Properties and Regulation of Biosynthesis of Cottonseed Storage Proteins

Comprehensive Progress Report

for period 1 December, 1976

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MASTER

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DISCLAIMER

PROGRESS REPORT

1. Main Research Accomplishment

For the past three years in research sponsored by this contract we have pursued our study of the regulation of gene expression in cotton seed embryogenesis by attempting to define what gene products are likely to be highly regulated during this developmental progression. To do this we measured the flow of nitrogen into the free amino acids pools of the developing cotyledons, and into the principal nitrogen nutritional reserve of the seed, the storage proteins. This was continued by following the flow of nitrogen from the storage proteins to the principal exported amino acid asparagine that occurs during the first several days of germination. In this fashion the rise and fall of certain enzymes of amino acid intermediary metabolism could be postulated, and in some cases, verified. These results were published in papers #1 and #6 of the publication list (section 4), and reprints enclosed in previous Progress Reports.

This work and other results contained in papers #2, 4 and 5 of the publication list prompted use to delineate the subsets of abundant mRNAs whose appearance and dissapearance coninsided with developmental events in cotyledon embryogenesis/germination with the short range goal of identifying proteins/enzyme activities and their mRNAs that represent specific developmental stages and the long range goal of using these representatives as probes for studying the mechanisms controlling the rise and fall of these mRNAs and their protein products.

Recent Results

Obvious mRNA products that characterize certain phases of the embryogenic developmental program of cotton cotyledons are the storage proteins,

and it has been their chemical characterization and the study of their biosynthesis that this contract has sponsored for the past twenty months. These proteins are of interest for a variety of reasons other than merely as representative tools for developmental biochemistry. They constitute the principal protein nutritive component of cotton seeds, and their amplitude makes cotton seed a very protein-rich food source.

Much of what we have found out about these proteins and their biosynthesis is presented in papers #7 and 8 of the Publication List. A preprint of #8 is enclosed as an appendix to this report. In previous progress reports we have presented some of the characteristics of these proteins, some of the most interesting of which are:

1. Comprised principally of two molecular weight species (about 46,000 and 52,000 daltons respectively) each of which seems to have 4-8 different isoelectric species identifiable by IEF.
2. Soluble in 8M urea, 2% SDS, 0.5 M NaCl or 2% DOC.
3. Comprise together about 30% of the total seed protein. (i.e. about 2.8 and 3.2 mgs each per dry seed embryo, which itself has a dry weight of about 60 mg and contains about 20 mg total protein)
4. The amino acid composition of these two species is notable in that over 20% of the nitrogen is in the form of arginine in both proteins which requires an increase in germination in the enzymes carrying out the movement of nitrogen from arginine to asparagine which is the form in which it is translocated from the cotyledons to the growing axis. The IEF data suggest that much of the glutamate and aspartate found on amino acid analysis is glutamine and asparagine in both proteins.

5. The 52,000 dalton species is glycosylated which means that its apparent molecular weight determined by electrophoresis in SDS is probably meaningless.

Our most recent results concerning the biosynthesis of these two proteins reveal a complex picture. These results have been obtained from 2D gels (IEF plus discontinuous electrophoresis in SDS) of stained protein, of fluorographs of radioactive protein labelled in vivo and fluorographs of radioactive protein produced by the wheat germ incorporating system programmed with isolated mRNA.

It appears that the 52,000 dalton protein first accumulated in vivo as a 70,000 dalton precursor which is also a glycoprotein, and which exhibits the same pI heterogeneity as do the mature storage proteins. The processing of this precursor to the final 52,000 dalton product is very slow and only by the final few days of embryogenesis has the process been completed. This slow process has been shown by stained gels and pulse - chase experiments involving in vivo labelling. The direct demonstration of the precursor - product relationship between these proteins was the cross reactivity between the 70,000 dalton protein and antibody made against the 52,000 dalton protein.

The identification of the initial products of mRNA translation corresponding to the 70,000 dalton precursor has been difficult to unequivocally demonstrate. At the moment it appears that the battery of mRNAs coding for the battery of isoelectric forms of this precursor produces a series of pI isomers all with a molecular weight of about 60,000 daltons. Glycosylation of these pI isomers, which does not occur in in vitro translation, presumably increases their "apparent" size to 70,000.

Most of these data are presented in the Appendix.

Finally, when immature cotton embryos are dissected from their seed coats and germinated precociously, the synthesis of the storage proteins ceases immediately, the processing of existing 70,000 dalton precursor to the 52,000 dalton set of pI isomers goes to completion and the germinative degradation of the storage protein insues. In contrast, in dissected embryos whose precocious germination is arrested by the plant growth regulator Abscisic Acid (as happens *in vivo* in the intact seed in late embryogenesis), storage protein synthesis stops, the processing of the precursor continues to completion, but no degradation of storage protein occurs. It is curious that storage protein synthesis ceases upon dissection of embryos from the maternal environment even when incubated in Abscisic Acid. It must indicate that the mRNA set for these proteins is not maintained by the growth regulator, but is controlled by other influences. Since the answers to these phenomena comprises our Proposed Research, they are discussed further in that section.

2. Plans for the continuation of present objectives and possible new objectives in consideration of past results. See Proposed Research in Renewal Proposal.

3. a. Graduate Students Trained.

Ms. Antonieta Capdevilla, M.S.

b. Post doctoral tenures completed.

(1) Dr. Larry Goldstein; present address: Department of Cell Biology, University of Texas Medical Center, Galveston, Texas.

(2) Dr. Machi Fukuyama Dilworth; present address: Assistant Program Director, Developmental Biology, National Science Foundation, Washington, D. C.

c. Current Post Doctoral Associates.

(1) Dr. Glenn Galau, Ph.D., California Institute of Technology

(2) Dr. Sally Greenway, Ph.D., University of Wales, Cardiff

4. Publications derived from Research during 3 years Contract period.

1. Capdevilla, A. M. and Dure, L. S. The developmental biochemistry of cotton seed embryogenesis and germination. VIII. Free amino acid pool composition during cotyledon development. Plant Physiol. 59, 268-273 (1977).

2. Dure, L. S. and Harris, B. Translation Control in Dicot Seeds. In Nucleic Acids and Protein Synthesis in Plants (L. Bogorad and J. Weil, eds.) Plenum Press, London, pp. 279-292 (1977).

3. Dure, L. S. and Walbot, V. Interspersion of Repetitive and Non-repetitive Sequence Elements in the Genome of Cotton. In CNRS Colloquim No. 261 Nucleic Acids and Protein Synthesis in Plants, pp. 27-33 (1977).

4. Dure, L. S. Stored messenger RNA in Germination. In The Physiology and Biochemistry of Seed Dormancy and Germination (A. A. Khan, ed.) Elsevier/North Holland Biomedical Press, Chapter 15, pp. 335-345 (1977).

5. Harris, B. and Dure, L. S. Developmental Regulation in Cotton-seed Germination: IX. Polyadenylation of Stored mRNA. Biochemistry 17, 3250-3256 (1978).

6. Dilworth, M. F. and Dure, L. S. The developmental biochemistry of cottonseed embryogenesis and germination. X. Nitrogen flow from Arginine to Asparagine in Germination. Plant Physiol. 61, 698-702 (1978).

7. Dure, L. S. Role of Stored Messenger RNA in late embryo development and germination. In Plant Seeds: Development, Dormancy and Germination (E. Rubenstein, ed.) Academic Press, New York (In Press).

8. Dure, L. S., Capdevilla, A. M. and Greenway, S. C. Messenger RNA domains in the embryo genesis and germination of Cotton Cotyledons. In Genome Organization and Expression in Plants (C. Leaver, ed.) Plenum Press, London. (In Press).

