

Evidence for De Novo Synthesis of Isocitratase and Malate Synthetase in Germinating Peanut Cotyledons¹

Claudio P. Longo

AEC Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48823

Received November 30, 1967.

Abstract. Evidence for *de novo* synthesis of isocitratase and malate synthetase in cotyledons of germinating peanut (*Arachis hypogaea* L.) was obtained by the density labeling method. When dry peanut cotyledons were cultured in $H_2^{18}O$, a 2.4% increase in the buoyant density of malate synthetase in a cesium chloride gradient was observed. In 100% D_2O the buoyant density shift was 5.5% for isocitratase and 3.5% for malate synthetase in comparison to the water controls. These data suggest that isocitratase and malate synthetase do not pre-exist in some inactive form in the cotyledons, but are completely synthesized after onset of germination from a pool of amino acids which do not derive directly from hydrolysis of storage proteins.

In most fat-storing seeds germination involves a massive breakdown of the fat reserves and their conversion to carbohydrates by means of the glyoxylate cycle (2). The 2 key enzymes of this cycle, isocitratase (E.C. 4.1.3.1) and malate synthetase (E.C. 4.1.3.2) appear in the fat storing tissue shortly after the seed has fully imbibed, reach a climax after a few days and decline thereafter (3). It is not known whether these enzymes are synthesized *de novo* or arise through the activation of some precursor already present in the dry seed, although data obtained with inhibitors of RNA and protein synthesis suggest that they are synthesized *de novo* after the start of germination in pumpkin cotyledons (7). The present work proposes an answer to this question by means of a different experimental approach: density labeling with heavy isotopes (6, 8). This technique consists in supplying the system under study with a substance containing a heavy isotope which can be incorporated into the amino acids used for the synthesis of new protein. The incorporation of the isotope results in an increase in mass of the protein which can be detected by isopycnic equilibrium centrifugation. In this way it is possible to decide whether a given protein is synthesized after the addition of the labeled compound or whether it had existed in the cells before isotope addition.

Materials and Methods

Virginia peanuts (*Arachis hypogaea* L.), variety R-56, 1965 crop (kindly supplied by Dr. R. L. Ory, New Orleans) were surface sterilized for 15 to 20

minutes in 1% sodium hypochlorite and were then soaked for 6 hours in sterile water before planting them in 1000 ml Erlenmeyer flasks containing moist, sterilized vermiculite. The seeds were allowed to germinate in a dark room at a constant temperature of 28°. In other experiments the seed coat and the embryo were removed from the dry seeds and the dry cotyledons were put, flat side down, in stoppered vials containing 0.5 to 0.6 ml water (H_2O , D_2O or $H_2^{18}O$ as specified in the results section). No bacterial or fungal contamination was observed when proper precautions were taken to maintain aseptic conditions. As already reported (11), the time course of appearance and the total amount of activity of the 2 glyoxylate cycle enzymes were approximately the same in cotyledons taken from intact seedlings or cultured separately.

For obtaining crude extracts 5 to 6 cotyledons were thoroughly washed in running distilled water, cut with a scalpel into fine slices and ground in a mortar with 0.05 M phosphate buffer pH 7.5 (2 ml per cotyledon). The resulting brei was squeezed through several layers of cheesecloth and centrifuged for 15 minutes at $14,800 \times g$. All operations were performed at a temperature of 0 to 4°. The clear supernatant was carefully separated from the fatty layer at the top of the centrifuge tube by means of a hypodermic syringe and used without further purification. Isocitratase was assayed by the method of Dixon and Kornberg (5) and malate synthetase by the method of Hock and Beevers (7). The assay procedure of Decker and Maitra (4) for estimation of glyoxylate was used as an additional check on the reliability of the Dixon-Kornberg test in the peanut extract. The agreement between data obtained by the 2 assay methods was good.

Density gradient centrifugation followed essen-

¹ Work carried out under United States Atomic Energy Commission Contract No. AT (11-1)-1338.

tially the procedure used by Filner and Varner (6). The gradient mixture used had the following composition: 2.42 ml CsCl solution having a density (ρ) of 1.56 g/cm³, 0.5 ml 0.1 M phosphate buffer pH 7.5 and water to a final volume of 4.5 ml. On this mixture, 0.1 ml of the crude peanut extract containing 2 to 3 mg of protein was layered together with 600 μ g of a highly purified preparation of α -amylase from barley aleurone layers. In some experiments a similar α -amylase preparation labeled with ¹⁴C was employed. The final gradient mixture was overlaid with 0.5 ml paraffin oil and spun for 65 to 72 hours at 62 to 65,000 rpm and 2° in the Spinco L2-65 ultracentrifuge using a SW-65 rotor. After centrifugation 6-drops fractions were collected by puncturing the tubes at the bottom and each alternate fraction was assayed for isocitratase or malate synthetase (odd numbers) and α -amylase (even numbers). If ¹⁴C-labeled α -amylase was used, all even fractions were collected in 5 ml of Bray's solution (1) and counted in a Beckman scintillation counter. The refractive index of 1 out of every 10 fractions was measured with a Bausch and Lomb refractometer and was converted to density by means of a standard curve.

