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Comparative Studies of Glyoxysomes from Various Fatty Seedlings[1]

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ABSTRACT

The separation of various organelles from cotton cotyledon (*Gossypium hirsutum* L.), cucumber cotyledon (*Cucumis sativus* L.), peanut cotyledon (*Archis hypogaea* L.), pine megagametophyte (*Pinus ponderosa* Laws), and watermelon cotyledon (*Citrullus vulgaris* Schrad.) by sucrose density gradient centrifugation was found to be similar to that described for castor bean endosperm (*Ricinus communis* L.). Equilibrium densities were 1.12 to 1.13 g cm^3 for endoplasmic reticulum, 1.17 to 1.19 g/cm^3 for mitochondria, and 1.25 g/cm^3 for glyoxysomes. Isolated glyoxysomes from different fatty seedlings have striking similar specific activities of individual enzymes. The only exception is alkaline lipase activity which, when assayed with an artificial substrate, varies some 10-fold in glyoxysomes from different fatty seedlings. The properties of individual enzymes in glyoxysomes from different fatty seedlings are qualitatively similar as regard to suborganelle localization and behavior in the presence of KCl and Triton X-100. In pine megagametophyte, the glyoxysomes and not the mitochondria are the intracellular site for the breakdown of stored lipid.

Glyoxysomes were first discovered and then studied intensively in the endosperm of germinating castor bean as discrete organelles housing a sequence of gluconeogenesis enzymes including alkaline lipase (18), fatty acid-activating enzyme (9), enzymes of the β-oxidation pathway (8, 15) and of the glyoxylate cycle (3), and the characteristic microbody marker enzymes (4, 19). It was found that glyoxysomes are also present in many other fatty seedlings (1, 5, 7, 11, 16, 19, 20).

In the studies of glyoxysomes, there are some discrepancies as to their possession of nucleic acids and protein-synthesizing ability (5, 10), their interrelationship with the leaf peroxisomes in greening cucurbit cotyledons (11, 16, 20), and the association of certain enzymes to the glyoxysomal membrane (14, 21). Different fatty seedlings have been used to study glyoxysomes, and the assumption is frequently made that glyoxysomes from these fatty seedlings are similar. Except for the demonstration that glyoxysomes from different fatty seedlings have an equilibrium density of 1.25 g/cm^3 in sucrose gradients and contain some marker enzymes, no thorough comparative study has been made.

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It was shown that in castor bean endosperm, the glyoxysomes house most if not all of the enzymes of the β-oxidation pathway and that only the glyoxysomes, and not the mitochondria, can degrade fatty acids (8). It was suggested recently that in the fat-containing megagametophyte of germinating pine seeds, mitochondria and glyoxysomes oxidize the fatty acids *in vivo* one-third and two-thirds respectively (6).

I now report experiments on the comparison of glyoxysomes isolated from different fatty seedlings and on the effect of salt and detergent on the glyoxysomal membrane-associated enzymes. The failure to locate alkaline lipase and fatty acyl CoA dehydrogenase in the mitochondria of pine megagametophyte is also reported.

MATERIALS AND METHODS

Plant Materials. Seeds were soaked in running water for 1 day and then allowed to germinate in moist vermiculite at 30 C in darkness. Endosperm of 5-day-old castor bean (*Ricinus communis L.* var. Hale), cotyledons of 4-day-old watermelon (*Citrullus vulgaris* Schrad.), cucumber (*Cucumis sativus* L.), and peanut (*Archis hypogaea* L.) seeds, cotyledons of 2-day-old cotton (*Gossypium hirsutum* L.) seeds, and megagametophyte of 9-day-old pine (*Pinus ponderosa* Laws var. Colorado) seeds were used.

Isolation of Organelles. The tissues were chopped in grinding medium with a razor blade followed by grinding in a mortar and pestle as described previously (14). The supernatant fraction obtained after centrifugation at 270*g* for 10 min was layered onto a sucrose gradient which was composed of 10 ml of 20% (w/w) sucrose on top of 40 ml of a 30 to 60% linear sucrose gradient. The gradient was centrifuged at 21,000 rpm for 4 hr in a Beckman L2-65B ultracentrifuge with Spinco rotor 25.2. Sufficient extract from each tissue was put onto the gradient so that 5 to 6 mg of glyoxysomal protein were obtained in the region of density 1.25 g/cm^3 in the gradient after centrifugation.

