Phytochemistry 1974 Vol 13 pp 59 to 64 Pergamon Press Printed in England

INTRACELLULAR SITE OF SUCROSE SYNTHESIS IN LEAVES

IVAN F BIRD, MARTIN J CORNELIUS, ALFRED J KEYS and CHARLES P WHITTINGHAM

Rothamsted Experimental Station, Harpenden, Hertfordshire AL5 2JQ

(*Received* 15 *July* 1973 *Accepted* 3 *September* 1973)

Key Word Index—*Pisum*, *Spinacia*, *Triticum*, *Vicia* pea spinach, wheat, bean, leaves, sucrose, biosynthesis, cytoplasm

Abstract—Improved conditions for extraction and assay increased rates of sucrose synthesis from uridine diphosphate glucose (UDPglucose) plus fructose 6-phosphate (F 6 P) catalysed by leaf extracts 20-fold Rates of 17 9 25 0, 9 2 and 27 7 μmol/hr/g fr wt respectively were obtained from pea shoots, spinach, wheat and bean leaves Chloroplasts isolated from pea shoots in which half the plastids were intact contained less than 4% of the total UDPglucose-fructosephosphate glucosyltransferase more than 30% of the ribulose diphosphate (RuDP) carboxylase, and more than 40% of the total chlorophyll of the leaf Although some of the UDPglucose-fructosephosphate glucosyltransferase was associated with particles smaller than chloroplasts at least 85% of the enzyme was not precipitated at 38 000 *g* UDPglucose pyrophosphorylase also thought to be essential for sucrose synthesis, was distributed between the cell fractions in a similar manner to UDPglucose-fructosephosphate glucosyltransferase It is concluded that sucrose synthesis in pea shoots and spinach leaves occurs mainly in the cytoplasm

INTRODUCTION

VARIOUS compounds formed from the immediate products of photosynthesis from CO_2 are rapidly exported from chloroplasts [1,2] During assimilation of $^{14}CO_2$ by tobacco leaves, glycine and serine were the compounds most rapidly and extensively labelled outside of the chloroplasts [3] Conversion of glycine to serine takes place in mitochondria[4] and is accompanied by oxidative phosphorylation[5,6] and evolution of carbon dioxide which probably largely accounts for the process of photorespiration in leaves Therefore, like dark respiration, photorespiration results in phosphorylation of ADP in mitochondria One possible benefit to the plant cell of ATP synthesis in the light in mitochondria (as distinct from that in the chloroplast) may be related to biosynthetic reactions located in the cytoplasm Miflin *et al* [7] and Tamas and Bidwell[8] have provided evidence to suggest that sucrose synthesis can occur elsewhere than in the chloroplast Isolated chloroplasts which are capable of photosynthesis from CO_2 do not usually produce sucrose [9] Although sucrose was a major product of photosynthesis by chloroplasts from *Acetabularia*,[10 11] it

[1] HEBER U SANTARIUS K A HUDSON M A and HALLIER U W (1967) *Z Naturforsch* **22b**, 1189
[2] BASSHAM J A KIRK M and JENSEN R G (1968) *Biochim Biophys Acta* **153**, 211
[3] ROBERTS, G R, KEYS, A J and WHITTINGHAM C P (1970) *J Exp Botany* **21**, 683
[4] KISAKI, T, IMAI, A and TOLBERT, N E (1971) *Plant Cell Physiol (Tokyo)* **12**, 267
[5] BIRD, I F, CORNELIUS, M J, KEYS, A J and WHITTINGHAM C P (1972) *Phytochemistry* **11**, 1587
[6] BIRD, I F, CORNELIUS, M J, KEYS, A J and WHITTINGHAM, C P (1972) *Biochem J* **128**, 191
[7] MIFLIN B J MARKER A F H and WHITTINGHAM C P (1966) *Biochim Biophys Acta* **120**, 266
[8] TAMAS I A and BIDWELL R G S (1971) *Can J Botany* **49** 299
[9] EVERSON R G COCKBURN W and GIBBS M (1967) *Plant Physiol* **42**, 840
[10] DODD, W A and BIDWELL R G S (1971) *Nature* **234**, 45
[11] WINKENBACH F PARTHASARATHY M V and BIDWELL R G S (1972) *Can J Botany* **50**, 1367

is clear that these chloroplasts were associated with cytoplasm and other cell constituents Furthermore, Heldt and Sauer[12] have shown that the inner membrane of the chloroplast is impermeable to sucrose, thus if sucrose were made in the chloroplast it would not be able to move out

