CYTOCHEMICAL LOCALIZATION OF MALATE SYNTHASE IN GLYOXYSOMES

RICHARD N. TRELEASE, WAYNE M. BECKER, and JOHN J. BURKE

From the Departments of Botany-Microbiology and Zoology, Arizona State University, Tempe, Arizona 85281 and the Department of Botany, University of Wisconsin, Madison, Wisconsin 53706

ABSTRACT

Cytochemical staining techniques for microbodies (peroxisomes) are limited at present to the enzymes catalase and α-hydroxy acid oxidase, and neither technique can distinguish glyoxysomes from other microbodies. Described here is a procedure using ferricyanide for the cytochemical demonstration by light and electron microscopy of malate synthase activity in glyoxysomes of cotyledons from fat-storing cucumber and sunflower seedlings. Malate synthase, a key enzyme of the glyoxylate cycle, catalyzes the condensation of acetyl CoA with glyoxylate to form malate and release free coenzyme A. Localization of the enzyme activity is based on the reduction by free CoA of ferricyanide to ferrocyanide, and the visualization of the latter as an insoluble, electron-opaque deposit of copper ferrocyanide (Hatchett's brown). The conditions and optimal concentrations for the cytochemical reaction mixture were determined in preliminary studies using a colorimetric assay developed to measure disappearance of ferricyanide at 420 nm. Ultrastructural observation of treated tissue reveals electron-opaque material deposited uniformly throughout the matrix portion of the glyoxysomes, with little background deposition elsewhere in the cell. The reaction product is easily visualized in plastic sections by phase microscopy without poststaining. Although the method has been applied thus far only to cotyledons of fat-storing seedlings, it is anticipated that the technique will be useful in localizing and studying glyoxylate cycle activity in a variety of tissues from both plants and animals.

INTRODUCTION

Microbodies are a recently discovered class of subcellular organelles which are widely distributed in the tissues of plants and animals (9, 19, 20, 27). Ultrastructurally, microbodies are characterized by a coarsely granular or fibrillar matrix delimited by a single membrane. When isolated and characterized biochemically, microbodies from both plant and animal tissues invariably contain at least one α-hydroxy acid oxidase and catalase and are called peroxisomes (5).

In the endosperm or cotyledons of fatty seedlings, organelles meeting these minimal criteria are found to contain in addition the enzymes of the glyoxylate cycle (3, 4, 11, 13, 22, 25, 31, 36, 37) and have therefore been termed glyoxysomes (2, 3)., The glyoxylate cycle enzymes are required to effect the net conversion of acetate to succinate which is required for biosynthesis of higher carbon compounds from the two-carbon level, a metabolic competence not otherwise found in higher organisms.

Cytochemical techniques currently available in

the literature for the detection of microbodies *in situ* are limited to staining procedures localizing catalase (8, 29, 39, 40) and α-hydroxy acid oxidase (32). Although these procedures are definitive for the identification of microbodies in general, they are not useful in distinguishing between glyoxysomes and other peroxisomes, since the enzymes involved are common to both types of organelles.

It is clear, therefore, that a specific cytochemical test for an enzyme unique to the glyoxysome would be useful in the specific detection of glyoxysomes *in situ*. Either malate synthase or isocitrate lyase might be suitable for this purpose, since both are key enzymes of the glyoxylate cycle.

We describe here a cytochemical staining procedure for malate synthase based on sulfhydryl-dependent ferricyanide reduction (16) and formation of a copper ferrocyanide deposit (23) detectable with both the light and electron microscope. The reaction sequence is as follows:

$$\text{acetyl-S-CoA} + \text{glyoxylate} \xleftarrow[\text{malate synthase}]{Mg^{++}} \text{L-malate} + \text{CoA-SH}$$

$$2\ \text{CoA-SH} + 2\ \text{ferricyanide} \leftrightarrow 2\ \text{ferrocyanide} + \text{CoA-S-S-CoA} + 2H^{+}$$

$$\text{ferrocyanide} + \text{cupric ion} \rightarrow \text{cupric ferrocyanide (Hatchett's brown)}$$

The development of this cytochemical procedure involved two sequential phases. Initially, a spectrophotometric assay based on ferricyanide reduction was used to devise an optimal incubation mixture under conditions simulating the eventual cytochemical reaction. Tissue blocks were then incubated in this reaction medium, embedded in plastic, and examined with both the light and electron microscope in order to modify the incubation conditions to meet the actual demands of the cytochemical process. Cotyledons from 3½- 4-day old cucumber seedlings were used in the test system because it was known that malate synthase activity is localized within glyoxysomes in this tissue and is maximal at this stage of postgerminative development (36). Using this procedure, an electron-opaque deposit attributable specifically to malate synthase activity was obtained reproducibly within the glyoxysomes of fat-storing cotyledons. A preliminary report of these findings has already appeared (35).

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus* L, cv. "improved long green") and sunflower seeds (*Helianthus annus*. L., cv. "Russian sunflower") were planted in moistened vermiculite overlying soil and grown under a 12-12-h light-dark cycle at 28°C. Fluorescent and incandescent lighting provided an intensity of about 800 foot candles. Cotyledons were harvested 3½-4 days after planting for use in enzyme assays and electron cytochemistry.

Enzyme Preparation

Homogenates were prepared as described previously (36) by dicing cotyledons thoroughly in 1 vol of grinding medium (0.5 M sucrose in 0.05 M sodium cacodylate, pH 7.1) using razor blades attached to a commercial electric knife handle. The homogenate was then strained through three layers of buffer-moistened Miracloth (Johnson & Johnson Filter Products Div., Chicago, Ill.) and the dicing procedure was repeated with the tissue residue. The pooled homogenate was differentially centrifuged three times, first at 500 *g* for 10 min to remove starch, nuclei, and cellular debris, then at 1,000 *g* for 15 min to remove most of the developing chloroplasts, and finally at 11,000 *g* for 30 min to sediment the glyoxysomes and mitochondria. Pellets were resuspended in grinding medium for assay purposes. Homogenates and subsequent fractions were assayed for malate synthase activity by the method of Dixon and Kornberg (6) and for protein by the modified Waddell method described by Murphy and Kies (28). The resuspended 11,000 *g* pellet was routinely used as the enzyme source for optimizing reaction conditions in the spectrophotometric assay described below.

