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Cottonseed Malate Synthase[1]

BIOGENESIS IN MATURING AND GERMINATED SEEDS

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ABSTRACT

The activity of malate synthase (MS) (EC 4.1.3.2) appears and increases during cotton (*Gossypium hirsutum* L.) seed maturation, persists through desiccation and imbibition, then increases again following germination. The research reported herein is a comparative study of the synthesis and acquisition of MS into glyoxysomes as they occur in maturing and germinated seeds. Rate-zonal centrifugation of cotyledon extracts revealed that the 5 Svedberg unit (S) cytosolic form of MS was the only form present at 42 days postanthesis (DPA) when activity was first detectable. At later stages (48 DPA, 0 day, 26 hours, and 48 hours), both the 5S and glyoxysomal 20S forms were present, with the 20S form becoming much more prevalent. Western blot analyses revealed that no other form(s) of MS were present in the phosphate-buffered gradients, and that 5S and 20S forms had the same subunit molecular weight in maturing and germinated seeds. Comparisons of radiospecific activity of MS immunoprecipitates following *in vivo* labeling with [^{35}S]methionine for varying time intervals provided strong evidence for a 5S-precursor to 20S-product relationship during both seed maturation and seedling growth. Comparisons of MS labeled *in vivo* and *in vitro* in wheat germ and rabbit reticulocyte lysates programmed with poly(A)$^+$RNA (from maturing and germinated seeds) revealed no detectable differences in subunit molecular weights. These results reinforced our other data indicating that MS was synthesized in the cytosol and acquired by glyoxysomes in both maturing and germinated cotton seeds without involvement of an intervening aggregate pool in the endoplasmic reticulum, or via processing of a cleavable precursor molecule. MS was translated from poly(A)$^+$RNA extracted from 28 DPA cotton seeds. This was nearly 2 weeks before MS activity or protein was detected *in vivo*. This finding invites further study on the regulation of RNA transcripts during maturation.

There is now considerable evidence that activities of glyoxysomal enzymes in cotyledons of oilseeds appear and rise during seed maturation before they exhibit their dramatic increase following seed germination (8, 11). MS[2] (EC 4.1.3.2), one of the key glyoxylate cycle enzymes, is especially interesting in that it appears and increases during seed maturation following the development of catalase and β-oxidation enzymes, but precedes the initial appearance of isocitrate lyase (4, 7). The rise in enzyme activities following seed germination is accompanied by an ultrastructurally obvious (29) and quantitatively measurable (21) increase in glyoxysome volume, whereas changes in glyoxysome volume are not perceptible during late seed maturation. We have been interested in the biogenesis of glyoxysomes during seed maturation and seedling growth for a number of years, especially in cotton seeds (Kunce *et al.* 21, and references therein). In this report, we focus on the biogenesis of MS, making comparisons of events occurring in maturing and germinated seeds. This study is facilitated by our capability to culture cottonseed embryos in the presence of ABA. Under these conditions, normal enzyme development occurs while germination is prevented; this is important because one can examine biogenesis events in immature seeds without the complications and potential misinterpretations related to germination and growth which do not occur during seed maturation.

Centrifugation of germinated seed homogenates on Tricine- or Tris-buffered sucrose gradients revealed the presence of three separable fractions of MS, *i.e.* soluble, ER-associated, and glyoxysomal (14, 15, 17, 19). Rate-zonal centrifugation of these fractions from cucumber seeds revealed that the soluble and glyoxysomal fractions consisted of both 5S monomeric and 19S octameric forms, with the 5S being the predominant form in the cytosol, and the 19S predominant in the glyoxysomes (19). Work with castor bean endosperm produced apparent conflicting results. In one study (10), the glyoxysomal form was characterized as a 5S form, while others (2) reported it was a 20S form. These apparent discrepancies can be resolved, however, when one considers the different methodology used to produce these data (see "Discussion"). The ER-associated MS in castor bean and cucumber fractions was characterized as an aggregate (10, 19). González (10) states that the aggregate was localized within the ER vesicles and therefore should be considered in the scheme of MS biogenesis. Kindl's group (12, 13, 19, 20), however, reported that the 5S monomer was the precursor to the 19S octamer, and that the MS aggregate was outside the ER simply exhibiting sedimentation behavior on gradients similar to ER vesicles. Therefore, ER in their view, did not have a bearing on the biosynthesis of glyoxysomal MS.

Only one study (with cucumber seeds) has been conducted on the radiolabeling of MS relative to its biogenesis during seed maturation (7). Immature seeds removed from the fruit, *i.e.* not cultured on ABA, were pulsed for 3 h with [^{35}S]methionine followed by a 15 h chase to assess relative radiospecific activity of MS in soluble, ER, and glyoxysomal fractions. Rate-zonal centrifugation (7) of immature-seed homogenates revealed only one form (S value not given) of MS. In the studies of immature cotton seeds reported herein, we pulse-labeled immature seeds cultured on ABA for much shorter times (*e.g.* 15 min) and compared changes in relative radiospecific activities with similar *in vivo* labeling of germinated seeds. Two forms of MS (5S cytosolic and 20S glyoxysomal) were identified in rate-zonal gradients; these forms varied in relative amount as the seeds matured. Under our conditions, aggregates of MS were not formed or radiolabeled; therefore, MS aggregates were not considered in the overall biogenesis of glyoxysomal MS.

