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Cytochemical Demonstration of Malate Synthase and Glycolate Oxidase in Microbodies of Cucumber Cotyledons[1]

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ABSTRACT

The cytochemical localizations of malate synthase (glyoxysomal marker) and glycolate oxidase (peroxisomal marker) have been examined in cotyledon segments and sucrose-gradient fractions from germinated cucumber (*Cucumis sativus* L.) seedlings. The seedlings were grown in the dark for 4 days, transferred to 4 hours of continuous light, then returned to the dark for 24 hours. Under these conditions, high specific activities for both glyoxysomal and peroxisomal enzymes are maintained in cotyledon homogenates and microbody-enriched fractions. Electron cytochemistry of the marker enzymes reveals that all or virtually all the microbodies observed in cotyledonary cells and sucrose-gradient fractions contain both enzymes. The staining in gradient fractions was determined from scoring a minimum of 600 photographed microbodies for each enzyme. After correcting for the number of particles stained for catalase reactivity (representing true microbodies), 94 and 97% of the microbodies were found stained for malate synthase and glycolate oxidase activity, respectively.

The results from these studies provide pertinent information toward understanding the succession from glyoxysomal to peroxisomal metabolism in cotyledons of fatty seedlings. The coexistence of two separate microbody types functioning at different stages of development apparently is not the case. The localizations of both marker enzymes within one microbody type strongly suggest that the metabolic transition involves a change in enzyme complement within an ongoing population of microbodies.

Microbodies are a class of morphologically similar subcellular organelles commonly found in plant and animal tissues (11, 26). They are bounded by a single delimiting membrane and contain a coarsely granular or fibrillar matrix. On a metabolic basis, plant microbodies have been divided into peroxisomes and glyoxysomes. Peroxisomes house enzymes involved in the metabolism of glycolate, a product of photosynthesis (22), whereas glyoxysomes, common to endosperm or cotyledons of fatty seedlings, contain enzymes of the glyoxylate cycle (21, 26).

Germinating fatty seedlings represent an excellent system for studying biogenesis and organization of microbodies. Activities of glyoxylate cycle enzymes increase steadily in microbodies (glyoxysomes) after the onset of germination, then decline rapidly as triglyceride reserves are depleted. Coincident with the lipid degradation, the cotyledons expand and become photosynthetically competent, and the microbodies gain enzyme activities characteristic of leaf peroxisomes (6, 13, 17, 24, 25). In these systems a change in metabolism occurs during postgerminative growth that directly involves enzymes contained within microbody particles. The evidence is not compelling whether cotyledonary glyoxysomes are degraded and replaced by a new population of microbodies that function as leaf peroxisomes or whether there is simply a change in enzymatic complement of the organelle, with the glyoxysomes persisting as functional peroxisomes.

A direct approach for determining whether one or two microbody types are present at the time of the transition is to localize cytologically the appropriate marker enzymes. This is now possible with the use of two recently introduced electron cytochemical stains: malate synthase for glyoxysomes (23) and glycolate oxidase for peroxisomes (18). The cytochemical evidence given in this paper shows that both enzymes are housed within one type of microbody in cucumber cotyledons grown under conditions permitting high levels of both glyoxysomal and peroxisomal enzyme activities.

MATERIALS AND METHODS

Cucumber seeds (*Cucumis sativus* L. cv. Improved Long Green) were surface-sterilized in 1% hypochlorite (Clorox) for 10 min and planted in moistened vermiculite overlying soil. The seedlings were grown in the dark (constant 25 C) for 4 days following planting, subjected to 4 hr of continuous fluorescent and incandescent light (29 C), returned to the dark for 48 hr, then placed back into continuous light. To ensure maximum uniformity among cotyledons used for enzymatic and cytochemical analyses, plants were selected according to their heights based on repeated observations of growth patterns.

Homogenate Assays. Cotyledons of cucumber seedlings were removed and diced thoroughly with razor blades in 2 volumes of grinding medium (24). The grinding medium consisted of 500 mM sucrose and 2.5% Ficoll in 20 mM potassium phosphate, pH 6.9. The slurry was further homogenized at 20,000 p.s.i. in a French pressure cell. Homogenates of 3-day to 8-day cotyledons obtained in this manner were assayed for catalase (14), glycolate oxidase (20), glyoxylate (hydroxypyruvate) reductase (20), malate synthase (2), and isocitrate lyase (2) activities, and for protein by the modified Waddell method described by Murphy and Kies (15).

Gradient Fractionation. Cotyledons used for gradient separation of intact organelles were diced thoroughly in 2 volumes of sucrose-Ficoll grinding medium without further homogenization in the French press. The slurry was filtered through three layers of buffer-moistened Miracloth and differentially centrifuged three times, first at 500*g* for 10 min to remove cellular debris, starch, and nuclei, then twice at 10,800*g* for 30 min to provide a pellet

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(P_3) rich in mitochondria and microbodies. The second 10,800g centrifugation serves to purify further the final pellet. P_3 was resuspended in grinding medium to a protein concentration of 7 to 9 mg/ml before application to a discontinuous sucrose gradient. This was prepared by layering 6 ml of sucrose (in 20 mM potassium phosphate, pH 6.9) at each of the following densities: 1.28, 1.26, 1.23, and 1.18 g/cm³.

The fractions were drained from the bottom of the tubes after a 4-hr centrifugation at 53,000g in a Beckman L2-50 ultracentrifuge with Spinco SW25.1 rotor. The fractions were collected and assayed for enzyme activities as for the homogenates, including Cyt c oxidase (7). For isocitrate lyase assays, a final concentration of 20 mM cysteine was immediately added to portions of differential pellets, supernatants, and gradient fractions.

Electron Microscopy and Cytochemistry. Malate synthase reactivity was detected cytochemically in cotyledon segments and gradient fractions following the method of Trelease *et al.* (23). Controls constituted either the deletion of sodium glyoxylate or acetyl-CoA from the reaction mixture. Glycolate oxidase was localized in cotyledon segments fixed and preincubated as for malate synthase cytochemistry (23). The incubation medium, developed by Shnitka (personal communication), was slightly modified and used as follows: 8.5 ml of 25 mM potassium phosphate (pH 7.2), 0.5 ml of 60 mM copper sulfate + 40 mM Na, K, tartrate (pH 7.2), 1 ml of 15 mM potassium ferricyanide, 5 mg of phenazine methosulfate, 2 mg of flavine mononucleotide, and 12.5 mg of sodium glycolate.

