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Characterization of Glyoxysomes from Castor Bean Endosperm[1]

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Abstract. **Electron micrographs are presented which establish the identity of the components of the 3 major bands observed after sucrose density centrifugation of the crude particulate fraction from the endosperm of germinating castor bean seedlings. These are: mitochondria (density 1.19 g/cc), proplastids (density 1.23 g/cc) and glyoxysomes (density 1.25 g/cc). Further evidence is provided on the enzymatic composition of the glyoxysomes. Essentially all of the particulate malate synthetase, isocitrate lyase, catalase, and glycolic oxidase is present in these organelles. The distribution of glyoxysomal enzymes on sucrose density gradients is contrasted with that of the strictly mitochondrial enzymes fumarase, NADH oxidase, and succinoxidase. Malate dehydrogenase and citrate synthetase are present in both organelles. The functional role of glyoxysomes and their relationship to cytosomes from other tissues is discussed.**

A previous report (5) established that when crude particulate preparations from germinating castor bean endosperm were centrifuged on sucrose density gradients, 3 major bands were obtained. The enzymes of the glyoxylate cycle were confined to the densest of these bands, and the particles contained in that band were therefore named glyoxysomes.

In this report electron micrographs are presented which show the morphology and degree of homogeneity of the particles in each of the bands, respectively mitochondria, proplastids and glyoxysomes. More detailed information is provided to show the distribution of mitochondrial and glyoxylate cycle enzymes in sucrose density gradients, and evidence for the association of catalase and glycolic oxidase with the glyoxysomes is presented.

Materials and Methods

Castor bean seeds were grown and their endosperms were harvested and homogenized as previously described (5). Pellets containing a mixture of cell particles were resuspended and placed on sucrose density gradients fabricated as described in reference 5 (noted in this paper as gradient system I) or on gradients consisting of 25 ml of sucrose grading from 60 % to 30 % (gradient system II) and contained in cellulose nitrate tubes for the Spinco SW25.2 rotor. All gradients contained EDTA at 10^{-3} M. Centrifugation was carried out for 4 hours at 25,000 rpm and 0° and 1 ml fractions were collected as described (5). Isocitrate lyase, malate synthetase, citrate synthetase, succinic dehydrogenase, and protein were assayed by the procedures previously reported (5). Catalase and glycolic oxidase were assayed using a Clark-type polarizing oxygen-electrode (Yellow Springs Instrument Company, Yellow Springs, Ohio) adapted to a Gilson Oxygraph (Gilson Medical Electronics, Madison, Wisconsin). Catalase was measured by rapidly injecting 1 to 30 μl of gradient fraction into a reaction mixture containing 380 μmoles of potassium phosphate buffer pH 7.0 and 1.8 μmoles H_2O_2 in a total volume of 4.0 ml. The buffer was boiled to remove O_2 and cooled under N_2. The assays were carried out at 2°. The same H_2O_2 solution was used for all of the assays and the concentration of H_2O_2 was checked periodically by measuring absorbancy of the solution at 230 mμ. The extinction coefficient of 0.067 cm^{-1} mM^{-1} given by Maehly and Chance (11) was used. Enzyme activity was determined from the nearly linear initial slope of the 1st order rate curves.

Glycolic oxidase was measured polarographically at 25° in 3.0 ml of reaction mixture containing 300 μmoles of sodium pyrophosphate buffer pH 8.2, 0.02 μmoles FMN, 10 γ of catalase (Sigma) and 20 μmoles of sodium glycolate.

Electron microscopy of the particulate bands separated in gradient system I was performed as follows: After ascertaining the distribution of protein in fractions from the gradient, 2 peak tubes from each of the major bands (*i.e.* mitochondrial, proplastid and glyoxysomal) were pooled to give 2 ml samples of each component. These samples were fixed during a 1 hour period by adding 0.28 ml of 50 % glutaraldehyde to each. The fractions were

