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ENZYMES OF STARCH AND SUCROSE METABOLISM IN *ZEA MAYS* LEAVES

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Abstract—Mesophyll and bundle sheath cells of maize leaves were separated and enzymes of starch and sucrose metabolism assayed. The starch content and activities of ADPglucose (ADPG) starch synthetase and phosphorylase expressed both on a chlorophyll and a protein basis were much lower in mesophyll cells compared to bundle sheath preparations. Exposure of the leaves to continuous illumination for 2·5 days caused the starch content of mesophyll cells to rise greatly and led to considerable increases in ADPG starch synthetase and phosphorylase activity. In glasshouse grown leaves the bulk of invertase, sucrose phosphate synthetase, sucrose phosphatase, UDPglucose pyrophosphorylase and amylase was situated in the mesophyll layer. Sucrose synthetase, ADPG starch synthetase and phosphorylase were largely confined to the bundle sheath. No enzyme could be completely assigned to one particular cell layer. Upon continuous illumination both ADPG starch synthetase and phosphorylase increased in the mesophyll by the same relative amount. The mesophyll is likely to be a major site for sucrose synthesis in maize leaves.

INTRODUCTION

LEAVES of C_4 plants are characterized by the presence of two concentric chloroplast-bearing cell layers about the vascular bundles. Plastids of the inner bundle sheath layer contain abundant starch; those of the outer mesophyll layer often do not.[1,2] In order to explain the low level of starch in mesophyll cells and to locate the site of sucrose synthesis in the leaf, we have separated the two tissues from leaves of *Zea mays* L. (maize) and studied the distribution of enzymes associated with starch and sucrose metabolism. Maize leaves were also subjected to prolonged continuous light to observe changes in the enzymes when mesophyll chloroplasts become loaded with starch. Huber *et al.*[3] have previously measured the activity of some enzymes of starch metabolism in aqueously isolated maize chloroplasts. The activities which they reported, however, were very low, partially attributable to significant losses of soluble protein from the plastids in aqueous medium.[4] To avoid this difficulty and to provide a more realistic basis for comparing mesophyll and bundle sheath cells, we have assayed the entire contents of the two cell layers for enzyme activity. Edwards and Black[5] have commented on a few enzymes concerned with starch metabolism in isolated mesophyll and bundle sheath cells of the C_4 plant *Digitaria sanguinalis* (crabgrass).

[1] DOWNTON, W. J. S. and TREGUNNA, E. B. (1968) *Can. J. Botany* **46**, 207.
[2] WOO, K. C., PYLIOTIS, N. A. and DOWNTON, W. J. S. (1971) *Z. Pflanzenphysiol.* **64**, 400.
[3] HUBER, W., DE FEKETE, M. A. R. and ZIEGLER, H. (1969) *Planta* **87**, 360.
[4] KIRK, J. T. O. and TILNEY-BASSETT, R. A. E. (1967) in *The Plastids*, p. 5, Freeman, London.
[5] EDWARDS, G. E. and BLACK, C. C. (1971) in *Photosynthesis and Photorespiration* (HATCH, M. D., OSMOND, C. B. and SLATYER, R. O., eds.), p. 153, Wiley–Interscience, New York.

RESULTS AND DISCUSSION

A buffer containing a reducing agent, diethyldithiocarbamate (DIECA) and Carbowax permits the release of enzymes in high activity from tissues rich in phenolic substances in grapevine.[6,7] Extraction of maize leaves with this medium (see Experimental) and one containing 10 mM dithiothreitol and 1% polyclar-AT (similar to that used by Osmond and Harris[8] for maize and sorghum leaves) resulted in the detection of identical activities for malate dehydrogenase (E.C. 1.1.1.37) and malic enzyme (E.C. 1.1.1.40). Osmond and Harris, and Huang and Beevers[9] have demonstrated that differences in enzyme composition between mesophyll and bundle sheath cells are not due to the selective inhibition of mesophyll extracts by endogenous phenolics as has been suggested by Baldry *et al.*[10] and Bucke and Long.[11] As a precaution against this possibility, however, we have employed the DIECA- and Carbowax-containing extraction medium throughout this study.

