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Control of Enzyme Activities in Cotton Cotyledons during Maturation and Germination

II. GLYOXYSOMAL ENZYME DEVELOPMENT IN EMBRYOS[1]

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ABSTRACT

The sequence of glyoxysomal enzyme development was investigated in cotyledons of cotton (*Gossypium hirsutum* L. cv. Deltapine 16) embryos from 16 to 70 days after anthesis (DAA). Catalase, malate dehydrogenase, and citrate condensing enzyme activities were barely detectable prior to 22 DAA, but showed dramatic increases from 22 to 50 DAA. Development of malate synthase activity, however, was delayed during this period, rising to peak activity from 45 to 50 DAA (just prior to desiccation) in the absence of any detectable isocitrate lyase activity. Substantial activities of all of these enzymes (except isocitrate lyase) persisted in the dry seeds. Isopycnic centrifugations on sucrose gradients demonstrated that the enzymes were compartmentalized within particles increasing in buoyant density with time of development (1.226 to 1.245 grams per cubic centimeter from 22 to 50 DAA). Of particular significance were the observations in 22-day embryos of smooth surfaced membrane dilations of rough endoplasmic reticulum having cytochemical catalase reactivity, and the demonstrations of catalase activities in microsomal fractions isolated throughout the 16- to 50-DAA period. Our data do not allow determination of the mechanism(s) for enzyme activation and/or addition to previously existing or newly formed microbodies, but do show that development and acquisition of enzyme activities within glyoxysomes occur sequentially and thus are not regulated in concert as previously thought.

Glyoxysomes isolated from endosperm and cotyledons of germinated fatty seeds have been extensively characterized (12, 22). Recent emphasis, however, has been on the biogenesis of glyoxysome, especially in castor bean endosperm (2, 10, 14, 15). The collective studies have supported the concept that glyoxysomes are derived directly from the ER by a process of vesiculation. Synthesis of the limiting membranes with their constituent enzymes and initial segregation of matrix proteins are believed to occur in the ER prior to detachment and formation of separate organelles (2).

In this paper we examined the events of glyoxysome (microbody) proliferation and enzyme development in a new system, *viz.* embryonic cotton cotyledons. This system has afforded the opportunity to study the critical stages of microbody ontogeny without the complications of large amounts of intracellular storage lipid and protein encountered with dry or early germinated seeds. We have ultrastructurally documented the microbody-ER association that has been inferred in the above cited studies. Furthermore, we have evidence that marker glyoxylate-cycle enzymes are not regulated in a coordinated fashion, a finding not previously noted in seed germination studies.

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MATERIALS AND METHODS

Growth and Selection of Plants. Cotton plants, *Gossypium hirsutum* L. cv. Deltapine 16, were grown under greenhouse conditions; flowers were tagged at anthesis to determine the age of the developing bolls. Ovules removed from bolls were selected mainly on the basis of a standard degree of seed coat sclerification (Fig. 1). Embryos were excised from these ovules and selected on the basis of age in DAA,[2] gross morphology (Fig. 1), and fresh wt. In some experiments, untagged field plants were used and material was selected on the basis of all parameters except age. Seeds were presoaked and germinated on Petri plates as described previously (18).

