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The Development of Microbodies (Glyoxysomes and Leaf Peroxisomes) in Cotyledons of Germinating Watermelon Seedlings[1]

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ABSTRACT

The ontogeny of glyoxysomes and leaf peroxisomes has been examined in the cotyledons of germinating watermelon (*Citrullus vulgaris*) seedlings. Organelles from the cotyledons were extracted by razor blade homogenization and microbodies were separated by sucrose density gradient fractionation. Both kinds of microbodies have the same mean equilibrium density on sucrose gradients.

The development of leaf peroxisomes was examined in seedlings transferred to light at 4 days and 10 to 12 days. In seedlings maintained in darkness to the age of 10 to 12 days, glyoxysomal enzymes virtually disappeared, and the losses were paralleled by a corresponding loss in microbody protein. During this period peroxisomal activity was low and changed only slightly. On transfer to light at this stage, the activity of peroxisomal enzymes rose strikingly. The residual glyoxysomal activity disappeared completely, and the developmental pattern of microbody catalase and microbody protein paralleled the light-induced glyoxysomal disappearance.

Similar patterns of microbody development were observed when 4-day-old dark-grown seedlings with maximum glyoxysomal activities were exposed to light. The activity of the peroxisomal enzymes increased and the glyoxysomal enzymes disappeared at a faster rate than in darkness. These changes were again paralleled by the accelerated demise of microbody catalase and microbody protein. Thus under both conditions glyoxysomes were selectively destroyed during peroxisomal development, and the amount of peroxisomes produced was insufficient to offset the loss of glyoxysomal protein. The results do not support the contention that glyoxysomes are transformed to leaf peroxisomes in developing cucurbit cotyledons and favor the view that the two kinds of microbody arise independently of each other.

There are two major functional classes of microbodies in higher plants: glyoxysomes, which are found in fatty tissues of germinating seedlings (1, 2), and leaf peroxisomes, which are found in green leaves (29, 32). These two classes of microbodies have been the object of detailed biochemical (4, 6–8, 10, 16, 17, 19, 28, 31, 35) and developmental (5, 11, 12, 13, 20, 21, 26, 27, 33, 34) studies. The relationship between glyoxysomes and leaf peroxisomes has attracted considerable interest because they are similar in their structure and physical properties (1, 10, 16, 30, 33) and because several enzymes are constituents of both kinds of organelles (1, 16, 30). The development of glyoxysomes in endosperm tissue (13, 34) and peroxisomes in greening leaves have been described (11, 14). In such materials, only one class of microbody is present and they arise independently of each other. The cotyledons of germinating cucurbit seedlings offer an interesting contrast, for in these organs glyoxysomes are present and functional during the early stages of growth when fat is converted to sucrose, but shortly thereafter, as fat utilization is completed and the distinctive enzymes of the glyoxysomes are lost, the cotyledons green and become photosynthetic and then have typical leaf peroxisomes. Thus the question naturally arises, do glyoxysomes in such organs give rise to peroxisomes by a selective loss of certain enzymes and the acquisition of others or is the development and demise of each class of microbody independent of the other (18, 33).

In a detailed study of the early growth of the cotyledons of *Citrullus vulgaris* (watermelon) under rigidly controlled conditions, the developmental patterns of some 19 enzymes involved in carbohydrate and fat metabolism were followed (20). The first 4 to 5 days of germination were characterized by the rapid conversion of stored fat to carbohydrates and the development of glyoxysomal activity. When the emerging 4-day-old dark-grown seedling was exposed to continuous illumination, three different enzymatic patterns were observed: (*a*) glyoxysomal activity declined more rapidly in the illuminated seedlings than in control seedlings still in darkness; (*b*) chloroplastic and peroxisomal activities increased dramatically; and (*c*) the development of enzymes of the tricarboxylic acid cycle and of several soluble enzymes was essentially unaffected.

When the germinating seedlings were maintained in darkness for prolonged periods (10–12 days) so that fat utilization was completed, glyoxysomal activity declined and virtually disappeared. Nevertheless, the subsequent illumination of these seedlings still brought about the development of photosynthesis and high levels of peroxisomal activity.

The development of peroxisomal enzymes can be experimentally disengaged from the development of the photosynthetic apparatus which normally accompanies it. Thus when 4-day-old dark-grown seedlings were exposed to brief periods of illumination (as little as 5 min), peroxisomal activity developed in a normal fashion, but no functional chloroplasts were produced (20).

