Chapter 29

SYNTHESIS AND COMPARTMENTATION OF ENZYMES DURING COTTONSEED MATURATION

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INTRODUCTION

An important event during postgerminative growth of oil seeds is gluconeogenesis from storage lipid (Figure 1). The process involves lipolysis of storage triglycerides within lipid bodies followed by activation and β-oxidation of fatty acids in the glyoxysomes. Acetyl-CoA, the product of β-oxidation, is shunted through the

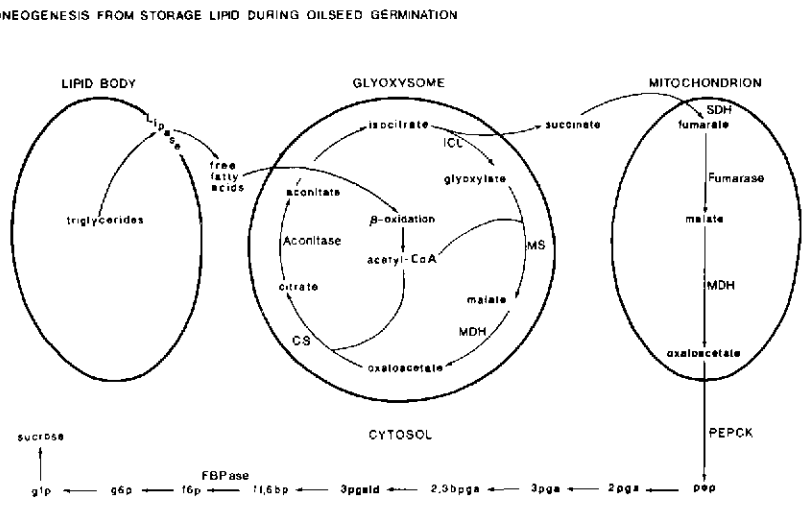

Figure 1. Diagrammatic representation of reserve lipid mobilization and gluconeogenesis following oilseed germination.

glyoxylate cycle producing succinate which presumably moves into the mitochondria and is converted to oxaloacetate (OAA). OAA is transported out of the

mitochondria and decarboxylated to phosphoenolpyruvate (PEP), which is converted to sucrose via several glycolytic enzyme reactions in the cytosol. This transport carbohydrate presumably moves from the cotyledons to the axis where it is used as a main carbon source for seedling growth.

Studies with several different oil seeds have shown that the two enzymes unique to the glyoxylate cycle, isocitrate lyase (ICL) and malate synthase (MS), develop *de novo* following germination (Beevers, 1979). Previous work with castor beans indicated that glyoxysomes housing these enzymes were synthesized together during postgerminative growth (see Beevers, 1979, for review). Interpretation of these studies led to the generalization that glyoxylate cycle enzymes are absent or have negligible activity in storage tissues of all ungerminated oil seeds (Beevers, 1979). Data presented in this paper, however, indicate that glyoxysomes containing all of the glyoxylate cycle enzymes (except ICL) are synthesized during cottonseed maturation and persist in dry cotyledons prior to germination. *De novo* synthesis of enzymes does occur during postgerminative growth but likely are added post-translationally to organelles synthesized during maturation and carried over into germinated seeds.

Ihle and Dure (1972) concluded from experiments with Actinomycin D (Act D) that the *de novo* synthesis of ICL and carboxypeptidase C during cottonseed germination was from pre-existing mRNAs stored in dry seeds. Since these enzymes were unique to germination, i.e., were not enzymes found in cotyledons during maturation, they postulated that ICL and carboxypeptidase C were representative of an entire body of similar "germination" enzymes whose synthesis was regulated in concert from mRNAs transcribed during maturation, but not translated until commencement of germination. In a proposed developmental scheme for cottonseed embryogenesis, Ihle and Dure (1972) suggested that transcription of this mRNA occurred shortly after natural atrophy of the vascular connection (funiculus) between the ovary wall and ovular tissue surrounding the developing embryos (at approximately 32 days postanthesis, DPA). Loss of vascular flow from the mother plant was presumed responsible for cessation of embryonic growth (cell division) and derepression of germination cistrons. Abscisic acid (ABA), apparently synthesized in the ovular tissues and absorbed by the embryos at about this time (DeLanghe and Vermeulen, 1972), was implicated in suppressing translation of the germination mRNAs, thus preventing vivipary of the embryos in maturing bolls. The overall scheme describes several developmental events which are thought to be part of the preprogramming of the seed for successful germination (Dure, 1975; Harris and Dure, 1978; Ihle and Dure, 1972).

Smith *et al.*, (1974) and Radin and Trelease (1976) independently reported that treatment of mature seeds and excised embryos with Act D *did* inhibit appearance of ICL activity. Both groups attributed the disparity between their results and those of Ihle and Dure to the method of treating embryos with Act D prior to germination. Furthermore, Schubert and associates (Chapter 22) and

Stewart (Chapter 20) have strong evidence that the funiculus does not break at 32 DPA, but remains intact, transporting carbon from the mother plant into the embryo sink until approximately 45-50 DPA. These results clearly are inconsistent with the developmental scheme proposed by Ihle and Dure (1972). There is ample evidence that a body of mRNA pre-exists in dry cotton seeds and that this mRNA needs to be further processed (e.g. by polyadenylation) for normal germination to commence (Dure, 1977; Hammett and Katterman, 1975; Harris and Dure, 1978). However, the conflicting results outlined above and data presented in this paper indicate that the specific role of this conserved mRNA should be reassessed. Moreover, it appears that the timing and control of developmental events related to preparation of the seed for germination must be reevaluated.

Over the past ten years our laboratory has been involved in studies aimed at understanding the relationship between activities of lipid-mobilizing and gluconeogenesis-related enzymes and their subcellular localization within cotyledons of various oil seeds. Results from this work have led us to conclude that there must be certain developmental events that occur during oilseed embryogenesis that would prepare the seed for the massive reserve mobilization that occurs during postgerminative growth. Dure's (1975) and Katterman's (Hammett and Katterman, 1975) laboratories have contributed considerable information on the molecular biology of nucleic acids involved in cottonseeds (see Chapter 28). Benedict's group (Chapter 22) has provided important information on carbon fixation and transport in developing bolls, and hopefully our laboratory can provide meaningful data on enzyme activity and organellar localization as they pertain to the physiology and biochemistry of developing cotton embryos.

METHODS

GROWTH AND SELECTION OF PLANTS

Cotton plants, *Gossypium hirsutum* L. cv. Deltapine 61 and 70, were grown under glasshouse conditions. Flowers were routinely tagged at anthesis to determine the age of developing bolls. Since the boll development period varied with the seasons, we selected embryos on the basis of age (DPA), a standard degree of ovule wall sclerification, and gross morphology and fresh weight of embryos (Choinski and Trelease, 1978).

