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Subcellular Localization and Developmental Changes of Aspartate-α-Ketoglutarate Transaminase Isozymes in the Cotyledons of Cucumber Seedlings[1]

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ABSTRACT

The total activity of aspartate-α-ketoglutarate transaminase in the cotyledons of cucumber (*Cucumis sativus* L.) seeds increased 17-fold during the first 2 days of germination in darkness and then declined gradually to 20% of the peak activity after 10 days. Exposure of the seedlings to light at day 3 accelerated the decline. The enzyme in the cotyledon extracts from seedlings at various ages was resolved into six distinct isozymes by starch gel electrophoresis. Isozymes 1 and 2 were glyoxysomal isozymes with different developmental patterns. Isozyme 1 followed the developmental pattern of the total enzyme activity in darkness, and was rapidly eliminated upon illumination. Isozyme 2 increased in activity to a peak at day 2 and declined rapidly thereafter, and disappeared completely at day 6; this developmental pattern was independent of light. No major difference in the optimal pH for activity, substrate specificity, and reversibility was observed between isozymes 1 and 2. The combined developmental pattern of isozymes 1 and 2 during germination correlated with that of the glyoxysomes. Isozyme 3 was located in the cytosol and its developmental pattern followed that of the total activity. Isozymes 4, 5, and 6 were plastid isozymes and appeared only after 2 days of germination. Unlike many other chloroplast enzymes, the appearance of the chloroplast transaminase isozymes was under temporal control and was independent of illumination. No enzyme activity was detected in isolated mitochondria. The findings illustrate a complicated cellular control system for the appearance of various organelle-specific transaminase isozymes and thus the amino acid metabolism during germination.

Aspartate-α-ketoglutarate transaminase (EC 2.6.1.1) has been considered to play important physiological roles in many plant tissues. Suggestion has been made that the enzyme participates in amino acid metabolism (5), electron shuttle (7), intercellular transport of metabolites during C_4 photosynthesis (6), and the proposed glycolate pathway (19). To carry out these functions, it is necessary for the enzyme to be present in different subcellular compartments. In spinach leaves, four distinct isozymes are present, each of which is restricted to one subcellular compartment, namely, the cytosol, chloroplasts, mitochondria, and peroxisomes (10, 15, 18, 21). In the fatty endosperm of castor bean, the glyoxysomes contain another unique isozyme (2).

Cucumber seeds contain protein and lipid in the cotyledons as food reserve. During germination, the protein and lipid are converted to amino acids and sugars, respectively, which are then transported to the growing embryonic axis. At a later stage when the seedlings are exposed to light, the cotyledons turn green and become photosynthetic (1). Thus, light induces a complete change in the function of the cotyledons during the later stage of germination.

The information on the existence and the developmental control of the organelle-specific AT[2] isozymes in the cotyledons of cucumber seedlings may be the first clue to understanding the roles of the transaminase in amino acid metabolism, interorganelle electron shuttle, and photosynthesis. Here, we report experiments on the developmental changes of organelle-specific AT isozymes in the cotyledons of cucumber seeds germinated in darkness. The effect of illumination on the various isozymes was monitored after exposing the seedlings to light at the peak stage of the food mobilization process.

MATERIALS AND METHODS

Plant Material. Seeds of cucumber (*Cucumis sativus* L., cv. "46765 Poinsett") were purchased from Food-Machine-Chemical Corp. (Modesto, Calif.) The seeds were soaked for 24 hr in running tap water and germinated at 30 C in trays of a 1:1 (v/v) mixture of moist vermiculite and soil. The trays were covered with aluminum foil to ensure that the seedlings were grown in complete darkness. The seedlings were grown for 10 days in complete darkness, or when 3 days old, were exposed to continuous illumination at 30 C in an environmental chamber, model 1-E-30 (Percival Co., Des Moines, Iowa) under GE cool white fluorescent lamps (intensity of 9.6×10^3 erg cm^{-2} sec^{-1}).