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[2] Abbreviations MS, malate synthase; PMSF, phenyl-methylsulfonyl fluoride; DPA, days postanthesis.

MATERIALS AND METHODS

Chemicals. Diethylpyrocarbonate, sarkosyl (*N*-lauroylsarcosine), and leupeptin were obtained from Sigma Chemical Co. Methionine, L-[^{35}S] (1100–1300 Ci/mmol, 1 mCi/0.1 ml 50 mM Tricine, pH 7.4) was purchased from NEN-DuPont. Fluoro-Hance was purchased from RPI. Nuclease treated rabbit reticulocyte and wheat germ lysate translation systems were obtained from Promega Biotec, Madison, WI. *Staphylococcus aureus* cells and aprotinin were purchased from Boehringer Mannheim Biochemicals. Guanidine thiocyanate was obtained from Fluka Chemical Co. Cesium chloride was purchased from Bethesda Research Lab. Ready-Solv MP scintillation fluid was obtained from Beckman Chemicals. Other chemicals used are listed in the companion paper (28).

Plant Material. Cotton plants, *Gossypium hirsutum* L. cv Deltapine 62 and Deltapine 70, were grown and immature seeds staged as described by Kunce *et al.* (21). Acid-delinted cotton seeds were surface sterilized in 1% (v/v) NaOCl for 10 min, rinsed thoroughly, then soaked with aeration for 6 h at 30°C in the dark. Age of the seedlings was measured from the initiation of soaking. For *in vivo* radiolabeling experiments, soaked seeds were hand decoated then plated on moist filter paper in covered Petri dishes. These decoated seeds were germinated and grown for 16 h in the dark at 30°C prior to incubation in [^{35}S]methionine (see later for amounts). For nonradiolabeling experiments soaked seeds were scrolled as described by Kunce and Trelease (22). For radiolabeling of maturing seeds, embryos were removed from ovules (42 DPA) and were cultured according to the method of Choinski *et al* (5) in nutrient medium containing 58 mM sucrose and 2.8 µM ABA. [^{35}S]Methionine was added to the embryos in the culture dishes after 2 d in culture.

Tissue Homogenization. Entire immature embryos (1 g) or cotyledons (1 g) excised from germinated seeds were homogenized in 1.5 ml of 100 mM K-phosphate, 8 mM $MgCl_2$, 2 mM glyoxylate (pH 7.2), 1 mM PMSF, and 1% (w/v) PVP. When seeds were radiolabeled *in vivo*, cotyledons from four immature or germinated seeds were homogenized in 1 ml of the same medium. Homogenization was performed in a motor-driven Teflon homogenizer. Homogenates were centrifuged for 30 min at 27,000*g* in a Beckman JA 20 rotor at 4°C. The supernatants were used for rate-zonal centrifugation, or were immunoprecipitated for analysis by SDS-PAGE.

Centrifugation Methods. Rate-zonal centrifugation and assay for MS activity were performed as described by Trelease *et al* (28). Sucrose-gradient centrifugation for the isolation of glyoxysomes was performed as described by Kunce and Trelease (22).

mRNA Preparation. All glassware required for the preparation of mRNA was heated at 220°C for at least 12 h. Aqueous solutions were treated with 0.1% (v/v) diethylpyrocarbonate then autoclaved for 30 min. Total RNA was isolated from 20-h germinated and 28-DPA immature cotton seeds. Cotyledon pairs (100) of each set of seeds were frozen in liquid N_2 and ground to a fine powder in a chilled mortar. The total-RNA isolation was performed using the guanidine thiocyanate/CsCl extraction procedure of Maniatis *et al* (24). Poly(A)$^+$RNA was isolated by applying the total RNA to an oligo(dT)-cellulose column and eluting with 10 mM Tris-HCl, 0.05% SDS (w/v) (pH 7.4).

***In Vitro* and *in Vivo* Radiolabeling.** For *in vitro* translations, aliquots of poly(A)$^+$RNA in sterile water were heated for 5 min at 65°C, then rapidly cooled in ice water. Cell-free translation was performed in two commercial systems, nuclease treated rabbit reticulocyte and wheat germ lysate. Translations with [^{35}S] methionine were accomplished as described by the supplier (Promega Biotec) except both mixtures were incubated at 29°C. The amount of poly(A)$^+$RNA was optimized for each mixture; 2.5 µg and 5.0 µg RNA per 50 µl mixture was used with the reticulocyte and wheat germ systems, respectively. For *in vivo* studies, immature and germinated seeds were incubated in the dark with [^{35}S]methionine for varying times at 30°C. The radioisotope was applied to four different areas of the cotyledons of each seed by adding 25-µl aliquots of 100 µl of H_2O containing either 10 or 30 µCi of label. For the rate-zonal and radiospecific activity experiments (Figs. 4–6), four seeds, incubated with 10 µCi each, were used for each time interval. For *in vivo* experiments, not involving rate-zonal centrifugation (Fig. 7), two seeds were incubated with 30 µCi of label for 1 h. The labeled seeds were washed thoroughly with 10 mM unlabeled methionine then frozen in liquid N_2 and held at −70°C until homogenized. Radiolabeled seeds that were frozen were always used within 1 d.