Assays. The assays of catalase, glycolate oxidase, Cyt oxidase, malate synthetase, citrate synthetase, isocitrate lyase, malate dehydrogenase, and fatty acyl CoA dehydrogenase (12–14) were as described previously. Alkaline lipase was assayed using N-methylindoxylmyristate as an artificial substrate (18). The assay of NADH-Cyt *c* reductase followed that described (17) using NADH to initiate the reaction. Triose phosphate isomerase was assayed according to Beisenherz (2). In the test of increase in enzyme activity by detergent or salt, 0.05% Triton X-100 or 0.2 M KCl was added to the reaction mixture.

Subfractionation. Two-ml glyoxysomal fraction in 54% sucrose was added to 4 ml of 0.05 M Tricine buffer, pH 7.5, with or without 0.3 M KCl and shaken in a Vortex mixer (14). After incubation in the cold for 30 min, the solution was centrifuged at 40,000*g* for 30 min in a Beckman rotor 65 to give a super-

natant fraction and a pellet fraction. The pellet was resuspended in 0.05 M Tricine buffer, pH 7.5, 18% sucrose, and as appropriate, 0.2 M KCl (14).

RESULTS AND DISCUSSION

The separation of various organelles in sucrose gradient centrifugation from different fatty seedlings was similar to that described from castor bean endosperm. Visible bands of glyoxysomes, mitochondria, and endoplasmic reticulum were observed in similar positions in the sucrose gradients. Equilibrium densities were 1.12 to 1.13 g/cm³ for endoplasmic reticulum, 1.17 to 1.19 g/cm³ for mitochondria, and 1.25 g/cm³ for glyoxysomes (Table I).

The preservation of intact glyoxysomes from the various fatty seedlings differs greatly. Using the recovery of the easily solubilized isocitrate lyase as an index, the per cent recovery of intact glyoxysomes at density 1.25 g/cm³ region in the gradients ranges from 13% in the watermelon preparation to 80% in the castor bean preparation. The recovery of Cyt *c* oxidase (mitochondrial marker) and NADH Cyt *c* reductase (endoplasmic reticulum marker) are in the range of 80 to 90%, but these enzymes have been shown to be tightly associated with the membrane of the respective organelles. There is a minor peak of NADH-Cyt *c* reductase in the mitochondrial region and that enzyme is antimycin A-sensitive.

The separation of organelles from pine megagametophyte in sucrose gradient is shown in Figure 1. The four protein peaks inside the gradient, corresponding to the four visible bands before fractionation, are endoplasmic reticulum at 1.12 g/cm³ (NADH Cyt *c* reductase as marker), mitochondria at 1.17 g/cm³ (Cyt oxidase as marker), proplastids at 1.21 g/cm³ (triose-P isomerase as marker), and glyoxysomes at 1.25 g/cm³ (catalase and isocitrate lyase as markers). Fatty acyl CoA dehydrogenase occurs in the glyoxysomes and not in the mitochondria, as its distribution follows those of catalase and isocitrate lyase. The activity at the top of the gradient is probably derived from broken glyoxysomes during organelle preparation, as indicated in the preparation of glyoxysomes from castor bean endosperm using refined grinding technique (17). In contrast, citrate synthetase and malate dehydrogenase (not shown) have double peaks, occurring in both the mitochondrial and the glyoxysomal regions. In a separate experiment, alkaline lipase was also found to be localized in the glyoxysomes and not in the mitochondria. It seems clear that the pine mitochondria lack the enzymatic capacity to carry out lipolytic activity or the β-oxidation of fatty acids.