Enzyme Preparation. All steps were performed at 0 to 4 C. Ten pairs of cotyledons were removed from the seedlings of various ages and ground with a glass homogenizer in a grinding medium consisting of 1 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 0.2% Triton X-100, and 0.15 M Tricine buffer adjusted with KOH to pH 7.5. A volume of 1.5 ml of grinding medium was used/g fresh weight. After centrifugation at 270*g* for 10 min to remove cell debris, the homogenate was recentrifuged at 10,000*g* for 30 min. More than 90% of the AT activity was recovered in the supernatant fraction and the pellet was discarded. Since the fresh weight of the cotyledons changed during germination, the volume of the supernatant fraction obtained from the cotyledons of different ages was raised with grinding medium to a standard of 3 ml/10 cotyledon pairs. The adjusted final solution was used as the total cotyledon extract for the assays of enzyme activities and for the separation of AT isozymes.

Organelle Preparation. All steps were performed at 0 to 4 C. The cotyledons were chopped into small pieces with a razor blade in a Petri dish containing grinding medium and then

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[2] Abbreviation: AT: aspartate-α-ketoglutarate transaminase.

ground with a mortar and pestle. The grinding medium was composed of 0.6 M sucrose, 1 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 10 mM dithiothreitol, and 0.15 M Tricine buffer adjusted with KOH to pH 7.5 (10). For the preparation of intact chloroplasts or proplastids, the crude extract was filtered through a Nitex cloth with a pore size of 35 μm^2. The filtrate was layered directly on top of a sucrose gradient composed of 30 ml 30 to 60% (w/w) sucrose over a cushion of 1 ml 60% sucrose. The gradients were placed in a Beckman rotor SW 27 and centrifuged at 10,000 rpm for 10 min in a Beckman L2-65B ultracentrifuge. For the preparation of mitochondria and glyoxysomes from 2-day-old dark-grown seedlings, the filtrate of the crude extract was first centrifuged at 270*g* for 10 min to remove intact proplastids, and the supernatant fraction was subjected to equilibrium sucrose gradient centrifugation as described before (8). In the preparation of intact chloroplasts, the 7-day-old light-grown seedlings were kept in darkness for 12 hr immediately before use.

The intact chloroplasts recovered from the sucrose gradients were in a dilute preparation and the activity of AT was not high enough to be resolved by starch gel electrophoresis. In order to obtain a high concentration of chloroplasts, 10 ml of the crude filtrate was layered onto a sucrose gradient composed of 25 ml 30 to 42% sucrose over a cushion of 1 ml of 42% sucrose, and centrifuged at 10,000 rpm for 10 min. The design was based on the finding that only intact chloroplasts were able to migrate through 42% sucrose in a similar rate sucrose gradient centrifugation (see Fig. 3 and description under "Results and Discussion"). The pellet, consisting of pure intact chloroplasts, was resuspended with 0.5 ml of the grinding medium plus 0.05% Triton X-100 to release the enzymes from the organelles. After incubation in the cold for 30 min, the resuspended pellet was centrifuged at 10,000*g* for 30 min, and the resulting supernatant fraction was used for the identification of AT isozymes by starch gel electrophoresis.

Assays. AT activity was assayed spectrophotometrically by coupling with NAD-malate dehydrogenase. The reaction mixture contained 0.06 M K-phosphate (pH 7), 3 mM aspartate, 0.12 mM NADH, 100 enzyme units of NAD-malate dehydrogenase (Sigma), 0.06 mM pyridoxal-5'-P, and 0.5 mM α-ketoglutarate (16). In the assay of the various organelle fractions, 0.05% of Triton X-100 was added to the reaction mixture, but no increase in activity was observed. The assays of catalase (9), NAD-malate dehydrogenase (13), NADP-glyceraldehyde-3-P dehydrogenase (4), Cyt oxidase (8), isocitrate lyase (13), glycolate oxidase (13), Chl (13), and triose-3-P isomerase (8) followed those described earlier. The sucrose density was read with a refractometer.

Electrophoresis. Horizontal starch gel electrophoresis was performed according to Meizel and Markert (14) using tris-citrate buffer (pH 7). The samples were subjected to electrophoresis for 15 hr at 10 v/cm at 4 C. The gel was sliced into pieces of 2 mm thickness, and after staining for AT activity at 30 C for 3 hr (3), it was fixed in 50% (v/v) glycerol for 1 hr. All of the isozyme patterns resolved from the cotyledon extracts of seedlings at various ages and from the subcellular fractions were not affected by the addition of 10 mM dithiothreitol or 0.1% Triton X-100 to the grinding medium or to the samples immediately before electrophoresis. The various isozymes were stable during the period of electrophoresis since they could be recovered quantitatively from the gel by the extraction method described earlier (10).

