DEVELOPMENTAL STUDIES ON

GLYOXYSOMES IN *RICINUS* ENDOSPERM

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ABSTRACT

The development of glyoxysomes and their associated enzymes, isocitrate lyase and malate synthetase, was studied in the endosperm of castor bean seeds during germination and early growth in darkness. The protein content of the glyoxysome fraction, separated by sucrose density centrifugation, increased linearly from day 2 to day 4 and declined subsequently, while maximum enzyme activities were reached at day 5. The specific activities of the enzymes in the glyoxysomes increased until day 5 and remained constant thereafter. At all stages of germination the only organelle with isocitrate lyase activity was the glyoxysome, but at the earlier stages a greater portion of the total activity was recovered in the soluble form. Malate synthetase was found primarily in the glyoxysomes after day 4, but at earlier stages part of the activity appeared at regions of lower density on the sucrose gradient. It was shown that this particulate malate synthetase activity was due to glyoxysomes broken during preparation, and that, as a result of this breakage, isocitrate lyase was solubilized. We conclude that both enzymes are housed in the glyoxysome in vivo throughout the germination period, and that the rise and fall in enzyme activities in phase with fat breakdown correspond to the net production and destruction of this organelle.

INTRODUCTION

During the germination of fatty seeds such as those of castor bean, the stored fat is converted to carbohydrate (1). One stage in the reaction sequence from fat to carbohydrate is the glyoxylate cycle (9) which converts acetyl CoA, arising from the breakdown of fatty acids, to succinate. During the germination period the key enzymes of the glyoxylate cycle, isocitrate lyase and malate synthetase, undergo striking changes in activity (4, 18). From extremely low levels in the ungerminated seeds, the activities of both enzymes increase rapidly until the time when the rate of conversion of fat to carbohydrate in the endosperm reaches its maximum at day 5. Subsequently the activities decline. The increase of these activities early in germination is now known to be due to a *de novo* synthesis of the enzymes (6, 8, 11, 15) that are present in the plants only during the period when fat is being utilized.

In castor bean seeds, the fat is localized in the endosperm, and the carbohydrate produced, principally sucrose, is absorbed by the cotyledons of the growing embryo (10). The rate of fat conversion reaches its maximum at the 5th day of germination. From the endosperm of 5-day-old seedlings Breidenbach and Beevers (2, 3) isolated a new kind of organelle carrying the enzymes of the glyoxylate cycle and therefore named the glyoxysome. This finding raised the possibility that the striking changes observed in the activities of

isocitrate lyase and malate synthetase during germination could be related to the formation and disappearance of this organelle.

MATERIALS AND METHODS

Preparative Procedures

Seeds of castor bean, Ricinus communis L. var. Baker 296 or Hale, were soaked for 12–16 hr in tap water at room temperature and then placed in moist vermiculite at 30°C and constant humidity in the dark (day 0). Seedlings were harvested at daily intervals, the testa and the embryo (root, hypocotyl, and cotyledons) were removed, and only the endosperm tissue was used as experimental material. This tissue was homogenized in a solution of 0.4 M sucrose, 0.167 M Tris buffer pH 7.5 or 0.167 M Tricine buffer (California Biochemical Corp., Los Angeles, Calif.) pH 7.8, 0.01 M dithiothreitol, 0.01 M KCl, 0.001 M MgCl$_2$, and 0.01 M ethylenediaminetetracetic acid (EDTA), pH 7.5. Usually 2 ml of medium was used for each gram of tissue. After the tissue was treated in an onion chopper, it was carefully ground in a mortar, and the resulting crude homogenate was filtered through two layers of cheesecloth. After centrifugation at 270 g for 10 min, the supernatant solution (supernatant I) was centrifuged at 10,800 g for 30 min. The resulting pellet (the crude particulate fraction) was resuspended in a few ml of 32% sucrose (w/v) and layered on the top of a sucrose density gradient. Discontinuous gradients were prepared by adding 5 ml of 60% sucrose, and 10 ml of 57% sucrose, 15 ml of 51% sucrose, 15 ml of 44% sucrose, and 5 ml of 32% sucrose, in sequence in cellulose nitrate tubes. Usually, the crude particulate fraction obtained from 40 g of tissue was applied to such gradients. Linear continuous gradients were prepared from 19.5 ml of 32% sucrose and 19.5 ml of 60% sucrose over a cushion of 11 ml of 60% sucrose, and on these gradients the particulate fraction from not more than 10 g of tissue was applied. For the experiments in which mitochondria were purified by flotation, the mitochondrial fraction from a discontinuous gradient was concentrated from 43 to 57% sucrose by adding 70% sucrose. This suspension was used as the cushion for a linear continuous gradient grading from 57 to 35% sucrose. All sucrose solutions for gradients contained 0.001 M EDTA pH 7.5. The gradients were centrifuged at 64,300 g in a Spinco rotor SW 25.2 (Beckman Instruments Inc., Spinco Div., Palo Alto, Calif.) for 5 hr; flotation gradients were spun for 8 hr. The gradients were fractionated from the bottom of the tubes. From discontinuous gradients the bands and interphases were usually collected as whole fractions; from continuous gradients 1 ml fractions were collected by an Isco density gradient fractionator (Instrumentation Specialties Company,

