.

We assume from previous experiments that the cotton total RNA preparations contain about 2% mRNA (10, 14). Thus, in the 25 μg total RNA used, there is assumed to be 0.5 μg mRNA. From this, the system incorporates 50 to 100 pmol leucine or 720 to 1440 pmol total amino acids into 2 to 4 pmol protein/μg mRNA. A μg mRNA is calculated to contain about 2.2 pmol mRNA. This is based on the average protein size of 40 kD and the inclusion of 250 nontranslated nucleotides in mRNA plus a poly(A) chain of 100 (10, 14). This yields an average mRNA length of about 1,500 nucleotides which is in agreement with the average size determined for cotton cotyledons by other means (10, 14, 29). Since the system is synthesizing only 2 to 4 pmol protein/2.2 pmol mRNA, each mRNA molecule is being translated on the average a little over 1 time in 90 min.

From these calculations, it should be expected that many false polypeptides would be seen on fluorographs of in vitro translation. Some mRNAs will not have been fully translated in 90 min, whereas others will have reinitiated for a second round. Both situations would give rise to incomplete protein chains whose mol wt are lower than those seen in in vivo synthesis, even if the RNA preparation used has no nuclease-shortened mRNA. This consideration should influence the interpretation of fluorographs of both in vitro synthesis and of the translation products immunoprecipitated by the antibody preparations.

We have also noted the marked stimulation of incorporation into wheat germ proteins (endogenous synthesis) by the added cotton RNA, presumably brought about by the protective effect of the cotton total RNA on the nuclease degradation of endogenous mRNA. To determine the intrinsic contribution of wheat germ products to fluorographs of 1D and 2D gels, reaction mixtures containing 25 μg Escherichia coli RNA in place of cotton RNA were carried through the electrophoretic/fluorographic procedures. The use of total RNA as the exogenous template source also has a protective effect on translation of exogenous mRNA. The large amount of RNA added appears to increase the survival time of the larger mRNAs since a greater amount of radioactivity is incorporated into the larger proteins when 25 μg total RNA is used in comparison to 0.5 μg mRNA-poly(A) (data not shown).

**Electrophoretic and Isoelectric Focusing Procedures.** The discontinuous 1D electrophoretic system in SDS was performed by the procedure of Laemmli (19) employing a 10 to 17% acrylamide gradient. For the 2D separation of proteins the procedure of O'Farrell (24) was used with the modifications given in the preceding paper (8); samples were prepared for these procedures as described in Ref. 8. Gels were stained with Coomassie Brilliant Blue R-250 and for carbohydrate by the periodic acid-Schiff reagent procedure as described by Segrest and Jackson (26). The radioactivity on 1D and 2D gels was analyzed by fluorography (2, 20) using fogged Kodak X-Omat R film exposed to the gels at −80 C.

**Immunological Techniques.** Antibodies to the mature 52- and 48-kD storage protein sets were prepared from rabbits as described in Ref. 8. To determine the immunoprecipitability of proteins separated in SDS by the 1D method of Laemmli, the 2D immunoelectrophoresis system of Converse and Papermaster (5) as modified by Chua and Blomberg (3) was used. This procedure causes protein SDS complexes from the 1D gel to migrate through a Lubrol-containing gel phase that sequesters the SDS into mixed micelles. The proteins, freed of SDS, are immunologically reactive as they enter the antibody-containing gel phase. For the immunoprecipitation of in vitro translation products, the IgG fractions were first subjected to ion exchange chromatography as described by Palacios et al. (25) to remove traces of ribonuclease. The IgG fractions were included in the 50-μl translation reaction mixtures at protein concentrations of 0.5 μg ml−1. After 90 min, the mixtures were diluted with 10 μl 2.5% Nonidet P-40, 750 mM NaCl, 250 mM Tris-HCl (pH 7.6), 5% sodium deoxycholate, 25 mM EDTA, and 10 mM leucine to stop protein synthesis. Additional IgG was added to each mixture at 0.5 mg protein ml−1 and the incubation continued for 20 h at 25 C at which time 40 μl of S. aureus suspension (30%), purified by the procedure of Kessler (18), was added to precipitate the antigen-antibody complexes. This precipitate was separated from the translation reaction mixture by centrifugation through a 2 M sucrose pad. The precipitated cell wall-antigen-antibody complex was washed by the procedure of Foster (9) and the antigen and antibody solubilized by incubation at 37 C in Laemmli sample buffer (19) for 30 min. Aliquots of the supernatant from this solubilization step were applied to the 1D gel without further treatment. The IgG fraction prepared in the same way from a nonimmunized rabbit was carried through the same procedure as a control.