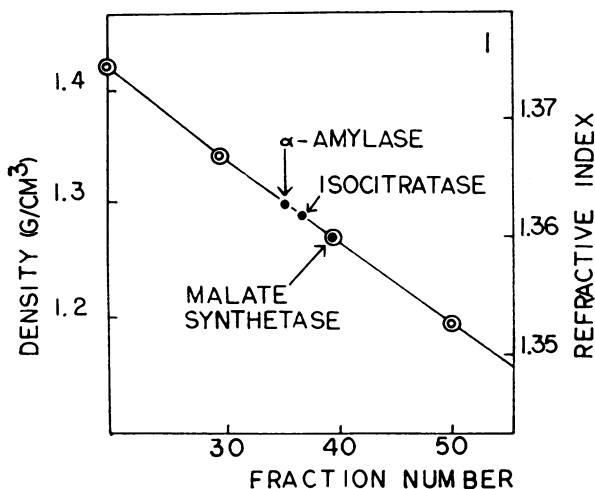


FIG. 1. Plot of density versus fraction number of a typical cesium chloride gradient. Experimental conditions as described in text. The density values were evaluated from refractive indices (shown on the right) by means of a standard curve. The position of the α -amylase, isocitratase and malate synthetase activity peaks are shown.

Results

Both the isocitratase and malate synthetase activities form well defined bands in a cesium chloride gradient under my experimental conditions. Their buoyant densities were evaluated by means of a "density gradient curve" obtained by plotting the densities of several fractions against fraction number. Such a curve had a constant shape from

one experiment to the next with only minor variations and was linear in the zone of the centrifuge tube where the enzymes banded (fig 1). The buoy-

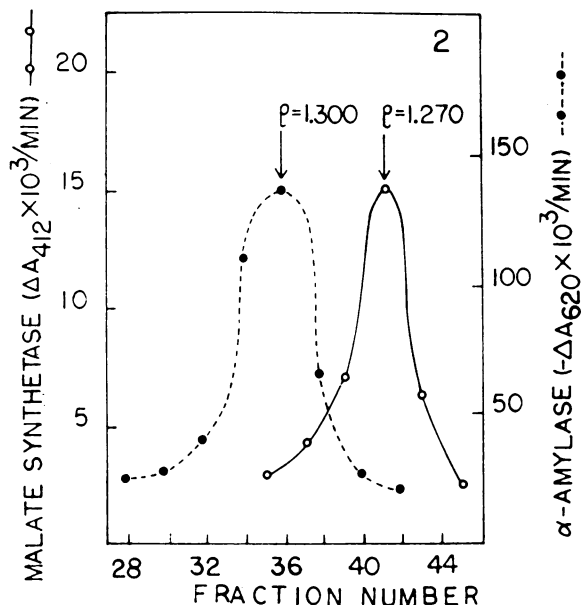


FIG. 2. Equilibrium distribution in a cesium chloride gradient of malate synthetase activity from cotyledons of 5-day-old peanut seedlings grown in 100% H₂¹⁶O. α -Amylase from barley aleurone layers was included in the gradient as a marker of known buoyant density. See text for other experimental conditions.

