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Citrate Cleavage Enzyme from Developing Soybean Cotyledons

INCORPORATION OF CITRATE CARBON INTO FATTY ACIDS[1]

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ABSTRACT

Data are presented which demonstrate a citrate cleavage enzyme in the supernatant of a developing soybean (*Glycine max* L. Merr., var. Harosoy 63) cotyledon homogenate following a 126,000*g* spin for 2 hours. Activity of the enzyme was observed directly in the supernatant enzyme preparation and in a desalted supernatant preparation by measuring the formation of acetylhydroxamate. Acetylhydroxamate production was dependent on citrate and coenzyme A. The reaction increased with time, citrate, and coenzyme A concentrations.

Involvement of the enzyme in lipid synthesis was investigated by the incorporation of carbon from citrate-1,5-^{14}C into fatty acids. Incorporation shows a pH optimum at 8.5, a temperature optimum at 30 C, and a dependence on ATP and coenzyme A. The reaction is linear throughout the range of extract concentrations tested and is linear as a function of time for 1 hour. Isotope was distributed primarily in unsaturated fatty acids.

In 1953, Srere and Lipmann (10) reported that an acetone powder of pigeon liver homogenate catalyzed a reaction between ATP, CoA, citrate, and hydroxylamine to form acetylhydroxamic acid. This led subsequently to the description of ATP citrate lyase (11). Since then, a form of the enzyme not requiring ATP or CoA has been found in bacteria (2).

It has been shown in animal systems that the cleavage of citrate is the source of acetyl CoA for fatty acid synthesis in the cytoplasm (9). Attempts to isolate this enzyme from plant tissue, have been unsuccessful (11), leaving unresolved the source of acetyl CoA for synthesis of plant fatty acids in the cytoplasm.

The object of the work presented is to determine if the citrate cleavage enzyme is present and to investigate the extent of its involvement in fatty acid synthesis in developing soybean cotyledons, which at maturity can be 25% triglycerides by dry weight.

MATERIALS AND METHODS

Materials. Coenzyme A was purchased from P-L Biochemicals,[2] ATP and NADPH were purchased from Sigma Chemical Company, and avidin and biotin from Nutritional Biochemical Co. Citrate-1,5-^{14}C and citrate-6-^{14}C were purchased from New England Nuclear Corporation.

Preparation of the Enzyme. Developing soybeans (*Glycine max* L. Merr. var. Harosoy 63) were picked at approximately 35 to 45 days after flowering (seed weight 255 to 355 mg fresh weight). Plants were grown at the South Agronomy Farm, University of Illinois, Urbana. The soybeans were removed from the pods; then the seed coats, plumules, hypocotyls, and radicles were detached from the cotyledons. The cotyledons were put in distilled H_2O at room temperature until grinding. The cotyledons were ground at room temperature in a volume of ice cold grinding buffer equal to twice the weight. The grinding medium consisted of 0.5 M sucrose, 10 mM MES, pH 6.1, and 0.1 mM EDTA. The homogenate was spun at 20,000*g* for 20 min at 6 C. The resulting supernatant was spun in a Beckman L-2 preparative ultracentrifuge at 126,000*g* for 120 min at 5 C. The supernatant was the source of enzyme used in all experiments (referred to as supernatant enzyme preparation), except to show a citrate dependency for acetylhydroxamate formation. A citrate-free enzyme preparation was prepared as follows. Two 1 cm × 10 cm columns of Sephadex G-25 in 10 mM MES pH 6.0 were spun in a swinging bucket rotor with an International PR-2 centrifuge at 500*g* for 10 min at 5 C. To each of the columns was then added 0.6 ml of the supernatant enzyme preparation. The columns were centrifuged again at 500*g* for 10 min at 5 C. The exclusion volume was collected and is referred to as the desalted enzyme preparation (4). An aliquot from each enzyme preparation was saved for protein (5) and citrate (8) determinations.

Assay Methods. The acetylhydroxamate reaction was run according to the method of Takeda *et al.* (13). The ferric chloride reagent for the assay and the hydroxamate standard curve were prepared according to the method of Stadtman (12).

