Cardini and coworkers (6) reported as one part of a plant materials survey that "negative or non-reproducible results" were obtained in sucrose synthesis studies with sugar beet leaves and roots. They stated that these results "may be attributed to the presence of interfering enzymes".

Burma and Mortimer (5), using isotopic tracer techniques, reported the synthesis of sucrose by sugar beet leaf homogenates when fructose-6-phosphate and uridine diphosphoglucose (UDPG) were present. They demonstrated that sucrose-phosphate was formed and assumed that it was subsequently dephosphorylated by phosphatase to sucrose. No sucrose synthesis occurred when fructose or fructose-1,6-diphosphate and UDPG were added to sugar beet leaf homogenates. Burma and Mortimer stated that their experiments with sugar beet leaves eliminated Leloir and Cardini's (11) first enzyme mechanism demonstrated with wheat germ, which was the interaction of UDPG and fructose for the direct formation of sucrose plus pyrophosphate.

This paper presents data showing that the assumption of Burma and Mortimer that only sucrose-phosphate is synthesized directly does not seem to be correct. Rather, it shows that sugar beet leaf tissue contains enzyme systems for the direct synthesis of sucrose as well as sucrose-phosphate through UDPG and fructose and UDPG and fructose-6-phosphate, respectively.

Any sucrose-phosphate which is formed is ultimately dephosphorylated by enzymes in sugar beet tissue at some stage prior to storage in the root. Thus the synthesis of both sucrose and sucrose-phosphate also has some bearing on the important unresolved question of whether sucrose or sucrose-phosphate is the principal sugar transported to the root for storage (4, 8, 9, 10, 18).

**Materials and Methods**

Preliminary experimentation resulted in adopting the following procedures for preparing beet leaf enzyme fractions active in the synthesis of sucrose and sucrose-phosphate. All operations were carried out at 0 to 5°C.

Six hundred grams of fresh leaves of 6 to 7 week old sugar beet seedlings (Beta vulgaris var GW 304) were used for preparing the enzyme fractions. A 100 g aliquot of seedling leaves was blended with 100 ml of 0.05 M, pH 7.2 phosphate buffer in a high speed blender. This homogenate was then squeezed in a fine nylon cloth. The liquid obtained was used for blending the next 100 g aliquot of leaves. This process was repeated until all 600 g of leaves had been homogenized, using only the original 100 ml of phosphate buffer diluent. The pH was periodically adjusted to 6.8 to 7.0 with NaOH during the blending procedure. Approximately 600 ml of filtered homogenate was obtained. This was centrifuged at 13,000 x G, and the precipitate obtained was discarded. The supernatant was gradually taken to pH 4.9 with acetic acid and immediately centrifuged at 13,000 x G for 15 minutes. The precipitate was discarded and the clear yellowish supernatant was left overnight at
4°C. The flocculent precipitate which then formed was collected by centrifugation and washed several times with 0.02 M, pH 4.9 acetate buffer. The re-centrifuged precipitate was then dissolved in sufficient 0.05 M, pH 7.2 phosphate buffer to make a thick slurry, and this final fraction was then dialyzed overnight in a continuous dializer against 0.02 M, pH 7.2 phosphate buffer. This enzyme fraction was used throughout these studies.

The complete reaction mixture for sucrose synthesis studies contained 1.5 μM UDPG, 4 μM fructose, 0.1 ml enzyme fraction (containing 1.4 mg protein nitrogen), 0.002 ml 0.1 M MgCl₂, and 0.01 ml of 1 M Tris buffer. If a pH above 9.0 was desired, ethanolamine-HCl buffer was substituted for the Tris buffer. The total volume of this reaction mixture was 0.2 ml. The complete reaction mixture for the study of sucrose-phosphate synthesis was identical to the mixture used for sucrose synthesis except that 4 μM of fructose-6-phosphate was substituted for the fructose and in addition 0.01 ml of 1 M KF was present as a phosphatase inhibitor. After 4 hours incubation at 37°C, the tubes containing the enzyme and substrates were placed in a boiling water bath for 5 minutes and then cooled. To each tube was added 0.8 ml of a solution of 0.025 N NaOH containing 15 mg NaBH₄. This was followed by adding a few drops of ethanol as a foam retardant. These tubes were left for 1 hour at room temperature and were then covered with a glass marble and placed in a boiling-water bath for 5 minutes to complete the reduction of the hexoses to their corresponding sugar alcohols. The use of NaOH only and heating to 100°C to eliminate reducing sugars (6) gave an excessive discoloration which was not observed in the borohydride reduction reaction. The reproducibility and sensitivity of the subsequent resorcinol procedure of Roe (17) for determinations of sucrose was greatly improved by using the borohydride procedure to eliminate fructose and glucose. It was necessary, however, to destroy the excess of borohydride by acidifying the mixture with a few drops of acetic acid before proceeding with the resorcinol method. For Roe’s procedure, the volumes were reduced to half and the color was measured in a 1 cm cell at 490 mμ in a Beckman Model B spectrophotometer.