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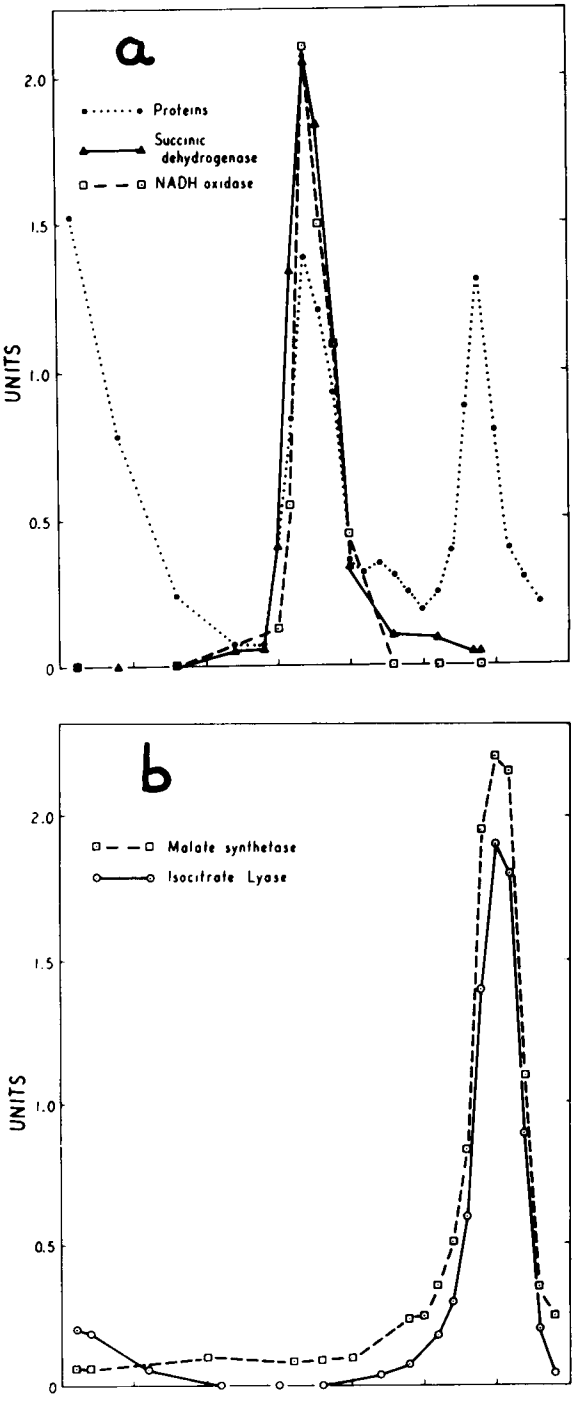
then diluted with 3 ml of a solution containing 5 % less (w/v) sucrose than that present initially in the gradient samples. Centrifugation for 15 minutes at 30,000 rpm in an SW39 Spinco rotor followed. After the supernatant solutions were decanted, the pellets were washed rapidly in 0.4 M sucrose buffered at pH 7.5 (0.1 M Tricine) and post-fixed for 15 minutes with 2.5 % OsO_4 in 0.4 M sucrose, 0.05 M Tricine, pH 7.5, made up immediately before use. This solution was then replaced with 1 % OsO_4 in 0.08 M phosphate, pH 7.5 for 1 hour, and then after renewal of the 1 % buffered OsO_4, the pellets remained in the solution overnight. All fixation steps were carried out at 0 to 4°.

The pellets were then dehydrated in a stepped series of increasing acetone concentrations, embedded in an Epon mixture, and sectioned with an LKB Ultrotome. Sections were stained with lead citrate or uranyl magnesium acetate and lead citrate in succession. Electron microscopy was conducted with a Philips EM200.

Results

Sucrose density gradient centrifugation of crude particulate pellets from the endosperm of castor beans resolves the enzymes that we have studied



