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Regulation of Glyoxysomal Enzymes during Germination of Cucumber

I. DEVELOPMENTAL CHANGES IN COTYLEDONARY PROTEIN, RNA, AND ENZYME ACTIVITIES DURING GERMINATION[1]

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ABSTRACT

Developmental patterns of glyoxylate cycle and photosynthetic activities have been correlated with electrophoretic profiles of cotyledonary RNA and protein in both light- and dark-grown cucumber seedlings (*Cucumis sativus* L.) Cytoplasmic rRNA increases 10-fold between days 0 and 5, and the steepest increase coincides with the most rapid rise in activities of the glyoxysomal enzymes, isocitrate lyase and malate synthase. Chloroplast rRNA and ribulose bisphosphate (RuBP) carboxylase begin rising at day 3, followed about a day later by increases in glyoxylate reductase activity and chlorophyll content. Of these phototrophic indicators, only chlorophyll requires light for its initial appearance. Sodium dodecyl sulfate gel electrophoresis of total and soluble cotyledonary protein showed several developmental patterns, including: (*a*) progressive disappearance of storage protein present initially in particulate form; (*b*) appearance and subsequent disappearance of a family of polypeptides identified by molecular weight, developmental profile, and density gradient centrifugation as subunits of glyoxysomal enzymes; and (*c*) appearance and progressive increase (in both light- and dark-grown cotyledons) of the large and small subunits of RuBP carboxylase, as well as other polypeptides presumably of chloroplast and peroxisomal origin.

Seed germination in fat-storing species requires a functional glyoxylate cycle to effect net gluconeogenesis from the acetyl-CoA derived by β oxidation of storage triglycerides (3). Although much is already known about the developmental physiology of the glyoxylate cycle enzymes and the glyoxysomal compartment in which they are localized (3, 8, 14, 15, 29, 35), little is understood about the regulatory mechanisms which underlie the developmentally orchestrated expression of this specific metabolic capability. We are interested both in the level(s) at which the activities of glyoxysomal enzymes are regulated during germination of cucumber (*Cucumis sativus*) and also in the control mechanisms involved in the subsequent transition from heterotrophy to autotrophy that takes place in the cotyledon upon emergence and greening.

This paper describes changes in size, cell number, metabolic function, and enzyme activities which occur in cotyledons of light- and dark-grown cucumber seedlings and attempts to relate these changes to developmental changes in cotyledonary protein and RNA, thus providing a characterization of the system necessary for further, more specific studies on the regulation of the glyoxysomal and peroxisomal enzymes. A preliminary report of this work has already been presented (2).

MATERIALS AND METHODS

Culture Conditions. Seeds of *C. sativus* L. var. Long Green Ridge or Improved Long Green were cold-imbibed (16 hr at 4 C) in distilled H_2O, then planted at a depth of about 1 cm in trays of Vermiculite underlaid with soil. Trays were either kept in continuous darkness (dark-grown) or illuminated for 12 hr/day with a mixture of fluorescent and incandescent lamps at an approximate intensity of 6,500 lux (light-grown). The temperature was in both cases maintained at 26 to 28 C for 12 hr, followed by a night depression to 22 C for 12 hr. Germination time was measured in days from planting of the cold-imbibed seeds.

Harvesting of Cotyledons. Cotyledons were harvested at daily intervals from day 0 (cold-imbibed only) to day 7, with harvesting initiated about 1 hr after the onset of illumination for the light-grown seedlings. The number of cells/cotyledon was determined by the method of Brown and Rickless (4). Fresh weight was determined on samples of 40 cotyledons harvested directly into tared, stoppered glass bottles. Oven dry weights were determined after drying the same samples for 24 hr at 90 C. Cotyledons for all other assays were harvested onto dry ice and stored at −80 C.

Homogenate Preparation. Cotyledons (20–100/sample) were first ground to a thick paste in a precooled mortar and pestle, without addition of medium. A volume of 0.05 M K-phosphate (pH 7.5) calculated to yield a supernatant protein concentration of about 5 to 10 mg/ml was then added and grinding was continued. Aliquots of the homogenate were removed for protein and RNA assays, for electrophoretic analysis, and for quantitation of RuBPC[4] protein. The remainder was then centrifuged at 10,000*g* for 10 min at 4 C, and samples of the resulting supernatant were taken for protein assay, gel electrophoresis, and enzyme assays.

Enzyme, Protein, and Chl Assays. Literature procedures were followed for the assay of isocitrate lyase (5), malate synthase (5),

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[4] Abbreviations: RuBPC: ribulose bisphosphate carboxylase; IL: isocitrate lyase; MS: malate synthase; Cat: catalase; GR: NADH-glyoxylate (hydroxypyruvate) reductase; Cyt Ox: cytochrome *c* oxidase; TEMED: N,N,N′,N′-tetramethylethylenediamine; EGTA: ethylene glycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid.

catalase (27), NADH-glyoxylate (hydroxypyruvate) reductase (34, with glyoxylate as substrate), and Cyt *c* oxidase (29). Results were expressed as enzyme units/cotyledon. For catalase, 1 unit is the amount of enzyme required to degrade 50% of the available H_2O_2 in 100 sec; for the other enzymes, 1 unit corresponds to the conversion of 1 nmol of substrate into product/min. Chl was determined by the method of Arnon (1). Protein was assayed by the procedure of Lowry *et al.* (26), after initial precipitation and washing of the protein with 5% (w/v) trichloroacetic acid.

Lipid Assay. Total lipid was determined by a modification of the methanol-chloroform extraction procedure of Radin (28). Cotyledons (3 g) were chopped with razor blades and ground with 10 ml of methanol-chloroform (2:1, v/v). Following low speed centrifugation, the supernatant was decanted and the pellet reextracted by grinding again with methanol-chloroform. Supernatants from both extractions were shaken with an equal volume of 2 M KCl. After phase separation, the organic phase was drained into a preweighed beaker, and the aqueous phase was then washed repeatedly with methanol-chloroform. The pooled organic phases were evaporated to dryness, and the amount of extracted lipid was determined by weight difference.

Electrophoretic Analysis of Cotyledonary Proteins. Proteins present in the total homogenate and in the 10,000*g* supernatant were analyzed by electrophoresis on 15% SDS-polyacrylamide slab gels, according to the method of Laemmli (17). Electrophoresis was carried out at room temperature with a current of 10 mamp (5 mamp during sample run-in) until the bromphenol blue marker reached the bottom of the gel. Gels were stained with 0.1% Coomassie blue in 50% methanol-5% acetic acid for 1 hr at 50 C, then destained by repeated changes of 40% methanol-7% acetic acid.