**Preparation of Cotton RNA for Translation.** Total high mol wt RNA was prepared from 50- and 100-mg embryo cotyledons and dry seed cotyledons by a protocol that involved solubilization by SDS, deproteinization with phenol and chloroform, and salt precipitations with LiCl and Na-aceate. The details of this procedure have been presented elsewhere (10).

**Nucleic Acid Hybridization.** mRNA-poly(A) was prepared from 50- and 100-mg cotyledons and from dry seed cotyledons by published techniques (10) as was DNA complementary to 50-μg embryo cotyledon mRNA-poly(A) (10). Their rate of reassociation in excess mRNA-poly(A) was measured by a protocol involving S, nuclease digestion and binding to DE-81 discs as described by Maxwell et al. (22). The details of this procedure have been published elsewhere (10).

**RESULTS**

**Identification of 70-kD Protein Set as a Storage Protein Precursor.** Figure 1A shows the 2D gel pattern of proteins of the
pellet fraction present in 50-mg embryo cotyledons. The pl isomers of the 52- and 48-kD storage protein sets are the most abundant proteins in this tissue at this developmental stage as they are in mature cotyledons (8). However, two other sets of proteins having pl heterogeneity are also present in this fraction that are absent in mature cotyledons (8). These have apparent mol wt of about 70,000 and 67,000. A companion 2D gel containing an identical sample was stained for carbohydrate by the PAS procedure. The only proteins of this fraction that reacted positively were the 52-kD storage protein set and the 70-kD set (PAS stain shown in the insert). We do not know the limits of detection of this staining procedure, and, thus cannot rule out the presence of much smaller numbers of glycosyl residues on other proteins in this fraction. The similarity in pl heterogeneity and in the degree of glycosyla-
tion between the 52- and 70-kD sets plus the fact that the 70-kD set disappears as the 52-kD set accumulates in late embryogenesis suggest a precursor-product relationship between the two protein sets.

The results of in vivo isotopic labeling of proteins for brief periods and of pulse-chase labeling proved to reinforce this possibility. Figure 1B shows the fluorograph of this same fraction from 50-mg embryo cotyledons labeled for 3 h after excision from the cotton boll. (Only the portion of the 2D gel containing the pertinent proteins are shown in parts B, C, and D of this figure). The 70-kD set has been heavily labeled during the 3-h pulse as has the 48-kD set. The 52-kD set, however, has an obviously lower specific radioactivity. Noteworthy here also is the observation that the individual members of 52-kD set have different specific radioactivities; that is, the more abundant members of the set by Coomassie staining have the least radioactivity. This rules out the possibility that the pl isomers of this set are artifactually produced during sample preparation and/or isoelectric focusing, which events would be random with regard to radioactivity content.

When the time period of pulse labeling is increased to 6 h, the specific radioactivity of the 52-kD set is increased and appears to be equal among the individual members of the set (Fig. 1C). If the 6-h incubation in isotope is followed by a 10-h further incubation without isotope, the 70- and 67-kD protein sets lose considerable radioactivity (Fig. 1D) but no reduction in stainable protein in these two sets is evident (data not shown) suggesting that radioactivity is “moving” through these protein sets. Longer “chase” periods, which show a complete loss of radioactivity from the 70-kD and 67-kD sets are complicated by the fact that the embryos begin to germinate precociously at which point the synthesis of these two sets stops and degradation of the 52- and 48-kD sets commences. Many pulse times have been used in these experiments and it has been possible to observe considerable radioactivity in the 70-, 67-, and 48-kD sets without detecting any radioactivity in the 52-kD set.