In my preparation of organelles by linear sucrose gradient centrifugation from pine megagametophyte, there is a distinct protein peak of proplastids at density 1.21 g/cm³ (Fig. 1). The activity of triose-P isomerase at this region of the gradient is high even though it represents only 5 to 10% of the total activity with the rest being in the soluble fraction. Much of the activity of this enzyme inside the gradient is confined to the density 1.21 g/cm³ region but it also spreads throughout the gradient, bringing slight contamination to other organelle fractions. In this experiment, the gradient was linear and the supernatant fraction obtained after centrifugation of the crude extract at 270*g* for 10 min was put directly onto the gradient. As a result, a cleaner separation of organelles was obtained than that described previously where resuspended particulate fraction was layered on a stepped gradient (5, 6). Yet, there was still some contamination of proplastids in the glyoxysomal region. This contamination may account for the finding that nucleic acids and protein-synthesizing capacity was present in the glyoxysome fraction from this material (5).

Table II shows the specific activities of the various enzymes in the glyoxysomal peak in the gradients. With the exception of alkaline lipase, individual enzymes in glyoxysomes from different fatty seedlings have strikingly similar specific activities after solubilization. Only the activities of isocitrate lyase and citrate synthetase show 2- to 3-fold difference in glyoxysomes from different fatty seedlings and the other enzymes appear within a 1-fold difference. Glyoxysomes from different sources thus appear to have a similar complement of the various enzyme activities. In contrast, alkaline lipase activity shows a 10-fold difference from the highest to the lowest values. This is the only enzyme assayed with an artificial substrate. It is quite likely that the enzyme may show some degree of substrate specificity toward the respective glycerides that are stored in the various fatty seeds.

Table I. *Densities and Per Cent Recovery of Various Organelles Isolated by Sucrose Gradient Centrifugation from Different Fatty Seedlings*

In the preparation from cotton cotyledon, catalase was used to calculate the per cent recovery of intact glyoxysomes. In the calculation of per cent recovery of Cyt reductase, the minor peak in the mitochondrial fraction was omitted.

	Castor Bean Endosperm	Watermelon Cotyledon	Peanut Cotyledon	Cucumber Cotyledon	Pine Megagametophyte	Cotton Cotyledon
	g/cm³					
Density of						
Endoplasmic reticulum	1.12	1.13	1.13	1.13	1.12	Not checked
Mitochondria	1.19	1.18	1.19	1.18	1.17	1.17
Glyoxysomes	1.24–1.25	1.25	1.25	1.25	1.25	1.25
	%					
Recovery of						
Cyt reductase at 1.12–1.13 g/cm³ density	98	92	94	97	100	Not checked
Cyt oxidase at 1.17–1.19 g/cm³ density	95	79	95	88	76	95
Isocitrate lyase at 1.25 g/cm³ density	80	13	31	48	64	50

Alkaline lipase in the castor bean glyoxysomes is associated tightly with the membrane and it resists solubilization by KCl and is inactivated by detergent or organic solvent (18). Malate synthetase and citrate synthetase are also membrane-associated, but they can be stripped off by KCl or detergent (14). In contrast, isocitrate lyase and catalase are readily released from the organelles by osmotic shock (14). Similar solubilization experiments were carried out with glyoxysomes isolated from other fatty seedlings (Table III). In general, 2-fold dilution of the isolated glyoxysomes solubilizes most of the isocitrate lyase but not the malate synthetase, citrate synthetase, and alkaline lipase. Citrate synthetase is less tightly bound to the membrane and shows various degrees of solubilization after osmotic shock. Treatment of the glyoxysomes with 0.2 M KCl solubilizes the two synthetases but not the alkaline lipase which is still associated with the membrane in the pellet. Thus, even though there are quantitative differences, the membrane-associated enzymes in glyoxysomes from different fatty seedlings respond quite similarly to these solubilization treatments.

The pellet obtained after solubilization of the enzyme from castor bean glyoxysomes was shown by electron microscopy and by equilibrium sucrose gradient centrifugation to contain the "ghosts" of the glyoxysomes (14). In the present studies,