The reciprocal rise of peroxisomal activity and fall in

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glyoxysomal activity in plants germinated under normal conditions has been the principle biochemical evidence for the suggestion that glyoxysomes are transformed into leaf peroxisomes (20, 27, 33). However, from the data obtained in experiments where seedlings were maintained for long periods in darkness before illumination, it is clear that the presence of glyoxysomal activity is not a requirement for the development of peroxisomal activity (20). Thus an alternative explanation for the reciprocal changes in glyoxysomal and leaf peroxisomal activities is that upon illumination, glyoxysomes are selectively destroyed and leaf peroxisomes are formed *de novo* (20).

In this paper, the ontogeny of the microbodies is followed under two experimental conditions, one in which leaf peroxisomes develop while glyoxysomes are still present and active, and one in which they develop after the glyoxysomes have essentially disappeared.

MATERIALS AND METHODS

Plant material (watermelon [*Citrullus vulgaris*] seedlings), the growing conditions, and the selection of cotyledons from the germinating seedlings have been described previously (20).

Preparation of Cellular Organelles from Cotyledons. Cotyledons were homogenized by chopping with a stainless steel razor blade for 12 min in a Petri dish on ice. Five ml of grinding medium were used for 3 g of tissue. The grinding medium consisted of 0.55 M sucrose, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA (pH 7.5), and 165 mM Tricine buffer (pH 7.5).

After chopping, the homogenate was passed through eight layers of cheesecloth into a centrifuge tube. Batches of cotyledons were chopped separately until a sample size of 160, 320, or 640 cotyledons were homogenized.

The homogenate was centrifuged at 480*g* for 10 min to remove cell debris. The resultant supernatant was centrifuged at 10,800*g* for 30 min to yield a supernatant fraction and a crude organelle fraction.

The crude organelle fraction was carefully resuspended in 3 ml of 32% (w/w) sucrose solution (made up in 1 mM EDTA, pH 7.5) and was layered onto a sucrose gradient.

The gradient consisted of 40 ml of sucrose solution increasing linearly from 32 to 60% (w/w) over a 10-ml cushion of 60% sucrose. Gradients were contained in 60-ml cellulose nitrate tubes. All sucrose solutions were prepared in 1 mM EDTA (pH 7.5).

Gradients were centrifuged for 4 hr at 48,000*g* (20,000 rpm) using an SW 25.2 rotor in a Beckman L2-65B ultracentrifuge. Following centrifugation, 1-ml fractions were collected from the top with an Isco Model D density gradient fraction collector. All procedures were carried out at 0 to 4 C.

Enzyme Assays. All enzyme assays were carried out spectrophotometrically at room temperature (24 C) except for glycolate oxidase, which was measured polarographically with a Clark oxygen electrode at 30 C. The methods employed were those described in the literature as follows: isocitrate lyase (9), malate synthetase (15), glycolate oxidase (3), hydroxypyruvate reductase (24), catalase (13, 23), fumarase (25).

Other Methods. Chlorophyll was assayed in acetone extracts at 663 nm. Protein was measured by the method of Lowry *et al.* (22). Sucrose concentrations were measured with a Bausch and Lomb Abbe 3-L refractometer.

RESULTS

Homogenization. The razor blade homogenization technique was the most satisfactory method of extracting organelles from the cotyledons. This method routinely extracted 25% of the total activity from the cotyledons, irrespective of the growth conditions. Secondly, this method systematically gave high yields of particulate glyoxysomal (~50%), leaf peroxisomal (~50%), and mitochondrial (~60%) activity. These recoveries did not vary as the size and geometry of the cotyledons changed during development. Thirdly, the activities of the enzymes under study remained stable during centrifugation and the time required for assay.

ISOLATION OF MICROBODIES BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

Isolation of Glyoxysomes. Glyoxysomal activity reaches its highest levels in 4-day-old dark-grown seedlings. A pure fraction of glyoxysomes can be isolated from the cotyledons by the fractionation of the crude organelle preparation on sucrose density gradients (Fig. 1). There is a major protein band between 39 and 44% sucrose and a second smaller protein band at 54% sucrose. The peak of fumarase activity coincides with the major protein band at 42% sucrose and identifies the mitochondrial fraction. The asymmetric distribution of protein about 42% sucrose indicates the presence of another fraction, identified as broken proplastids by electron microscopy.