Electrophoretic Assay for RuBPC. RuBPC protein was determined by electrophoretic fractionation of total homogenate protein under denaturing conditions, followed by recovery and quantitation of the stained protein from the appropriate excised gel region. Gels of 5% acrylamide, 0.125% bis-acrylamide, and 0.0875 M Tris-glycine (pH 9.5) were polymerized with TEMED and NH_4HSO_4, prerun for 30 min at 1 to 1.5 mamp/tube, and loaded with 100 to 200 μg of homogenate protein in 50 mM Tris-glycine (pH 9.5) containing 10% sucrose and bromphenol blue as marker. Gels were run at 1 to 1.5 mamp/tube for 2.5 hr and then stained for 20 hr in 0.5% Amido black 10B in 20% ethanol-7% acetic acid, followed by destaining in 20% ethanol-7% acetic acid. The carboxylase band (identified by coelectrophoresis of the purified enzyme) was excised from the gel and incubated overnight at 25 C in 1 ml of 1 N NaOH. The protein content of the NaOH extracts was quantitated from the *A* at 615 nm, using a standard curve prepared by electrophoresis and NaOH extraction of graded amounts (10–100 μg) of spinach D-ribulose-1,5-diphosphate carboxylase (Sigma Chemical Co). Values obtained can therefore be regarded as relative approximations only.

Electrophoretic Analysis of Proteins from Microbodies Isolated by Density Centrifugation. For analysis of organellar proteins, light-grown cotyledons from days 1 through 6 (5–8 g/day) were harvested fresh and homogenized by chopping with razor blades and subsequent grinding in a mortar and pestle with 2.5 volumes of 0.40 M sucrose in TKME buffer (0.05 M Tris-HCl [pH 7.2] at 25 C, 50 mM potassium acetate, 10 mM magnesium acetate, and 1 mM EGTA). The homogenate was filtered through Miracloth and centrifuged at 600*g* for 10 min. Thereafter 12.5 ml of the supernatant was layered onto a 45-ml linear 16 to 60% (w/w) gradient of sucrose in TKME buffer. Gradients were centrifuged at 58,400*g* for 4 hr in a Spinco SW 25.2 rotor at 4 C. Fractions (1.5 ml) were assayed for sucrose density (determination of a refractive index at 20 C), and for isocitrate lyase, malate synthase, Cyt *c* oxidase, and protein as described above. For SDS-polyacrylamide gel electrophoresis, the protein in each gradient fraction was precipitated with 80% (v/v) acetone, redissolved in sample buffer, and loaded on a per-cotyledon basis onto 15% SDS-polyacrylamide slab gels. To facilitate direct comparison of proteins present in the microbody region of the gradient at each day, fractions corresponding to a buoyant density of 1.26 g/cm^3 (peak activities of isocitrate lyase and malate synthase; one fraction/gradient) were analyzed in adjacent lanes of the same slab gel, with loading again on a per-cotyledon basis.

RNA Assay. Homogenate samples to be assayed for RNA were precipitated with an equal volume of cold 1 N perchloric acid, allowed to stand on ice for 60 min, and then centrifuged at 3,000*g* for 10 min. The pellets were washed twice by resuspension in 5 ml of 0.5 N perchloric acid, then twice with 5 ml of ethanol/chloroform-ether, 2:1:1 (v/v/v). The pellets were allowed to drain and were then dissolved in 1 ml of fresh 0.3 N NaOH. After incubation for 18 hr at 37 C, tubes were cooled on ice and neutralized with cold 70% perchloric acid. The resulting precipitate was removed by centrifugation (3,000*g* for 10 min) and the supernatant was assayed for RNA content by determination of A_{260}, assuming an *A* of 1.0 to correspond to an RNA concentration of 31.7 μg/ml.

Electrophoretic Quantitation of RNA. Cotyledonary RNA was extracted at 0 to 4 C by the phenol procedure of Leaver and Ingle (20). Fractionation was achieved by electrophoresis on 2.4% polyacrylamide gels (25 μg of RNA/gel) as described by Loening (22), but at 5 C to maintain the integrity of the 23S chloroplast rRNA (19). Gels were run for 4 hr with a current of 3 mamp/gel. The 7.5-cm gels were scanned at 265 nm using a Joyce Loebl UV scanner. To quantitate the various species of nucleic acids (25S and 18S cytoplasmic rRNA, 23S and 16S chloroplast rRNA, and 4S + 5S RNA), individual peaks were cut from the scans, weighed, and expressed as a fraction of the total area under the curve for a particular scan. Each such fraction was then multiplied by the total RNA content (in μg/cotyledon) determined as described above for cotyledons of the same stage and growth conditions.

RESULTS

Seeding Development. The characteristic appearance of light-grown cucumber seedlings is illustrated in Figure 1. To ensure maximum uniformity among cotyledons used for analyses, plants were selected to correspond as closely as possible to the morphological stages shown in Figure 1. Dark-grown seedlings were similar to those shown in Figure 1 for days 0, 1, 2, and 3, but thereafter were characterized by marked hypocotyl elongation, retention of the hypocotyl hook, and lack of cotyledonary expansion or greening.

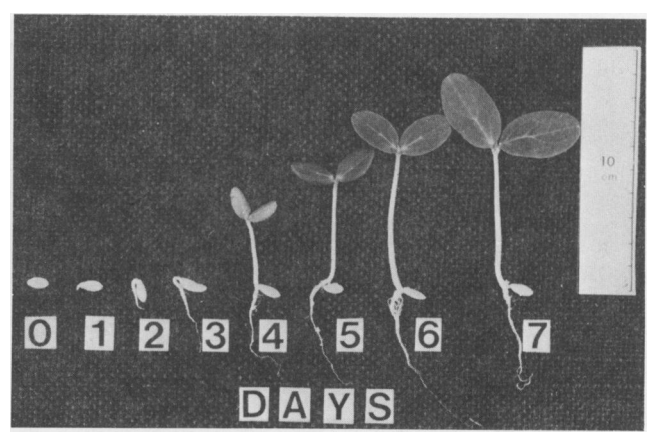

FIG. 1. Growth and development of light-grown cucumber seedlings. Seeds were cold-imbibed (16 hr at 4 C), then planted in Vermiculite and germinated under a 12/12 hr light/dark cycle, with time of germination measured in days from planting. Greening of cotyledons begins at the proximal end upon emergence from the seed coat at day 3 and is essentially complete by day 5.

Upon emergence from the seed coat, light-grown cotyledons undergo a rapid increase in size and fresh weight not seen in the dark (Fig. 2A); between days 1 and 7, fresh weight increases 10-fold in the light, but only 2-fold in the dark. This increase is due entirely to water uptake, as seen from the dry weight data (Fig. 2B), which actually decreases through day 4, reflecting the progressive depletion of both fat and protein stores. (The modest increase in weight seen in light-grown cotyledons after day 5 is presumably due to the accumulation of photosynthetically derived mass.) The decrease in cotyledonary dry weight is almost exactly balanced by an increase in the dry weight of the rest of the plant axis; the combined dry weight of axis plus two cotyledons is 22.6 mg at day 0, 22.1 mg at day 4 in the light, and 22.6 mg at either day 4 or day 7 in the dark.