In the present system of starch gel electrophoresis, the intensity of the isozyme bands in the gel remained at its maximum for several hours after 3 hr of staining. Within the limit of the amount of enzyme extract that we applied to the gel, the intensity of an isozyme band in one gel was proportional to the amount of enzyme extract applied initially. Thus, a direct comparison of the developmental change of each individual isozyme was made by applying the cotyledon extracts from seedlings of various ages to different slots in one starch gel for electrophoresis. The intensity of each individual isozyme band in the starch gel was monitored quantitatively by scanning spectrophotometrically at 625 nm and computing the area under each peak. Scanning was performed in a quartz cuvette in a Gilford gel scanner (model 2520) attached to a Gilford spectrophotometer and recorder.

RESULTS AND DISCUSSION

Total AT Activity in the Cotyledons of Dark- and Light-grown Seedlings. The total AT activity increased 17-fold during the first 2 days of germination in darkness and then declined gradually to about 20% of the peak activity after 10 days (Fig. 1).

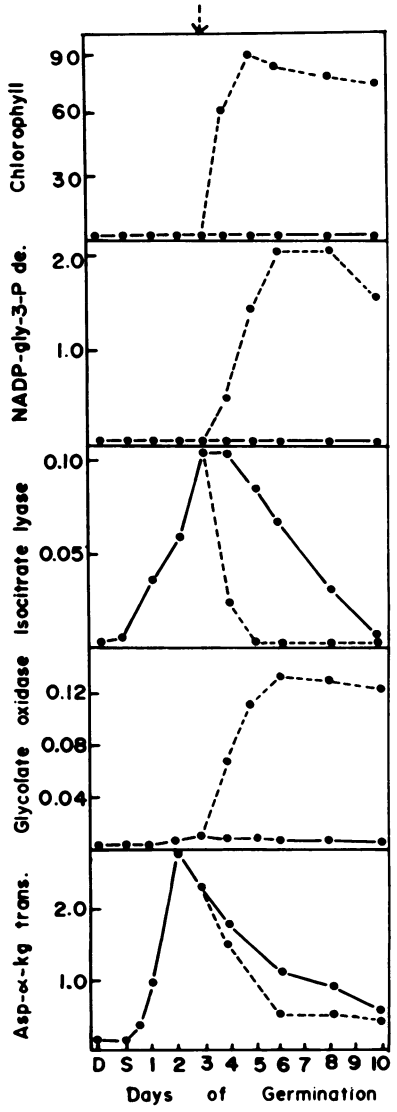

FIG. 1. Changes in the amount of Chl and the total activities of various enzymes in the cotyledons of cucumber seedlings during germination. After germination in darkness for 3 days, the seedlings were either maintained in prolonged darkness (●) or exposed to continuous light (○). Chl content is expressed in μg/cotyledon pair. Enzyme activities are expressed in μmol of substrates reacted/min·cotyledon pair. D and S indicate cotyledon extracts from dry seeds and soaked seeds, respectively. The data on Chl, glycolate oxidase, and isocitrate lyase have been reported (13).

When the dark-grown seedlings were exposed to light at day 3, the decrease in activity was accelerated. Only 20% of the peak activity was found after exposure of the seedlings to light for 3 days, but this residual activity remained fairly constant from day 6 to day 10. The drastic effect of light on the general physiology of the cotyledons was demonstrated (Fig. 1) by the appearance of Chl, NADP-glyceraldehyde-3-P dehydrogenase (markers for chloroplasts), and glycolate oxidase (a marker enzyme for peroxisomes), and the accelerated disappearance of isocitrate lyase (a marker enzyme for glyoxysomes), as also reported by others (11, 20).

Isolation of Organelles Containing AT Activity. The supernatant fraction obtained after centrifugation at 270*g* for 10 min of the crude cotyledon extract of 2-day-old dark-grown seedlings was resolved into various organelle fractions in a sucrose gradient (Fig. 2). Only one distinct peak of AT activity was present at density 1.25 g/cm³ and the rest of the activity remained at the top of the gradient. The peak of AT activity coincided with that of catalase, a marker enzyme for glyoxysomes, and thus represented the glyoxysomal AT. No distinct peak of AT activity was associated with that of Cyt *c* oxidase, a marker enzyme for mitochondria, at density 1.18 g/cm³. The minor activity at fraction 32 should represent glyoxysomes trapped on top of the mitochondrial band since a small amount of catalase activity was also present. Solubilization of the glyoxysomes and mitochondria with 0.05% Triton X-100 did not change the activity. In contrast to the distribution of AT, there were two major peaks of NAD-malate dehydrogenase in the gradient, representing those of the mitochondria and glyoxysomes. Furthermore, even though the mitochondria contain a distinct isozyme of NAD-malate dehydrogenase (13), no AT isozyme was resolved in starch gel from the isolated mitochondria. We conclude that, unlike those in many plant and animal tissues, the mitochondria from cucumber cotyledons do not contain AT activity.

