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Development of Enzymes in the Cotyledons of Watermelon Seedlings[1]

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ABSTRACT

Changes in hypocotyl length, cotyledon weight, lipid content, chlorophyll content, and capacity for photosynthesis have been described in seedlings of *Citrullus vulgaris*, Schrad. (watermelon) growing at 30 C under various light treatments. Corresponding changes in the levels of 19 enzymes in the cotyledons are described, with particular emphasis on enzymes of microbodies, since during normal greening, enzymes of the glyoxysomes are lost and those of leaf peroxisomes appear. In complete darkness enzymes of the glyoxysomes reach a peak at 4 days and decline as the fat is depleted. Enzymes of mitochondria and of glycolytic pathways also peak at 4 to 5 days and either remain unchanged or decline to a lesser extent. Exposure to light at 4 days, when the cotyledons emerge, results in a selectively greater destruction of enzymes of the glyoxylate cycle; chlorophyll synthesis and capacity for photosynthesis increase in parallel, and there is a striking increase in the activities of chloroplast enzymes and in those of the leaf peroxisomes, hydroxypyruvate reductase and glycolate oxidase. The reciprocal changes in enzymes of the glyoxysomes and of leaf peroxisomes can be temporally dissociated, since even after 10 days in darkness, when malate synthetase and isocitrate lyase have reached very low levels, hydroxypyruvate reductase and glycolate oxidase increase strikingly on exposure to light and the cotyledons become photosynthetic. Furthermore, the parallel development of enzymes of leaf peroxisomes and functional chloroplasts is not immutable, since hydroxypyruvate reductase and glycolate oxidase activity can be elicited in darkness following a 5-minute exposure to light at day 4 while chlorophyll does not develop under these conditions.

During the early growth of cucurbit and some other fatty seedlings, an interesting transition occurs in the metabolism of the cotyledons. For the first 5 to 6 days, the utilization of stored fat and its conversion to sucrose is the dominant event. Under normal conditions the cotyledons have emerged into the light by this time and they expand into fully functional leaves as the fat reserves are depleted. Thus the onset of photosynthetic metabolism is reciprocally related to the decline of fat metabolism.

Changes in enzyme activities during greening of normal leaves have been examined (7–11, 23, 24, 33, 34), and some information is available for the special case of cotyledons of fatty seedlings (14, 17, 22, 32, 35–37). Particular interest attaches to those enzymes characteristic of microbodies, since during fat utilization one class of microbody, the glyoxysome, is present, whereas during greening, leaf peroxisomes appear.

An increase in glycolate oxidase activity accompanying the decline of isocitrate lyase has been demonstrated in cotyledons of sunflower grown under light-dark cycles (14). The effect of transfer to light at various stages of growth in the dark on glycolate oxidase, hydroxypyruvate reductase, catalase, and isocitrate lyase have been described in this material (32). In cucumber cotyledons too, changes in enzymes of the glyoxylate cycle and those of leaf peroxisomes have been measured during greening under light-dark cycles and after transfer to light after 5 days in darkness (35). Furthermore, it is known from the work of Van Poucke and his associates (36, 37) that the development of glycolate oxidase and glyoxylate reductase in cotyledons of *Sinapis* seedlings is under the control of the phytochrome system as it is in the young developing leaves of *Phaseolus* (11, 23) and also, apparently, of wheat (8).

It is clear from these reports and our own previous work with watermelon cotyledons (20, 21, 27, 28), that during normal development and greening, the loss of enzymes of the glyoxylate cycle is accompanied by striking increases in the levels of glycolate oxidase and hydroxypyruvate reductase. However, the two aspects of the transition can be experimentally separated by withholding light until later stages of germination; under appropriate conditions the development of the latter enzymes can be elicited by applying light to seedlings which have been grown in darkness until the rate of fat utilization and the levels of glyoxysomal enzymes have declined.