The incorporation of citrate-1,5-^{14}C into fatty acids was carried out in 25-ml Erlenmeyer flasks. The reaction was stopped with 5 ml of 15% (w/v) methanolic KOH. The samples

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[2] Mention of a trademark or proprietory product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

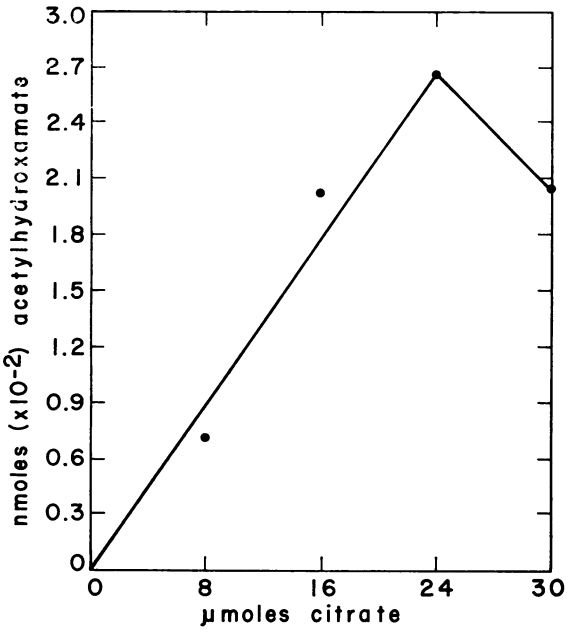

FIG. 1. Acetylhydroxamate formation as a function of citrate concentration by the desalted enzyme preparation. The reaction mixture contained, in a volume of 2 ml, the following in μmoles: tris, 200 (pH 8.5); ATP, 10; β-mercaptoethanol, 2; hydroxylamine, 2; CoA, 0.24 (0.2 mg); $MgCl_2$, 0.5; citrate as specified; and 1 ml of desalted enzyme preparation. The reaction was run for 1 hr at 30 C.

Table I. *Effect of Varying CoA Concentration on Acetylhydroxamate Formation*

The reaction mixture contained, in a volume of 2.5 ml, the following in μmoles: tris, 200 (pH 8.5); ATP, 10; β-mercaptoethanol, 2; hydroxylamine, 2; $MgCl_2$, 0.5; citrate, 8; with varying amounts of CoA as indicated and 1.5 ml of supernatant enzyme preparation. The reaction was run for 1 hr at 30 C.

CoA Added to Reaction Mixture	Acetylhydroxamate Formed
μmole	*nmoles*
0	375
0.24	1230
0.48	1750

were transferred to 100-ml round bottom flasks and refluxed on a steam bath for 2 hr. The solution was cooled with 5 ml of distilled H_2O and the 3 ml 6 N HCl were added to the flask. The aqueous phase was extracted three times with 10 ml of petroleum ether (b.p. 60–68 C)-diethyl ether (2:1 v/v). The petroleum ether-diethyl ether extracts were then combined in a 100-ml round bottom flask with 5 ml of glacial acetic acid and then were taken to dryness on a flash evaporator at 25 C. The fatty acid residue was brought up to a known volume in a petroleum ether-diethyl ether (2:1 v/v), then counted in a modified Brays scintillation solution in a Packard liquid scintillation spectrometer. All counts were corrected for quenching by the channels ratio method.

Analysis of Fatty Acids. Methyl esters of the fatty acids were prepared by refluxing the samples for 1 hr in 2% (v/v) H_2SO_4 in methanol. The methyl esters of the saturated fatty acids were separated from the methyl esters of the unsaturated fatty acids by silver nitrate TLC (3). The solvent system used was petroleum ether (b.p. 60–68 C)-diethyl ether (93:7 v/v). The resulting methyl ester bands were scraped directly into scintillation vials and counted using a modified Brays scintillation fluid. Recovery of counts in methyl esters from the thin layer plates was above 90%.

RESULTS

Citrate Cleavage Enzyme. Figure 1 shows the stimulation of acetylhydroxamate production with increasing concentrations of citrate using the desalted enzyme preparation (see "Materials and Methods"). The desalted enzyme preparation did not contain any citrate. After the addition of 24 μmoles of citrate, the activity declined. When the reaction is run with the supernatant enzyme preparation, the point at which activity no longer increases is at 8 μmoles of citrate added (total of 22 μmoles of citrate, see "Citrate Determination"). This verified the level of endogenous citrate in the supernatant enzyme preparation. The desalted enzyme preparation contains 15 mg of protein per ml.

Table I demonstrates the dependency of acetylhydroxamate production on CoA using the supernatant enzyme preparation. An addition of 0.24 μmoles of CoA to the reaction mixture increased the acetylhydroxamate production 3.3-fold, while the addition of 0.48 μmole of CoA caused a stimulation of 4.7-fold over the background value.

