Formation of Sucrose From Malate in Germinating Castor Beans [1, 2]
II. Reaction Sequence From Phosphoenol-Pyruvate to Sucrose

C. R. Benedict [3] & Harry Beevers

Department of Biological Sciences, Purdue University, Lafayette, Indiana

Malate is a key intermediate in converting acetyl units to sucrose in germinating castor beans (8). In an earlier paper evidence was presented to show that a mechanism exists for the production of phosphoenol-pyruvate from malate in this material and that phosphoenol-pyruvate is an intermediate in the production of sucrose from malate (6). In this paper individual reactions which form a sequence leading from phosphoenol-pyruvate to sucrose are described.

Material & Methods

The procedures for preparation of extracts, chromatographic and counting procedures were as previously described (6). The methods for following individual enzyme reactions are outlined in the text.

Results

As described earlier, castor bean extracts contain phosphoenol-pyruvate carboxylase, which in the presence of its substrate phosphoenol-pyruvate and $C^{14}O_2$ produces oxalacetate-4-C^{14} (6). The results in table I show that oxalacetate-4-C^{14} is also produced when 3-phosphoglycerate is provided as the 3-carbon unit. This demonstrates that the extracts can interconvert 3-phosphoglycerate and phosphoenol-pyruvate, presumably through the action of 3-phosphoglyceric acid mutase and enolase.

In the presence of ATP, phosphoenol-pyruvate is produced from oxalacetate by phosphoenol-pyruvate carboxykinase in the preparations (6). When such preparations were incubated with oxalacetate and ATP^{32} (produced by photophosphorylation by spinach chloroplasts) both P^{32} labeled phosphoenol-pyruvate and phosphoglycerate were detected on chromatograms and identified by co-chromatography. These intermediates, labeled with C^{14}, were also isolated from endosperm tissue which was converting malate-C^{14} (produced in vivo by $C^{14}O_2$ fixation) to sucrose.

Table I

Oxalacetate Formation From Phosphoglyceric Acid

Time (min)	cpm $HC^{14}O_3^-$ Incorporated into OAA-4-C^{14}/ml
0	13.5
8	304.0
20	1,502.0
30	2,695.0
30 (minus PGA)	63.5

The reaction mixture contained in micromoles: Phosphoglyceric acid (PGA) 10; $KHC^{14}O_3$, 50 (5×10^6 cpm); 0.4 ml cytoplasmic particles, and 1.0 ml supernatant enzymes in 200 micromoles potassium phosphate pH 7.2. Total volume 3.7 ml. Reaction time as indicated at 25 C.

Table II

Formation of 1,3-Diphosphoglyceric acid From 3-Phosphoglyceric Acid

Time (min)	PGA Added (micromoles)	O.D.$_{540}$ (1,3-diPGA)
0	50	0.000
10	50	0.051
20	50	0.088
30	50	0.111
30	0	0.00
30	10	0.018
30	50	0.097
30	100	0.145

Reaction mixture contained in micromoles; ATP, 10; $MgCl_2$, 7.5; hydroxylamine (NH_2OH), 250; 0.5 ml supernatant enzymes in 150 micromoles potassium phosphate pH 7.2. Total volume 4.0 ml. Reaction time as indicated at 25 C.

Phosphoglyceryl kinase was demonstrated directly by incubating aliquots of the soluble fraction with PGA and ATP in the presence of hydroxylamine (4). The hydroxamate formed from 1, 3-diphosphoglycerate was estimated spectrophotometrically. As shown in table II the reaction proceeds at a diminishing rate with time and is strongly dependent on PGA concentration.

► Triosephosphate Dehydrogenase. Endosperm extracts brought about a rapid reduction of DPN when

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[3] Present address: Department of Microbiology, Dartmouth Medical School, Hanover, N. H.

fructose-1, 6-diphosphate was added, and the presence of aldolase and DPN-linked triosephosphate dehydrogenase can be deduced from this. It was of some interest to find that in addition, TPN was reduced under these conditions and the fact that iodoacetate (0.01 M) inhibited the reduction showed that a triosephosphate dehydrogenase (rather than a glucose-6-phosphate dehydrogenase) was responsible (7). Subsequent experiments with phosphoglyceraldehyde as substrate have established beyond doubt that both DPN- and TPN-linked triosephosphate dehydrogenases are present in this material (table III). This finding raises the possibility that the TPN-linked enzyme is primarily responsible for the production of triosephosphate from PGA, as it is supposed to be in photosynthesis. However, whereas the reaction 1, 3-diphosphoglyceric acid → phosphoglyceraldehyde can readily be demonstrated with DPNH as the coenzyme in our extracts, we have been unable consistently to demonstrate such a reduction with TPNH. It will be recalled that an arsenate requirement (implying reversibility) became evident only during the later stages of purification of the spinach enzyme (11) and also that a non-reversible TPNH-linked triosephosphate dehydrogenase has been described from leaves (2, 3). The interplay and significance of these various enzymes in leaves where they coexist is not established. Clearly the demonstration that TPN is reduced on adding triosephosphate is not by itself an adequate basis for supposing that the TPN-linked enzyme rather than the DPN-linked one is the functional enzyme for the reverse reaction in the endosperm. The aldolase which is present in the endosperm is no doubt responsible for the conversion of triosephosphate to hexose phosphate; the labeling patterns in glucose produced from acetate (8) and from glycerol (5) are consistent with such a condensation.