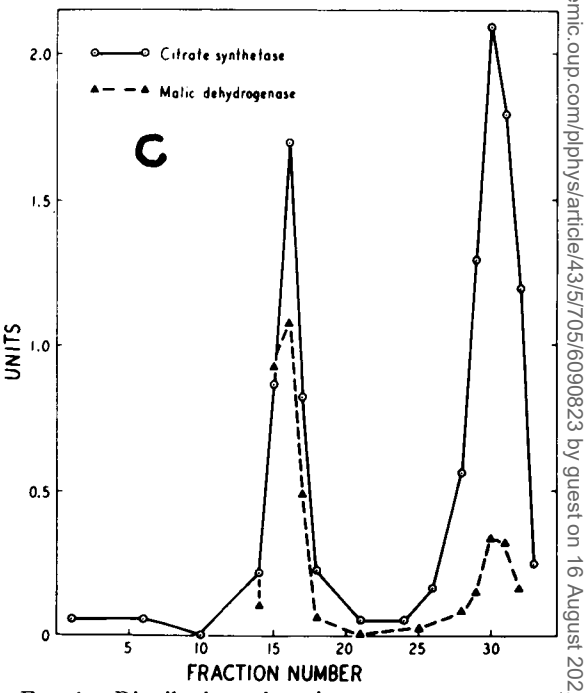


FIG. 1. Distribution of various enzymes associated with mitochondria and glyoxysomes in 1 ml fractions from sucrose density gradients (System I). a) Enzymes detected only in the mitochondrial band. b) Enzymes detected only in the glyoxysomal band. c) Enzymes detected in both the mitochondrial and glyoxysomal bands. Units are: Succinic dehydrogenase, μmoles DCPIP reduced per minute per ml (× 0.4); NADH oxidase, μmoles NADH oxidized per minute per ml (× 0.1); citrate synthetase, μmoles *p*-nitrothiophenol ion formed per minute per ml; malic dehydrogenase, μmoles NADH oxidized per minute per ml (× 0.01); malate synthetase, μmoles *p*-nitrothiophenol ion formed per minute per ml; isocitrate lyase, μmoles of glyoxylate-phenylhydrazone formed per minute per ml; protein is shown in units of mg per ml. Fraction 1 is the top of the gradient.

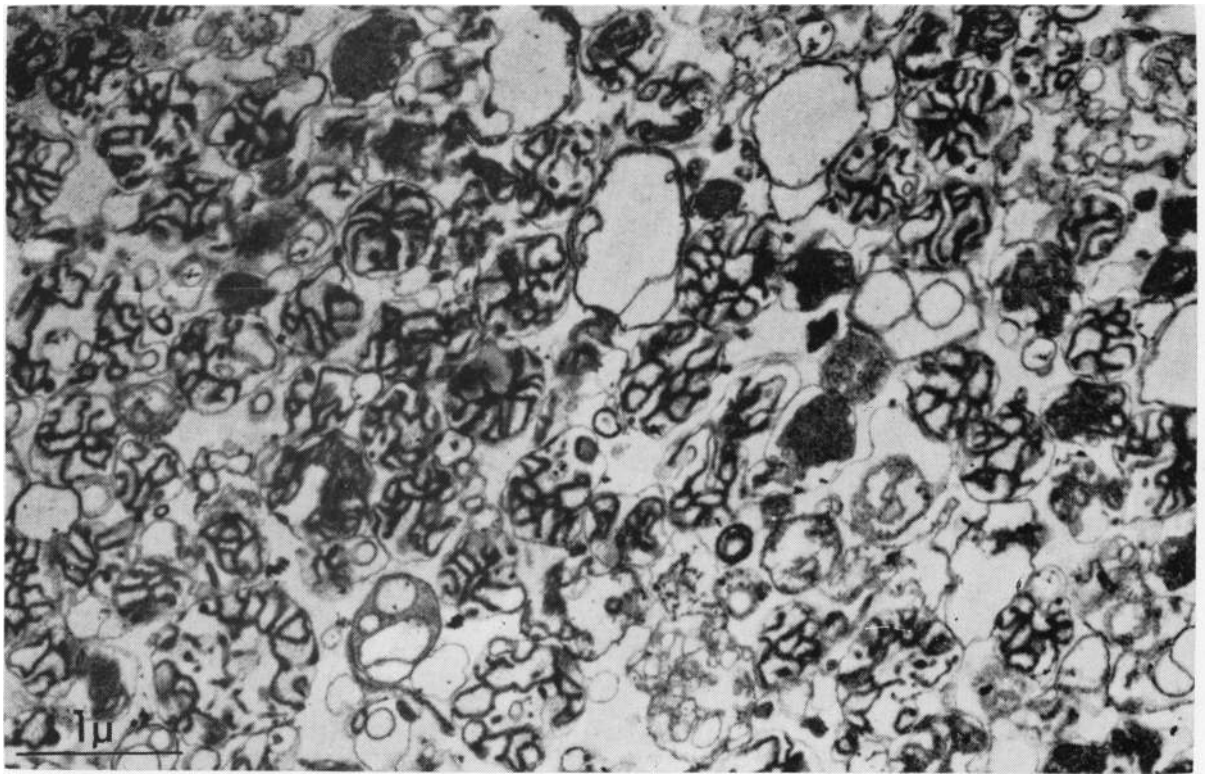


FIG. 3. Mitochondrial fraction. 22,000 ×.