Isocitrate lyase and malate synthetase, the two glyoxysomal enzymes, and catalase are confined to a single peak coinciding with the protein band at 54% sucrose. The localization of the distinctive glyoxysomal marker enzymes and of catalase in this single protein band identifies it as the glyoxysomal fraction.

Isolation of Leaf Peroxisomes. Leaf peroxisomal activity develops to its highest levels in the cotyledons of 8-day-old light-treated seedlings (4 days dark and 4 days light). Glyoxysomal activity is no longer detectable at this stage.

When the crude organelle fraction from these seedlings is fractionated on sucrose density gradients, a single broad protein band is found between 38 and 46% sucrose (Fig. 2). The Chl distribution (A_{663}) identifies this as chloroplasts. Fumarase is found at 44% sucrose and identifies the protein shoulder of the protein band as the mitochondrial fraction. Another small protein peak appears at 54% sucrose and the localization of glycolate oxidase, hydroxypyruvate reductase, and catalase within this protein peak identifies this fraction as the leaf peroxisomes.

The location of the microbody fraction is defined by the distribution of the marker enzymes of the glyoxysomes and leaf peroxisomes. The nine fractions between 51 and 56% sucrose were selected as representing the microbody fraction and were used for the measurement of microbody protein and the activities of the isolated glyoxysomes and leaf peroxisomes.

DEVELOPMENT OF MICROBODIES IN COTYLEDONS OF DARK-GROWN SEEDLINGS

After the stored fat has been consumed, glyoxysomal activity rapidly declines in the cotyledons. A comparison of the organelles extracted and fractionated from 4-, 8-, and 12-day-old dark-grown seedlings shows that the decline of glyoxysomal activity is accompanied by a net loss of microbody protein (Fig. 3). The losses in microbody protein represent the general demise of glyoxysomes. This is shown by the fact that the distribution profiles of isocitrate lyase, malate synthetase, and catalase remain essentially unchanged during development. The losses in microbody protein cannot be explained as resulting from a progressive fragility of the microbodies with age. In addition to the decline of glyoxysomes from the

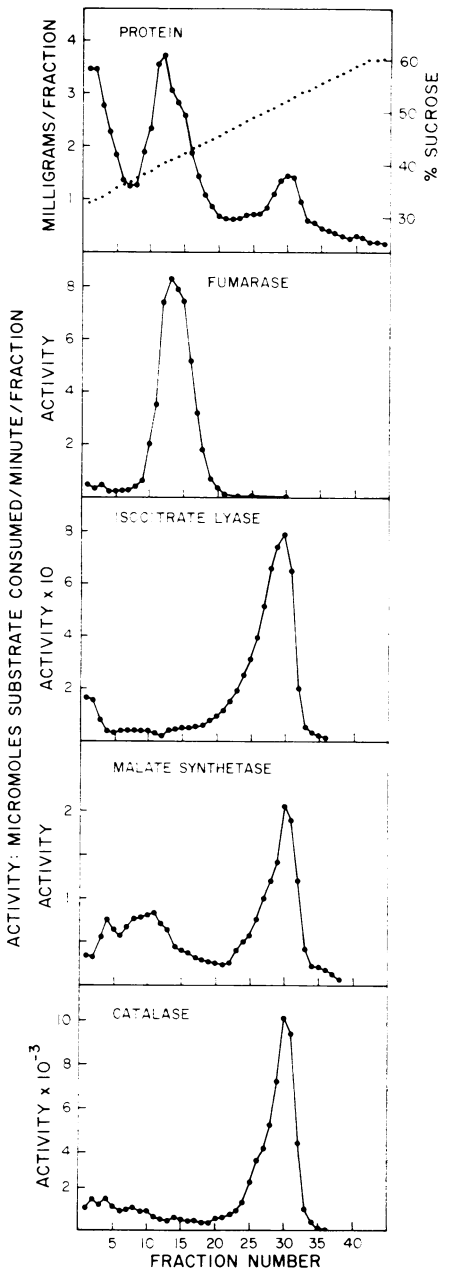

FIG. 1. Distribution of organelle protein anad the activities of the mitochondrial enzyme, fumarase, and the glyoxysomal enzymes, isocitrate lyase, malate synthetase and catalase after sucrose density gradient separation of the components of the crude particulate fraction from cotyledons of 4-day-old dark-grown watermelon seedlings. The distribution profiles represent the recovery from 640 cotyledons.

cotyledons, there is a loss of protein from the mitochondrial-proplastid fraction (Fig. 3).