Figure 2 shows the acetylhydroxamate reaction as a function of time. There is a doubling between 0.5 hr and 1 hr, then a decline in activity. Since there is a large quantity of citrate in the supernatant enzyme preparation, we might assume that the stability of the enzyme or the limitation of some other substrate is the cause rather than a depletion of citrate.

Citrate Determination. Results of the citrate determination on the supernatant enzyme preparation showed that each ml contained 13 to 15 μmoles of citrate. When citrate-1,5-^{14}C was used as the substrate with the supernatant enzyme preparation, 2 μmoles of unlabeled citrate and 0.5 μmole of citrate-1,5-^{14}C were added to the reaction mixture. Therefore, before calculating nanomoles of citrate cleaved, the dpm were multiplied by a correction factor of 64.

Incorporation Patterns of Citrate into Fatty Acids. Table II shows characteristics of the incorporation of citrate-1,5-^{14}C into fatty acids using the supernatant enzyme preparation from developing soybean cotyledons containing 20 to 25 mg protein/ml. The incorporation from citrate, as with the system incorporating acetate into fatty acids (6), is dependent on the presence of both ATP and coenzyme A. Neither a dependence here nor at the point where the acetylhydroxamate is formed is a confirmation that the cleavage itself requires CoA and ATP. We cannot say if this reaction is akin to the animal or the bacterial enzyme. As would be expected, citrate-6-^{14}C was not incorporated into fatty acids. Regardless of the cleavage point, carbon 6 should be in oxaloacetate.

Avidin Inhibition. Citrate provides acetyl CoA by the citrate cleavage enzyme reaction. The acetyl CoA formed is carboxylated by a biotin-dependent enzyme, acetyl CoA carboxylase, to form malonyl CoA. The dependency of this enzyme on biotin makes the incorporation from citrate into fatty acids vulnerable to avidin inhibition.

The results of avidin inhibition (Table III) compare favorably with acetate incorporating systems from developing soybean cotyledons (6), and similarly, the reversal of the inhibition with biotin was complete.

Product of the Reaction. Analysis of the distribution of the incorporated citrate-1,5-^{14}C carbon by TLC showed that 12% of the total ^{14}C in the fatty acids was in palmitic and stearic (saturated fatty acids), 48% in oleic, 23% in linoleic, and 17% in linolenic. This pattern is similar to that shown for developing soybean cotyledons *in vivo* (7).

Effect of pH. As with the soluble system which incorporates acetate, reported by Rinne (6), there are two peaks of activity, one at pH 8.5 in tris buffer and one at pH 9.5 in glycine buffer. The purified system from rats showed a pH optimum of 8.4 in tris buffer (13). It should be emphasized here that a pH optimum for a linked system will not necessarily reflect the optimum for the citrate cleavage enzyme since we are measuring the result of a multienzyme pathway.

Enzyme Concentration. The incorporation of citrate-1,5-^{14}C carbon into long chain fatty acids was directly proportional to the amount of supernatant enzyme preparation added to the reaction mixture throughout the protein range from 13 to 52 mg.

Time. Figure 3 demonstrates the incorporation from citrate as a function of time. The rate of incorporation was linear for the 1st hr and then decreased.

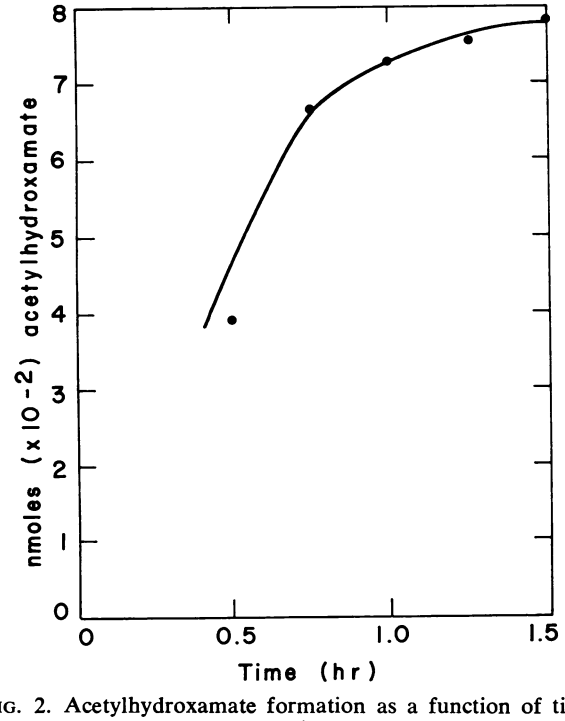

FIG. 2. Acetylhydroxamate formation as a function of time by the supernatant enzyme preparation. The reaction mixture contained, in a volume of 2 ml, the following in μmoles: tris, 200 (pH 8.5); ATP, 10; β-mercaptoethanol, 2; hydroxylamine, 2; CoA, 0.24 (0.2 mg); $MgCl_2$, 0.5; citrate, 8; and 1 ml of supernatant enzyme preparation. The reaction was run for 1 hr at 30 C.