5. Present state of knowledge in this area. See Renewal Proposal.

6. Present division of federal support for our overall research program. See Renewal Proposal.

STATEMENT OF CURRENT EXPENDITURES

	Total to Date	Expected to Spend to End of Contract	Total Actual & Estimated Cost	Balance on Hand End of Contract
Personal Services	7,184	4,316	11,500	-0-
Staff Benefits	415	1,481	1,896	+347
Travel (Domestic)	-0-	300	300	-0-
Expense	959	-0-	959	-347
Equipment	-0-	-0-	-0-	-0-
Indirect Costs	1,910	4,645	6,555	-0-

Total Expenditures to Date	10,468
Total Estimated Expense to End of contract	10,742
Total Actual & Estimated Cost for Budget Period	21,210
Balance on Hand at End of Contract	-0-

Appendix

MESSENGER RNA DOMAINS IN THE EMBRYOGENESIS AND GERMINATION OF COTTON COTYLEDONS

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INTRODUCTION

We have begun the task of describing the development of cotton cotyledons through embryogenesis and germination in terms of the mRNA subsets that, through their appearance and disappearance, determine this developmental sequence. This, of course, represents an attempt to describe a developmental sequence in terms of sequential gene activity.

Initially, we wish to establish a fundamental molecular basis for this developmental sequence so that its regulation takes on a more definitive biochemical perspective. Ultimately, we hope to isolate mRNAs representative of discrete developmental stages and, through them to isolate their genes, so as to examine their unique features that govern their expression.

Our approach to this description has been, in part, to delineate cotyledon development in terms of the changing protein populations that determine the ontogenic progression. Due to the limitations of the techniques available for studying protein populations only the rather abundant proteins of the total population can be catalogued. The total genetic complexity of the mRNA population existing at a given developmental stage, and the extent to which it is included in the population from other stages, is being determined by other means. These measurements will not be presented today. What we wish to present here are some preliminary findings concerning the mRNA subsets active during cotyledon development as described by catalogs of their protein products.

CATALOGS OF PROTEINS DURING DEVELOPMENT

We have begun the description of the abundant members of mRNA subsets by compiling the following protein catalogs at selective points during the developmental sequence.

1. Catalog of extant proteins . . Coomassie staining of 2D gels
2. Catalog of proteins synthesized in vivo . . . fluorography of 2D gels
3. Catalog of proteins synthesized in vitro from purified RNA fractions . . . fluorography of 2D gels.
4. Determination of RNA complexity and sequence overlap during development. . . RNA:cDNA hybridization.

The first of the catalogs involves extracting protein from cotyledons in various solvent systems at time points during development and separating them by electrophoresis in one dimension by the discontinuous sodium dodecyl sulfate (SDS) system of Laemmli[1] or by the two dimensional electrophoretic system of O'Farrell[2] in which the proteins are first separated on cylindrical gels by migration to their respective isoelectric points and then electrophoresed in the presence of SDS into a discontinuous slab gel where they separate by virtue of their differences in molecular weight. The separated proteins are visualized by staining with Coomassie Brilliant Blue R-250. This technique displays only the more abundant proteins of the tissue, and the thousands of enzymes whose concentration in the tissue is likely to be low are not observed. Furthermore, such a display of proteins is not a direct measure of mRNA population since many proteins are likely to be stable and to accumulate and persist after their mRNAs have disappeared. Likewise rapidly turning over proteins would under-represent their mRNA concentration. Nevertheless, such displays do allow for a developmental description of the tissue in terms of extant proteins.

The second catalog presents a measure of the mRNA population present at a given developmental point in terms of the proteins being synthesized in vivo during the time interval that the tissue is exposed to radioactive amino acids. Here, the tissue is exposed to the labelled amino acids for brief periods at points during ontogeny after which the proteins are extracted and separated by the two dimensional electrophoretic system. The location on the slab gel of the proteins synthesized during the incubation period (radioactive proteins) are displayed by the fluorographic techniques of Lasky and Mills[3]. In order to follow the processing or degradation of certain proteins, the cotyledons are further incubated in the absence of radioactive isotopes in some instances (pulse-chase labelling).

The third catalog involves purifying RNA fractions from cotyledons at discrete developmental points and allowing these fractions to direct *in vitro* translation using both the rabbit reticulocyte and wheat germ systems[4,5]. The radioactive protein products are separated and visualized as are the *in vivo* synthesized products.

DEVELOPMENTAL STAGES CATALOGUED

The basis for choosing particular time points in the ontogeny of cotton cotyledon tissue for study stems from earlier work on the developmental biochemistry of this tissue in our laboratory[6,7,8]. This developmental framework is depicted diagrammatically in Figure 1. The upper half of the figure indicates the developmental points at which the extant proteins are extracted for the stain catalog and at which cotyledons are pulse-labelled for the catalog of the proteins being synthesized *in vivo*. The lower half shows that at the same developmental points RNA is extracted for translation in the cell-free systems to establish the mRNA populations directly by *in vitro* protein synthesis.

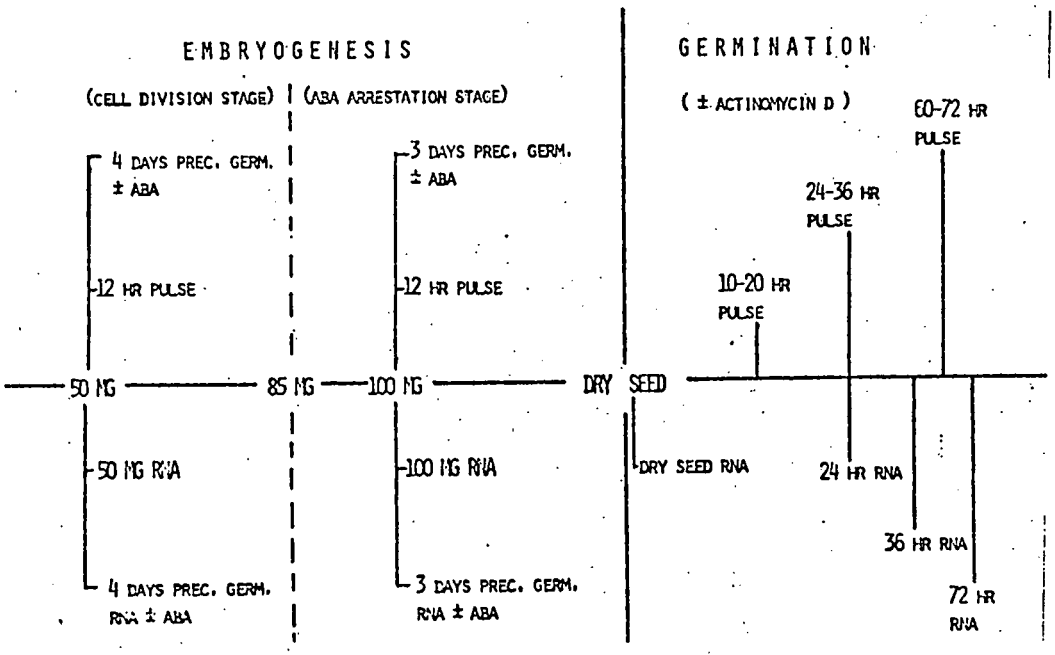

Fig. 1. Diagrammatic presentation of the developmental points studied. Top half indicates preparations made for stain and *in vivo* synthesis catalogs. Bottom half indicates preparations of mRNA made for *in vitro* synthesis catalog.