EFFECT OF LIGHT ON MICROBODY DEVELOPMENT

Effect of Light on Organelle Development in Cotyledons of 10-day-old Dark-grown Seedlings. Through the development of the seedling grown in darkness, only small changes in glycolate oxidase and hydroxypyruvate reductase activity were detected in the microbodies (Fig. 4). But the activity of these peroxisomal enzymes in the microbody fraction rose dramatically when 10-day-old dark-grown seedlings were exposed to continuous illumination.

The activity of the distinctive enzymes of the glyoxysomes in this microbody fraction, followed a distinctly different

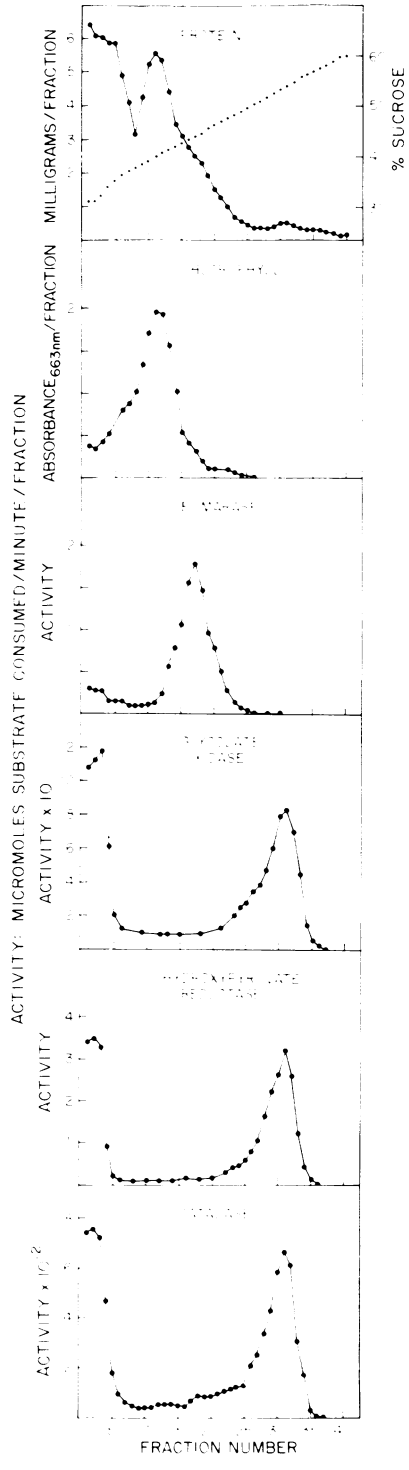

FIG. 2. Distribution of organelle protein, Chl, and the activities of the mitochondrial enzyme, fumarase, and the leaf peroxisomal enzymes, glycolate oxidase, hydroxypyruvate reductase, and catalase after sucrose density gradient separation of the components of the crude particulate fraction from cotyledons of 8-day-old light-treated watermelon seedlings (4 days dark + 4 days light). The distribution profiles represent the recovery from 640 cotyledons.

developmental pattern. Between the ages of 4 and 12 days, malate synthetase and isocitrate-lyase in the microbodies declined to very low levels (Fig. 4), and the small amount of glyoxysomal activity remaining in the microbodies of 10-day-old dark-grown seedlings disappeared completely when these seedlings were transferred to light.

The decline of microbody catalase and microbody protein paralleled the steady demise of glyoxysomal activity during the period when the seedlings were maintained in darkness, and also when the 10-day-old seedlings were exposed to light (Fig. 4).

The disappearance of microbody protein is graphically illustrated in Figure 5, where the protein profiles of sucrose gradient separations of crude organelle preparations from the cotyledons of 12-day-old light- and dark-grown seedlings are compared. This gradient profile shows the net increase in total particulate protein due to the development of chloroplasts, and the net loss in microbody protein.