Table II. *Requirements for Fatty Acid Synthesis from Citrate*

The reaction mixture contained, in a volume of 2 ml, the following in μmoles: tris, 100, (pH 8.5); ATP, 10; β-mercaptoethanol, 2; $MgCl_2$, 0.5; NADPH, 0.25; $KHCO_3$, 30; citrate, 2; coenzyme A, 0.24 (0.2 mg), and 1 ml of supernatant enzyme preparation. Isotope used, 1 μCi of citrate-1,5-^{14}C or citrate-6-^{14}C (both 2 μCi/μmole) as specified. The reaction was run for 1 hr at 30 C with shaking.

Reaction Mixture	Incorporation from Citrate into Fatty Acids	Decrease
	nmoles	%
Complete (citrate-1,5-^{14}C)	1776	0
−CoA	510	72
−NADPH	925	48
−ATP	315	83
−Mg	1774	0
Complete (citrate-6-^{14}C)	0	100

Table III. *Effect of Avidin on Incorporation from Citrate*

Reaction mixture and conditions are the same as for Table II except as noted below. Avidin preincubated with enzyme for 5 min at room temperature.

Reaction Mixture	Incorporation from Citrate into Fatty Acids	Decrease
	nmoles	%
Complete (citrate-1,5-^{14}C)	1260	0
+62 g avidin	988	21.6
+125 g avidin	692	45.1
+250 g avidin	318	75.0
+250 g avidin + 0.25 μmole biotin	1246	1.2

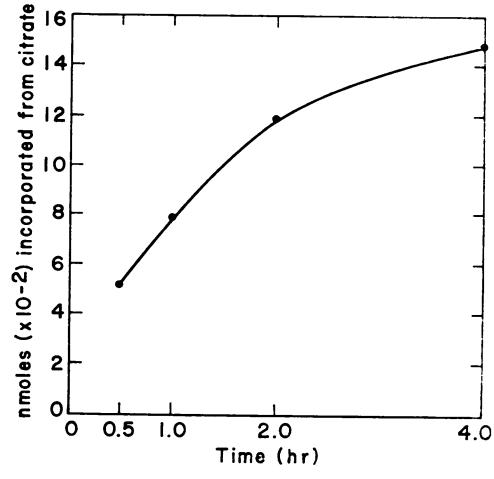

FIG. 3. Incorporation from citrate-1,5-^{14}C into fatty acids as a function of time by the supernatant enzyme preparation. The reaction mixture contained, in a volume of 2 ml, the following in μmoles: tris, 100 (pH 8.5); ATP, 10; β-mercaptoethanol, 2; $MgCl_2$, 0.5; NADPH, 0.25; $KHCO_3$, 30; citrate, 2; citrate-1,5-^{14}C 1 μCi (0.5 μmole); coenzyme A, 0.24 (0.2 mg), and 1 ml of supernatant enzyme preparation. The reaction was run at 30 C with shaking at the times indicated.

Temperature. We have received maximum results and repeatability at 30 C. Good activity has been observed at 37 C, the optimum for the ATP citrate lyase in animals (13), though the results were quite erratic. All experiments were run at 30 C.

DISCUSSION

In animal tissues, it has been shown several times that the source of extramitochondrial acetyl CoA is citrate (1, 9). Spencer and Lowenstein (9) have shown in animals that citrate plays a regulatory role in fatty acid synthesis. In both plants and animals, acetate and acetyl CoA have proved to be excellent substrates for cytoplasmic fatty acid synthesis (6, 9). We have shown here that acetyl CoA can be produced from citrate by a cell-free extract of developing soybean cotyledons and that the incorporation of citrate carbon into fatty acids follows a pattern similar to the incorporation of acetate into the same preparation. We found that developing soybean cotyledons have a pool of citrate which is large enough to be the axial carbon source for both fatty acid synthesis and the substantial

amino acid demands for storage protein. Whether such a large pool would be present in the cytoplasm or in the vacuole is not known, but it is presumed to be available.

We propose that the source of acetyl CoA for fatty acid synthesis in developing soybean cotyledons is citrate via citrate lyase.

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