Cell number remains constant through the time period under study. Ungerminated cucumber embryos contain about 6.78×10^5 cells/cotyledon, and this does not change significantly during the first 7 days of development in either light or dark. (Averaged over all stages and expressed as $\bar{x} \pm s_{\bar{x}}$, cell numbers for light- and dark-grown seedlings were, respectively, $6.78 \pm 0.08 \times 10^5$ and $6.84 \pm 0.15 \times 10^5$ cells/cotyledon.) This agrees well with electron microscopic studies (35) showing no cell division in cucumber cotyledons. Similar conclusions have been reached for cotyledons of other species (24, 36). Table I is a summary of the data for light-grown cotyledons, expressed on a per-cell basis (assuming 6.78×10^5 cells/cotyledon for all stages).

Heterotrophic Indicators. Developmental profiles for lipid utilization and for several glyoxysomal enzymes are shown in Figure 3. Lipid metabolism begins at day 1 and is essentially complete by day 6. This correlates well with the progressive loss of lipid bodies seen in ultrastructural studies (35). As expected (15, 29, 35), isocitrate lyase and malate synthase rise from near-zero levels in the ungerminated seed to peak activities at a time (day 4) which coincides with the period of most rapid lipid depletion. Activities then decrease rapidly and disappear within a week (slightly longer in the dark; about 10% of the peak activity of each enzyme is still left at day 8 in the dark). The same rise and fall of glyoxylate cycle activities are seen in half-cotyledons with and without the embryonic axis attached (D. G. Kerley and W. M. Becker, unpublished observations), confirming the findings of others (7, 32) that these enzymes are not under control of the axis. Catalase resembles isocitrate lyase and malate synthase in its activity profile during early stages, but retains about half of its peak activity at later stages, presumably because it is an enzyme common both to the glyoxysomes involved in heterotrophic metabolism during early stages and to the peroxisomes present at later stages.

Autotrophic Indicators. Several specific indicators of autotrophic function are presented in Figure 4. Their appearance coincides with the arrival of the seedling above ground and the emergence of the cotyledons from the seed coat. RuBPC precedes Chl by at least 24 hr in its initial appearance and, unlike Chl, does not depend upon light as a trigger, although illumination obviously affects the levels of RuBPC protein present at later stages (*i.e.* after day 4). This accords well with the report of Dockerty *et al.* (6) that light is not essential for RuBPC development in either the cotyledons or the endosperm of germinating castor bean. A similar pattern, though somewhat delayed in time, is seen for glyoxylate (hydroxypyruvate) reductase. Like RuBPC, this peroxisomal enzyme seems to depend upon light for attainment of full activity, but not for its initial appearance. Other workers have also reported that both this reductase and glycolate oxidase, a companion peroxisomal enzyme, can undergo at least some increase in activity in dark-grown cotyledons at a time in development when activity can be promoted by light (8, 9, 29–31, 33).

Cotyledonary Protein. The protein content of the cotyledonary homogenate and of the 10,000*g* × 10-min supernatant fraction is depicted as a function of germination time for light-grown seedlings in Figure 5A. In the dark (data not shown), the pattern is essentially the same, except that the decrease continues throughout the experiment, dropping by day 7 to 1.57 mg/cotyledon, com-

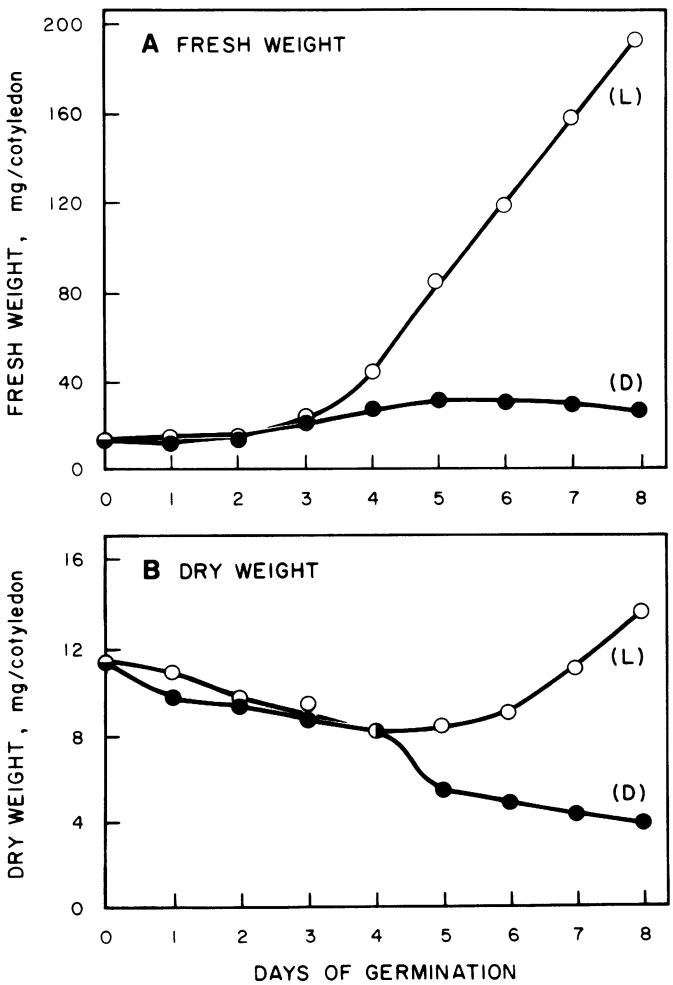

FIG. 2. Developmental changes in weight of cotyledons from cucumber seedlings grown in the light (○——○) or in the dark (●——●). A: fresh weight; B: dry weight.

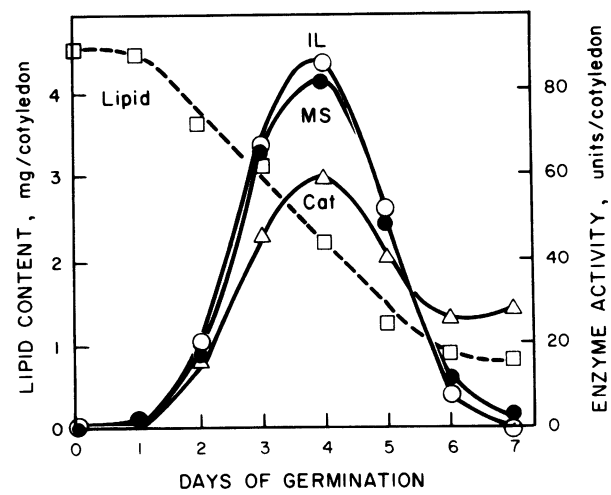

FIG. 3. Developmental changes in lipid content and glyoxysomal enzyme activities in cotyledons of light-grown cucumber seedlings. Homogenates were prepared from cotyledons of each seedling stage and were assayed as described in the text for isocitrate lyase (IL; ——○——), malate synthase (MS; ——●——), catalase (Cat:b—△——), and total lipid content (——□——), with all values expressed on a per-cotyledon basis.