Homogenization and Centrifugation Procedures. To determine total enzyme activities, embryos and seeds were homogenized thoroughly in 100 mM K-phosphate (pH 6.9) with 3 mM $MgCl_2$ (containing 3 mM DTT in experiments testing for isocitrate lyase activity) with a Potter-Elvehjem motor-driven Teflon homogenizer, then centrifuged at 30,900*g* for 20 min. The volume (ml) of grinding medium/embryo varied with the age examined ranging from 1:4 at 22 DAA to 1:1 at 50 DAA; seeds were done at 1:1. The supernatant was used directly for assays. For differential centrifugation experiments, embryos were chopped with razor blades attached to an electric knife in grinding medium containing 20 mM K-phosphate (pH 6.9), 2.5% Ficoll, 0.5 M sucrose, and 3 mM EDTA. The resulting homogenate was then filtered through three layers of Miracloth into cold centrifuge tubes and spun at 270*g* for 10 min in a Beckman JA-20 rotor to remove cell debris, followed by two additional spins of 10,800*g* for 30 min to obtain a microbody pellet, and 150,000*g* for 60 min with a Beckman TI-50 rotor in a Sorvall OTD-2 ultracentrifuge to obtain a microsomal pellet. For zonal rotor gradients, approximately 120 whole embryos were homogenized in 50 ml of grinding medium as described for the differential experiments. The supernatant from the 270*g* centrifugation was applied to a 900-ml continuous sucrose gradient generated by adding 100 ml each of 20, 25, 30, 35, 40, 45, 50, 55, and 60% (w/w) sucrose dissolved in 20 mM K-phosphate (pH 6.9) to a Beckman JCF-Z zonal rotor spinning at 2,000 rpm. The gradient was constructed on 200 ml of 60% sucrose in the same buffer. After sample application, an additional 50 ml of grinding medium diluted 1:1 with distilled H_2O was injected followed by 700 ml of 20 mM K-phosphate (pH 6.9) as an overlay. After loading, the contents were centrifuged at 25,000*g* (calculated at the radius of the rotor corresponding to a buoyant density of 1.25 g/cm^3) for 2.5 hr in a Beckman J-21B centrifuge. The gradient was fractionated by pumping 62% sucrose through the edge line of the rotor; fractions were collected in 25-ml aliquots by hand for enzyme assay and cytochemistry.

[2] Abbreviations: DAA: days after anthesis; OAA: oxaloacetic acid; DAB: 3,3'-diaminobenzidine; MDH: malate dehydrogenase.

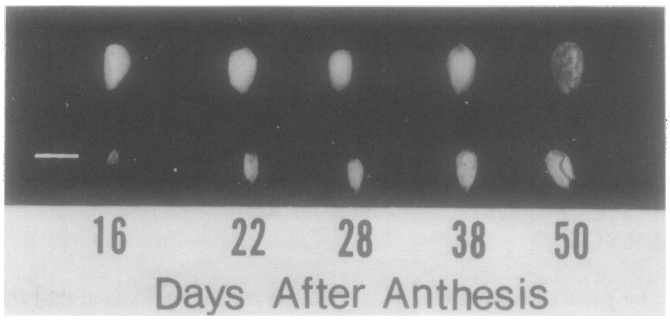

FIG. 1. Morphology of ovules and embryos excised from bolls at various ages postanthesis. Upper row illustrates excised ovules. Incipient seed coat sclerification is apparent at the chalazal end of ovules at 38 DAA. Sclerification of the seed coat is uniform at 50 DAA. Lower row illustrates embryos excised from ovules. Note the increased size with development time. Bar in the lower left corner equals 1 cm.

Enzyme Assays. Zonal gradient fractions and differential fractions obtained as described were assayed for catalase (17), isocitrate lyase (6, 19), citrate condensing enzyme (7), and NADH Cyt *c* reductase (15). In the malate synthase assay (7), the reaction was initiated with glyoxylate after measuring endogenous deacylase activity on acetyl-CoA. The volumes and concentrations of components in the reaction mixtures were adjusted to allow for larger sample aliquots in preparations with low activities. MDH was assayed by the addition of the following components: 0.85 ml of 60 mM HEPES (pH 7.4) containing 0.13% Triton X-100, 0.05 ml of crude extract, and 0.05 ml of 1.25 mM NADH in 60 mM HEPES (pH 7.4). The reaction was started with 0.05 ml of 10 mM OAA (neutralized with NaOH). Absorbance change was recorded with a Coleman 124 spectrophotometer every 30 sec for 90 sec, and enzyme activity calculated on the basis of a molar extinction coefficient of NADH equal to 6.22×10^3 (7). Protein was measured by the method of Lowry *et al.* (16). Total nitrogen was measured by a two-step procedure: first, 10 mg of dry wt of cotyledon tissue was completely digested in H_2SO_4 to convert all cell nitrogen to ammonium (4). The NH_4-N was then quantified by the use of a modified Conway microdiffusion technique (21). Total lipid was determined gravimetrically after extraction and drying according to Christie (6).