To test further the putative precursor-product relationship between the 70- and 52-kD sets, we have made use of the 2D immunoelectrophoretic technique (3, 5) that allows proteins separated in the 1D system of Laemmli in the presence of SDS (19) to react immunochemically when electrophoresed into an agarose gel containing an appropriate IgG fraction. Figure 2 shows the immunoprecipitates formed when the pellet protein fraction from 50-mg embryo cotyledons separated by the 1D system was electrophoresed into the IgG fraction from the serum of rabbits immunized against the 52-kD protein set (top) and against the 48-kD set (bottom). A duplicate 1D slab, stained with the Coomassie stain, showing the position of the pellet proteins in the 1D gel is shown in place across the bottom. (The Lubrol-containing section of the agarose slab is not shown.) We demonstrated (8) that the 52- and 48-kD protein sets share common antigenic determinants. Thus, it was anticipated that rocket immunoprecipitates would be formed by both of these protein sets with both IgG preparations as is found in Figure 2. The figure also shows that the 70-kD protein set forms immunoprecipitates with both sets of antibodies. As mentioned, the kinetics of labeling and the common glycosylation intensity shown (Fig. 1A) suggests that the 52-kD set is derived from the 70-kD set; however, the derivation of the 48-kD set is not so clear since they also are seen to share common immunological determinants. In most of the repetitions of this experiment we have observed the faint rocket immunoprecipitate formed by the 67-kD protein set with both IgG fractions which is visible in Figure 2. The relative paucity of this protein set in the 1D gels (in embryonic cotyledons) makes this interaction less certain.

If the 70-kD (and probably the 67-kD) protein sets are intermediates in the synthesis of the principal storage proteins of the mature seed, the smaller fragments produced by the proteolytic

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**Fig. 1.** Two dimensional electrophoretic display of stained proteins and proteins synthesized in vivo in 50-mg embryo cotyledons. A, pellet protein stained with Coomassie. Insert, section of a companion gel stained for carbohydrate. B, C, and D, fluorographs of pellet protein after 3 h incubation (B) and after 6 h incubation (C) in 14C-amino acids. In D, cotyledons were incubated 6 h in 14C-amino acids followed by 20 h incubation without the isotopes. In B, C, and D, only the section of the gel containing pertinent proteins is shown. Numbers on the left give approximate molecular weights in kilodaltons. Low mol wt proteins circled in A become radioactive only after 6 h incubation in the isotopes.
cleavages involved may persist in the tissue. In this regard we have observed that some of the small proteins in the pellet fraction of 50-mg embryo cotyledons become radioactive only in the longer pulse incubations. These proteins are circled in Figure 1A. Their possible derivation from the 70- and/or 67-kD sets has not been pursued to date.

Storage Protein Synthesis Decreases during Maturation Phase of Embryogenesis. Several lines of evidence indicate that storage protein synthesis falls off as embryos go from the cell division phase of development into the maturation phase. First, the amount of the 70-kD precursor protein set decreases relative to other pellet proteins during the maturation phase, and it and the 67-kD set are absent in mature cotyledons as noted before. Figure 3, A through C, shows this disappearance from stained 2D gels. (Only the portion of the slabs corresponding to the segment of the isoelectrofocusing gel from pH 5.8 to 7.4 is shown in this figure.) Part A shows the pellet protein from 50-mg embryo cotyledons; part B, from 100-mg embryo cotyledons; and part C, from mature seed cotyledons.

