PYRUVATE DEHYDROGENASE COMPLEX ACTIVITY IN PROPLASTIDS AND MITOCHONDRIA OF DEVELOPING CASTOR BEAN ENDOSPERM.

E.E. Reid, C.R. Lyttle, D.T. Canvin, and D.T. Dennis*

Department of Biology, Queen's University
Kingston, Ontario, Canada

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Summary: Proplastids and mitochondria from developing castor bean endosperm have been separated using both prolonged centrifugation in continuous gradients and brief centrifugation in discontinuous sucrose density gradients. Continuous gradients showed some contamination of proplastids by mitochondria but no contamination was found in the discontinuous gradients. Catalase, a microsomal marker, was found between the mitochondria and proplastids in the discontinuous gradients. The pyruvate dehydrogenase complex activity was found coincident with both the mitochondria and proplastid peaks in both gradients.

Long chain fatty acid biosynthesis has been shown to be associated with a particulate fraction from developing castor bean endosperm (1). Further resolution of this fraction by sucrose density gradient centrifugation has indicated that this synthetic activity is associated with the proplastid fraction (2,3,4). Acetate, acetyl-CoA and malonyl-CoA (1,4) can act as substrates for fatty acid synthesis. When malonyl-CoA is used as substrate, acetyl-CoA, NADH and NADPH are also required whereas when acetyl-CoA is used, ATP, HCO_3^-, and Mn^{2+} are required in addition to NADH and NADPH (1). It has been suggested that NADH is the primary reductant in chain elongation and that NADPH is required for desaturation (1,4,5). These results raise the problem of the source of acetyl-CoA, ATP and reduced cofactors for fatty acid biosynthesis. A 10,000 x g pellet from developing castor beans has been shown to incorporate radioactivity from sucrose into fatty acids even after repeated washings (5). By the use of intermediates and inhibitors, it was shown that the entire pathway from sucrose to fatty acids is located in the 10,000 x g fraction (5). It was suggested that NADH and ATP are supplied by the oxidative

* Present address until July 1975: Department of Chemistry, University of California, Los Angeles, California 90024

breakdown of sucrose to acetyl-CoA (5). Assuming that the organelle responsible for both the breakdown of sucrose to acetyl-CoA and for fatty acid biosynthesis in the 10,000 x g fraction is the proplastid, the pyruvate dehydrogenase complex should be present in the proplastids as well as the mitochondria.

Methods and Materials:

Castor bean plants, *Ricinus communis* L., variety Baker 296, (Baker Castor Oil Company, Plainview, Texas) were grown in a greenhouse and developing seeds were harvested 25-30 days after pollination. Endosperm tissue was homogenized according to the method of Drennan and Canvin (1) except that a 500-10,000 x g pellet was resuspended in grinding medium.

Both linear and discontinuous gradients were used and in all cases the sucrose solutions were buffered with 0.01 M TES [N-tris-(hydroxymethyl)-methyl-2-amino ethane sulfonic acid], pH 7.5. The continuous gradients were 30 ml of 35-55% sucrose cushioned on 4 ml of 60% sucrose. After layering on a 1 ml aliquot of the resuspended 500-10,000 x g particles, the gradients were centrifuged for 3 hr at 104,000 x g using an SW27 rotor in a Spinco L-2, type D ultracentrifuge. The discontinuous gradients contained 1 mM $MgCl_2$ and were composed of 10 ml of 35%, 14 ml of 45%, and 10 ml of 60% sucrose. Two milliliters of sample were layered on and the discontinuous gradients centrifuged as above with the time reduced to 10 min. All gradients were collected dropwise from the bottom, the continuous gradient in 1.2 ml fractions and the discontinuous in 1.1 ml fractions. The fractions were numbered in the order they were collected.

Catalase was assayed by the method of Breidenback et al. (6). All other enzyme activities were monitored using a Gilford modified Beckman DU spectrophotometer. Succinic dehydrogenase was determined spectrophotometrically by the method of Hiatt (7) except that 0.6 mM 2,6-dichlorophenolindophenol (DCIP) and 0.15 mg/ml phenazine methosulfate were used and a 15 min substrate preincubation at 25° was included. Triose phosphate isomerase

activity was detected by the method of Beisenherz (8). The pyruvate dehydrogenase complex was assayed by a modification of the method of Schwartz et al. (9). The assay mixture minus pyruvate was incubated for 10 min at 25° and included 0.1% Triton X-100. Sucrose concentration in the gradients was determined by an Abbe refractometer. Protein was determined by the absorbance at 280 nm or by the method of Lowry et al. (10) after precipitating the protein with 10% trichloroacetic acid.

Results:

Three major protein peaks were found in the continuous gradient. A peak having an equilibrium density of 1.18 contained most of the succinate dehydrogenase activity and was, therefore, the location of the mitochondria. A peak with an equilibrium density of 1.21 contained most of the triose phosphate isomerase activity which moved into the gradient, and this peak was assumed to be the location of the proplastids. An equilibrium density of 1.21 has been shown for proplastids by other workers (11). The separation of the proplastids from the mitochondria was not complete. Pyruvate dehydrogenase complex activity was found in both peaks, and it was unlikely that the activity coincident with the proplastid peak could be accounted for solely by mitochondrial contamination.