Studies on mature seeds were done mostly with commercial Deltapine 61 and 70 seed. Similar results were obtained when seeds harvested from our glasshouse plants were acid delinted and germinated on plates. Germination and postgerminative growth of seedlings in Petri dishes was done according to the presoaked and decoated procedure previously described (Radin and Trelease, 1976).

ORGANELLE ISOLATION AND ENZYME ASSAYS

Total enzyme activity in immature embryos and germinated seeds was determined from clarified homogenates of French-pressed samples (Choinski and

Trelease, 1978). Isolation of organelles was accomplished with a Beckman JCF-Z zonal rotor. The procedure is described by Choinski and Trelease (1978) for immature embryo organelle isolation, and essentially the same procedure for germinated seeds is outlined by Bortman *et al.* (1981). Enzyme activity was measured according to procedures in Bortman and Trelease (1981) and Miernyk and Trelease (1981a, b). Total nitrogen was measured as described by Choinski *et al.* (1981). Total lipid was gravimetrically determined after chloroform-methanol extraction and Folch washing; neutral lipid was that fraction collected after passing the total lipid extract through a silica gel G column in chloroform (Miernyk and Trelease, 1981a). Proteins in germinated-seed gradients were estimated by the Lowry *et al.* (1951) procedure; those in embryo gradient fractions and embryo cultures were determined by the Bradford method (1976). BSA was the standard for Lowry, and human gammaglobulin for Bradford procedures. Close agreement between the two methods was found, but the Bradford technique was more convenient.

EMBRYO CULTURE

Complete details of the culture procedure are given in Choinski *et al.* (1981). Excised ovules (with fibers removed) were surface sterilized in 1.3 percent hypochlorite, and embryos were aseptically removed with a scalpel and soaked in sterile solution (water, ABA or ABA plus an inhibitor.) Following a 3-hour presoak, 15 embryos were placed in a 150 x 25 mm plastic Petri dish containing 75 ml of culture medium in 0.8 percent agar. Cultures were maintained in the dark at 30C. The cotton ovule medium of Beasley and Ting (1973) was used with the following exceptions: 0.058 M sucrose was substituted for glucose plus fructose, and 3 mM L-aspargine and 4 mg/ml NH_4-malate were added. ABA and Act D solutions were autoclaved separately before addition to the autoclaved medium. Cordycepin and cycloheximide solutions were filter-sterilized.

ENZYME DEVELOPMENT AND ORGANELLE LOCALIZATION

IN GERMINATED SEEDS

Mature seeds decoated after a 4-hour imbibition period and germinated on moist Petri plates for several days (see Figure 1, Bortman *et al.*, 1981, for morphology) developed glyoxylate cycle and gluconeogenesis-related enzyme activities which peaked at about 38 to 48 hours after initial imbibition (Table 1). Isolation of organelles on sucrose-density gradients from cotyledons of 48-hour germinated seedlings revealed that a fatty acid β-oxidation marker enzyme (3-hydroxyacyl CoA dehydrogenase), catalase, glyoxylate cycle enzymes (ICL, MS and citrate synthase), and alanine and aspartate aminotransferases (not shown) all banded in the 1.26 g/cm^3 region of the gradient, separate from mitochondria

Table 1. Development of enzyme activity in cotyledons of *Gossypium hirsutum* (cv. Deltapine 61, 1977 harvest) germinated and grown in the dark on moist filter paper at 30C. Values are means from at least 4 separate experiments wherein 12 to 16 cotyledon pairs were homogenized per experiment. (From Bortman *et al.*, 1981).

Enzyme	Hours after initial soaking					
	0	10-12	22-26	36-40	45-50	70-75
	nmol min^{-1} cotyledon $pair^{-1}$ $X10^{-1}$					
Isocitrate lyase	0	0	12	41	42	38
Malate synthase	32	43	71	200	200	160
Catalase[1]	4	11	22	59	55	56
Malate DH	840	1,190	1,650	1,840	1,970	1,850
Citrate synthase	28	42	45	52	84	51
Aconitase	6	8	24	55	51	29
Enoyl hydratase	110	350	490	620	187	23
Hydroxyacyl-CoA DH	38	78	116	208	147	42
Oxoacyl thiolase	3	4	17	26	16	6
Aspartate AT	38	33	110	137	160	112
Alanine AT	32	28	41	42	36	21
Triosephosphate isomerase	1,840	1,900	1,950	1,950	2,160	1,880
Fructose-bisphosphatase	7	7	15	122	96	32
NADP-isocitrate DH	6	6	14	23	27	20

[1]Lück units

marker enzymes equilibrating at 1.19 g/cm^3 (Figure 2). The 1.26 g/cm^3 region of the gradient is where glyoxysomes characteristically equilibrate when isolated from other oil seeds (Beevers, 1979). Electron microscopic examination of these fractions showed that glyoxysomes were the predominant organelles (Bortman *et al.*, 1981).