Spectrophotometric (Ferricyanide Reduction) Assay for Malate Synthase

The need for a spectrophotometric assay which could simulate the cytochemical reaction conditions was not met by existing procedures (6, 18), due to interference by the ferricyanide and ferrocyanide present or generated in the cytochemical reaction mixture. Instead, an assay based on the decrease in absorbance of ferricyanide at 420 nm was used. The reaction mixture contained 60 μmol potassium phosphate (pH 7.6), 8 μmol magnesium chloride, 0.5 μmol potassium ferricyanide, 0.2 μmol acetyl CoA, 0.4 μmol sodium glyoxylate, and enzyme in a total volume of 1.0 ml. The reaction was initiated by the addition of the sodium glyoxylate and the decrease in absorbance at 420 nm was monitored using a Coleman model 124 spectrophotometer (Coleman Instruments Div., Perkin Elmer Corp., Maywood, Ill.). Sodium glyoxylate and CoA were purchased from the Sigma Chemical Co., St. Louis, Mo., and acetyl CoA was prepared by the method of Stadtman (34). Tris-HCl (pH 7.8) can be substituted for potassium phosphate without deleterious effect in this assay (but not in the cytochemical reaction).

Cytochemical Assay for Malate Synthase: Fixation of Tissues

Cotyledons from 3½–4-day old seedlings were segmented into 1-mm squares in fixing solution with the use of a double-edged razor blade. The fixation solution found most reliable was 4% formaldehyde (prepared fresh from paraformaldehyde) plus 1% glutaraldehyde in 0.05 M sodium cacodylate, pH 7.1. Fixation was at 4°C for 5, 10, 15, or 30 min (the time being varied among experiments). After fixation, cotyledon segments were rinsed for 20-30 min at room temperature in at least three changes of 20 mM potassium phosphate, pH 6.9.

INCUBATION: Following aldehyde fixation and rinsing, the cotyledon segments were preincubated at room temperature with 3.0 mM potassium ferricyanide (in 20 mM potassium phosphate, pH 6.9) for 30 min to remove endogenous oxidizable material (16, 17) and were then rinsed in the same buffer at least three times for a total 20–30 min. The tissue blocks were then incubated for 40 min at 37°C in corked glass tubes. The tubes were agitated in the incubator using an Ames Lab-Tek aliquot mixer (Ames Co., Div. of Miles Lab., Inc., Elkhart, Ind.) to ensure adequate exchange of reactants between the medium and tissues.

The incubation medium for the demonstration of malate synthase reactivity was patterned after that of Higgins and Barrnett (16), using the optimal conditions established with the spectrophotometric (ferricyanide reduction) assay. The cytochemical medium was prepared immediately prior to use by adding the following components in the indicated order and with stirring between each addition (μmoles are given in parentheses): 0.3 ml 65 mM potassium phosphate, pH 6.9 (19.5); 0.2 ml of a solution containing 50 mM copper sulfate and 500 mM sodium potassium tartrate, pH 6.9 (10 μmol copper sulfate, 100 μmol tartrate); 0.25 ml double-distilled water; 0.03 ml 50 mM potassium ferricyanide (1.5); 0.1 ml 50 mM magnesium chloride (5.0); 0.02 ml 150 mM sodium glyoxylate (3.0); and 0.1 ml 10 mM acetyl CoA (1.0). When dimethyl sulfoxide (DMSO) was used, 0.05 ml was added after ferricyanide and before magnesium chloride, and the amount of water was reduced to 0.20 ml. As a control, tissue segments were incubated in media from which either the glyoxylate or acetyl CoA had been omitted. After the incubations, tissues were rinsed for 20–30 min with several changes of 50 mM sodium cacodylate, pH 7.1.

POSTFIXATION AND PREPARATION OF TISSUES FOR VIEWING. Cotyledon segments were postfixed in 2% OsO_4 in 0.05 M sodium cacodylate, pH 7.1, for 1 h at room temperature. They were then dehydrated in an acetone series and embedded in either a mixture of low viscosity epoxy resins (33) or Araldite-Epon. The tissues were flat embedded in aluminum pans so that they could be specifically oriented on plexiglass rods (cemented with Eastman 910 adhesive; Eastman Chemical Products, Inc., Kingsport, Tenn.). The orientation was such that when sectioned, the cotyledons were cut in a peridermal plane through palisade cells. This allowed observation of the depth of penetration of the reactants into the tissues on four sides. Silver-gray sections were cut on a Sorvall MT-1 ultramicrotome (Ivan Sorvall, Inc., Newtown, Conn.), poststained in 2% aqueous uranyl acetate for 10 min followed by lead citrate for 5 min and observed with a Philips EM-300 electron microscope. For light microscopy, sections about 0.5 μm thick were used and reaction product was clearly visible without poststaining.

RESULTS

Spectrophotometric Studies

The basis for the spectrophotometric assay used in these studies is the sulfhydryl-dependent reduction of ferricyanide. That ferricyanide reduction is proportional to sulfhydryl concentration under our assay conditions and can therefore be used as a reliable assay for liberation of free coenzyme A is illustrated by the data shown in Fig. 1; the slope of the line (1.03) represents the micromolar absorbancy index of ferricyanide.

Crucial to a cytochemical reaction is the pH at which it is performed, since pH affects not only

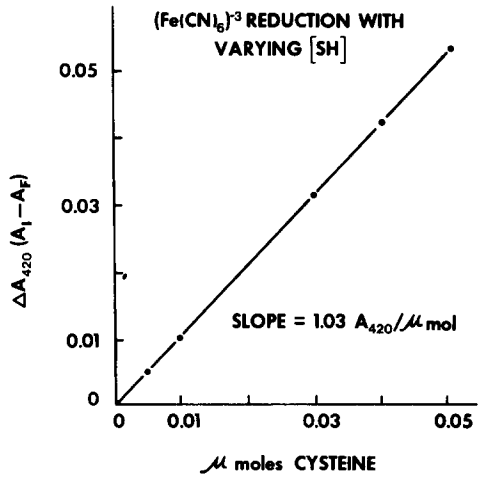


FIGURE 1 Dependence of ferricyanide reduction on sulfhydryl (cysteine) concentration. The change in absorbance at 420 nm (ΔA_{420}) was determined by subtracting the final absorbance of ferricyanide plus sulfhydryl (A_F) from the initial ferricyanide absorbance (A_I) in the absence of sulfhydryl.

enzyme activity, but also interactions of components in the reaction mixture and precipitation of the final electron-dense product. Fig. 2A shows the activity to be highest at pH 8.3, but the instability of the cytochemical reaction mixture at this pH dictated the use of a lower pH. The reaction was positive in tissues incubated at pH 7.6, but problems were often encountered due to intracellular crystal formation. The most reproducible results were obtained at pH 6.9; this pH was therefore adopted for routine use.