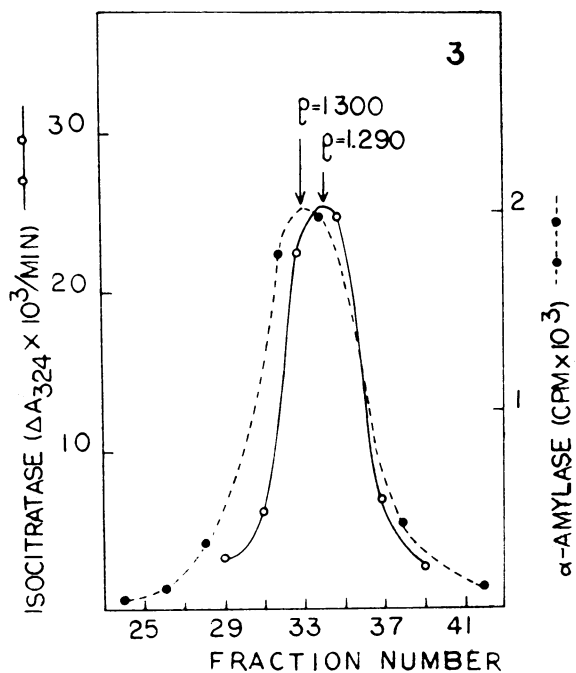


FIG. 3. Equilibrium distribution in a cesium chloride gradient of isocitratase activity from cotyledons of 5-day-old peanut seedlings grown in 100% H₂¹⁶O. See text for other experimental conditions.

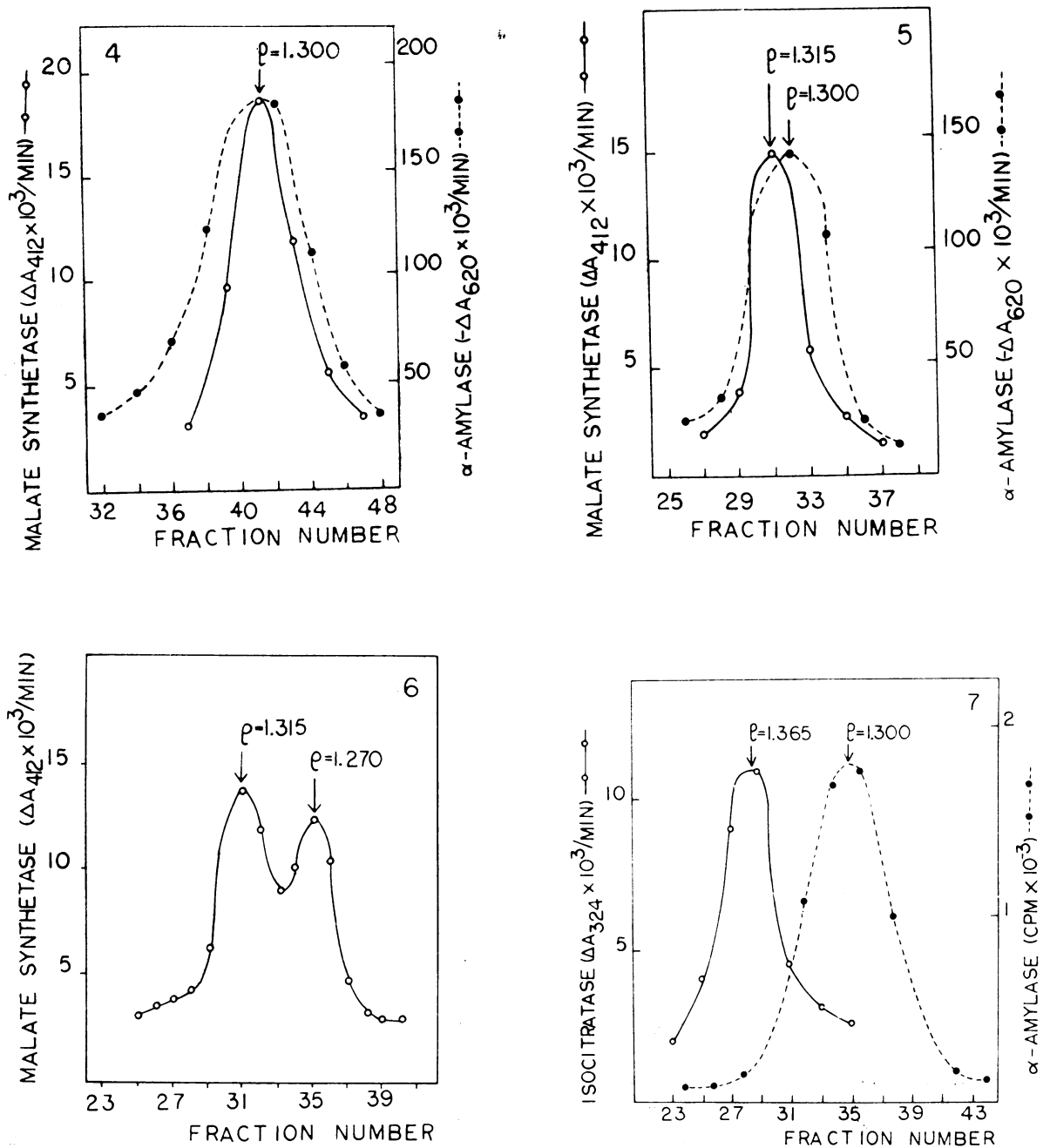


FIG. 4. Equilibrium distribution in a cesium chloride gradient of malate synthetase activity from a single, excised peanut cotyledon grown for 5 days in 93% H_2^{18}O . See text for other experimental conditions.