UDPglucose-fructosephosphate glucosyltransferase is probably the main enzyme responsible for sucrose synthesis in plants Bird *et al*[13] found that about 50% of this enzyme activity was associated with chloroplasts isolated in non-aqueous media[14] However the total amount of enzyme activity recovered from the whole tissue was less than 1 μmol/hr/g fr wt which suggests that considerable enzyme inactivation may have taken place during the isolation procedure In addition, it is now known that chloroplasts isolated by non-aqueous methods are contaminated with cytoplasm to a greater extent than was previously supposed[15] The combination of these two factors resulted in too much of the enzyme activity being apportioned to the chloroplasts and too little to the cytoplasm Haq and Hassid[16] found very small (less than 0 1 nmol/hr/g fr wt) activities of UDPglucose-fructosephosphate glucosyltransferase activity associated with chloroplasts from sugar cane leaves prepared by the method of Whatley *et al*[17] No information was provided about the amount of enzyme in other leaf fractions Early attempts to extract active UDPglucose-fructosephosphate glucosyltransferase from leaves proved difficult[18-20] Subsequently Hawker[21] was more successful and found higher (up to 8 2 μmol/hr/g fr wt) activities, especially in pea leaves, but, in a medium containing 0 3 M mannitol not more than 10–14% of the activity was associated with particles We have modified further both extraction and assay conditions and have found rates of sucrose synthesis in excess of 15 μmol hr/g fr wt catalysed by UDPglucose-fructosephosphate glucosyltransferase in leaves Little of the activity was associated with intact chloroplasts from either pea shoots or spinach leaves

RESULTS

UDPglucose-fructosephosphate glucosyltransferase from pea shoots like the similar enzyme in the scutellum of wheat,[22] is stimulated by magnesium ions but is inhibited by excessive concentrations of various salts, e g Tris hydrochloride For the enzyme in pea shoots, the optimum concentration of magnesium chloride was about 10 mM Sodium fluoride inhibited the reaction strongly at the concentration employed by Lyne and ap Rees[23] Extracts of pea shoots had little fructose 6-phosphatase or UDPglucose-fructose glucosyltransferase activity Therefore the true activity present of UDPglucose-fructosephosphate glucosyltransferase was more nearly measured when fluoride EDTA and much of the Tris buffer were omitted and $MgCl_2$ was added to reaction mixtures described by Lyne and ap Rees[23]

[12] HELDT H W and SAUER F (1971) *Biochim Biophys Acta* **234**, 83
[13] BIRD I F PORTER H K and STOCKING C R (1965) *Biochim Biophys Acta* **100**, 366
[14] STOCKING C R (1959) *Plant Physiol* **34**, 56
[15] BIRD I F CORNELIUS M J DYER T A and KEYS A J (1973) *J Exp Botany* **24**, 211
[16] HAQ, S and HASSID W Z (1965) *Plant Physiol* **40**, 591
[17] WHATLEY F R ALLEN M B and ARNON D I (1959) *Biochim Biophys Acta* **32**, 32
[18] MENDICINO J (1960) *J Biol Chem* **235**, 3347
[19] DUTTON J V CARRUTHERS A and OLDFIELD J F T (1961) *Biochem J* **81**, 266
[20] RORIM E S WALKER H G and MCCREADY R M (1960) *Plant Physiol* **35**, 269
[21] HAWKER J S (1967) *Biochem J* **105**, 943
[22] KEYS A J (1959) Ph D Thesis Univ London
[23] LYNE R L and AP REES T (1972) *Phytochemistry* **11** 2171

Shoots from peas grown with light intensities of 18000 lx contained more UDPglucose-fructosephosphate glucosyltransferase than shoots grown with light of 3500 lx Most of the activity in the shoots was in the leaflets In Table 1 are shown measured activities in extracts of shoots of pea, and leaves of spinach, wheat and field bean Results of the colorimetric and radioisotope assays are in good agreement In reaction mixtures containing UDP-[^{14}C]glucose and F 6 P no evidence was found for sucrose phosphate formation Therefore, all of the tissue extracts contained sufficient sucrose phosphatase to hydrolyse all of the sucrose phosphate formed Under the conditions of assay, there was no detectable hydrolysis of F 6 P to fructose and activities of UDPglucose-fructose glucosyltransferase in extracts were low (Table 1) The synthesis of sucrose from UDPglucose and F 6 P must, therefore, have been almost entirely the result of sucrose phosphate synthesis and its subsequent hydrolysis catalysed by the two enzymes UDPglucose-fructosephosphate glucosyltransferase and sucrose phosphatase The overall measured rates were determined by the former enzyme