Immunoprecipitation. The immunoprecipitation and washing procedures were optimized for enzyme yield and to give minimal sample contamination. All steps were performed in 1.5 ml microfuge tubes and centrifuged at 12,000*g* for 1 min unless otherwise stated. All *S. aureus* cells were washed and resuspended to a 10% suspension in 1× buffer (100 mM K-phosphate, 1% Triton X-100 (v/v), 1 M NaCl, 8 mM $MgCl_2$, 1 mM EDTA, 10 mM methionine, 1 mM benzamidine-HCl, 1 mM iodoacetamide, pH 7.2, 1 mM PMSF).

Aliquots (0.3 ml) of the rate-zonal fractions (for Figs. 4–6) were diluted with 0.3 ml of 2× buffer, except it contained 100 mM K-phosphate, 8 mM $MgCl_2$, 25 µg/ml leupeptin, and 25 µg/ml aprotinin. IgGs, as prepared by Trelease *et al.* (28), were added (150 µg) and then incubated for 1 h at 25°C, then for 22 h at 4°C. *S. aureus* cells (80 µl) were added and mixed by inversion for 1 h at 25°C before pelleting. The *S. aureus* pellets were washed five times in 1× buffer, except it contained 150 mM NaCl. The pellets were then washed two times in this modified 1× buffer, except with 0.1% Triton X-100 (v/v) and 0.02% SDS (w/v). The two final washes were in 100 mM K-phosphate, 150 mM NaCl, and 2 mM EDTA (pH 7.2); these pellets were then prepared for SDS-PAGE (22).

The *in vitro* translation mixtures were diluted from 50 µl to 1 ml with 1× buffer, except with 150 mM NaCl, and placed in ice water for 15 min. These solutions were then centrifuged for 15 min at 4°C, and the supernatants were removed and added to new tubes containing 50 mg NaCl to adjust the final NaCl concentration to 1 M. The mixtures were then chilled in ice water for 15 min and centrifuged again for 15 min at 4°C; the supernatants were removed and added to new tubes. *S. aureus* cells (20 µl) were added to preadsorb undesirable material for 30 min with continual inversion at 25°C. Following centrifugation the supernatants were added to new tubes containing 50 µg IgG then incubated for 1 h at 25°C and 22 h at 4°C. *S. aureus* cells (50 µl) were added and mixed by inversion for 1 h at 25°C. The suspension was then centrifuged and the pellet washed nine times (see above). The supernatants (1 ml) from homogenized *in vivo* radiolabeled cotyledons of immature and germinated seeds, were added to 50 mg NaCl and vortexed for 10 s. *S. aureus* cells (30 µl) were added to preadsorb undesirable materials as above. The supernatants were removed, 75 µg (15 µl antisera) of IgGs were added, and the mixture incubated for 1 h at 25°C, then 22 h at 4°C. A suspension of *S. aureus* (100 µl) was added and mixed by inversion 1 h at 25°C. The solution was then centrifuged and the pellet washed nine times as described above.

SDS-PAGE and Western Blotting. SDS-PAGE and Western blotting procedures were performed according to Kunce and Trelease (22). Gels consisted of 8% acrylamide, except for the 7 to 13% gradient gel used for the fluorograph in Figure 7B. SDS-PAGE gels were prepared for fluorography using Fluoro-Hance as described by the supplier. For the determinations of [^{35}S] methionine incorporation into the two forms of MS (Fig. 6), immunoprecipitates of rate-zonal fractions were subjected to SDS-PAGE and stained with Coomassie brilliant blue (22). MS

protein bands were cut out of the gel and placed into 7-ml capped tubes containing 0.5 ml H_2O_2 (30%) and incubated at 50°C for 18 h. Glacial acetic acid (150 μl) was added to each tube, and the mixtures were transferred to 15 ml of Ready-Solv MP in scintillation vials (20 ml). Radioactivity was quantified by scintillation spectroscopy in a Beckman LS 8000 liquid scintillation counter. Quenching of the individual samples was not significantly variable, hence data are reported in cpm.