We present here day-to-day changes in the activities of microbody enzymes in watermelon seedlings grown under rigidly controlled conditions and selected for uniformity. These changes are placed in the context of the changing physiology of the cotyledons by data on the growth, fat content, chlorophyll content, and capacity for photosynthesis. Corresponding changes in enzymes concerned with other metabolic sequences and the response to a variety of light treatments are described.

MATERIALS AND METHODS

Plant Material and Growth Conditions. Watermelon seeds (*Citrullus vulgaris*, Schrad., purchased from Vaughn Seed Co., Ovid, Mich.) were soaked for 24 hr in running water and sown in boxes on a vermiculite-soil mixture (2:1) (27). The seeds were covered with 2 to 3 cm of vermiculite, thoroughly moistened, and placed in a Controlled Environment seed germinator, at 30 C and 60% relative humidity. To insure that the germinating seedlings were grown in complete darkness, the

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boxes were covered with aluminum foil, secured by masking tape.

At 4 days or 10 days from the start of soaking, the dark-grown seedlings were exposed to continuous illumination provided by Sylvania cool white fluorescent tubes supplemented with 100 w incandescent white light at a total average intensity of 5.2×10^4 ergs per cm^2 per sec. To protect the illuminated seedlings from desiccation, the boxes were covered with a glass plate, one-sixteenth inch thick.

Selection of Plant Material. At intervals from the start of soaking, seedlings were selected for uniformity by both chronological age and hypocotyl length. The age of the seedlings was defined as the number of days from the beginning of soaking, and dark-grown seedlings with the following hypocotyl lengths were used: day 3 (1–3 cm radicle), day 4 (5–7 cm), day 5 (8–13 cm), day 6 (14–20 cm), day 7 (20–22 cm), day 8 ($\geqslant$22 cm), day 10 ($\geqslant$24 cm), day 12 ($\geqslant$25 cm). Seedlings whose light treatment commenced at day 4 were identified by India ink marks at the base of the hypocotyl, and seedlings whose light treatment commenced at day 10 were selected if their hypocotyl length was $\geqslant$25 cm.

Estimation of Total Lipid. Samples of 20 to 40 cotyledons were homogenized in 80% (v/v) ethanol in a VirTis 45 homogenizer at full speed for 1 min. The homogenate was filtered through a Whatman No. 1 filter, and the residue was washed three times with hot 80% ethanol. The residue was dried at 60 C for 1 hr and extracted with petroleum ether (boiling point 60–75 C) in a Soxhlet apparatus for 12 to 16 hr. The filtrate from the ethanol extraction was taken to dryness under reduced pressure at 40 C, dissolved in water, and extracted three times with equal volumes of ethyl ether. The ether fractions were combined and brought to dryness in a weighed vessel under reduced pressure at 40 C. The lipid fraction was then oven-dried at 80 C for 24 hr and weighed.

Dry Weight. Samples of 20 to 40 cotyledons were detached from the seedlings, dried for 96 hr at 80 C, and weighed.

Measurement of Photosynthesis by the Greening Cotyledons. Photosynthesis was measured as the rate of $^{14}CO_2$ fixation in a closed system. The apparatus consisted of a Plexiglas chamber for the plant tissue (inner dimensions 5 × 5 × 25 cm), a $^{14}CO_2$ generating chamber, a gas mixing chamber, a gas circulating pump, a needle valve, and a flowmeter. The chamber was illuminated from above by a 300-w photoflood lamp at an intensity of 1.5×10^5 ergs per cm^2 per sec. The temperature of the chamber during the fixation period was 30 C.