Cotyledons from embryos 50 mg in weight are in the midst of their logarithmic phase of growth, and the outer seed tissues are

in vascular connection with the mother plant. Cell number is increasing at this point and the storage nutrients are accumulating. ABA levels are very low at this point. If embryos are removed from the boll at this point and placed on moist filter paper they will precociously germinate[7]. However, removal brings about an immediate cessation of cell division in the cotyledons which causes the resultant seedling to have half-sized cotyledons. An ABA solution of 10^{-6}M applied to the filter paper totally prevents this precocious germination. For this reason the catalogs have been extended to include the possible changes in mRNA subsets related to the cessation of cell division, to precocious germination or to ABA arrestation. All of these phenomena have occurred by the 4th day after removal of embryos from the seed tissue.

When the embryos have reached 100 mg in wet weight they have entered the maturation phase of embryogenesis. (Final embryo wet weight is about 125 mg). The vascular connection between the mother plant and the seed has atrophied and cell division has stopped. ABA levels have risen markedly, presumably to prevent "vivipary"[8]. These embryos precociously germinate readily when excised from the seed tissues, and, again this is prevented if the immature embryos are placed on filter paper moistened with an ABA solution. Again the catalogs have been extended to include measurements of changes occurring during precocious germination. Cotyledons from excised embryos at this stage of development incubated in the ABA solution are of interest since these embryos have been removed from a high ABA environment in the seed.

The dessicated dry seed represents a special developmental stage. The mature cotton seed has no form of dormancy to overcome. Its cotyledons contain mRNApoly(A)[9], and it is of interest to know if this RNA is still functional after several hours of germination or whether it is simply residual embryonic RNA that is rapidly degraded as has been suggested by earlier experiments[9].

With germination many new enzyme activites are required in cotyledons, and many of these activities are known to result from the de novo synthesis of both mRNA and protein during this period[10,11,12]. Other new enzyme activities appear as the result of de novo protein synthesis but without requiring concomittant RNA synthesis[8,13]. For this reason the catalogs include data from cotyledons germinated in the presence of actinomycin D so as to determine those proteins that may arise from pre-existing but unexpressed mRNA (stored mRNA).

Finally, during the 3rd day of germination, proteins coded for by the chloroplast genome become detectable, and, since their

mRNAs are not likely to have poly(A) sequences, they may be made apparent by the fact that all of the in vitro cell-free translation is carried out with poly(A)-RNA as well as polyA+ RNA at all the developmental stages.

With this developmental scheme in mind, it is possible to hypothesize the existance of certain mRNA subsets at the outset; each subset under different regulatory influences as to time of synthesis and disappearance but whose concerted, integrated and overlapping functioning insures cotyledon embryogenesis and germination. It is anticipated that the catalogs will indicate the members of such subsets. A rather small number of such putative subsets are listed below.

CONCEIVABLE mRNA SUBSETS

Embryogenesis	Representative protein
1. functional only during cell division stage	actins
2. functional only during ABA arrestation stage (ABA induced?)	
3. functional throughout embryogenesis	
4. non-functional during ABA arrestation	stored mRNA derivatives
5. constitutive (independent of developmental events)	enzymes of intermediary metabolism
Germination	
1. Residual	
2. Stored	carboxypeptidase C
3. Newly synthesized, unique to germination	asparagine synthetase, glyoxylate cycle enzymes
4. Newly synthesized, constitutive	enzymes of intermediary metabolism

LIMITATIONS AND POTENTIAL ARTEFACTS OF TECHNIQUES

Before presenting some of our data it is well to emphasize the constraints and limitations of the techniques used and some of the sources of artefact in these procedures so that the data may be viewed with the proper perspective.

First, as has been mentioned, only the 200-400 most abundant proteins are made readily visible in these procedures. Furthermore, only those proteins whose pIs are between pH 7.5 to 4.5 are focused in the first dimension. Many proteins have pIs beyond this range as indicated by the pile up of stain and radioactivity often seen on the edges of the slab gels. Technique for visualizing these proteins have been developed ("NEPHGE gels"[14]), but have not yet been incorporated into these studies. Next, quite a different display of proteins is observed when radioactive methionine is used as the protein precursor in comparison with radioactive leucine or amino acid mixtures. Proteins with inordinate amounts of methionine attain very high specific radioactivity that is not a true indication of their rate of synthesis in vivo or in vitro. The presence of the N-terminal methionine on in vitro labelled proteins, but its likely absence on these labelled in vivo contributes to this distortion of synthesis.

A further difficulty is encountered when in vivo and in vitro synthesis patterns are compared. Not a great deal of match-up is observed. This makes identifying identical proteins detected by the two methods hazardous. The failure of the two displays to indicate common radioactive proteins is likely to be due to the absences of the processing events in the in vitro translating systems. Large differences in the intensity of identical proteins between the two types of measurements may also indicate a rapid turnover of certain proteins in vivo (failure to accumulate although their mRNA is in high concentration) or simply result from the fact that translation of certain mRNAs may be regulated in some fashion in vivo, whereas such regulation is missing in in vitro translation.

RESULTS

Our catalogs are far from complete at present. Much of the in vivo synthesis catalog during germination remains to be completed. Much of the data is yet to be analyzed in detail. However, from the data collected to date, several interesting observations have accrued, and it is these observations we wish to present here.

PROCESSING OF THE PRINCIPAL STORAGE PROTEINS

Figure 2A presents a one dimensional view of the proteins present in the cotyledons of a mature cotton seed. Early on we found it convenient to divide the total protein of this tissue to that which is readily soluble (extracted in 0.1 M NaCl, pH 8.3) from that which requires high salt (0.5 M NaCl) or 6 M urea or 2% SDS or 2% deoxycholate to make soluble. The left hand gel well

contains total cotyledon protein (extracted 2% SDS), the center well contains the readily soluble fraction (0.1 M NaCl extract) and the right hand well contains the protein that can be pelleted at low speed from the 0.1 M NaCl homogenate. The two principal storage proteins are seen to be in the pellet fraction and to comprise together about 25% of the total cotyledon protein.