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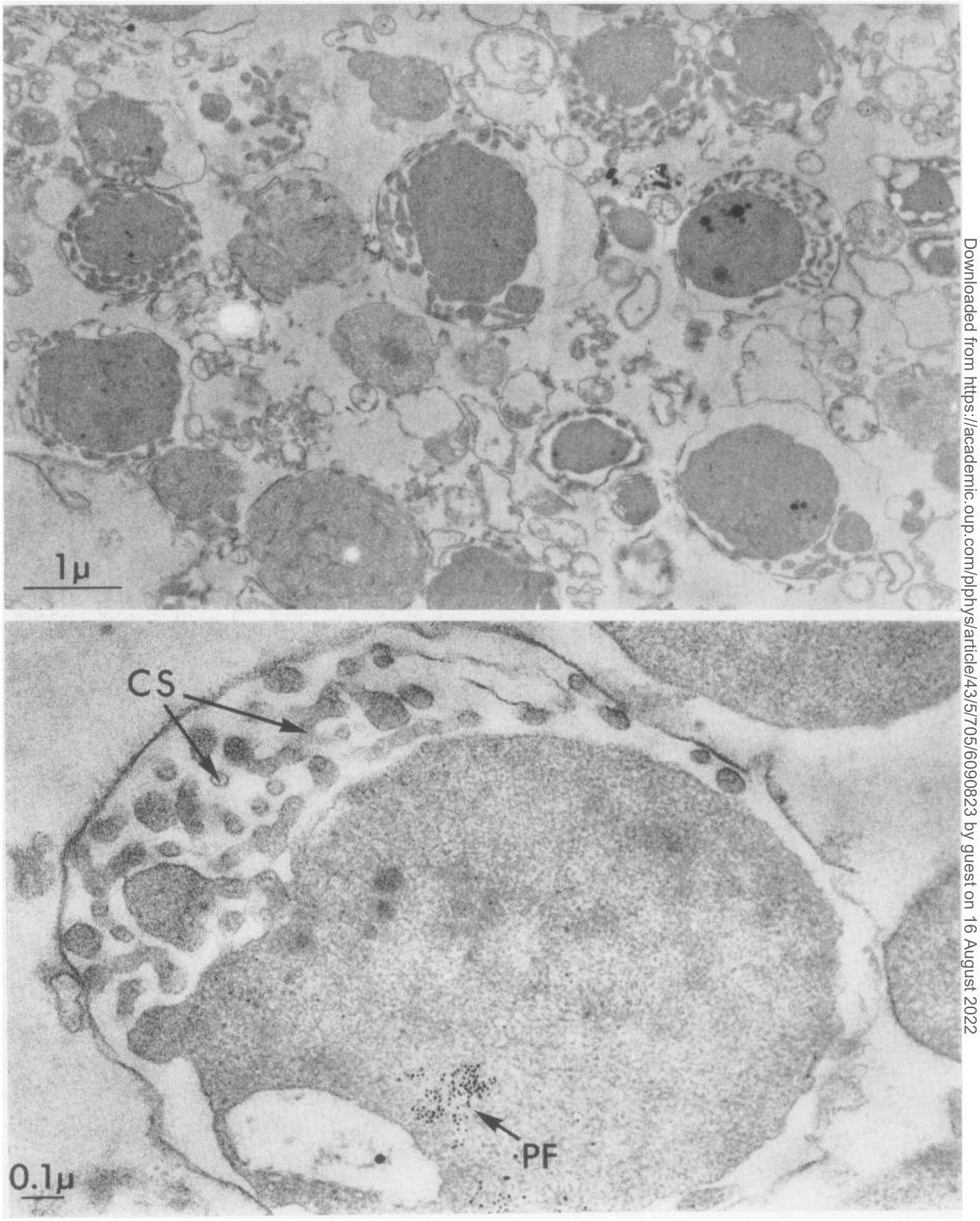

FIG. 4a (top). Proplastid fraction. 16,500 ×.

FIG. 4b (bottom). Section from a proplastid showing iron-containing micelles of phytoferritin (PF) and canaliculate system (CS). 73,000 ×.