At suitable intervals during the greening of the cotyledons, 10 seedlings were harvested, the hypocotyl was cut 2.5 cm from the apex, and the primary leaves were discarded. The seedlings were placed into the apparatus in 5-ml beakers containing 2.5 mM potassium phosphate solution at pH 3.5. With the apparatus open to air and a flow rate of 3 liters per min, the cotyledons were preincubated for 15 min. The apparatus was purged and equilibrated with a calibrated air mixture of 380 μl/l of CO_2 at a flow rate of 3 liters per min. The system was closed and measurement of photosynthetic CO_2 fixation was initiated by injecting 5 μc of $NaH^{14}CO_3$ (20 mc/mmole prepared by ICN) into 1 ml of 1 N HCl solution in the gas-generating chamber. The tissue was exposed to $^{14}CO_2$ gas for varying lengths of time (from 10–30 min), removed from the chamber, and thoroughly extracted with 80% boiling ethanol. The extract was brought to dryness under reduced pressure at 40 C, and taken up in 25 ml of 80% ethanol. Samples of 50 to 300 μl were assayed for ^{14}C in a scintillation fluid composed of 6 g of 2,5-diphenyloxazole and 100 g of naphthalene in 1.0 liter of dioxane. The rate of CO_2 fixation was expressed as cpm fixed per hr per cotyledon; 10^6 cpm represent 1.77 μmoles CO_2.

Preparation of Cell-free Extracts. Twenty cotyledons were removed and placed into 20 ml of chilled grinding medium containing 10 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM EDTA, 165 mM Tricine buffer, pH 7.5. The tissue was homogenized for 60 sec using a VirTis 45 homogenizer at full speed. Two ml of 5% Triton X-100 solution was added to the grinding medium and allowed to stand for 20 min with occasional stirring. The homogenate was centrifuged at 20,000*g* to remove cell debris. After centrifugation, the floating fat was removed, and the resultant supernatant was used for enzyme assays. The detergent treatment released virtually all activity from the cell debris. 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 (6), malate synthetase (18), citrate synthetase (18), glycolate oxidase (3), hydroxypruvate reductase (27, 38), catalase (13, 25), fumarase (31), succinate dehydrogenase (16), NADP-linked isocitrate dehydrogenase (30), NAD-linked malate dehydrogenase (29), NAD-linked triose-P dehydrogenase (15), NADP-linked triose-P dehydrogenase (15), 3-phosphoglycerate kinase (15), triose-P isomerase (2), aldolase (12), enolase (4), phosphoglycerate mutase (4), pyruvate kinase (5), NADP-linked glucose-6-P dehydrogenase (19). Chlorophyll was assayed according to Arnon (1) and MacKinney (26).

RESULTS AND DISCUSSION

Growth and Development of the Watermelon Seedling. There are distinctive relationships between the chronological age and the development of germinating watermelon seedlings when they are grown under carefully controlled conditions of temperature, humidity, and illumination. The changes in morphology and physiology with age are summarized in Figure 1.

Growth in Darkness. When the seedling germinates, the radicle emerges at day 3 and hypocotyl elongation begins. In darkness, rapid hypocotyl elongation takes place between day 4 and day 7, after which elongation continues at a slow rate.

Rapid hypocotyl elongation is accompanied by a rapid loss in the dry weight of the cotyledons. Between day 0 and day 3, there is almost no loss in dry weight. From day 3 to day 7, the bulk of dry weight is lost, and from day 8 to day 12, the dry weight declines very slowly.

Most of the loss of cotyledon dry weight is due to the utilization of the stored lipid. This begins at day 3 and is nearly completed by day 7. The lipid is converted into carbohydrates which are rapidly translocated from the cotyledons and used by the growing seedling axis.

There is no significant change in fresh weight of the cotyledons throughout the development of the seedling in darkness. Microscopic studies show that at day 4, the cell is filled predominantly with storage lipid bodies; by day 10 the volume previously occupied by the lipid bodies is replaced by a large central vacuole.

Changes Initiated by Light. The seedling emerges from the soil, and the cotyledons are exposed at the age of 4 days. When 4-day-old dark-grown seedlings are transferred to continuous illumination, dramatic changes in the morphology of the seedling are initiated. The fresh weight of the cotyledons increases rapidly due to expansion. The rates of hypocotyl elongation and loss of cotyledon dry weight are slowed down while the rate of lipid utilization increases slightly. The cotyledons turn green and become photosynthetic (Fig. 1). The apical meristem is activated, and the first and second true leaves begin to develop.