Organelles from gradient fractions were fixed for 10 min by adding 10% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, Pa.) to a final concentration of 3%. Preserved organelles were then collected by centrifugation for 20 min at 10,800g (4 C), preincubated in buffered 3 mM potassium ferricyanide, rinsed, and finally resuspended in the appropriate incubation medium (40 min at 37 C). Pellets from these incubation media were rinsed, uniformly resuspended, and collected on 0.45-μm cellulose triacetate filters using a Swinney adaptor attached to a 10-ml syringe. The material was held in place with a similar filter, embedded in 2% agar, postfixed for 1 hr at room temperature with 2% OsO_4 in 50 mM sodium cacodylate (pH 7.1), dehydrated in graded acetone solutions, and embedded in a mixture of low viscosity resins (19).

RESULTS

Microbody Enzyme Activities. Enzyme profiles for cucumber cotyledons grown under alternating dark and light conditions are shown in Figure 1. The enzymes investigated were selected for their involvement in glyoxysomal and peroxisomal metabolism. Malate synthase activity (glyoxysomal) rises dramatically both in the dark and during the 4-hr exposure to light. High activity is still apparent for at least 24 hr after being returned to the dark, then it decreases gradually between days 5 and 6. After placing the seedlings in continuous light, malate synthase activity drops sharply through day 8.

Glycolate oxidase and glyoxylate (hydroxypyruvate) reductase activities increase only slightly during growth in the dark, then rise dramatically upon exposure to light (day 4–5) (Fig. 1). From day 5 to 6, these activities remain nearly constant; glycolate oxidase exhibits a 6-fold greater response to light than glyoxylate reductase. Upon further exposure to continuous light (at day 6), both activities increase again to near maximal levels within 24 hr.

Under these conditions (Fig. 1), catalase activity closely resembles the malate synthase profile during the first 6 days of growth, but upon exposure to continuous light (at day 6), the activity does not decrease to zero, but eventually levels off at approximately 50% maximal activity.

These profiles show that on day 5, 24 hr after the 4-hr exposure to light, significant levels of both glyoxysomal and peroxisomal activities are maintained in cotyledon homogenates. The specific activities of these enzymes separated on sucrose gradients are given in Table I. The bands obtained at 1.26 to 1.28 g/cm³ consistently have the highest specific activity of microbody enzymes. Activities of glyoxylate reductase and catalase are nearly the same in the 1.23 to 1.26 g/cm³ band. Electron microscopy of these fractions reveals a greatly enriched microbody fraction at 1.26

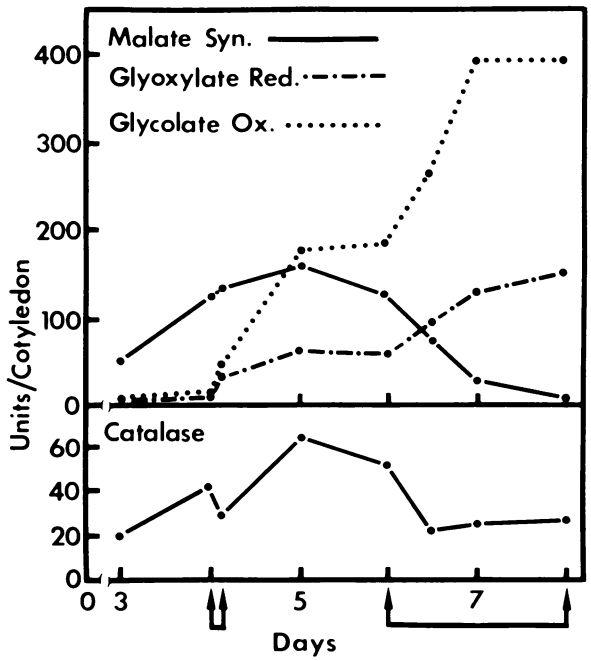


FIG. 1. Changes in peroxisomal and glyoxysomal enzyme activities in homogenates of cucumber cotyledons. Seedlings were grown in the dark (constant 25 C) for 4 days, subjected to 4 hr of continuous light (between arrows), returned to the dark for 48 hr, then placed back into continuous light (between arrows). Enzyme activity: malate synthase and glyoxylate reductase, nmoles substrate consumed/min·cotyledon; glycolate oxidase, 0.5 nmoles substrate consumed/min·cotyledon; catalase, units/cotyledon (a unit of catalase is that amount of enzyme required to catalyze the decomposition of 50% of the H_2O_2 present per 100 sec at 25 C).

Table I. *Activities of Microbody Enzymes and Cytochrome Oxidase on Discontinuous Sucrose Gradient*

Cucumber seedlings were grown in the dark (constant 25 C) for 4 days following planting, subjected to 4 hr of continuous light (29 C), and returned to the dark for 24 hr prior to homogenization. Particles were separated on the discontinuous gradient, and the distribution of microbody enzymes and Cyt oxidase was determined.

Density	Malate Synthase	Isocitrate Lyase	Glycolate Oxidase	Glyoxylate Reductase	Cyt Oxidase	Catalase
(g/cm³)	nmoles substrate consumed/min·mg protein				activity[1]	units[2]
1.26–1.28	668.2	110.2	169.2	365.9	1.35	221.7
1.23–1.26	480.3	61.3	64.4	334.5	0.97	222.7
1.18–1.23	206.2		39.4	58.2	10.09	51.5
Soluble	183.9	22.8	48.3	91.7	3.94	59

[1] Activity of Cyt oxidase is expressed as the first-order rate constant for the disappearance of reduced Cyt c (reduced with sodium dithionite) at 550 nm/mg protein.

[2] One unit of catalase activity is that amount of enzyme required to catalyze the decomposition of 50% of the H_2O_2 present per 100 sec at 25 C/mg protein.

to 1.28 g/cm³, abundant microbodies, membranes, and some mitochondria at 1.23 to 1.26 g/cm³, and principally mitochondria at 1.18 to 1.23 g/cm³.