RESULTS

MS Forms in Cotton Seeds. In the accompanying paper (28), two forms of MS, a 5S monomer and 20S dodecamer, were identified and immunocharacterized in extracts of germinated cotton seeds. Under the conditions employed, an aggregate was not present in gradient fractions of cotton seeds. In this study, a series of rate-zonal experiments was designed to assess the relative amounts of the two forms at different ages in maturing and germinated seeds (Fig. 1). Table I shows the total activity per seed at each age, and gives values for the relative percent activity in the two forms at each age. Both forms were present at all ages except at 42 DPA (Fig. 1A). This was the stage at which MS activity was first detectable in the immature seeds (Table I). The 20S form was predominant (nearly two-thirds of the total gradient MS) after only 6 more d of seed maturation (48 DPA). At later ages, the relative percent of the 20S MS increased up to almost 90% of the total gradient MS. The decreasing percent of 5S MS was not due to a significant decrease in activity in this region of the gradients (note scale differences in Fig. 1), but to an increase in 20S MS. This form of the enzyme was shown to be localized in glyoxysomes of germinated seeds (28); it also was in the glyoxysomes of immature seeds (Fig. 1C). Western blot analyses of the 5S and 20S forms from maturing and germinated seeds showed a single band in 63 kD region (Fig. 2) indicating that the subunit mol wt of 20S and 5S forms was not different.

The data in Figure 1 and Table I provided information only on active forms of MS. To identify possible inactive forms of MS, fractions from rate-zonal gradients were subjected to SDS-PAGE and the electrophoresed proteins were electroblotted to nitrocellulose. Blots probed with anti-MS antiserum (*e.g.* Fig. 3) showed MS existed only in the two regions corresponding to the 5 and 20S activity peaks, hence no inactive forms were detected. The blot in Figure 3 was reprobed with anticatalase antiserum allowing us to identify 11S catalase, thereby confirming the integrity of the gradients and reliability of our estimated S-value calculations.

***In Vivo* Radiolabeling of MS.** Radiolabeling patterns of MS forms in extracts of maturing and germinated seeds separated by rate-zonal centrifugation are shown in Figures 4 and 5, respectively. The patterns for both maturing and germinated seeds indicated qualitatively that during the 30 or 45 min radiolabeling period, the 5S accumulated more label than the 20S form, whereas during the 120 min radiolabeling period, the 20S form accumulated more label. The similar patterns shown in these Figures support a 5S-precursor to 20S-product relationship in both maturing and germinated seeds.

Values for radiospecific activities of the MS forms in maturing and germinated seeds after incubation with [^{35}S]methionine for varying times are shown in Figure 6, A and B. Radiospecific activity was calculated using the activity of MS as a basis for comparison rather than protein. In both maturing and germinated seeds, the 5S form rapidly incorporated [^{35}S]methionine for the first hour (when peak radiospecific activity was reached). Radiospecific activity of the 20S form in both maturing and germinated seeds exhibited an initial lag period before it increased over the next 120 min. The relative radiospecific activity of the 5S form to total radiospecific activity was calculated from data given in Figure 6, A and B, and is shown in Figure 6C.

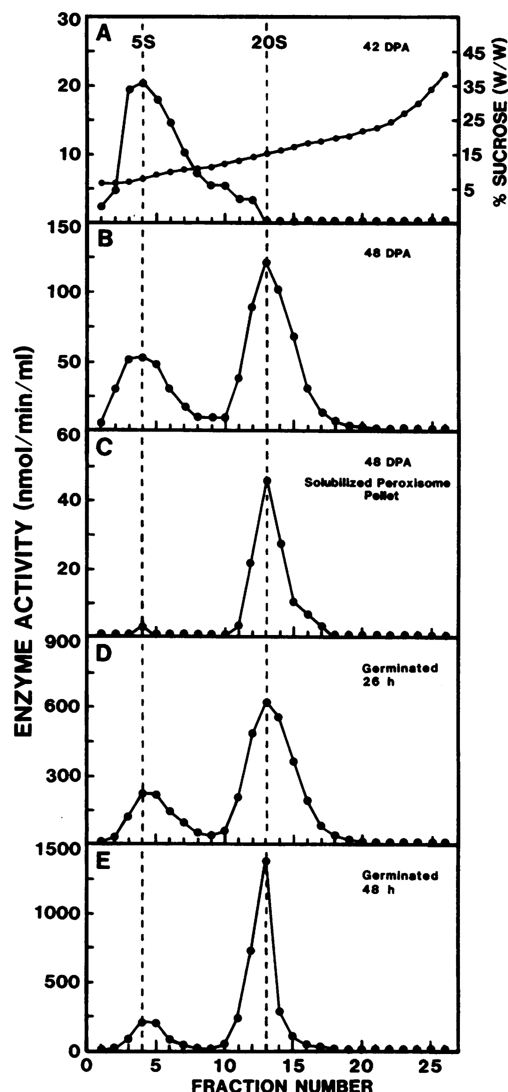

FIG. 1. Profiles of malate synthase activity following rate-zonal centrifugation of extracts from cotton cotyledons excised from varying aged immature and germinated seeds. The extracts were layered onto sucrose gradients (5–25%, w/w) and centrifuged for 19 h (5°C) in a Sorvall AH-627 rotor at 24,000 rpm. Between 80 to 100% of the activity applied was recovered in the various gradients. Sedimentation coefficients were calculated by the method of McEwen (25).