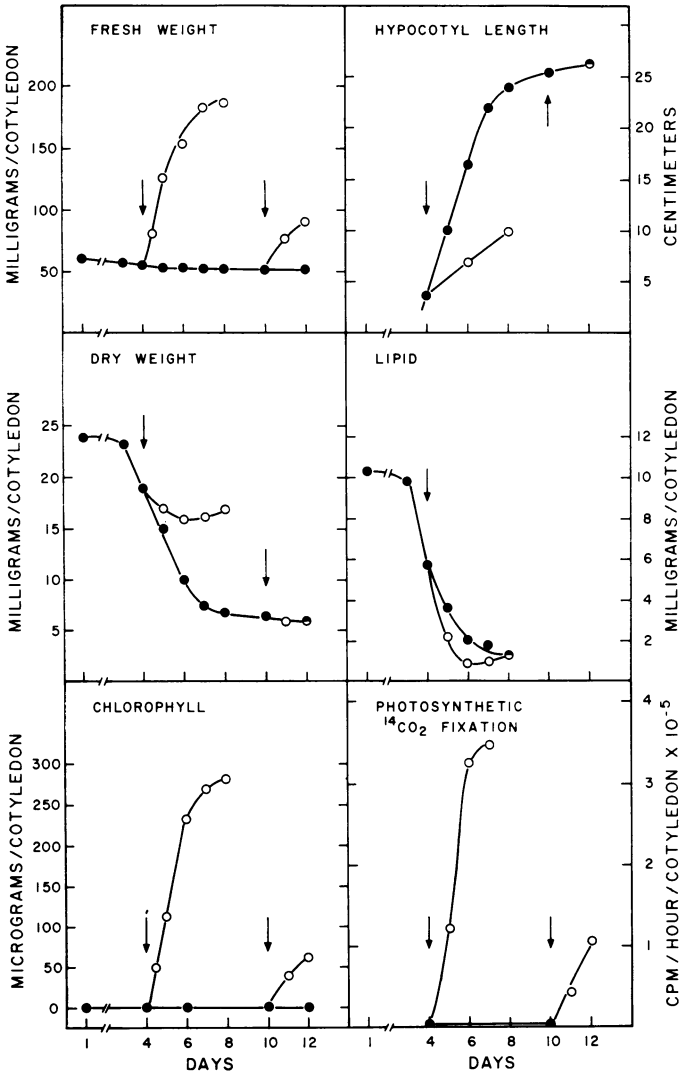

FIG. 1. Growth and development of germinating watermelon seedlings. Effect of age on the development of hypocotyl length and cotyledon fresh weight, dry weight, lipid content, chlorophyll content, and photosynthesis in dark- and light-grown seedlings. ●: Seedlings grown in darkness; ○: seedlings transferred to continuous illumination at 4 days and 10 days as indicated by arrows.

The cotyledons of seedlings maintained in darkness until day 10, develop into functional leaves under continuous illumination in a pattern similar to that measured in younger seedlings. The cotyledons expand and become leaf-like, although no measurable changes can be detected in cotyledon dry weight or hypocotyl elongation. Even at this late stage in development, 10-day-old cotyledons are still metabolically active as shown by their capacity to become photosynthetic (Fig. 1).

Development of Enzyme Activity in Cotyledons of Dark-grown Seedlings. The developmental patterns of enzymes of the watermelon cotyledon are also related to the age of the seedling. Under carefully controlled growth conditions, these patterns can be readily reproduced.

Enzymes involved in various aspects of carbohydrate metabolism have been investigated. The enzymes chosen are characteristic of glyoxysomes, leaf peroxisomes, mitochondria, chloroplasts, and the soluble fraction. Characteristic patterns of enzyme development are shown in Figure 2.

When seedlings are grown in darkness, the activity of many enzymes rises dramatically, from low levels at day 2, to a maximum at day 4 or day 5. This is followed by a period of declining enzyme activity. This pattern is shown by malate synthetase, isocitrate lyase, fumarase, and NADP-linked glucose-6-P dehydrogenase.