Since, when 50-mg embryos are pulse-labeled for 6 h with radioactive amino acids, the 70-, 52-, and 48-kD protein sets are by far the most heavily labeled proteins found in the total protein of cotyledons (see Fig. 7, top), it seems likely that the storage protein mRNA should be among the very abundant mRNA sequences in the tissue. Should these sequences decrease in late embryogenesis, this should be demonstrable by cDNA:mRNA hybridization experiments designed to measure the relative concentrations of mRNA sequences (Fig. 4). Here cDNA synthesized to mRNA-poly(A) from 50-mg embryo cotyledons has been reassocited to the homologous RNA from which it was synthesized, to mRNA-poly(A) from 100-mg embryo cotyledons, and to mRNA-poly(A) from mature seed cotyledons, and the rates of reassociation measured. A computer analysis of the kinetics of these reassociation reactions along with many others dealing with mRNA populations in cotton embryogenesis and germination will be published elsewhere (11). However, Figure 4 shows that there is a superabundant mRNA subset in 50-mg embryo cotyledons that has decreased 10- to 15-fold in concentration in 100-mg cotyledons and has vanished as an abundant component in mature seed cotyledons. It should be noted that the members of this subset have not disappeared completely in mature seed cotyledons since the mRNA-poly(A) from this tissue drives all the 50-mg embryo cotyledon cDNA into hybrid.

Kinetically, this superabundant mRNA subset behaves as if it were comprised of only one to three different sequences. This very low number possibly indicates again extensive sequence homology between these storage proteins that is suggested by the immunochemical cross-reactivities; that is, this protein sequence homology is reflected in Figure 4 as sequence homology among mRNAs.

Finally, it should be possible to follow the concentration of the mRNAs for these storage proteins directly by examining the products of in vitro translation programmed by mRNA obtained from cotyledons of the three developmental stages. When this was done utilizing the translating system from wheat germ, no products

![Fig. 2. Immunelectrophoresis of pellet protein from 50-mg embryo cotyledons. Proteins were separated in the first dimension by the SDS-gel system of Laemmlli (19) as shown by stained gel across the bottom on which the approximate molecular weights of the pertinent proteins are indicated. Proteins in the 1D gels were electrophoresed into slabs containing the anti-52-kD protein IgG fraction (top) and the anti-48-kD protein IgG fraction (bottom). The Lubrol-containing zone of the agarose slab used to sequester SDS is not shown. Protein from 0.005 cotyledon pairs was electrophoresed in the first dimension. IgG fractions contained 5 mg protein ml⁻¹.](image)

![Fig. 3. Stained 2D gels of pellet protein from: A, 50-mg embryo cotyledons; B, 100-mg embryo cotyledons; C, mature seed cotyledons; D, 50-mg embryo cotyledons that have been precociously germinated 4 days; E, 50-mg embryo cotyledons that have been incubated in ABA 4 days. Only the section of the gels containing the pertinent proteins is shown. Pellet protein equivalent to 0.05, 0.02, 0.01, 0.1, and 0.1 cotyledon pairs was electrophoresed in A through E, respectively.](image)
SYNTHESIS OF STORAGE PROTEINS

The rate of reassociation of cDNA synthesized to 50-mg embryo cotyledon mRNA-poly(A) and its homologous mRNA-poly(A), △; 100-mg embryo cotyledon mRNA-poly(A), □; and mature seed cotyledon mRNA-poly(A), ○. Rate is expressed as per cent hybridized (S, resistant) versus log DNA concentration in mol nucleotide pairs 1^{-1} (C_o) $\times$ time of incubation in s (τ).

![Figure 4](image-url)

**Fig. 4.** The rate of reassociation of cDNA synthesized to 50-mg embryo cotyledon mRNA-poly(A) and its homologous mRNA-poly(A), △; 100-mg embryo cotyledon mRNA-poly(A), □; and mature seed cotyledon mRNA-poly(A), ○. Rate is expressed as per cent hybridized (S, resistant) versus log DNA concentration in mol nucleotide pairs 1^{-1} (C_o) $\times$ time of incubation in s (τ).