FIG. 5. Equilibrium distribution in a cesium chloride gradient of malate synthetase activity from excised peanut cotyledons grown for 5 days in 100% D_2O . See text for other experimental conditions.

FIG. 6. Equilibrium distribution in a cesium chloride gradient of malate synthetase activity from peanut cotyledons grown in 100% D_2O and 100% H_2O . The mixture of crude extracts layered on the gradient contained 3.2 mg protein of D_2O -grown cotyledons and 0.8 mg protein of H_2O -grown cotyledons. See text for other experimental conditions.

FIG. 7. Equilibrium distribution in a cesium chloride gradient of isocitratase activity from excised peanut cotyledons grown for 5 days in 100% H_2O . See text for other experimental conditions.

ant densities observed were 1.300 ± 0.005 for α -amylase, 1.290 ± 0.005 for isocitritase and 1.270 ± 0.005 for malate synthetase (figs 2 and 3). No difference in buoyant densities could be detected between enzyme activities of cotyledons incubated in isolated conditions and those of cotyledons attached to the seedling. The steep gradients used throughout all experiments were chosen in order to obtain sufficiently high enzyme activities for estimation by reducing the variance of the peaks. No precipitation of proteins was observed in the cesium chloride gradients despite the relatively high amounts of cotyledon extract used. By the use of Gaussian paper it could be shown that all 3 of the enzymic activities tested followed a Gaussian distribution in the gradient with good approximation.

As can be seen in figure 4 the malate synthetase band of cotyledons grown in $H_2^{18}O$ (93 ^{18}O atoms %) is markedly shifted towards the heavy side of the gradient as compared to the $H_2^{16}O$ control (fig 2). The shift in buoyant density is 2.4 %.

While $H_2^{18}O$ did not have any inhibitory effect on the development of malate synthetase and isocitratase activity, a 60 % reduction was observed in cotyledons incubated in pure D_2O . Whole seeds grew poorly under these conditions and the reduction in the level of the glyoxylate cycle enzymes was even more severe than in isolated cotyledons. In extracts from 5-day-old peanut cotyledons incubated in 100 % D_2O a 3.5 % increase in the buoyant density of the malate synthetase band was observed (fig 5). If crude extracts from cotyledons incubated in 100 % H_2O were mixed with comparable deuterated extracts in equal proportions (on the basis of specific enzyme activity) it was possible to resolve the heavy and light malate synthetase bands in the gradient (fig 6). In cotyledons incubated in 100 % D_2O , a 5.5 % shift in buoyant density was found for isocitratase activity (fig 7). If 80 % rather than 100 % D_2O was used, the density shift was 1.2 % for both isocitratase and malate synthetase and no reduction of enzyme activity was observed in the cotyledons.

Discussion

Filner and Varner (6) calculated that $H_2^{18}O$ labeling of α -amylase from aleurone layers should increase its mass by 0.72 % if the enzyme was synthesized only from amino acids provided by the hydrolysis of reserve proteins. It cannot be estimated what the percent mass increase of malate synthetase would be under similar conditions because the amino acid composition of this enzyme in higher plants is not known. The magnitude of the observed shift (about 3 times greater than that calculated for α -amylase) suggests, however, that introduction of ^{18}O only through breakage of peptide bonds of reserve proteins is insufficient to

account for the total increase found. It may be assumed therefore that the ^{18}O label is introduced into the malate synthetase molecule also through additional metabolic events, e.g. conversion of sugars to amino acids, so that all of the carboxyl oxygens become fully equilibrated with the oxygen of the medium. The observed buoyant density of unlabeled malate synthetase, which is lower than that of most other proteins, tends to rule out the presence in the enzyme of a carbohydrate portion which would become heavily labeled if synthesized in presence of $H_2^{18}O$. The buoyant densities of glycoproteins are indeed higher than those of other proteins because of the high buoyant densities of polysaccharides (about 1.6).