Inc., Lincoln, Nebraska). All operations were carried out at 0–4°C.

Biochemical Assays

All enzymes were assayed spectrophotometrically. Isocitrate lyase and malate synthetase were assayed essentially as described by Hock and Beevers (8). Succinic dehydrogenase was assayed by the method of Hiatt (7) with the use of an extinction coefficient 1.56 × 10^7 cm^2/mole for dichlorophenol indophenol (16). Catalase was assayed by a slight modification of the method of Lück (12) with the use of an extinction coefficient 6.7 × 10^4 cm^2/mole for H$_2$O$_2$ (13). Protein was determined by a modification of the Lowry procedure (5). Sucrose concentrations (w/v) were determined with a Bausch & Lomb Abbe Refractometer (Bausch & Lomb, Inc., Rochester, N.Y.).

Labeling Experiments

0.25 μc of leucine-^{14}C (8.0 × 10^{-4} μmoles in 5 μl) or 2.5 μc of leucine-^{3}H (6.25 × 10^{-5} μmoles in 5 μl) were applied directly to each of 10–20 endosperm halves (17). The tissue was incubated at 30°C in moist atmosphere in Petri dishes for a definite time of treatment (2–6 hr) and then homogenized together with unlabeled endosperm. Organelle fractions were isolated as described above. The incorporation of radioactivity into these fractions was determined by the method of Mans and Novelli (14) and the precipitated protein samples were assayed on a Beckman DPM Scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). For the double labeling experiments, the window of the ^{3}H-channel was adjusted so that the overspill of ^{14}C was about 15% (internal standard). No ^{3}H was detected in the ^{14}C-channel.

RESULTS

Protein

During the first 3 days of germination of the castor bean seeds the protein content of the whole endosperm remains constant, but, as shown in Fig. 1, the protein content of the crude particulate fraction increases rapidly until the third day. After the third day, when the total protein of the endosperm begins to decrease, the protein of the crude particulate fraction accounts constantly for about 13% of the total protein. Thus, early in germination a portion of the protein of the seed is transformed into newly synthesized organelles, and at later stages, when a net loss of protein occurs, total protein and particulate protein are degraded at the same rate (Fig. 1). Eventually the products are completely absorbed by the growing embryo (17).
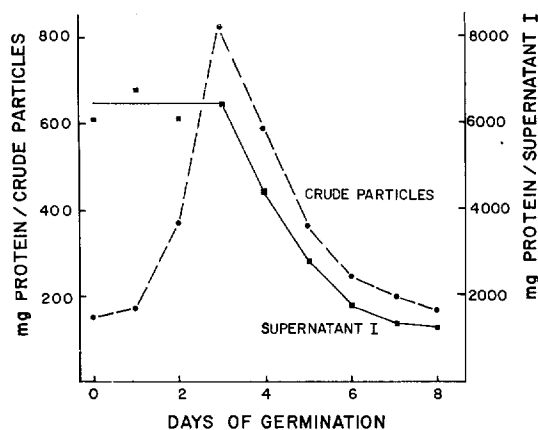
FIGURE 1 Changes in the protein content of the endosperm (supernatant I and of the crude particulate fraction during the germination of castor bean seeds. The results are expressed as mg protein/100 g fresh weight of endosperm and as mg protein/crude particulate fraction from 100 g fresh weight of endosperm.
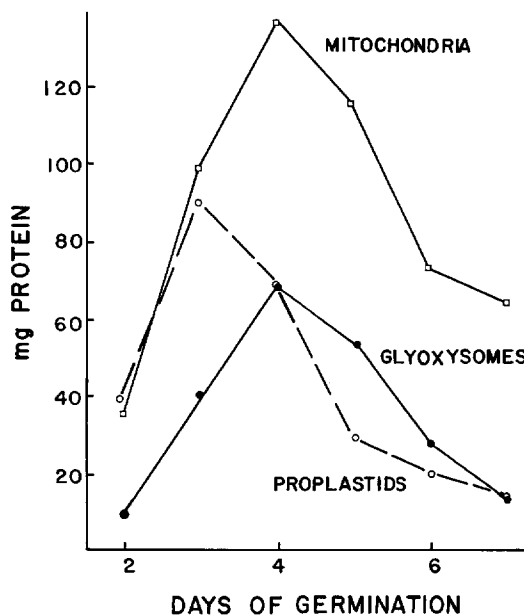

