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Development of Microbodies in Sunflower Cotyledons and Castor Bean Endosperm during Germination[1]

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ABSTRACT

In cotyledons of sunflower seedlings glyoxysomal and peroxisomal enzymes exhibit different rates of development during germination. The total activity of isocitrate lyase, a glyoxysomal marker enzyme, rapidly increased during the first 3 days, and then decreased 89% by day 9. Exposure to light accelerated this decrease only slightly. The specific activity of glyoxysomal enzymes (malate synthetase, isocitrate lyase, citrate synthetase, and aconitase) in the microbody fraction from sucrose density gradients increased between days 2 and 4 about 2- to 3-fold, and thereafter it remained about constant in light or darkness.

Total activity of the peroxisomal enzymes increased slowly in the dark during the first 4 days of germination and thereafter remained at a constant level of activity in the dark or increased 2-fold in 24 hours of light. The specific activties of glycolate oxidase, hydroxypyruvate reductase, and serine-glyoxylate aminotransferase in the isolated microbody fraction increased about 10-fold between days 2 and 4 in the dark and then remained constant or increased again 10-fold after an additional 48 hours in the light.

The total activity of the common microbody marker, catalase, developed similarly to isocitrate lyase, but decreased only 72% by day 9. The specific activities of enzymes (catalase, malate dehydrogenase, and aspartate aminotransferase) common to both microbody systems were 10- to 1000-fold greater than those of other enzymes. It is proposed that malate and aspartate may be involved in hydrogen transport between microbodies and other cellular sites.

Glutamate-glyoxylate aminotransferase was very active in microbodies from castor bean endosperm and sunflower cotyledons. The specific activity of this aminotransferase developed similarly to glyoxysomal enzymes in the dark but further increased in the light, as did peroxisomal enzymes.

The microbody fraction of castor bean endosperm germinated in the dark for 5 days contained both glyoxysomal and peroxisomal enzymes of similar specific activity.

Adjacent to the microbody fraction on sucrose gradients from sunflower cotyledons were etioplasts at slightly lower densities and protein bodies at similar and higher densities. Their presence in the microbody fractions resulted in artificially low specific activities.

Two biochemical systems have been established in plant microbodies (29). Glyoxysomes are present in germinating tissues which are converting storage fat to sugars, such as the endosperm of the castor bean (2, 3, 6, 9) and the cotyledons (11, 23, 24) of several germinating seeds. These particles contain the β-oxidation system for fatty acids (7, 14) and the glyoxylate cycle (6). Catalase, isocitrate lyase, and malate synthetase have been used most often as marker enzymes for this microbody. Peroxisomes are present in photosynthetic tissue and contain enzymes for the glycolate pathway of metabolism from the photosynthetic carbon reduction cycle (29, 30, 34, 35, 41). Catalase, glycolate oxidase, and hydroxypyruvate reductase have been used as marker enzymes for this type of microbody activity. In cotyledons of germinating seedlings capable of developing into photosynthetically active tissue in the light, a complex situation exists where the microbody fraction contains glyoxysomal enzymes as well as peroxisomal enzymes (11, 15, 23, 24, 26). It is not clear whether there is one population of microbodies with a changing enzymatic composition or whether there are two types of microbodies, which appear morphologically similar but biochemically different. This paper deals only with enzymatic changes during germination and greening. Rather than to assume two microbody populations, we are using the terms glyoxysomal system and peroxisomal system to connote two specific and different metabolic pathways in microbodies from germinating tissue.

Numerous recent investigations have contributed to the understanding of the development of microbodies in cotyledons. Glyoxysomal marker enzymes increase rapidly in activity during the first days of germination and then decline and disappear in either light or darkness when the lipid bodies have been consumed (4, 9, 11–13, 16, 39). The increase in isocitrate lyase and malate synthetase has been shown to be *de novo* synthesis (10, 20). Peroxisomal marker enzymes were found to be low, or even absent, in the dark, whereas light enormously increases their activity (5, 8, 11, 15, 19, 23, 24, 28, 31, 32, 37, 38). In this paper we report upon the development and the effect of light on many of the microbody enzymes.

METHODS

Plants. Sunflower seeds, *Helianthus annuus* L., var. Mammouth Russian, from Vaughn's Seed Co., Ovid, Mich., were dehusked, soaked for about 15 min in a suspension of 5 mg/ml of Captan (Chevron Chemical Company, Ortho Division, San Francisco, Calif.), and then washed with water. Castor bean seeds, *Ricinus communis* L., var. Hale, from Baker Castor Oil Compay, Plainview, Texas, were treated similarly. Sunflower

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seeds were germinated in sand saturated with half-strength Hoagland's solution at 25 C in the dark or in white light of 800 to 1000 ft-c. Castor beans were germinated at 30 C in moist vermiculite, as reported previously (4). Germination was considered to be initiated at the time of the first water imbibition. If water saturating conditions were not maintained, germination rates were slowed, and the days required for development of enzyme activity were not reproducible.

Preparation of Homogenates and Particles. Two procedures were used, and all steps were performed at 0 to 5 C. A complete homogenization of the tissue was necessary for determining total enzyme activity, and an incomplete gentle homogenization had to be used for the isolation of particles (30). For total enzyme activity, cotyledons from five seedlings were ground vigorously for 2 min with a mortar, pestle, and sand in 3 ml of grinding medium, which consisted of 0.1 M sodium phosphate at pH 7.4, 2 mM EDTA, and 5 mM DTT.[3] After the volume was adjusted to 6 ml, the homogenate was centrifuged at 27,000*g* for 20 min. It was assumed that all the microbody enzymes were solubilized, since the concentration of catalase activity in the pellet was equivalent to the supernatant volume associated with the pellet. A 1-ml aliquot of the aqueous solution between the pellet and the fat layer was placed on a Sephadex G-25 column (0.7 × 16.6 cm) which had been equilibrated with a buffer of 0.1 M phosphate, pH 7.4, and 2 mM EDTA. All enzyme activity was collected in a volume of 1.8 ml, and this fraction was used for subsequent assays. The addition of DTT to the grinding medium was necessary to prevent rapid loss of some enzyme activities, particularly isocitrate lyase. Removal of DTT by Sephadex G-25 filtration was necessary, since it interfered with some assays, particularly glycolate oxidase. By this procedure no appreciable lag phases were observed during enzyme assays.