Electron Microscopy and Cytochemistry. Portions of zonal gradient fractions were fixed for 1 hr by the addition of an equal amount of 40% (w/w) sucrose containing 4.5% (v/v) glutaraldehyde in 20 mM K-phosphate (pH 6.9). The fixed suspension was diluted slowly (1:1) with gradient buffer and spun at 10,800*g* for 30 min. Segments of cotyledons were fixed with 3% glutaraldehyde in 50 mM K-phosphate (pH 6.9) for 1 hr. Cotyledon segments and sucrose gradient fractions were cytochemically stained for catalase via the technique of Frederick and Newcomb (9). Handling of the fixed material was as described previously (3).

RESULTS

Ultrastructural Aspects of Embryonic Cotyledons. Preservation of cellular material in embryos examined at various stages between 16 to 28 DAA was especially good for detailed ultrastructural observations. Of interest in cells at 16 DAA was the preponderance of free ribosomes in the cytoplasm as compared to those attached to the widely dispersed profiles of ER. Storage lipid bodies were not present in any appreciable number/cell and the infrequently observed small vacuoles exhibited no deposition of dark-staining material. Views of typical microbodies among other organelles were not apparent, nor was there any suggestion of vesiculation from the infrequent RER profiles. At 22 DAA, lipid bodies were more prevalent in the cells (Fig. 2A) and a conspicuous increased number of RER profiles were commonly observed (Fig. 2A, arrows). Careful observations of thin sections revealed smooth surfaced dilated portions of RER profiles (Fig. 2B, inset). The dilations were difficult to discern because of their finely granular matrix and relatively low contrast compared with surrounding cytoplasm (Fig. 2B, inset). When the cotyledons were cytochemically stained for catalase reactivity with H_2O_2 and DAB as the substrates, the RER dilations were considerably more apparent in the sections (Fig. 2B). An unevenly distributed granular reaction product was characteristic of the matrix portion of the smooth surfaced blebs; the boundary membranes did not appear to be stained (note arrow on left). Several favorable section views showed that the dilations or blebs were directly connected to the RER (note arrow on right).

Examinations of a later development stage, 28 DAA, revealed that a considerable portion of the cell's volume was occupied by lipid bodies and vacuoles containing electron-dense material (Fig. 3). Evidence of dilated connections to RER were not noted in 28-day material and microbody profiles separate from ER were seen only infrequently. Spherical particles, superficially resembling microbodies, were commonly observed in the cytoplasm of 28-day cells (Fig. 3). However, their matrices were more electron-dense than would be expected for newly synthesized microbodies and similar, but smaller, bodies were seen in spatial proximity with dictyosomes (note double arrows and inset, Fig. 3). These bodies were of identical electron density to the storage protein bodies. These observations strongly suggest that dictyosomes are involved in processing the protein body material. This is a reasonable hypothesis based on known dictyosome function and has precedent from the work of Harris and Boulter (11) on developing *Vigna* seeds.

Little definitive data on microbody associations with RER could be obtained from cotyledon sections of 38- and 50-day material. Lipid and protein bodies occupied essentially all of the cytoplasmic area and obscured detailed subcellular features.

Development of Glyoxysomal Enzymes during Embryogenesis and Germination. Total enzyme activities/embryo were determined from 20 to 70 DAA (Fig. 4, upper panel). Isocitrate lyase was assayed with continuous (7) and a discontinuous (19) method, but was not found at any developmental stage. Catalase and citrate condensing enzyme activities exhibited a dramatic increase from 22 to 50 DAA. A substantial per cent of the total particulate catalase activity was recovered in microsomal fractions (Table I). These obvious changes in enzyme activities occurred at the same time, and with similar increases, as did the dry wt and deposition of total lipid and nitrogen in the embryos (Fig. 4, lower panel).