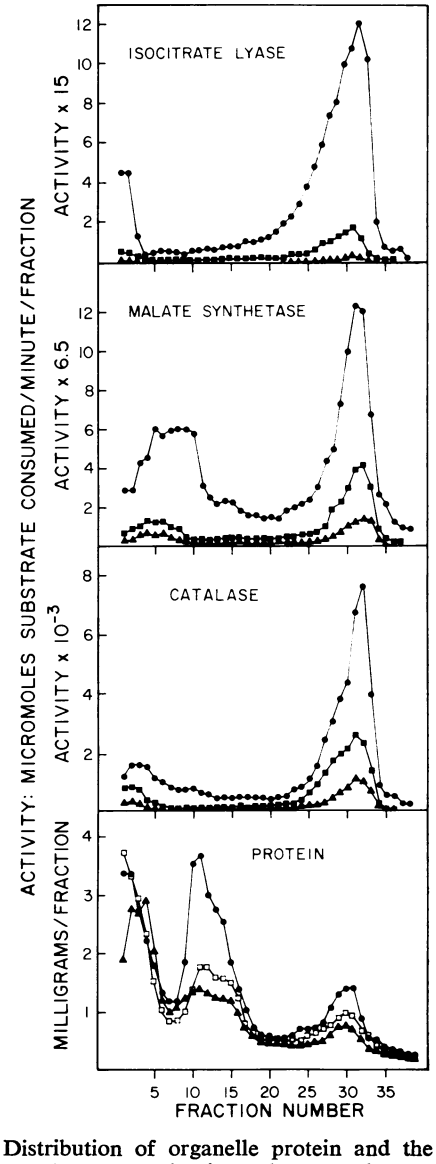

FIG. 3. Distribution of organelle protein and the activities of the glyoxysomal enzymes, isocitrate lyase, malate synthetase, and catalase after sucrose density gradient separation of the crude particulate fraction from the cotyledons of 4- (●), 8- (■), and 12- (▲) day-old dark-grown watermelon seedlings. Gradients representative of each stage of development are superimposed so that 42% sucrose (1.19 g/cm³) is aligned at fraction 14 and 54% sucrose (1.25 g/cm³) is aligned at fraction 31. Each distribution profile represents the recovery from 640 cotyledons.