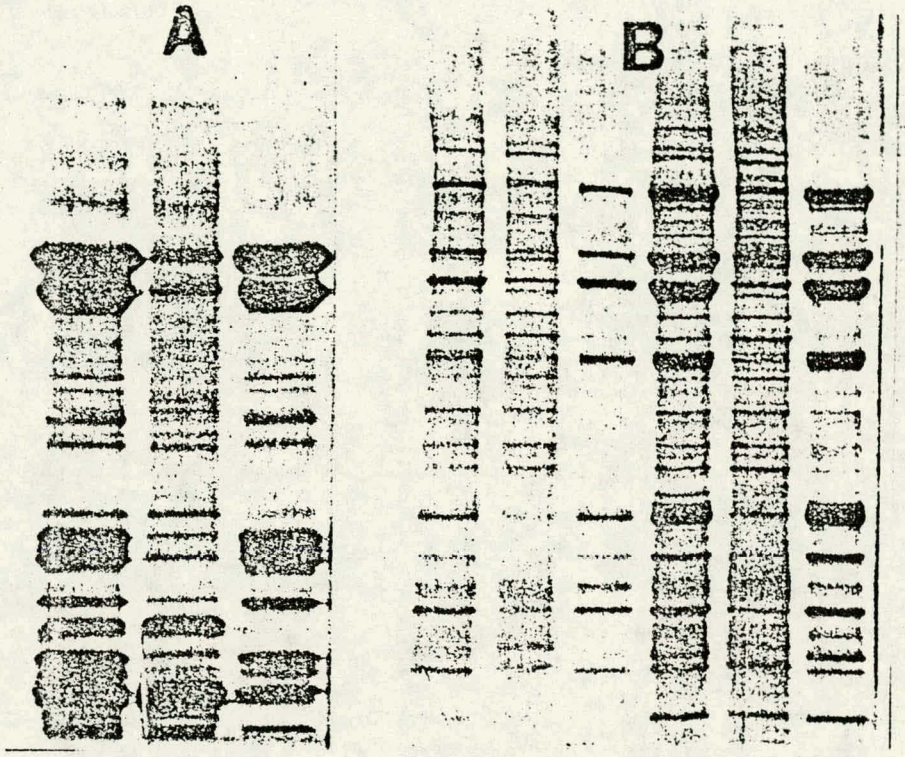

Fig. 2. Display of cotyledon proteins separated by discontinuous gel electrophoresis in SDS and stained for protein. In A, proteins from dry seed cotyledons; left well = total protein, center well = readily soluble protein, right well = pellet protein. In B, proteins from cotyledons of immature embryos. Left three wells are total, readily soluble and pellet proteins from cotyledons of 50 mg embryos. Right three wells are the same preparations from cotyledons of 100 mg embryos.

Figure 3 shows the one dimensional electrophoretic separation of total protein extracted from cotyledons during points in embryogenesis and germination. Several interesting phenomena are observed in this gel. Two principal storage proteins have apparent molecular weight of 53,000 and 48,000 daltons. It is apparent that the smaller of the two storage proteins appears first and accumulates faster initially than does the larger storage protein. It is also degraded much more rapidly in germination.

Further, several proteins that are abundant in embryogenesis vanish during the last days of embryogenesis and are undetectable in dry seed cotyledons. Two of these have molecular weights of about 70,000 and 40,000 daltons and are seen in Figure 2B to be confined to the pellet fraction.

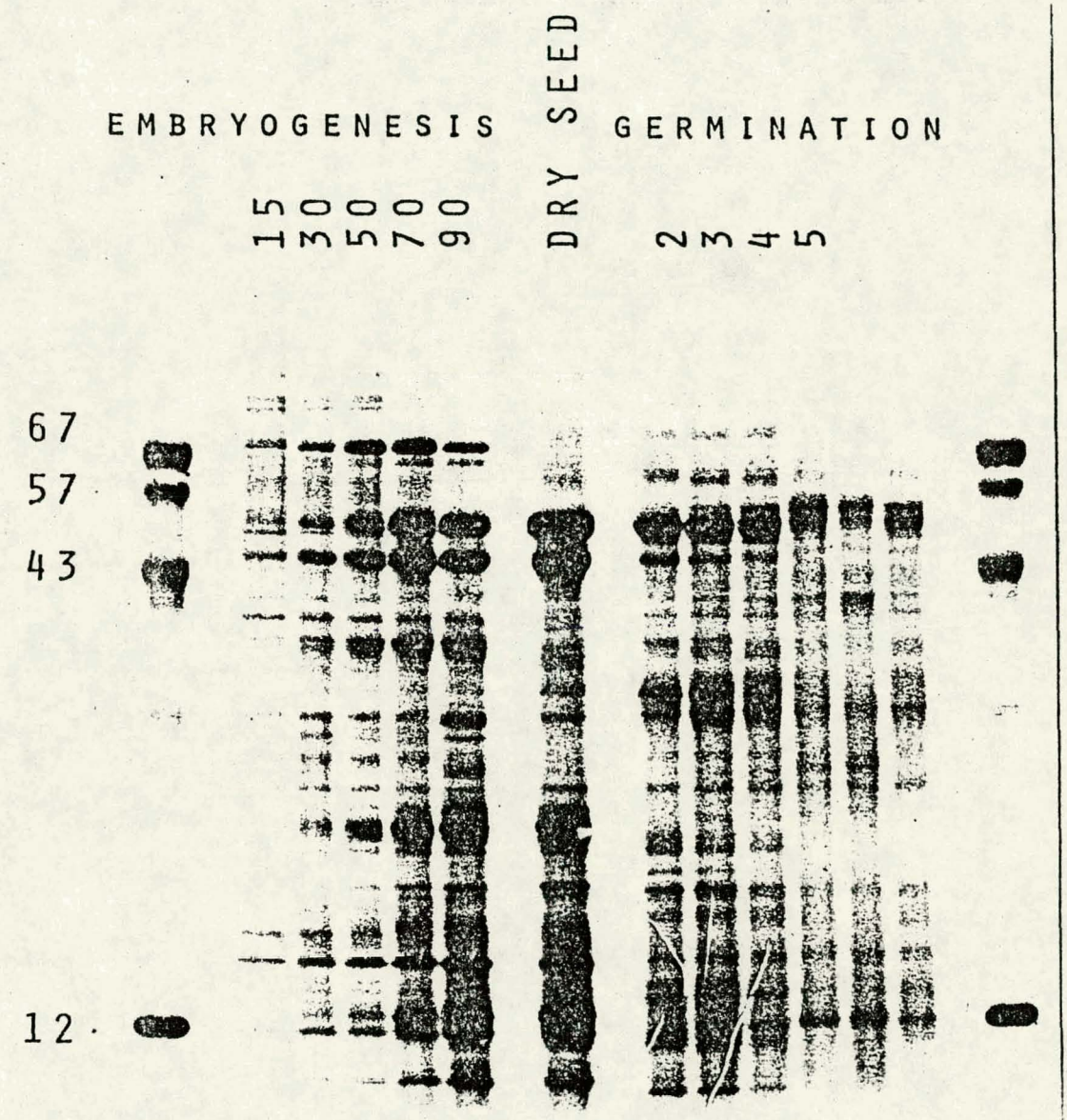

Fig. 3. Display of total cotyledon protein extracted at points during development. Electrophoresis as in Fig. 1. Numbers under EMBRYOGENESIS refer to the size in mgs of the embryos used. Numbers under GERMINATION refer to the days that the embryos were germinated. Numbers on the left margin are the molecular weights in kilodaltons of reference proteins.