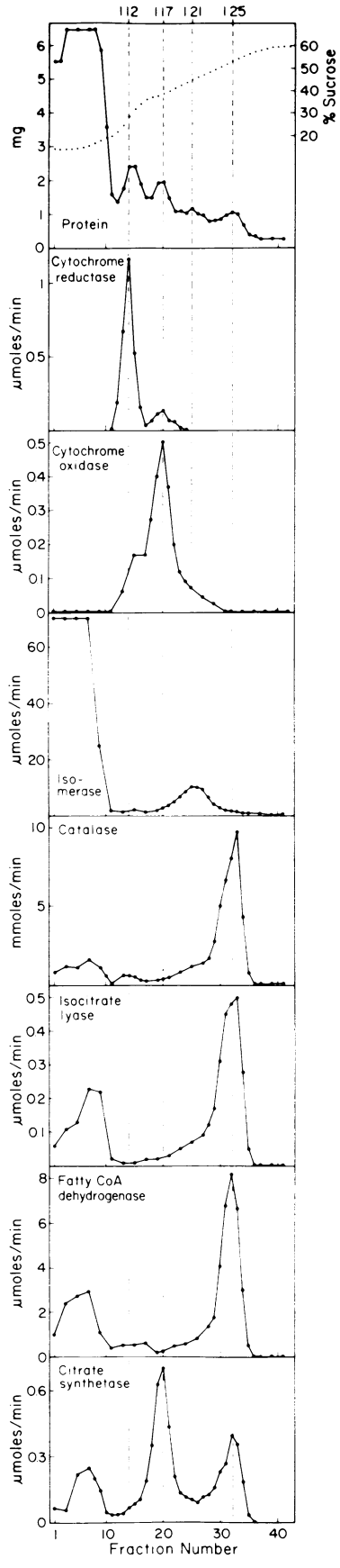

FIG. 1. Separation of organelles from the megagametophyte of pine seeds after seven days of germination. The dashed lines mark the peak activities of Cyt *c* reductase (endoplasmic reticulum marker), Cyt *c* oxidase (mitochondrial marker), triosephosphate isomerase (proplastid marker), and catalase (glyoxysomal marker).

peanut glyoxysomes after the osmotic treatment were subjected to further sucrose gradient centrifugation. Alkaline lipase and malate synthetase migrated together during centrifugation and most of the activity reached a density of 1.21 g/cm^3 after centrifugation for 4 hr (data not shown). The peak did not move further into the gradient after 17 hr of centrifugation and thus 1.21 g/cm^3 is the equilibrium density. Isocitrate lyase was solubilized and it remained at the top of the gradient. Thus, the membrane fraction of the peanut glyoxysomes also behaves similarly to that of the castor bean glyoxysomes in sucrose gradient centrifugation.

The activities of the enzymes associated with the glyoxysomal membrane can be increased by salt or detergent (Table IV), presumably due to the solubilization of the enzymes from the membrane. Triton X-100 has no effect on the activities of isocitrate lyase and catalase (two easily solubilized enzymes), whereas KCl increases their activities slightly. Triton X-100, but not KCl, increases the activity of the castor bean glyoxysomal malate dehydrogenase, an enzyme associated loosely with the membrane (14). The detergent and salt have no effect on the activities of malate dehydrogenase from other sources. KCl, but not Triton X-100, increases 2- to 3-fold the activities of fatty acyl CoA dehydrogenase in the castor bean and peanut glyoxysomes, but the salt has no effect on the enzymes in the glyoxysomes from other sources. Triton X-100, but not KCl, increases slightly the activities of citrate synthetase in glyoxysomes from all sources except that from pine megagametophyte; the activity of pine glyoxysomal citrate synthetase is increased 7-fold by KCl. KCl or Triton X-100 increases 2-fold the activity of malate synthetase in the castor bean glyoxysomes, but a combination of the salt and detergent still gives a 2-fold increase, suggesting that the mechanism of increase in enzyme activity by salt and detergent may be similar. KCl or Triton X-100 increases slightly the activities of malate synthetase in glyoxysomes from other sources. Triton X-100 completely inactivates the alkaline lipase in glyoxysomes from all sources and KCl increases only the activity of the enzyme from castor bean while it inactivates those from other sources. In general, the activities of the membrane-associated enzymes of glyoxysomes from different fatty seedlings are increased somewhat by salt or detergent, and alkaline lipase, the most tightly membrane-associated enzyme, may be a lipoprotein, since its activity is completely abolished by detergent or organic solvent (18).