The dependence of reaction rate on concentrations of ferricyanide, glyoxylate, and acetyl CoA is shown in Fig. 2B-D. Ferricyanide concentration was limiting up to about 75 nmol/ml and both glyoxylate and acetyl CoA concentrations were limiting up to approximately 200 nmol/ml. Cytochemical reactions were performed with various concentrations of these compounds in the ranges obviously not limiting the spectrophotometric assays. This work resulted in adopting 1,500 nmol/ml ferricyanide, 1,000 nmol/ml acetyl CoA, and 3,000 nmol/ml glyoxylate for the routine incubation medium. Glyoxylate became inhibitory in the spectrophotometric assay (though not, apparently, in the cytochemical reaction) at concentrations above 4,000 nmol/ml; comparable information is not available for acetyl CoA.

A final criterion used to assess the feasibility of the ferricyanide assay was the dependence of reac-

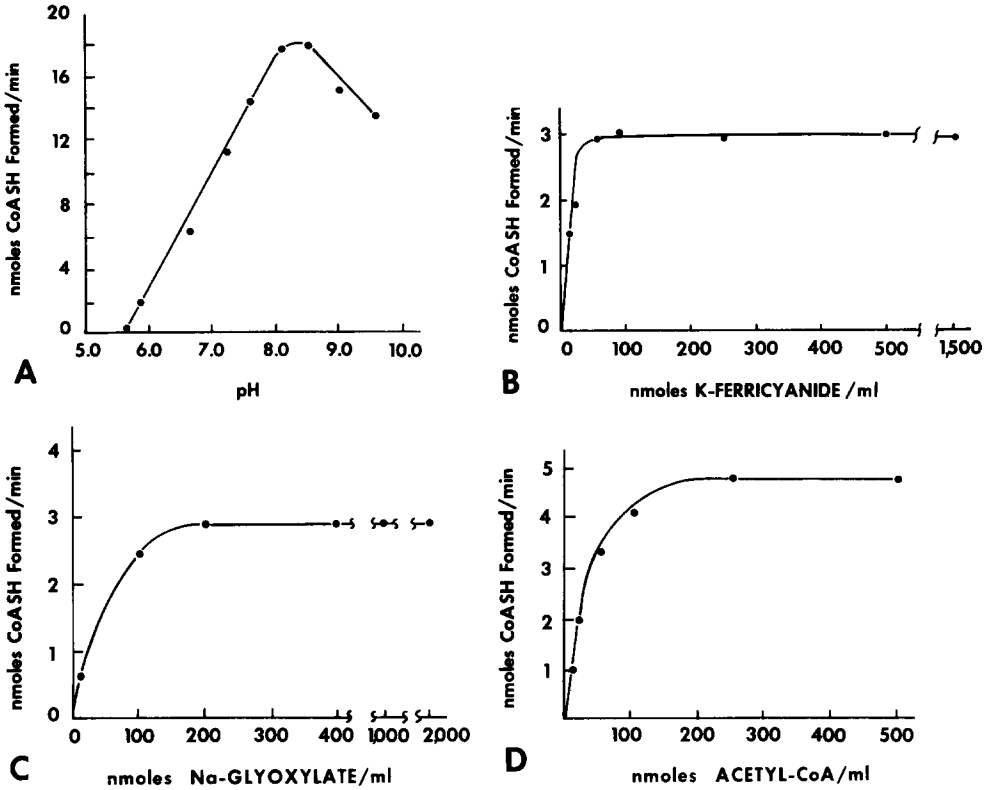


FIGURE 2. Change in malate synthase activity with varying pH (A), and substrate concentrations (B–D). All reaction mixtures in B-D contained 60 mM potassium phosphate, pH 7.6, and 8 mM $MgCl_2$ and were assayed by monitoring ferricyanide reduction. A. pH activity curve determined with 50 μg protein using the 5,5′dithiobis (2-nitrobenzoic acid) assay in 60 mM Tris-HCl and 60 mM potassium phosphate. B. Varying ferricyanide concentration; the 1-ml reaction mixture contained 200 nmol sodium glyoxylate, 250 nmol acetyl-CoA, and 13 μg protein. The high concentration of ferricyanide required assaying at 455 nm instead of 420 nm (ϵ_{455} = 150 M^{-1} cm^{-1}, reference 38). C. Varying glyoxylate concentration; the 1-ml reaction mixture contained 100 nmol potassium ferricyanide, 250 nmol acetyl-CoA, and 13 μg protein. D. Varying acetyl-CoA concentrations; the 1-ml reaction mixture contained 100 nmol potassium ferricyanide, 200 nmol sodium glyoxylate, and 20 μg protein.

tion rate on enzyme concentration. Shown in Fig. 3 are the results obtained by varying the enzyme concentration over a 10-fold range. Note that the reaction proceeds linearly with time for at least 3 min (for 6 min in most cases) and that the amount of product formed is directly proportional to enzyme concentration.

Cytochemical Studies

Incubation of formaldehyde/glutaraldehyde-fixed cucumber cotyledons in medium containing acetyl CoA, glyoxylate, magnesium chloride, ferricyanide, and copper tartrate chelate at the concentrations indicated by the preliminary spectrophotometric assays resulted in pronounced deposition of light- and electron-opaque material within structures clearly recognizable as glyoxysomes. In Fig. 4A and B, 0.5-μm sections of cotyledons are shown as they appear in the phase microscope. Fig. 4A represents a control tissue block (without glyoxylate) and Fig. 4B an experimental block (complete medium). In Fig. 4A, the glyoxysomes are not stained, whereas in Fig. 4B, they are clearly visible as discrete dark bodies distributed throughout the cytoplasm. The copper ferrocyanide reaction product imparts only a pale brown color to glyoxysomes when viewed with bright-field illumination, but gives remarkable clarity and resolution under phase optics.

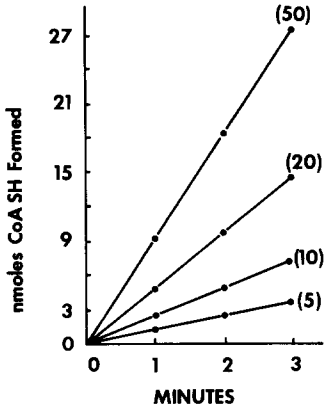


FIGURE 3 Malate synthase activities measured by ferricyanide reduction at 420 nm with varying enzyme concentration (numbers in parentheses indicate micrograms of protein). The 1-ml reaction mixture contained 100 nmol potassium ferricyanide, 400 nmol sodium glyoxylate, 250 nmol acetyl-CoA, 8 μmol $MgCl_2$, and 60 μmol potassium phosphate, pH 7.6. Using 20 μg protein, the same velocity shown in this figure was obtained with 25, 50, 75, and 100 nmol tartrate in the reaction mixture.