In order to prepare proplastids free from mitochondrial contamination, advantage was taken of the fact that proplastids have a larger sedimentation coefficient in sucrose gradients than do mitochondria or microbodies (11). A discontinuous gradient was also used to enhance this separation. Four protein bands were found on the discontinuous gradient (Fig. 1A). Band A represented soluble proteins, bands B and C were particulate matter held back at the interfaces between sucrose concentrations, and D was a pellet with little of the activities assayed for. Succinate dehydrogenase was found in band B with no activity in band C (Fig. 1A). Triose phosphate isomerase was found in bands A, B, and C (Fig. 1B). Band C, therefore, contained proplastids free from mitochondrial contamination. The triose phosphate isomerase activity in band B probably represents proplastids held up at the interface and that in band A

is soluble activity and activity from broken proplastids. Pyruvate dehydrogenase complex activity was found in both the proplastid and mitochondrial fractions (Fig. 1B). This indicates that the pyruvate dehydrogenase complex is found in proplastids and is not a contaminating activity from mitochondria since no succinate dehydrogenase was found in this band. The majority of the catalase activity, a microbody marker (11), was found between bands B and C, although some activity was found throughout the gradient. Microbodies have a similar equilibrium density to proplastids but a smaller sedimentation coefficient than proplastids (11).

In experiments with a 500 x g supernatant fraction and a 500-10,000 x g

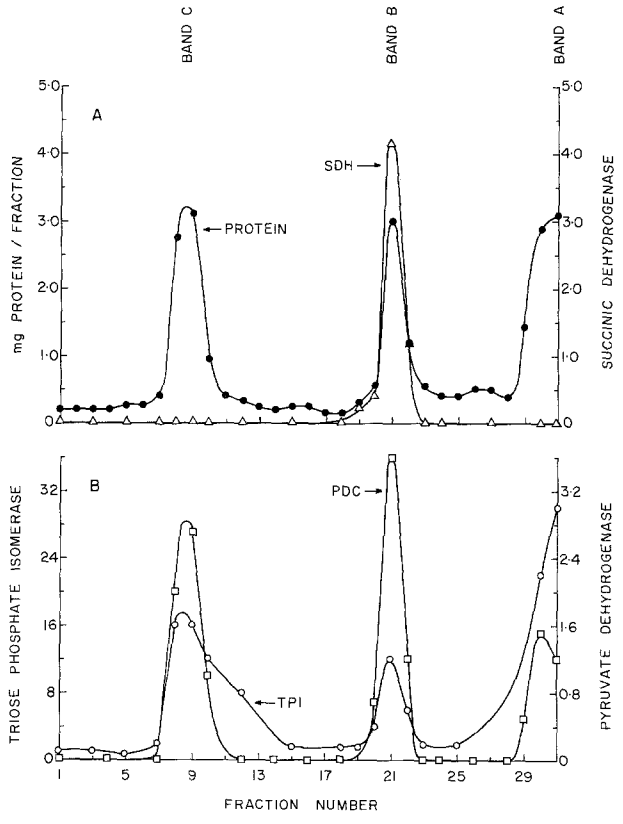

Figure 1: Distribution of protein, succinic dehydrogenase (SDH), triose phosphate isomerase (TPI), and pyruvate dehydrogenase complex (PDC) on a discontinuous sucrose density gradient. (A) succinic dehydrogenase - μmoles DCIP reduced/h/fraction (Δ) and protein (●). (B) triose phosphate isomerase - μmoles NADH oxidized/h/fraction (o) and pyruvate dehdyrogenase - μmoles NAD^+ reduced/h/fraction (□).

pellet from castor bean endosperm it was found that 1-5-^{14}C citrate, in the presence of ATP and CoA, was not incorporated into fatty acids although it was metabolized to $^{14}CO_2$ by these fractions whereas ^{14}C-pyruvate was incorporated into fatty acids which indicates the absence of citrate lyase in these fractions.

Discussion:

The results in this paper demonstrate that a significant amount of the pyruvate dehydrogenase complex activity from developing castor bean endosperm appears to be associated with the proplastid fraction. Together with earlier data (2-4) this means that the proplastid has the complete pathway for the conversion of pyruvate to oleic acid. The location of the complex within the proplastid allows the generation of acetyl-CoA inside the organelle and eliminates the requirement for transport of acetyl-CoA from the mitochondrion. The apparent absence of citrate lyase activity in the endosperm also makes such a transport system unlikely. If the reported incorporation of sucrose into fatty acids (5) is also an activity of the proplastids, and since NADH is required for stearic acid biosynthesis (1,4), the conversion of sucrose to acetyl-CoA would supply all the reducing power and ATP for fatty acid biosynthesis. It is assumed this organelle can also supply the NADPH required for the desaturation of stearic acid to oleic acid. The further conversion of oleic acid to ricinoleic acid, the storage fatty acid in castor bean endosperm, appears to be associated with the microsomal fraction (12) so that it is assumed that the oleic acid is transported from the proplastid. The regulatory properties of the mitochondrial and proplastid pyruvate dehydrogenase complexes should be quite different since they are serving different functions in the cell. The kinetic and structural properties of these two enzymes are at present under investigation.

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