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Synthesis of Isocitrate Lyase in Sunflower Cotyledons during the Transition in Cotyledonary Microbody Function[1]

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ABSTRACT

Density-labeling with 10 millimolar $K^{15}NO_3$/70% ^2H_2O has been used to investigate isocitrate lyase synthesis during greening of sunflower (*Helianthus annuus* L.) cotyledons when the glyoxysomal enzyme activities sharply decline and the transition in cotyledonary microbody function occurs. A density shift of 0.0054 (kilograms per liter) was obtained for the profile of isocitrate lyase activity in the CsCl gradient with respect to the ^1H_2O control. Quantitative evaluation of the density-labeling data indicates that about 50% of the isocitrate lyase activity present towards the end of the transition stage in microbody function is due to enzyme molecules newly synthesized during this stage.

Fat-storing cotyledons of certain seeds, including those of the sunflower, become functional as leaves in the normal course of germination. During early stages of development the stored fat is mobilized, and if the cotyledons emerge from the soil and gain exposure to light they develop into photosynthetic organs. During this changeover in cotyledonary metabolism, the microbody population of the mesophyll cells loses its glyoxysomal activities and acquires the enzymic characteristics of leaf peroxisomes. It is still a matter of discussion as to how this transition in microbody function occurs (1, 7, 8). One reason for the current difficulty in interpreting published biochemical data as unequivocal evidence either for or against any one of the discussed hypotheses, is our ignorance of the existence and, if so, extent of turnover of glyoxysomes during the transition stage in microbody function. Results which demonstrate the occurrence of isocitrate lyase synthesis during normal greening of fatty cotyledons are presented in this paper. These results were obtained by density-labeling the glyoxysomal marker enzyme and quantitatively evaluating the data obtained.

MATERIALS AND METHODS

Plant Material and Standard Growth Conditions. Achenes of sunflower (*Helianthus annuus* L., var. Spanners Allzweck) were soaked 12–14 h and then germinated in moist Vermiculite at 30 C. After 2.5 days of growth in darkness the seedlings were exposed to continuous light. The counting of days of germination was begun with the planting of the soaked achenes.

Density-Labeling. For density-labeling, a solution of 10 mM $K^{15}NO_3$ in 70% ^2H_2O was used. The procedure for introducing the isotope solution into the cotyledons of embryos and of 2.5-day-old, dark-grown seedlings, as well as the growth conditions for labeled embryos and seedlings, were those described by Betsche and Gerhardt (3). The length of the labeling period at each developmental stage was 2.5 days.

Partial Purification of Isocitrate Lyase. The cotyledons were ground in a chilled mortar with quartz sand. The homogenization medium (0.5 ml/cotyledon) consisted of 170 mM Tricine (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 10 mM dithioerythritol. In most cases the medium contained in addition 3 mM digitonin. The homogenate was filtered and centrifuged (12,000*g*, 20 min). To the supernatant (crude extract) ammonium sulfate was added to 34% saturation. The precipitated protein was collected by centrifugation (48,000*g*, 20 min) and dissolved in homogenization medium (without digitonin). Protein precipitated again from this solution between 20 and 36% ammonium sulfate saturation was collected by centrifugation and dissolved in 2 ml gradient medium (see below). The resulting solution was clarified by centrifugation (100,000*g*, 15 min). The supernatant contained 40–50% of the isocitrate lyase activity of the crude extract. The specific activity of the enzyme was increased 8- to 9-fold by the partial purification. All operations were carried out at 0–4 C.

Density Gradient Centrifugation. CsCl (Supra grade from Merck) was added to the enzyme solution (0.422 g/ml) and 2 ml of this solution were then mixed with 3 ml of a CsCl solution of density 1.31 kg/l prepared in gradient medium. This medium consisted of 17 mM Tricine (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 10 mM dithioerythritol. The final enzyme/CsCl solution which contained ≤ 2 mg protein/5 ml was centrifuged in a Beckman fixed-angle rotor Ti-65 for 40 h at 45,000 rpm (177,000g_{max}) at 5 C. Since the tubes of the Ti-65 rotor have a total volume of 13.5 ml the final enzyme/CsCl solution was overlayed with paraffin oil to fill the tubes to the top. After centrifugation the gradients were fractionated into 190 one-drop fractions (26 μl/drop) by means of a peristaltic pump. Every 10th fraction was taken for refractive index determination. The refractive indices were converted into density units by the equation of Bruner and Vinograd (4). The recovery of isocitrate lyase from gradients was 60%.