The situation for malate synthase was quite different; essentially no activity was noted until just prior to desiccation at 50 DAA, at which time a sharp rise in activity was recorded (Fig. 4, upper panel). The peak activity, attained in a considerably shorter time period than the other enzymes, represented about 20% of the peak malate synthase activity found in germinated seeds (Table II). Comparable activities for all enzymes examined were found in dry seeds (Table II). Isocitrate lyase activity was absent in seeds, but following germination it developed rapidly after a lag period with malate synthase activity. Catalyase activity developed rapidly between 0 and 30 hr prior to glyoxylate-cycle enzyme increases, a situation similar to that found during embryogenesis (Fig. 4, upper panel).

Density Gradient Experiments. From the above data, two distinct types of microbody populations seemed to exist during development: those lacking (22-DAA) and those containing (50-DAA) malate synthase. Organelle fractionation was attempted using standard sucrose density gradient technique with a Beckman SW 25.2 rotor as described by Huang and Beevers (13); however, despite this and many other variations in grinding media, gradient buffer, centrifugation time, etc., reproducible results were not

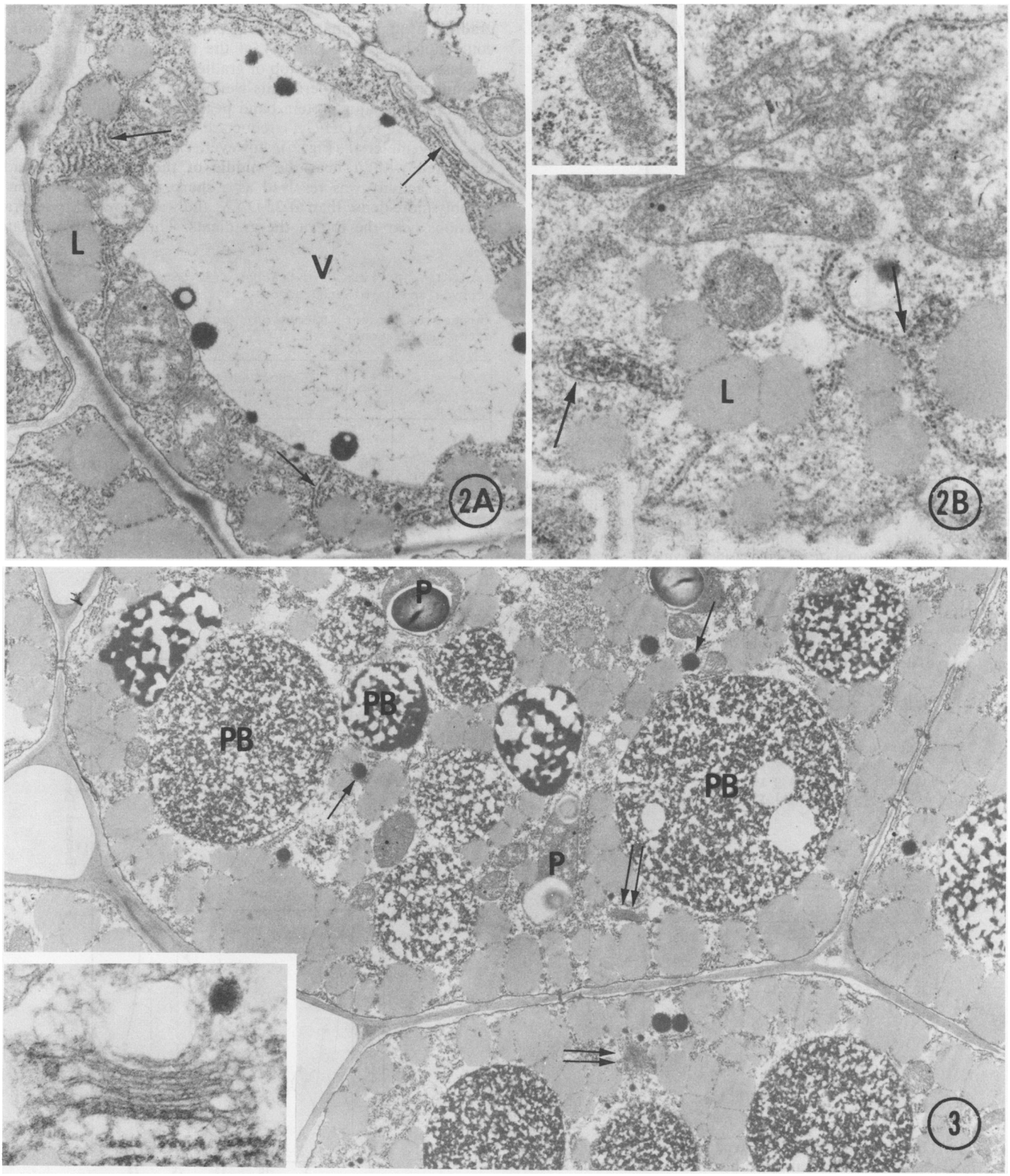

FIG. 2. Electron micrographs of cotyledonary cells from 22-DAA embryos. A: cells typically contain large vacuoles (V) lacking electron-dense material. Numerous segments of RER (arrows) and lipid bodies (L) are noted throughout the cytoplasm at this stage. B: high magnification of a portion of a tissue segment incubated in DAB medium to localize catalase reactivity. Reactivity