For the preparation of organelles, 10 g fresh weight of sunflower cotyledons or castor bean endosperm (without the seedling) were homogenized in 15 ml of a grinding medium by a Sorvall OmniMixer. The grinder was run first at a low speed to chop the tissue into small pieces and then for 5 to 8 sec at high speed. The grinding medium was similar to that described by Cooper and Beevers (6) but contained 1 M sucrose for sunflower cotyledons and 0.8 M sucrose for castor bean endosperm. The homogenate was filtered through six layers of cheesecloth and applied directly to the top of sucrose gradients. A grinding medium without DTT gave the same amount of particulate-bound enzymes, as measured by catalase activity, but loss of solubilized enzyme was significant. Density gradients were prepared with sucrose in 10 mM EDTA at pH 7.5. A typical gradient for isolating particles from sunflower cotyledons contained 2.5-ml layers of 60, 60, 56.5, 51.5, 50, 47.5, 45, 42.5, 40, 37.5, 35, 32.5, 30, 27.5, and 25% sucrose (w/w). Gradients for particles from castor bean endosperm contained two additional layers of 22.5 and 20% sucrose and smaller fractions on the upper part of the gradient. These gradients gave better results than step gradients with larger density differences as described previously (6). The gradients were centrifuged at 25,000 rpm in a SW 25.2 rotor with a Beckman L-2 ultracentrifuge. Centrifugation for 2 hr was sufficient for preparations from sunflower cotyledons, but 4 hr of centrifugation was needed for particle separation from castor bean endosperm.

After centrifugation 2.5-ml fractions were drained from the bottom of the gradient, except for the uppermost fraction of 11 to 14 ml which represented the supernatant fraction. A 0.5-ml aliquot of the top fractions was run through Sephadex G-25 columns to remove DTT before being assayed for some enzymes.

Assays. All enzyme assays were performed at 25 C and within 60 hr after homogenizing the tissue. Spectrophotometric assays were run with a Gilford recording spectrophotometer with an automatic changer for four cuvettes. After endogenous rates were recorded, reactions were initiated with substrates. Specific activities are expressed as nmoles min^{-1} mg^{-1} of protein in a gradient fraction, and a total activity during the time course of development as nmoles min^{-1} pair cotyledons^{-1}. Protein was determined by the Lowry procedure (21), and chlorophyll by its absorption at 652 nm (1). The refractive indices for sucrose concentrations were measured at 25 C in a Bausch and Lomb refractometer and reported as a sucrose density (*d*) at 10 C.

Glycolate oxidase (EC 1.1.3.1) was measured spectrophotometrically at 600 nm by the anaerobic reduction of DCPIP (34). Since sufficient DCPIP could not be used for maximal enzyme activity, all rates were multiplied by a factor of 3.15 in order to obtain the maximal rates for saturating DCPIP conditions (30).

NADH-hydroxypyruvate reductase (EC 1.1.1.29) was assayed in gradient fractions by following the oxidation of NADH at 340 nm (35) after addition of hydroxypyruvate (1 mM final). In time course studies of total activity (Fig. 1), glyoxylate (75 mM final) was used as a substrate, which is reduced at a slower rate than hydroxypyruvate (35).

The isocitrate lyase (EC 4.1.3.1) assay measured the formation of glyoxylate phenylhydrazone by following the increase of absorbance at 324 nm (13). The molar extinction coefficient of glyoxylate phenylhydrazone was determined as 1.67×10^4 cm^{-1} $mole^{-1}$.

Catalase (EC 1.11.1.6) was assayed by the initial disappearance of 12.5 mM H_2O_2 as measured by the decrease of absorbance at 240 nm (22).

Cytochrome *c* oxidase (EC 1.11.1.5) was assayed by the oxidation of cytochrome *c* at 550 nm (33). Cytochrome *c* was reduced by dithionite so that the absorbance ratio 550 nm/565 nm was about 9 to 10. Activity (nmoles min^{-1}) as the initial rate of the first order reaction (27), was calculated from the equation

$$\frac{dA_{550}}{dt} = \frac{\epsilon_{red} - \epsilon_{ox}}{\epsilon_{red}} \frac{dA\ red_{550}}{dt}.$$

dA_{550} is the absorption change from both the decrease of the reduced and the increase of the oxidized form of cytochrome *c*. dA red_{550} is the absorption change due to the decrease of reduced cytochrome *c*. Molar extinction coefficients of cytochrome *c* at 550 nm are $\epsilon_{red} = 28.4 \times 10^3$ cm^{-1} $mole^{-1}$ and $\epsilon_{ox} = 8.1 \times 10^3$ cm^{-1} $mole^{-1}$ (36).

Malate dehydrogenase (EC 1.1.1.37) was assayed spectrophotometrically by the oxidation of NADH (34).

The aconitase (EC 4.2.1.3) assay was based on the disappearance of the double bond of *cis*-aconitate, which results in a decrease of absorbance at 240 nm (25).

Malate synthetase (EC 4.1.3.1) and citrate synthetase (EC 4.1.3.7) were assayed by a modified procedure of Cooper and Beevers (6), which is dependent upon the formation of a yellow complex between DTNB and the sulfhydryl group of CoA, as it is formed from acetyl-CoA. A molar extinction coefficient of 1.36×10^4 cm^{-1} $mole^{-1}$ at 412 nm was used. The assay mixture in a final volume of 1 ml contained 100 μmoles of Tricine at pH 8.0, 0.1 μmole of DTNB, 5 μmoles of $MgCl_2$, 0.2 μmole of acetyl CoA, 1 to 20 μl of enzyme, and 5.0 μmoles of glyoxylate or oxaloacetate. The malate synthetase assay was

[3] Abbreviations: DTT: dithiothreitol; DCPIP: 2,6-dichlorophenolindophenol; DTNB: 5,5′-dithiobis-(2-nitrobenzoic acid).

initiated by the addition of glyoxylate and the citrate synthetase assay by addition of enzyme.

Aspartate aminotransferase (EC 2.6.1.1) was assayed by coupling the formation of oxaloacetate to malate dehydrogenase activity and measuring NADH oxidation (41).

Glutamate glyoxylate aminotransferase (EC 2.6.1.4) and serine glyoxylate aminotransferase (EC 2.6.1.–) were assayed by the formation of glycine-^{14}C from glyoxylate-1,2-^{14}C (17). The reaction mixture contained, in a final volume of 1.27 ml, 15 μmoles of glyoxylate-^{14}C, 23 μmoles of L-glutamate or L-serine at pH 7.0, 75 nmoles of pyridoxal phosphate at pH 7.0, 19 μmoles of phosphate at pH 7.5, and enzyme. The assay was started by addition of glyoxylate-^{14}C, incubated for 15 min at 30 C, and stopped by boiling for 3 min. The boiled mixture was run through a Dowex 50 acetate column to absorb unreacted glyoxylate-^{14}C. The radioactivity in the effluent from the column was counted as glycine-^{14}C (17).