TABLE 1 GLUCOSYLTRANSFERASE ACTIVITIES EXTRACTED FROM GREEN PLANT TISSUES

Source of extract	UDPglucose-fructosephosphate glucosyltransferase (μmol sucrose/hr/g fr wt) Colorimetric assay	^{14}C assay	UDPglucose-fructose glucosyltransferase Colorimetric assay
Pea shoots 18 000 lx	17 9	19 0	4 5
Spinach leaves	25 0	23 3	0 4
Wheat leaves	9 2	7 4	1 7
Field bean leaves	27 7	25 8	3 3

In all experiments, chloroplasts isolated by the method of Cockburn *et al* [24] contained only very low activities of UDPglucose-fructosephosphate glucosyltransferase Most of the activity was soluble and could be recovered from supernatant liquids Table 2 shows a detailed examination in which RuDP carboxylase was also measured to show how a soluble chloroplast enzyme was distributed between fractions Less than 4% and usually less than 1% of the UDPglucose-fructosephosphate glucosyltransferase was in the fraction containing whole chloroplasts Rather more activity was associated with the fraction containing mitochondria and peroxisomes but most of the activity was not associated with cell particles By contrast, more than 30% of the RuDP carboxylase was associated with the fraction containing whole chloroplasts, least was in the fraction containing small cell particles and most was soluble UDPglucose pyrophosphorylase was distributed between the fractions in a similar manner to UDPglucose-fructosephosphate glucosyltransferase If it is assumed that RuDP carboxylase is retained only by intact chloroplasts an alternative measure of intactness can be made for the 0–2000 *g* fraction In the case of spinach leaf this measure compares well with that obtained by measuring oxygen evolution in the presence of ferricyanide but with shoots of pea it gave a higher value than that obtained with ferricyanide We assume that some chloroplasts had ruptured membranes but had not lost all their stroma material [25] We concluded that neither UDPglucose-fructosephosphate glucosyltransferase nor UDPglucose pyrophosphorylase are soluble enzymes of the

[24] COCKBURN, W, WALKER D A and BALDRY, C W (1968) *Plant Physiol* **43**, 1415
[25] LEECH R M (1964) *Biochim Biophys Acta* **79**, 637

chloroplast stroma and that they are not firmly bound to chloroplast membranes The slight association with smaller particles pelleted between 2000 and 38000 *g* may be significant However even when different media from that of Cockburn *et al*[24] were employed for homogenization, e g that of Pierpoint[26] or Medium B of Dalling *et al*[27] we found nearly 95% of the UDPglucose-fructosephosphate glucosyltransferase was soluble

Table 2 Enzymic activities in fractions obtained by differential centrifugation from homogenates of pea shoots and spinach leaves

Fraction	Chlorophyll (mg)	Intact chloroplasts (%)	UDPglucose-fructosephosphate glucosyltransferase	RuDP carboxylase	UDPglucose pyrophosphorylase
			(activity as μmol hr)		
Pea shoots 3500 lx					
0 2000 *g*	4 58	50 (88)*	0 74	273	
2000 38 000 *g*	5 70		2 60	74	
Supernatant			19 30	347	
Pea shoots 18 000 lx					
0 2000 *g*	4 70	52 (69)*	0 64	265	8
2000 38 000 *g*	4 68		1 96	74	101
Supernatant			52 6	476	2900
Spinach leaf					
0 2000 *g*	12 4	31 (35)*	2 60	251	79
2000 38 000 *g*	7 5		8 50	34	98
Supernatant			255 0	860	9980

* Figures in parentheses are values calculated from RuDP carboxylase activities

DISCUSSION

Both UDPglucose pyrophosphorylase and UDPglucose-fructosephosphate glucosyltransferase are essential enzymes for sucrose synthesis in plants Our data suggest that neither enzyme is present in intact chloroplasts and if, *in vivo* these enzymes are associated with any particle in the cell the association must be readily broken during homogenization of the tissue If one accepts that the enzyme localization observed *in vitro* occurs also *in vivo*, sucrose synthesis in leaves cannot take place in the chloroplasts Since carbon from CO_2 is rapidly incorporated into sucrose during photosynthesis under a wide range of conditions[28] sucrose must be synthesized from a product of photosynthesis that can easily move from the chloroplast into the cytoplasm At the present time either triose phosphate or certain intermediates of the glycollate pathway or both appear to be the products of photosynthesis that could fulfil this role