FIGURE 2 Changes in the protein content (mg protein/100 g fresh weight of endosperm) of the mitochondrial, proplastid, and glyoxysome bands isolated on discontinuous sucrose density gradients during the germination of castor bean seeds.

Centrifugation of the crude particulate fraction on a sucrose density gradient results in three major protein bands–the mitochondria, the proplastids and the glyoxysomes (2). From the 5th day onward, the protein present in these bands accounted for about 60% of that added as crude particulate protein. Clearly defined bands of organelles were obtained only after 2 days of germination, and between days 2 and 3 only 30% of the crude particulate protein layered on the gradient was recovered in the three bands. The rest was present as soluble protein at the top of the gradient and as a background throughout the gradient.

Fig. 2 shows the protein content of the major organelles for days 2–7 during germination; at 8 days the endosperm has almost completely disappeared. The protein recovered in the proplastid fraction accounts throughout for about 10% of that present in the crude particulate fraction and thus reaches a peak at day 3. The protein in the glyoxysome and mitochondrion fractions increases until the 4th day.

During the 2–4 day period there was a sevenfold increase in the amount of protein recovered as glyoxysomes. After the 4th day the protein in each organelle fraction declines. Nevertheless, it was found that when leucine-^{14}C was introduced into the intact endosperm of 5-day-old seedlings, it was

incorporated into trichloroacetic acid-precipitable protein from each of the separated organelle fractions.

Isocitrate Lyase

The results in Fig. 3 establish that at all stages of germination the glyoxysomes account for almost all of the isocitrate lyase activity present in the organelles separated from the crude particulate fraction. The same distribution was observed on both continuous and discontinuous gradients. The small activity in the proplastids between days 4 and 6 probably represents a contamination of this fraction by glyoxysomes. The isocitrate lyase activity of the glyoxysome fraction reaches a peak at day 5, corresponding to the peak of the total enzyme activity (that present in supernatant I, before sedimentation of the crude particulate fraction) and that of the crude particulate fraction (Fig. 4). However, the proportion of the total isocitrate lyase activity that is recovered in the crude particulate fraction is not constant throughout the germination period. From day 5 onward, most of the enzyme activity was recovered
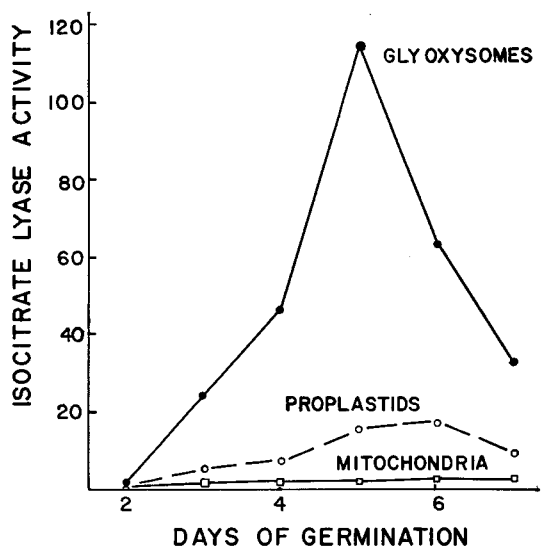
FIGURE 3 Changes in the isocitrate lyase activity of the three major particle bands from the endosperm of germinating castor bean seeds isolated on discontinuous sucrose density gradients. Activity: μmoles isocitrate reacted/min per 100 g fresh weight of endosperm.
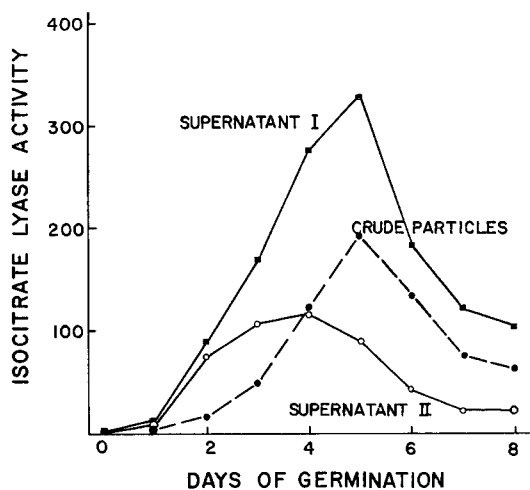