RESULTS

Time Course of Enzyme Development. After 1 to 9 days of germination, total enzyme activities in sunflower cotyledons were determined after complete homogenization of the tissue (Fig. 1). After 3 days isocitrate lyase, a glyoxysomal marker enzyme, reached maximal activity of about 380 nmoles min^{-1} pair of cotyledons^{-1}. During the following 6 days this activity declined by 89%. Peroxisomal marker enzymes, glycolate oxidase and hydroxypyruvate reductase, developed more slowly during the first 4 days of germination. When the cotyledons were kept in the dark, these activities thereafter remained at a nearly constant level of 120 nmoles min^{-1} pair cotyledons^{-1},

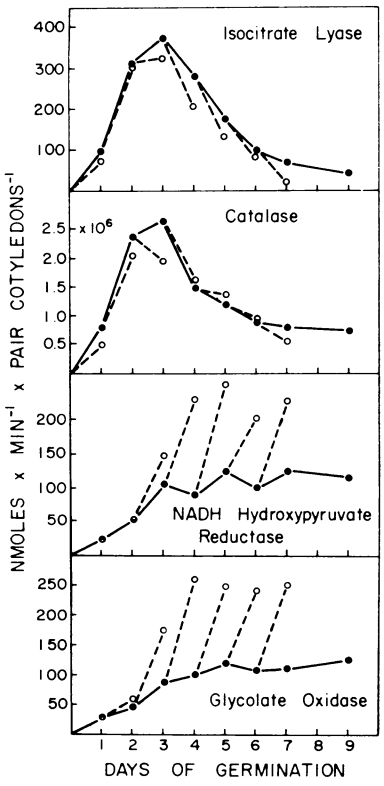

FIG. 1. Total enzymatic activity in homogenates of sunflower cotyledons during germination. Seeds were germinated in continuous darkness (●——●) for 1 to 9 days or placed in the light for 1 day before assaying (○-----○).

which is one-third of the isocitrate lyase peak activity. These results demonstrate a different developmental pattern for glyoxysomal and peroxisomal enzymes. Such differences are suggestive of two biochemical systems in microbodies of the cotyledons.

Catalase, which is common to both microbody systems, increased to peak activity within 3 days, as did isocitrate lyase, but thereafter it declined to only 28% of its maximal activity. This result implies that in the first days of development catalase activity was part of the glyoxysomal system. That catalase did not decrease later as much as other glyoxysomal enzymes must be related to its presence also in the peroxisomal system, which becomes more evident later in development.

Exposure to white light for 24 hr at different times during germination has a markedly different effect on glyoxysomal and peroxisomal enzymes. The total enzyme activity in the light is shown by the dashed lines in Figure 1. Glyoxysomal isocitrate lyase activity was only slightly lower after irradiation than in the seedlings kept always in the dark. Peroxisomal glycolate oxidase and hydroxypyruvate reductase activities doubled in 24 hr in the light any time after the 2nd day of germination as compared to the dark controls. Although 24 hr of light stimulated peroxisomal enzymes after only 2 days of dark germination, the final amount of activity was greatest in response to light after 3 to 4 days of dark germination, as if maximal peroxisomal development were not possible earlier. Light had little effect upon the level of catalase activity. Since catalase is a constituent of peroxisomes from green sunflower leaves (34), and since the other peroxisomal enzymes increased considerably in light, the absence of a change in total catalase in the light is noteworthy.

Activities in Particles from Sunflower Cotyledons. Particles from homogenates prepared by limited and gentle grinding were separated on sucrose gradients by isopycnic centrifugation (Figs. 2 and 3). Microbodies, marked by catalase, banded at a density of 1.265 g cm^{-3} and mitochondria, marked by cytochrome *c* oxidase, at a density of 1.18 g cm^{-3}. On gradients of light-grown seedlings the main peak of chlorophyll, representing broken chloroplasts, was at a density of 1.16 g cm^{-3}, and a small peak, representing intact, whole chloroplasts was at 1.22 g cm^{-3}. From dark-grown seedlings a yellow band of whole etioplasts or proplastids was visible just above the microbodies, and these organelles were only partially separated from the microbodies (unpublished).

The method of grinding the sunflower cotyledons and directly centrifuging the whole homogenate on a sucrose gradient generally yielded 15 to 35% of each microbody enzyme in the particulate band (Fig. 2). Six to 12% of the activity was smeared on the gradient, and the rest was solubilized in the supernatant fraction. The yield of microbodies was better from older seedlings germinated in the dark than from younger seedlings, and best from seedlings that had been in the light for 2 days. A better yield of microbodies from older tissue has previously been reported from castor bean endosperm (9). After 4 days in the dark, isocitrate lyase was recovered in the microbody band in somewhat higher yields than the other microbody enzymes shown in Figure 2. Another phenomenon was a poor yield of hydroxypyruvate reductase in microbodies from 2-day-old, dark-grown seedlings.

The separation of the microbodies from the mitochondria and chloroplasts was highly significant on the basis of the specific activities of their marker enzymes (Table I). In order to estimate maximal cross contamination among these three particles, the specific activity of a marker enzyme in other bands of organelles is cited as a percentage of the maximal specific activity in its own particulate band (Table II). Thus,