TABLE 1. ACTIVITY OF ENZYMES IN MESOPHYLL AND BUNDLE SHEATH CELLS OF GLASSHOUSE GROWN *Zea mays* LEAVES

Enzyme	μmol/hr/mg chlorophyll		μmol/hr/mg protein	
	Mesophyll	Bundle sheath	Mesophyll	Bundle sheath
Malic enzyme	86·9	924·0	6·5	32·3
Phosphorylase	8·3	31·1	0·59	0·97
ADPG starch synthetase	2·6	16·1	0·19	0·52
ADPG pyrophosphorylase	7·6	67·5	0·61	2·6
UDPG pyrophosphorylase	826·6	1163·7	60·5	41·3
Amylase	274·4	198·5	22·0	7·8
Sucrose synthetase	0·42	4·2	0·03	0·14
Sucrose-P synthetase	2·8	2·9	0·20	0·19
Sucrose phosphatase	87·9	126·5	6·9	4·2
Invertase	11·6	13·8	0·93	0·53
Starch (mg glucose/mg chlorophyll)	0·58	6·2		
Protein (mg/mg chlorophyll)	13·4	29·2		
Chlorophyll (mg/g fr. wt leaf)	2·1			

Enzymes of Starch and Sucrose Metabolism in Glasshouse Grown Maize Leaves

Much evidence favors the hypothesis that the various enzymes associated with the C_4 pathway of photosynthesis are differentially compartmentalized between mesophyll and bundle sheath cells.[5,8,9,12–14] Malic enzyme is largely confined to the bundle sheath. This enzyme was routinely assayed in each experiment to provide an estimate of the purity of mesophyll cell preparations. The enzyme could not be entirely assigned to the bundle sheath since it was detected in an extract derived almost entirely from the mesophyll cells, obtained when whole leaf sections were gently ground in a mortar. With this method malic

[6] HAWKER, J. S. (1969) *Phytochemistry*, **8**, 9.
[7] DOWNTON, W. J. S. and HAWKER, J. S. (1973) *Phytochemistry* **12**, 1557.
[8] OSMOND, C. B. and HARRIS, B. (1971) *Biochim. Biophys. Acta* **234**, 270.
[9] HUANG, A. H. C. and BEEVERS, H. (1972) *Plant Physiol.* **50**, 242.
[10] BALDRY, C. W., BUCKE, C. and COOMBS, J. (1971) *Planta* **97**, 310.
[11] BUCKE, C. and LONG, S. P. (1971) *Planta* **99**, 199.
[12] BJORKMAN, O. and GAUHL, E. (1969) *Planta* **88**, 197.
[13] SLACK, C. R., HATCH, M. D. and GOODCHILD, D. J. (1969) *Biochem. J.* **114**, 489.
[14] BERRY, J. A., DOWNTON, W. J. S. and TREGUNNA, E. B. (1970) *Can. J. Botany* **48**, 777.

enzyme was 9% as active as the bundle sheath extracts on a chlorophyll basis and 20% as active on a protein basis. The enzymes in mesophyll and bundle sheath cells listed in Table 1 are presented both on a chlorophyll and a protein basis since protein: chlorophyll ratios differ significantly between the two cell types.

Leaves from glasshouse grown plants when stained with I_2/KI gave an intense reaction for starch only in bundle sheath plastids. The concentrations of starch present are given in Table 1. Malic enzyme, ADPglucose (ADPG)pyrophosphorylase (ATP:α-D-glucose-1-phosphate adenyltransferase) and sucrose synthetase (E.C. 2.4.1.13) had only about 0·1 of the specific activity (per unit chlorophyll) in mesophyll cells compared to bundle sheath cells.

Other enzymes such as UDPglucose (UDPG) pyrophosphorylase (E.C. 2.7.7.9), invertase (E.C. 3.2.1.26), sucrose phosphate synthetase (E.C.2.4.1.14) and sucrose phosphatase (sucrose phosphate phosphohydrolase) when taken on a chlorophyll basis were much closer in specific activity in the two cell layers. When they were considered on a protein basis, however, the mesophyll extracts were 1·5–2 times more active than bundle sheath preparations. Sucrose phosphatase activity in both mesophyll and bundle sheath cells was inhibited 35–37% when incubated with 100 mM sucrose and inhibited 85–90% with 100 mM maltose indicating the presence of a specific sucrose phosphatase.[15] Huber *et al.*[3] were unable to detect sucrose synthetase and sucrose phosphate synthetase in maize chloroplasts isolated in aqueous solvent.