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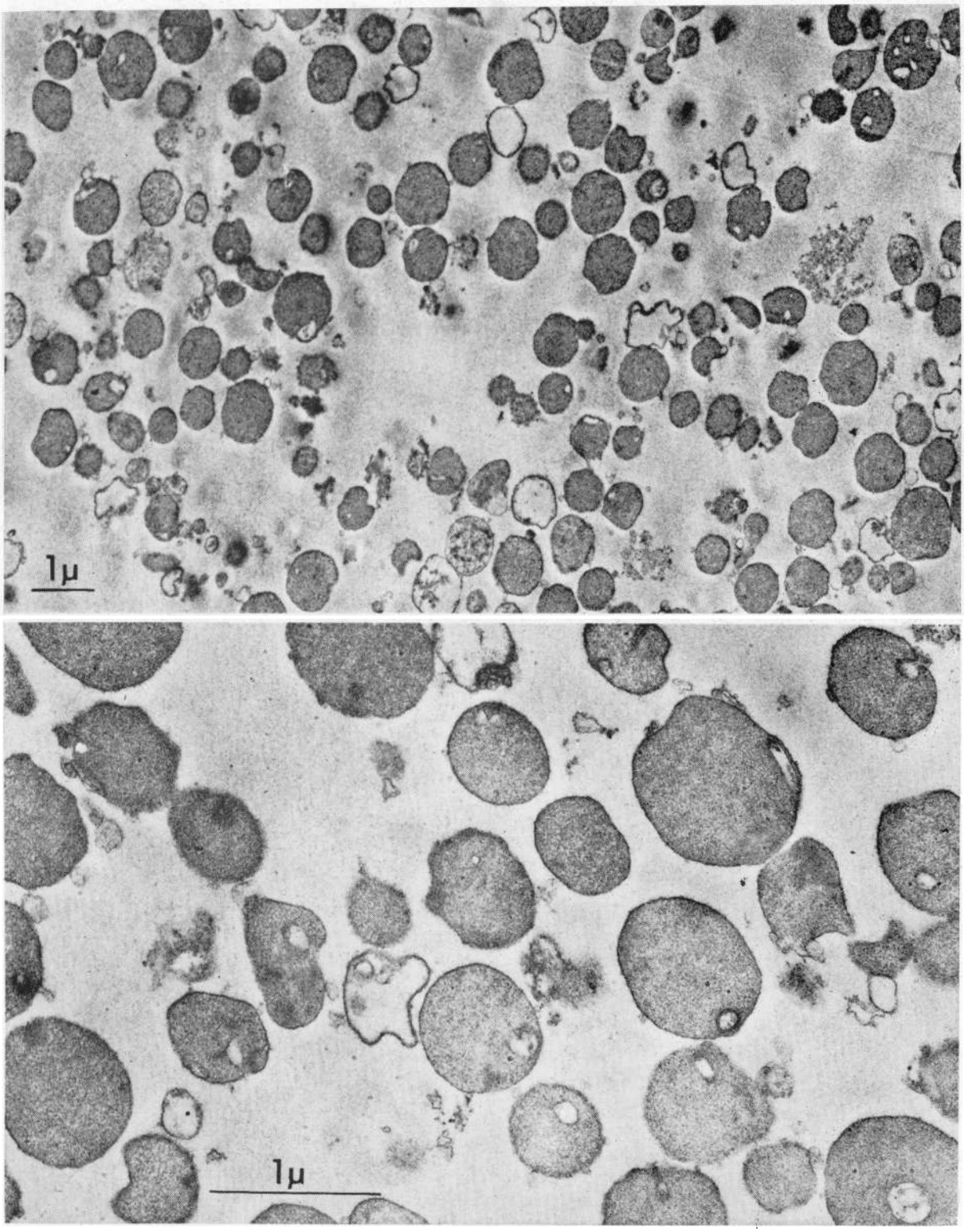

FIG. 5a (top). Glyoxysome fraction. 10,000 ×.
FIG. 5b (bottom). Glyoxysome fraction. 28,000 ×.

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into 3 distinct groups: 1) Those associated only with mitochondria, as shown for NADH oxidase and succinic dehydrogenase in figure 1a and including fumarase and cytochromes (5); 2) Those associated only with glyoxysomes as shown for isocitrate lyase and malate synthetase, in figure 1b; and 3) Those associated with both organelles as shown for citrate synthetase and malate dehydrogenase in figure 1c. As can be seen, the distribution patterns for the enzymes within a group parallel each other closely.

The distribution of catalase and glycolic oxidase, shown in figure 2 parallels the distribution of malate synthetase and isocitrate lyase in a very striking manner. Except for the soluble activity remaining at the top of the gradient, these enzymes are almost entirely restricted to the glyoxysome band.