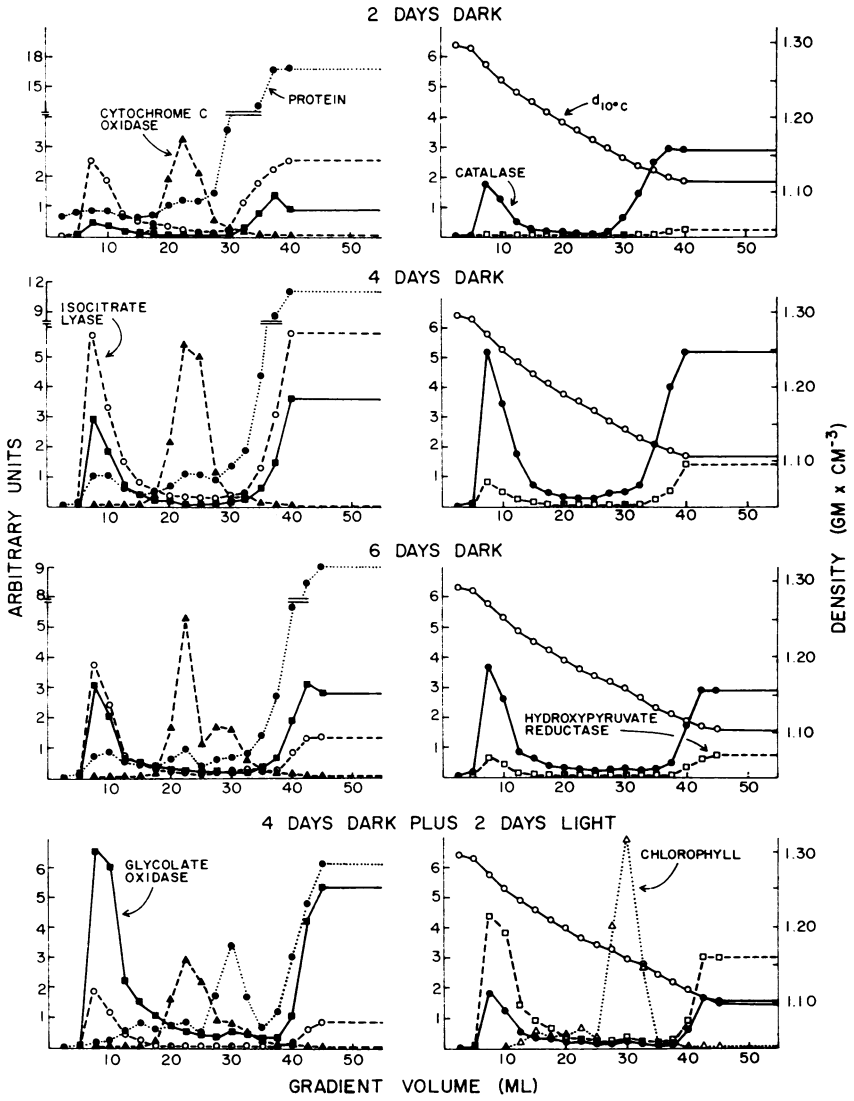

FIG. 2. Particle separation from sunflower cotyledons by sucrose density gradients. The marker enzyme for mitochondria is cytochrome *c* oxidase; for microbodies, glycolate oxidase and hydroxypyruvate reductase; and for chloroplasts, chlorophyll. The soluble or supernatant fraction was at the top of the gradient from about 40 ml to 56 ml and represented the volume of homogenate placed on the gradient. A fatty layer of 1 to 4 ml on the very top of the gradient was skimmed off and discarded. ○——○: Sucrose density (10 C) as g cm^{-3}; ●——●: catalase; graph unit as 2,000 μmoles min^{-1} ml^{-1}; ■——■: glycolate oxidase: graph unit as 0.05 μmole min^{-1} ml^{-1}; □-----□: hydroxypyruvate reductase; graph unit as 0.5 μmole min^{-1} ml^{-1}; ▲-----▲: cytochrome *c* oxidase; graph unit as 1 μmol min^{-1} ml^{-1}; ○-----○: isocitrate lyase; graph unit as 0.2 μmole min^{-1} ml^{-1}; ●····●: protein; graph unit as 1.50 mg ml^{-1}; △····△: chlorophyll; graph unit as 100 μg ml^{-1}.

there was less than 1% of the chlorophyll and cytochrome *c* oxidase in the microbody fraction.

The protein profiles of the gradients varied according to the age of the seedlings. Since 10 g fresh weight of cotyledons were always used, the total protein per gradient decreased as the seedlings aged. This was due mainly to water uptake by the cotyledons, as the total protein per seedling changed only little (11). Most of the protein was in the supernatant. In all gradients a protein peak occurred at the density of mitochondria and of whole and broken chloroplasts, if present. The amount of protein in the microbody band was small, and the total protein peak in this part of the gradient spanned the microbodies and whole etioplasts, if etiolated cotyledons were used (unpublished). In addition, on the gradient from seedlings germinated for 2 days in the dark, a significant amount of protein was observed at densities higher than 1.27 g cm^{-3} and thus below the microbodies. This region of the gradient appeared cloudy and contained storage protein bodies (unpublished). Very little or none of this protein was present after 6 days of germination.

Since glyoxysomal and peroxisomal enzymes were not separated (Figs. 2 and 3), the gradients did not differentiate between two kinds of microbodies. However, the developmental difference between a glyoxysomal and peroxisomal enzyme system can clearly be seen by comparing specific activities in the microbody fraction (Fig. 4). In the dark the specific activities of glyoxysomal enzymes, such as isocitrate lyase, malate synthetase, citrate synthetase, and aconitase, rose 2- to 3-fold between days 2 and 4 after germination and declined slightly by day 6. Specific activities of peroxisomal enzymes, such as glycolate oxidase, hydroxypyruvate reductase, and serine glyoxylate aminotransferase, were very low at day 2 but increased about 10-fold between days 2 and 4 and then stayed the same at day 6 in the dark. After exposure of the seedlings to light between days 4 and 6, specific activities of glyoxysomal enzymes increased slightly over the dark controls, while the spe-

Table I. *Specific Activities of Enzymes in Particulate Fractions from Sunflower Cotyledons*
Values are from the peak of each fraction from graidents in Figure 2 and 3.

	2 Days Dark Germination			4 Days Dark Germination			6 Days Dark Germination			4 Days Dark and 2 Days Light Germination			
	Microbodies	Mitochondria	Supernatant	Microbodies	Mitochondria	Supernatant	Microbodies	Mitochondria	Supernatant	Microbodies	Mitochondria	Chloroplasts	Supernatant
	nmoles min^{-1} mg^{-1} protein												
Glyoxysomal enzymes													
Malate synthetase	561	33	21	1,560	273	10	944	200	12	1,380	117	70	10
Isocitrate lyase	350	25	18	820	36	62	618	20	18	926	15	4	17
Citrate synthetase	176	153	10	426	223	15	231	167	24	406	87	43	2
Aconitase	20	496	136	44	776	269	32	827	249	94	441	56	215
Peroxisomal enzymes													
Glycolate oxidase	53	4	5	327	7	31	402	20	30	2,330	70	17	83
Hydroxypyruvate reductase	15	3	8	539	7	76	554	17	52	10,560	256	65	255
Serine glyoxylate aminotransferase	2	6	22	210	17	27	90	12	27	14,100	221	62	396
Glyoxysomal and peroxisomal enzymes													
Catalase (× 10^3)	2,430	186	207	7,440	355	551	6,110	390	390	9,000	314	88	300
Malate dehydrogenase	6,130	3,580	2,460	16,000	4,980	5,550	11,600	5,700	720	16,300	3,400	720	510
Aspartate aminotransferase	1,930	7,160	223	5,330	11,400	552	3,820	11,700	447	6,540	7,340	550	419
Glutamate glyoxylate aminotransferase	310	180	65	923	255	89	1,100	419	111	17,000	454	113	567
Mitochondrial enzyme													
Cytochrome *c* oxidase	8	1,500	2	11	3,200	2.3	10	3,420	3	18	2,080	139	3