FIGURE 4 Changes in the isocitrate lyase activity of the crude fractions from the endosperm of germinating castor bean seeds. Supernatant I, 270 g supernatant; supernatant II, 10,800 g supernatant; crude particles, 10,800 g pellet. Activity: μmoles isocitrate reacted/min per 100 g fresh weight of endosperm.
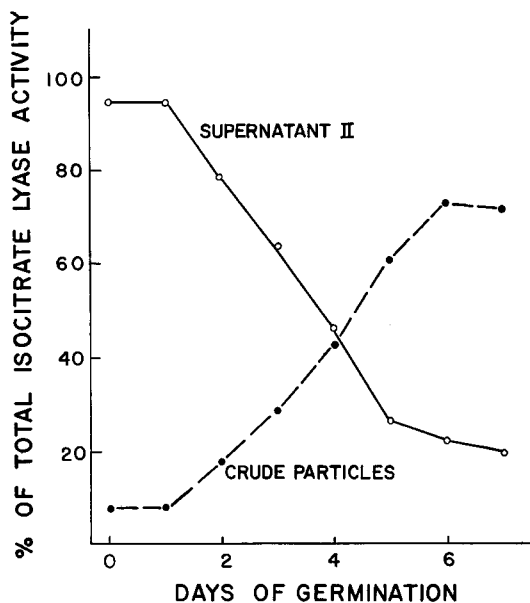

FIGURE 5 Changes in the distribution of the total isocitrate lyase activity (supernatant I) between soluble and particulate fractions. Supernatant II, 10,800 g supernatant; crude particles, 10,800 g pellet.

in the particulate fraction. However, at earlier stages more of the enzyme activity was present in the supernatant solution (supernatant II), remaining after sedimentation of the crude particulate material. The enzyme present in supernatant II was sedimented only after 10 hr at 250,000 g. As shown in Fig. 5, the percentage of total isocitrate lyase associated with the crude particulate fraction increased from 5 to 75% between days 1 and 6. Repeated experiments showed that the portion of the enzyme activity not associated with organelles was characteristic for the stage of germination. Various modifications of the homogenization procedure and of the grinding medium (including the substitution of Tricine for Tris buffer) failed to increase the proportion of particulate isocitrate lyase activity at the earlier stages.

As shown in Fig. 6, the specific activity of the isocitrate lyase in the glyoxysomes increases 10-fold between the 2nd and 5th days of germination and remains constant after that. Thus, the decline of the particulate enzyme activity parallels the decline of the protein in the glyoxysomes.