Instead of the extensive *in vitro* synthesis of protein having a mol wt of about 60,000 and the somewhat less extensive synthesis of protein of about 69 kD, the synthesis of these species, which seem to be the only products with mol wt appropriate to be possible precursors of the storage proteins, is not as extensive as might be expected in view of the apparent superabundance of these mRNA sequences shown in Figure 4. The inconsistency is probably due to the large number of incomplete protein chains present in the reaction after 90 min and to nuclease/protease shortening of mRNA and translation products that plague the wheat germ-translating system.

We have attempted to establish that these large translation products represent the initial translates of the mRNAs for the mature 52- and 48-kD proteins by immunoprecipitation of these products with the antibodies raised against the 52- and 48-kD protein sets. Lanes 4 and 5 of Figure 5 show the translation products precipitated by the anti-52- and anti-48-kD protein IgG fractions, respectively, by the double immunoprecipitation procedure. Both sets of antibodies precipitate the 69- and 60-kD translation products; however, lower mol wt protein species are precipitated also; one of about 50 kD and others in the 35- to 40-kD range. The specificity of these immunochemical reactions is indicated by the fact that none of these translation products are precipitated when the IgG fraction from nonimmunized rabbits is used in the procedure. The diffuse radioactivity in lanes 4 and 5 conceivably represent incomplete protein chains of the 69- and 60-kD proteins that, although of much lower mol wt, contain the antigenic determinants. The immunoprecipitable 50-kD protein is inexplicable as yet. It cannot be identified as a specific protein or protein set on 2D gels and may represent incomplete chains or the products of ersatz processing reactions that the wheat germ systems can carry out on the higher mol wt translation products. It should be noted that the 60-kD translation product is a poor antigen with either of the antibody preparations. It is precipitable in high yield in the double immunoprecipitation procedure only if the antibody preparations are included in the translation reaction and the immunochemical reaction allowed to continue for many hours after the translation reactions have been stopped. If the translation mixture is first treated with TCA or acetone to precipitate protein and then antibodies added to the solubilized precipitate after the manner of Chua (3) and many others, the 69- and 50-kD translation products are immunoprecipitable in good yield, whereas only traces of the 60-kD protein can be precipitated. This phenomenon in which antibodies to mature proteins react poorly with initial translation products that are destined to undergo extensive modification is not unusual (9) and has been found in the case of storage proteins of peas (16).

When the radioactive translation products programmed with mRNA from the three developmental points are separated on the 2D system and fluorographed (Fig. 6), the 69- and 60-kD proteins are found to be protein sets having pI heterogeneity. The 60-kD set is indicated by the arrow. Furthermore, these proteins, which are among the abundant products of 50-mg embryo cotyledon RNA (top), are much less abundant among the products of 100-mg embryo cotyledon RNA (middle) and are not found among the products of mature seed cotyledon mRNA.

From these results, and those given in Figure 4, it seems apparent that the decrease in storage protein synthesis is due to a loss of its mRNA sequences.

Abscisic Acid Does Not Influence Storage Protein Synthesis.

We have found that when cotton embryos are removed from the boll during the cell division stage, cell division ceases and precocious germination ensues unless it is prevented by incubation of the embryos in ABA (17). Storage protein synthesis also stops upon removal from the boll as would be expected in embryos that are switching from the embryogenic developmental program to the germination program. This synthesis also ceases in embryos...
whose precocious germination is arrested by AbA. In both instances, the conversion of the 70-kD precursor set to the 52-kD set goes to completion which is shown in Figure 3, parts D and E. In part D, the stained pellet protein from 50-mg embryo cotyledons that have been excised and incubated on moist filter paper for 4 days is shown. These embryos have begun visible precocious germination. Comparing this gel with that in part A reveals that the 70- and 67-kD protein sets have disappeared as abundant proteins and that the 52- and 48-kD sets have also decreased in amount. (Protein from twice as many cotyledon pair equivalents has been used in part D as in part A.) In the case of nongerminating embryos incubated 4 days in an AbA solution (part E), the 70- and 67-kD protein sets have disappeared, but no decrease in the 52- and 48-kD protein sets is noticeable. (Again, protein from twice the cotyledon pair equivalents has been used in part E relative to part A to emphasize the disappearance of the precursor protein sets.)