Table I. *Amount of Malate Synthase Activity in Maturing, Dry, and Germinated Cotton Seeds, and Relative Percent of Gradient Activity in the 5S and 20S Forms at Each Age*

Age	Total Activity	Relative Activity[a]	
		5S	20S
	nmol/min·seed	%	
42 DPA	45	100	0
48 DPA	219	37.9	62.1
Dry	380	36.1	63.9
26 h	552	25.3	74.7
48 h	1922	12.4	87.6

[a] Ratio of enzyme activity in the 5S region of rate-zonal gradients (Fig. 1) to total gradient activity.

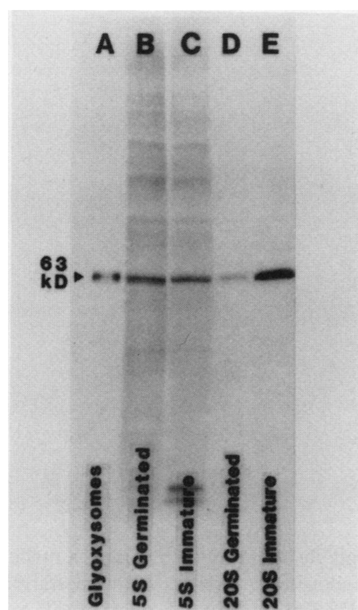

FIG. 2. Western blot showing comparative electrophoretic (SDS-PAGE) migration of 5S and 20S malate synthase subunits from immature and germinated seeds. Samples B through E were taken from peak activity tubes following rate-zonal centrifugation on sucrose gradients (5–25%). Sample A was prepared from glyoxysomes isolated from germinated seeds by isopycnic centrifugation on sucrose gradients. SDS-PAGE was done on 4% stacking, 8% separating, 17 cm long gels. All lanes had 4 nmol/min MS activity except lane D which had 1.6 nmol/min.

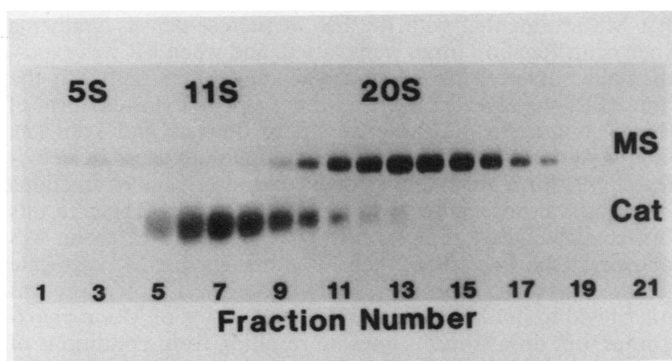

FIG. 3. Western blot of fractions from a rate-zonal gradient similar to Figure 1E (48-h germinated seeds) probed for malate synthase (MS) and catalase (CAT) following transfer from a SDS-PAGE gel (as in Fig. 2). A constant amount of gradient sample (50 μl) was applied to each lane.

These values are normalized ratios, thus potential errors in assessing addition and uptake of the [^{35}S]methionine did not affect these values. The trends shown in Figure 6, A and B, and the decreasing slopes in Figure 6C (indicative of increasing radiospecific activity of the 20S form) provide quantitative evidence for the qualitative patterns shown in Figure 4 and 5.

Lanes A-e and B-a in Figure 7 show the comparative migrations of *in vivo* labeled MS subunits following SDS-PAGE on 8% and 7 to 13% gradient (B-a) separating gels relative to *in vitro* radiolabeled MS (see below). MS in immature seeds (42 DPA) cultured on nutrient media in the presence of ABA readily took up applied [^{35}S]methionine over a 60 min time period, whereas much less label was incorporated into MS during the same incubation period for germinated seeds.

***In Vitro* Labeling of MS.** Figure 7 illustrates the results obtained when wheat germ and reticulocyte translation systems were programmed with poly(A)$^+$RNA isolated from 28-DPA and 20-h-germinated seeds and compared to *in vivo* radiolabeled MS from both germinated and maturing seeds. All samples were immunoprecipitated then subjected to SDS-PAGE. Fluorographic analyses showed a single band in the 63 kD region for all samples and treatments. Proteolysis of mature MS or a possible MS precursor(s) was avoided by rapidly boiling (Fig. 7A, lane c) and incubating and washing immunoprecipitates in the presence of protease inhibitors (all samples). The translation of MS from poly(A)$^+$RNA isolated from 28-DPA seeds indicated that mRNA for MS existed in immature seeds 12 d before activity (4) or the protein (28) was detected. The data in Figure 7 also showed that the subunit mol wt of the enzyme purified by Trelease *et al.* (28) (lane A-d) was not altered during purification.