Table II. *Percentage of Maximal Specific Activity in Fractions from Sucrose Gradients of Sunflower Cotyledons*

The specific activity in the most active fraction is equated to 100% and in other fractions as a percentage of the maximal specific activity. These values represent the maximal cross contamination in other fractions.

Germination Time	Glycolate Oxidase	Hydroxypyruvate Reductase	Catalase	Isocitrate Lyase	Cytochrome *c* Oxidase	Chlorophyll
	%	%	%	%	%	%
2 days dark						
Microbodies	100	100	100	100	0.6	
Mitochondria	8	19	8	7	100	
Supernatant	9	53	9	5	0.1	
4 days dark						
Microbodies	100	100	100	100	0.3	
Mitochondria	2	1	5	4	100	
Supernatant	9	14	7	8	0.1	
6 days dark						
Microbodies	100	100	100	100	0.3	
Mitochondria	5	3	6	3	100	
Supernatant	8	9	6	3	0.1	
4 days dark plus 2 days light						
Microbodies	100	100	100	100	0.8	<0.1
Mitochondria	3	2	3	2	100	10
Broken chloroplasts	1	1	1	0.4	6.7	100
Supernatant	4	2	3	2	0.1	<0.1

cific activities of peroxisomal enzymes increased about 10-fold over the dark controls. The specific activities of enzymes common to both microbody systems, such as catalase, malate dehydrogenase, and aspartate aminotransferase, exhibited a developmental pattern similar to that of other glyoxysomal enzymes.

Aconitase activity in the microbody fraction was very low, probably due to extensive solubilization of this enzyme as previously reported for castor bean endosperm by Cooper and Beevers (6). However, the specific activity of the remaining aconitase in the microbody fraction was higher than would be expected if this activity were only due to a contaminating mitochondrial enzyme. The maximal cross contamination of mitochondrial cytochrome *c* oxidase in the microbody fraction was less than 1% (Table II), whereas the specific activity of aconitase in the microbody fraction was 4 to 20% of that in the mitochondria.

Malate synthetase was the least solubilized of the microbody enzymes, for less of this activity was in the supernatant fraction compared with other microbody enzymes. However, at the microbody peak a shoulder of malate synthetase activity on the side with less dense sucrose was present, which suggests that part of this protein was being lost from the microbodies as they approached their final density. No NADPH-isocitrate dehydrogenase activity was found in the microbody fractions of the gradients, although low levels of this enzyme have been reported in other types of microbodies (41).

Glutamate-Glyoxylate Aminotransferase. The site-specific location of this irreversible transaminase has been shown for leaf peroxisomes (41), but it was reported absent in castor bean microbody preparations (6, 17). In the present studies we found that this transaminase was present in microbodies of sunflower cotyledons (Fig. 3) and castor bean endosperm (Fig. 5). The specific activity of the enzyme in microbody fractions from both tissues was very high (Tables II and III). In fact, its specific activity in the microbodies was as high or higher than malate synthetase, isocitrate lyase, and citrate synthetase and also much greater than other peroxisomal enzymes in the microbody fraction of dark-germinated tissue. The response of the enzyme to light in microbodies of sunflower cotyledons indicates peroxisomal characteristics, whereas the initial high activity during dark germination suggests a glyoxysomal function. Glutamate-glyoxylate aminotransferase