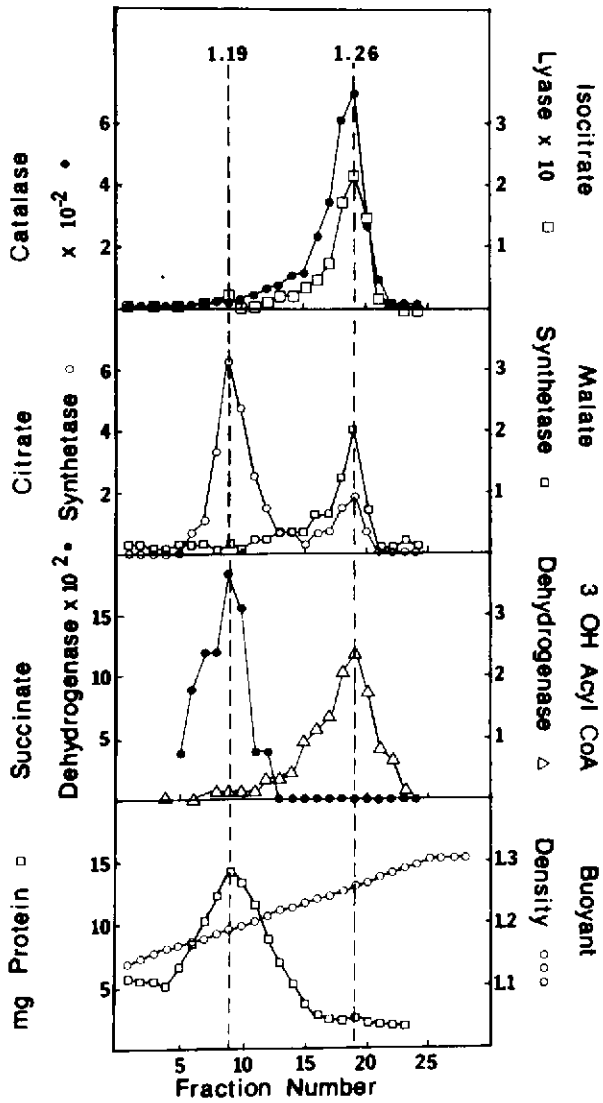

Figure 2. Distribution of protein and enzyme activities on a continuous sucrose density gradient after centrifuging a clarified homogenate of cotyledons removed from 48-hr-old seedlings. Enzyme activities are μmol/min/fraction, except for ICL which is nmol/min. Mitochondria (1.19 g/cm³) are clearly separated from glyoxysomes (1.26 g/cm³).

IN MATURING EMBRYOS

Selected enzyme activities were assayed in homogenates of embryos excised from bolls at various times after anthesis. Figures 3 and 4 and Table 2 show development of enzyme activities in maturing embryos compared to that in germinated mature seeds. Catalase, a constituent enzyme of glyoxysomes, and β-oxidation enzymes (known to be in glyoxysomes, Miernyk and Trelease, 1981a) increased in activity from 22 to 50 DPA. Activity of MS did not parallel these increases in activity; it was not detectable in embryos excised from 22 to 40 DPA

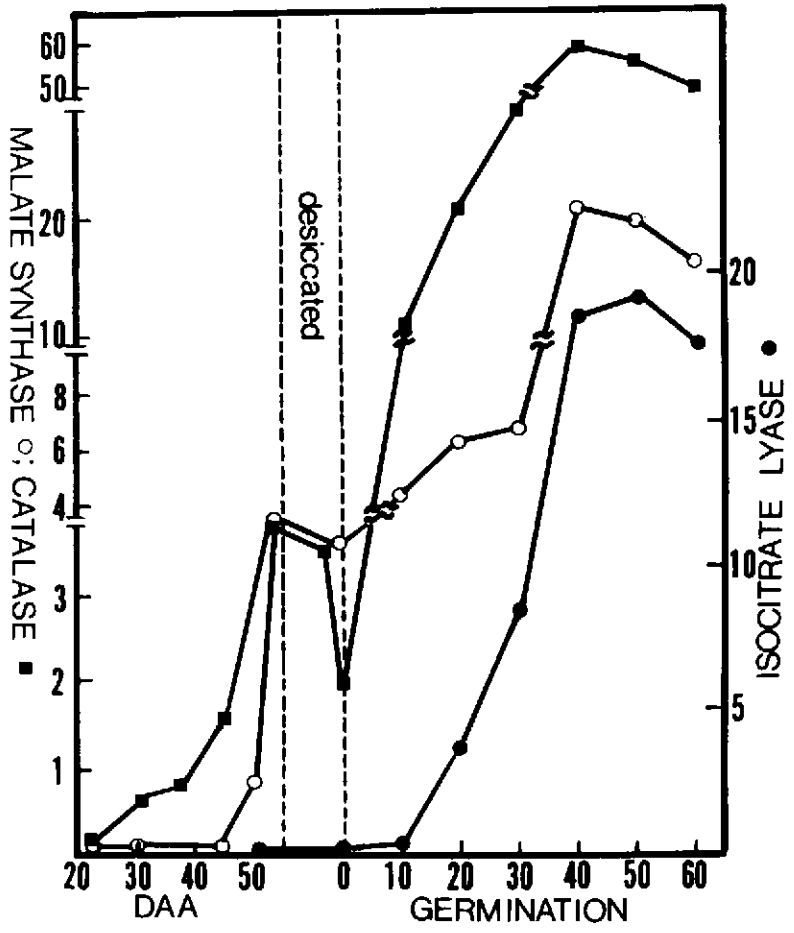

Figure 3. Development of selected glyoxysomal enzyme activities in embryos and germinated seeds. For actual activities (nmol/min/embryo), multiply ordinate values by 10 for catalase, by 100 for malate synthase and by 20 for isocitrate lyase.

but increased dramatically from approximately 42 DPA to 50 DPA, reaching a level nearly 20 percent of peak activity found in germinated mature seeds. Activity of ICL, a companion enzyme to MS in the glyoxylate cycle, was not detectable at any stage of maturation; it developed only after a 10-hour lag period following germination of mature seeds (Figure 3). The lack of ICL activity in immature

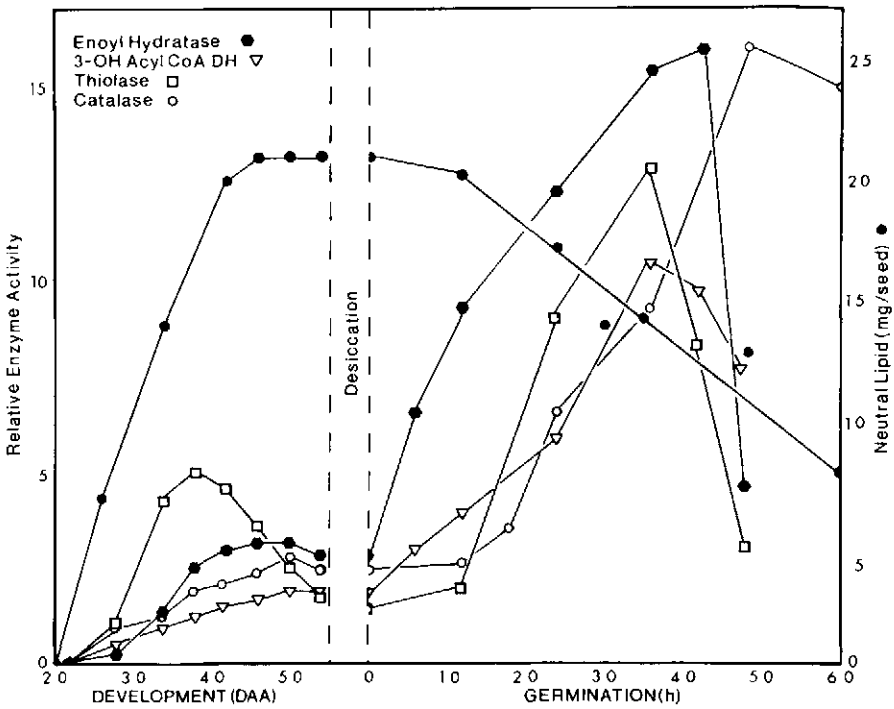

Figure 4. Development of catalase and β-oxidation enzyme activities in embryos and germinated seeds in relation to the synthesis and degradation of neutral lipid. For actual enzyme activity (nmol/min/embryo), multiply ordinate values by 200 for enoyl hydratase, by 100 for hydroxyacyl-CoA DH, by 20 for thiolase and by 18 for catalase. (From Miernyk and Trelease, 1981a).