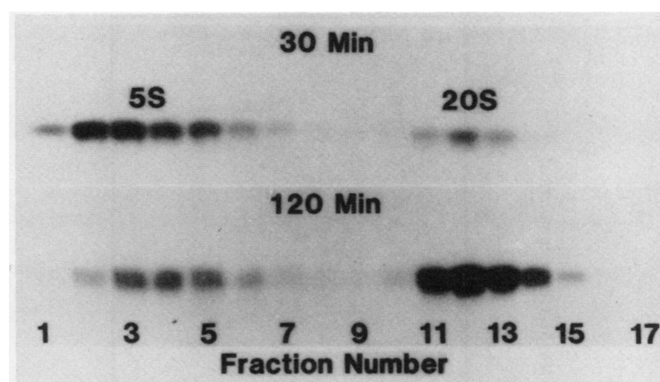

FIG. 4. Fluorograph illustrating comparative radiolabeling patterns of 5S and 20S forms of malate synthase in maturing seeds following *in vivo* incubation for 30 or 120 min in [^{35}S]methionine. MS was immunoprecipitated from 0.3 ml of each rate-zonal (5–25%, w/w sucrose) fractions and subjected to SDS-PAGE as in Figure 2.

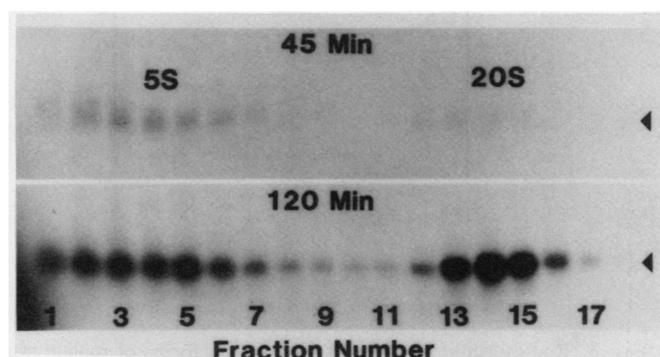

FIG. 5. Fluorograph illustrating comparative radiolabeling patterns of 5S and 20S forms of malate synthase in germinated seeds following *in vivo* incubation for 45 or 120 min in [^{35}S]methionine. MS was immunoprecipitated from 0.3 ml of each rate-zonal (5–25%, w/w sucrose) fractions and subjected to SDS-PAGE as in Figure 2. Arrows indicate migration of purified MS (63 kD).

DISCUSSION

Our results strongly indicate a 5S-precursor to 20S-product relationship between the two forms of MS found in both immature and germinated cotton seeds. This is in agreement with interpretations of other studies on this relationship, but those studies were done only on germinated cucumber seeds (19, 20). We employed homogenizing and centrifugation conditions that prevented formation of MS aggregates (28), thereby allowing us

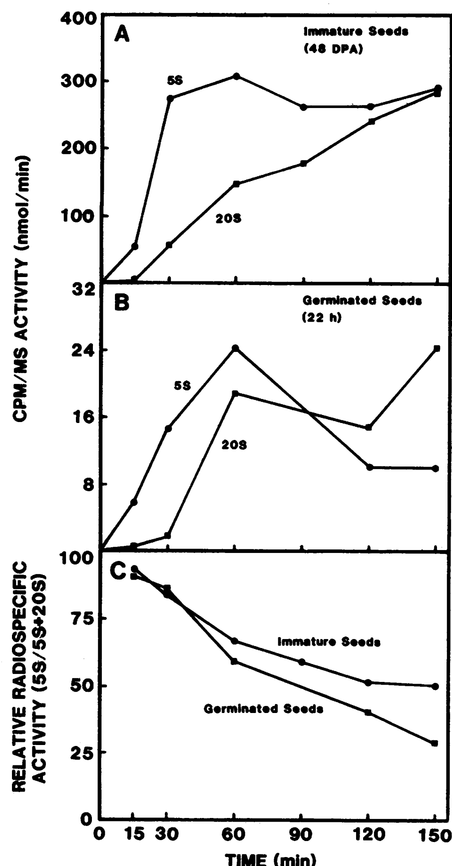

FIG. 6. Radiospecific activities of 5S and 20S forms of malate synthase following incubation of immature (panel A) or germinated (panel B) seeds in [^{35}S]methionine for varying time periods. Panel C is a computed ratio of the data presented in panels A and B. Seeds were homogenized after each incubation period, the extracts subjected to rate-zonal centrifugation, peak-activity fractions in the 5S and 20S regions were immunoprecipitated and subjected to SDS-PAGE, and the Coomassie-stained MS bands were cut from the gels and radioactivity determined by scintillation counting.