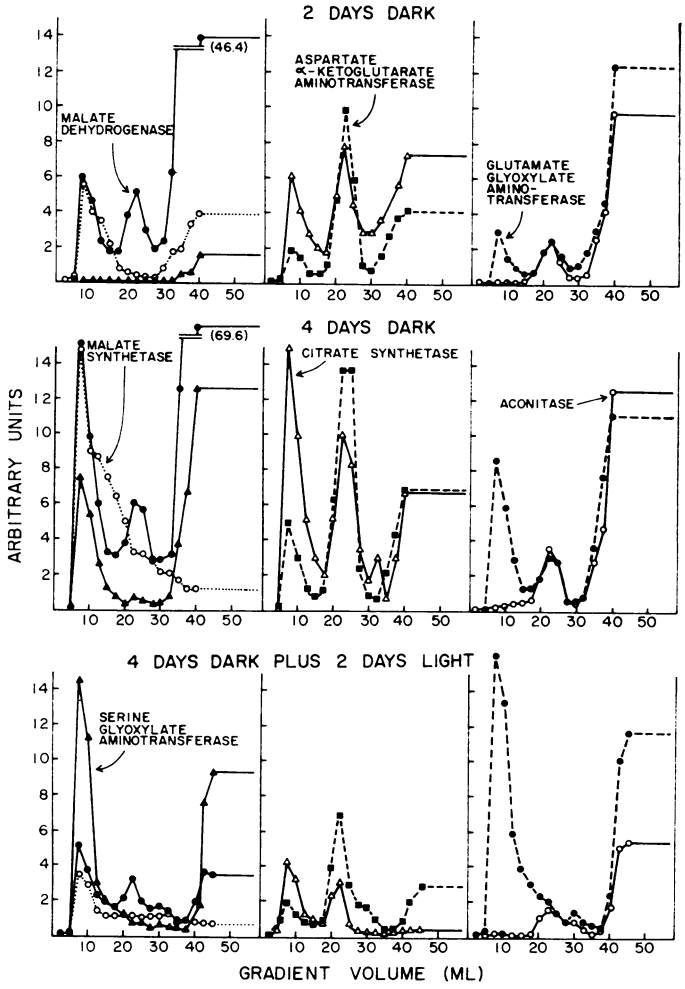

FIG. 3. Particulate enzymes from sunflower cotyledons isolated on sucrose density gradients. Data from seedlings germinated 6 days in the dark are not shown, since the distribution patterns on the gradient was similar to that after 4 days of dark germination. Assays were run on the same gradient as used in Fig. 2. Units in parentheses for malate dehydrogenase are off scale. ●——●: Malate dehydrogenase; 1 graph unit is 1.5 μmoles min^{-1} ml^{-1}; ▲——▲: serine-glyoxylate aminotransferase; 1 graph unit is 0.04 μmole min^{-1} ml^{-1}; ○···○: malate-synthetase; 1 graph unit is 1.5 μmoles min^{-1} ml^{-1}; ■-----■: aspartate-α-ketoglutarate aminotransferase; 1 graph unit is 1.5 μmoles min^{-1} ml^{-1}; △——△: citrate synthetase; 1 graph unit is 0.04 μmole min^{-1} ml^{-1}; ●-----●: glutamate glyoxylate aminotransferase; 1 graph unit is 0.05 μmole min^{-1} ml^{-1}; ○——○: aconitase; 1 graph unit is 0.4 μmole min^{-1} ml^{-1}.

may therefore be designated as an enzyme common to both microbody systems. Activity of this enzyme is also associated with the mitochondria.

Mitochondrial Enzymes. Citrate synthetase, aconitase, glutamate-glyoxylate aminotransferase, malate dehydrogenase, and aspartate aminotransferase activities were associated with both the mitochondria and microbodies, which were well separated from each other on the sucrose density gradients (Fig. 3). The specific activity of the enzymes associated with the mitochondria doubled between days 2 and 4 in the dark and then remained constant at day 6 in the dark (Table I). After illumination of the seedlings between days 4 and 6 the specific activity of these mitochondrial enzymes decreases about 50%. All of the mitochondrial enzymes exhibited similar developmental changes in activity. Different developmental sequences indicate that in a plant cell mitochondrial and microbody enzymes may be regulated differently. Variation in the activity of glutamate-glyoxylate aminotransferase in response to light is an example. The specific activity of the enzyme in the microbodies increased 10-fold in the light over the dark control, whereas the specific activity of the enzyme in the mitochondria was about 50% lower in the light-grown seedlings as compared with the dark control. On the other hand, none of the enzymes associated with the glyoxylate cycle in the microbodies or the citric acid cycle in the mitochondria showed enhanced activity in the light.

Microbody Enzymes in Castor Bean Endosperm. In earlier reports (3, 6) glycolate oxidase had been detected in germinating castor bean endosperm. We have repeated the isolation and enzymatic analysis of particles from castor bean endosperm germinated for 5 days in the dark (Fig. 5) and found both glyoxysomal and peroxisomal enzymes as in the dark-germinated sunflower cotyledons. The presence of seven glyoxysomal enzymes—malate synthetase, isocitrate lyase, citrate synthetase, catalase, malate dehydrogenase, aconitase, and

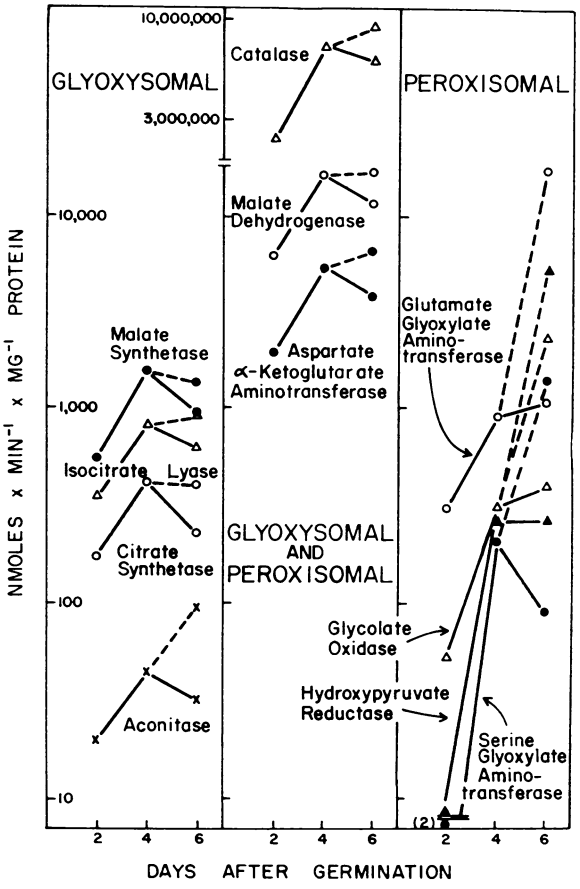

FIG. 4. Specific activities of enzymes in microbody fraction from sunflower cotyledons. Germination time is designated as days in the dark (——) or after 4 days in the dark and additional 2 days in the light (-----). The enzymes are grouped into three categories. On the left are glyoxysomal enzymes: malate synthetase (●), isocitrate lyase (△), citrate synthetase (○), and aconitase (×). On the right are peroxisomal enzymes which increase in activity in light: glycolate oxidase (△), hydroxypyruvate reductase (▲), serine-glyoxylate aminotransferase (●), and glutamate-glyoxylate aminotransferase (○). In the middle are enzymes which belong to both the peroxisomal and glyoxysomal systems: catalase (△), malate dehydrogenase (○), and asparate aminotransferase (●). Glutamate glyoxylate aminotransferase is probably common to both systems, but in this figure it is placed with enzymes with specific activities which increase in the light.

aspartate aminotransferase—confirms earlier reports by Beevers' group (2, 3, 6). The specific activities of the enzymes on a protein basis are presented in Table III. The values are of the same magnitude as from 4-day-old, dark-grown sunflower seedlings (Table II). Variations were a higher specific activity for isocitrate lyase and a lower one for malate synthetase in castor bean microbodies as compared to those from sunflower seedlings.

Specific peroxisomal enzymes—glycolate oxidase, hydroxypyruvate reductase, and serine-glyoxylate aminotransferase—were also found in the microbody fraction from castor bean endosperm. The specific activities of these enzymes were very significant. This is the first report of hydroxypyruvate reductase and serine-glyoxylate aminotransferase in microbodies from castor bean endosperm after 5 days of germination. The peroxisomal enzymes from castor bean endosperm had specific activities 1.5 to 4.5 times lower than in microbodies from sunflower cotyledons germinated in the dark for 4 days. Glutamate-glyoxylate aminotransferase is also reported for the first time from microbodies of castor bean endosperm, and its specific activity is 4 times higher than in microbodies of 4-day-old, dark-grown sunflower cotyledons.