Electron microscopic examination of the centrally located fractions from each band showed a degree of purity obtained by sucrose density-gradient centrifugation that was consistent with the distribution pattern of enzymic activities. The morphological characters of the fractions are shown in the accompanying electron micrographs. Figure 3 shows the homogeneity of the mitochondrial fraction. Figure 4a illustrates the proplastid fraction. The designation of these particles as proplastids rests upon the following observations: 1) Red fluorescence concentrated in spots at the limit of resolution and indicative of chlorophyll was seen in a portion of the non-fixed particles by fluorescence microscopy; 2) Electron transparent areas diagnostic for small starch grains are evident in some of the sectioned particles, and osmiophilic globules reminiscent of those found in plastids are common (*cf.* fig 4a); 3) Electron dense profiles of the iron rich micelles of phytoferritin, a macromolecule apparently limited to plastids (9), were seen in some of the particles (*cf.* fig 4b); and 4) Internal membranes are sparse, as is evident in figure 4. Glyoxysomes and mitochondria were seen as occasional contaminants of the proplastid fraction. The canaliculate systems commonly seen at the peripheries of the proplastids (fig 4) are similar to those seen less frequently in proplastids isolated from leaves of *Phaseolus vulgaris* and are considered to be an artefact of the preparation procedures, since they are lacking in sections of proplastids fixed *in situ*.

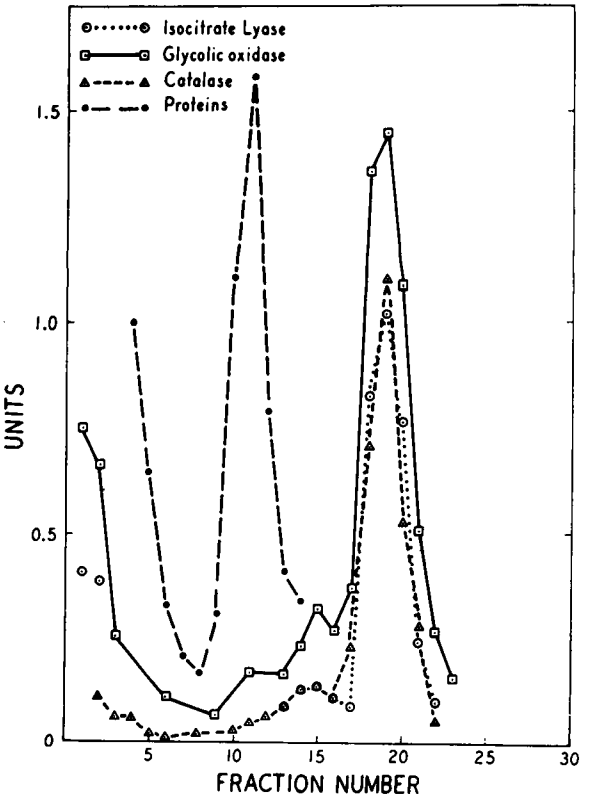

FIG. 2. Comparison of the distribution of glycolic oxidase and catalase with isocitrate lyase in 1 ml fractions from sucrose density gradients (System II). Units are: Glycolic oxidase, μmoles O_2 consumed per minute per ml; catalase, μmoles O_2 produced per minute per ml ($\times$ 0.001); isocitrate lyase and protein as in figure 1.

The degree of homogeneity of the glyoxysome fraction can be assessed from figure 5a. Clearly, a large proportion of the particles are morphologically identical. Contamination of the fraction by proplastids and mitochondria was exceedingly low. Figure 4b depicts a section of a rare proplastid found in the glyoxysome fraction. Figure 5b gives a more detailed image of sectioned glyoxysomes. These isolated organelles are bounded by a single unit membrane and often display small internal electron transparent spaces surrounded by a single, or rarely, a double membrane. These latter spaces could represent artefacts that arose during the preparation procedures. The stroma of the glyoxysomes has a finely granular appearance.

Particles resembling castor bean endosperm glyoxysomes by virtue of their similar enzyme composition and density have been separated also from particulate preparations from the cotyledons of germinating peanut and watermelon seedlings. The glyoxylate cycle is known to operate in these organs. No protein band of equivalent density was found when the same procedure was applied to corn roots which lack the glyoxylate cycle. However, catalase was localized in a broad band in a region of the gradient intermediate in density between that of mitochondria and glyoxysomes from castor bean preparations. Organelles clearly resembling the isolated glyoxysomes are recognizable in electron micrographs of sections of castor bean endosperm (Tanner and Beevers, unpublished) and squash cotyledon (Ashton, personal communication).