In most experiments, glyoxysomes were stained in the first three to five cells into the tissue block on all sides. Based on an average diameter of 14 μm per palisade cell cut in the peridermal plane, reactivity could be observed to a depth of approximately 40–70 μm into the tissue blocks after incubation for 40 min. Increasing the incubation time to 60 min did not appear to increase penetration of the reactants significantly.

In Fig. 5, typical glyoxysomes are shown as they appear in formaldehyde/glutaraldehyde-fixed tissue, without incubation in any reaction mixture. The glyoxysomes are seen as discrete, round to oval bodies bounded by a single membrane and containing a finely granular matrix of moderate electron opacity. Glyoxysomes at this stage of seedling development characteristically exhibit invaginations of ribosome-containing cytoplasm into the particle, as has been noted previously (35).

When such tissue is incubated in the complete cytochemical reaction medium, the glyoxysomes appear heavily and specifically stained by the deposition of copper ferrocyanide as shown in Fig. 6. The reaction product is always distributed uniformly throughout the entire matrix and does not appear to be specifically associated with the boundary membrane.

Controls designed to delineate the specificity of copper ferrocyanide deposition included omission of the substrates (glyoxylate or acetyl CoA) or the cofactor, magnesium (7). Fig. 7 illustrates the results obtained when glyoxylate was omitted from the reaction mixture. Small background deposits are present in the cells, but substrate omission completely eliminates specific glyoxysomal staining. Comparison with Fig. 5 demonstrates that there is also no detectable background matrix staining by the ferricyanide reduction reaction. The same observations were made when acetyl CoA was eliminated. Omission of magnesium chloride from the reaction mixture did not provide an adequate control, however, since in its absence weaker but nonetheless significant reactivity was observed within glyoxysomes even though no activity could be measured in the spectrophotometric reaction. Presumably, intracellular magnesium concentrations in the fixed tissue are adequate to support enzyme activity.

Remarkably good ultrastructure is obtained with tissues fixed for only 5 min prior to incuba-

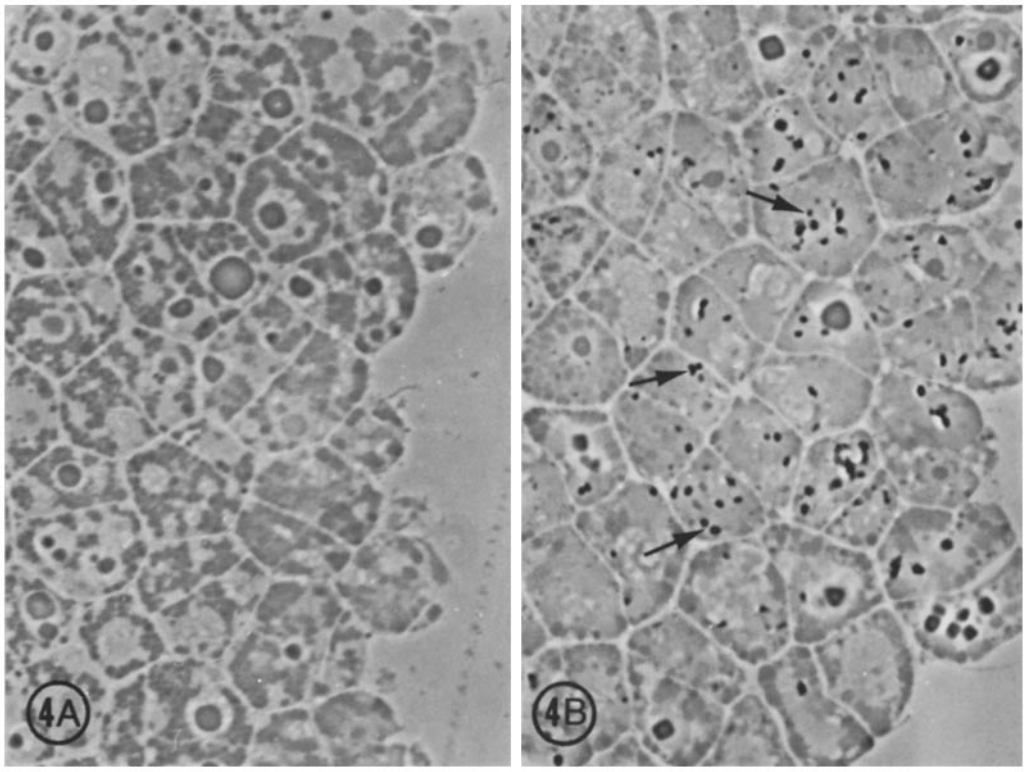


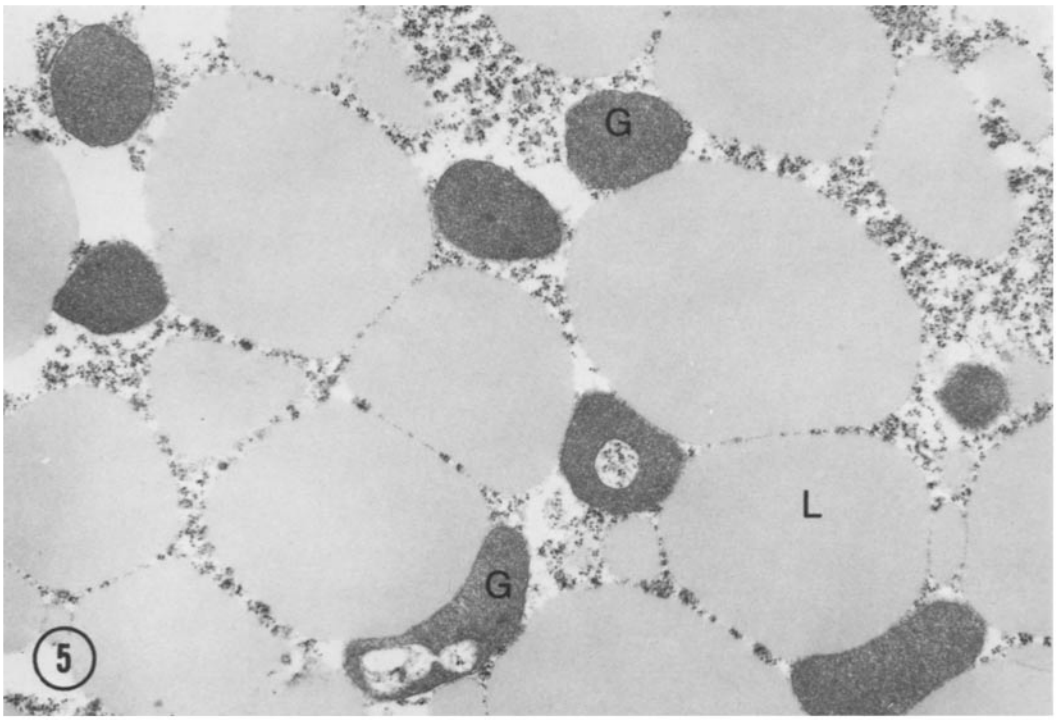
FIGURE 4 Light micrographs (phase microscopy) of peridermally sectioned cucumber cotyledons incubated in ferricyanide media for malate synthase activity. A. Control, glyoxylate omitted from the media. B. Complete medium; glyoxysomes (arrows) clearly have a reaction product not visible in the control (A). Note that the reactivity is visible five to six cells into the tissue block. Both × 2,000.