embryos is consistent with Dure's findings (Dure, 1975), but the noncoordinated development of MS and the other enzyme activities during maturation has not been shown previously. All of these enzymes are required for successful postgerminative lipid mobilization but appear in immature embryos prior to desiccation. In addition, activity of these enzymes (except ICL) developed in the presence of ABA which has been shown to accumulate in maturing embryos (Radin and Hendrix, personal communication). These data are inconsistent with two aspects of Dure's developmental scheme: (a) ABA prevents vivipary through suppression of mRNA translation of enzymes required for "germination", and (b) the synthe-

Table 2. Developmental changes in enzyme activity during cotton embryo maturation. French-pressed, clarified homogenates were used directly for assays. Values are means ± standard errors of eight separate experiments. (From Miernyk and Trelease, 1981b).

Enzyme	Days postanthesis					
	34	38	42	46	50	54
	nmol/min/embryo					
Malate synthase	0	0	43 ± 44	115 ± 38	208 ± 45	319 ± 24
Malate DH	8310 ± 2004	7770 ± 2005	8330 ± 2263	7960 ± 2736	8230 ± 2419	8390 ± 2628
Citrate synthase	83 ± 28	142 ± 30	150 ± 49	167 ± 47	205 ± 55	279 ± 42
Aconitase	40 ± 8	48 ± 8	75 ± 21	79 ± 16	79 ± 12	57 ± 7
NADP-isocitrate DH	55 ± 24	53 ± 10	74 ± 12	63 ± 19	66 ± 19	62 ± 17
Fumarase	103 ± 47	148 ± 11	134 ± 1	131 ± 42	141 ± 27	146 ± 64
Aspartate AT	327 ± 47	314 ± 40	348 ± 54	323 ± 48	338 ± 15	358 ± 34
Alanine AT	327 ± 68	341 ± 18	367 ± 32	333 ± 85	325 ± 28	322 ± 21

Table 3. Total malate synthase activity in crude homogenates of dry cotton seeds representing several species and cultivars. Enzyme preparation and assay as in Methods. Seed dry weight is derived from decoated seeds previously stored at room temperature. Values are the mean of four separate preparations. Units are nmol/min. (From Miernyk *et al.*, 1979).

Seed	Units/seed	Units/mg dry seed wt	Units/mg as a per cent of Deltapine 16
Gossypium barbadense L. cv. Pima (tetraploid)	747	8.53	129
Gossypium hirsutum L. cv. Deltapine 61 (tetraploid)	451	7.84	118
Gossypium hirsutum L. cv. Deltapine 16 (tetraploid)	380	6.63	100
Gossypium herbaceum L. var. Africanum (diploid)	91	5.33	80
Gossypium hirsutum L. cv. Stoneville 213 (tetraploid)	252	4.32	65
Gossypium arboreum L. (diploid)	125	3.70	56
Gossypium thurberi Tod. (diploid)	38	3.25	49
Gossypium davidsonii Kell. (diploid)	77	3.15	48
Gossypium australe F.v.M. (diploid)	12	2.30	35

sis of an entire body of "germination" enzymes is regulated in a coordinated fashion (Dure, 1975). These aspects will be discussed in more detail later.

The discovery of substantial MS activity in mature Deltapine cotton seeds appeared to be an anomaly among oil seeds since it had been generally assumed that glyoxysomal enzyme activity was absent or negligible in oil seeds (Beevers, 1979). This prompted us to examine other ungerminated oil seeds, including several species and cultivars of cotton, for MS activity (Miernyk *et al.*, 1979). The results from a survey of ungermination cotton seeds is shown in Table 3. All seeds had relatively high activity, ranging from 35 to 129 percent of the activity

(units/mg dry seed weight) found in *Gossypium hirsutum*, cv. Deltapine 16. None of the seed homogenates had detectable ICL activity. A survey of other ungerminated oil seeds, including mono- and dicotyledons representing 11 families, showed that appreciable MS activity was present in homogenates of all oil seeds examined (Miernyk *et al.*, 1979). Thus, it was concluded that the synthesis of MS during seed development is universal among oil seeds, apparently in the absence of glyoxylate-cycle associated ICL activity.

The appearance of β-oxidation enzyme activities in the maturing embryos deserves further discussion. Figure 4 is a more detailed view of the development of these enzyme activities in embryos and germinated seeds, plotted in relation to synthesis and utilization of neutral lipids. Activity of three β-oxidation enzymes increased with catalase activity beginning at about 22 DPA. Enoyl hydratase and 3-hydroxyacyl CoA dehydrogenase rose to a peak with catalase at 50 DPA and remained essentially the same during the desiccation period. Thiolase activity, however, increased initially with the other enzymes, but consistently showed a peak activity at approximately 38 DPA. The significance of this is not understood. Although more enzyme activity develops following germination of mature seeds (Figure 4), clearly demonstrable β-oxidation enzyme activity developed in the maturing embryos during the period of neutral lipid synthesis and deposition in lipid bodies. The near parallel synthesis of neutral lipids and the enzyme system used to degrade (β-oxidize) fatty acids from neutral lipids seemed inconsistent with the anabolic metabolism normally associated with maturing cotyledon cells. This apparent inconsistency was addressed earlier by Hutton & Stumpf (1969) who had shown the presence of β-oxidation activity in maturing castor beans. Considering the known toxicity of free fatty acids and their high rate of synthesis during this period, they proposed that the B-oxidation system served as a "scavenger" system for those fatty acids not incorporated into triglyceride moieties. We do not have any additional evidence for or against this hypothesis and, therefore, consider it to be a viable explanation for the concurrent existence of β-oxidation enzymes and neutral lipid synthesis.