Two possible precursors of sucrose synthesis may be considered firstly, triose phosphate formed in the chloroplasts by carboxylation of RuDP and subsequent reduction of the resulting PGA There is good evidence that triose phosphate can freely pass in both directions through the chloroplast membrane[1,2] Secondly sucrose may be synthesized from intermediates of the glycollate pathway and this synthesis may take place entirely

[26] Pierpoint W S (1959) *Biochem J* **71**, 518
[27] Dalling M J Tolbert N E and Hageman R H (1972) *Biochim Biophys Acta* **283** 505
[28] Calvin M and Benson A A (1949) *Science N Y* **109** 140

in the cytoplasm Intermediates of the glycollate pathway are readily used for sucrose synthesis by leaf tissue [29] Also, when the concentration of CO_2 in the atmosphere is 300 ppm or less, carbon from photosynthesis appears more rapidly in glycine and serine than in phosphate esters in the cytoplasm [3] Under these conditions, glycine and serine will be more readily available as substrates for sucrose synthesis in the cytoplasm than triose phosphates As the concentration of CO_2 is increased the glycollate pathway is suppressed but synthesis of sucrose continues.[30] It appears probable that some sucrose is made from intermediates of the glycollate pathway and some from triose phosphate, the proportions from each source will vary according to the conditions of the experiment

Intermediates of the glycollate pathway become uniformly labelled very quickly during photosynthesis from $[^{14}C]CO_2$,[31] hence sucrose made from them should quickly become uniformly labelled When the concentration of CO_2 is high enough to suppress the glycollate pathway, sucrose synthesized from triose phosphate will be initially labelled non-uniformly The latter has been demonstrated experimentally by Calvin[32] and by Gibbs [33] It has not yet been shown whether sucrose is more uniformly labelled when the concentration of CO_2 is lower We are presently undertaking experiments to determine the distribution of ^{14}C in sucrose and in triose and hexose phosphates after photosynthesis with different concentrations of $[^{14}C]CO_2$

Two roles have been suggested as to the functional significance of photorespiration and the glycollate pathway [34] Either the glycollate pathway provides useful metabolites for further biosynthesis or it removes excess reductant from the photosystems of the chloroplasts A third possibility is that when the concentration of CO_2 in the atmosphere is low, and intermediates of the Calvin cycle are relatively depleted, the rate of export of triose phosphate is not sufficient to supply the cytoplasm with substrate for maintenance, respiration and essential synthesis Under these circumstances, glycollate is the main compound transferring carbon from chloroplast to cytoplasm

EXPERIMENTAL

Seeds of *Pisum sativum* (var Feltham First) were sown in washed moist, vermiculite each week and kept at 18° with 11 hr light periods at 3500 lx Shoots of the seedlings were used either after 21 days or after 17 days in the above conditions followed by 4 days at 20° with 12 hr light periods at 18000 lx Leaves of *Spinacia oleracea* (var Victoria Longstanding Summer) were from plants grown for 3 weeks in a glasshouse with supplementary illumination Leaves from *Vicia faba* (var Maris Bead) and *Triticum aestivum* (var Kolibri) were from plants grown in a glasshouse for 3 and 2 weeks respectively

To obtain an extract in which total UDPglucose-fructosephosphate glucosyltransferase was measured tissues (5 g) were ground in a mortar at 0 for 2 min with 3 g acid-washed sand and 7 5 ml 0 01 M Tris buffer pH 7 0 containing 0 1% (w/v) bovine serum albumin (Sigma) Homogenates were filtered through 2 layers of nylon gauze and 1 layer of nylon filter-cloth (50 μ mesh) Filtrates were centrifuged at 38 000 g for 10 min and the supernatant liquids were freed from substances of low MW by passing them through columns of Sephadex G25 (coarse) previously equilibrated with 0 01 M Tris buffer pH 7 0

For fractionation plant material (40 g) was homogenized for 15 sec in partly frozen medium (160 ml) of the composition described by Cockburn *et al* [24] but with 0 1% (w/v) bovine serum albumin added After squeezing the homogenate quickly through muslin and filtering through cotton wool and 50 μ mesh nylon filter-cloth, a fraction containing many intact chloroplasts was centrifuged from the filtrate at 2000 g for 1 min at 0° (0–2000 g fraction) The supernatant liquid from this centrifugation was centrifuged at 38 000 g for 10 min to give a 2000–