tion in the cytochemical reaction mixture. In Fig. 8, the organelles are shown to be well preserved as compared with cytoplasmic components in tissues fixed for 30 min (cf. Fig. 6). The enzyme reactivity is also of similar intensity to that shown in tissues fixed for longer times.

The sharp localization of reaction product within glyoxysomes is better illustrated at higher magnification. Fig. 9 shows a glyoxysome in section view containing an invaginated pocket of ribosomes. The copper ferrocyanide is restricted exclusively to the matrix and has not diffused beyond the boundaries of the limiting membrane or into the cytoplasmic inclusion. The rather amorphous nature of the reaction product is also obvious at this magnification.

The unambiguous restriction of copper ferrocyanide to glyoxysomes is further illustrated by sections that have not been contrasted with uranyl acetate and lead citrate (Fig. 10). Omission of poststaining emphasizes differences in electron opacity between structures with and without accumulated reaction product, since the density of the copper ferrocyanide is inherent in the compound and is not dependent upon additional heavy metals. In Fig. 10, only the glyoxysome possesses substantial electron opacity; the cytoplasmic matrix and the mitochondrion are much more electron transparent.

Cotyledons of sunflower seedlings were also tested for malate synthase reactivity (Fig. 11). The reaction product in these cells was again restricted primarily to the matrix of the glyoxysomes, with small background deposits occurring in other cytoplasmic components. The sole difference between the staining of sunflower and cucumber cotyledon segments was the poor penetration of reactants experienced with sunflowers. The average diameter of the cells is about two to three times that of the cucumber cells, however, which



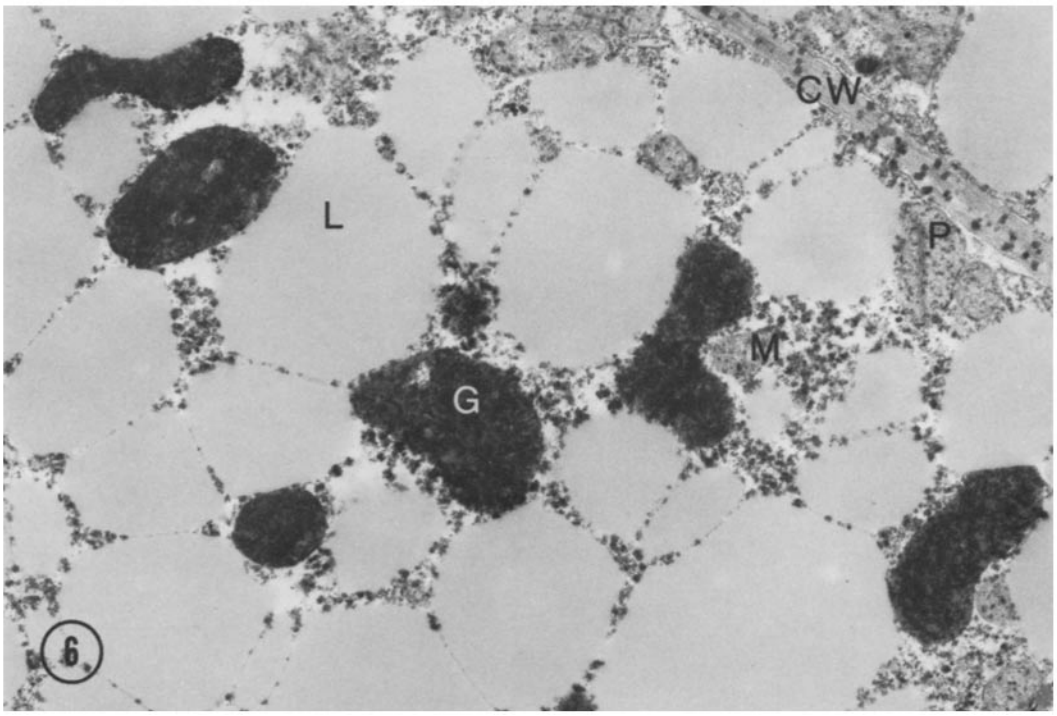
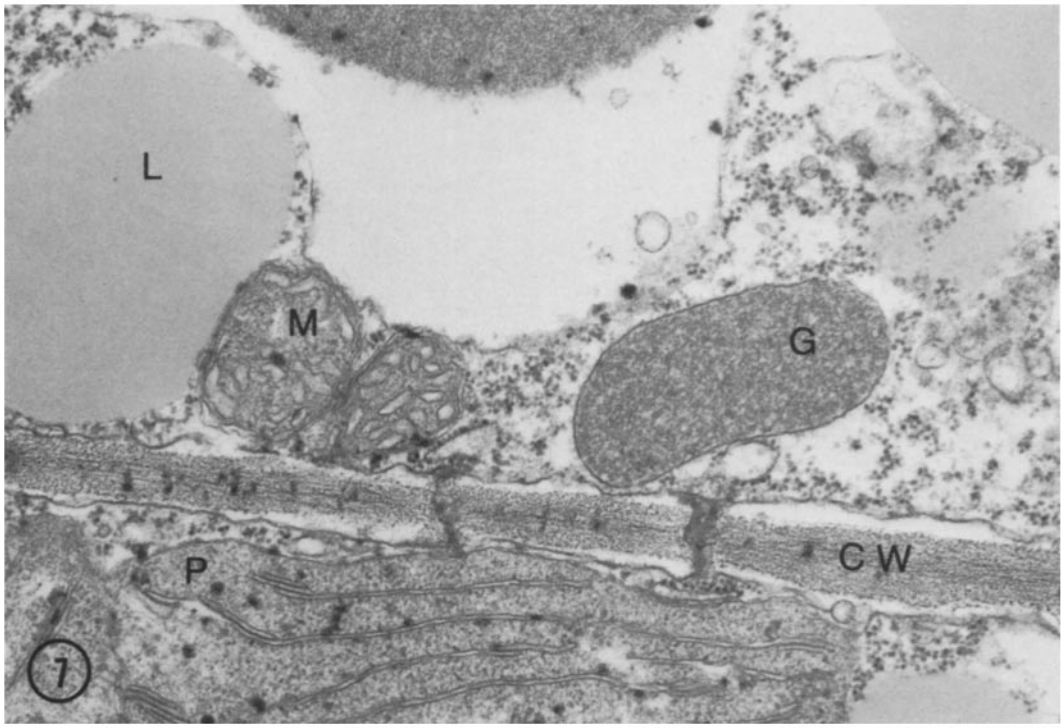