Electron Microscopy and Cytochemistry. Malate synthase cytochemistry was performed on cotyledon segments taken from 3-, 4-, 4.5- and 5-day-old seedlings to determine whether all, or only a portion of the microbodies in cells were stained for enzyme reactivity. Several segments for each experiment were examined in both longitudinal and peridermal views (including palisade and spongy cells). At each stage of postgerminative growth, all microbodies in a zone 3 to 5 cells from the edge of the tissue blocks exhibit electron-dense product attributable to malate synthase reactivity. Typical results are illustrated in Figure 2; reaction product is apparent in all seven microbodies exposed in this section. No other recognizable microbodies are observed unstained. Background deposition is evident throughout the cytoplasm; this amount is also observed in control sections (deletion of substrates).

Similar results are obtained when cotyledon segments from 4-, 4.5- and 5-day-old seedlings are treated for glycolate oxidase activity. All palisade- and spongy-cell microbodies in a zone of penetration (only one to two cells into tissue segments) are positive for enzyme reactivity (Fig. 3). The reaction product is most often distributed throughout the microbody matrix, but in some cells the product is observed as a continuous ring around the periphery of microbodies. This phenomenon is interpreted as positive reactivity, since controls never produce such images. On occasion, both types of microbody staining are observed in the same cell. Background deposits are somewhat different from those seen in malate synthase experiments; needle-shaped deposits, rather than particulate granules, are the rule for glycolate oxidase. These different forms are probably due to copper ion interactions with different compounds in the two reaction media.

Electron cytochemistry of isolated microbodies overcomes problems of reactant penetration, and allows analysis of numerous particles in any given section view. Positive identification of these organelles is often difficult. Certain cellular components can be cut in a number of different planes giving a superficial resemblance to microbodies. Gradient fractions were stained for catalase reactivity with the diaminobenzidine technique (5) to distinguish between true microbodies and those organelles only resembling microbodies (Fig. 4). Osmium black deposition is clearly evident in microbody matrices except in areas where invaginated pockets occur. Other particles morphologically similar to microbodies are present in significant number, but are not stained (circled in Fig. 4). The proportion of stained microbodies to unstained microbody-like organelles was scored (Table II) and used as a guide to determine the number of malate synthase- and glycolate oxidase-stained microbodies in similar gradient fractions (see below).

Figure 5 represents 1 of 20 different micrographs used to score the number of microbodies stained for malate synthase activity. Positive staining is judged by comparing deposition in samples incubated in a complete reaction mixture with the control samples (Fig. 6). Enzyme-dependent staining is clearly evident in a majority of the microbodies; those particles having a similar appearance to microbodies, but not judged as stained, are circled on the micrograph.

Glycolate oxidase reactivity (Fig. 7) is also found in a majority of microbodies taken from a fraction similar to that shown in Figure 5. Again enzyme reactivity was determined by comparison with controls (Fig. 8). Microbodies stained for glycolate oxidase contain product either in their matrices or at their peripheries. Those particles considered unstained are circled.

In an attempt to quantitate the above microscopic results, all particles morphologically resembling microbodies were scored separately for either catalase, malate synthase, or glycolate oxidase reactivity (Table II). A minimum of 600 microbodies treated for each enzyme were scored for positive staining according to criteria given above. Eighty-six per cent of the particles are stained for catalase, while 80 and 83% are stained for malate synthase and glycolate oxidase activity, respectively. Since only catalase-positive particles qualify as microbodies, then approximately 14% of the particles scored must be other cellular components only resembling microbodies. If the 86% catalase value is corrected to 100% and similar adjustments are made for the other enzymes (Table II), it becomes apparent that essentially all catalase-containing particles share malate synthase and glycolate oxidase activities. These data corroborate interpretation of cytochemical staining in intact tissue segments.