Hutton & Stumpf (1969) also investigated subcellular localization of β-oxidation enzymes in developing castor beans. Their data showed enzyme activities in both the mitochondrial and glyoxysomal regions of sucrose density gradients. In the endosperm of germinated castor beans, however, the activities clearly were restricted to glyoxysomes (Cooper and Beevers, 1969). In scutellum from germinated corn seedlings (Longo and Longo, 1975) and megagametophyte of germinated pine (Beevers, 1979) activities were found in both the mitochondria and glyoxysomes. Isolation of particles and distribution of enzyme activities on sucrose gradients from cotton embryos harvested 38 DPA is shown in Figure 5. The β-oxidation enzymes and catalase were clearly segregated in the 1.25 g/cm^3 region of the gradient, separate from mitochondria equilibrating at 1.21 g/cm^3. Activities of MDH and citrate synthase peaked in both regions of the gradient (not shown). This indicates that glyoxysomal-type particles were synthesized

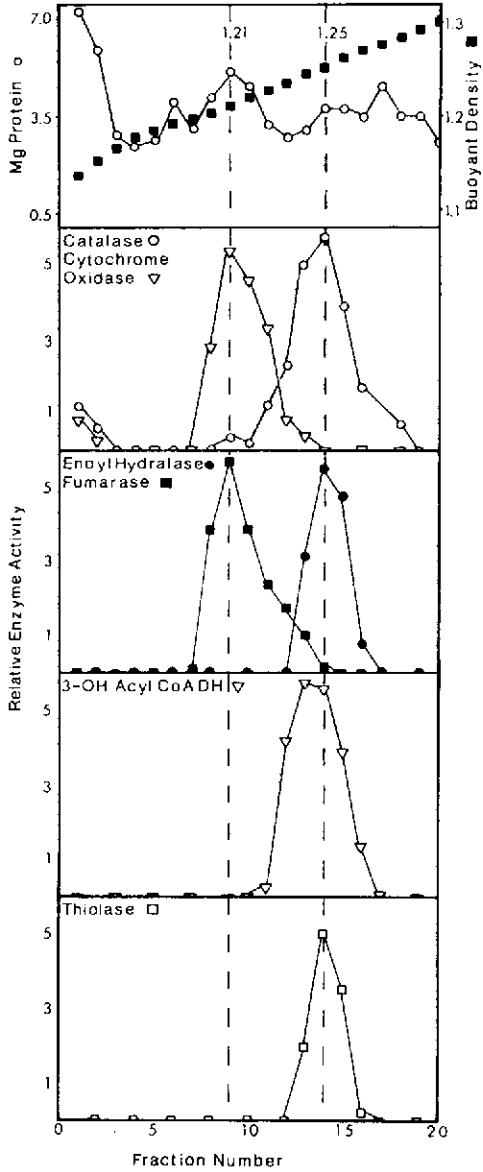

Figure 5. Distribution of protein and enzyme activity on a sucrose gradient after centrifuging a clarified homogenate of 38 DPA embryos. For actual activity (nmol/min/fraction), multiply ordinate values by 60 for enoyl hydratase, 2800 for fumarase, 85 for hydroxyacyl-CoA DH and 12 for thiolase. (From Miernyk and Trelease, 1981a).

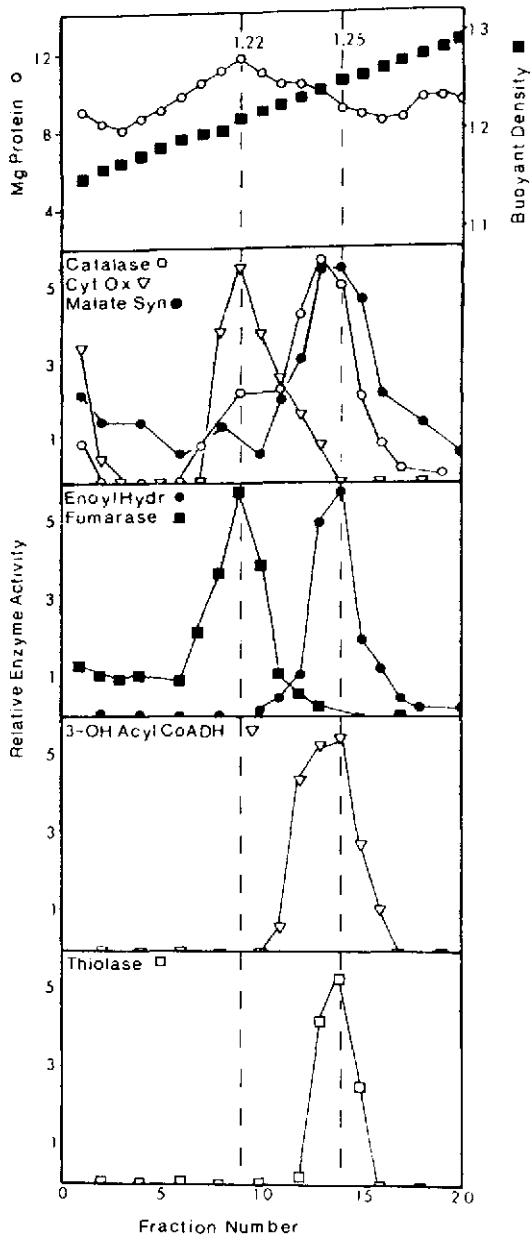

Figure 6. Distribution of enzyme activity from mature embryos on a sucrose gradient. Activity: enoyl hydratase x 150, fumarase x 4000, 3-OHAcCoA DH x 100, thiolase x 20 malate synthase x 45. (From Miernyk and Trelease, 1981a).

during maturation, notably in the absence of either marker glyoxylate cycle enzyme (ICL or MS). Isolation of particles from mature embryos (54 DPA) showed the same β-oxidation enzyme distribution, i.e., an exclusive localization in glyoxysomal-type particles (Figure 6). Citrate synthase, MDH and aspartate aminotransferase (not illustrated) also were localized in glyoxysomes isolated from mature embryos.

The results outlined above show that fatty-acid β-oxidation and related gluconeogenic enzymes are synthesized during embryogenesis and that these enzymes are compartmentalized into organelles which have a similar buoyant density on sucrose gradients, as do glyoxysomes isolated from germinated seeds (see Table 4 for a summary). This comparison does not necessarily show that the

Table 4. Summary of enzyme activities from mature embryos (50-54 DPA) equilibrating in the mitochondrial and/or glyoxysomal regions of sucrose density gradients.

Enzymes	Mitochondria 1.20-1.22 g/cm³	Glyoxysomes 1.23-1.26 g/cm³
	nmol/min/fractions	
Isocitrate lyase	0	0
Hydroxyacyl CoA DH	0	1,487
Enoyl hydratase	0	1,528
Thiolase	0	158
Catalase	26	127
Malate synthase	37	734
Aspartate AT	483	1,125
Citrate synthase	1,452	333
Malate DH	9,848	5,226
Alanine AT	80	0
NADP isocitrate DH	160	0
NAD isocitrate DH	80	0

embryo-synthesized organelles are involved in gluconeogenesis, as are glyoxysomes in germinated seeds. The absence of detectable ICL activity in embryo-synthesized organelles suggests that gluconeogenesis from acetyl-CoA is not likely. An alternate hypothesis would be that these organelles have some role in seed maturation. Considering the known enzyme content of the organelles, it seemed possible that they are centrally involved in organic acid metabolism (see Miernyk and Trelease, 1981b, for more data and discussion).

