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The Activity of Uridine Diphosphate Glucose–D-Fructose 6-Phosphate 2-Glucosyltransferase in Leaves

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1. By using EDTA in reaction mixtures it was possible to determine the activity of sucrose phosphate synthetase in freshly prepared leaf extracts without the complications caused by sucrose phosphatase. 2. EDTA was found also to increase the activity of sucrose phosphate synthetase by as much as 100%. 3. High sucrose phosphate synthetase activities were found in leaf preparations in which sucrose phosphatase was inhibited by EDTA. By contrast with previous reports, the activities were sufficient to allow sucrose synthesis in leaves during photosynthesis to occur via sucrose phosphate. 4. Sugar-cane plants having different rates of photosynthesis also had different activities of sucrose phosphate synthetase in their leaves. 5. It is suggested that the activity of sucrose phosphate synthetase in leaves may play a role in the control of the rate of photosynthesis.

The pathway of synthesis of sucrose in leaves during photosynthesis is still not known. Of the two sucrose-synthesizing enzymes known to occur in leaves [sucrose synthetase (UDP-glucose–D-fructose 2-glucosyltransferase, EC 2.4.1.13) and sucrose phosphate synthetase (UDP-glucose–D-fructose 6-phosphate 2-glucosyltransferase, EC 2.4.1.14)] several workers favour the latter as being the enzyme involved (Mendicino, 1960; Bird, Porter & Stocking, 1965). The presence of sufficient specific sucrose phosphatase (sucrose phosphate phosphohydrolase) in leaves to allow sucrose phosphate to be an intermediate in sucrose synthesis is consistent with the above view (Hawker, 1966).

The previously reported activity of sucrose phosphate synthetase extracted from leaves has been too low to account for the rate of sucrose synthesis occurring *in vivo* (Leloir & Cardini, 1955; Mendicino, 1960; Hatch, 1964; Bird *et al.* 1965; Haq & Hassid, 1965). However, precise assay has been hampered by the presence of several interfering enzymes, particularly phosphatases.

In the absence of Mg^{2+} ions sucrose phosphatase is inactive (Hawker & Hatch, 1966); thus the addition of EDTA to the assay medium allows determination of the activity of sucrose phosphate synthetase in freshly prepared crude preparations from leaves.

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MATERIALS

Leaves were obtained from sugar cane (var. Pindar), peas (*Pisum sativum* var. Massey Gem), tobacco (*Nicotiana* sp.), and silver beet (*Beta vulgaris*) grown in nutrient culture in a phytotron in sunlight.

[U-^{14}C]Fructose 6-phosphate was prepared from [U-^{14}C]-fructose by using yeast hexokinase, and purified by passage through a column (100 cm. × 1·5 cm.) of Sephadex G-10, followed by a 1 ml. column of charcoal (washed with 5 mN-HCl) and a 10 ml. column of Dowex 50 (Na^+ form).

METHODS

Preparation of crude leaf extracts. Leaf laminae (5 g.) were cooled to 2°, cut into thin slices with scissors and ground in a mortar with 10 ml. of medium containing tris–HCl buffer, pH 7·6 (100 mM), mannitol (300 mM), $MgCl_2$ (10 mM), EDTA (20 mM), cysteine hydrochloride (20 mM) and sodium diethyldithiocarbamate (20 mM). With sugar-cane leaves 2 g. of acid-washed sand was also added. All operations were carried out at 2°. The filtrate obtained by squeezing the homogenate through muslin was centrifuged for 15 min. at 20000 *g*. A sample (4 ml.) of the supernatant solution was desalted by passage through a column (15 ml.) of Sephadex G-25 that had been washed with 10 mM-tris–HCl buffer, pH 7·0. The pellet was suspended in 1 ml. of the same buffer and frozen and thawed. The desalted supernatant solution and suspended pellet were used for enzyme studies.

Preparation of partially purified sucrose phosphate synthetase from wheat germ. The method of Mendicino (1960) was used.

Assay of sucrose phosphate synthetase. This was carried

out by measuring the incorporation of labelled fructose 6-phosphate into sucrose phosphate at 22°. Reaction mixtures contained UDP-glucose (1·0 μmole), KF (1·25 μmoles), potassium phosphate buffer, pH 6·7 (2·5 μmoles), tris–HCl buffer, pH 7·0 (2·5 μmoles), [U-^{14}C]fructose 6-phosphate (0·086 μmole; 10^6 disintegrations/min.) and 30 μl. of enzyme in a total volume of 75 μl. The reaction was stopped by heating at 100° for 2 min. A sample (20 μl.) was applied to a chromatogram. To the remainder was added 0·1 ml. of glycine–NaOH buffer, pH 10·6 (Gomori, 1955), containing $MgCl_2$ (5 μmoles), alkaline phosphatase (Sigma type III) (2 μl.) and 10 μl. of toluene. After incubation overnight at 30°, 20 μl. was spotted on a chromatogram. The percentage of the total activity in sucrose before and after phosphatase treatment was determined with a Geiger–Müller tube after chromatography in ethyl acetate–pyridine–water (8:2:1, by vol.) (Hatch, 1964).