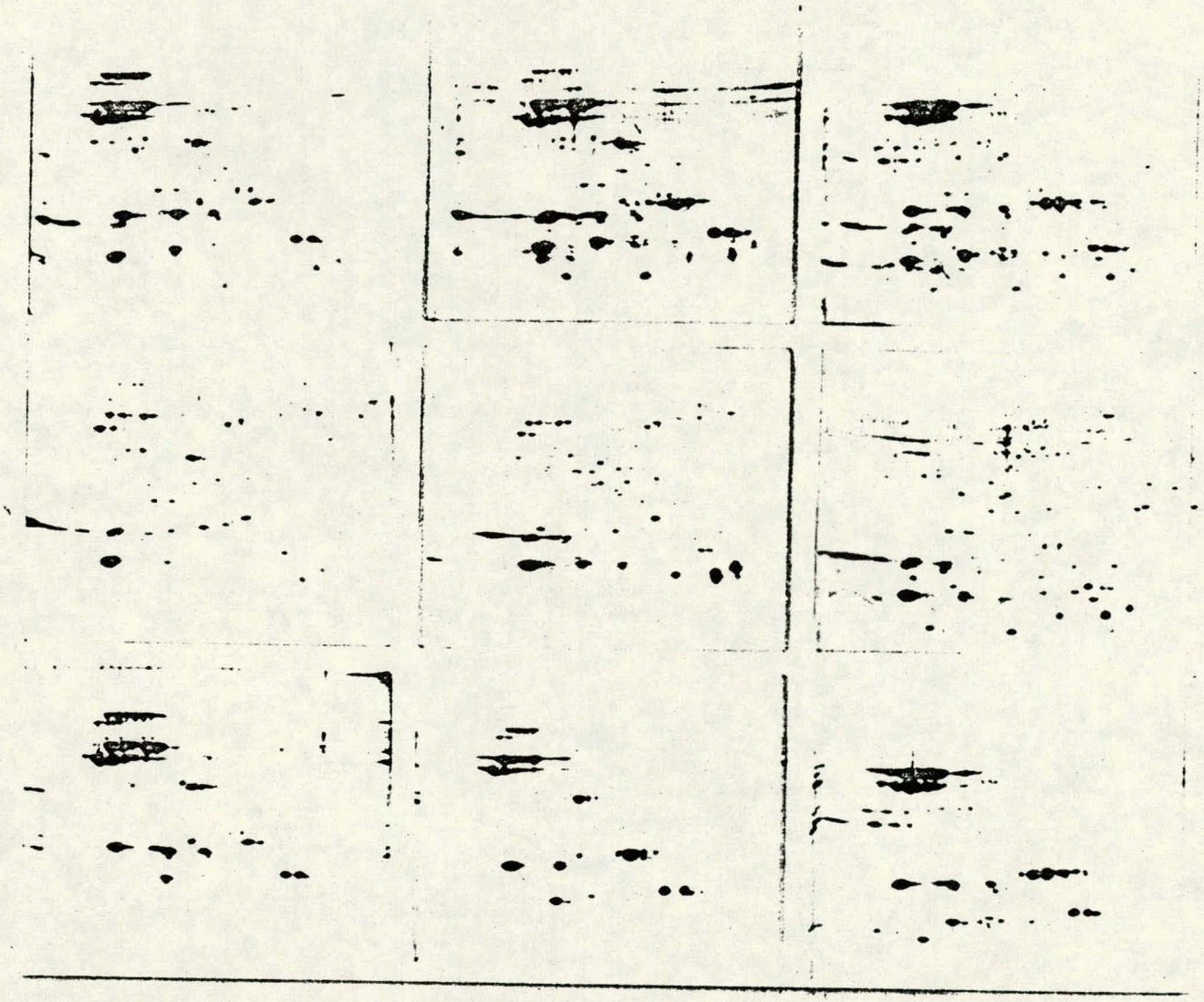

Fig. 4. Display of stained cotyledon proteins separated by the 2D system. Left hand panels are: top, total protein from 50 mg embryos; center, readily soluble protein from the same; bottom, pellet protein from the same. Middle panels are the same preparations from 100 mg embryos and the right hand panels are the same preparations from dry seeds.

Figure 4 is the two dimensional display of proteins from cotyledons from 50 mg, 100 mg and dry seed embryos. The principle storage proteins are found to have a range of pI heterogeneity, which subsequent data will show is probably not introduced during solubilization or electrophoresis artefactually. Again the 70,000 dalton protein is seen to disappear as the tissue matures as well as does the 40,000 dalton protein. The same pI heterogeneity found in the storage proteins is observed in the 70,000 dalton protein. The readily soluble fraction (supernatant fraction) is seen to have fewer abundant proteins than does the pellet fractions, and these are of rather low molecular weight. A number of changes are seen in this display of extant proteins during embryogenesis, but only a few of these have been studied in detail to date.

A number of changes occur in these populations during cotyledon embryogenesis; however, we have concentrated principally on the changes in the dominant storage proteins and the two disappearing proteins. Our initial in vivo translation studies for the second catalog yielded rather intriguing results which are presented in Figure 5. Here, pellet proteins from 100 mg cotyledons that have been incubated 6 hours in ^{14}C amino acids have been electrophoresed in two wells in the Laemmli one dimensional gel system. An autoradiogram of these two wells is on their right. (The gel is highly overloaded to increase its radioactivity). The two principal storage proteins are grotesquely obvious in the stained wells. The autoradiograph shows extensive synthesis of the smaller storage protein, but no apparent radioactivity in the larger. This was curious at the time, since, at this point in embryogenesis, the larger protein is accumulating faster than the smaller one (Figure 3). Furthermore, the figure shows extensive labeling of the 70,000 dalton protein, which, although an abundant protein at this developmental stage, is in the process of vanishing from the tissue at this time. Obviously its synthesis continues into the period of its degradation or processing.

A reasonable conjecture at this point would be that the larger storage protein is synthesized initially as the 70,000 dalton species which is cleaved in a rather slow process. To test this idea we carried out a series of pulse-chase incubations of 50 mg cotyledons in which they were pulsed for 6 hours, and incubated thereafter for varying time periods without isotopes before being harvested. These and other data are presented in Figure 6. At the top left of this composite is shown a stained two dimensional gel of pellet protein from 50 mg cotyledons. At the top right is an identical gel stained for carbohydrate by the PAS method[15]. The larger storage protein and its 70,000 dalton putative precursor are found to be glycoproteins, and to be the only glycoproteins demonstratable in the pellet fraction by this method. In the middle of the left panel a fluorograph of pellet protein

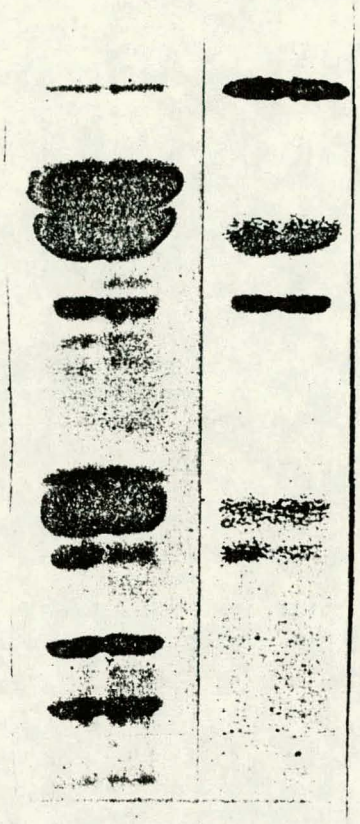

Fig. 5. In vivo protein synthesis in cotyledons of 100 mg embryos. Left two wells are the electrophoretic separation of pellet proteins. The right two wells are autoradiographs of the left wells.

from 50 mg cotyledons that have been pulsed 6 hrs and chased 12 additional hours is presented. Radioactivity is seen here in the larger storage proteins, but the various pI isomers do not have the same specific radioactivity. To the right of this gel is given an expanded view of fluorographs of the storage protein region of the two dimensional gel from gels containing protein from cotyledons pulsed 6 hrs and chased 6, 9, 15 and 24 hrs. From this it is apparent that the larger storage proteins gain radioactivity with time, and the 70,000 dalton proteins appear to lose radioactivity. These data reinforce the precursor-product idea; however, to firmly establish this relationship we utilized immunochemical techniques.