Recent evidence obtained by Kindl's group with cucumber seeds bears directly on our work with cotton seeds. They, like others, have been unable to assay ICL *activity* in dry seed extracts, but they did detect a protein in the dry-seed extracts which was precipitable with an antibody made to purified ICL obtained from germinated seeds (Köller *et al.*, 1979). Thus, ICL could exist in an inactive form

in vivo, or it may be inactivated during extraction. We did a number of experiments with cottonseed extracts to test for possible inactivation of the enzyme during homogenization (Miernyk *et al.*, 1979). Addition of a protease inhibitor, gel filtration, etc., did not yield any ICL activity. These treatments did not rule out the possible existence of an inactive enzyme *in vivo*. However, we have prepared an antibody to germinated-seed ICL and could not detect any immunological reactants in extracts from immature embryos (Miernyk *et al.*, 1982). We believe the protein is not present in dry seeds but is synthesized *de novo* following germination, then added post-translationally to organelles synthesized during seed maturation. This delayed synthesis and compartmentation may be an important control on lipid mobilization.

Work in several laboratories on castor beans has led to a generalization that glyoxysomes are synthesized *de novo* following germination (Beevers, 1979). The data favor the hypothesis that the constituent enzymes are inserted into newly formed glyoxysomes as they vesiculate from segments of rough endoplasmic reticulum. New evidence generated by Kindl's group indicates that enzyme addition to cucumber cotyledon glyoxysomes does not proceed in this fashion. Rather, their data show that several enzymes are added to glyoxysomes from cytoplasmic pools (Frevert *et al*, 1980; Kindl, 1982). We do not have any direct biochemical data on glyoxysomal biogenesis in cotton seeds, but we have made cytochemical electron-microscopic observations for catalase reactivity in cotyledon cells of maturing embryos and germinated seeds and found nearly the same number of glyoxysomes per cell in immature and germinated cotyledons, plus an increased size of glyoxysomes in germinated seeds (Kunce *et al.*, unpublished). Taken together, these data allow speculation that the organelles synthesized during maturation are direct precursors of glyoxysomes in germinated seeds. The renewed synthesis of gluconeogenic enzymes following germination likely reflects the need for more of the same enzymes to process the carbon liberated from stored triglycerides. Hence, the embryo-synthesized organelles may serve as "primer glyoxysomes" already constructed and available to accept the new enzymes.

Such a hypothesis is not conceptually different from Ihle and Dure's (1972) original hypothesis that cotton embryos are programmed to prepare the seed for successful germination (and/or postgerminative growth). Their postulate, however, was based on the idea that the mRNAs for the germination enzymes (actually postgerminative enzymes, since they studied ICL and a protein degrading enzyme) were transcribed during maturation and were available for translation following germination. Our work with these and numerous other enzymes has shown that the enzymes increase in activity and are incorporated into organelles prior to germination. It is clear from Dure's work that mRNAs exist in dry cotton seeds and that they must be processed before normal germination and growth can occur (Dure, 1977; Harris and Dure, 1978). It seems appropriate now to learn which of the three subsets of mRNAs described by Dure (stored, residual, and newly-synthesized mRNA) code for the various enzymes involved in germination and seedling establishment.

IN CULTURED EMBRYOS

There are few data in the literature describing the influence of hormones, metabolites, drugs, etc., on the development of enzymes in embryos during cotton boll maturation. Such information is needed to construct or modify working hypotheses on the mechanisms of developmental processes leading to preparation of the seed for germination. Due to the inherent difficulty in treating intact bolls with these compounds, we devised an *in vitro* culture system for excised embryos (Choinski *et al.*, 1980). Our efforts focused on culturing embryos excised about 40 DPA, since MS activity developed from zero activity subsequent to 40 DPA.

A modified version of the cotton ovule culture medium of Beasley and Ting (1973) was used. Inclusion of sucrose and L-aspargine in the media was based on high *in vivo* levels of these compounds (Mauney *et al.*, 1967) and their known role as primary transportable forms of carbon and nitrogen. The ammonium salt of malate was added in part because of the high level of malate noted in cotton endosperm tissue, and because NH_4-malate plus sucrose and salts in the media

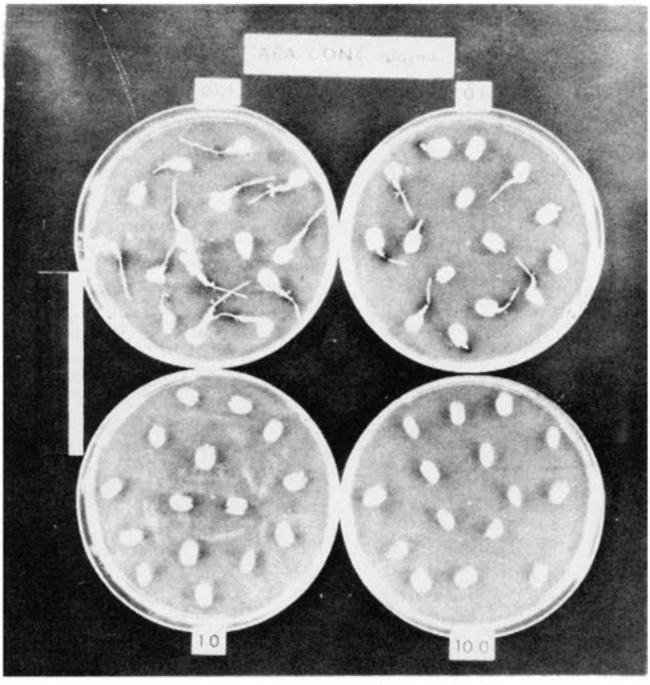

Figure 7. Appearance of embryos cultured *in vitro* for 4 days on nutrient media containing various concentrations of ABA. 1.0 μg/ml ABA equals 3.8 μm ABA. Bar on left equals 10 cm. (From Choinski *et al.*, 1981).

were reported to greatly improve culturing efficiency of young cotton embryos (Mauney *et al.*, 1967). ABA was included in the media since it prevents precocious germination *in vivo* (vivipary).