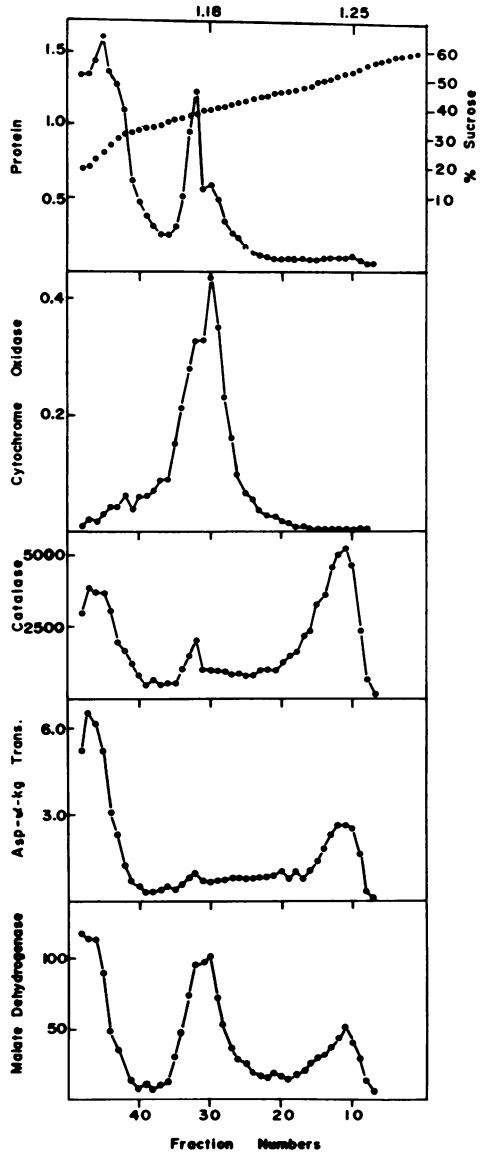

FIG. 2. Separation of organelles from an extract of cotyledons of 2-day-old dark-grown cucumber seedlings in a sucrose gradient. The supernatant fraction obtained after centrifugation of the crude extract at 270*g* for 10 min was applied directly onto the sucrose gradient and centrifuged at 21,000 rpm for 4 hr. Units/gradient fraction: protein, relative absorbance at 280 nm; all enzymes, μmol/min.

The chloroplasts in the total cotyledon extract of 7-day-old light-grown seedlings were isolated in a sucrose gradient after a rate centrifugation. The migration of intact chloroplasts (Chl and NADP-glyceraldehyde-3-P dehydrogenase as markers), broken chloroplasts (Chl as a marker), microbodies (catalase as a marker), and mitochondria (Cyt *c* oxidase as a marker) in the gradient followed those of the spinach leaf extract reported earlier (10) (Fig. 3). In the present preparation, two small but distinct peaks of AT activity were present at densities 1.21 g/cm³ and 1.16 to 1.18 g/cm³, corresponding to intact chloroplasts and microbodies, respectively. Again, no activity was associated with the mitochondria at density 1.14 g/cm³ (fraction 31). The absence of AT activity in the mitochondria of green cotyledons was also revealed (data not shown) when the mitochondria were isolated by the conventional equilibrium sucrose gradient centrifugation (10). The peak of chloroplast AT activity in the gradient (Fig. 3) was small because of the low recovery of the intact organelles, as indicated by the distribution of Chl and NADP-glyceraldehyde-3-P dehydrogenase. Using a similar isolation procedure employing triose-3-P isomerase as the marker, the intact proplastids from cotyledons of 6-day-old dark-grown seedlings were also found to contain AT activity (data not shown).