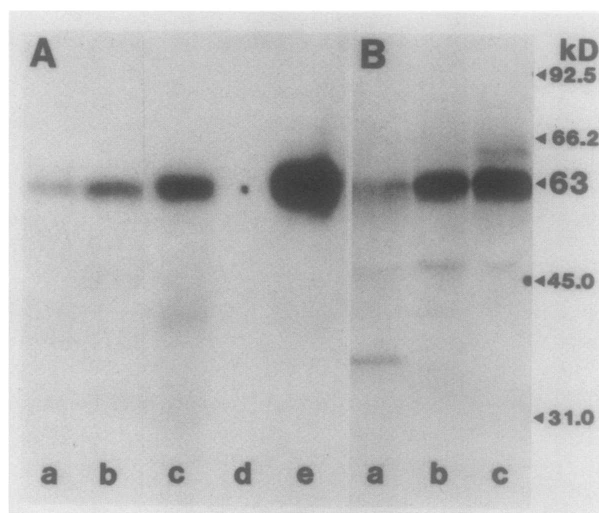

FIG. 7. Fluorographs of immunoprecipitates made with antimalate synthase serum following *in vivo* or *in vitro* translations with [^{35}S] methionine. A, SDS-PAGE on a 4% stacking, 8% separating (17 cm long) gels; B, SDS-PAGE on 4% stacking, 7-13% gradient separating (17 cm long) gel. *In vivo* translations are lanes A-e and B-a of maturing and germinated seeds, respectively. *In vitro* translations with reticulocyte lysate are A-b and B-b,c, and with wheat germ lysates are A-a,c. Poly(A)$^+$RNA is from germinated seeds (A-a,b,c; B-b) or immature seeds (B-c). Lane A-d is a radioactive ink spot marking the migration of purified MS. The wheat germ translation mixture used for A-c was boiled in 2% SDS for 15 min before immunoprecipitation.

to examine biogenesis of MS without the complication of putative nonphysiological aggregates (12), which likely would result in indiscriminate aggregation of varying form(s). A 5S form was shown to be the major form of MS in the cytosol (soluble fraction on isopycnic gradients of organelles) of cucumber (20), castor bean (10), and cotton (RB Turley, RN Trelease, unpublished results). In addition, a form having estimated S values between 18 to 21S was found to be the major form in glyoxysomes isolated from cotton (28; Fig. 1C), cucumber (20), and castor beans (2). In another study on castor beans (10), the glyoxysomal form was identified as a 5S form. However, this was determined by analyzing enzyme that was released from these glyoxysomes by gentle mechanical disruption in a low-salt medium. Significantly more MS activity was released when castor bean glyoxysomes were osmotically shocked in a 0.2 M KCl medium (2). Collectively then, our results support the concept advanced previously by others (14, 17, 20) that MS is synthesized in the cytosol before being incorporated into glyoxysomes.

An aggregate form(s) of MS shown to be associated with ER in Tris- or Tricine-buffered sucrose-density gradients was considered in the biogenesis route of MS. Bowden and Lord (2) reported that a cytosolic form of MS did not occur in castor beans. Their data showed that microsomal MS was labeled with [^{35}S]methionine before the glyoxysomal enzyme, indicating that precursor MS was in the ER (23). Gonzalez (10) published data showing that MS sedimented with the ER in sucrose-density gradients when centrifugation times were varied, and when ER was experimentally subjected to the so-called 'magnesium shift.' In the same study she revealed the occurrence of a 5S cytosolic form of MS in contrast to the earlier report of Bowden and Lord (2). Kindl and coworkers (14, 20) compared the radiospecific activities of MS forms in both castor bean and cucumber fractions following varying pulses with [^{35}S]methionine. Their results showed the highest radiospecific activity in the cytosolic MS following a short pulse (less than 120 min). The ER-MS aggregate had the lowest radiospecific activity in both seeds, therefore was interpreted to not be involved in the trafficking of MS to glyoxysomes, but an artificial aggregate resulting from conditions of seed homogenization. Further support for the contention was given in their report where they used flotation gradients and centrifugation to separate the MS aggregate from ER vesicles (15). González (10) was concerned that these results reflected a mechanical dissolution of MS from the inside of the ER vesicles. The point of discussion is that there is considerable controversy between groups as to the biogenetic importance of this recognized aggregate associated by some physical means with ER in these seed fractions.

We also have found MS associated with the ER fractions from cotton seeds when the seeds were homogenized and centrifuged in Tricine-buffered media for 3 h (3). However, MS aggregates did not sediment with ER when the centrifugation time (22 h) was increased nor when Hepes was substituted for Tricine. In related studies, MS and glyoxysomal MDH translated *in vitro* in the absence of microsomal vesicles were posttranslationally imported *in vitro* into cucumber (18) and castor bean (9) glyoxysomes, respectively. Becker *et al.* (1) attempted co-translational transport of MS as well as other glyoxylate-cycle enzymes into dog pancreas microsomes, but were not successful. The data considered collectively at this stage favor a cytosolic synthesis and posttranslational import of MS into glyoxysomes without

the involvement of ER. Our contribution to the trafficking of MS is that we have studied another seed system, involving both maturing and germinated seeds, wherein conditions were employed such that an aggregate was not formed. This made it possible to provide unambiguous data which showed that a 5S cytosolic precursor form of MS was somehow converted to an oligomerized (dodecamer) (28) form in glyoxysomes in the absence of an intermediate aggregate form.

The only other studies involving radiolabeling of MS in maturing seeds were done by Kindl and coworkers with cucumber (7, 16). However, the immature seeds were radiolabeled in the absence of ABA, thus the data reflected events that occurred during a germination mode, rather than one where precocious germination was blocked by the presence of ABA. They (7) concluded that MS was synthesized in the cytosol prior to import into glyoxysomes. MS found associated with ER in the Tris-buffered gradients was believed to be an aggregate of soluble glyoxysomal MS, forming an additional cytosolic pool. This pool was not necessarily thought to be an intermediate precursor pool, but rather a reservoir for the import of glyoxysomal MS into glyoxysomes.