Assays. Enzyme assays were performed spectrophotometrically at 20 C. Isocitrate lyase activity was determined according to Hock and Beevers (10) with minor modifications. The assay mixture contained, in a total volume of 1.1 ml, 170 mM Tricine (pH 7.4), 3 mM phenylhydrazine hydrochloride, 5 mM $MgCl_2$, 2 mM EDTA, and a suitable amount of enzyme. CsCl gradient fractions were assayed by adding the assay mixture to the one-drop fractions. The reaction was started with 10 μl of 0.33 M DL-isocitrate (allo-free), and 3 min after the addition of the substrate the reaction rate was recorded. Lactate dehydrogenase was assayed as described (2). Protein was determined by the Lowry method.

RESULTS

Development of Isocitrate Lyase Activity. Previous experiments have shown that in sunflower cotyledons the activities of glyoxysomal enzymes rise to a maximum around day 2 of germination

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and subsequently decline (3, 5, 11). In the course of the present studies it was determined that isocitrate lyase reaches its maximum activity after 2.5 days of germination (Table I). The time from the planting of the achenes up to this time will be denoted as stage I of development.

As already documented (3, 5, 6, 11), coincident with the loss of glyoxysomal enzyme activities a rise in enzyme activities characteristic of leaf peroxisomes occurs in the light. These contrasting activity changes illustrate the transition in microbody function. The time when it occurs (from day 2.5 through day 5 of germination) will be defined as stage II of development.

Growth of the sunflower seedlings on 10 mM $K^{15}NO_3$/70% ^2H_2O had no serious effect on the developmental pattern of isocitrate lyase activity (Table I). Similar results have already been reported (9, 13).

Addition of digitonin (3 mM final concentration) to the homogenization medium increased the measurable isocitrate lyase activity in the crude extract (Table I). The increase was more pronounced in crude extracts from 5-day-old cotyledons than in those from 2.5-day-old cotyledons.

Density-Labeling of Isocitrate Lyase. Labeling of isocitrate lyase during stage I resulted in a density increase of the enzyme (Fig. 1) averaging 0.0160 kg/l (1.2% density shift). If the labeling experiments were carried out at stage II, an incorporation of label into isocitrate lyase was also obtained (Fig. 2). The peak position of the activity profiles, in the CsCl gradient, of isocitrate lyase from unlabeled and labeled cotyledons were separated by six to eight fractions. On the average, the density difference between the activity peaks amounted to 0.0054 kg/l (0.4% density shift).

Corresponding results were also obtained in experiments in which the CsCl gradients were centrifuged (36,000 rpm ≙ 170,000g_{max}, 63 h) in a Beckman swinging-bucket rotor SW-56 and where lactate dehydrogenase was used as density marker (Fig. 3). However, due to the much shallower gradients obtained in fixed-angle rotors the use of the Ti-65 rotor resulted in an increased number of fractions separating the activity peaks and in a greater number of fractions contributing to each peak. All of the

Table I. *Change of Isocitrate Lyase Activity in Cotyledons of Sunflower Seedlings*

Seedlings were transferred into light after 2.5 days of growth in the dark. The values in parentheses were obtained with the homogenization medium containing 3 mM digitonin.

Time of Germination	Growth Medium	
	$K^{14}NO_3/^1H_2O$	$K^{15}NO_3$/70% ^2H_2O
	Isocitrate Lyase Activity	
days	*pkat/cotyledon*	
0	133	133
2	1,834	1,934
2.5	2,784 (3,367)	2,601 (2,884)
3	1,867	2,167
5	350 (984)	383 (1,017)

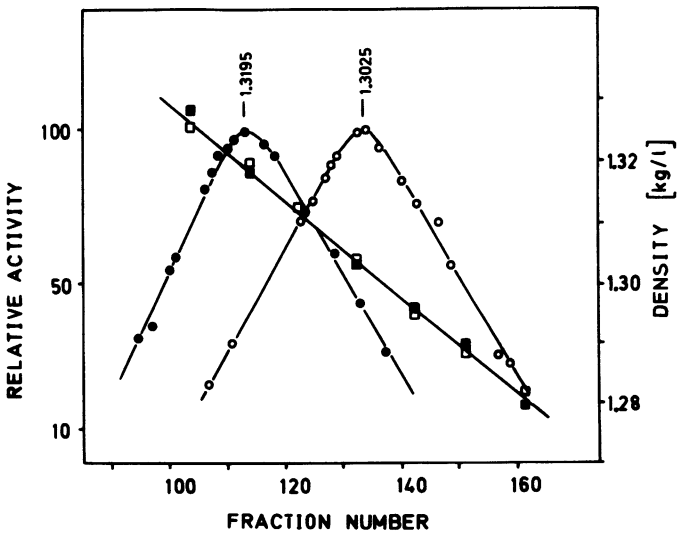

FIG. 1. Activity profiles of isopycnically banded isocitrate lyase. Unlabeled enzyme (○); enzyme labeled during stage I (●). CsCl gradients centrifuged in the fixed-angle rotor Ti-65. Relative activity: all points of each individual activity profile are expressed as a percentage of the respective profile maximum. Density of CsCl gradients (□, ■).