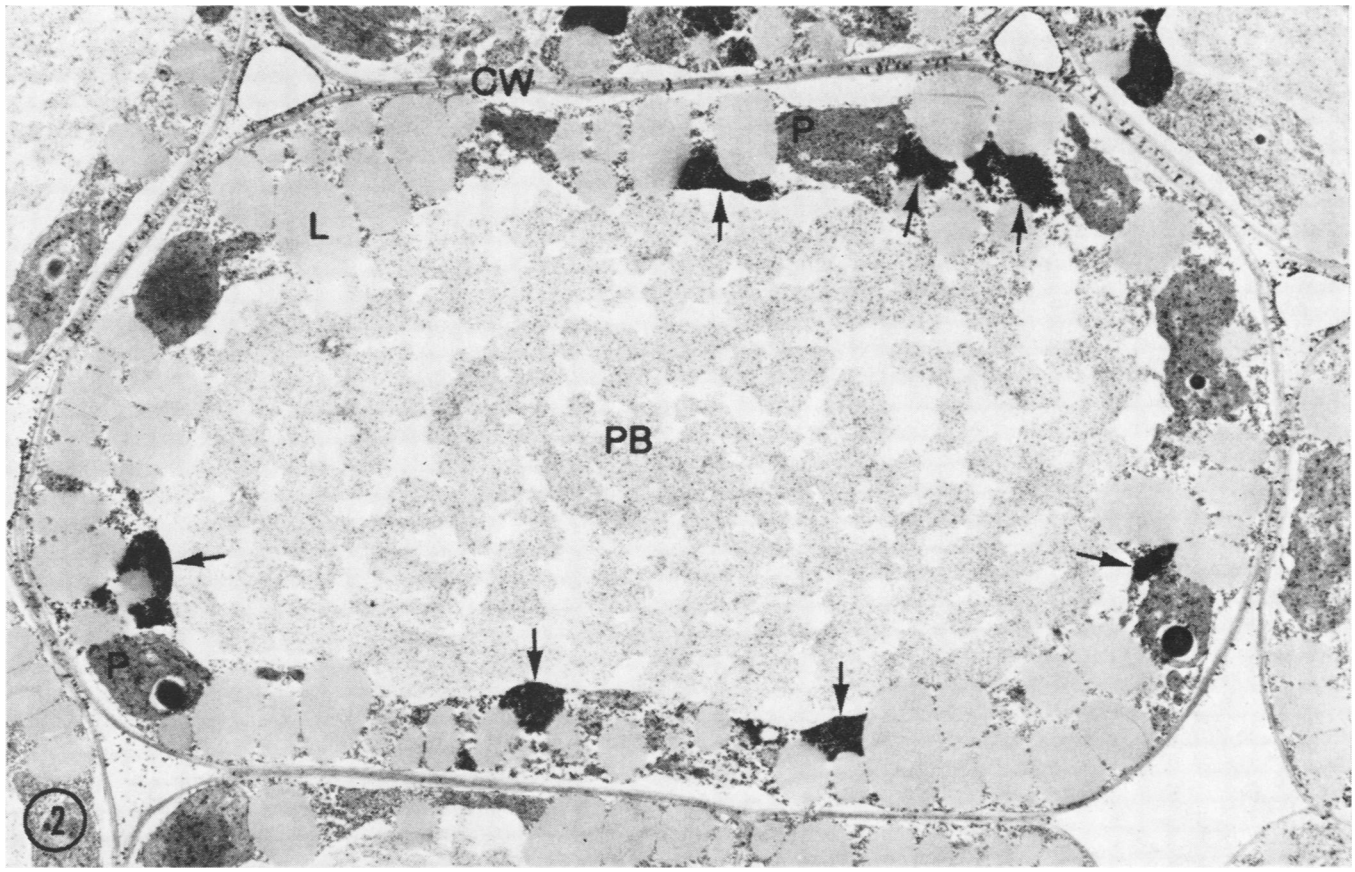
DISCUSSION

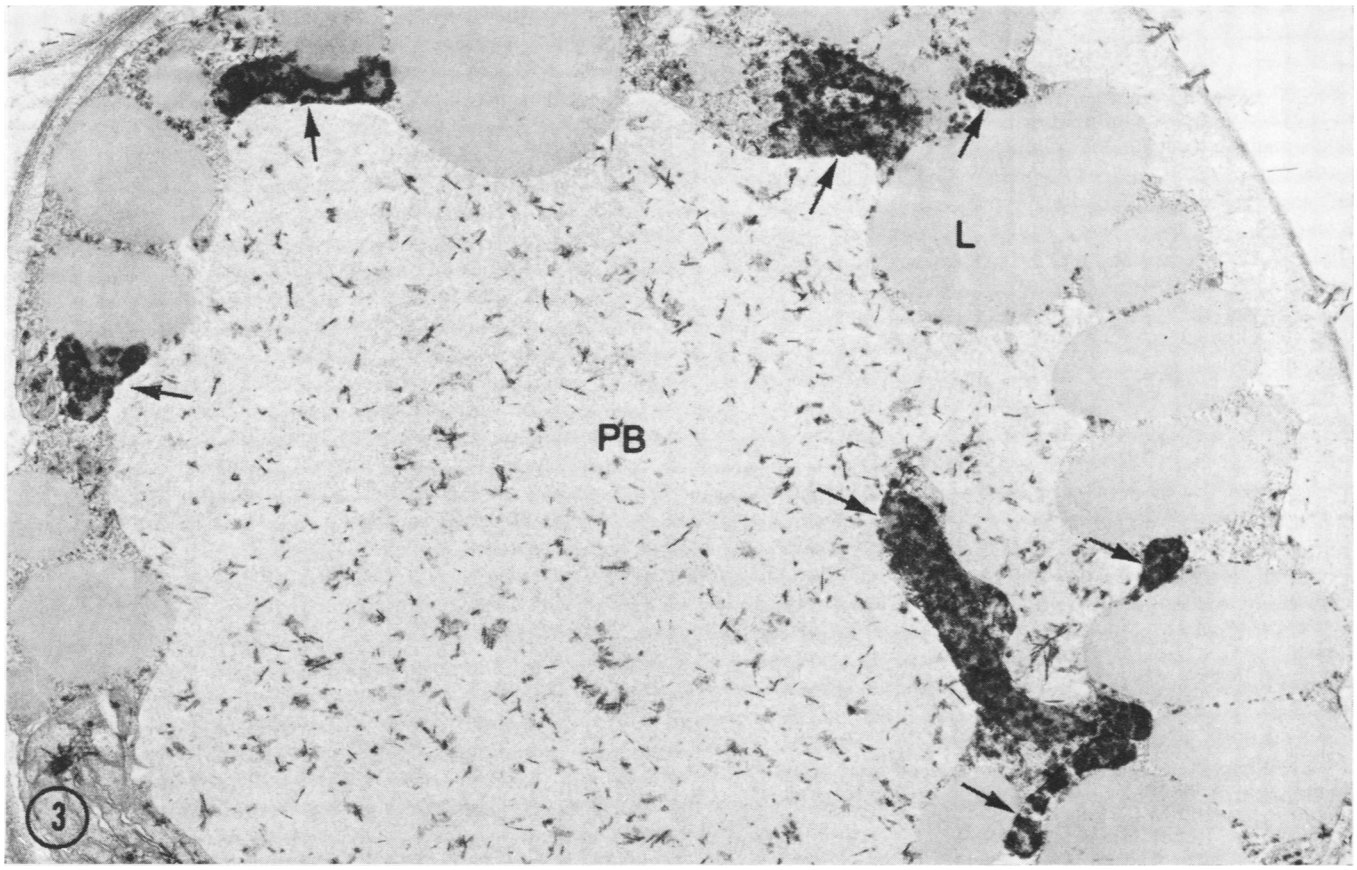
Three models have been postulated to explain the succession from glyoxysomes present in fat-storing cotyledons during early lipid-degrading stages to the peroxisomes found later at photosynthetic stages (24). The first model assumes glyoxysomes and peroxisomes to be separate populations of microbodies with glyoxysomes being degraded and replaced by a new population of microbodies that function as leaf peroxisomes. The second model is also based on the concept of two distinct kinds of particles, but presumes that coexistence of both populations of microbodies, each active in microbody metabolism at a different stage of development. A third mechanism of developmental ontogeny may involve a change in the enzymatic complement of the organelle, with glyoxysomes persisting as functional peroxisomes. Using cucumber seedlings as a study system, Trelease *et al.* (24) could not find ultrastructural evidence either for glyoxysomal degradation or for peroxisomal formation in light-grown seedlings (model 1). No evidence was found to indicate that a *de novo* synthesis of peroxisomes occurs upon exposure of etiolated seedlings to light (when glycolate oxidase activity greatly increases). Their data are most consistent with the concept of succession within existing particles (models 2 and 3). Definitive data are not available to determine whether peroxisomal enzymes are located in nonfunctional particles at the time of glyoxysomal metabolism (model 2) or in the same particles that house glyoxysomal enzymes (model 3).