The cessation of storage protein synthesis is more compellingly demonstrated in Figure 7 which shows the products of in vivo synthesis contained in the total protein fraction from cotyledons of pulse-labeled 50-mg embryos (top), from those excised and allowed to germinate precociously 4 days (middle), and from those excised and incubated in an AbA solution for the same period (bottom). The labeling period for the excised embryos was the last 12 h of the 4-day period. In this set of fluorographs, the synthesis of some of the abundant proteins that are synthesized in 50-mg embryo cotyledons continues in both sets of dissected embryos. Several of these are circled for orientation. The AbA-treated cotyledons are found to continue the synthesis of many proteins seen in the 50-mg embryo cotyledon synthesis, yet the synthesis of proteins not seen in the starting material is notable in the AbA-treated cotyledons. (In other experiments, we have observed that these proteins appear in stained gels as well as in the fluorographs of pulse-labeled proteins from cotyledons further along in embryogenesis when the endogeneous AbA concentration has increased. A strong case can be made that some of these proteins (and their mRNAs) become members of the abundant set in response to high levels of AbA. These data will be published separately.)

The most pronounced difference in the abundant proteins being synthesized in vivo between the 50-mg embryo cotyledons and both sets of excised cotyledons is the absence in the latter of storage protein synthesis. Identical results have been obtained with all age groups of embryos treated in this way (from 35–110 mg fresh weight). Furthermore, when RNA extracted from excised embryos of any age precociously germinated ± AbA is used to program protein synthesis in the wheat germ system, the 69- and 60-kD translation products are not detectable (data not shown). Thus, the cessation of storage protein synthesis in both sets of embryos is due to the loss of its mRNA.

DISCUSSION

From these results it is clear that the synthesis of the principal storage proteins of cotton involves a complex sequence of events that will finally be described in detail with difficulty. It is apparent that the 52- and 48-kD mature protein sets are not synthesized from mRNA as molecules of these sizes. The 70-kD precursor set appears to be a very long lived intermediate in the synthesis of the 52-kD set based on the kinetics of in vivo labeling of the two sets, on the occurrence of common antigenic determinants, and on the fact that both sets are glycosylated. That the 70-kD set also gives rise to the 48-kD set cannot be ruled out since they also share antigenic determinants, but the apparent lack of substantial glycosyl residues on the 48-kD set makes this seem unlikely. Also conceivable from the immunochemical experiments is that the 48-kD set is derived from the 67-kD precursor set that does not accumulate to an appreciable extent perhaps because it is more rapidly processed to the 48-kD set.
In either case, the size of the peptide cleaved off is extremely large (about 15–20 kD), far too large to represent a signal peptide that is involved in the co-translational passage of nascent proteins into vesicles (1) such as protein bodies in this case. Furthermore, the size is much larger than has been found for the transit peptides involved in the post-translational passage of proteins through organelar membranes (4, 7, 15). In fact, a case can be made for the cleavage events taking place inside the protein body itself. The existence of glycosyl residues on the 70-kD set suggests that it has already passed into a vesicle and is found in toto in crude protein body preparations from immature embryos (8). Further, its long life time is not common to known examples of co-translational and post-translational processing. It is likely that the smaller polypeptides resulting from these postulated in situ cleavages of the 70- and 67-kD sets persist in protein bodies without undergoing further degradation. As mentioned before, we have observed that some of the smaller polypeptides of the pellet protein fraction that are abundant in stained gels are labeled only slowly in in vivo synthesis. They would not be expected to react immunologically with antibodies made to the 52- and 48-kD sets. Evidence is accumulating that an analogous case of in situ cleavage of long lived precursors may take place in the synthesis of some of the storage proteins of peas (6, 16) and of soybeans (27, 28).