Amylase (E.C. 3.2.1.1 and 3.2.1.2) differed from the other enzymes we studied in being more active per unit chlorophyll in mesophyll extracts. Bourne *et al.*[16] reported that aqueously isolated chloroplasts from bundle sheath cells of sugarcane had twice the amylase activity of mesophyll plastids per unit protein. When considered on a protein basis, the amylase in our mesophyll preparations was about 3 times greater than in our bundle sheath preparations. Mesophyll extracts contained 16% of ADPG starch synthetase (ADPG-starch glucosyltransferase) activity and 27% of phosphorylase (E.C. 2.4.1.1) activity compared to bundle sheath activities per unit chlorophyll.

UDPG starch synthetase (UDPG-starch glucosyltransferase) was not detected in mesophyll cell preparations and extracts from bundle sheath cells gave variable results, the maximum activity being 2·7 μmol/hr/mg chlorophyll, which was less than 20% of the activity measured with ADPG as substrate. Substitution of rabbit liver glycogen for potato starch as a primer did not change the results. Edwards and Black[5] did not detect either ADPG or UDPG starch synthetase in mesophyll cells of crabgrass.

Activity of Enzymes in Maize Grown under Continuous Light

The mesophyll cells of maize leaves grown under glasshouse conditions contained little starch. These cells also had comparatively low levels of ADPG starch synthetase and phosphorylase, both of which have been implicated in starch synthesis. Following the transfer of the plants to continuous illumination for 2·5 days, plastids of the mesophyll stained intensely with I_2/KI. The concentration of starch in both the mesophyll and bundle sheath layers increased markedly, particulatly in mesophyll cells (Table 2).

With the exception of phosphorylase and ADPG starch synthetase, activity for all enzymes studied increased by a factor of 1·2–1·6 in both mesophyll and bundle sheath cells (Table 2). ADPG starch synthetase and phosphorylase increased 3- and 4-fold respectively

[15] HAWKER, J. S. (1966) *Phytochemistry* **5**, 1191.
[16] BOURNE, E. J., DAVIES, D. R. and PRIDHAM, J. B. (1970) *Phytochemistry* **9**, 345.

on a chlorophyll basis and 2- and 3-fold on a protein basis in mesophyll cells under continuous illumination. Bundle sheath activities for these enzymes changed little.

TABLE 2. ACTIVITY OF ENZYMES IN MESOPHYLL AND BUNDLE SHEATH CELLS OF *Zea mays* LEAVES GROWN UNDER CONTINUOUS LIGHT FOR 2·5 DAYS

Enzyme	μmol/hr/mg chlorophyll		μmol/hr/mg protein		Increase in activity under continuous light*	
	Mesophyll	Bundle sheath	Mesophyll	Bundle sheath	Mesophyll	Bundle sheath
Malic enzyme	136·3	1306·8	8·4	41·7	1·6	1·4
Phosphorylase	31·5	42·5	1·9	1·4	3·8	1·4
ADPG starch synthetase	7·1	19·7	0·44	0·63	2·8	1·2
ADPG pyrophosphorylase	8·9	81·3	0·51	2·6	1·2	1·2
UDPG pyrophosphorylase	1275·9	1904·0	76·6	60·1	1·5	1·6
Amylase	372·6	308·0	20·7	8·9	1·4	1·6
Starch (mg glucose/mg chlorophyll)	40·8	67·6			70·3	10·9
Protein (mg/mg chlorophyll)	16·6	31·8			1·2	1·1
Chlorophyll (mg/g fr wt leaf)	1·84					

* Enzyme activity (per unit chlorophyll) in plants grown under continuous light divided by activity in glasshouse grown plants (Table 1).