Malate Synthetase

The developmental pattern and intracellular localization of this second key enzyme of the

glyoxylate cycle is, in general, the same as that for isocitrate lyase. The total malate synthetase activity and that present in the glyoxysomes
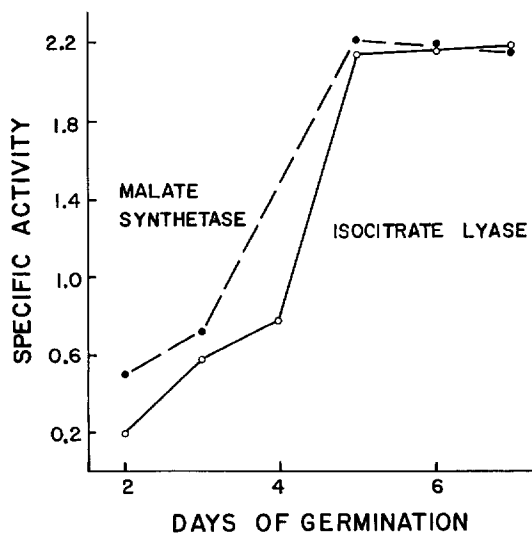
FIGURE 6 Changes in the specific activities of iso-citrate lyase and malate synthetase of the glyoxysomes during germination of castor bean seeds. Specific activity: μmoles substrate consumed/min per mg glyoxysome protein.
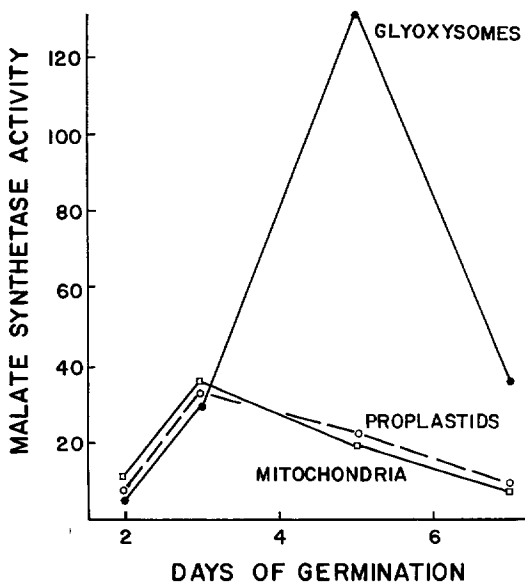

FIGURE 7 Changes in the malate synthetase activity of the three major particle bands from the endosperm of germinating castor bean seeds isolated on discontinuous sucrose density gradients. Activity: μmoles glyoxylate reacted/min per 100 g fresh weight of endosperm.

TABLE I

Ratio of Isocitrate Lyase to Malate Synthetase in the Castor Bean Endosperm (Supernatant I) and in the Isolated Glyoxysomes at Different Days of Germination

	Days			
	2	3	5	7
Supernatant I	0.65	0.73	0.85	1.08
Glyoxysomes	0.40	0.80	0.88	0.92

increases to day 5 and then declines (Fig. 7), and the specific activity in the glyoxysomes remains constant after day 5 (Fig. 6). The ratio of the two enzyme activities in the original extract (supernatant I) and in the glyoxysomes is shown in Table I.

As shown in Fig. 7 most of the particulate malate synthetase is present in glyoxysomes from day 4 onward. However, in contrast to the finding for isocitrate lyase, considerable amounts of malate synthetase activity were recovered in the mitochondrial and proplastid regions of the discontinuous gradient during the early stages of germination. When Tris buffer was used in place of the