In the present work, exposure of etiolated cucumber seedlings to 4 hr of continuous light resulted in simultaneously high levels of glyoxysomal and peroxisomal enzyme activities (day 5, Fig. 2). These physiological conditions avoided the confusing concomitant loss or gain of enzymes apparent in light-grown plants (13, 17, 24). Electron cytochemistry of the marker enzymes at this 5-day growth period allowed us to determine the number of microbody types involved in the transition. The following reasoning was used to make this distinction. If two types of microbodies occur together in cells, then some portion of them should stain for malate synthase (glyoxysomal marker), and a separate portion should stain for glycolate oxidase (peroxisomal marker). If on the other hand, these enzymes are contained within the same particle, then all the microbodies should stain for both enzymes. From the data obtained, it is clear that all or virtually all of the microbodies present during the transition period contain both glyoxysomal and peroxisomal marker enzymes.

Cotyledons were taken at several different growth intervals to determine whether there was a change in staining pattern of *in situ* microbodies. The observations were consistent in that all microbodies at each stage were stained for both enzyme activities. Relatively poor penetration of reactants into tissue segments and the limited number of microbodies seen in cell sections required cytochemistry of microbodies obtained from sucrose-gradient fractions.

Using discontinuous-gradient separation, microbodies at the





FIGS. 2 and 3. Electron micrographs of cells from the cotyledons of 5-day-old cucumber seedlings. Tissue segments were incubated in ferricyanide media for enzyme reactivity. Fig. 2: Reaction product attributable to malate synthase reactivity is distributed in all microbodies (arrows). Some nonspecific deposits occur in the cell wall (CW), plastids (P), and vacuolating protein bodies (PB), but not in lipid bodies (L). × 8,200. Fig. 3: Reaction product attributable to glycolate oxidase reactivity is distributed in the matrix of all microbodies (arrows). Nonspecific needle-shaped deposits are apparent in other organelles and the vacuolating central protein bodies (PB). × 20,400.

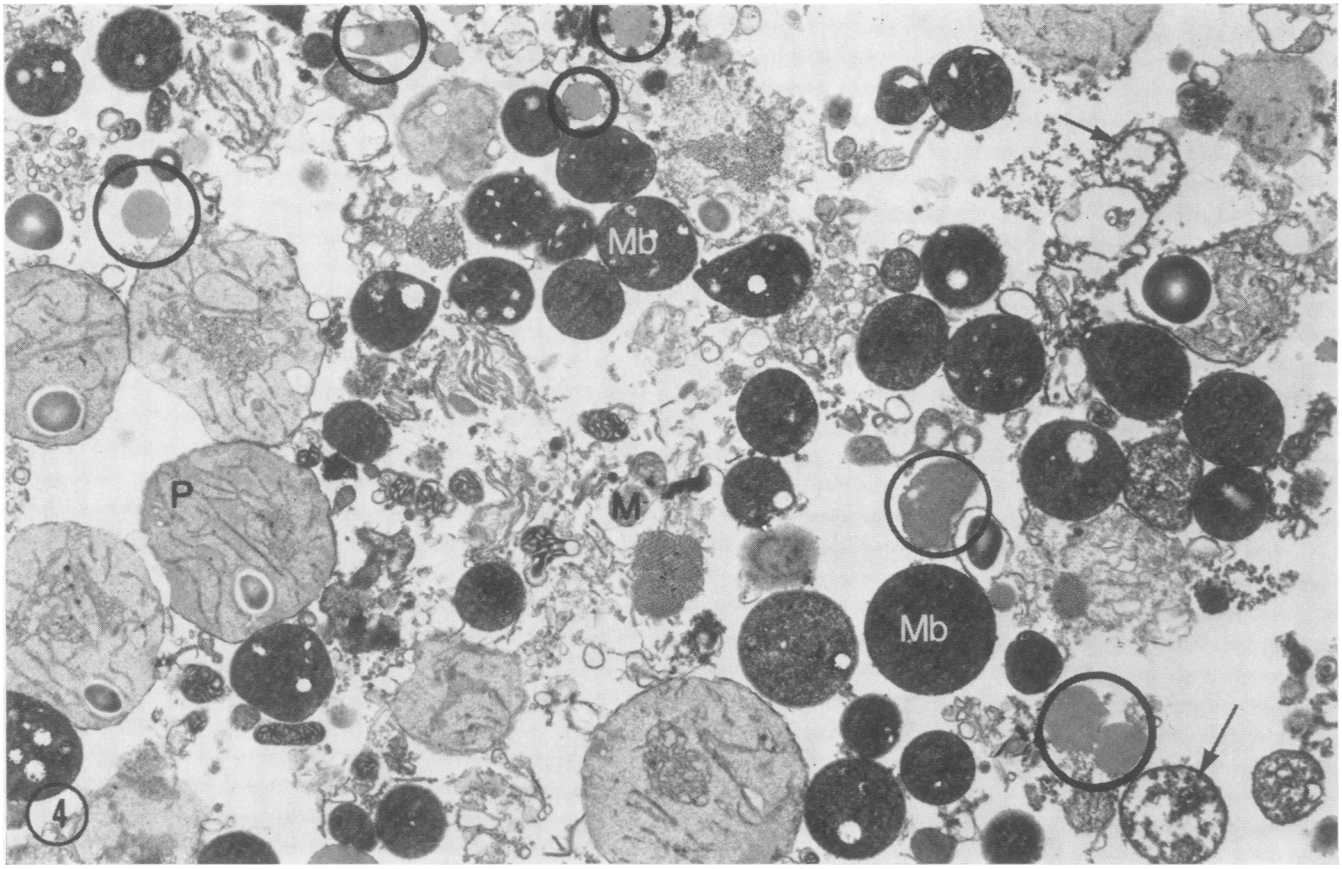


FIG. 4. Electron micrograph of a portion of the microbody band (1.26–1.28 g/cm³) incubated in the diaminobenzidine media for the detection of catalase. Dense reaction product occurs throughout the matrix of the microbodies (Mb). Broken microbodies (arrows) show dense staining in their membranes and in the sparse matrix material. Several particles, other than plastids (P) and condensed mitochondria (M), resemble microbodies, but are not stained for catalase (circled). Of the circled and stained particles, 40 of the total 47, or 85%, are considered microbodies because of catalase staining. × 11,700.