AT Isozymes and Their Subcellular Location. The total extracts of cotyledons were subjected to starch gel electrophoresis for the separation of AT isozymes. The subcellular locations of these isozymes were identified by using various isolated subcellular fractions. The supernatant fraction obtained after centrifugation of the crude extract at 10,000*g* for 30 min was used as a crude cytosol fraction. Since breakage of organelles was unavoidable during the isolation procedure, both the cytosol enzymes as well as enzymes derived from broken organelles were present in the crude cytosol fraction.

Six isozymes were resolved from the cotyledon extracts of 2- and 6-day-old dark-grown seedlings (Fig. 4). Isozymes 1 and 2 were identified as the glyoxysomal isozymes. Isozyme 3 was not associated with any organelle fraction and was enriched in the crude cytosol fraction. We suggest that isozyme 3 represented the cytosol isozyme, and the other isozymes in the crude cytosol fraction were derived from broken organelles. Isozymes 4, 5, and 6 were those of the proplastids. Whereas all of the latter three isozymes were present in the total cotyledon extract of 6-day-old seedlings, isozyme 6 was missing in that of 2-day-old seedlings.

During the germination of cucurbit seeds, light induces a change in the functional role of the cotyledons from a storage tissue to a photosynthetic one (11, 13, 20). The glyoxysomes rapidly disappear whereas the chloroplasts and the peroxisomes emerge. In the present study, isozymes 3, 4, 5, and 6 were resolved from the total extract of green cotyledons of 7-day-old light-grown seedlings (Fig. 4). Again, isozyme 3, the cytosol isozyme, was enriched in the crude cytosol fraction. Isozymes 4,

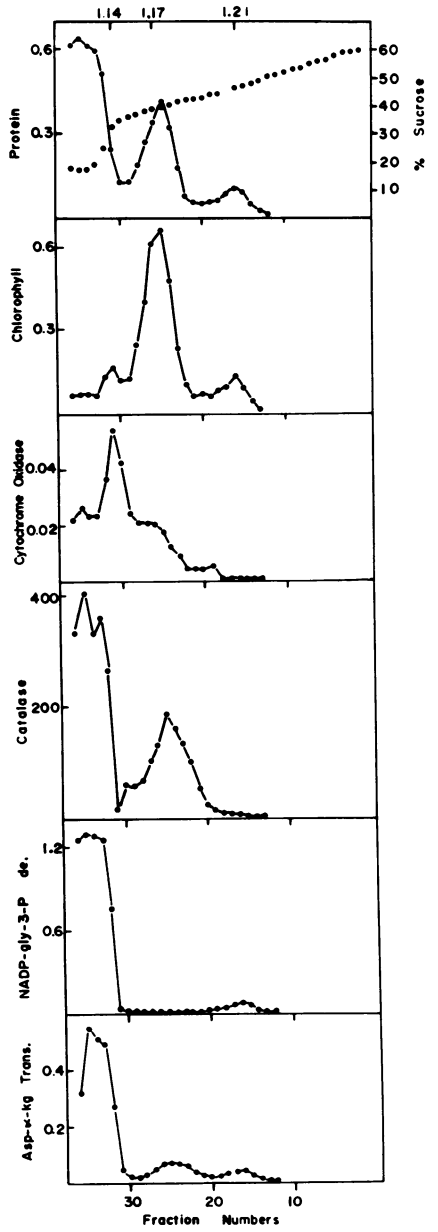

FIG. 3. Separation of organelles from an extract of cotyledons of 7-day-old light-grown seedlings in a sucrose gradient. The total extract was applied directly onto the sucrose gradient and centrifuged at 10,000 rpm for 10 min. Units/gradient fraction: protein, relative absorbance at 280 nm; Chl, relative absorbance at 652 nm; all enzymes, μmol/min.

5, and 6 were identified as the chloroplast isozymes. The two glyoxysomal isozymes (isozymes 1 and 2) were absent in the extract. Isolated peroxisomes contained a trace amount of AT activity which was detected spectrophotometrically but was too low to be revealed in the starch gel. Also, no extra isozyme of the peroxisomes was resolved from the total cotyledon extract in starch gel electrophoresis since the peroxisomal AT activity was calculated to be very low as compared with the activity in the chloroplasts and cytosol. Whether or not the peroxisomes contained a minor but unique isozyme is unknown. The minor amount of the peroxisomal AT activity in the cell is also revealed in other plant tissues. In spinach leaves, the peroxisomal AT represents only a few per cent of the total AT activity (10), and the isolated peroxisomes possess a low AT specific activity which is only 6% of that in isolated glyoxysomes of castor bean (9).