Table III

Pyridine Nucleotide Reduction by Triose Phosphate Dehydrogenase

Enzyme ml	Δ O.D.$_{340}$/min TPN Reduction	Δ O.D.$_{340}$/min DPN Reduction
0.05	0.009	...
0.10	0.020	0.06
0.20	0.041	...
0.20 (Boiled)	0.0	0.0

The reaction mixtures contained in micromoles, tris, pH 7.2, 100; DPN or TPN, 0.15; phosphoglyceraldehyde, 2; supernatant enzymes solution as indicated and water to a volume of 3 ml.

► Sucrose Synthesis From Hexose Derivatives. Two systems for sucrose synthesis have been elucidated from higher plant tissues. Uridine diphosphoglucose-fructose transglucosylase is the enzyme responsible for one of these reactions and free sucrose is the end product (9). In the other, uridine diphosphoglucose-fructose-6-phosphate transglucosylase produces sucrose phosphate (12). The first enzyme was shown to be present by a method based on procedures of the Buenos Aires group (9) and Turner (13).

Endosperm material from 4 to 5 days old castor beans (30 g) was blended with 2 volumes of 0.08 M KH_2PO_4 and 0.01 M cysteine, pH 7.0. The supernatant solution was collected after centrifuging at 18,000 × *g* for 15 minutes. To 20 ml of this, 80 ml of saturated $(NH_4)_2SO_4$ were added dropwise over 2 to 3 hours, with stirring. The precipitate was collected and dissolved in 7 ml cold water and dialyzed at 3 C for 2 hours against 300 ml 0.025 M potassium phosphate, pH 7.0. In the incubation mixture (table IV) 1 ml of the dialyzed solution was used. It is evident that the added fructose had been converted to sucrose and a smaller amount of C^{14} was recovered as free glucose.

Since labeled fructose-6-phosphate was not available, a test of similar sensitivity for the uridine diphosphoglucose-fructose-6-phosphate transglucosylase was not possible. It was hoped that by incubating glucose-C^{14}, ATP, and uridine triphosphate with the enzyme, sufficient uridine diphosphoglucose-C^{14} might be produced to allow the production of sucrose-C^{14} with unlabeled fructose-6-phosphate as the glucosyl acceptor. However, no sucrose-C^{14} was produced under these conditions.

Table IV

Sucrose-C^{14} Production From Fructose-C^{14}

Sugar	C^{14} cpm
Sucrose	49,980
Fructose	71,410
Glucose	2,356

The digest contained, in micromoles, potassium phosphate, pH 7.0, 50; cysteine, pH 7.0, 10; fructose-1,6-C^{14}, 10 (3.2 × 10^6 cpm); uridine diphosphoglucose, 10; sucrose, 3; and 1.0 ml enzyme (see text) in a volume of 2.1 ml. The reaction was allowed to proceed for 12 hours under N_2. The mixture was deproteinized and the salts removed with resins. The neutral fraction was run on chromatograms with ethyl acetate, acetic acid water (6/2/4 v/v/v) as solvent and the free sugars from a portion of the chromatogram were eluted and counted.

Discussion

The results presented in this and a previous paper (6) show that the castor bean endosperm is equipped with enzymes which, working in sequence, can bring about the formation of sucrose from malate. The reaction sequence suggested; (malate → oxalacetate → CO_2 + phosphoenol-pyruvate → ---> hexose phosphate → sucrose) is consistent with previous observations on the utilization of individual carbons of acetate and malate during sucrose formation by intact tissues (8).

The enzymes of the conventional Embden-Meyerhof-Parnas sequence of glycolysis are present. The functioning of this pathway in reverse would require the provision of ATP and reduced nucleotide as well as phosphoenol-pyruvate. The concomitant reactions of β-oxidation of long chain fatty acids are believed to be the source of this assimilatory power. Although a TPN-linked triosephosphate dehydrogenase is present in the endosperm tissue, we have no evidence that this enzyme, rather than the DPN-linked dehydrogenase, participates in the reductive step during sucrose formation from phosphoenol-pyruvate.

Summary

► 1. Evidence is presented that the following enzymes are present in the endosperm tissue of germinating castor beans: 3-phosphoglyceric mutase, enolase, phosphoglyceryl kinase, DPN- and TPN-linked triosephosphate dehydrogenases, aldolase, and uridine diphosphoglucose → glucose transglucosylase. ► 2. These enzymes, acting in the presence of ATP and reduced nucleotide generated during fatty acid oxidation are believed to be responsible for the formation of sucrose from phosphoenol- pyruvate, which is itself produced from oxalacetate by phosphoenol-pyruvate carboxykinase.

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