is shown in two smooth surfaced membrane-bound ER dilations (arrows). One of the dilations is shown directly connected to RER (right arrow). Inset: view of a typical dilation seen in tissue segments not treated with DAB. Matrix shows substantially less electron density than that seen in DAB-treated cells.

FIG. 3. Electron micrograph of cotyledon cells from 28-DAA embryos. Extensive protein body (PB) development is observable at this stage. Dictyosomes (double arrows and inset) are often noted in spatial proximity with electron-dense vesicles. Larger vesicles of identical density are noted throughout the cytoplasm (single arrows). Plastids (P) with starch grains are common.

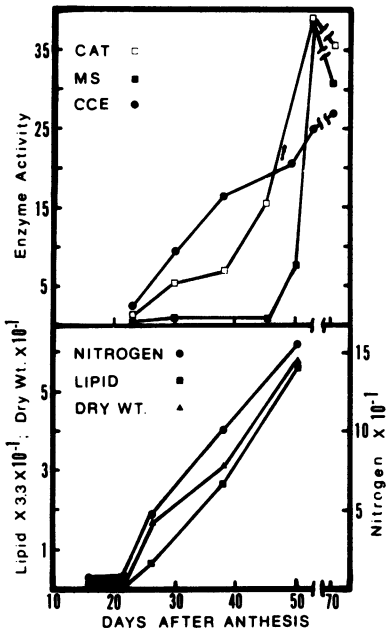

FIG. 4. Changes in glyoxysomal enzyme activities, dry wt, total lipid and nitrogen during embryogenesis and seed desiccation. Upper panel: whole embryos of various ages postanthesis were homogenized and centrifuged at 30,900*g* for 20 min. Supernatant was used directly for assay. Malate synthase (MS) and citrate condensing enzyme (CCE) activities are expressed as nmol of substrate consumed/min·embryo × 10^{-1}. Catalase (CAT) units: that amount of enzyme required to decompose 50% of the H_2O_2 present/120 sec at 25 C (17). Lower panel: dry wt (mg/embryo) was determined after drying for at least 48 hr in a 70 C oven. Portions of the dried embryos were used for nitrogen (μmol of N/embryo) and lipid (mg/embryo) determinations.

Table I. Pelletable catalase activity during embryogenesis.

Differential centrifugation of chopped embryo homogenates yielded S_1 (270*g* supernatant), P_2 (10,800*g* pellet) and P_3 (150,000*g* microsomal pellet). Percent in P_3 equals $P_3/P_2 + P_3$.