Table II. *Percentages of Gradient Particles Stained for Catalase, Glycolate Oxidase, and Malate Synthase Reactivity*

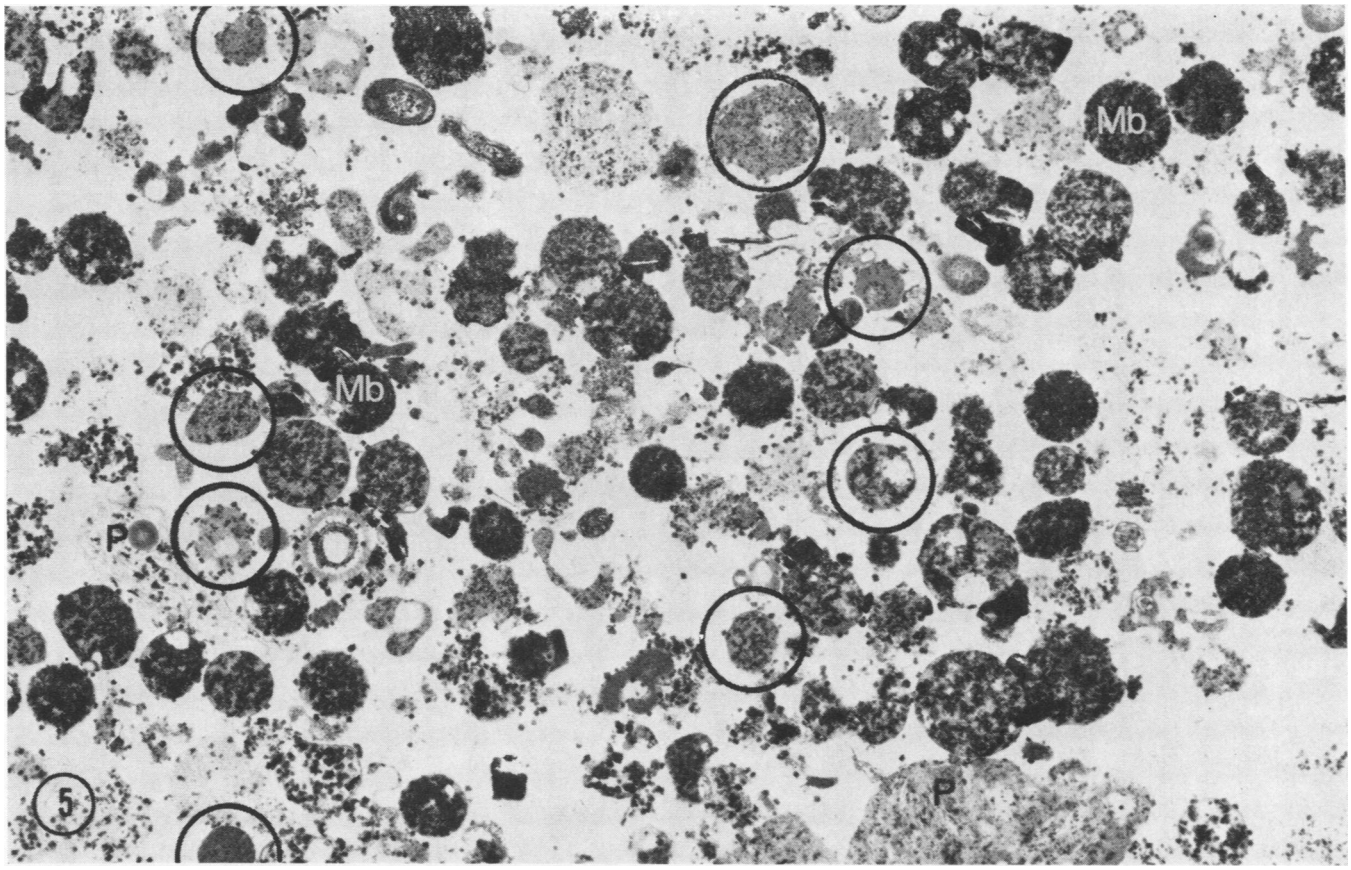
The microbody gradient fractions (1.26–1.28 g/cm³) were incubated in cytochemical media for separate detections of catalase, glycolate oxidase, and malate synthase. A minimum of 20 electron micrographs taken from several different experiments were used for each enzyme. Particles were scored for reactivity by comparison with controls. The percentages given below are a calculation of the number of stained particles divided by the total number of particles in the gradient fraction resembling microbodies. Catalase values are corrected to 100% since only microbodies are catalase positive. Using the same criteria, the other values are corrected by the same ratio to determine the number of microbodies stained for glycolate oxidase and malate synthase activity.

Gradient Cytochemistry	Organelles	Per Cent with Reactivity	Corrected for Catalase Reactivity
	No.		%
Catalase	732	85.9	100
Glycolate oxidase	628	83.4	97
Malate synthase	873	79.7	94

1.26 to 1.28 g/cm³ step could be reproducibly separated from the less pure organelles at the 1.23 to 1.26 g/cm³ layer. Brown *et al.* (1) analyzed pumpkin microbodies from essentially the same fraction by removing only the heavier portion of bands resolved on continuous gradients. Use of this heavier band for cytochemical staining is valid since high specific activities of both peroxisomal and glyoxysomal enzymes are mutually present (Table I). Thus, this band represents a major portion of cotyledon microbodies which are readily amenable to enzyme cytochemistry. Some cytochemical tests were performed on the lighter band, but contamination with nonmicrobody components made rigorous scoring of stained microbodies impossible.

Cytochemical staining of microbodies in gradient fractions was analyzed by comparing the number of stained microbodies to the total number of microbodies in a large field of view. Catalase cytochemistry provided the index as to what proportion of the fractionated particles consisted of true microbodies. After correction with the catalase value (Table II), the data show that essentially all the microbodies contain both malate synthase and glycolate oxidase, thus corroborating the tissue staining observations. Given even a 20% error in scoring technique, *i.e.* 80% stained for malate synthase and 80% for glycolate oxidase with the remaining 20% in each case having only one enzyme, 60% of the total number of microbodies would still have to contain both enzymes. These experiments with gradient fractions would have clearly shown two separate microbody types if they had been present.