FIGURE 5 Portion of a cucumber cotyledon showing the appearance of glyoxysomes (*G*) when tissue is fixed for 10 min and poststained in the usual manner without incubation in the ferricyanide medium. Compare their matrix of moderate opacity with that of stained glyoxysomes in Fig. 6. Some glyoxysomes exhibit cytoplasmic invaginations and are generally distributed among storage lipid bodies (*L*). × 20,000.

FIGURE 6 Portion of a cucumber cotyledon cell that has been fixed for 30 min and incubated for 40 min in a complete ferricyanide medium (pH 6.9). Dense reaction product, attributable to malate synthase activity, is uniformly distributed in the matrix of glyoxysomes (*G*). Some nonspecific deposits can be seen in the cell wall (*CW*), plastids (*P*), and mitochondria (*M*), but not in lipid bodies (*L*). × 15,000.



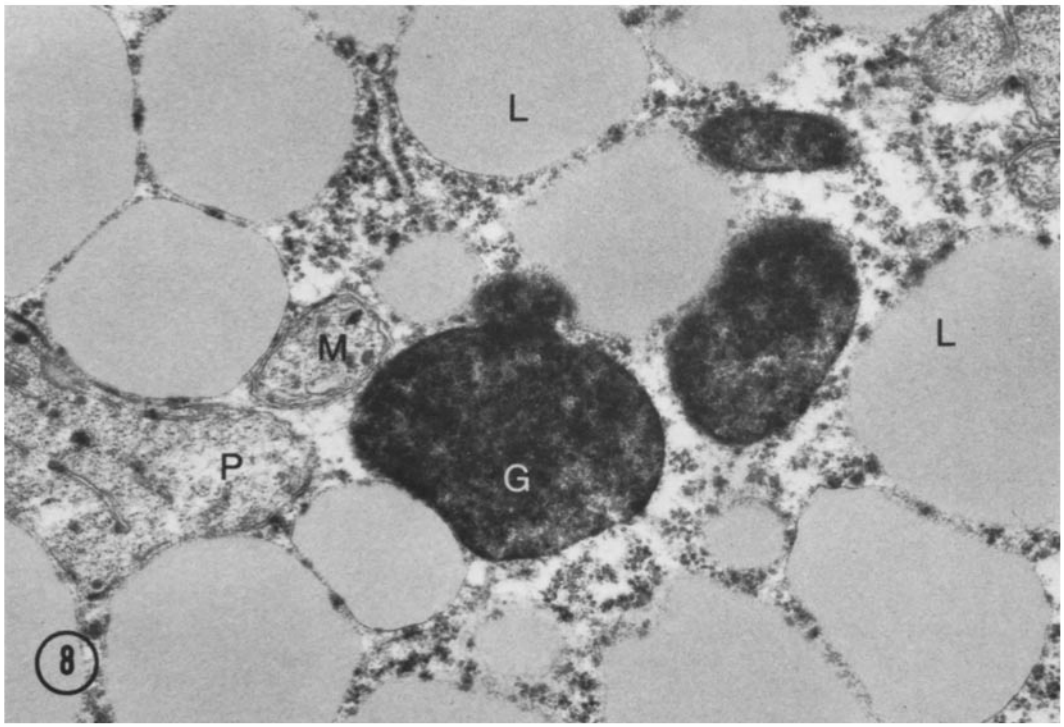
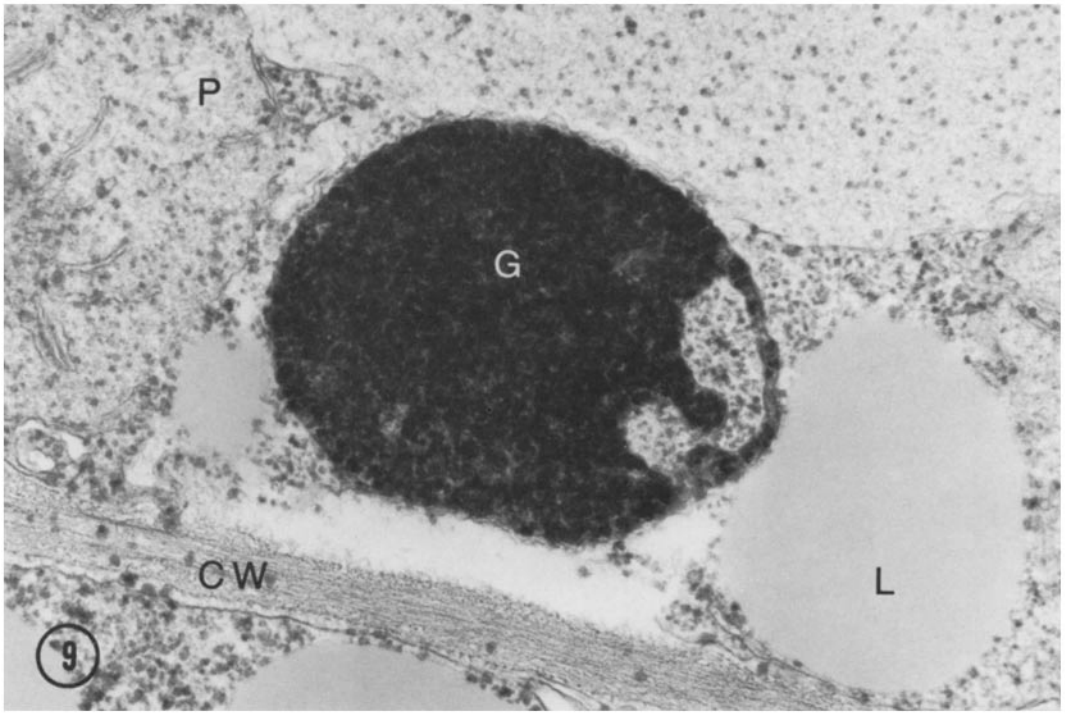
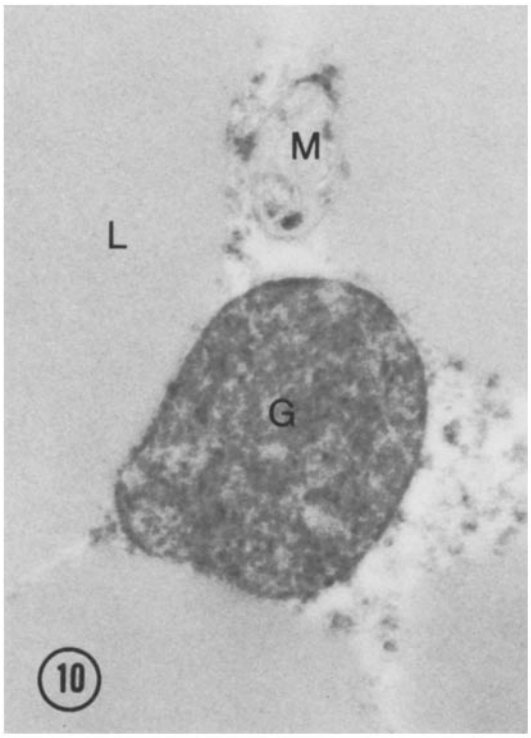