In a previous report (6), the crude extract of pine megagametophyte was incubated with radioactive triglycerides and subsequent separation of the particulate fraction by stepped sucrose gradient centrifugation revealed that the mitochondrial band and the glyoxysomal band contained one-third and two-thirds, respectively, of the radioactive breakdown products of triglycerides in the gradient. From these data (6), it was suggested that in pine megagametophyte, the mitochondria and the glyoxysomes oxidize the fatty acids *in vivo* one-third and two-thirds, respectively. In that work, the mitochondria were not checked for the presence of the necessary enzymes for the oxidation of the fatty acids and only the easily solubilized isocitrate lyase, but not an enzyme that is associated with the glyoxysomal membrane, was checked for possible contamination of the mitochondrial band by broken glyoxysomes. The present work shows that alkaline lipase and fatty acyl CoA dehydrogenase are present only in the glyoxysomes but not in the mitochondria. It is possible, of course, that other enzyme systems for fat oxidation may be present in the mitochondria.

Table II. *Enzyme Activities in Glyoxysomes from Different Fatty Seedlings*

Enzyme activities were assayed in the presence of suitable amount of KCl or Triton X-100.

Enzymes	Castor Bean Endosperm	Watermelon Cotyledon	Peanut Cotyledon	Cucumber Cotyledon	Pine Megagametophyte
	nmoles/min·mg protein				
Catalase	13,000,000	9,900,000	11,000,000	11,000,000	9,500,000
Malate dehydrogenase	38,000	55,000	34,000	80,000	60,000
Fatty acyl CoA dehydrogenase	6,500	7,100	6,200	5,600	6,400
Malate synthetase	2,100	1,100	1,300	1,600	2,100
Isocitrate lyase	930	520	300	330	480
Citrate synthetase	840	700	330	800	350
Glycolate oxidase	95	70	58	89	Not checked
Alkaline lipase	19	4	7	2	8

Table III. *Solubilization of Glyoxysomal Enzyme Activities after Osmotic Breakage with or without 0.2 M KCl of Glyoxysomes Isolated from Various Fatty Seedlings*

Enzyme activities were assayed in the presence of suitable amount of KCl or Triton X-100. Results are expressed as the amount in the supernatant fraction divided by the sum of the amounts in the supernatant fraction and in the pellet. Recovery of each activity after treatment was within 100 ± 30%.

Enzymes	Castor Bean Endosperm		Watermelon Cotyledon		Peanut Cotyledon		Cucumber Cotyledon		Pine Megagametophyte	
	H_2O	KCl	H_2O	KCl	H_2O	KCl	H_2O	KCl	H_2O	KCl
	%									
Isocitrate lyase	98	98	94	92	97	96	97	98	98	98
Citrate synthetase	11	72	20	73	26	90	63	99	89	98
Malate synthetase	6	82	16	88	21	85	28	96	10	93
Alkaline lipase	11	20	8	8	6	11	15	11	9	28

Table IV. *Effect of 0.05% Triton X-100 or 0.2 M KCl on Activities of Glyoxysomal Enzymes*

Glyoxysomes isolated in sucrose gradients were used directly for enzyme assays and the salt or detergent was added to the assay mixture. Data are expressed as per cent of the original activities. For simplicity, 100% is used to represent those values of 100 ± 10%.

Enzymes	Castor Bean Endosperm		Watermelon Cotyledon		Peanut Cotyledon		Cucumber Cotyledon		Pine Megagametophyte	
	KCl	Triton	KCl	Triton	KCl	Triton	KCl	Triton	KCl	Triton
	%									
Isocitrate lyase	66	100	84	100	100	100	88	100	57	100
Catalase	81	100	86	100	73	100	100	100	100	100
Malate dehydrogenase	67	120	60	100	56	100	62	100	65	100
Fatty acyl CoA dehydrogenase	300	130	72	100	240	100	88	100	100	100
Citrate synthetase	100	160	84	170	45	120	100	120	750	100
Malate synthetase	220	200	160	120	160	120	170	120	150	100
Alkaline lipase	130	0	75	0	54	0	56	0	29	0

The present work also shows that the glyoxysomes from pine megagametophyte are easily broken as are those from castor bean. Broken glyoxysomes appear at a lighter density in sucrose gradient centrifugation (14, present work) and may contaminate the mitochondrial fraction, especially in a stepped gradient. The possibility that incubation of the pine extract at 30 C might break a substantial amount of the fragile glyoxysomes is supported by the comparatively low recovery of isocitrate lyase in the glyoxysomal fraction in the gradient after incubation (6). These facts should be taken into consideration of the conclusion that in pine megagametophyte, mitochondria can oxidize fatty acids at rate one-half of that in the glyoxysomes (6).