Some enzymes, such as enolase, reach their maximum activity at day 4 or day 5 and persist for periods up to day 8 with little or no loss in activity.

Others, such as glycolate oxidase, are found in relatively low levels throughout the developmental period of dark-grown seedlings. No activity of the NADP-linked triose-P dehydrogenase was detected in cotyledons of dark-grown seedlings.

Development of Enzyme Activities in the Cotyledons when 4-day-old Dark-grown Seedlings are Exposed to Continuous Illumination. Continuous illumination of dark-grown seedlings effects marked changes in the developmental pattern of several enzymes. Certain enzymes such as malate synthetase are selectively destroyed. In contrast, glycolate oxidase and NADP-

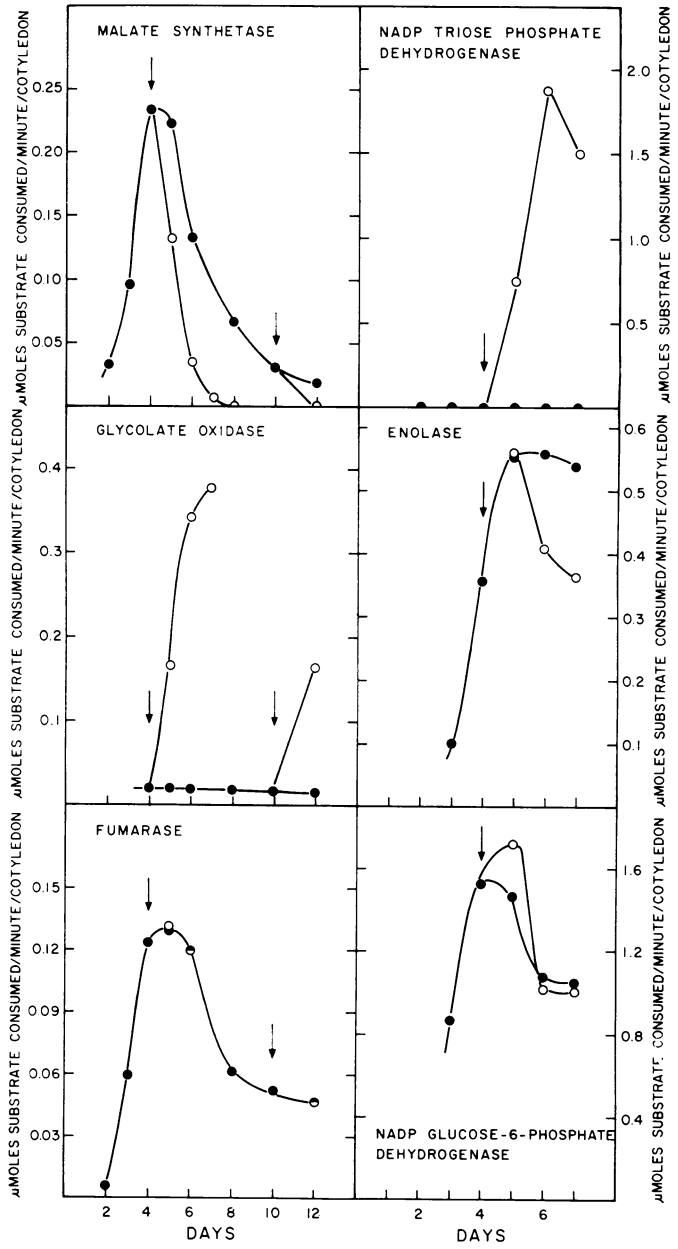

FIG. 2. Developmental changes of enzymes in the cotyledons of dark- and light-grown watermelon seedlings. Symbols as in Figure 1.