Figure 7 shows the appearance of embryos cultured for four days after boll excision on media containing various concentrations of ABA, and Table 5 gives the values for percent germination, radicle growth and change in enzyme activity. Embryos cultured without ABA (not pictured) appeared the same as embryos cultured with 0.01 μg/ml (0.038 μM) ABA. Both precociously germinated (95-100 percent) (Figure 7) and produced radicles of approximately the same length and fresh weight (Table 5). Embryos on media with 0.1 μg/ml (0.38 μM) ABA

Table 5. Influence of varied ABA concentrations on enzyme development, germination and radicle growth of cultured cotton embryos. Embryos were presoaked for 3 hours in the designated ABA concentration prior to culturing on plates. (From Choinski *et al.*, 1981)

	0 Day[1]	4 Days culture				
		ABA concentration (μM)				
Enzyme		0	0.038	0.38	3.8	38
		nmol/min/embryo				
Malate synthase	0	659	596	572	514	513
Isocitrate lyase	0	5	5	3	0	0
Catalase[2]	32	87	103	109	96	86
Hydroxyacyl-CoA DH	208	888	852	896	843	846
		Percent				
Germination	—	95	100	74	2	0
		mg/embryo				
Radicle[3] growth (fresh wt)	5	60	56	32	5	5
		mm				
Length	9-10	30-35	25-40	12-28	9-10	9-10

[1]Embryos harvested 38-40 DPA; Average fresh wt = 93 mg/embryo
[2]Catalase units are Lück units—see Methods
[3]The term radicle as used here includes radicle and hypocotyl tissue

also precociously germinated, but the average percent germination (74 percent) and radicle growth (Table 5) was less than for embryos cultured on 0.038 μM ABA. Precocious germination of embryos was essentially nil in cultures with 1.0 and 10.0 μg/ml (3.8 and 38 μM) ABA.

Comparing the enzyme activity in 0 day (excised) embryos with that in embryos cultured for 4 days on 3.8 or 38 μM ABA (Table 5) shows that considerable development of MS, catalase and hydroxyacyl-CoA dehydrogenase activity oc-

curred in cultures without any appearance of ICL activity. Detectable ICL activity and elevated levels of the other enzymes were apparent in those embryos cultured on ABA concentrations $\leq$0.38 μM ABA (Table 5). However, these embryos exhibited radicle growth indicating that the ICL and elevated activities were due to appearance of enzymes related to postgerminative growth.

Literature values for endogenous ABA concentrations are expressed as an amount per boll (Davis and Addicott, 1971) or μg ABA/kg embryo (DeLanghe and Vermeulen, 1972). Thus, it is difficult to determine whether the concentration of ABA preventing precocious germination (3.8 μM) in our system is similar to an *in vivo* concentration. An estimate of molar concentration in 34 DPA embryos from DeLanghe and Vermeulen's data is 0.08 μM ABA per embryo. Walbot *et al.*, (1975) and other reviewers (e.g., Walton, 1977) refer to "combined

Table 6. Comparison of changes in enzyme activity and other parameters during development of cotton embryos cultured *in vitro* or maintained on plants *in vivo*. (From Choinski *et al.*, 1981)

	38 DPA	4-Day culture (*in vitro*)	54 DPA (*in vivo*)
Enzyme		nmol/min/embryo	
Malate synthase	0	432 ± 18[1]	385 ± 23
Isocitrate lyase	0	0	0
Catalase[2]	27 ± 2	61 ± 3	80 ± 2
Hydroxyacyl-CoA DH	168 ± 11	557 ± 17	426 ± 28
Citrate synthase	255 ± 20	406 ± 17	388 ± 25
Malate DH[2]	21.4 ± 2	23.1 ± 1	26.6 ± 3.2
Aspartate AT	500 ± 16	563 ± 27	466 ± 17
Weight		mg/embryo	
Fresh	81 ± 3	139 ± 5	55 ± 1.2
Dry	46 ± 3	53 ± 4	52 ± 0.6
Protein (Bradford)		mg/embryo	
Total	30.6 ± 0.7	31 ± 0.7	34.5 ± 0.2
Insoluble	21 ± 0.5	23 ± 0.5	25 ± 0.7
		μmol/embryo	
Nitrogen	222 ± 17	261 ± 17	286 ± 4
Lipid		mg/embryo	
Total	13.8 ± 0.9	15.0 ± 0.6	
Neutral	11.9 ± 0.6	13.9 ± 0.5	16.0 ± 0.8

[1]Values for enzyme activity are means ± S.E. of replicates from at least three separate experiments. Other values are means ± S.E. of replicates from one or two experiments.

[2]Malate dehydrogenase units are μmol/min/embryo; catalase units are Lück units.

gas chromatography and bioassay" values for ABA concentration in cotton ovules and embryos that ostensibly are included in a published report by Ihle and Dure (1970). However, careful examination of this reference does not reveal any such values; thus, if these data are published, their source is not known to us. Since enzyme development occurred without precocious germination in embryos cultured on 3.8 μM ABA, this concentration was used in media for all subsequent experiments.

A comparison of various developmental changes in cultured embryos and *in vivo* embryos is shown in Table 6. Activity of all enzymes increased in the presence of 3.8 μM ABA to levels comparable to those found in embryos removed from bolls 54 DPA. Dry weight increase of cultured and *in vivo* embryos was similar, whereas protein and neutral lipid accumulations in cultured embryos were less than in *in vivo* embryos. Overall, the data show that embryos cultured on nutrient media containing 3.8 μM develop similarly to maturing embryos within bolls.

Table 7 shows the influence of adding various protein-synthesizing inhibitors (Act D-transcription, cordycepin-polyadenylation, cycloheximide-translation) to ABA-containing cultures. All three inhibitors essentially prevented the development of MS activity, and they suppressed additional development of catalase,

Table 7. Influence of protein synthesis inhibitors on enzyme development and fresh weight of cultured cotton embryos. Concentration of inhibitors was: ABA—3.8 μM; Act D—8.0 μM; cordycepin—300 μM; cycloheximide—178 μM. (From Choinski *et al.*, 1981)

	0 Day[1]	3 Days culture			
		ABA	ABA Act D	ABA Cordycepin	ABA Cycloheximide
		nmol/min/embryo			
Enzyme					
Malate synthase	0	400	24	29	37
Isocitrate lyase	0	0	0	0	0
Catalase[2]	25	55	18	22	12
Citrate synthase	243	452	192	234	160
Malate DH[2]	26.2	28.0	25 4	27.8	33.4
Aspartate AT	1012	1977	1630	1537	1672
Enoyl hydratase	2072	3625	2022	1818	1620
Hydroxyacyl-CoA DH	138	300	154	126	106
Thiolase	164	100	117	74	76
		mg/embryo			
Embryo (fresh wt)	74	131	101	104	98

[1]Embryos harvested 38-40 DPA

[2]Catalase units are Lück units; malate DH units are μmol/min/embryo

citrate synthase, enoyl hydratase, hydroxyacyl-CoA dehydrogenase and thiolase activity. Further development of MDH and aspartate aminotransferase activity was apparent in the presence of these drugs, but some inhibition was noted. It may be significant that the latter two enzymes exist as multiple forms (Beevers, 1979). The results may reflect a selective inhibition of the glyoxysomal, and not the mitochondrial or cytoplasmic, forms. The complete inhibition of citrate synthase development would be an exception to this idea, since it apparently also exists in multiple forms.