Discussion

On the basis of enzymes now known to be localized in the glyoxysome, the plausible functional scheme in figure 6 is suggested. The steps catalyzed by glyoxylate reductase and aconitase are indicated with dashed lines because, although these enzymes are present in the tissue (5, 10, 12), their association with glyoxysomes has not been definitely established. The above sequence places reactions causing the net formation of succinate from acetyl CoA in the glyoxysome. The location and positive identity of the reaction sequence carrying out the further conversion of succinate to carbohydrate is not completely known. The existing evidence (3) shows that the major link between dicarboxylic acid and more direct carbohydrate precursors is the conversion of oxalacetate to phosphopyruvate, mediated by phosphoenol pyruvate carboxykinase. For the moment we must conclude that the conversion of succinate to malate occurs in the mitochondria, since this is the only organelle containing succinic dehydrogenase and fumarase. However the role of non-particulate enzymes and also proplastids in the conversion of succinate to sucrose is currently being examined.

The relationship between glyoxysomes and certain particles isolated from other tissues and organisms requires comment. Muller and Hogg (14) reported recently that the enzymes isocitrate lyase and malate synthetase (but not citrate synthetase and malate dehydrogenase) were specifically associated with particles, denser than mitochondria, isolated from *Tetrahymena pyriformis*. The particles also contained catalase and α-hydroxy acid oxidase, which are distinctive enzymes of the peroxisomes isolated from liver and kidney and extensively studied by de Duve and his collaborators (1, 2, 6). Because of this and their very similar sedimentation behavior, the *Tetrahymena* particles were referred to as peroxisomes (14).

Clearly, the peroxisomes from mammalian tissues (which of course lack the enzymes of the glyoxylate cycle), the *Tetrahymena* particles (which have some of these enzymes) and the glyoxysomes from castor bean (which have an essentially complete cycle) have different metabolic functions. Nevertheless they have some common features. The 3 kinds of particles show nearly identical equilibrium densities on sucrose gradients, and all contain catalase and at least 1 flavin-linked oxidase. Peroxisomes from mammalian tissues were conclusively shown (1) to be identical with microbodies described by Rhodin (15) in electron micrographs of mouse kidney. Mollenhauer, Morre, and Kelley (13) reported that cytosomes, resembling microbodies, were widely distributed in cells of various plant species. Many of these are known to lack isocitrate lyase and malate synthetase. Bonner (4) recently reported that when a particulate fraction from mung bean hypocotyl was centrifuged on a sucrose gradient, the catalase activity was concentrated at a density greater than that of the mitochondria, and in preliminary experiments we have found this to be true also for preparations from corn and castor bean roots. None of these tissues have glyoxylate cycle activity.

It thus appears that particles containing catalase (and in this respect resembling the peroxisomes described by deDuve 7, 8) may be widely distributed in plant tissues. In the organs of fatty seedlings where fat is being converted to carbohydrate a biochemically distinct particle is present which houses the machinery of the glyoxylate cycle, and is therefore referred to as the glyoxysome.