Glycolate oxidase staining in both tissue segments and gradient fractions occasionally shows reaction product at the periphery of microbodies rather than throughout their matrices. This ringing effect is noticeably absent in controls (lacking substrate) and therefore is considered positive staining. These observations are



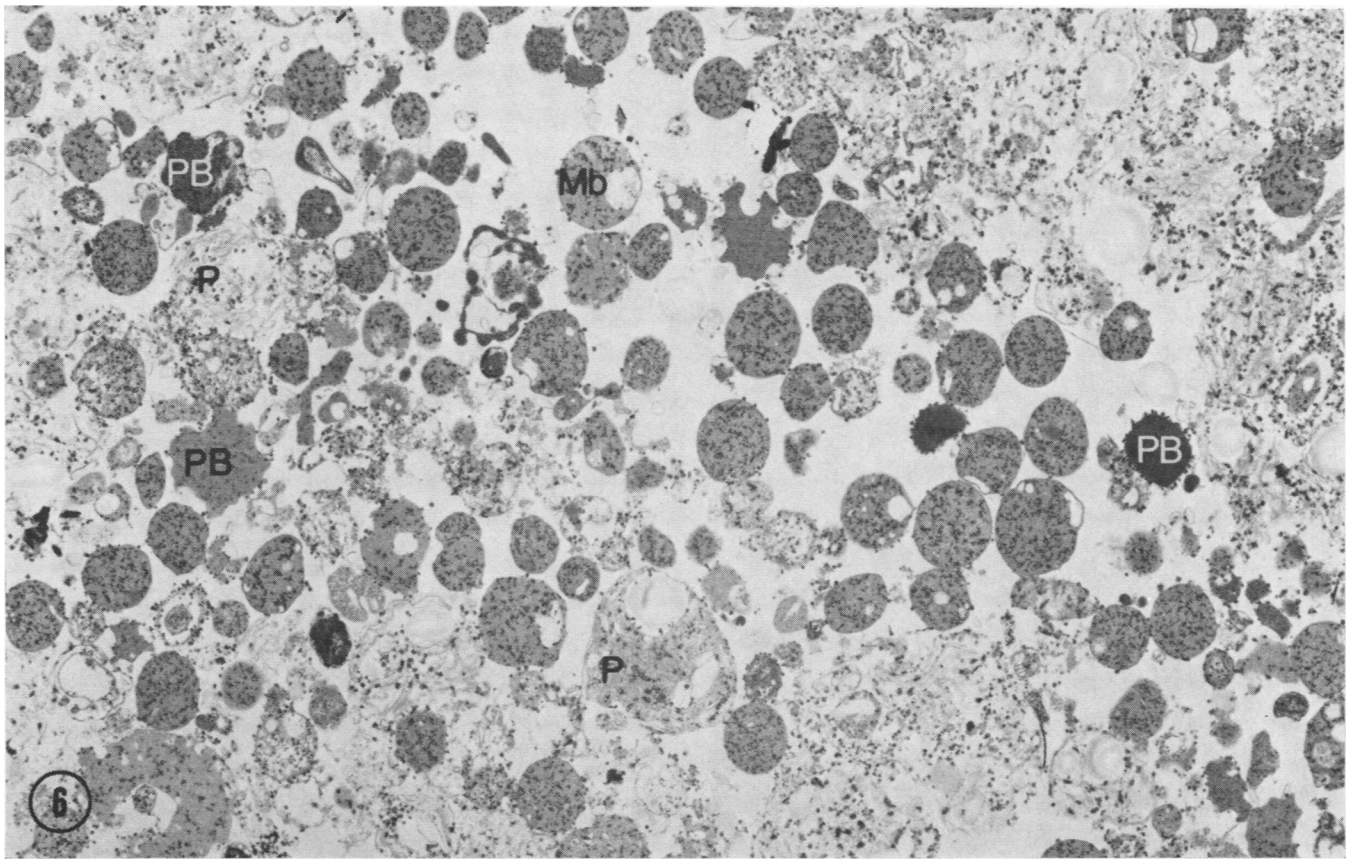


FIG. 5. Representative electron micrograph of a portion of the microbody band (1.26–1.28 g/cm³) incubated in complete ferricyanide medium for the detection of malate synthase. Reaction product attributable to malate synthase reactivity is distributed throughout the matrix of microbodies (Mb). Several particles, other than plastids (P), resemble sectioned microbodies but are not stained (circled). In this figure 83% of all particles identifiable as possible microbodies are stained for malate synthase reactivity. × 11,500.

FIG. 6. Electron micrograph of a portion of the microbody band incubated in ferricyanide medium without glyoxylate (control) for the detection of malate synthase. Nonspecific deposits occur in plastids (P), protein bodies (PB), and microbodies (Mb). These background deposits are clearly distinguishable from enzymic staining in Figure 5. × 9,500.