The data in Table 7 provide good evidence that enzymes known to be incorporated into glyoxysomes during maturation (Table 4) can be transcribed and translated in the presence of ABA. Inhibition by cordycepin indicates that the mRNAs for these enzymes must be polyadenylated prior to translation. Harris and Dure (1978) also have reported the requirement for such processing of mRNAs conserved in the dry seeds. Development of ICL activity again is conspicuous by its absence. These and other results previously described for ICL development explain the rationale for Dure's proposal that "germination enzyme" development is suppressed by ABA, and, hence, so is vivipary. None of the embryos in this experiment precociously germinated. In this regard, our results are again inconsistent with Dure's finding that addition of Act D to ABA-treated embryos overcame the effect of ABA and permitted germination. The difference in results is not presently understood. We have cultured excised embryos on agar plates containing only ABA and found that the embryos did not germinate. Thus, we believe that ABA is important in preventing vivipary, but we do not accept the hypothesis that its action is through the prevention of mRNA translation of enzymes unique to germinated seeds, since these enzymes are translated in immature embryos in the presence of ABA (Tables 5-7). One cannot ignore the lack of ICL development, however. It is entirely possible that ABA blocks ICL appearance and thereby blocks germination. This reasoning is clouded by the fact that ICL activity appears in precociously-germinated embryos and germinated seeds several hours after germination (protrusion of the radicle) commences, not concomitant with germination. (See Chapter 28 for additional information on ABA control of mRNAs.)

SUMMARY

Assaying for activity of several key gluconeogenesis-related enzymes in maturing cotton embryos has revealed new information pertinent to understanding developmental events that occur during boll maturation. First and foremost is the fact that all of the gluconeogenesis-related enzymes examined, except ICL, appear at some time during maturation and are incorporated into organelles (glyoxysomes and mitochondria). Their localization in organelles within mature embryos is the same as in germinated seeds. The function of the embryo-synthesized glyoxysomes is not clearly understood. They may play a non-gluconeogenic

role during the late stages of maturation, such as in the metabolism of organic acids (Miernyk and Trelease, 1981b), and/or they may be needed as direct precursors for the glyoxysomes centrally involved in lipid mobilization and gluconeogenesis during postgerminative growth. Other functions are equally possible but have not been adequately explored.

Our data generally reenforce the concept originally proposed by Dure that certain developmental events occur which prepare the seed for successful germination and/or postgerminative growth. However, the data presented in this paper coupled with results from Benedict's laboratory (Chapter 22) strongly indicate that substantial modification of Dure's proposed developmental scheme is in order. The following is a list and brief discussion of major modifications suggested by the new information. Figure 8 is a diagrammatic summary of these events and

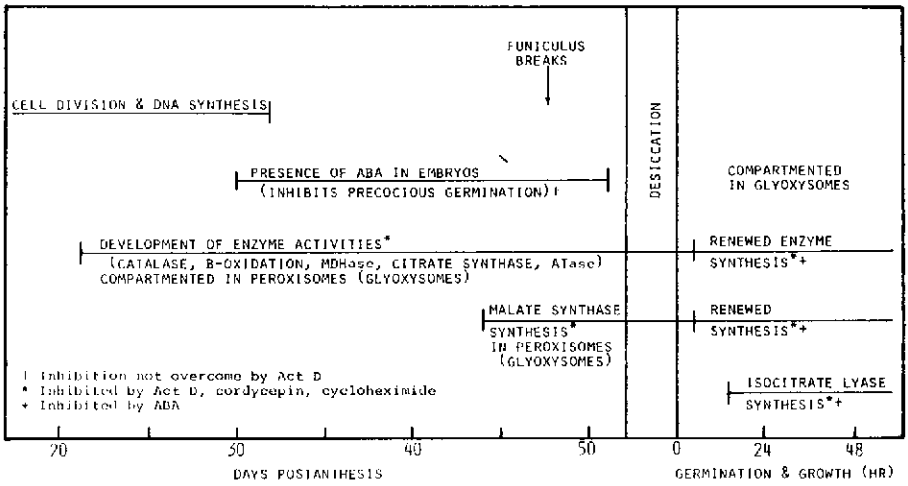

Figure 8. Schematic summary of the sequence of events in maturing and germinated cotton seeds. (From Choinski *et al.*, 1981)

our results.:

1. The list of developmental events associated with the natural atrophy of the funiculus thought to occur at 32 DPA should be abandoned and described again in a revised temporal map. Benedict's group has strong evidence that funiculus atrophy occurs much later (45 to 50 DPA), and our data show the appearance of several enzymes beginning at about 22 DPA;
2. The concept that ABA prevents vivipary should be preserved. The postulated mechanism of ABA inhibition of germination needs to be revised based on new knowledge that numerous "germination" enzymes are synthesized during maturation. The proposal that immature embryos and mature seeds are differentially sensitive to ABA must be reconsidered since two independent reports

have shown that mature seeds also do not germinate in the presence of ABA (Halloin, 1976a; Radin and Trelease, 1976);

3. The synthesis of glyoxysome-type organelles and timing of their appearance with different enzymes should be integrated into the developmental scheme. The early appearance of catalase and β-oxidation enzymes likely is important in relation to successful lipid synthesis and deposition. The delayed appearance of MS activity and absence of any development of ICL activity probably are important to maturation, but more work is needed to understand the significance of these phenomena; and,
4. The concept of mRNAs being transcribed during maturation and conserved in dry seeds should be retained. However, the timing of this mRNA synthesis and suggestions that they are not translated until germination should be reconsidered. Knowledge of the proteins coded for by these mRNAs would be helpful in this regard. Finally, the proposition that the pre-existing mRNAs code for the entire body of enzymes unique to germination in a coordinated fashion is no longer tenable. Many of these enzymes are synthesized during maturation, some at different times. Some or all of the conserved mRNA may code for renewed synthesis of these enzymes, or initial synthesis of ICL, but more data are needed to explore these possibilities.

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