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LITERATURE CITED

1. Beevers, H. 1969. Glyoxysomes of castor bean endosperm and their relation to gluconeogenesis. Ann. N. Y. Acad. Sci. 168: 313-324.
2. Beisenherz, G. 1955. Triosephosphate isomerase from calf muscle. Methods Enzymol. 1: 427-435.
3. Breidenbach, R. W. and H. Beevers. 1967. Association of the glyoxylate cycle enzymes in a novel subcellular particle from castor bean endosperm. Biochem. Biophys. Res. Commun. 27: 462-469.
4. Breidenbach, R. W., A. Kahn, and H. Beevers. 1968. Characterization of glyoxysomes from castor bean endosperm. Plant Physiol. 43: 705-713.
5. Ching, T. M. 1970. Glyoxysomes in megagametophyte of germinating ponderosa pine seeds. Plant Physiol. 46: 475-482.
6. Ching, T. M. 1973. Compartmental utilization of carboxyl-14C-tripalmitin by tissue homogenate of pine seeds. Plant Physiol. 51: 278-284.
7. Cooper, T. G. and H. Beevers. 1969. Mitochondria and glyoxysomes from castor bean endosperm: enzyme constituents and catalytic capacity. J. Biol. Chem. 244: 3507-3513.
8. Cooper, T. G. and H. Beevers. 1969. β-Oxidation in glyoxysomes from castor bean endosperm. J. Biol. Chem. 244: 3514-3520.

9. Cooper, T. G. 1971. The activation of fatty acids in castor bean endosperm. J. Biol. Chem. 246: 3451-3455.
10. Douglass, S. A., R. S. Criddle, and R. W. Breidenbach. 1973. Characterization of deoxyribonucleic acid species of castor bean endosperm. Plant Physiol. 51: 902-906.
11. Gerhardt, B. 1973. Untersuchugen zur Funktionsanderung der Microbodies in den Keim Blattern von *Helianthus annuus* L. Planta 110: 15-28.
12. Huang, A. H. C. and H. Beevers. 1971. Isolation of microbodies from plant tissues. Plant Physiol. 48: 637-641.
13. Huang, A. H. C. and H. Beevers. 1972. Microbody enzymes and carboxylases in sequential extracts from C_4 and C_3 leaves. Plant Physiol. 50: 242-248.
14. Huang, A. H. C. and H. Beevers. 1973. Localization of enzymes within microbodies. J. Cell Biol. 58: 379-389.
15. Hutton, D. and P. K. Stumpf. 1969. Characterization of the oxidation systems from maturing and germinating castor bean seeds. Plant Physiol. 44: 508-516.
16. Kagawa, T. and H. Beevers. 1975. The development of microbodies (glyoxysomes and leaf peroxisomes) in cotyledons of germinating watermelon seedlings. Plant Physiol. 55: 258-264.
17. Lord, J. M., T. Kagawa, T. S. Moore, and H. Beevers. 1973. Endoplasmic reticulum as the site of lecithin formation in castor bean endosperm. J. Cell Biol. 57: 659-667.
18. Muto, S. and H. Beevers. 1974. Lipase activities in castor bean endosperm during germination. Plant Physiol. 54: 23-28.
19. Theimer, R. R. and H. Beevers. 1971. Uricase and allantoinase in glyoxysomes. Plant Physiol. 47: 246-251.
20. Trelease, R. N., W. M. Becker, P. J. Gruber, and E. H. Newcomb. 1971. Microbodies (glyoxysomes and peroxisomes) in cucumber cotyledons. Correlative biochemical and ultrastructural study in light and dark-grown seedlings. Plant Physiol. 48: 461-475.
21. Trelease, R. N. and W. M. Becker. 1974. Cytochemical localization of malate synthetase in glyoxysomes. J. Cell Biol. 60: 483-495.