usual Tricine, the percentage of the total malate synthetase activity recovered in the particulate fraction increased from 30 to 70% between days 2 and 5, as was found for isocitrate lyase. However, when Tricine was used, 60% of the total malate synthetase was particulate, even at the younger stages. Regardless of which buffer was used, roughly equal amounts of malate synthetase were present in each of the three organelle fractions prepared from younger seedlings on discontinuous gradients. The fact that isocitrate lyase did not accompany the malate synthetase recovered in the mitochondrial and proplastid bands shows that intact glyoxysomes are not present at these less dense regions of the gradient. It was demonstrated that added soluble malate synthetase was not absorbed by mitochondria and that the activity associated with mitochondria and pro-plastids was not removed by washing the crude particulate fraction with grinding medium before centrifugation on the gradient. However, when the mitochondrial fraction from 3 day old endosperm with associated malate synthetase was recentrifuged on a linear flotation gradient, about 80% of
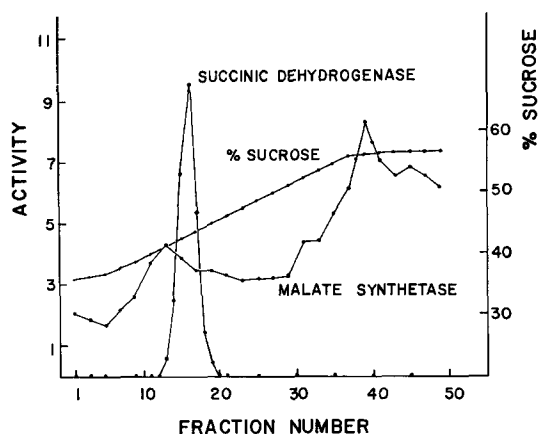
FIGURE 8 Resolution of the mitochondrial fraction prepared from 3-day-old endosperm on a discontinuous sucrose density gradient. A linear continuous sucrose density was constructed over the concentrated mitochondrial fraction before centrifuging for 8 hr at 64,300 g. Gradient collected in 1 ml fractions. Activity: succinic dehydrogenase, μmoles $\times$ 10^{-1} dichlorophenol indophenol reduced/min per ml; malate synthetase, μmoles $\times$ 10^{-2} glyoxylate reacted/min per ml.

the activity was clearly separated from the succinic dehydrogenase peak, which marks the location of the mitochondria (Fig. 8). It thus appears that part of the malate synthetase recovered in the crude particulate fraction at day 3 is present on particles that are not intact glyoxysomes and that are recovered on the usual discontinuous gradients in both mitochondrial and proplastid fractions. The distribution of malate synthetase, observed when the crude particulate fraction from 3 day old endosperm was separated on a continuous gradient, lends additional weight to this conclusion. As shown in Fig. 9, where the distribution of succinic dehydrogenase marks the mitochondrial fraction and that of isocitrate lyase and catalase marks the glyoxysomal protein, the malate synthetase distribution is complex. Part of the activity is clearly in the glyoxysome peak, but a second protein peak with malate synthetase activity at density 1.21–1.22 is present, not coinciding precisely with the mitochondria but actually overlapping the mitochondrial and the proplastid bands. In discontinuous gradients, this form of particulate malate synthetase is distributed between the proplastid and the mitochondrial fractions but is not a true component of either.

From the following considerations we conclude

that the malate synthetase appearing at higher regions of the gradient can be ascribed to broken glyoxysomes. When the glyoxysome fraction recovered in 54% sucrose was diluted with an equal volume of EDTA (10^{-3} M) disruption of the organelles occurred, since on centrifuging at 27,000 g for 30 min 70% of the isocitrate lyase and 70% of the catalase were recovered in the supernatant solution. However, the resulting pellet contained 60% of the protein and 70% of the malate synthetase. When this pellet of deliberately broken glyoxysomes was centrifuged on a continuous gradient, the malate synthetase distribution was close to that illustrated in Fig. 9. That is, some activity was recovered in the glyoxysome region but most was present as a broad peak at density 1.21–1.22.

An alternative possibility, that the less dense particles containing malate synthetase but lacking isocitrate lyase were precursors rather than breakdown products of glyoxysomes, was disposed of as follows. To the same sample of endosperm tissue (3 days old) leucine-^{3}H was provided, followed 2 hr later by leucine-^{14}C. It was reasoned that if the particles of lower density containing malate synthetase were precursors of glyoxysomes, their ^{3}H:^{14}C ratio should be significantly lower than that in the glyoxysomes themselves. 4 hr after adding leucine-^{3}H, organelles from the labeled endosperm were separated on a discontinuous gradient. The glyoxysome and the mitochondrial fractions, both containing malate synthetase, were collected. A portion of the glyoxysome fraction was precipitated with trichloroacetic acid and assayed for ^{14}C and ^{3}H. The rest of this fraction was diluted with EDTA and only the pellet (deliberately broken glyoxysomes) was precipitated and assayed. The malate synthetase in the mitochondrial band was separated from the mitochondria on a flotation gradient (as in Fig. 8), and the precipitated protein was assayed. As shown in Table II, the three protein fractions, i.e. whole glyoxysomes, deliberately broken glyoxysomes, and the particles with malate synthetase separating at lower density on the original gradient, showed closely similar ^{3}H:^{14}C ratios.