In maturing cotton seeds, we identified only two forms of MS (Figs. 1, 2, and 4). Only the 5S form was present in the 42-DPA seeds corresponding to the age when MS activity was first detectable (Table I). This MS exists solely as the cytosolic form. Because it was the earliest-appearing form, and the 20S form (found only in the glyoxysomes) appeared later in seed maturation (Fig. 1), it is logical to speculate that the 5S form is the precursor to glyoxysomal MS without going through a reservoir of aggregated ER-associated MS. This hypothesis is supported by our qualitative (Fig. 4) and quantitative (Fig. 6A) data derived from studies of ABA-cultured embryos. Essentially the same patterns were observed from *in vivo* studies with germinated seeds (Fig. 5). Comparisons of MS forms in immature and germinated seeds showed that sedimentation coefficients were the same (Fig. 1) as were subunit mol wt (Fig. 2) and immunological characters (Fig. 5A; Ref 28). From these collective data, we conclude there are no appreciable structural differences between the forms in maturing and germinated seeds, and that the precursor to product relationship of MS does not differ in maturing and germinated seeds.

This conclusion is interesting because the cellular events occurring in immature and germinated seeds are not the same. Kunce *et al.* (21) did a morphometric analysis of glyoxysomal size, volume, and number per cell in maturing and germinated cotton seeds. Throughout the maturation phase (30–50 DPA), the glyoxysomes remained nearly spherical (about 0.5 μm diameter). Following germination, when MS activity increased again, the glyoxysomes became elongate and highly pleiomorphic with the volume increasing nearly 7-fold over the organelles found in cotyledons at late-stage maturation. Thus, it appears that MS is synthesized and added to spherical, relatively unchanging organelles during seed maturation by similar trafficking events that occur when the organelles are increasing in volume during postgerminative seedling growth.

To our knowledge, poly(A)$^+$RNA has not been extracted (at any stage) from any other maturing seeds for the purpose of translating glyoxysomal enzymes in an *in vitro* translation system. RNA was extracted from 28 DPA cotton seeds originally to examine the product(s) of *in vitro* translated catalase for another study. MS was immunoprecipitated from this cell free translation mixture (Fig. 7). The data showed that for both immature and germinated seeds, the *in vitro* translated products banded in the same region of gels as did *in vivo* radiolabeled products. *In vitro*/*in vivo* comparisons of radiolabeled immunoprecipitates from germinated seeds have been made previously for cucumber (13, 26) and castor bean (6), giving the same results. Collectively, these results indicate a larger mol wt precursor of MS is not involved in the trafficking of MS into oilseeds glyoxysomes. We employed added precautions by including several protease inhibitors in the first five washing steps, and prepared a sample which was boiled in SDS immediately following *in vitro* translation (Fig. 7). Glycosylation of MS could alter the mobility of the *in vivo* labeled product and compensate for cleavage of a putative presequence giving the result in Figure 7. We did several tests for detecting sugar residues on the enzyme and evaluated the incorporation of [^{3}H]glucosamine (3). Results of all these experiments failed to show glycosylation of cottonseed MS, therefore we believe the results in Figure 7 reflect the absence of a cleavable peptide *in vivo*.

The results from *in vivo* radiolabeling of ABA-cultured embryos indicate *de novo* synthesis of MS when its activity is first detected. Köller *et al.* (16) reported similar results after labeling immature cucumber seeds (−ABA) with [^{3}H]leucine. Western blot analysis of immature cotton seed extracts (28) indicated MS protein was not present before activity appeared. Choinski *et al.* (5) found that addition of inhibitors to ABA-cultured cotton embryos known to block transcription (actinomycin D), polyadenylation (cordycepin), and translation (cycloheximide) essentially prevented development of new MS activity. Collectively, these data reinforced the notion of *de novo* synthesis of MS in the immature embryos via transcription and translation when it was needed, *i.e.* at 42 DPA. *In vitro* translations in this study, however, showed that at least some poly(A)$^+$RNA transcripts for MS were already present at 28 DPA. This raises the question as to how translation of MS is regulated in maturing seeds. Inhibition of the appearance of MS activity by actinomycin D treatment reported by Choinski *et al.* (5) probably did not reflect an inhibition of the transcription of MS transcripts, but possibly transcriptional inhibition of other component(s) which activated or derepressed the translation of 'masked' MS mRNA. During germination and seedling growth of cucumber, measurements of the changes in amounts of MS (measured either by assaying MS activity or using immunological reactions with specific antisera) indicated that the developmentally regulated synthesis of MS was brought about primarily by changes in the amount of MS gene transcripts, rather than a control of translation (27, 30). Further studies are needed to decipher the potentially complex mechanism of initial MS appearance in maturing seeds.

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