In summary, we identified isozymes 1 and 2 as the glyoxysomal isozymes, isozyme 3 as the cytosol isozyme, isozymes 4, 5, and 6 as the proplastid and chloroplast isozymes.

The two glyoxysomal isozymes, isozymes 1 and 2, were extracted from the starch gel and their properties were studied according to the procedure described earlier (10). No major difference was observed between the two isozymes. They exhibited optimal activity at pH 7 to 8.5. The transamination reaction was freely reversible, and α-ketoglutarate was preferred to pyruvate, glyoxylate, or hydroxypyruvate as the amino group acceptor. With glutamate as the amino group donor, oxaloacetate was superior to pyruvate or glyoxylate as the acceptor. It appeared that both isozymes have similar properties as those of AT described in many plant and animal tissues (2, 15, 16). The

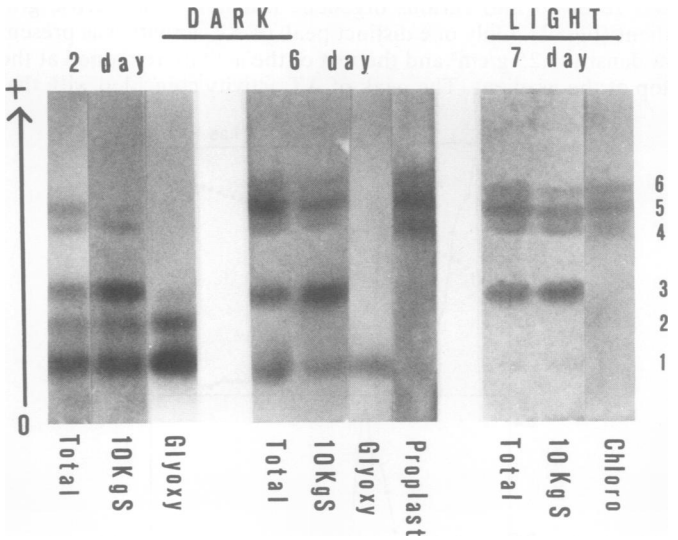

FIG. 4. Separation and subcellular localization of aspartate-α-ketoglutarate transaminase isozymes from cucumber cotyledons. The total extract (total), the supernatant fraction obtained after centrifugation of the total extract at 10,000*g* for 30 min (10 Kg S), isolated glyoxysomes (glyoxy), isolated proplastids (proplast), and isolated chloroplasts (chloro) were obtained from the cotyledons of dark- and light-grown seedlings. The organelles were isolated by rate or equilibrium sucrose gradient centrifugation as described under "Materials and Methods."

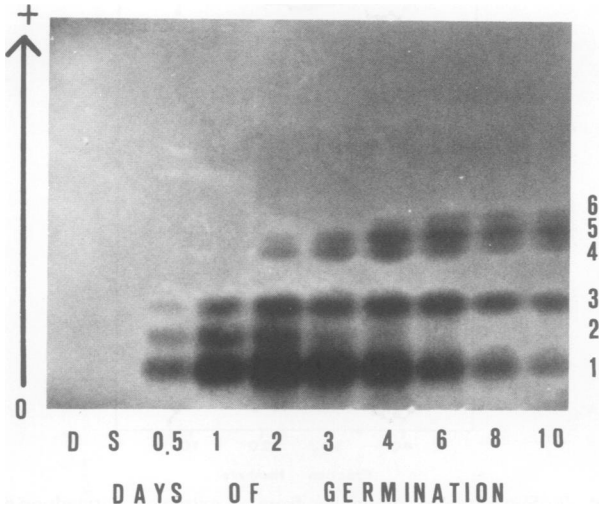

FIG. 5. Developmental changes of aspartate-α-ketoglutarate transaminase isozymes in the cotyledons of dark-grown cucumber seedlings. The total extracts of the cotyledons from dry seeds (D), soaked seeds (S), and seedlings germinated in complete darkness from day 0.5 to day 10 were used.

significance of having two isozymes in the glyoxysomes was not resolved in the present study.