First, the large storage proteins were purified and antibodies against them prepared in rabbits by conventional means. Figure 7, left side, shows a typical titration of the antibody preparation by the "rocket" technique[16]. Shown in the right hand side of this figure is the result obtained when total cotyledon protein from 50 mg cotyledons, separated by one dimensional electrophoresis, is electrophoresed into the antibody containing region of the

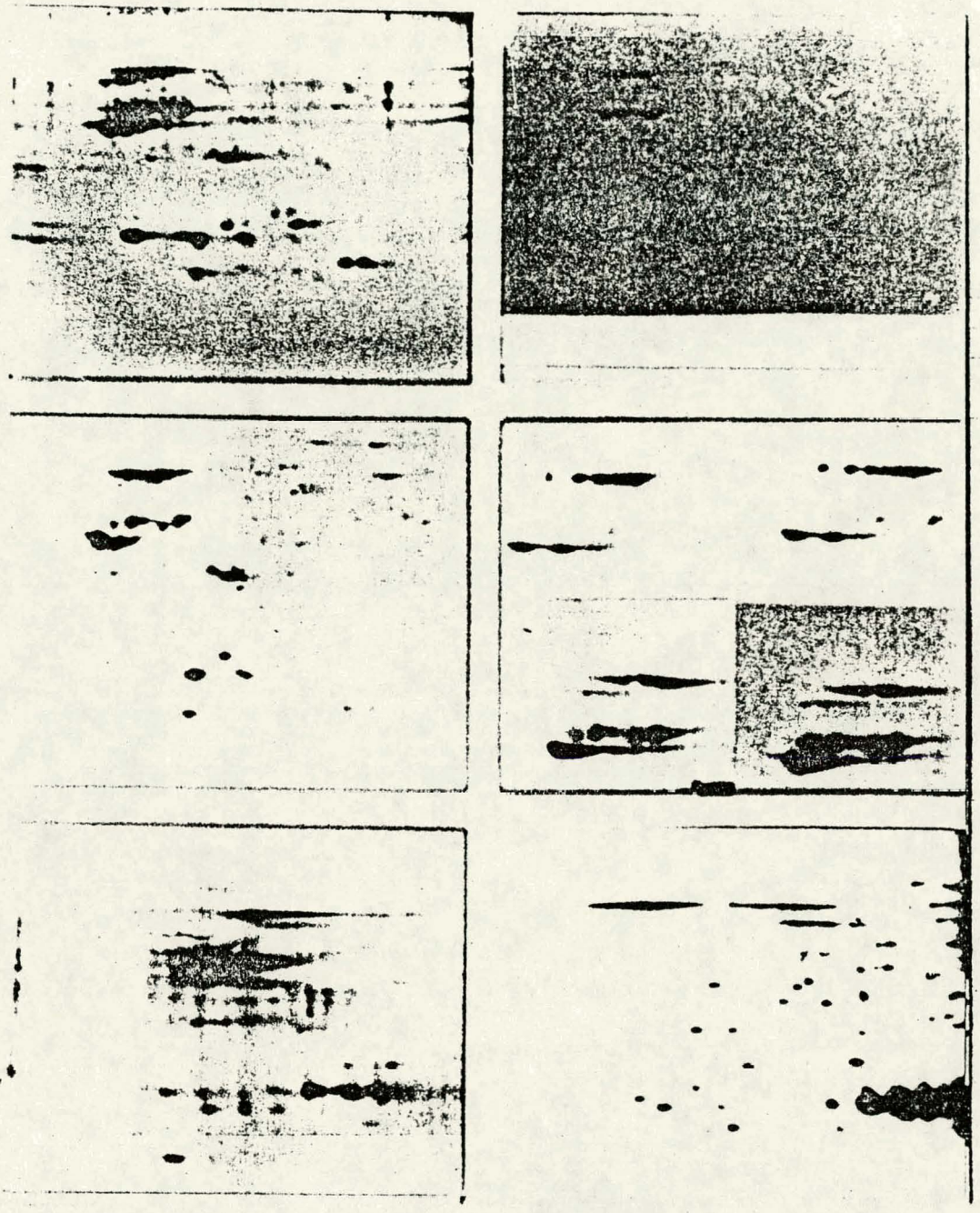

Fig. 6. Top, left: stained pellet protein from 50 mg embryos. Top, right: same preparation stained for carbohydrate. Middle, left: Fluorograph of the same preparation incubated in radioactive amino acids for 6 hrs and chased for 12 hrs. Middle, right: expanded views of fluorographs of storage protein area of gels. Bottom panels: In vitro synthesized proteins from total RNA from 50 mg cotyledons. Left = ^{3}H leu; right = ^{35}S met.

gel by the method of Chua and Blomberg[17]. Two regions of the gel containing the separated cotyledon proteins are seen to contain proteins that immunoprecipitate the antibodies, and these regions contain the 70,000 dalton protein and the larger storage protein which served as the antigen. From this it is apparent that the larger storage protein species are derived from the 70,000 dalton species.

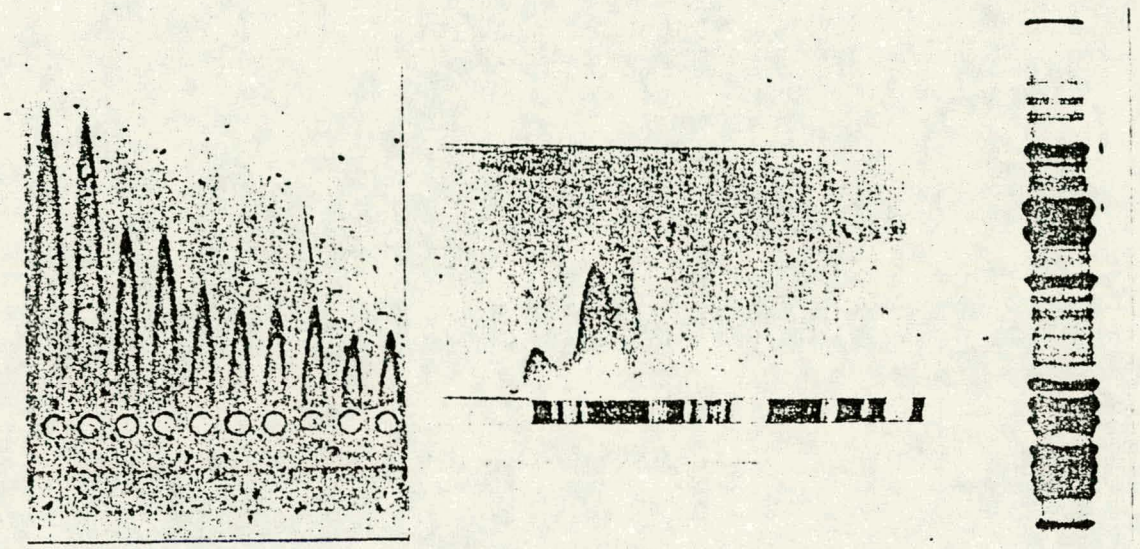

Fig. 7. Left: Rocket immunoelectrophoresis of antigen (large storage protein) vs. rabbit antiserum. Stained gel. Middle and right: Total protein from 50 mg cotyledons was electrophoresed as shown on far right. A longitudinal section of the 1D gel was electrophoresed into antiserum to the large storage protein[17]. Both are stained gels.

Data gathered for the third catalog (in vitro translation products) allowed us to attempt to identify the initial translation products that give use to the 70,000 dalton species. Since the processing of initial translation products into glycoproteins probably does not occur in the wheat germ systems, the initial products of the mRNA for the 70,000 dalton pI isomers would not be expected to co-migrate with these proteins. The bottom left of Figure 6 is the fluorograph obtained when total RNA from 50 mg cotyledons is translated in the wheat germ system and its products electrophoresed. In this instance ^{3}H leucine was used as the radioactive precursor. A band of proteins of about 60,000 molecular weight and showing pI heterogeneity is found to the acidic side of the storage proteins and could be considered the unglycosylated precursor of the 70,000 dalton

proteins. (In this fluorograph, non-radioactive protein from dry seed cotyledons has been included in the preparation separated by the two dimensional system. The mature unlabelled storage proteins can be seen on the fluorograph as white areas since very abundant proteins tend to exclude other proteins from their region of the gel). However, when the experiment is carried out with ^{35}S methionine (bottom right-hand panel) other potential precursor bands show up. These proteins obviously have a different ratio of methionine to leucine than does the band seen on the left hand panel. We have not yet resolved the question of the identity of the precursor of the 70,000 dalton proteins. There does not appear to be an easily demonstrable immunochemical relationship between the larger and smaller storage proteins. However, the glycosyl residues of the larger storage proteins may have played a determinative role in antibody production, which might leave the unglycosylated smaller storage proteins unreactive.