The biosynthesis of sucrose and sucrose-phosphate was confirmed by paper chromatography of aliquots of the inactivated enzyme reaction mixture following passage of the mixture through a cold mixed bed ion exchange column containing equal amounts of Dowex-50 (H+ form) and Duolite A-4 (OH− form) resins. Sucrose-phosphate remained on the column. Pre-treatment at pH 9.7 with invertase-free alkaline phosphate dephosphorylated sucrose-phosphate and produced sucrose in the column eluate. Aliquots of the ion-exchanged solution were concentrated, spotted, and developed on paper in a solvent of ethyl acetate, pyridine, and water (8:2:1 v/v) (19). Some chromatograms were developed in a solvent of n-propanol, ethyl acetate, and water (7:1:2 v/v) (1). Fructose and sucrose spots were detected on the paper by a dip of 0.5% resorcinol in ethyl acetate made 0.2 N with concentrated HCl, followed by air drying and heating at 85°C for 5 minutes.

The enzyme fraction used for the sucrose and sucrose-phosphate synthesis studies was assayed for invertase activity in a mixture consisting of 0.01 ml pH 7.0 Tris buffer, 30 μM sucrose, 0.003 ml 0.1 N MgCl₂, and 0.3 ml of enzyme (4.2 mg of protein N) in a total volume of 1 ml.

This enzyme fraction was also assayed for phosphatase activity at pH 7.0 in a mixture identical to that used for the invertase assay, except that 10 μg of fructose-6-phosphate was substituted for the sucrose. Inorganic phosphate resulting from hydrolysis of the fructose-6-phosphate was determined colorimetrically by a modification of the method of Allen (2) in which the samples were diluted to 15 to 18 ml with water before adding perchloric acid, anisol reagent, and molybdate. The tubes were read at 660 mμ after 10, but not more than 20 minutes, and were compared with known standards.

**Results and Discussion**

Assays of the pH 4.9 precipitated enzyme fraction revealed that some invertase was still present. After 2 hours incubation at pH 7.0, 0.3 ml of the enzyme inverted 5 and 10 μM of sucrose in 2 and 4 hours, respectively. Additional studies with this enzyme fraction at pH 9.0 revealed a 40-fold decrease in invertase activity from that found at pH 5.0. The described purification procedures decreased the amount of invertase present in the enzyme fraction to a level where detectable amounts of sucrose could now be accumulated. Studies of pH effect on sucrose synthesis with our enzyme fraction are shown in figure 1. Optimum sucrose synthesis occurred at pH 8.7 and then decreased sharply up to pH 9.7. This does not mean that the pH optimum of the uridine diphosphoglucose fructose transglycosylase is 8.7, but that at this pH the rate of the transglycosylase exceeds the action of invertase by the greatest amount. One enzyme preparation gave optimum sucrose synthesis at pH 8.4. This may have been due to the presence of a lesser amount of invertase. Other small variations were also found on replication of the enzyme fractionation procedure. In several cases it was difficult to get a good precipitate after leaving the yellowish supernatant overnight at pH 4.9. More consistent precipitates, but of lower activity for sucrose synthesis, were obtained if the yellowish supernatant was dialyzed against distilled water prior to the adjustment to pH 4.9. When only 100 ml of buffer was used to homogenize 600 g of leaf tissue, an adequate
precipitate of active protein following pH adjustment was usually obtained.

This enzyme fraction was active in both sucrose and sucrose-phosphate synthesis. Under the described assay conditions, from 0.15 to 0.25 micromoles of sucrose-phosphate were synthesized. Optimum sucrose-phosphate synthesis was observed when 0.01 to 0.03 ml of 1 M KF was present as a phosphatase inhibitor. The same amount of KF inhibited sucrose synthesis by approximately 25% at pH 8.7. Ammonium molybdate markedly inhibited sucrose-phosphate synthesis when used as a phosphatase inhibitor at a concentration of 0.001 M.

Phosphatase activity observed under the described assay conditions was fairly low, with less than 10% of the fructose-6-phosphate present being dephosphorylated even after 4 hours incubation.

The identities of sucrose and sucrose-phosphate were verified in several ways. When the enzyme-substrate mixture used for sucrose synthesis was passed through the mixed bed ion exchange column and spotted on paper, a neutral substance moving with an Rf identical to that of sucrose was found to have passed through the column. Chromatography of the material in this spot after treatment with analytical invertase resulted in spots which moved identically with known glucose and fructose. The incubation mixture before passage through the ion exchange column and the eluate of the column contained the same amounts of sucrose as determined by the resorcinol procedure of Roe (17).