Table III. *Specific Activity of Enzymes in Particulate Fractions from Castor Bean Endosperm*

Values are from the peak of each fraction as shown in Figure 5.

	Microbodies	Mitochondria	Supernatant
	nmoles min^{-1} mg^{-1} protein		
Glyoxysomal enzymes			
Isocitrate lyase	1,495	100	33
Citrate synthetase	627	455	14
Malate synthetase	507	15	0
Peroxisomal enzymes			
Glycolate oxidase	70	3	6
Hydroxypyruvate oxidase	145	9	27
Serine glyoxylate aminotransferase	164	247	638
Glyoxysomal and peroxisomal enzymes			
Catalase	5,104 × 10^3	360 × 10^3	83 × 10^3
Malate dehydrogenase	15,274	6,163	1,322
Aspartate aminotransferase	7,689	8,288	366
Glutamate glyoxylate aminotransferase	3,798	4,165	1,591
Mitochondrial enzyme			
Cytochrome *c* oxidase	26	4,307	1

DISCUSSION

Time Course of Development. In cotyledons from sunflower seedlings germinated in the dark and in the light the developmental sequence of enzymes for glyoxysomal metabolism differs in two major ways from enzymes related to peroxisomal metabolism. (*a*) Glyoxysomal enzymes increase rapidly to maximal total activity during the first 3 days of germination in the dark and then decrease to very low levels by the 9th day. Peroxisomal enzymes increase slowly during the first 4 days of dark germination and then remain at a constant level until day 9. (*b*) When the germinating seedling is exposed to light for 24 hr, the amount of glyoxysomal enzymes decreases slightly faster, but peroxisomal enzymes double in activity compared to the dark controls.

In order to demonstrate quantitative changes of microbody enzymes, specific activities in the peak microbody fraction were plotted on a logarithmic scale in Figure 4. The enzymes are grouped into three categories for which consistent trends were evident. In one group of enzymes necessary for glyoxysomal activities are malate synthetase, isocitrate lyase, citrate synthetase, and aconitase, which increased in specific activities between days 2 and 4 and began to decrease only a little between days 4 and 6. The specific activities of these enzymes were only slightly higher when the seedlings were exposed to light between days 4 and 6. A second group of enzymes specific for peroxisomal activity are glycolate oxidase, hydroxypyruvate reductase, and serine-glyoxylate aminotransferase. These enzymes in the microbody fraction increased 10-fold in specific activity between days 2 and 4 in the dark. After an additional 2 days in the light the specific activity of these enzymes increased again 10-fold, whereas they remained at about the same level in dark controls. The enzymes of this group are directly involved in the glycolate pathway of metabolism in peroxisomes from green spinach leaves (29).

Enzymes in the third group of Figure 4 were initially found in both glyoxysomes from castor bean endosperm and peroxisomes of green leaves (29). To this group belong catalase, malate dehydrogenase, and aspartate aminotransferase. Changes in their specific activity during germination are similar to other glyoxysomal enzymes. However, the logarithmic plot in Figure 4 emphasizes that the specific activities of these three enzymes in the microbody fraction are higher (10- to 1000-fold) than the other microbody enzymes. Thus, little change in their specific activities later during greening may

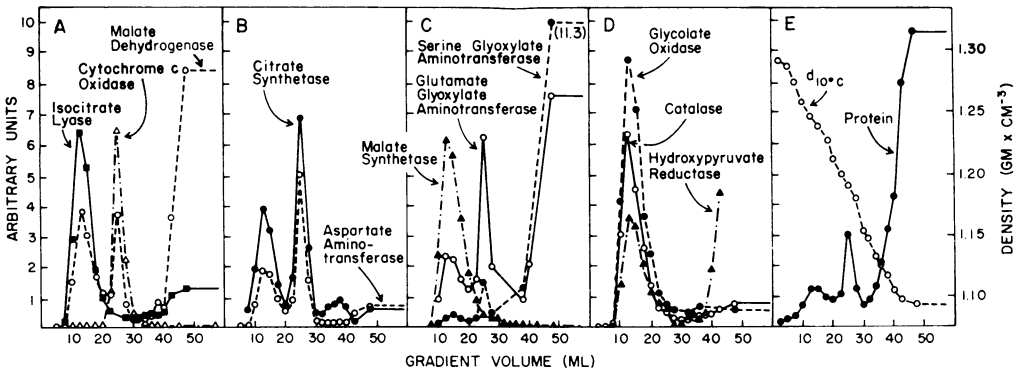

FIG. 5. Distribution of particulate enzymes on sucrose gradients from homogenates of castor bean endosperm. The seedlings had been germinated for 5 days in the dark. A: ○-----○: Malate dehydrogenase; graph units as 5 μmoles min^{-1} ml^{-1}; △-·-·-△: cytochrome *c* oxidase; graph units as 2 μmoles min^{-1} ml^{-1}; ■——■: isocitrate lyase; graph units as 0.25 μmole min^{-1} ml^{-1}; B: ●——●: citrate synthetase; graph units as 0.2 μmole min^{-1} ml^{-1}; ○-----○: aspartate α-ketoglutarate aminotransferase; graph units as 0.5 μmole min^{-1} ml^{-1}; C: ●-----●: serine-glyoxylate aminotransferase; graph units as 0.5 μmole min^{-1} ml^{-1}; ○——○: glutamate-glyoxylate aminotransferase; graph units as 2 μmoles min^{-1} ml^{-1}; ▲-·-·-▲: malate synthetase; graph units as 0.1 μmole min^{-1} ml^{-1}; D: ●-----●: glycolate oxidase; graph units as 0.01 μmole min^{-1}; ○——○: catalase; graph units as 1.0 mmole min^{-1} ml^{-1}; ▲-·-·-▲: hydroxypyruvate reductase; graph units as 0.05 μmole min^{-1} ml^{-1}; E: ○——○: density (d 10 C) as g cm^{-3}; ●——●: protein; graph units as 1 mg ml^{-1}.

be related to the fact that they were present in great excess at all times. In contrast, other peroxisomal enzymes of lower specific activity increased greatly in the light. No increase in catalase in the light might mean that the glyoxysomal catalase disappeared as fast as the peroxisomal system was formed from either *de novo* synthesis of catalase or reutilization of the glyoxysomal catalase.