PROTEIN CHANGES DURING PRECOCIOUS GERMINATION

As previously mentioned our catalogs include changes in cotyledon proteins occurring during the precocious germination of 50 and 100 mg embryos. This portion of the catalogs also includes protein changes that may take place in cotyledons from these dissected embryos whose precocious germination is artifically arrested by ABA. Figure 8 shows the stained catalog of these changes. The left hand column shows from top to bottom, total protein from cotyledons of ungerminated 50 mg embryos, from these cotyledons after 4 days of precocious germination and from cotyledons incubated 4 days in ABA. In both sets of dissected embryos the 70,000 dalton precursor has vanished. The 40,000 dalton protein is also disappearing in both sets. Some loss of the two principal storage proteins is evident in the precociously germinating cotyledons as well as of the many other abundant proteins. This loss has not occurred in the ABA treated cotyledons. The middle column shows the same sequence for the 100 mg embryo cotyledons. In this case precocious germination ±ABA was carried out for 3 days. Again the 70,000 dalton and 40,000 dalton proteins disappear in the germinating cotyledons and are decreased in those treated with ABA. When these two sets of embryos are incubated for 2 additional days (bottom two panels of the right hand column of the figure) the changes are more apparent. The germinating cotyledons show a great loss of storage protein and other abundant proteins, whereas those treated with ABA have lost the 70,000 and 40,000 dalton proteins, but none of the others.

Of particular interest in this figure is the accumulation of several new proteins in the ABA-treated cotyledons (enclosed in pen). Some of these proteins are not apparent in 50 or 100 mg embryos nor in those precociously germinating. However, they are

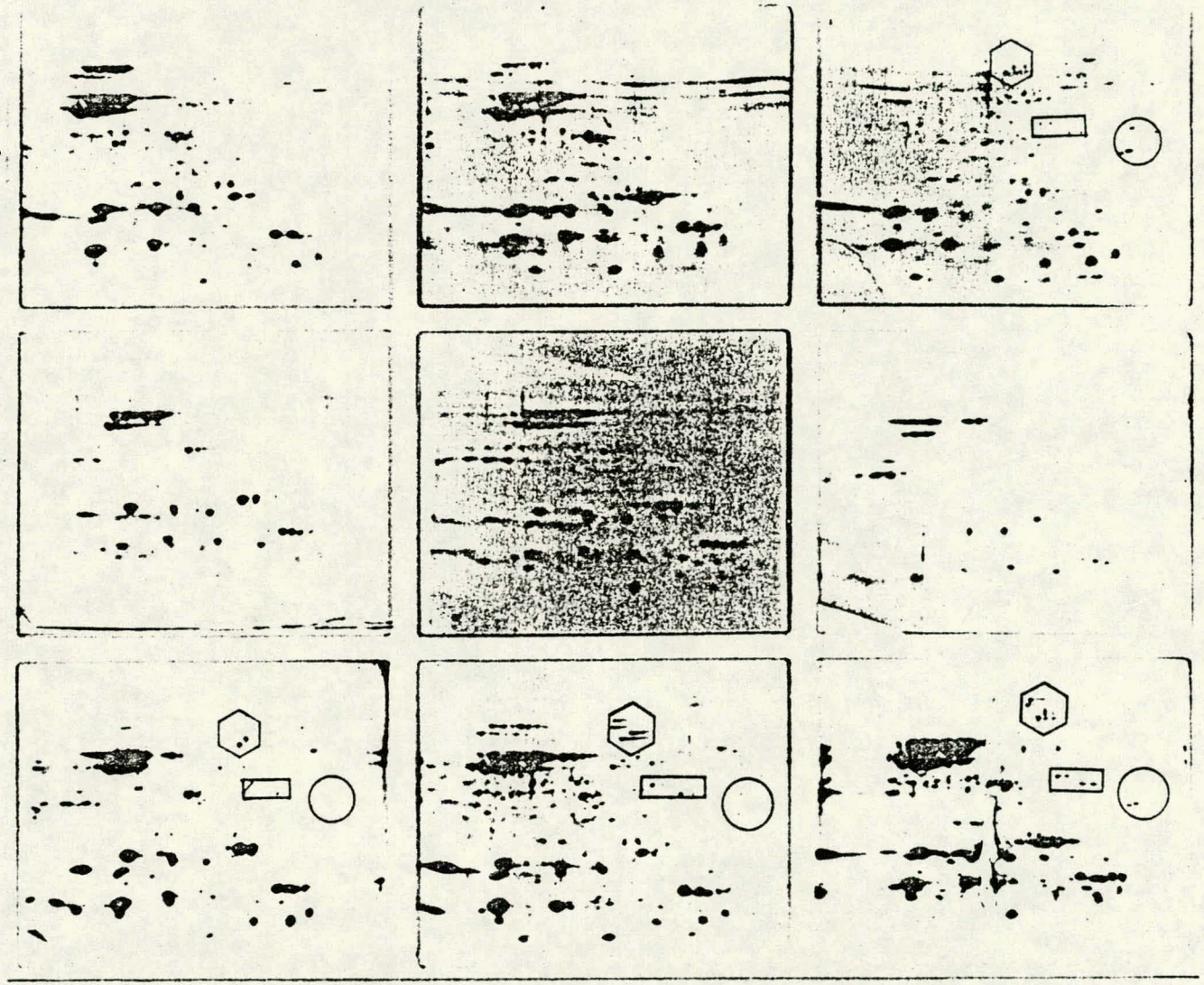

Fig. 8. Stained gels of total cotyledon protein. Left top, from 50 mg embryos; center, from those germinated 4 days; bottom, from those incubated 4 days in ABA. Center. top, from 100 mg embryos; center, from 100 mg embryos germinated 3 days; bottom. from 100 mg embryos incubated 3 days in ABA. Right top, from the readily soluble fraction of dry seeds; center, from 100 mg embryos germinated 5 days; bottom. from 100 mg embryos incubated 5 days in ABA.

found in the mature seed as shown by the upper right hand panel which is the readily soluble protein from dry seed cotyledons.

Figure 9 shows the protein synthesized in vivo by cotyledons from 50 and 100 embryos during precocious germination ±ABA. The 50 mg sequence is on the left hand side. From top to bottom are shown total radioactive proteins from cotyledons of embryos transferred to isotope containing solutions for 6 hours immediately after dissection and then harvested (these show the proteins being synthesized in situ at these stages in development) followed by the radioactive proteins from precociously germinating cotyledons and finally those from ABA arrested cotyledons. In the latter two cases the 50 mg embryos were incubated 4 days and the 100 mg embryos 3 days. They were exposed to the isotope for the last 12 hours of the incubation period so as to determine the proteins being synthesized during the last phase of incubation.

The top panels show that both 50 and 100 mg cotyledons are synthesizing the 70,000 and 40,000 dalton proteins, although this synthesis is somewhat less in the older cotyledons. By the end of the incubation period, all of this synthesis has stopped in both sets of cotyledons, even in those treated with ABA. Many new proteins are being synthesized in the unarrested cotyledons; however, in the ABA arrested cotyledons the pattern is quite different. Much of the radioactivity is found in those proteins that were found to accumulate in late embryogenesis in the stain catalog. The salient question here is are any/all of these proteins induced by ABA? Some of these proteins are being synthesized in 50 mg cotyledons in situ when the endogenous ABA level is low. Their synthesis continues when their precocious germination is arrested by ABA but ceases when germination begins. Thus these proteins may be considered products of a mRNA subset that functions during the latter half of embryogenesis before and after ABA arrestation and that disappeared upon germination.