Assay of sucrose synthetase. This was carried out by measuring the incorporation of labelled fructose into sucrose at 22°. Reaction mixtures contained [U-^{14}C]-fructose (0·046 μmole; 5×10^5 disintegrations/min.), UDP-glucose (1·0 μmole) and 50 μl. of enzyme in a total volume of 70 μl. The percentage conversion of fructose into sucrose was determined as described by Hatch, Sacher & Glasziou (1963).

Assay of sucrose phosphatase. This was carried out by measuring the rate of hydrolysis of [U-^{14}C-*fructosyl*]sucrose phosphate as described by Hawker & Hatch (1966) except that the tris–maleate buffer was adjusted to pH 6·7.

Determination of sucrose phosphate synthetase/sucrose synthetase activity ratio and percentage of each enzyme in the precipitate. Under the conditions employed the kinetics of the reactions were essentially first-order. Ratios of activities and distribution between the supernatant solution and precipitate could therefore be calculated from the percentage conversion of supplied fructose or fructose 6-phosphate when these values were less than 20%. For the assay of sucrose phosphate synthetase, EDTA (2·5 μmoles) was added to the reaction mixtures described above.

RESULTS

Effect of EDTA and malate on the assay and activity of sucrose phosphate synthetase. Even in the presence of potassium fluoride and inorganic phosphate there was a considerable amount of sucrose formed in the reaction mixtures for sucrose phosphate synthetase determinations before phosphatase treatment (Table 1). Previous workers have not been able to determine whether sucrose formed under these conditions was due to hydrolysis of the product (sucrose phosphate) or hydrolysis of the substrate (fructose 6-phosphate) and subsequent synthesis of sucrose by sucrose synthetase. There was considerably less sucrose formed in the presence of EDTA (Table 1), probably owing to the inhibition of sucrose phosphatase (Hawker, 1966). At the same time EDTA increased the percentage conversion of fructose 6-phosphate into sucrose phosphate by as much as 100% (Table 1). Malate did not always inhibit the phosphatase but did increase the synthesis of sucrose phosphate. Activity of the partially purified wheat-germ enzyme was almost doubled by EDTA and malate, the kinetics being essentially first-order. The identity of the sucrose was confirmed by co-chromatography of unlabelled sucrose and by treatment with yeast invertase, which yielded radioactive fructose. When UDP-glucose was omitted from the reaction mixtures no sucrose or sucrose phosphate was formed.

Apparent Michaelis constant. Reaction conditions were adjusted to give less than 15% conversion of fructose 6-phosphate into sucrose phosphate with the sugar-cane-leaf enzyme with a range of con-

Table 1. *Effect of EDTA and malate on the assay and activity of sucrose phosphate synthetase*

Reaction mixtures and methods are described in the Methods section. EDTA (2·5 μmoles) or sodium malate (10 μmoles) was added to some reaction mixtures.

Enzyme source	Time (hr.)	Sucrose formed (percentage conversion of [U-^{14}C]fructose 6-phosphate into sucrose)		
		Standard mixture	EDTA added	Malate added
Pea leaf				
Before phosphatase treatment	0·5	9	1	13
After phosphatase treatment	0·5	12	19	16
Pea leaf				
Before phosphatase treatment	4	—	17	—
After phosphatase treatment	4	—	70	—
Sugar-cane leaf				
Before phosphatase treatment	3	10	4	3
After phosphatase treatment	3	13	24	18
Tobacco leaf				
Before phosphatase treatment	3	4	1	5
After phosphatase treatment	3	6	12	8
Wheat germ				
Before phosphatase treatment	3	1	1	5
After phosphatase treatment	3	84	97	96

Table 2. *Activity of sucrose phosphate synthetase in crude extracts from leaves*

Reaction mixtures and methods are described in the Methods section. EDTA (2·5 μmoles) and additional unlabelled fructose 6-phosphate (1·2 μmoles) were added to reaction mixtures for the determination of sucrose phosphate synthetase activity listed in the first column. All values are means of determinations on at least eight leaves.

Source of enzyme	Sucrose phosphate synthetase activity (μmoles of sucrose phosphate synthesized/g. fresh wt./hr.)	Percentage of activity in precipitate		Sucrose phosphate synthetase/sucrose synthetase activity ratio
		Sucrose phosphate synthetase	Sucrose synthetase	
Sugar-cane leaf	2·6	10	14	0·6
Pea leaf	8·3	19	34	10
Silver-beet leaf	1·6	—	24	2·7
Tobacco leaf	5·3	—	—	—

centrations up to 15·1 mM in the presence of EDTA. The apparent Michaelis constant determined by the procedure of Lineweaver & Burk (1934) gave a value of 8·4 mM. However, the leaf preparations contained phosphoglucose isomerase, which rapidly converted the fructose 6-phosphate into glucose 6-phosphate, and at equilibrium about 30% of the activity was in fructose 6-phosphate. Correction of the above Michaelis constant gives a value of 2·4 mM, which agrees well with the values obtained by Leloir & Cardini (1955) and Mendicino (1960). The rate of the reaction in the presence of 15·1 mM-fructose 6-phosphate (concentration supplied) was 90% of the calculated maximum rate and this concentration was used to determine the activity of the enzyme in leaf preparations.