Changes in the AT Isozymes during Germination. A direct comparison of the development of each individual isozyme was made by applying the cotyledon extracts from seedlings of various ages to different slots in one starch gel for electrophoresis. The resulting starch gels of the dark-grown and of the light-grown samples are shown in Figures 5 and 6, respectively. A scanning profile of the isozyme pattern of the cotyledon extract from 2-day-old seedlings is shown in Figure 7. Within the limit of the amount of extract that we applied to the gel, the intensity of each isozyme in one gel was roughly proportional to the amount of enzyme extract applied initially. When diluted 1:1 with grinding medium (v/v), the extract yielded a similar isozyme pattern and the area under each peak was within 40 to 60% of the corresponding peak in the control (Fig. 7). The change in the amount of each individual isozyme was followed quantitatively by computing the area under each peak in the scanning profiles throughout the period of germination (Fig. 8). The three plastid isozymes, isozymes 4, 5, and 6, were treated as one group.

In the cotyledon of dry and soaked seeds, no detectable amount of any isozyme was resolved. As germination proceeded, isozymes 1, 2, and 3 gradually accumulated and peaked around day 2. Thereafter, isozyme 2 (glyoxysomal) dropped off rapidly and disappeared completely at day 6; this developmental pattern was independent of illumination. The amount of isozyme 1 (glyoxysomal) and isozyme 3 (cytosol) gradually decreased after their peaks of development, and at day 10, only 10 to 20%

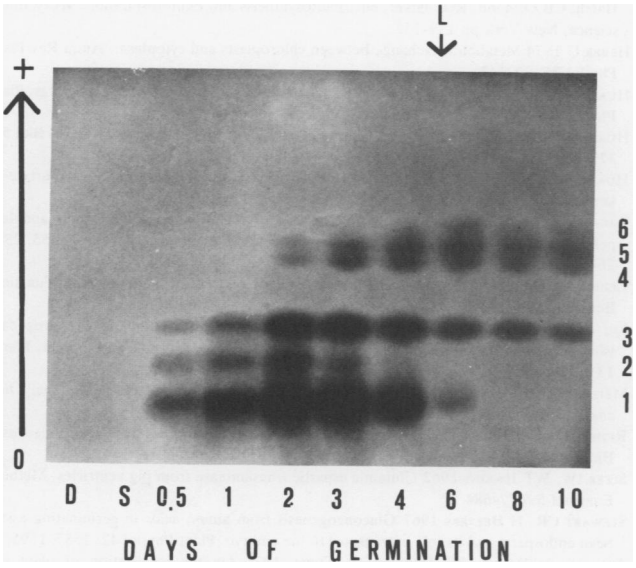

FIG. 6. Developmental changes of aspartate-α-ketoglutarate transaminase isozymes in the cotyledons of light-grown cucumber seedlings. The total extracts of the cotyledons from dry seeds (D), soaked seeds (S), and seedlings germinated from day 0.5 to day 10 were used. Light was applied after 3 days of germination.

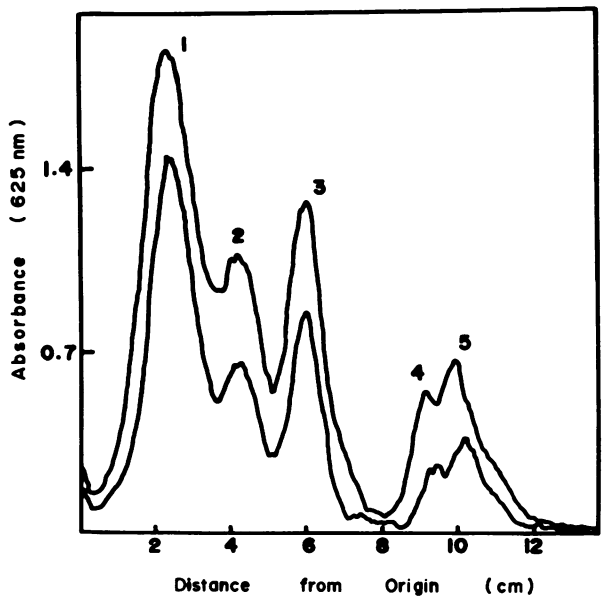

FIG. 7. Scanning profile of the aspartate-α-ketoglutarate transaminase isozymes in the cotyledon extract of 2-day-old cucumber seedlings. The lower curve is a scanning profile of the isozymes when the amount of cotyledon extract applied to the starch gel was reduced to half. Isozyme 6 was not present at this stage of germination.