Some of the proteins found in the ABA treated cotyledons, however, are not synthesized in the younger cotyledons and may indeed result from exposure to ABA, appearing in late embryogenesis in situ and upon exposure of young embryos to ABA after dissection. These proteins we feel are the products of the mRNA subset induced by ABA and whose function is to prohibit immature embryos from germinating before seed maturation is complete. It has been known for some time that ABA inhibition of the synthesis of several developmentally important enzymes is, itself, a phenomenon that requires continued RNA synthesis[8,18].

We have said very little about the 40,000 dalton protein that disappears upon the dissection of immature embryos and disappears normally in late embryogenesis. We believe this protein to be one

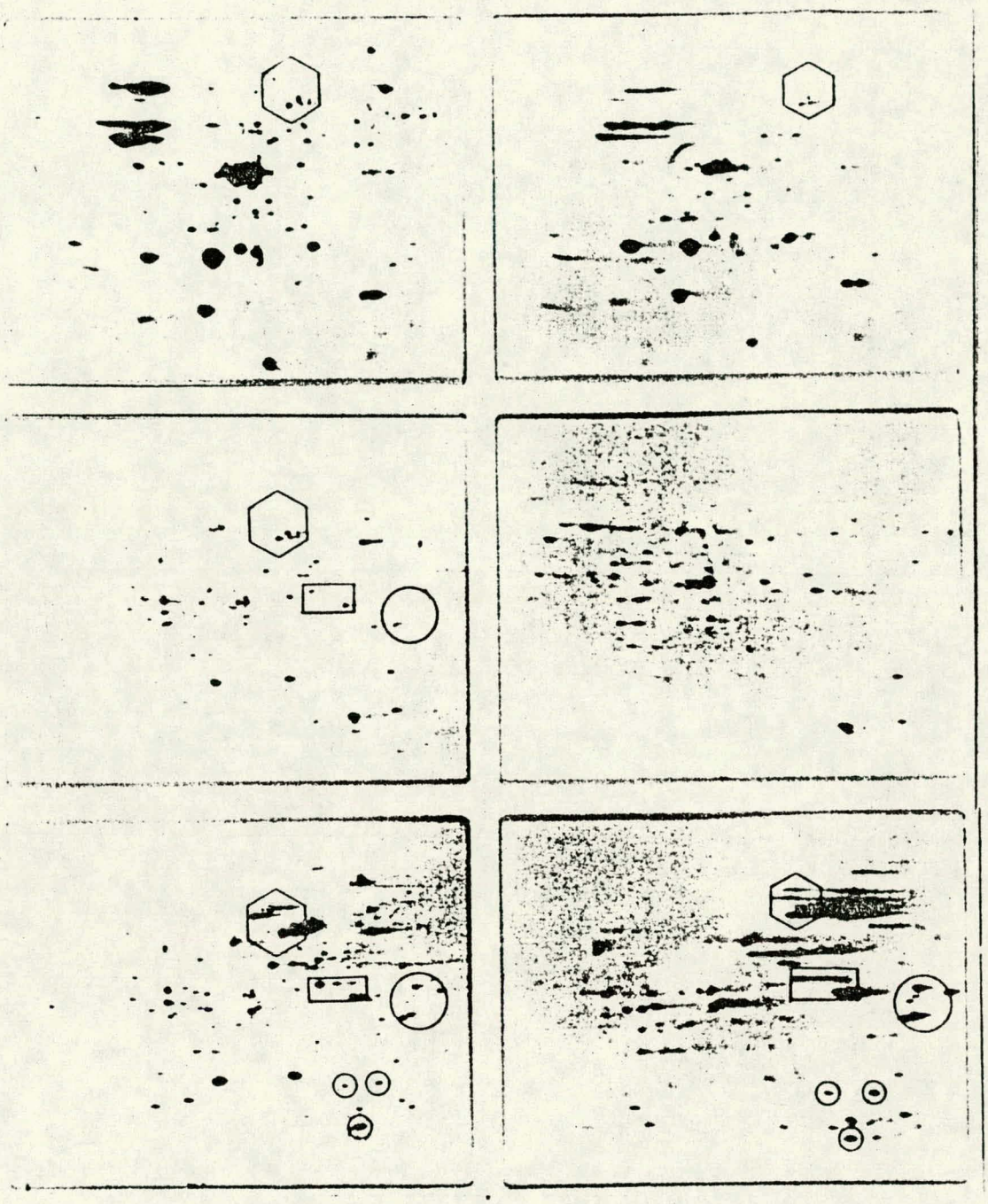

Fig. 9. Fluorographs of proteins synthesized in vivo. Left panels are from cotyledons of 50 mg embryos. Top, from embryos labelled briefly after dissection; middle from those labelled after 4 days germination; bottom, from those labelled after 4 days in ABA. Right panels are from cotyledons from 100 mg embryos. Treatments are the same, except germination and incubation was for 3 days.

of the plant actins whose presence is linked to cell division. Our rationale for this belief is summarized as follows:

PROPERTIES OF PURATIVE COTTON ACTIN

Chemical

1. Molecular weight = about 40,000 daltons
2. pI = 6.2 in 8 molar urea
3. Insoluble in dilute salts or Triton X-100
4. Soluble in SDS, DOC, 6 M urea, half molar salts
5. Solubilized by millimolar ATP, Ca^{++}
6. Precipitated by the addition of KCl at 0.1 M
7. Binds weakly to DNAse I-Sepharose

Biological

1. Abundant protein during cell division phase of embryogenesis.
2. Disappears during maturation phase.
3. In vivo synthesis demonstratable.
4. Synthesis ceases (as does cell division) and protein disappears when very young embryos are removed from seed.
5. Synthesis not maintained by ABA

SUMMARY

These data from both the stain and in vivo synthesis catalogs can be diagrammatically summarized as follows:

PROTEIN CHANGES IN COTYLEDON EMBRYOGENESIS

at 50 mg stage

1. low endogenous ABA
2. cell division occurring
3. storage protein synthesis and processing from 70K dalton precursor occurring
4. actin synthesis occurring

at 100 mg stage

1. high endogenous ABA
2. no cell division
3. storage protein synthesis and processing continuing
4. actin synthesis decreasing, actin disappearing

upon precocious germination

1. cell division stops prematurely in 50 mg embryos.
2. storage protein synthesis stops, degradation begins
3. processing of 70K dalton precursor continues until complete
4. actin synthesis stops, actin disappears
5. synthesis of embryogenic proteins stops, they disappear

6. germination proteins appear

upon incubation in exogenous ABA
1. cell division stops prematurely in 50 mg embryos
2. storage protein synthesis stops but no degradation occurs
3. processing of 70K dalton precursor continues until complete
*4. actin synthesis stops, actin disappears
**5. many embryogenic proteins continue to be synthesized
***6. ABA proteins appear

in the mature seed
1. all storage protein processed, 70K dalton precursor gone
2. actin has disappeared
3. embryogenic proteins evident
4. ABA proteins evident

*member of cell division mRNA subset which may depend on vascular connection

**members of embryogenic mRNA subset which functions during and after cell division phase, and is not dependent upon vascular connection or high endogenous ABA, disappears when germination program begins

***members of ABA induced mRNA subset, functions to prevent initiation of the germination program.

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