Peroxisomal Enzymes in Germinating Seedlings. Our data do not clarify whether there is one type of microbody with enzymes for both the glyoxysomal system (glyoxylate cycle) and the peroxisomal system (glycolate pathway) or whether there are two biochemically different types of microbodies. The role of the glyoxysomal system in a germinating seedling is clearly established for fat conversion to succinate. The role of peroxisomes in green leaves is associated with photorespiration during photosynthesis. The significance of peroxisomal enzymes in dark-grown tissue is unknown, particularly in castor bean endosperm which does not develop into a green leaf. In germinating tissue the peroxisomal system may be involved in the biosynthesis of glycine, serine, and C_1 compounds as in green leaves. It may also function to dispose of excess reducing capacity generated by the glyoxysomal system (29).

In experiments establishing the glyoxysomal system in germinating castor bean endosperm, Beevers' group (3, 6) reported a low level of glycolate oxidase in the microbody fraction and the absence of glyoxylate reductase, which in this paper is designated hydroxypyruvate reductase (35). The role of glycolate oxidase in glyoxysomes of castor bean endosperm had previously (3) been implicated in the oxidation of excess NADH in the glyoxysomes together with glyoxylate reductase and catalase. In our re-examination of enzymes in the castor bean microbodies after 5 days of germination, nearly as much peroxisomal enzyme activity has been found as for the glyoxysomal enzymes. Since all peroxisomal enzymes are present, it is likely that glycolate oxidase acts as part of a specific peroxisomal system, which may, however, be involved in NADH oxidation. The latter hypothesis is unlikely because in 2-day-old sunflower seedlings the activities of glycolate oxidase and hydroxypyruvate reductase are too low to reoxidize much of the NADH when the enzymes of the glyoxylate cycle have almost reached maximal activity.

Malate-Aspartate Hydrogen Transport System. Malate dehydrogenase and aspartate aminotransferase in leaf peroxisomes suggested that malate and aspartate were hydrogen carriers between the peroxisomes and chloroplasts or mitochondria (30, 41). The same transport system could occur from the glyoxysomes. During glyoxysomal metabolism of fatty acids, excess NADH is produced in large amounts and must be reoxidized, preferably in a manner which would conserve the energy for growth, as in gluconeogenesis. The 10- to 100-fold higher activity of malate dehydrogenase and aspartate aminotransferase in glyoxysomes, compared to other enzymes associated with the glyoxylate cycle, suggests that these two enzymes may fulfill multiple functions. Besides the glyoxylate cycle, they could be a part of a transport system for movement of reducing equivalents out of the glyoxysomes as malate. Elsewhere in the cell where NADH is needed, malate oxidation to oxalacetate would occur, and then oxalacetate would return to the microbody as aspartate. In the microbody the aspartate would be reconverted to oxalacetate by the microbody aspartate aminotransferase. This system for reoxidation of NADH generated in microbodies seems preferable to its oxidation by hydroxypyruvate (glyoxylate) reductase mentioned above, because the enzymes for malate and aspartate transport are present in sufficient activity and the energy is not wasted by a terminal oxidation. However, catalase in glyoxysomal metabolism would still be essential for the oxidation of the reduced flavin generated during the β-oxidation of fatty acids.

Errors in Specific Activities of Microbody Enzymes. The changes in the specific activities of enzymes in the microbody fraction showed certain anomalies which required further consideration. Total isocitrate lyase activity, for instance, was equal at days 2 and 4 (Fig. 1); however, the specific activities of this enzyme in the microbody fraction increased 3-fold. After 4 days of germination when total isocitrate lyase activity dropped, the specific activity in the microbody fraction did not decline and was even higher in the light than in the dark. One explanation for these contradictions is that the apparent specific activities of microbody enzymes in the gradient band are too low, because total protein is dependent upon other particles in this area of the gradient. Bands of storage protein bodies of slightly higher density and of etioplasts of slightly lower densities overlapped into the microbody fraction. Consequently, the calculated specific activities of all the microbody enzymes from this area of the gradient were erroneously low, particularly at early stages of development. After 6 days of germination the storage protein bodies had disappeared (Schnarrenberger, Oeser, and Tolbert, unpublished), and this decrease in total protein could account for the increase of specific activity for isocitrate lyase between days 2 and 4. The density of etioplasts between days 2 and 6 was about 1.26 g cm^{-3}. In light, etioplasts developed into chloroplasts with a lighter density (Schnarrenberger, Oeser, Tolbert, unpublished), but microbodies remained at a density of about 1.265 g cm^{-3}. As a result, the microbody band was less contaminated with plastids, and the apparent specific activity of enzymes in microbodies increased. If the microbodies could have been obtained pure, there might have been different changes in specific activities. The specific activities of the microbody enzymes were most nearly correct after 6 days of germination in the light, when the protein bodies and etioplasts had mainly disappeared. These considerations apply equally to all microbody enzymes during development. Thus the great increase in specific activities of peroxisomal enzymes in the light is a valid comparison with the specific activities of glyoxysomal enzymes, because both were affected in the same way.

Formation of Glycine and Serine by Microbodies. The presence of high glutamate-glyoxylate aminotransferase activity in microbodies of castor bean endosperm as well as in germinating sunflower seedlings is emphasized by our data. Both the glycolate pathway in leaf peroxisomes and the glyoxylate cycle in glyoxysomes produce glyoxylate, and this transaminase would function for the biosynthesis of glycine in these microbodies. Likewise the microbodies in germinating tissue, as well as in leaf peroxisomes, contained the two enzymes, hydroxypyruvate reductase and serine-glyoxylate aminotransferase, for serine-glycerate interconversion. Glycine and serine are used for protein, glycine for porphyrin biosynthesis, and serine for all C_1 moieties needed for cell wall and nucleic acid synthesis. These syntheses are essential for growth, and glycine and serine formation should be considered a vital part of metabolism in microbodies (29).

The developmental pattern for glutamate-glyoxylate aminotransferase in germinating sunflower cotyledons in the dark was similar to the increases for other enzymes common to both the glyoxysomal and peroxisomal metabolic sequences. The activity in the dark initially increased rapidly as a glyoxysomal enzyme to a higher specific activity than the peroxisomal enzymes. After exposure to light, however, its activity in the microbody fraction further increased as did other specific peroxisomal enzymes. In this respect the specific activity of glutamate-glyoxylate aminotransferase exhibited a differ-

ent developmental sequence than catalase, malate dehydrogenase, and aspartate aminotransferase, which are also common to both the peroxisomal and glyoxysomal systems.

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