[29] WANG D and WAYGOOD E R (1962) *Plant Physiol* **37**, 826
[30] MORTIMER D C (1959) *Can J Botany* **37**, 1191
[31] RABSON, R, TOLBERT N E and KEARNEY, P C (1962) *Archs Biochem Biophys* **98**, 154
[32] CALVIN M BASSHAM J A BENSON A A LYNCH V H OUELLET C SCHOU L STEPKA W and TOLBERT N E (1951) *Symp Soc Exp Biol* **5**, 284
[33] GIBBS, M (1951) *Plant Physiol* **26**, 549
[34] COOMBS J (1971) *Proc R Soc* **179B**, 221

38 000 *g* pellet and the corresponding supernatant liquid. Soluble protein was extracted from the pelleted fractions by suspending them for 15 min at 0° in 0·01 M Tris buffer pH 7·0 containing 0·1% bovine serum albumin. The suspensions were centrifuged at 38 000 *g* for 10 min and the supernatant liquids assayed for the various enzymes. Soluble protein from the original homogenate (supernatant liquid) was separated from substances of low MW by using columns of Sephadex G25 (coarse) and assayed for enzyme activity.

Part of the 0–2000 *g* fraction was resuspended and assayed for CO_2 fixation[35] and under similar conditions but with added ADP (1 mM) and $MgCl_2$ (5 mM) for stimulation of O_2 evolution by ferricyanide. The increase in the rate of O_2 evolution caused when all plastids were ruptured following brief exposure to a hypotonic soln was taken as a measure of intact plastids present.[36,37] Chlorophyll was measured by the method of Arnon.[38] RuDP carboxylase was determined by the method of Andrews and Hatch[39] and UDPglucose pyrophosphorylase by the method of Feingold.[40] Reaction mixtures for assay of UDPglucose-fructosephosphate glucosyltransferase activities contained 1·5 μmol of UDPglucose, 1 μmol of $MgCl_2$ and 1·5 μmol of F 6 P with 0·05 ml of the protein soln in 0·01 M Tris buffer pH 7·0 containing 0·1% (w/v) bovine serum albumin. The total vol was 0·10 ml. Control reaction mixtures contained either no UDPglucose or 1·5 μmol fructose instead of F 6 P. Usually synthesis of sucrose plus sucrose phosphate was measured colorimetrically by the method of Cardini *et al.*[41] Leloir and Cardini[42] Synthesis was also checked in reaction mixtures containing UDP-[^{14}C]glucose by chromatography before and after treatment with alkaline phosphatase[21,23] using Whatman 3 MM paper and *n*-BuOH–HOAc–H_2O (12:3:5). The sucrose on the chromatograms was eluted with H_2O and the ^{14}C present was measured by liquid scintillation counting. To determine whether or not F 6 P was extensively hydrolysed to fructose, reaction mixtures without UDPglucose were examined by TLC cellulose using EtOH–1M ammonium acetate pH 3·8 (7:5:3).[43] Sugars and sugar phosphates were detected by spraying with 1% resorcinol in EtOH–2N HCl (1:9) and heating at 110°.

[35] Walker D A (1971) *Methods in Enzymology* (San Pietro A ed.) Vol XXIII Part A pp 211–220. Academic Press: New York.
[36] Heber U and Santarius K A (1970) *Z. Naturforsch.* **25b**, 718.
[37] Werdan K, Heldt H W and Geller G (1972) *Biochim. Biophys. Acta* **283**, 430.
[38] Arnon D I (1949) *Plant Physiol.* **24**, 1.
[39] Andrews T J and Hatch M D (1971) *Phytochemistry* **10**, 9.
[40] Feingold D S, Neufeld E F and Hassid W Z (1964) *Modern Methods of Plant Analysis* (Linskens H F, Sanwal B D and Tracey M V eds.) Vol VII pp 474–519. Springer: Berlin.
[41] Cardini C E, Leloir L F and Chiriboga J (1955) *J. Biol. Chem.* **214**, 149.
[42] Leloir L F and Cardini C E (1955) *J. Biol. Chem.* **214**, 157.
[43] Paladini A C and Leloir L F (1952) *Biochem. J.* **51**, 426.