The identification of the initial translation products of the storage protein genes is also complicated by the common antigenic determinants (sequence homologies) that must characterize these products as they do the mature protein sets. In vitro synthesized products with apparent mol wt of about 69,000 and 60,000 are precipitated by antibodies to both the 52- and 48-kD sets. The simplest picture that can be drawn to date, based on current concepts of protein processing, is schematized in Figure 8. Here, the 69-kD set of in vitro products are shown to give rise to the 67-kD precursor set seen in stained gels and in fluorographs of in vivo labeled protein. The small change in mol wt would be the result of processing via the co-translational passage into the protein body vesicle. In situ cleavage would produce the 48-kD set of the mature seed cotyledon. The 52-kD set is shown in this scheme to arise from the 60-kD in vitro products by glycosylation that accompanies passage into the vesicles. A signal peptide could also be lost in this passage but not noticed because of the apparent increase in molecular weight caused by the sugar residues. The very slow in situ cleavage produces the 52-kD set that, along with the 48-kD set, characterize the mature seed cotyledon.

Some of the events postulated in Figure 8 are not without analogies in other higher plants. Differences in the degree of glycosylation among members of storage protein sets that appear to represent a multigene family because of immunological cross-reactivity, and that result in large differences in apparent molecular weights, have been found in Phaseolus vulgaris (13). In this

![Figure 7: Two dimensional electrophoretic separation of total protein synthesized in vivo in 50-mg embryo cotyledons (top), 50-mg embryo cotyledons precociously germinated 4 days (middle), and 50-mg embryo cotyledons incubated 4 days in AbA (bottom). Circled proteins are proteins common to all three syntheses for orientation. The pH range of the first dimension is as in Figure 6.](image)

![Figure 8: Scheme of possible processing events in the accumulation of the principal storage proteins. A, co-translational loss of signal peptide; B, co-translational loss of signal peptide and glycosylation; C, in situ cleavage.](image)
case, the mature proteins can be traced back to initial translation products by deglycosylation (30) and by assuming the loss of a signal peptide. No subsequent in situ cleavages are indicated by the data in this case. The existence of signal peptides on the in vitro translation products of storage proteins from many other higher plants has been demonstrated (collated in Ref. 21). In many cases, these peptides can be removed by incubation in vitro with membranes from a number of sources.

Clearly, an understanding of the regulation of the expression of storage protein genes in many organisms will be clouded by the extensive processing that occurs between translation and accumulation of the final products, and by the existence of the many members of the multigenic families that are probably products of individual cistrons but share a great deal of sequence homology.

An interesting aspect of storage protein synthesis in cotton from a developmental point of view is that it is principally a midembryogenesis process. The presumptive mRNA for these proteins declines in amount relative to other mRNA after the cell division phase progresses into the maturation phase as shown by the kinetics of cDNA:RNA hybridization and by the fluorographs of in vitro synthesized proteins. A similar disappearance of storage protein mRNA from the abundant class has been observed in late embryogenesis in soybean embryos by hybridization techniques (12).

It is not surprising that AbA, although preventing the viviparous germination of immature embryos, does not maintain storage protein synthesis in dissected embryos. In vivo, storage protein synthesis is seen to begin and reach a maximum when AbA levels are low and to decline during the period of AbA increase. Thus, factors regulating earlier embryo development must be involved in the expression of storage protein genes.

The processing of the larger precursors to the mature storage protein forms continues in dissected embryos when their precocious germination is arrested by incubation in AbA, yet further degradation of these proteins does not take place. This also is not surprising since one aspect of the AbA prevention of vivipary must be the repression of the enzyme systems that carry out this degradation in germination.

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