Table I. *Enzyme Activities at Different Stages of Development of Watermelon Cotyledons*

	Intracellular Location	Activity in Cotyledons of 4-day-old Dark-grown Seedlings	Part of 4-day Activity Remaining at 7 Days	
			7 days dark	4 days dark + 3 days light
		μmoles substrate consumed/min·cotyledon)	%	
Isocitrate lyase	Mb(G)[1]	0.16	19	0
Malate synthetase	Mb(G)	0.23	51	4
Glycolate oxidase	Mb(G, LP)	0.020	100	1100
NADH-hydroxypyruvate reductase	Mb(G, LP	0.19	105	2260
Catalase	Mb(G, LP)	17,100	49	35
Fumarase	M	0.13	64	68
Succinate dehydrogenase	M	0.042	74	78
Citrate synthetase	Mb(G), M	0.11	91	120
Malate dehydrogenase	Mb(G, LP), M, S	47	66	60
NADP-isocitrate dehydrogenase	M, S	0.05	50	80
NADP-triose phosphate dehydrogenase	C	ND[2]	ND	(10.2)[3]
Phosphoglycerate kinase	C, S	5.5	102	310
Triose phosphate isomerase	C, S	21	76	160
Aldolase	C, S	0.16	106	380
Enolase	S	0.39	140	102
Phosphoglycerate mutase	S	0.75	79	87
NAD-triose phosphate dehydrogenase	S	2.2	45	50
Pyruvate kinase	S	0.17	88	222
NADP-glucose-6-P dehydrogenase	S	1.52	76	65

[1] Mb (microbody), G (glyoxysome), LP (leaf peroxisome), M (mitochondria), C (chloroplast), S (soluble).

[2] ND: not detectable.

[3] Value in parenthesis denotes activity recovered from 7-day-old light-treated tissue.

linked triose-P dehydrogenase rise dramatically. Fumarase and NADP-linked glucose-6-P dehydrogenase characterize a third developmental pattern, since their activities remain unchanged on transfer to light.

Other enzymes, such as enolase, follow essentially the same developmental pattern as those in dark-grown seedlings for 1 or 2 days before any differences can be measured.

A summary of the behavior of the enzymes studied is presented in Table 1. In this table a comparison is given of the activities found in cotyledons of 4-day and 7-day dark-grown seedlings and 7-day light-treated seedlings (4 days dark + 3 days light). The cytoplasmic location of each enzyme is given. The activities of isocitrate lyase and malate synthetase, enzymes unique to glyoxysomes, and of catalase, an enzyme common to both glyoxysomes and leaf peroxisomes decline more rapidly in seedlings exposed to continuous illumination than in seedling grown in darkness.

In contrast the activity of glycolate oxidase and hydroxypyruvate reductase, present in leaf peroxisomes, increases dramatically in the cotyledons of seedlings exposed to light. A similar dramatic rise in activity is seen in those enzymes associated with the chloroplasts. These enzymes include NADP-linked triose-P dehydrogenase, phosphoglycerate kinase, triose-P isomerase, and aldolase.

Enzymes whose developmental patterns are essentially no different in dark-grown and light-treated seedlings include the mitochondrial marker enzymes: fumarase and succinate dehydrogenase; the soluble glycolytic-gluconeogenic enzymes; phosphoglycerate mutase and NAD-linked triose-P dehydrogenase; and the soluble enzyme of the oxidative pentose-P sequence, NADP-linked glucose-6-P dehydrogenase. The activity of another glycolytic enzyme, pyruvate kinase, rises surprisingly under the influence of light.

Relatively small effects of transfer of light are shown on the activities of citrate synthetase, and NADP-linked isocitrate dehydrogenase.

Thus, three major patterns can be recognized in the response of enzyme activities on transfer to light. Enzymes of the glyoxysomes are selectively destroyed, whereas there is a marked increase in the activity of peroxisomal and chloroplastic enzymes. Mitochondrial and soluble enzymes are generally unaffected by transferring the seedling to light.