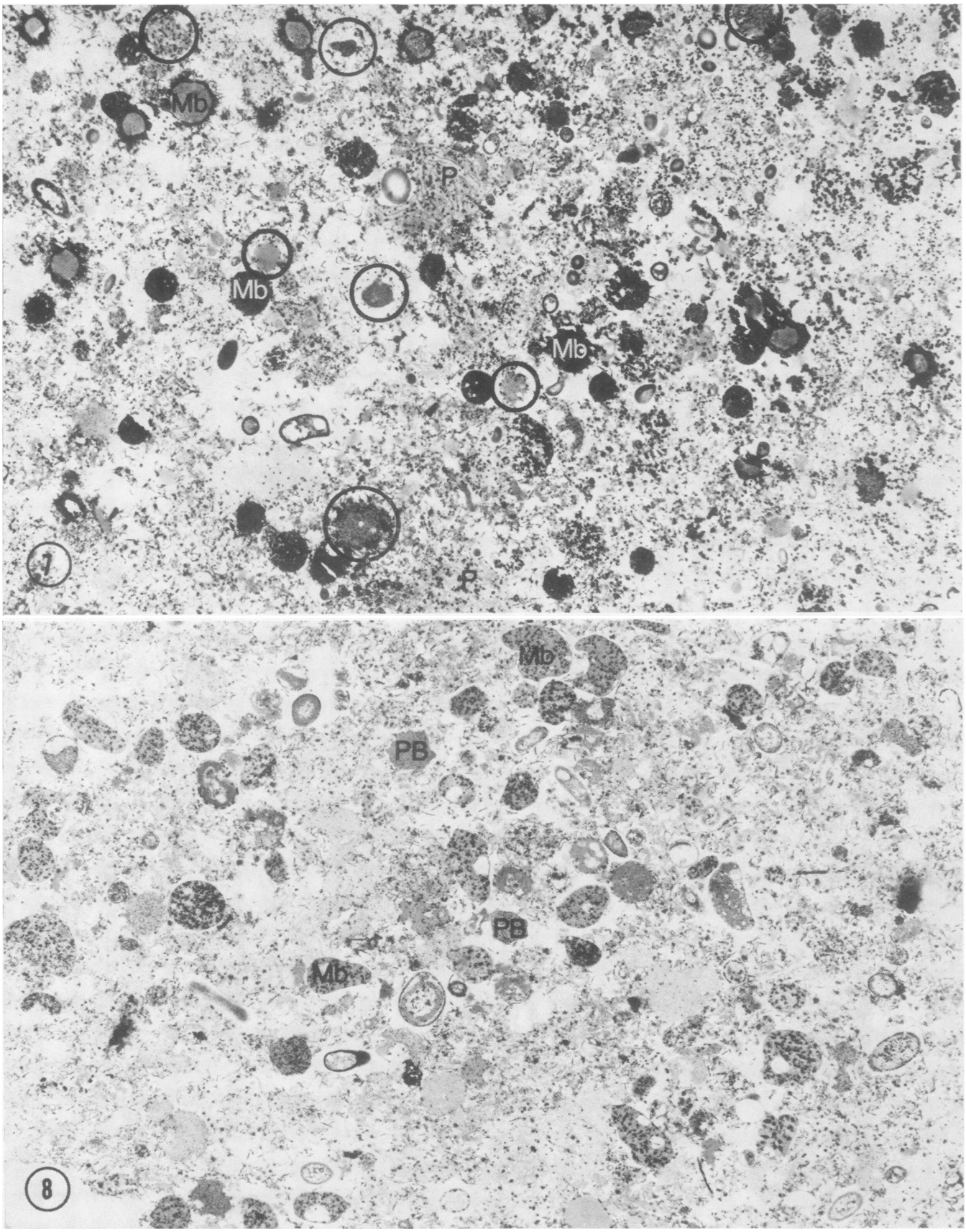


FIG. 7. Representative electron micrograph of a portion of the microbody band (1.26–1.28 g/cm³) incubated in complete ferricyanide medium for the detection of glycolate oxidase. Reaction product attributable to glycolate oxidase reactivity is distributed throughout the matrix of some microbodies and surrounding the periphery of others (Mb). Several particles, other than plastids (P), resemble microbodies but are not stained (circled). In this figure, 87% of all particles identifiable as possible microbodies are stained for glycolate oxidase reactivity. × 7,260.

FIG. 8. Electron micrograph of a portion of the microbody band incubated in ferricyanide medium without glycolate (control) for the detection of glycolate oxidase reactivity. Nonspecific deposits occur in protein bodies (PB) and microbodies (Mb). These background deposits are clearly distinguishable from enzymic staining shown in Figure 7. Reaction product surrounding the periphery of some microbodies is noticeably absent in the control. × 9,900.

not unique to cucumber seedlings since similar findings were obtained in rat liver and *Hydra* tissues stained for glycolate oxidase (8, 9). Hand (9) suggests this ringing is a consequence of reducing equivalents building up within microbody matrices prior to ferricyanide penetration. As ferricyanide enters the organelles, it is reduced in the presence of copper to produce copper ferrocyanide only on the peripheries. This is most prevalent when lactate is used as a substitute substrate for glycolate. Lactate also produces considerable peripheral staining in cucumber cotyledons; we have preincubated microbody fractions with ferricyanide and failed to eliminate the peripheral staining. At the moment this effect is not understood; perhaps it represents some physiological aspects of microbodies not yet considered.

Several other approaches with a variety of fatty seedlings have been used to study the ontogenetic relationship between glyoxysomes and peroxisomes. Radin and Breidenbach (16) examined catalase turnover in safflower seedlings. Hoch (10) prepared antibodies to glyoxysomal membranes in developing watermelon seedlings. Brown *et al.* (1) compared biochemical properties of microbody proteins in pumpkins and castor bean germlings. In each case the authors addressed the problem of the number of microbody types involved in their systems, but could not draw definitive conclusions based on the data. Drumm and Schopfer (3) believe their comparison of the time course of catalase activity with time courses of glyoxysomal and peroxisomal enzymes in dark-grown and far red-irradiated watermelon cotyledons provides further circumstantial evidence favoring the microbody transformation hypothesis.

As an alternate view, Kagawa and Beevers (12) favor the concept that glyoxysomes are selectively destroyed and peroxisomes are formed *de novo*. Their conclusions are based on work with germinated watermelon seedlings where they point out (*a*) loss of microbody protein correlates with declines in glyoxysomal activity, and (*b*) the amount of microbody protein associated with fully developed peroxisomes is considerably less than that present when glyoxysomal activity is at its height. It should be pointed out that one major event also occurring rapidly in light-grown fatty seedlings and more slowly in dark-grown plants is the breakdown of protein bodies (17, 24). We always see section views of these organelles in our microbody gradient fractions and are constantly aware of them as common contaminants. Through hundreds of observations and serial sectioning, we can now recognize that many of the single membrane-bound particles cytochemically unstained are portions of protein bodies. Schnarrenberger *et al.* (17), also cognizant of this contamination, states that calculated specific activities of microbody enzymes in gradient fractions are erroneously low because total protein is dependent upon other particles in this area of the gradient, namely etioplasts and storage protein bodies. Ericson and Chrispeels (4) also report sedimentation of protein bodies at a density of 1.26 to 1.28 g/cm^3.

In view of the above data and evaluation of the gradient region described by Kagawa and Beevers (Fig. 7 in ref. 12), it appears their results can be interpreted in another way. The protein loss correlated with glyoxysome enzyme decline may not represent degradation of microbody protein. This could explain why the amount of glyoxysomal protein is significantly greater than peroxisomal protein (most of this protein represents unhydrolyzed protein bodies), and why protein values never drop to zero (glyoxysomes are still present). Their data could be entirely consistent with ultrastructural events described by Trelease *et al.* (24) and support interpretations of microbody events put forth in this paper.

The data presented here show that during a selected transition period when glyoxysomal and peroxisomal enzymes are present in cucumber cotyledons, all the microbodies contain both malate synthase and glycolate oxidase. This information almost certainly rules out the possible coexistence of two different kinds of microbodies active at different stages of development. Instead, the localization of both marker enzymes in one microbody type strongly suggests the metabolic changeover occurs by a change in enzyme complement within existing microbodies without loss of organelle integrity, rather than through the actual replacement of degraded glyoxysomes by newly formed peroxisomes.

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