DAA	Catalase units[1]/embryo x 10^2			% in P_3
	S_1	P_2	P_3	
20	23.0	15.0	4.2	21.8
25	N.D.[2]	31.6	15.9	33.4
30	120.0	80.2	27.5	25.5
40	205.0	63.0	13.1	17.2
50	399.0	121.0	28.0	18.7

[1] See figure 4

[2] Not determined

obtained. The difficulties were resolved by using a zonal rotor as described under "Materials and Methods." The results of such an experiment with 22-day embryos is illustrated in Figure 5. A broad band of protein was apparent in the middle of the gradient from approximately 1.150 to 1.250 g/cm^3. Catalase activity sedimented as a wide peak from 1.204 to 1.230 g/cm^3. Considerable activity was also present in the 1.17 g/cm^3 region of the gradients coincident with a majority of the NADH Cyt *c* reductase activity (an ER marker). MDH showed a peak activity of 1.204 g/cm^3 with a slight shoulder at 1.226 g/cm^3. Of interest were the high values for all enzyme activities and protein within the light region of the gradient at approximately 1.100 g/cm^3. Malate synthase activity could not be detected in any of the fractions containing peak catalase activity, or at the top of gradients.

Similar gradient experiments were done with 50-day material (Fig. 6). One major protein band peaked near 1.200 g/cm^3. The distribution of NADH Cyt *c* reductase activity was similar to that in 22-day material (Fig. 5), however, catalase activity was not associated with it near the middle of the gradients. Instead, catalase activity was resolved as a sharp peak at 1.245 g/cm^3, notably more dense than at 22 DAA, and was dispersed in several fractions near the top of the gradients. The profile for malate

Table II. Activities extracted from cotyledons of 50 DAA embryos and germinated seeds[1].

Percent is percent in embryos: activities at 50 DAA/40 hr after imbitition.

Enzyme	DAA	Hours after Imbibition							
	50	0	10	20	30	40	50	60	%
	nmoles/min/cotyledon pair								
Malate Synthase	380	350	431	636	676	2040	1990	1674	19
Isocitrate Lyase	0	0	3	38	85	187	192	177	0
Cit. Cond. Enzyme	248	276	250	277	294	349	410	363	71
Catalase[2]	37	18	109	205	274	590	550	480	6

[1] Collected from greenhouse grown plants approx. 120 DAA

[2] See Figure 4 for units

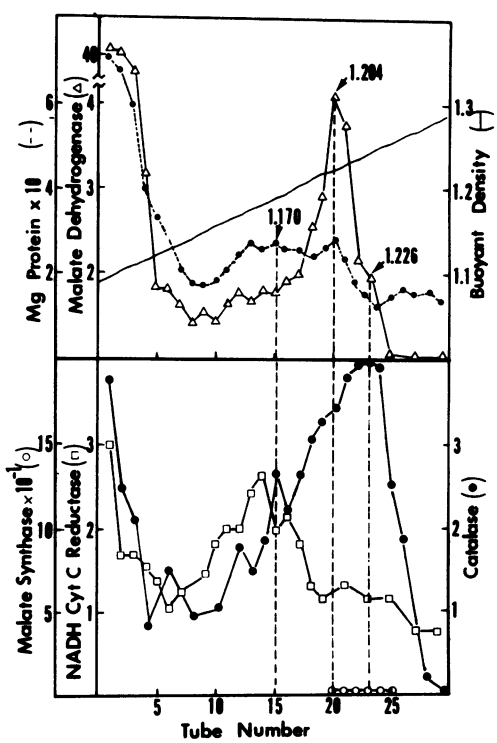

FIG. 5. Isopycnic gradient centrifugation of 22-DAA embryo-clarified homogenates. Whole 22-DAA embryos were chopped with an electric knife, and centrifuged at 270*g* for 10 min. The supernatant was injected onto a dynamically loaded sucrose gradient in a JCF-Z zonal rotor, and centrifuged for 2.5 hr at 25,000*g*. Catalase activities in tubes 10 through 25 represent 52% of that applied. Activities: nmol/min·fraction for all enzymes except catalase; see Figure 4 for units.