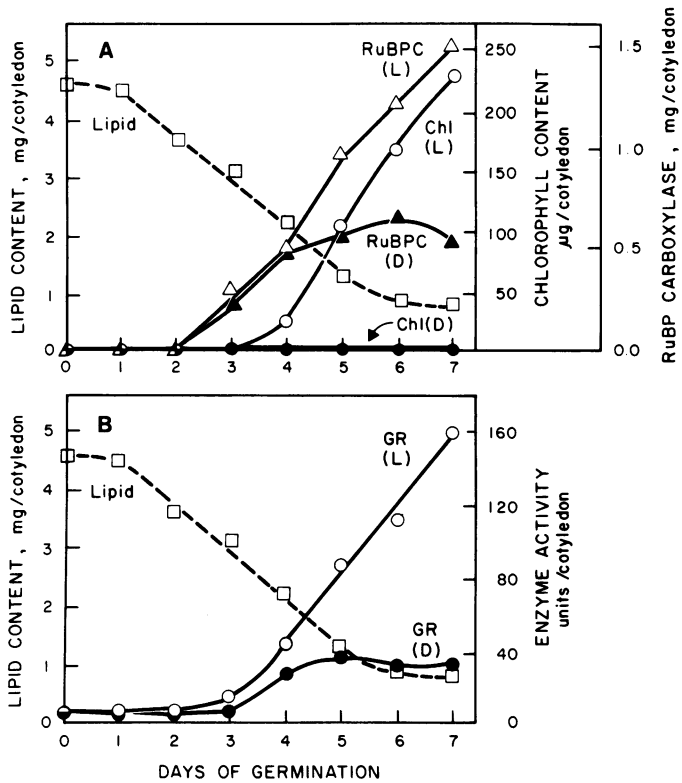

FIG. 4. Developmental changes in chloroplast and peroxisomal markers in cotyledons of light-grown (open symbols) and dark-grown (closed symbols) cucumber seedlings. A: amounts of RuBPC protein (△——△) and Chl (○——○) per cotyledon. B: activity of glyoxylate (hydroxypyruvate) reductase (GR; ○——○) per cotyledon. Lipid content (□——□) as in Figure 3.

pared with 2.23 mg/cotyledon in the light. Prior to day 2, most (75–85%) of the homogenate protein sediments upon centrifugation at 10,000*g* for 10 min, presumably because it is present in storage protein bodies. Protein bodies are prominent ultrastructural features of cucurbit cotyledons (25, 35) and are recovered with glyoxysomes at a density of 1.26 g/cm^3 upon sucrose density centrifugation of organellar preparations from early stages (see below). A striking decrease in sedimentable protein occurs between days 2 and 3, such that by day 4 more than 80% of the total protein is present in a form which no longer sediments at 10,000*g*. The same pattern of progressive mobilization of cotyledonary protein is seen in both the light and the dark, as shown in Figure 5B.

Shown in Figure 6 are SDS-polyacrylamide gel profiles of homogenate and supernatant protein for both light- and dark-grown cotyledons, loaded on a per-cotyledon basis to facilitate direct comparison of band intensities. The most prominent developmental feature of the homogenate gels (Fig. 6, A and B) at early stages is the progressive disappearance of a cluster of low mol wt polypeptides (mol wt 20,000–35,000), almost certainly representing storage proteins located initially in protein bodies. The striking change in sedimentable protein shown in Figure 5 is mirrored in the supernatant profiles of Figure 6, C and D by the sudden appearance at day 3 of a series of low mol wt bands, probably corresponding to solubilized but as yet incompletely digested storage polypeptides. Although the initial solubilization of particulate storage proteins occurs at the same rate in both light and dark, the resulting soluble polypeptides persist significantly longer in the dark (Fig. 6).

Also discernible on the gels of Figure 6 is a family of polypeptide bands which can by their mol wt range (45,000–75,000) and developmental pattern be tentatively identified as glyoxysomal enzyme subunits. This identification is facilitated by the subunit

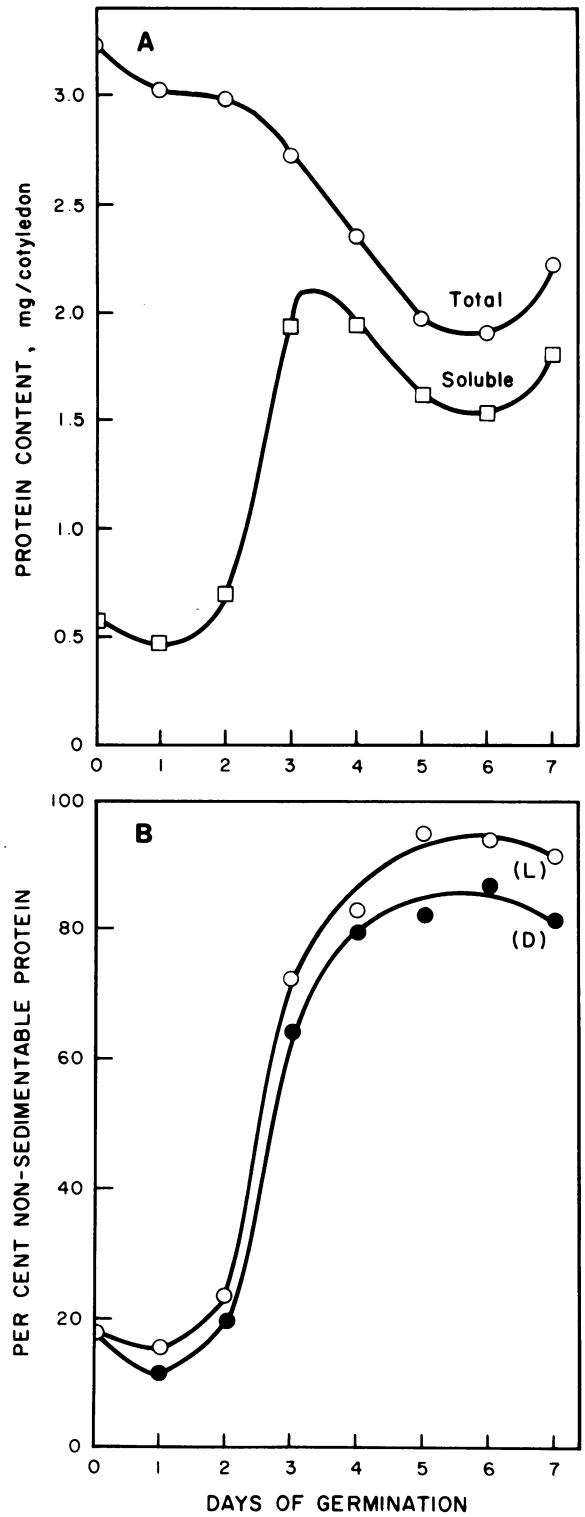

FIG. 5. Developmental changes in protein content of cucumber cotyledons during germination. A: amount of total (○——○) and soluble (□——□) protein per cotyledon. The difference between the two values at any given time point represents the amount of total homogenate protein removed by centrifugation at 10,000*g* for 10 min. B: nonsedimentable (10,000*g* supernatant) protein expressed as a per cent of total cotyledonary protein for light-grown (○——○) and dark-grown (●——●) seedlings.