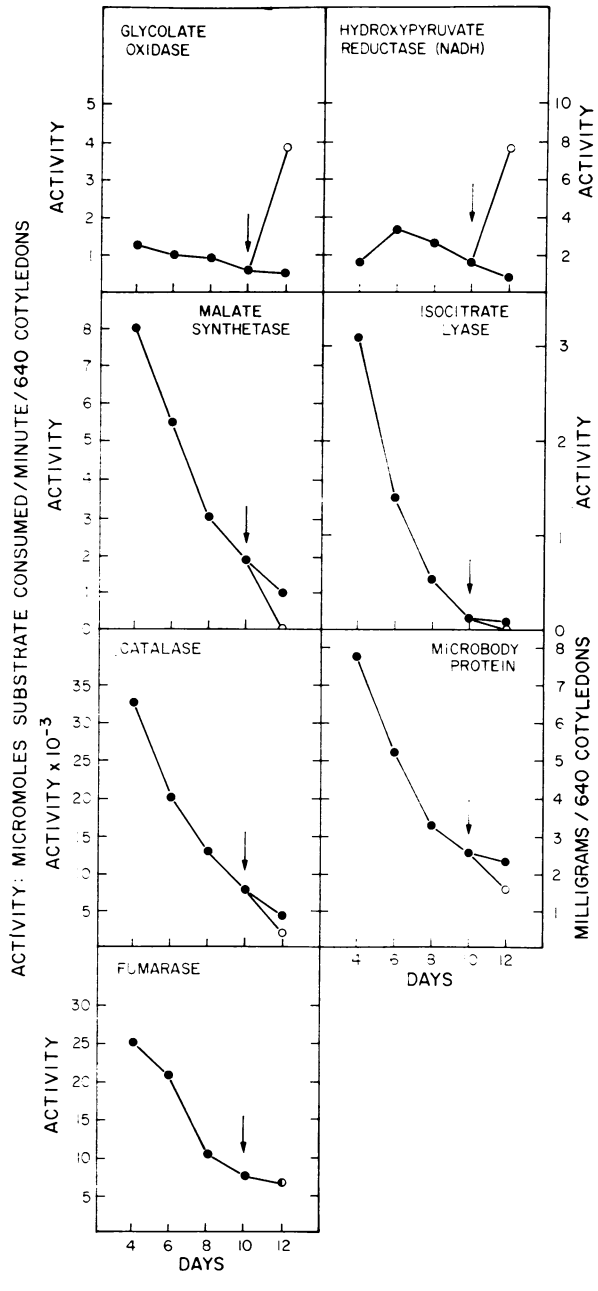

FIG. 4. Effect of light on the developmental changes in fumarase activity and the activities of glycolate oxidase, hydroxypyruvate reductase, malate synthetase, isocitrate-lyase, catalase and of microbody protein in the cotyledons of germinating watermelon seedlings between the ages of 10 and 12 days. Dark-grown seedlings (●), light-treated seedlings (○), arrow indicates the age at which the seedlings were transferred to light.

The mitochondrial enzyme, fumarase, is included in this study to demonstrate the selective nature by which light effects various developmental changes in the cotyledon. Whereas the levels of particulate glyoxysomal and peroxisomal activities and microbody protein are altered when the watermelon seedling is transferred to light, particulate fumarase activity is virtually unaffected (Fig. 4).

Effect of Light on Organelle Development in the Cotyledons of 4-day-old Dark-grown Seedlings. Microbody development

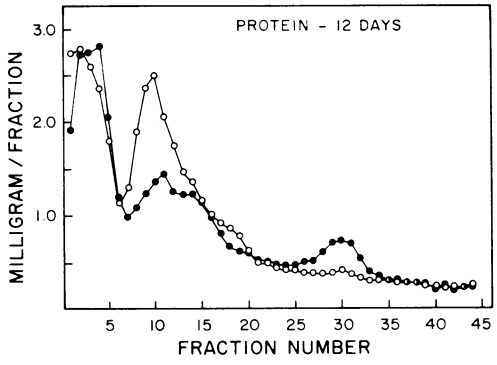

FIG. 5. Distribution of protein after sucrose density gradient separation of the components of the crude particulate fractions from the cotyledons of 12-day-old dark-grown seedlings (●) and 12-day-old light treated seedlings (10 days dark + 2 days light) (○). Gradients representative of preparations from each stage of development are superimposed so that 42% sucrose (1.19 g/cm^3) is aligned at fraction 14 and 54% sucrose (1.25 g/cm^3) is aligned at fraction 30. Each distribution profile represents the particulate protein recovered from 640 cotyledons.

was also studied at the age when peroxisomes are formed in the presence of functionally active glyoxysomes. The activity of the peroxisomal enzymes, glycolate oxidase, and hydroxypyruvate reductase in the microbody fraction increase dramatically upon illumination (Fig. 6). Conversely, the microbody activity of isocitrate-lyase and malate synthetase disappear at an accelerated rate compared to that found in the dark-grown seedlings.

The same accelerated demise is found in catalase activity and microbody protein (Fig. 6). The magnitude of these losses closely parallels the disappearance of glyoxysomal activity. Fumarase activity in the mitochondria behaved quite differently. As shown in Figure 6, exposure to light at day 4 prevented most of the loss in activity which occurred in the dark controls.

Figure 7 illustrates the differences in microbodies and other particulate fractions recovered from light- and dark-grown seedlings. This figure shows clearly the increases in particulate protein due to the chloroplast development. In addition, the loss of microbody protein is striking (Fig. 7).

DISCUSSION

The experimental data in this paper bear on the question whether the changes in microbody function during the greening of cucumber cotyledons are due to the modification of existing microbodies (glyoxysomes) or to the destruction of glyoxysomes and the production of a different class of microbodies, the leaf peroxisomes. Based largely on information from electron micrographs, Newcomb's group (33) has argued for the first possibility.

It should be noted that this model entails the selective destruction, within the microbodies, of all of the enzymes of the glyoxylate cycle and those associated with fatty acid breakdown, and the simultaneous acquisition of enzymes typical of leaf peroxisomes such as glycolate oxidase and hydroxypyruvate reductase. Because it is established that much of the cellular catalase is lost during the transition (20, 27, 33), a *partial* destruction of catalase within the glyoxysome must be invoked.