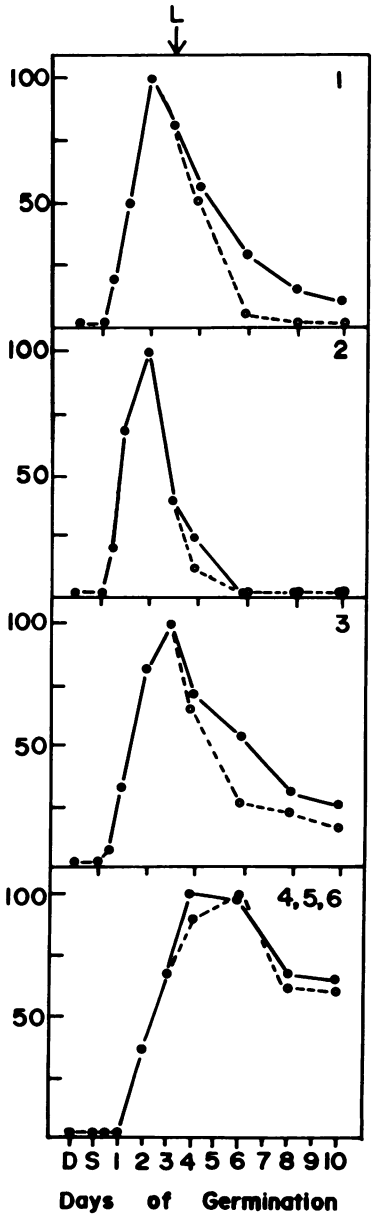

FIG. 8. Changes in the relative amount of each individual aspartate-α-ketoglutarate transaminase isozyme in the cotyledons of cucumber seedlings during germination. Isozymes 4, 5, and 6 were treated as one group. After germination in darkness for 3 days, the seedlings were either maintained in prolonged darkness (●) or exposed to continuous light (○). The amount of each isozyme is expressed in per cent of that at the peak stage of development. D and S indicate the cotyledon extracts from dry seeds and soaked seeds, respectively.

remained. The decrease in isozymes 1 and 3 was accelerated by illumination of the seedlings at day 3, and isozyme 1 was eliminated completely at day 8. The combined rise and fall of the two glyoxysomal isozymes (1 and 2) and the effect of illumination on them are in agreement with the ontogeny of the glyoxysomes in cucumber (20) and other cucurbit seedlings (11). Isozymes 4, 5, and 6, the plastid isozymes, appeared at a later stage of germination. Isozymes 4 and 5 began to accumulate at day 2 and isozyme 6 did not appear until day 4. The accumulation of these plastid isozymes was independent of illumination at a later stage of germination. Such a development pattern was in contrast to that of other chloroplast enzymes which were induced by light (*e.g.* NADP-glyceraldehyde-3-P dehydrogenase in Fig. 1, and see review 22).

The per cent distribution of AT activity in different cellular compartments, as estimated from the scanning profile of the isozyme patterns, changed drastically during germination. In the 2-day-old cotyledon, the two glyoxysomal isozymes accounted for more than 60% of the total enzyme activity and thus the glyoxysomes were the major intracellular site of AT activity (Fig. 7). Some 25% of the total activity was represented by the cytosol isozyme. The proplastid isozymes at this stage of germination occupied only a small per cent of the total activity. At a later stage of germination under illumination (day 10), the cotyledon changed completely to a photosynthetic tissue. After all of the glyoxysomal isozymes had been removed, the chloroplasts, which contained three isozymes, became a major intracellular site of AT activity (Fig. 6). The rest of the AT activity was represented only by the cytosol isozyme.

Comparison with the Castor Bean Endosperm. The cucumber cotyledon is very similar to the endosperm of castor bean, which has been well studied, in that both tissues contain protein and lipid as the food reserve. The subcellular location of AT in the cucumber cotyledon is different from that in the castor bean endosperm where the majority of the enzyme is distributed evenly between the mitochondria and the glyoxysomes (2). We confirmed such an enzyme localization in the castor bean endosperm and also showed that the glyoxysomes and the mitochondria possessed distinct AT isozymes (data not shown). Whether or not the difference in the subcellular localization of AT between the two tissues reflects a difference in the pattern of amino acid metabolism (17) or the interorganelle electron shuttle (12) requires further investigation.

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