mol wt now available (16) for several glyoxysomal enzymes from cucumber, including isocitrate lyase (63,000), malate synthase (63,000), citrate synthase (46,000), malate dehydrogenase (37,000), crotonase (75,000), thiolase (45,000), and catalase (54,000). Bands in this mol wt range become detectable by about day 2, peak

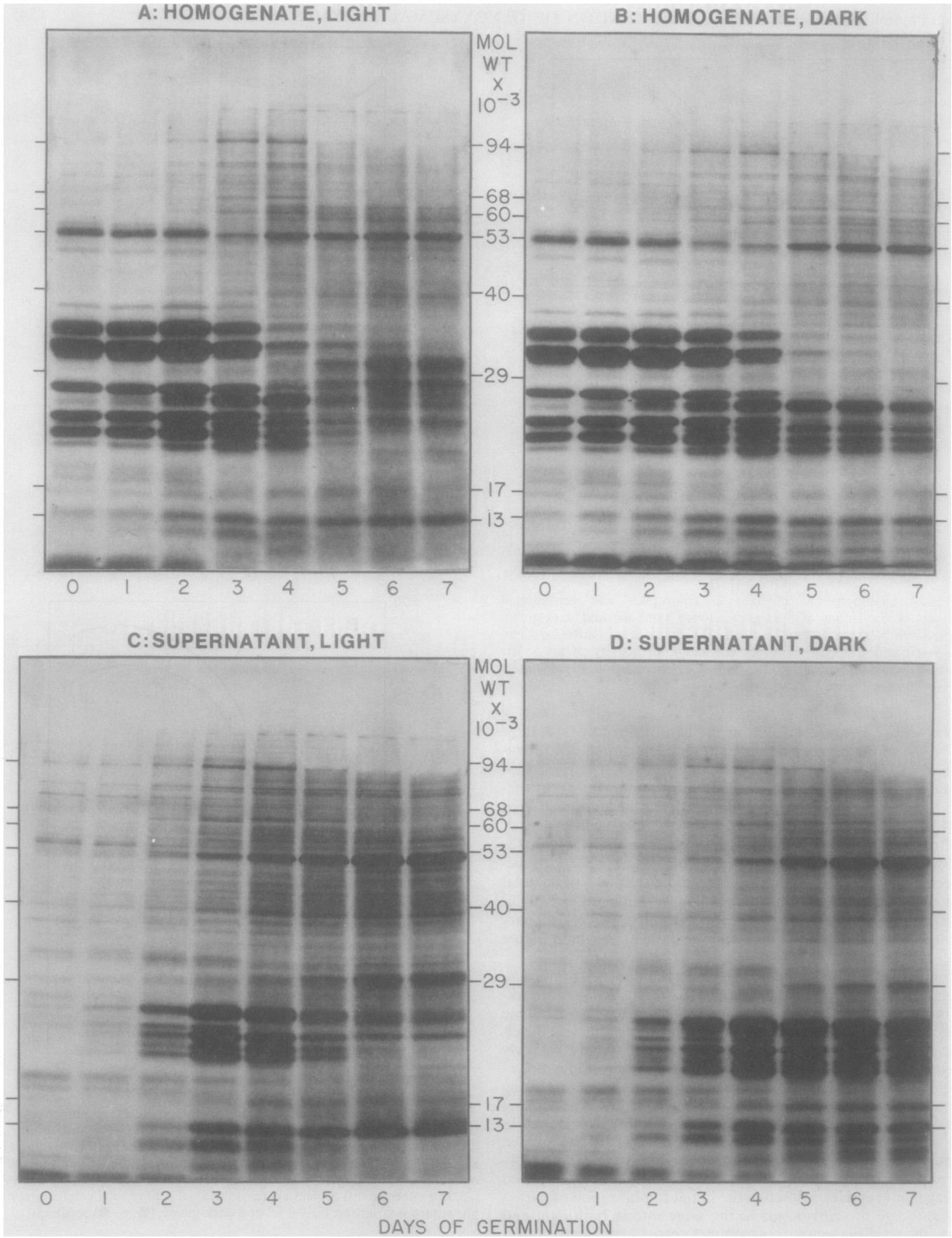

FIG. 6. SDS-polyacrylamide gels of total and supernatant protein from cotyledons of light-grown and dark-grown cucumber seedlings. Samples were loaded on a per cotyledon basis onto 15% polyacrylamide slab gels and subjected to electrophoresis as described in the text. Actual amounts of protein applied corresponded to 2.0% (homogenate) or 5.0% (supernatant) of the protein content of a single cotyledon at a given stage. Upper gels: homogenate proteins from light-grown (A) and dark-grown (B) seedlings. Lower gels: supernatant proteins from light-grown (C) and dark-grown (D) seedlings. Mol wt markers are, from top to bottom: phosphorylase (94,000), BSA (68,000), bovine catalase (60,000), carbonic anhydrase (29,000), and myoglobin (17,200). Positions of the large (53,000) and small (13,000) subunits of RuBPC are also indicated.

intensities at day 4, and decrease in prominence thereafter, just as would be expected for glyoxysomal enzymes, assuming a direct correlation between activity and enzyme protein. Specifically, bands of the appropriate mol wt for the subunits of isocitrate lyase, malate synthase, and catalase are readily identifiable and correlate well in intensity across the gels of Figure 6 with the activity profiles for these enzymes in Figure 3. A further family of bands can be identified which increase in intensity after about day 3 and appear to reflect the developmental patterns of the autotrophic functions depicted in Figure 4. Especially prominent among these polypeptides are the large (53,000) and small (13,000) subunits of RuBPC.

Microbody Proteins. To identify specific polypeptides of Figure 6 more directly with glyoxysomal function, cotyledonary homogenates were prepared in the presence of 0.4 M sucrose and subjected to equilibrium density centrifugation on linear sucrose gradients. As seen from the representative gradient profile (for day 3) shown in Figure 7, glyoxysomal marker enzymes (isocitrate lyase and malate synthase) band at a density of 1.26 g/cm^3, while the mitochondrial marker (Cyt *c* oxidase) peaks at about 1.185 g/cm^3, with minimal cross-contamination. Figure 8 shows the gel profiles for the peak microbody fraction (ρ = 1.26 g/cm) from gradients corresponding to days 1 through 6 (light-grown), all loaded on a per-cotyledon basis. Initially (days 1–2), the most prominent polypeptides present in this region of the gradient are those tentatively identified from Figure 6 as storage proteins. The most prominent of these storage polypeptides have mol wt of 21,000, 23,000, 26,000, 30,500, 33,500, and 35,500. Their recovery in this region of the gradient confirms their presence in particulate form at early stages. Their subsequent mobilization during germination is substantiated by progressive disappearance of these polypeptides from this region of the gradient, accompanied by a reciprocal appearance at the top of the gradient of soluble polypeptides of the same or slightly lower mol wt (gels not shown). More relevant to our specific interest in the glyoxysomal enzymes is the family of higher mol wt polypeptides which appear at the 1.26 g/cm^3 region of the gradient at day 2, peak in intensity at day 4, and disappear thereafter. This accords well not only with the over-all pattern expected of glyoxysomal enzymes (Fig. 3) but also with the relative activities of isocitrate lyase and malate synthase actually recovered in this region of the density gradient at each stage (*cf.* Fig. 7; other gradients not shown).