The other possibility, which mechanistically appears infinitely simpler, requires that the glyoxysomes are destroyed after fat utilization is complete (as indeed they are in fatty endosperm tissue [13]) and that leaf peroxisomes are produced during greening (just as they are in the development of normal leaves [11]).

A major point made by the Newcomb group is that the electron micrographs failed to reveal evidence of glyoxysome destruction or of new microbody synthesis during the acquisition of peroxisomal enzymes (33). For quantitative evidence they provide counts of microbody profiles in electronmicrograph sections, and conclude from these that the fall in numbers of microbodies is not commensurate with the loss of glyoxysomal enzyme activities which occurs when the seedlings

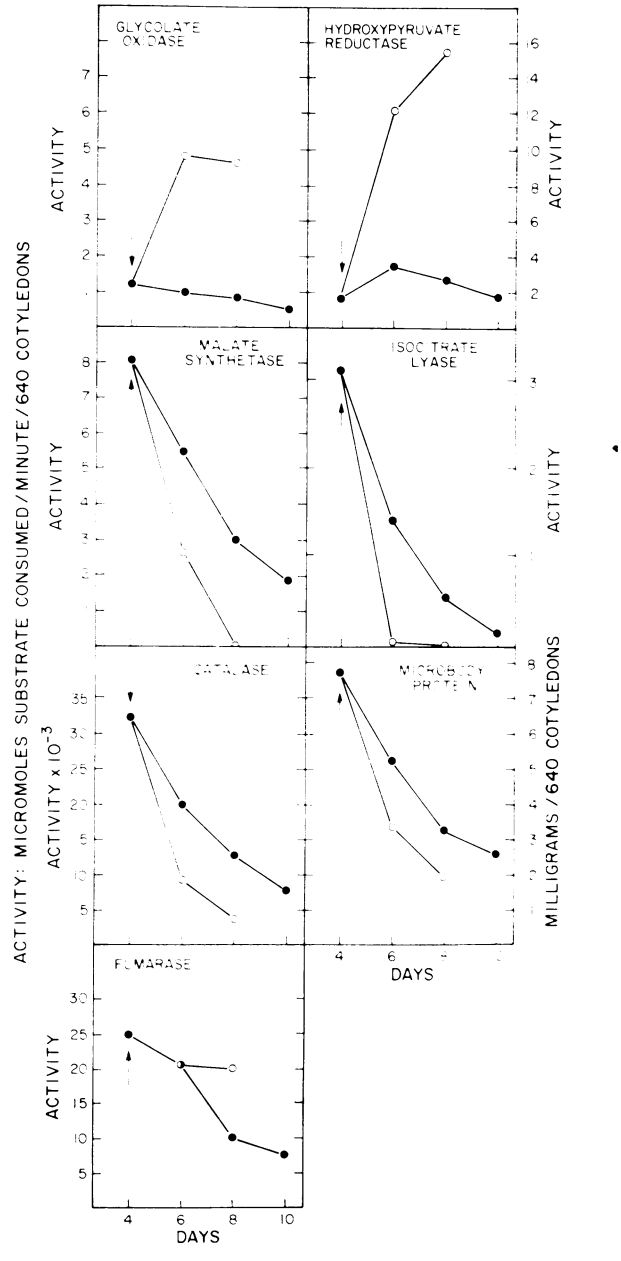

FIG. 6. Effect of light on the development changes in the mitochondrial fraction fumarase activity and the microbody fraction activities of glycolate oxidase, hydroxypyruvate reductase, malate synthetase, isocitrate-lyase, catalase, and of microbody protein in the cotyledons of germinating seedlings between the ages of 4 and 8 days. Dark-grown seedlings (●), light-treated seedlings (○), arrow indicates the age at which the seedlings were transferred to the light.