Development of Enzyme Activities in Cotyledons when 10-day-old Dark-grown Watermelon Seedlings are Exposed to Continuous Illumination. As shown in Figure 1 the utilization of stored fat in dark-grown seedlings was completed by day 8. After this time there is little change in weight of the cotyledon or in hypocotyl length. In the cotyledons of these seedlings, malate synthetase activity declined to very low levels following the completion of fat utilization. In contrast, little change in the levels of glycolate oxidase were measured between day 4 and day 12.

When 10-day-old dark-grown seedlings were transferred to continuous light, there was the same kind of dramatic increase in glycolate oxidase activity as observed when 4-day-old dark-grown seedlings were transferred, and the cotyledons became green and photosynthetic (Fig. 1). The residual activity of malate synthetase declined to zero under the influence of light, but fumarase activity was virtually unaffected by the transfer.

Effect of Brief Light Exposures on Enzymes of Glyoxysomes and Leaf Peroxisomes. In the experiments showing that exposure to light induced the formation of enzymes of leaf peroxisomes in parallel with that of chlorophyll and the capacity for photosynthesis the earliest measurements were made 12 hr after transfer to light. In Figure 3 the early kinetics of the development of chlorophyll, glycolate oxidase, and hydroxy-

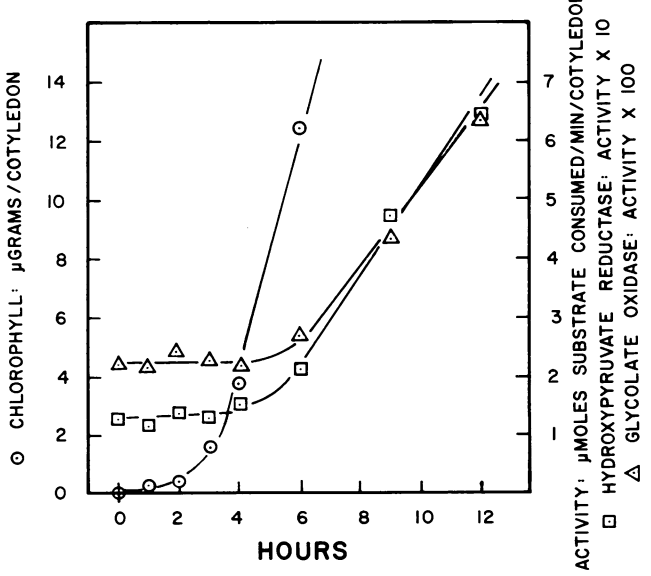

FIG. 3. Changes in the amount of chlorophyll (○) and in the activities of glycolate oxidase (△) and hydroxypyruvate reductase (□) in cotyledons immediately following the transfer of 4-dav-old dark-grown seedlings to light.