Cotyledonary RNA. One of the most striking features of cotyledonary development is the large increase in cellular RNA, due mainly to the accumulation of rRNA, as shown in Figure 9.

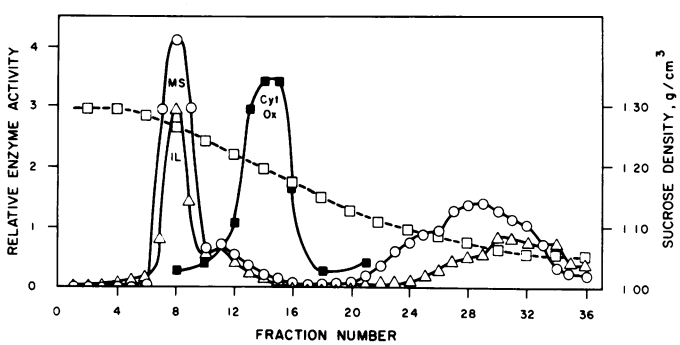

FIG. 7. Distribution of glyoxysomal and mitochondrial marker enzymes on a sucrose density gradient. The 600*g* × 10 min supernatant from a homogenate of 3-day light-grown cotyledons was applied to a 45-ml sucrose gradient (16–60%, w/w) and centrifuged to equilibrium (4 hr at 22,000 rpm in a Spinco SW 25.2 rotor). Fractions (1.5 ml) were assayed for isocitrate lyase (IL; △——△), malate synthase (MS; ○——○), Cyto oxidase (Cyt Ox; ■——■), and for sucrose density (□——□; determined from the refractive index at 20 C). To calculate actual enzyme activities in units/fraction, multiply ordinate values by 1,000 for isocitrate lyase and malate synthase and by 150 for Cyt *c* oxidase.

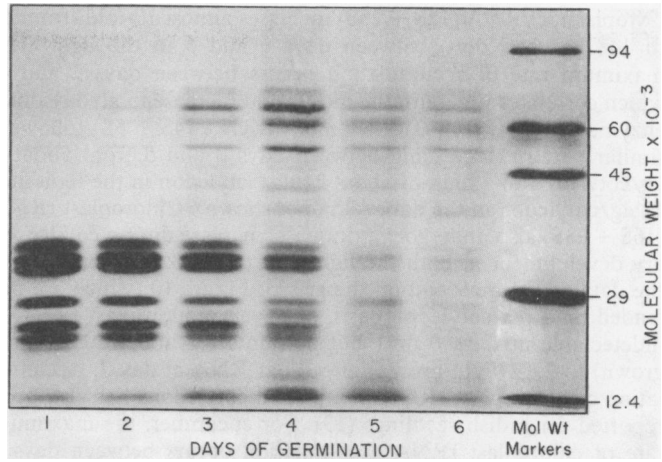

FIG. 8. SDS-polyacrylamide gels of protein from the peak microbody fractions of sucrose density gradients for light-grown cucumber cotyledons. Cotyledons from days 1 through 6 were ground in the presence of 0.4 M sucrose and the 600*g* × 10-min supernatant from each stage was centrifuged to equilibrium on a 45-ml sucrose gradient (16–60%, w/w). Conditions of centrifugation, collection, and assay were as in Figure 7. Protein was precipitated from each fraction with 80% acetone, redissolved in electrophoresis sample buffer, and loaded on a per cotyledon basis onto 15% polyacrylamide slab gels for electrophoresis as described in the text. The actual amount of protein applied corresponded to that portion of the protein from two cotyledons which was recovered from the sucrose gradient in the 1.5-ml fraction corresponding most closely to a density of 1.26 g/cm^3. Gels correspond, from left to right, to days 1, 2, 3, 4, 5, and 6. Mol wt markers in the right lane are, from top to bottom: phosphorylase (94,000), bovine catalase (60,000), ovalbumin (45,000), carbonic anhydrase (29,000) and Cyt *c* (12,400).

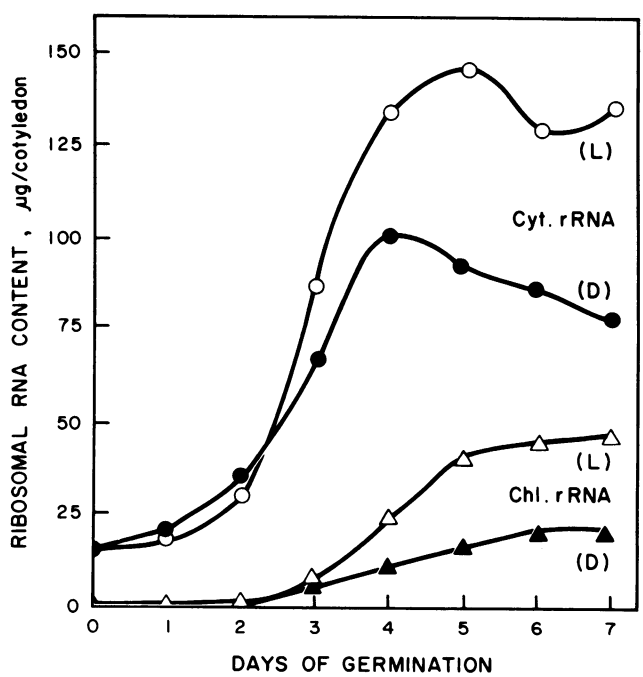

FIG. 9. Developmental changes in rRNA content of cucumber cotyledons during germination. Amounts of cytoplasmic (18S + 25S) rRNA (○——○) and chloroplast (16S + 23S) rRNA (△——△) per cotyledon were calculated for light-grown (open symbols) and dark-grown (closed symbols) seedlings by subjecting total phenol-extracted RNA from each stage to electrophoresis and scanning as described in Figure 10. The area under each rRNA peak was expressed as a fraction of the total area under the scan curve, and that fraction was then multiplied by the total RNA content (in µg/cotyledon) determined as described in the text.