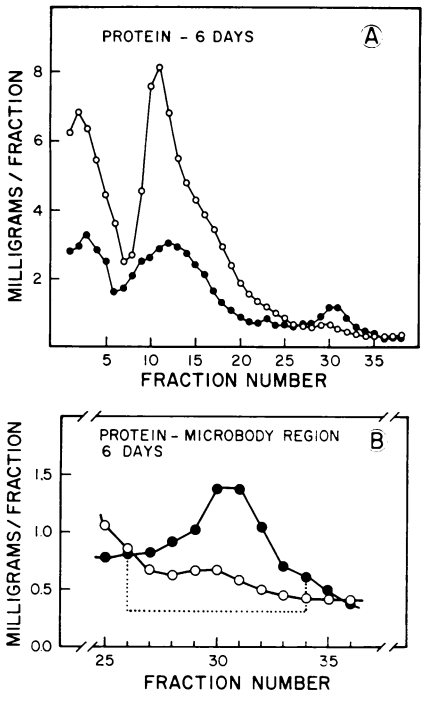

FIG. 7. A: Distribution of protein after sucrose density separation of the components of the crude particulate fraction from the cotyledons of the 6-day-old dark-grown seedlings (●) and 6-day light-treated seedlings (4 days dark + 2 days light) (○——○). Gradients representative of each stage of developement are superimposed so that 42% sucrose (1.19 g/cm³) is aligned at fraction 14 and 54% sucrose (1.25 g/cm³) is aligned at fraction 30. Each distribution profile represents the recovery from 640 cotyledons. B: A detailed comparison of the protein distribution in the microbody region. The area enclosed by the curves and the dotted line indicates the region of the gradient used to estimate the protein content in the fraction extracted from the cotyledons of the dark-grown (●) and light-treated (○) seedlings.

are maintained in darkness. No information was provided on changes in numbers of microbodies during the crucial time of transition or on the numbers existing after glyoxysomal function was completed and leaf peroxisomes were fully developed.

Some question may be raised for the data for the dark-grown material. It is clear from the electron micrograph in Fig. 17 of Trelease *et al.* (33) that storage lipid was still present in the oldest cotyledons examined (10 days). Further, almost one-half of the peak level of malate synthetase was present in the 10-day-old cotyledons. Thus it is not surprising that substantial numbers of glyoxysomes remained at this stage in their material.

Although we have used watermelon in our experiments, the behavior is generally similar to the cucumber used by the Newcomb group. It should be emphasized that all of our seedlings were grown under rigidly controlled conditions and that samples were selected for uniformity of size at precisely timed intervals (20). The growth characteristics of the material have been described; under our conditions storage lipid has been completely utilized after 8 days in darkness (20). The activities of malate synthetase and isocitrate lyase rise to a sharp peak at 4 days and decline to very low levels by day 10. (We have never observed uncoordinated changes in these activities of the kind shown by Newcomb's group [33] in their Fig. 13.) The provision of light at any time after 4 days results in an accelerated decline in these activities and in that of catalase, and at the same time the activities of the glycolate oxidase and hydroxypyruvate oxidase are strikingly increased (20).

Two important observations in the present paper are (*a*) that the declines in the activities of glyoxysomal enzymes are shown to be due to losses in activity from the microbody fraction, and that the total amount of microbody protein shows a corresponding decline; (*b*) the amount of microbody protein after the leaf peroxisomes are fully developed is less than one-fourth of that present when glyoxysomal activity is at its height (4 days). These changes are not due to changes in fragility of microbodies during growth of the cotyledon since, as we have shown, the percentage recovery of microbody enzymes in the particulate form does not change appreciably. Moreover, because there is no evidence for size changes or differences in sedimentation behavior of the microbodies during the transition, we conclude that the numbers of microbodies per cotyledon decrease during the declining phase of fat utilization and that the numbers present after greening has occurred are only a fraction of those present at the 4-day stage. It follows then that no model for the transition of microbodies is tenable which holds that any sizeable fraction of the glyoxysomes is transformed into leaf peroxisomes.

In support of the proposition that the decline in numbers of glyoxysomes once begun, continues to their ultimate exclusion and that none are transformed into leaf peroxisomes, the following considerations are pertinent. (*a*) Leaf peroxisomes are formed in the cotyledons regardless of whether they are allowed to green at the time when glyoxysomes are present in their largest numbers or when, after withholding light for 10 days, the level of microbody protein has declined drastically and the glyoxysomal enzymes have essentially disappeared. (*b*) The provision of light results not only in the accelerated loss of enzymes of the glyoxylate cycle but also of catalase from the microbodies.

Leaf peroxisomes are formed when light is provided but the amount of protein they represent is so small that it is insufficient to offset the loss in microbody protein due to glyoxysome decline. The catalase response is interpreted similarly; the synthesis of catalase and its incorporation into developing leaf peroxisomes induced by light is insufficient to offset the loss due to the accelerated destruction of glyoxysomes.

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