Rate against time. The rate of synthesis of sucrose phosphate in the presence of EDTA was linear with time when the percentage conversion of supplied fructose 6-phosphate into sucrose phosphate was less than 20%. For higher percentage conversions, values corrected for kinetics of a first-order reaction were linear with time up to at least 70% conversion of supplied fructose 6-phosphate. From this result it is obvious that the reaction catalysed by phosphoglucose isomerase rapidly reached equilibrium.

Activity and distribution of sucrose phosphate synthetase and sucrose synthetase in crude extracts from leaves. The activity of sucrose phosphate synthetase in crude leaf preparations measured at 15·1 mM-fructose 6-phosphate is shown in Table 2. By using the data presented by Hatch (1964) it is possible to estimate approximately the activity at saturating concentrations of fructose 6-phosphate of sucrose phosphate synthetase extracted from sugar-cane leaves. Extraction and assay procedures now employed for sugar-cane leaves give values tenfold greater than that reported by Hatch (1964).

Some of the sucrose phosphate synthetase and sucrose synthetase remained associated with the precipitate in the leaf preparations (Table 2). In comparison it was found that only 7% of the total sucrose phosphatase remained in the unwashed precipitate of pea-leaf preparations.

The sucrose phosphate synthetase/sucrose synthetase activity ratio ranged from 10 in pea-leaf preparations to 0·6 in sugar-cane-leaf extracts.

Activity of sucrose phosphate synthetase in leaves having different rates of photosynthesis. Waldron, Glasziou & Bull (1967) carried out experiments in which the rates of photosynthesis of sugar-cane plants were measured during growth at different temperatures. When the temperature was lowered, stalk growth and the rate of photosynthesis declined, but when the same plants were moved to higher temperatures growth and the rate of photosynthesis increased. Leaves from this experimental material were used for the extraction of sucrose phosphate synthetase and were fully expanded on both dates of harvest and did not change macroscopically during the temperature shift. The activity of sucrose phosphate synthetase had increased from 0·9 to 3·1 μmoles of sucrose phosphate synthesized/g. fresh wt./hr. (Table 3), during which time the rate of photosynthesis increased 2·5-fold.

DISCUSSION

Evidence pointing to sucrose phosphate as being an intermediate in sucrose synthesis during photosynthesis, including the presence of sucrose phosphatase in leaves, has been discussed previously (Hawker, 1966). The values currently obtained for the activity of sucrose phosphate synthetase in leaves are much higher than those calculated from previous reports and would permit sucrose production during photosynthesis to occur by a pathway involving sucrose phosphate. However, sucrose

Table 3. *Activity of sucrose phosphate synthetase and the rate of photosynthesis of sugar-cane leaves from plants grown at different temperatures*

Enzyme preparations were made from sugar-cane leaves of plants growing at 14° that were then moved to 22° (Waldron *et al.* 1967). Sucrose phosphate synthetase activity was determined as described in Table 2. Values are means of determinations on three extracts.

Sample date	Sucrose phosphate formed (μmoles/g. fresh wt. of leaf/hr.)	Sucrose phosphate synthetase/sucrose synthetase activity ratio	Rate of photosynthetic CO_2 fixation* (expressed as μmoles sucrose synthesized/g. fresh wt. of leaf/hr.)
10 August 1966	0·9	0·46	4·0
31 August 1966	3·1	0·66	10

* Calculated from data of Waldron *et al.* (1967).

synthetase was also present in the leaves studied and no conclusions can be drawn as to the relative importance of the two enzymes in sucrose synthesis during photosynthesis.

Inhibition of sugar-cane stalk growth by low temperature may be accompanied by a rise in sugar concentration and a fall in the rate of photosynthesis. This effect may be reversed by raising the temperature, and, since there is no short-term effect of temperature changes on photosynthesis, these results may be indicative of a regulatory mechanism for controlling photosynthesis (Waldron *et al.* 1967). The results given in Table 3 were obtained on leaves from the experimental material used by these workers and show correlated changes in sucrose phosphate synthetase activities and photosynthetic rates. Demonstration of the occurrence of end-product inhibition of sucrose phosphatase activity by sucrose in sugar-cane leaves (Hawker, 1967) and stimulation of ADP-glucose pyrophosphorylase activity in spinach leaves by intermediates of sucrose synthesis (Ghosh & Preiss, 1966) shows that intermediate metabolites may participate in regulating photosynthesis by controlling enzyme activity. The present results demonstrate that control of enzyme synthesis might also be involved.

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