The increase in density of both isocitratase and malate synthetase after incubation of the tissue in D_2O cannot be explained by the introduction of deuterium through amino acids derived from D_2O -hydrolysis of reserve proteins. In this instance deuterium would become attached to the peptide nitrogen of the protein backbone and would be easily exchangeable with the hydrogen of water (9). Such an exchange could have an adequate opportunity to occur during grinding and centrifugation. On the other hand deuterium attached to carbon atoms is known to be quite stable (with the possible exception of the carbon bearing the α -amino group in amino acids). Attachment of deuterium to carbon atoms of the glyoxylate cycle enzymes could be due to synthesis of amino acids from other compounds (e.g. glucose) or to extensive reworking of the carbon skeletons of the amino acids resulting from breakdown of storage protein. Ignorance of the amino acid composition of these enzymes again precludes any calculation of the expected mass increase. For an enzyme molecule with side chains containing a comparatively large number of hydrogen atoms, an increase in mass of 5 to 6 % upon substitution of every stable hydrogen by deuterium can be estimated (8). It must be recalled, however, that in concentrated salt solutions (as in a cesium chloride gradient) the change in buoyant density may not be an exact index of the increase in mass of the enzyme molecule (10). For this reason only qualitative conclusions may be drawn from my results. The high increase in buoyant density of both isocitratase and malate synthetase suggests that these enzymes are synthesized from a pool of amino acids which do not derive directly from hydrolysis of reserve proteins. This suggestion is confirmed by the results obtained with ^{18}O -labeling of malate synthetase. The different increases in buoyant densities of isocitratase and malate synthetase could tentatively be explained by assuming that the 2 enzymes have a very different amino acid composition or that they are synthesized from amino acids of 2 separate pools.

Even if account is taken of the possible pitfalls in interpreting density labeling data, it may be confidently concluded that isocitratase and malate

synthetase are synthesized in peanut cotyledons after imbibition. The possibility of a mixed origin (*de novo* synthesis and activation of preexisting enzyme molecules) cannot be completely ruled out, but it does not seem very likely. In this case one would expect to see the enzyme activity from the cotyledons grown in heavy water to form 2 distinct bands as in figure 6 or at least 1 single band having a greater width than the water control. The experimental data show, on the contrary, that the shape and the width of the bands are not affected by the density label.

Acknowledgment

The author is indebted to Drs. P. Filner and J. E. Varner for many helpful discussions.

Literature Cited

- BRAY, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. *Anal. Biochem.* 1: 279-85.
- CANVIN, D. T. AND H. BEEVERS. 1961. Sucrose synthesis from acetate in the germinating castor bean: kinetics and pathway. *J. Biol. Chem.* 236: 988-95.
- CARPENTER, W. D. AND H. BEEVERS. 1959. Distribution and properties of isocitritase in plants. *Plant Physiol.* 34: 403-09.
- DECKER, E. E. AND U. MAITRA. 1964. Conversion of γ -hydroxyglutamate to glyoxylate and alanine: purification and properties of the enzyme system. *J. Biol. Chem.* 237: 2218-27.
- DIXON, G. H. AND H. L. KORNBERG. 1959. Assay methods for key enzymes of the glyoxylate cycle. *Biochem. J.* 72: 3p.
- FILNER, P. AND J. E. VARNER. 1967. A test for *de novo* synthesis of enzymes: density labeling with $H_2^{18}O$ of barley α -amylase induced by gibberellic acid. *Proc. Natl. Acad. Sci. U. S.* 58: 1520-26.
- HOCK, B. AND H. BEEVERS. 1966. Development and decline of the glyoxylate-cycle enzymes in watermelon seedlings. (*Citrullus vulgaris* Schrad.). Effects of dactinomycin and cycloheximide. *Z. Pflanzenphysiol.* 55: 405-14.
- HU, A. S. L., R. M. BOCK, AND H. O. HALVORSON. 1962. Separation of labeled from unlabeled proteins by equilibrium density gradient sedimentation. *Anal. Biochem.* 4: 489-504.
- HVIDT, A. AND S. O. NIELSEN. 1966. Hydrogen exchange in proteins. *Advan. Protein Chem.* 21: 287-386.
- IFFT, J. B. AND J. VINOGRAD. 1966. The buoyant behaviour of bovine serum mercaptalbumin in salt solution at equilibrium in the ultracentrifuge. II. Net hydration, ion binding, and solvated molecular weight in various salt solutions. *J. Phys. Chem.* 70: 2814-22.
- MARCUS, A. AND J. FEELEY. 1964. Isocitric lyase formation in the dissected peanut cotyledon. *Biochem. Biophys. Acta* 89: 170-71.