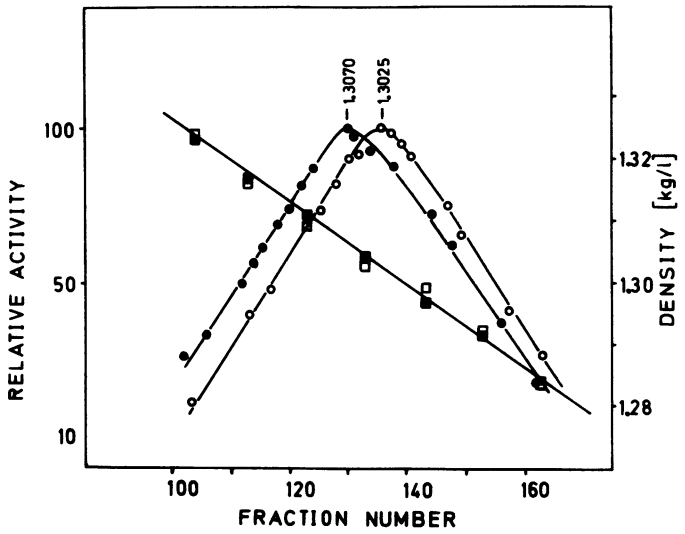

FIG. 2. Activity profiles of isopycnically banded isocitrate lyase. Unlabeled enzyme (○); enzyme labeled during stage II (●). Details as in Figure 1.

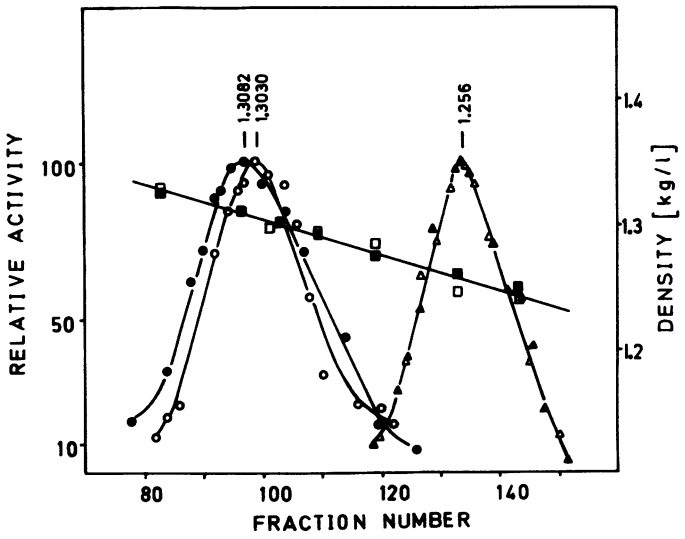

FIG. 3. Activity profiles of isopycnically banded isocitrate lyase. Unlabeled enzyme (○); enzyme labeled during stage II (●). CsCl gradients centrifuged in the swinging-bucket rotor SW-56. Activity profiles have been aligned so that profiles of control enzyme (△, ▲: lactate dehydrogenase) coincide. Relative activity: all points of each individual activity profile are expressed as percentages of the respective profile maximum. Density of CsCl gradients (□, ■).

nine labeling experiments performed during stage II resulted in a density shift of the profile of isocitrate lyase activity in the CsCl gradient with respect to the ^1H_2O control.

Apparent Synthesis of Isocitrate Lyase during Stage II. The density shift obtained for the activity profile of isocitrate lyase in the CsCl gradient following labeling of cotyledons during stage II demonstrates a synthesis of the enzyme at this developmental stage. The amount of isocitrate lyase, which at this stage results from the balance between actual enzyme synthesis and the degradation of newly synthesized enzyme (*i.e.* the apparent synthesis of isocitrate lyase) was calculated from the density-labeling data following the mathematical treatment outlined by Betsche and Gerhardt (3). It was found that 45–55% (depending on the individual experiment) of the isocitrate lyase activity present at the end of stage II was due to enzyme molecules synthesized during this stage. The calculation of apparent enzyme synthesis is based on the fundamental presuppositions that the activity profile, in the CsCl gradient, of unlabeled enzyme and at least each half-section of the activity profile of enzyme labeled during stage I are describable by a Gaussian equation (3). By transforming the Gaussian equation into a linear expression, the bell-shaped curve is converted to a straight line. Figure 4 shows that these presuppositions are fulfilled; the activity profile of unlabeled isocitrate lyase and each half-section of the activity profile of isocitrate lyase labeled during stage I gave straight lines when they were treated mathematically as Gaussian curves.