Sucrose-phosphate synthesized by the incubation mixture was adsorbed on the mixed bed column. A negative resorcinol test was obtained with the eluate. A positive test for sucrose was observed in the eluate when the heat-inactivated incubation mixture containing sucrose-phosphate was taken to pH 9.7, treated with alkaline phosphatase and then passed through the mixed bed column.

Studies with Canna indica leaves (16) demonstrated that when labeled glucose was administered to leaf discs, the percentage of total activity in the glucose decreased while the activity of sucrose showed a parallel increase. No activity was detected in the free fructose, but both moieties of sucrose were found to have been labeled. Infiltration of labeled fructose to the leaf discs resulted in a similar C14 labeling pattern of the sucrose, but free glucose was not labeled. The same experiments of Putman and Hassid (16) also gave very strong evidence for the existence of the endogenous free hexoses in inert pools. Edelman, Ginsburg, and Hassid (7) state that glucose and fructose taken up by Canna leaves and wheat seedlings may become phosphorylated at the time of entry into the leaf tissue and that these sugars do not enter the monosaccharide compartments of the plant. These workers obtained results with wheat seedlings which were in agreement with the Canna leaf disk studies. Sucrose synthesized following introduction of labeled glucose was labeled in both moieties but free labeled fructose did not appear. However, the participation of free fructose in sucrose synthesis cannot be ruled out on the basis of these experiments. Leloir and Cardini (12) state that studies on green leaves with labeled substrates would prove that free fructose is not involved directly in the synthesis of sucrose except for the fact that the same experiments demonstrated storage of sugars in leaves in metabolically inert compartments. In other words, administered glucose and fructose do not equilibrate with the corresponding endogenous pools of these sugars and thus cannot be considered as metabolically equivalent to the endogenous stored glucose and fructose. When glucose is administered, the labeling rate of the fructose and its incorporation into sucrose at the site of sucrose synthesis is probably much faster than labeling fructose and its transport into the metabolic pools. The labeling experiments have demonstrated only that administered free hexoses do not enter the endogenous free hexose pools and have not shown, conversely, that the hexoses of the pools cannot participate, either free or through subsequent phosphorylation, in sucrose synthesis. In sugar beet leaf tissue, the endogenous sucrose and fructose pools have been reported as 1.7 and 4.7 mg, respectively, per gram of fresh leaf tissue (13). Pavlinova (15) has reported similar values for these pools. Because of the small amounts of hexose phosphates in sugar beet leaf (14) and the fact that one would expect these sugar phosphates to be in a state of equilibrium since phosphohexoisomerase is present and active, it is logical to assume that following the introduction of either labeled glucose, fructose, or of CO2, any deviation from equal labeling of the hexose moieties constitutes fair evidence that the route of sucrose synthesis is not entirely through phosphorylated fructose. There are several examples of a significant disparity of label of the sucrose hexoses, including the studies of Axelrod and Seegmiller (3) with apple tissue and those of Putman
and Hassid (16) with Canna indica. Nelson and Mortimer (13) found that the specific activity of the fructose moiety of sucrose was lower than that of the glucose moiety when excised sugar beet leaves were killed after carrying on photosynthesis in the presence of C¹⁴O₂ followed by C¹²O₂ and this labeling pattern was interpreted on the assumption that sucrose can be formed in sugar beet leaves only from UDPG and fructose-6-phosphate. In earlier studies, Burma and Mortimer (5) reported that following the introduction of labeled glucose-1-phosphate to a sugar beet leaf, the resulting sucrose on inversion had “approximately equal activities in both glucose and fructose.”

Even if the label of sucrose in a plant tissue is found to be equal in the two hexose moieties, one still cannot rule out the participation of free fructose in sucrose synthesis because of the possibility of dephosphorylation of fructose-6-phosphate at the site of synthesis. It seems impossible to assess the degree of participation in sucrose synthesis of either fructose or fructose-6-phosphate on the basis of only the final labeling of sucrose in leaf or whole plant experiments.

**Summary**

It was demonstrated that sugar beet leaf tissue contains active systems for the synthesis of both sucrose and sucrose-phosphate as indicated below. This is the first time that reaction I has been shown to be operative in green leaf tissue.

I. UDPG + fructose \( \rightarrow \) sucrose + UDP

II. UDPG + fructose-6-phosphate \( \rightarrow \) sucrose-phosphate + UDP

Methods for the purification and assay of the enzyme fraction are described as are conditions for demonstrating its maximum activity in the presence of invertase. All attempts to prepare enzyme fractions from sugar beet leaves which were free of invertase failed. Methods are described for the differentiation of sucrose and sucrose-phosphate formed in enzyme-substrate incubation mixtures.

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**Literature Cited**