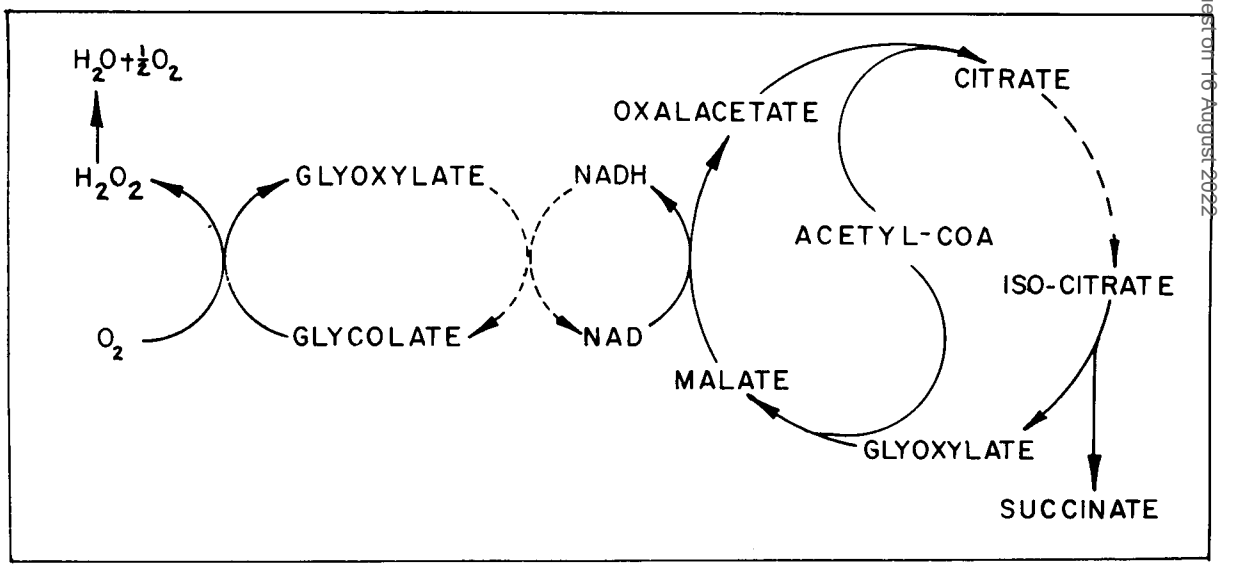

FIG. 6. Proposed reaction sequence in the glyoxysome.

The fact that this particle also contains catalase and glycolic oxidase places it in the same general category as the peroxisomes. A role for these 2 enzymes in NAD regeneration in the glyoxysomes is suggested (fig 6), but the possibility remains that their association with the glyoxysome is incidental to its major function. Further information is certainly required to establish the relationship between cytosomes from different sources and their functional roles in metabolism.

Acknowledgment

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Notes added in proof. (a) By providing SH protectants in the sucrose gradient we have now shown that aconitase is indeed associated both with mitochondria and glyoxysomes. (b) The unidentified cytoplasmic organelles in the EM pictures of fatty tissue of Yucca seedlings (Horner, H. T. and J. J. Arnott, 1966. Botan. Gaz. 127: 48–64 fig 46, and H. T. Horner, personal commun.) are almost certainly glyoxysomes.

Literature Cited

1. Baudhuin, P., H. Beaufay, and C. E. DeDuve. 1965. Combined biochemical and morphological study of the particulate fractions from rat liver. J. Cell. Biol. 26: 219–43.
2. Beaufay, H., P. Jacques, P. Baudhuin, O. Z. Sellinger, J. Berthet, and C. E. DeDuve. 1964. Tissue fractionation studies 18. Resolution of mitochondrial fractions from rat liver into three distinct populations of cytoplasmic particles by means of density equilibration in various gradients. Biochem. J. 92: 184–205.
3. Beevers, H. 1961. Metabolic production of sucrose from fat. Nature 191: 433–36.
4. Bonner, W. D. 1967. Peroxidase associated with higher plant mitochondria. Plant Physiol. 42: 366–70.
5. 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: 462–69.
6. DeDuve, C. E. and P. Baudhuin. 1966. Peroxisomes (Microbodies and related particles.) Physiol. Rev. 46: 323–57.
7. DeDuve, C., H. Beaufay, and P. Baudhuin. 1963. Intracellular localization of catalase in rat liver. Acta Chem. Scand. 17: S210–S215.
8. DeDuve, C., H. Beaufay, P. Jacques, Y. Rahman-Li, O. Z. Sellinger, R. Watiaux, and S. Deconinck. 1960. Intracellular localization of catalase and of some oxidases in rat liver. Biochim. Biophys. Acta 40: 186–87.
9. Hyde, B. B., A. J. Hodge, A. Kahn, and M. L. Birnstiel. 1963. Studies on phytoferritin. I. Identification and localization. J. Ultrastruct. Res. 9: 248–58.
10. Kornberg, H. L. and H. Beevers. 1957. The glyoxylate cycle as a stage in the conversion of fat to carbohydrate in castor beans. Biochim. Biophys. Acta 26: 531–37.
11. Maehly, A. C. and B. Chance. 1954. The assay of catalases and peroxidases. In: Methods of Biochemical Analysis. D. Glick, ed. Interscience Publishing Inc., New York. p 357–424.
12. Marcus, A. and J. Velasco. 1960. Enzymes of the glyoxylate cycle in germinating peanuts and castor beans. J. Biol. Chem. 235: 563–67.
13. Mollenhauer, H. H., D. J. Morré, and A. G. Kelley. 1966. The widespread occurrence of plant cytosomes resembling animal microbodies. Protoplasma LXII: 44–52.
14. Müller, M. and J. F. Hogg. 1967. Occurrence of protozoal isocitrate lyase and malate synthase in the peroxisome. Federation Proc. 26: 284 abst. #158.
15. Rhodin, J. 1954. Correlation of ultrastructural organization and function in normal and experimentally changed proximal convoluted tubule cells of the mouse kidney. Aktiebolaget Godvil. Stockholm.