DISCUSSION

The rising and falling pattern of activity of the key enzymes of the glyoxylate cycle (isocitrate lyase and malate synthetase) in germinating castor bean and other fatty seedlings is well documented.
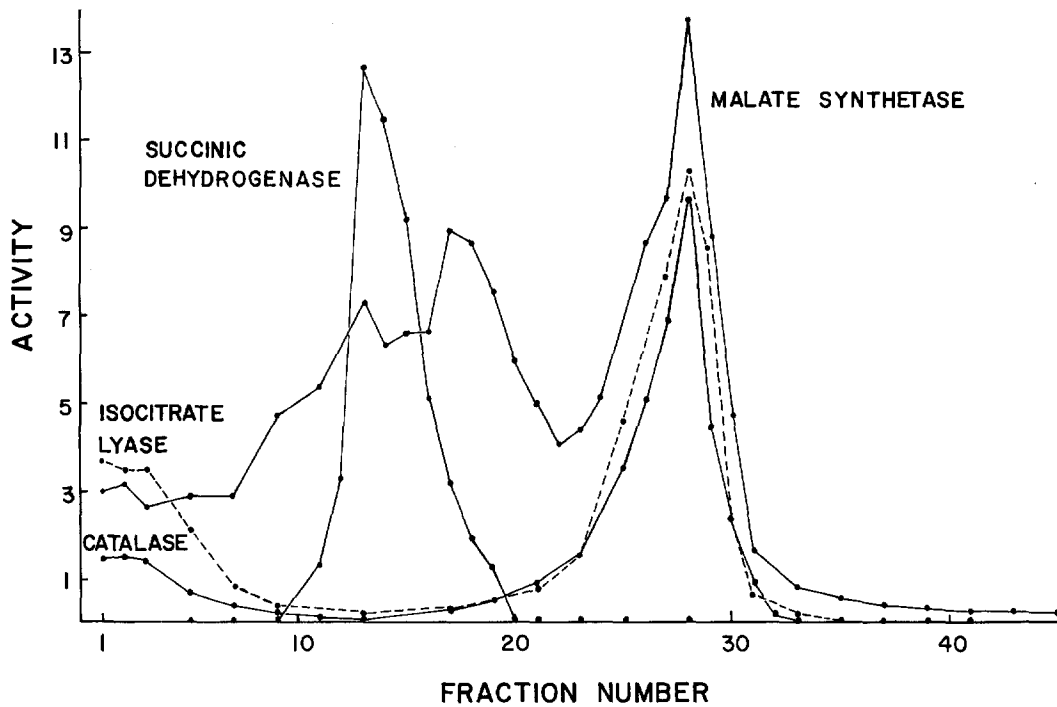
FIGURE 9 Distribution of isocitrate lyase, malate synthetase, catalase, and succinic dehydrogenase on a linear sucrose density gradient. The crude particulate fraction applied to the gradient was prepared from the endosperm of 3-day-old castor bean seedlings. Gradient collected in 1 ml fractions; fraction 1 is the top of the gradient. Activity: isocitrate lyase and malate synthetase, μmoles $\times$ 10^{-1} substrate reacted/min per ml; succinic dehydrogenase, μmoles $\times$ 10^{-2} dichlorophenol indophenol reduced/min per ml; catalase, μmoles $\times$ $3.3 \times 10^2 H_2O_2$ consumed/min per ml.

TABLE II

Incorporation of leucine-^{3}H and leucine-^{14}C into different fractions from the endosperm of 3-day-old castor bean seedlings (var. Hale). Endosperm halves were treated for 2 hr with leucine-^{3}H, and leucine-^{14}C was added in addition. After a total incubation time of 4 hr, the fractions were isolated from the endosperm (see text). The values given are averages of four separate samples.