FIGURE 7 Appearance of a glyoxysome (*G*) and other organelles of a cucumber cotyledon when glyoxylate is omitted from the ferricyanide reaction mixture. Fixation and incubation as in Fig. 6, but without glyoxylate. The general appearance of the glyoxysome matrix closely resembles those in cells not exposed to copper and ferricyanide (Fig. 5). Mitochondria (*M*), cell wall (*CW*), plastids (*P*), and lipid bodies (*L*) show the same background deposits as observed in cells exposed to complete medium (Fig. 6). × 40,000.

FIGURE 8 Portion of a cucumber cotyledon fixed for 5 min and incubated in a complete ferricyanide reaction mixture (pH 6.9). Dense reaction product, attributable to malate synthase activity, is distributed in the matrix of glyoxysomes (*G*). Note the remarkably good fixation of the ground plasm and plastids (*P*), mitochondria (*M*), and lipid bodies (*L*). × 40,000.





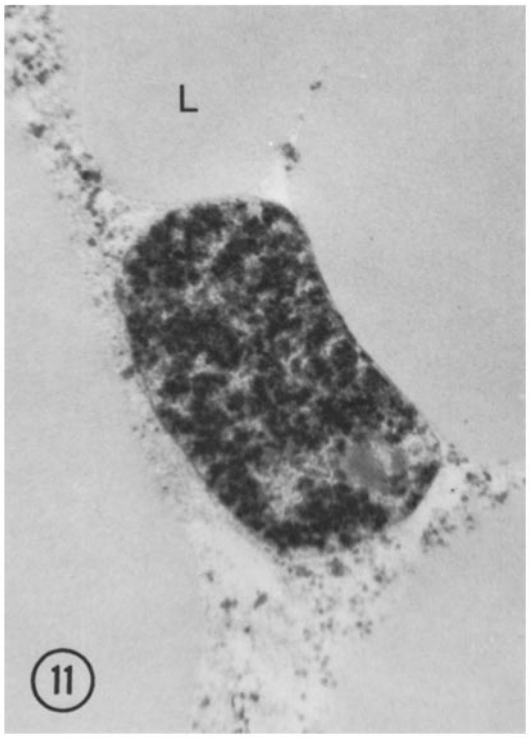


FIGURE 9 Appearance of a glyoxysome (*G*) in a cell of a cucumber cotyledon fixed for 15 min and incubated in a complete ferricyanide reaction mixture, pH 7.6. The pocket containing ribosomes is evident within the glyoxysome; note the sharp restriction of reaction product to the glyoxysome matrix, not into the pocket. Plastids (*P*), lipid bodies (*L*), and cell wall (*CW*) are nearby. × 48,500.

FIGURE 10 Portion of a cucumber cotyledon incubated in complete ferricyanide medium as in Fig. 6, but section is not poststained in uranyl acetate and lead citrate. The nearby mitochondria (*M*) and ground plasm are quite electron transparent compared to the glyoxysome (*G*) matrix. *L*, lipid droplet. × 45,000.

FIGURE 11 Appearance of a glyoxysome in a cell of a sunflower cotyledon fixed 30 min and incubated in a complete ferricyanide reaction mixture, pH 6.9. The boundary membrane is clearly visible around the matrix, which contains reaction product. *L*, lipid droplet. × 45,000.

may account at least partially for the difference in staining.

DISCUSSION

The development of this cytochemical procedure was greatly facilitated by preliminary spectrophotometric studies conducted to delineate optional conditions of pH and reactant concentration for the eventual cytochemical reaction medium. Using an assay based on ferricyanide reduction, we were able to vary conditions systematically to maximize the reaction rate and ensure stability of the system before beginning actual cytochemical studies. When the conditions derived in this way were then applied to fixed tissue segments, only a few modifications in the incubation medium were required before reproducible electron-opaque deposits of copper ferrocyanide were localized specifically within the glyoxysomes.

One of the most critical components of the reaction mixture is the buffer, since the choice, pH, and concentration greatly affect the quality and specificity of deposition product. A variety of buffers appropriate to the pH range 7.0–8.0 were tried, but only potassium phosphate gave satisfactory results. Lukaszyk (26) had previously noted phosphate is needed to ensure deposition of copper ferrocyanide apparently by preventing a rise in pH which can lead to its solubility (14); a requirement for phosphate is now confirmed by our own work.

In some of the initial cytochemical experiments, however, nonenzymatic crystal formation was noted in all cytoplasmic components, apparently due to precipitation of magnesium phosphate. This problem was especially acute at high pH (7.6) or high concentrations of phosphate (60 mM) and/or magnesium (10 mM) and was eliminated by reducing the phosphate concentration to 20 mM, the magnesium concentration to 5 mM, and the pH to 6.9 (which corresponds to about 50% peak activity on the pH curve, see Fig. 2A).