DISCUSSION

The extent of apparent isocitrate lyase synthesis during stage II has been calculated by a strictly mathematical evaluation of the density-labeling data. Due to a possibly changing contribution of unlabeled precursors to the enzyme syntheses during cotyledon development, some of the parameters on which the calculation depends may not have been quite correctly determined by the approaches used for their estimation. This problem has been discussed in detail by Betsche and Gerhardt (3). By taking the possible limits of error into account it was shown (3) that the calculated rates of apparent enzyme synthesis are essentially the same. This fact was confirmed for the apparent synthesis of isocitrate lyase. The result that about 50% of the isocitrate lyase activity measurable at the end of stage II was due to enzyme newly synthesized during this stage is considered to be a reasonably accurate estimate.

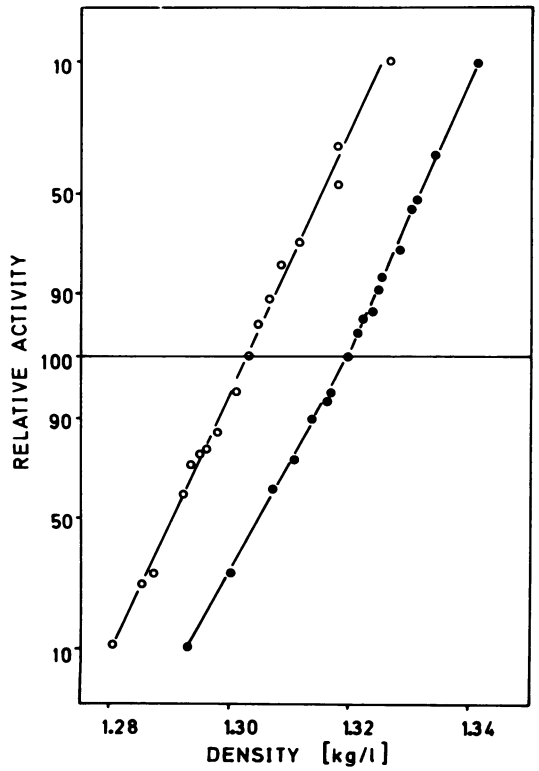

FIG. 4. Linear expression of the isocitrate lyase profile in Figure 1. The regression lines calculated for the linearized activity profile of unlabeled enzyme (○) and for each half-section of the linearized activity profile of labeled enzyme (●) are shown. Relative activity expressed as a percentage of the maximum value of the corresponding activity profile.

Results on the density-labeling of isocitrate lyase during the developmental stage of fatty cotyledons when this enzyme activity declines have already been published (9). In contrast to our data, a density shift in the activity profile of isocitrate lyase in the CsCl gradient with respect to the ^1H_2O control had not been observed. This discrepancy can be explained on the basis that, in those studies, the resolution of the corresponding gradient region was about 0.007 density units per fraction, while the density shift reported in this paper for stage II averaged 0.0054 density units.

The labeling of isocitrate lyase during stage II occurred after the peak of enzyme activity had been reached. The activity loss of total isocitrate lyase follows first-order kinetics during stage II (8) and at the end of this stage about 50% of the existing enzyme had been labeled. Therefore, if the degradation of unlabeled, preexisting isocitrate lyase and the degradation of newly synthesized, labeled enzyme were not substantially different, it can be concluded that the synthesis of isocitrate lyase at stage II was not restricted to the early phase of this developmental stage. If it had been so, the activity peak would have occurred later than observed.

At present, no unambiguous answer can be given to the question of what process causes the synthesis of isocitrate lyase during stage II. The synthesis can be due either to turnover of the enzyme and/or to a delayed formation of glyoxysomes. Assuming that the mesophyll cells of a cotyledon develop out of synchrony, individual mesophyll cells will be at different stages of development at a defined stage of cotyledon development. While isocitrate lyase activity in the more developed cells declines during stage II, that in less developed cells may still be undergoing synthesis in the course of glyoxysome formation. Delayed formation of glyoxysomes in part of the cotyledonary mesophyll cells would also result in catalase synthesis since on a cotyledon basis the development of catalase and glyoxysomal enzymes occurs concomitantly (3, 5, 7, 11). However, the apparent catalase synthesis during stage II is not greater than that occurring in much older cotyledons (3). On the basis of this fact, we favor the interpretation that the synthesis of isocitrate lyase during stage II indicates a turnover of the enzyme.

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