	Size of sample counted	^{3}H	^{14}C	^{3}H : ^{14}C
	λ	cpm	cpm	
Fractions from the discontinuous gradient				
background		20.4	8.4	
glyoxysomes	200	942.8	41.9	27.5
deliberately broken glyoxysomes	200	1797.7	81.7	24.4
mitochondria	100	2293.1	101.9	24.3
Fractions from the flotation gradient of the mitochondria				
background		20.7	9.2	
mitochondria	300	2867.1	126.7	24.2
separated malate synthetase peak	300	200.5	17.2	22.5

The major fact established in this paper is that the changes in the amount of glyoxysome protein recovered from the endosperm during the germination of the castor bean follow a closely similar pattern. In addition, it is concluded that throughout the germination period the glyoxysome is the only organelle housing these enzymes.

A sevenfold increase in glyoxysome protein occurs between days 2 and 4, and there is a subsequent steady decrease when total isocitrate lyase and malate synthetase are declining. It is not possible to state with certainty that glyoxysomes exist in the ungerminated seed, since it is only after 2 days of germination that clearly identifiable bands of organelles can be separated on the sucrose gradient. The occurrence of very low levels of isocitrate lyase in the crude particulate fraction obtained after 12 hr of soaking may be due to glyoxysomes existing at this time. However, by an extrapolation of the glyoxysome curve in Fig. 2, it can be confidently concluded that if glyoxysomes are present before germination begins, they are present in very small numbers, and we interpret the rising curve to mean that the number of these organelles increases strikingly during germination.

Nevertheless, the production of the two enzymes occurs more rapidly than that of the glyoxysomes themselves, since the specific activities in the glyoxysomes rise sharply during the first 5 days (Fig. 6.). These results show that the synthesis of the enzymes and of the organelle itself are independently controlled. After the 5th day, however, when the amount of glyoxysome protein is declining, the specific activities of malate synthetase and isocitrate lyase remain constant. That is to say, the decline in the levels of these enzymes is directly related to the loss of the organelles. The disappearance of the glyoxysomes is probably the inevitable consequence of the overall proteolytic process in older endosperm tissue. However, it is important to note that in organs like watermelon cotyledons, which take on a photosynthetic function after the fat has disappeared, there is clearly a selective loss of glyoxysomes (D. I. McGregor and H. Beevers, unpublished observations). From experiments with cycloheximide, Hock and Beevers (8) deduced that the synthesis of the two enzymes in watermelon cotyledons stops when the peak activities are reached. Nevertheless, we have shown that incorporation of leucine-^{14}C into glyoxysomes (and other particulate proteins) occurs in endosperm tissue from 5-day-old seed-

lings (B. Gerhardt and H. Beevers, unpublished observations). Thus, even when there is a net disappearance of glyoxysomes, there is protein turnover in these organelles.

At all stages of germination the only particulate band with isocitrate lyase activity was the glyoxysomal one (Fig. 3) sedimenting at density 1.25. By the use of this enzyme as marker it can be concluded that no change in the density of the glyoxysomes occurs during germination. However, during the early stages of germination, only part of the particulate malate synthetase was recovered at density 1.25, and a second peak of enzyme activity was found in the mitochondrial-proplastid region (Fig. 7, 8). Neither isocitrate lyase nor catalase, another enzyme of the glyoxysome, was present in this peak.

The solution to this anomaly was suggested by experiments in which intact glyoxysomes were deliberately broken. When isolated glyoxysomes (in 54% sucrose) were diluted or treated with deoxycholate (0.4%), isocitrate lyase was readily released into the soluble phase, while malate synthetase was retained on the particulate matter (broken glyoxysomes) recovered in a subsequent centrifugation. When the broken glyoxysomes with malate synthetase activity were centrifuged on a sucrose gradient, protein and enzyme banded together higher in the gradient than the original glyoxysomes. It therefore seems likely that the particulate malate synthetase not sedimenting with glyoxysomes in preparations from young tissue (Fig. 7) is due to glyoxysomes broken during preparation.

Such breakdown of glyoxysomes during preparation of organelles from younger endosperm would also account for the fact that most of the isocitrate lyase is not recovered in the crude particulate fraction; the percentage in the glyoxysomes increases from 5 to 75% between days 2 and 6, while that recovered in the soluble fraction decreases correspondingly.

After 5 days of germination, the glyoxysomes are apparently more stable during the preparative procedure, and it is clear that both enzymes are contained only in these organelles. The above yonsiderations allow us to conclude that at the counger stages also, the enzymes in the native condition are present only in the glyoxysome.

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