Cytoplasmic rRNA (18S + 25S) increases almost 10-fold (from 15 to 148 μg/cotyledon) between days 0 and 5 in the light. The maximum rate of accumulation occurs between days 2 and 4, which correlates well with the most rapid increase in glyoxysomal enzyme activities (Fig. 3). Low mol wt RNA (4S + 5S) follows a similar pattern, increasing between days 0 and 5 from about 3 μg/cotyledon to a high of about 24 μg/cotyledon in the light and 12 μg/cotyledon in the dark (data not shown). Chloroplast rRNA (16S + 23S) also undergoes a dramatic increase during cotyledonary development in both the light and the dark, as seen both in the data of Figure 9 and on the gels of Figure 10 (which are not loaded on a per-cotyledon basis, however). Chloroplast rRNA is indetectable at days 0 or 1 but accounts for about 20% (dark-grown) to 25% (light-grown) of cellular RNA at day 7. Accumulation of chloroplast rRNA in etiolated cotyledons has also been reported for radish seedlings (13). For cucumber, the maximum rate of chloroplast rRNA accumulation occurs between days 3 and 5 in both light and dark, about 24 hr after the most rapid increase in cytoplasmic rRNA. This coincides with the most rapid increases in the several autotrophic indicators of Figure 4.

DISCUSSION

Much of the physiological data presented here have been reported previously for cucumber (35) or other (8, 14, 15, 29) fat-storing species, but few prior attempts have been made to correlate developmental changes in enzyme activities with electrophoretic protein and RNA patterns. In addition, it seems useful to have all of this information compiled for the same species as a data base for further studies on the molecular mechanisms underlying glyoxysomal enzyme appearance and organellar biogenesis.

Cotyledonary metabolism during cucumber germination is characterized initially by gluconeogenic utilization of stored fat via the glyoxylate cycle. By subjecting cotyledonary protein to SDS-polyacrylamide gel electrophoresis, specific bands can be identified which by size and developmental pattern appear to be subunits of glyoxylate cycle enzymes. This identification is strengthened by recovery of the same family of bands with the same developmental profile at a density of 1.26 g/cm^3 in sucrose gradients. Further confirmation is afforded by the observation that antiserum against either isocitrate lyase (18) or malate synthase from cucumber cotyledons reacts specifically with a single protein present in both the homogenate and a 1.26 g/cm^3 gradient fraction (J. E. Lamb and H. Riezman, unpublished observations). The good agreement in developmental profile between glyoxysomal enzyme activities and actual amounts of subunit protein detectable on gels is consistent with reports linking the appearance of glyoxysomal enzyme activities to *de novo* enzyme synthesis in a variety of species (10–12, 23), and suggests in addition that the subsequent disappearance of activity is a consequence of enzyme degradation.

The rapid increase in glyoxysomal enzyme activities between days 2 and 4 correlates well with the accumulation of cytoplasmic rRNA, and therefore presumably with the capacity of the cotyledonary cell for cytoplasmic protein synthesis. This is accompanied by increases in several indicators of messenger RNA availability, including a 9-fold enhancement in the level of poly(A)-containing RNA between days 0 and 5, a marked increase in the polysome content between days 1 and 5, and a dramatic increase, especially between days 2 and 3, in the ability of total cotyledonary RNA to stimulate amino acid incorporation in a cell-free protein-synthesizing system derived from wheat germ (21; E. M. Weir and C. J. Leaver, unpublished observations).

Concomitant with fat metabolism, protein reserves of the cotyledon are also mobilized and degraded. Initially, most of the protein of the cotyledon appears to be present as storage protein bodies which sediment rapidly at 10,000*g* and band in sucrose at or near the glyoxysomal density of 1.26 g/cm^3. The progressive mobilization of this particulate protein apparently involves initial solubilization to a nonsedimentable form, followed by gradual degradation of the solubilized polypeptides, probably to the amino acid level. The initial solubilization occurs at the same rate and to the same extent in both light- and dark-grown cotyledons, but the resulting polypeptides then disappear more rapidly in the light than in the dark. The significance of this observation is not yet clear, but it may suggest a light dependence of the proteolytic enzymes required for further digestion. Also unresolved is the extent to which the resulting amino acids are reutilized for protein synthesis within the cotyledon or are instead translocated to the growing axis. That substantial protein synthesis must occur within the cotyledon is clear, however, from the appearance of enzyme activities known to depend upon *de novo* protein synthesis (*e.g.* the glyoxysomal enzymes) and especially from the accumulation of large amounts of the single protein, RuBPC.

The accumulation of carboxylase protein and the appearance of chloroplast rRNA are among the most striking manifestations of the transition to autotrophy that occurs upon seedling emergence. From the rRNA data, it can be calculated (Table I) that the number of chloroplast ribosomes increases from essentially none at days 0, 1, and 2 to about 26 million/cell by day 7 (compared to about 60 million cytoplasmic ribosomes/cell at day 7). Most of this increase occurs between days 3 and 5, which

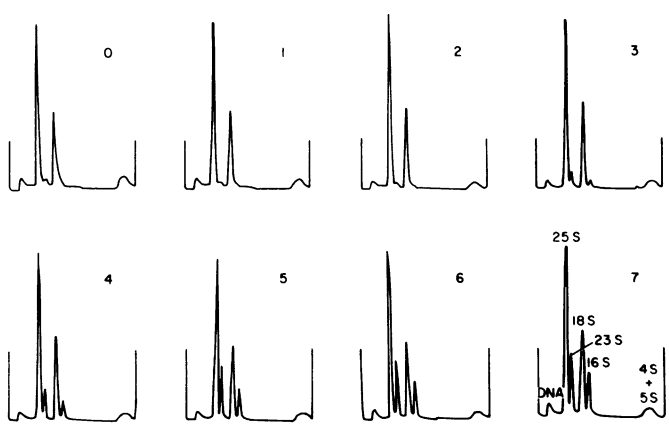

FIG. 10. Acrylamide gel profiles for RNA from cotyledons of light-grown cucumber seedlings. Each gel (10 cm; 2.4% polyacrylamide) was loaded with about 25 μg of phenol-extracted total nucleic acid, subjected to electrophoresis at 5 C for 4 hr with a current of 3 mamp/gel and then scanned at 265 nm.

Table I. Postgerminative changes in cotyledonary cells of cucumber

Data of Figs. 2, 3, 4, 5 and 9 are expressed here on a per-cell basis, assuming 6.78 x 10^5 cells per cotyledon at all stages. Number of ribosomes per cell calculated by assuming both 70S and 80S ribosomes to be 50% rRNA (16S + 23S and 18S + 25S, respectively).

	Age of seedling (Light-grown)			
	day 1	day 3	day 5	day 7
Weight				
Total, ng/cell	23	36	125	233
Dry, ng/cell	16	14	12	16
Water, ng/cell	7	22	113	217
Protein				
Total, ng/cell	4.5	4.0	2.9	3.3
Sedimentable (10000g), ng/cell	3.8	1.1	0.5	0.6
Soluble, ng/cell	0.7	2.9	2.4	2.7
RuBP Carboxylase, ng/cell	~0	0.2	0.7	1.1
Lipid, ng/cell	6.6	4.6	1.9	1.2
Chlorophyll, pg/cell	~0	2	154	332
RNA				
Total, pg/cell	30	154	319	314
Cyt. rRNA (18S + 25S), pg/cell	26	130	217	201
Chl. rRNA (16S + 23S), pg/cell	~0	11	67	75
4S & 5S, pg/cell	4	13	35	37
Ribosomes				
Cytoplasmic (80S), millions/cell	8	39	65	60
Chloroplast (70S), millions/cell	~0	4	24	26

coincides well with the most rapid increases in the several autotrophic indicators investigated. RuBPC is quantitatively the most significant of these indicators; within a few days, its large (53,000) and small (13,000) subunits become the most prominent polypeptides on the gels. Closer examination of the gels reveals a family of polypeptides which appear and increase in synchrony with the carboxylase subunits, presumably because they are components of proteins involved in photosynthesis or related autotrophic functions.