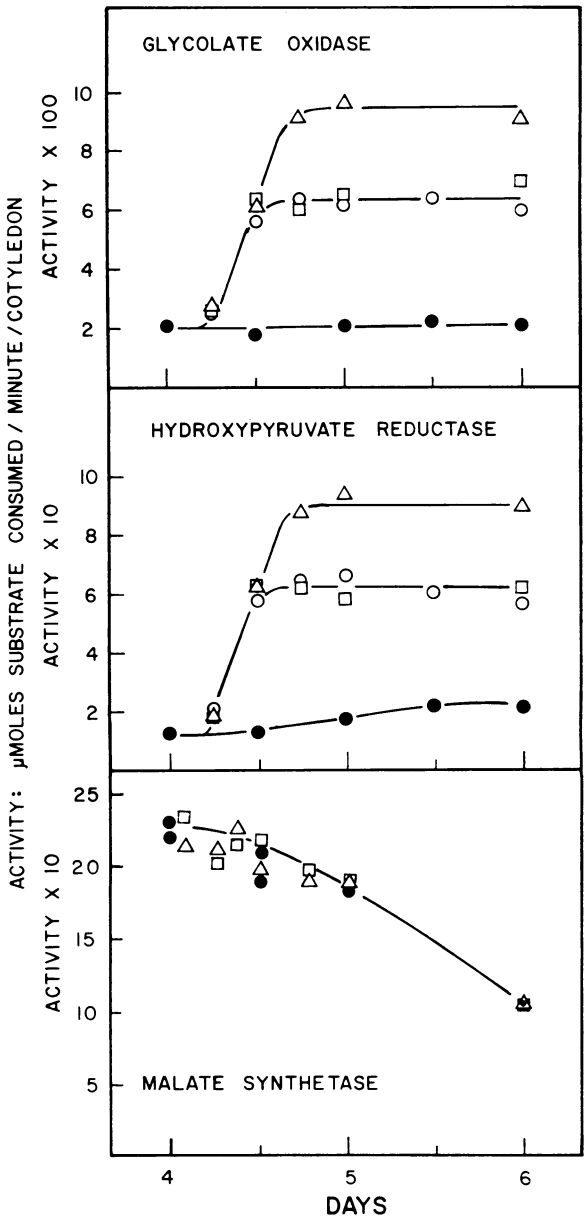

FIG. 4. Effect of brief periods of illumination on the development of glycolate oxidase, hydroxypyruvate reductase, and malate synthetase in 4-day-old seedlings. Illumination periods are indicated as follows: none (●); 5 min (○); 1 hr (□); 3 hr (△).

pyruvate reductase in continuous light is shown. Rapid accumulation of chlorophyll began after 3 hr under these conditions and continued at this rate for some 24 hr. A linear increase in the production of the two enzymes of the leaf peroxisomes began only after about 5 hr.

Nevertheless, as shown by the data in Figure 4, the formation of the two enzymes can take place in darkness following quite brief light treatment. A single 5-min exposure on day 4 resulted in the development of the two enzymes to levels some 25% of those eventually reached in continuous light. One hr of light had essentially the same effect, but after a 3-hr exposure the levels reached during subsequent growth in darkness were considerably higher, and similar levels were achieved when a single 5-min light exposure was given on each of days 4 and 5. These treatments are essentially ineffective in bringing about the synthesis of chlorophyll. Thus, immediately after a 5-min light treatment on day 4, the amount of chlorophyll was 0.2 μg per cotyledon and no increase followed in darkness. These levels are only a fraction of 1% of those achieved in continuous light (>280 μg per cotyledon, Fig. 1). Thus while the development of the enzymes of leaf peroxisomes and that of chlorophyll both require light, they are under separate control, and the enzymes can be produced in the absence of functional chloroplasts (8, 11, 23, 36, 37). It can also be seen from Figure 4 that the brief light treatments which elicit the development of the enzymes of the leaf peroxisomes have no effect on the rate of loss of the glyoxysomal enzyme malate synthetase. Catalase and isocitrate lyase were similarly unaffected, and it is of interest that these enzymes did not respond to the light treatments in the phytochrome experiments of Karow and Mohr (22) and Drumm (36).

CONCLUSIONS

The striking changes in morphology and development which are initiated when the cotyledons of 4-day-old watermelon seedlings are illuminated are accompanied by concomitant changes in enzymatic activity and function as follows: (*a*) enzymes of the glyoxylate cycle decline more rapidly than they do in continuous darkness, (*b*) enzymes of the tricarboxylic acid cycle and glycolytic pathways follow a similar pattern to that observed in continuous darkness; (*c*) enzymes of the chloroplasts and leaf peroxisomes increase strikingly in activity and the cotyledons become photosynthetic.

The fact that the changes described in (*c*) can be elicited after enzymes of the glyoxysomes have reached very low levels by withholding light until day 10 strongly suggests that a population of glyoxysomes is not a necessary prerequisite for the formation of leaf peroxisomes in the cotyledons; nor, of course, is this true in normal leaf development (9). Furthermore development of enzymes of the leaf peroxisomes in the cotyledons is under separate control from that of chloroplasts, since glycolate oxidase and hydroxypyruvate reductase increase strikingly in darkness following brief exposure of the 4-day cotyledons to light, whereas chlorophyll does not develop under these conditions.

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