The concentrations of copper and tartrate were not varied in the cytochemical studies, but graded levels of tartrate up to 100 mM concentration used in the cytochemical assay did not inhibit the ferricyanide spectrophotometric assays. Copper frequently inhibits enzymes whose action involves sulfhydryl groups. The possible effects of copper on the reaction rate could not be tested with the spectrophotometric assay, since at all copper concentrations tried, precipitation was observed upon addition of the enzyme, even though the copper was precomplexed with tartrate. The experiments with fixed tissue, however, revealed definitive reaction products only in the presence of all substrates and the required cofactor, thus demonstrating copper does not completely inhibit malate synthase reactivity. Furthermore, we have recently demonstrated malate synthase activity in polyacrylamide gels using a slight modification of this copper-containing ferricyanide reaction mixture (41).

DMSO is often added to cytochemical incubation media to accelerate penetration or increase the precision of localization (32) and has also been reported to facilitate electron transfer to artificial acceptors such as ferricyanide (15). In the tests with cotyledons, however, DMSO had no effect on malate synthase reactivity, penetration, or precision of localization. Amplification of the original Hatchett's brown deposition with 3,3'diaminobenzidine (DAB) as described by Hanker and co-workers (14, 15) also was not apparent with our procedure for malate synthase localization.

Although the reaction product obtained with our procedure is largely confined to the matrix of the glyoxysomes, some electron-opaque material always occurs in other parts of the cytoplasm. Background deposits attributable to acyl CoA hydrolase (deacylase) activities have been reported in cytochemical procedures which depend upon the liberation of free coenzyme A (16, 17). Some of the background seen in the present work may be attributable to hydrolase activity or possibly to endogenous substrates. However, we are not aware of a specific malate synthase inhibitor which could be used to distinguish between these two possibilities. Moreover, the possibility exists that the background deposits encountered here are primarily a consequence of using copper as the precipitant for ferrocyanide, since a similar background has been seen in other cytochemical studies which depend on the presence of cupric ions, whereas in instances in which uranyl or manganous ions have been used as capture metals (16, 17), little or no background is obvious. Nevertheless, deposition in the glyoxysomes was clearly dependent on glyoxylate, since controls lacking only this substrate showed no electron opacity within glyoxysomes (Fig. 7).

Localization of Malate Synthase Within the Glyoxysome

The enzymatic deposition of reaction product within glyoxysomes occurs uniformly throughout the matrix of the organelle, indicating strongly that the enzyme occurs within the matrix and is not restricted to the membrane. This finding was not entirely expected, however, in view of what had been deduced previously from sedimentation studies with isolated glyoxysomes. It has usually been assumed that matrix enzymes will be readily released into the soluble phase upon organelle breakage, while membrane-bound enzymes will be retained with the disrupted organelle. Isocitrate lyase, for example, is readily released to the soluble phase when glyoxysomes are broken and is therefore assumed to be located in the matrix. By the same token, the results shown in Figs. 6, 8, 9, and 11 suggest that malate synthase should also be solubilized when glyoxysomes are broken. However, Gerhardt and Beevers, studying glyoxysomal enzymes from castor bean endosperm (12), found that although most of the malate synthase activity banded in sucrose gradients with other glyoxysomal enzymes when the sample was obtained from seedlings more than 4 days of age, at earlier stages part of the activity appeared at regions of lower density. This they interpreted to be due to broken glyoxysomes which retained malate synthase activity as a membrane-bound component. Similar results were obtained in our own work with developing cucumber cotyledons (unpublished observations). Furthermore, a recent preliminary report by Huang and Beevers (21) indicates that greater than 90% of the malate synthase is associated with a pelletable fraction containing most of the glyoxysomal membrane label ([^{14}C]choline) when glyoxysomes isolated in 54% sucrose are diluted twofold with water.

A possible explanation to rationalize these conflicting findings is that malate synthase is in fact associated with the boundary membrane and that the reaction product seen within the glyoxysomal matrix after cytochemical staining is simply a consequence of diffusion away from the actual enzyme site. However, this interpretation seems unlikely for several reasons. First, reaction product is always sharply restricted within the limiting membrane, yet if one is to invoke diffusion of product away from the membrane to the center of the glyoxysome, one might also expect product to be visible outside the membrane, unless, of course, substrates and trapping agents can enter the glyoxysome but the cupric ferrocyanide cannot diffuse back out. Second, if the product were diffusing into the matrix, then gradients of reactivity should be seen in at least some ultrastructural section views, particularly in cells nearer the center of the tissue block where penetration of reactants becomes limiting. Repeated observations failed to confirm this expectation, however, because when the matrix is not entirely electron opaque from the reaction product, deposits are uniformly scattered throughout the matrix. This is illustrated well in Fig. 11, which also shows the boundary membrane to be clearly visible without reaction product deposits.

Thus the explanation which appears to be most consistent with all data is that malate synthase is bound to or comprises a substantial portion of the matrix material but that this matrix, whatever its nature, remains attached to the limiting membrane when the organelle is broken. It should be noted that catalase has also been demonstrated cytochemically to be associated with the matrix of glyoxysomes (40), leaf peroxisomes (8), and animal peroxisomes (29) while α-hydroxy acid oxidase reaction product was deposited throughout the organelle in heavily stained preparations, but was restricted to the outer margin of the matrix and to the nucleoid in lightly stained specimens (32).

Potential Uses of Malate Synthase Cytochemistry

The availability of a cytochemical assay for a glyoxysome-specific enzyme is likely to prove of considerable value in studies on the origin and fate of glyoxysomes in fat-storing seedlings. Of particular interest are species such as sunflower or the cucurbits, since fat reserves in these seeds are stored in cotyledons, which, after depletion of lipid reserves expand, become photosynthetically competent and are found to contain microbodies with an enzyme complement characteristic of leaf peroxisomes (36). The resulting concomitant decrease in glyoxysomal enzyme activities and increase in peroxisomal enzyme activities could be due to a replacement of one population of microbodies by another or to a change in the enzyme complement of an ongoing population of organelles. Studies designed to distinguish between these and other possibilities remain inconclusive

(11, 22, 31, 36); malate synthase cytochemistry may well provide a definitive answer.

This cytochemical technique may also be especially useful in studying the possible existence of glyoxysomes in animal tissues for which glyoxylate cycle enzymes or activities have been reported (1, 10, 24, 30) but no intracellular localization has yet been established, probably due to difficulties in isolating organelles from these sources. The cytochemical demonstration of malate synthase, therefore, may open new avenues of research in gluconeogenic metabolism and perhaps extend knowledge of the occurrence of glyoxysomes to animal tissues. Studies of this nature are presently underway in our laboratories.

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