We are at present interested in whether the developmental changes in the levels of specific cotyledonary proteins as documented here are accompanied by, and perhaps dependent upon, comparable changes in the levels of translatable mRNAs, and in the manner in which the synthesis of such proteins is coupled to enzyme packaging and glyoxysomal biogenesis.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BECKER WM, CJ LEAVER, EM WEIR 1977 Developmental changes in cotyledonary RNA and protein during germination of cucumber. Plant Physiol 59: 5–57
3. BEEVERS H 1969 Glyoxysomes of castor bean endosperm and their relation to gluconeogenesis. Ann NY Acad Sci 168: 313–324
4. BROWN R, P RICKLESS 1949 A new method for the study of cell division and cell extension with some preliminary observations on the effects of temperature and of nutrients. Proc R Soc Lond B 136: 110–115
5. COOPER TG, H BEEVERS 1969 Mitochondria and glyoxysomes from castor bean endosperm. Enzyme constituents and catalytic capacity. J Biol Chem 244: 3507–3513
6. DOCKERTY A, JM LORD, MJ MERRETT 1977 Development of ribulose-1,5-diphosphate carboxylase in castor bean cotyledons. Plant Physiol 59: 1125–1127
7. FORD MJ, P SLACK, M BLACK, JM CHAPMAN 1976 A reexamination of the reputed control of cotyledonary metabolism by the axis. Planta 132: 205–208
8. GERHARDT B 1973 Untersuchungen zur Funktionsänderung der Microbodies in den Keimblättern von *Helianthus annuus*. Planta 110: 15–28
9. GERHARDT B 1974 Studies on formation of glycolate oxidase in developing cotyledons of *Helianthus annuus* L. and *Sinapis alba* L. Z Pflanzenphysiol 74: 14–21
10. GIENTKA-RYCHTER A, JH CHERRY 1968 *De novo* synthesis of isocitritase in peanut (*Arachis hypogaea* L.) cotyledons. Plant Physiol 43: 653–659
11. HOCK B 1970 Die zeitliche Dauer der Isocitrat-lyase-synthese in Kotyledonen von Wassermelonenkeimlingen. Planta 93: 26–38
12. HOCK B, H BEEVERS 1966 Development and decline of glyoxylate-cycle enzymes in watermelon seedlings (*Citrullus vulgaris*, Schrad.). Effects of dactinomycin and cycloheximide. Z Pflanzenphysiol 55: 405–414
13. INGLE J 1968 Effect of light and inhibitors on chloroplast and cytoplasmic RNA synthesis. Plant Physiol 43: 1850–1854
14. KAGAWA T, H BEEVERS 1975 Development of microbodies (glyoxysomes and leaf peroxisomes) in cotyledons of germinating watermelon seedlings. Plant Physiol 55: 258–264
15. KAGAWA T, DI MCGREGOR, H BEEVERS 1973 Development of enzymes in the cotyledons of watermelon seedlings. Plant Physiol 51: 66–71
16. KÖLLER W, H KINDL 1977 Glyoxylate cycle enzymes of the glyoxysomal membrane from cucumber cotyledons. Arch Biochem Biophys 181: 236–248
17. LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
18. LAMB JE, B ROBERG, WM BECKER 1977 Purification and characterization of isocitrate lyase (EC 4.1.3.1) from cucumber cotyledons. Plant Physiol 59: S-19
19. LEAVER CJ 1973 The molecular integrity of chloroplast ribosomal ribonucleic acid. Biochem J 135: 237–240
20. LEAVER CJ, J INGLE 1971 The molecular integrity of chloroplast ribosomal ribonucleic acid. Biochem J 123: 235–244
21. LEAVER CJ, EM WEIR, R WALDEN, SW WIETGREFE, WM BECKER 1977 Developmental changes in cytoplasmic and organellar RNAs during germination of cucumber. Plant Physiol 59: S-57
22. LOENING UE 1967 The fractionation of high-molecular-weight ribonucleic acid by polyacrylamide-gel electrophoresis. Biochem J 102: 251–257
23. LONGO CP 1968 Evidence for *de novo* synthesis of isocitritase and amalate synthetase in germinating peanut cotyledons. Plant Physiol 43: 660–664
24. LOTT JNA 1970 Changes in the cotyledons of *Cucurbita maxima* during germination. I. General characteristics. Can J Bot 48: 2227–2231
25. LOTT JNA, CM VOLLMER 1973 Changes in the cotyledons of *Cucurbita maxima* during germination. IV. Protein bodies. Protoplasma 78: 255–272
26. LOWRY OH, NJ ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275
27. LÜCK H 1963 Catalase. In HU Bergmeyer, ed, Methods of Enzymatic Analysis. Academic Press, New York, pp 885–894
28. RADIN NS 1969 Preparation of lipid extracts. Methods Enzymol 14: 245–254
29. SCHNARRENBERGER C, A OESER, NE TOLBERT 1971 Development of microbodies in sunflower cotyledons and castor bean endosperm during germination. Plant Physiol 48: 566–574
30. SCHOPFER P, D BAJRACHARYA, R BERGFELD, H FALK 1976 Phytochrome-mediated transformation of glyoxysomes into peroxisomes in the cotyledons of mustard (*Sinapis alba* L.) seedlings. Planta 133: 73–80
31. SCHOPFER P, D BAJRACHARYA, H FALK, W THIEN 1975 Phytochromgesteuerte Entwicklung von Zellorganellen (Plastiden, Microbodies, Mitochondrien). Ber Deutsch Bot Ges 88: 245–268
32. SLACK PT, M BLACK, JM CHAPMAN 1977 The control of lipid mobilization in *Cucumis* cotyledons. J Exp Bot 28: 569–577
33. THEIMER RR, G ANDING, P MATZNER 1976 Kinetin action on the development of microbody enzymes in sunflower cotyledons in the dark. Planta 128: 41–47
34. TOLBERT NE 1971 Isolation of leaf peroxisomes. Methods Enzymol 23: 665–682
35. TRELEASE RN, WM BECKER, PJ GRUBER, EH NEWCOMB 1971 Microbodies (glyoxysomes and peroxisomes) in cucumber cotyledons. Correlative biochemical and ultrastructural study in light- and dark-grown seedlings. Plant Physiol 48: 461–475
36. WEIDNER M 1967 Der DNS-Gehalt von Kotyledonen und Hypokotyl des Senfkeimlings (*Sinapis alba* L.) bei der phytochromgesteuerten Photomorphogenese. Planta 75: 94–98