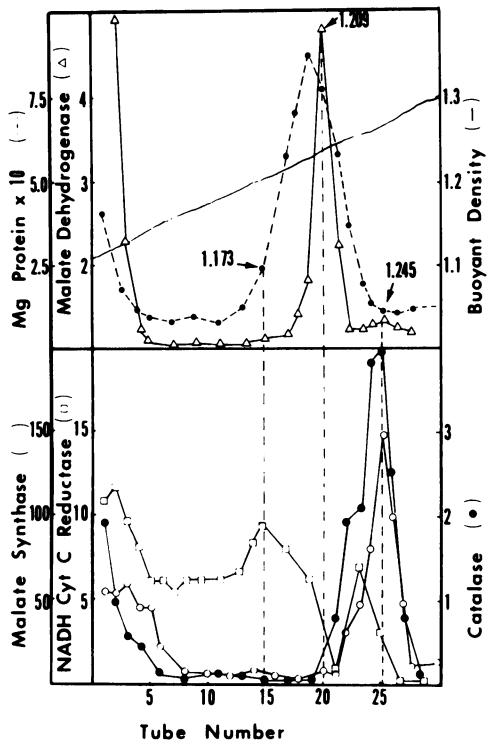

FIG. 6. Isopycnic gradient centrifugation of 50-DAA embryo-clarified homogenates. Catalase activities in tubes 21 through 27 represent 30% of that applied. Conditions and units are the same as in Figure 5.

synthase activity closely followed catalase activities, significantly separated from the major MDH peak which marked the mitochondria.

DISCUSSION

Our study of cotton embryos has shown that certain enzymes known to be compartmentalized within cotton glyoxysomes (1) are also active during seed formation. The levels of activity and their temporal expressions varied, however, depending on the developmental period. The low catalase activity measured prior to 22 DAA appears to be a "base" level similar to that found in achlorophylous tissues (13). At 22 DAA, a significant change in cotyledon metabolism was initiated. Synthesis and deposition of storage proteins and lipids were accompanied by a dramatic rise in catalase activity. The latter suggests a synthesis of microbodies. This postulate is supported by: (*a*) the observation of RER-derived dilations showing catalase cytochemical reactivity; (*b*) pelletable catalase activity (in P_2 and P_3, Table I) after differential centrifugation; and (*c*) equilibration of catalase-containing particles on sucrose gradients. These particles, however, are not competent glyoxysomes because particulate malate synthase activity developed approximately 20 days later without expression of isocitrate lyase activity.

The demonstration of catalase in ER dilations provides the first direct cytochemical evidence to support the concept (2, 10, 14, 15) that microbody matrix proteins are sequestered by the RER prior to vesiculation. Gonzalez and Beevers (10) could not show catalase activity in ER fractions of 2-day-old castor bean endosperm, although they believe that the enzyme was released during fractionation. Our success in showing catalase activity in ER both cytochemically and biochemically most likely is attributable to working with embryonic cotyledon cells lacking massive deposits of reserve protein and lipid as occurs in mature seeds.

The development of malate synthase activity in embryos is unique among the fatty seedlings studied thus far. Its behavior following germination, however, is characteristic (22), *i.e.* an initial lag is followed by a rise to a peak concurrent with isocitrate lyase activity. Considering the model for regulation of "germination enzymes" in cotton (8), the embryonic activity could represent a premature appearance of normal seed activity as a consequence of decreased ABA concentration allowing an escape from ABA arrestment of mRNA translation. This is not a likely explanation since we have found that malate synthase activity developed normally in cultured embryos supplied with exogenous ABA (10 μg/ml) (5) which prevented precocious germination. Isocitrate lyase activity, on the other hand, does not appear when germination is prevented by ABA (18, 20). This indicates that the two glyoxylate cycle enzymes are regulated independently and that the malate synthase activity is probably not a simple slippage in timing of concerted regulation of germination enzymes.

We have no direct evidence to determine whether the malate synthase was added to or activated within microbodies formed earlier, or sequestered within ER lumens and vesiculated as part of newly synthesized microbodies. The increased buoyant density of microbodies at 50 DAA may indicate that protein was added to preexisting particles. Of interest here are the observations that in both developing embryos and germinated seeds, development of considerable catalase activity preceded the appearance of new malate synthase activity. Follow-up efforts are being made to discover the mode of malate synthase accumulation in microbodies.

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