ADPG pyrophosphorylase, which is responsible for ADPG synthesis, did not show an increase in activity in mesophyll cells parallel to that found for starch synthetase. The activity of the pyrophosphorylase, however, was in excess of the synthetase under the continuous light condition, and in leaves it has been shown that this enzyme is regulated by photosynthetic intermediates, especially 3-phosphoglyceric acid.[17]

Distribution of Total Enzyme Activity between Mesophyll and Bundle Sheath Cells in Maize

The relative distribution of total leaf chlorophyll between mesophyll and bundle sheath cells can be determined from equations[2] in which the ratio chlorophyll *a*/chlorophyll *b* in plastids of each layer and the whole leaf is known. It was calculated that the mesophyll layer of glasshouse-grown leaves contained 70% of the total leaf chlorophyll. From the ratio of protein:chlorophyll in each cell layer, the percentage distribution of protein between the two cell layers was also calculated. Mesophyll cells were found to have 51% of the protein. Crabgrass leaves show a similar distribution of protein although chlorophyll is about equally distributed between mesophyll and bundle sheath cells.[5] Utilizing information on the distribution of either chlorophyll or protein between the two cell layers, it was possible to determine the relative distribution of total enzyme activity between mesophyll and bundle sheath cells for each enzyme studied. The distribution of enzymes associated with sucrose metabolism are given in Table 3. No enzyme was entirely restricted to one particular cell layer. While 60–70% of the total leaf complement of invertase, sucrose phosphate synthetase, sucrose phosphatase and UDPG pyrophosphorylase was associated with mesophyll cells, only about 20% of the leaf complement of sucrose synthetase was located in the mesophyll. Malic enzyme was distributed 18%:82% between the mesophyll and bundle sheath. The association of sucrose synthetase with the bundle sheath fraction in maize and with vascular

[17] PREISS, J. and KOSUGE, T. (1970) *Ann. Rev. Plant Physiol.* **21**, 433.

bundles in stems of sugarcane[18] raises the possibility that this enzyme is involved in the translocation of sucrose in plants. Since the bulk of UDPG pyrophosphorylase, sucrose phosphate synthetase and sucrose phosphatase was confined to the mesophyll layer, this tissue may be the main site of sucrose synthesis in maize leaves. Over 65% of the sucrose phosphate synthetase present in crabgrass leaves is also located in mesophyll cells.[5]

Table 3 also summarizes the distributions of activity between mesophyll and bundle sheath cells for some enzymes of starch metabolism. In leaves grown under continuous light the distributions of ADPG pyrophosphorylase, UDPG pyrophosphorylase and amylase remained about the same as for glasshouse-grown leaves. The mesophyll component of both ADPG starch synthetase and phosphorylase, on the other hand, increased markedly, both enzymes showing the same relative change in mesophyll distribution. Accompanying these changes in enzyme distribution was an increase in mesophyll starch content from 18 to 55% of the total.

TABLE 3. PERCENTAGE DISTRIBUTION OF STARCH AND CHLOROPHYLL AND OF ENZYMES ASSOCIATED WITH SUCROSE AND STARCH METABOLISM IN GLASSHOUSE-GROWN MAIZE LEAVES

Enzyme	Activity* No additional light	Activity* With continuous light for 2·5 days before analysis	Enzyme or Compound	Activity* No additional light	Activity* With continuous light for 2·5 days before analysis
Invertase	67	—	ADPG starch synthetase	27	42
Sucrose synthetase	20	—	ADPG pyrophosphorylase	22	18
Sucrose phosphate synthetase	70	—	Amylase	76	70
Sucrose phosphatase	62	—	Starch	18	55
UDPG pyrophosphorylase	62	58	Chlorophyll	70	67
Phosphorylase	38	60			

*Results are expressed as activity in mesophyll as a percentage of total activity in mesophyll and bundle sheath (=100%). Where no value is presented no determination was made.

Comparing rates of starch synthesis and activities of phosphorylase and ADPG starch synthetase in maize leaves, Fekete and Vieweg[19] concluded that only phosphorylase was sufficiently active to account for rates of starch formation (1·24 μmol glucose/hr/g fr. wt). The activity of ADPG starch synthetase that we were able to detect in glasshouse grown leaves (13·6 μmol/hr/g fr. wt), however, does not eliminate starch synthetase as an agent responsible for starch synthesis. Our activities were also 2-fold in excess of rates for starch synthesis later reported by Vieweg and Fekete.[20] At present there appears to be no compelling evidence to suggest that either phosphorylase or ADPG starch synthetase is more important for starch synthesis in maize leaves. Upon continuous illumination both enzymes increased in mesophyll by an equivalent amount. ADPG starch synthetase has often been suggested as the enzyme responsible for starch synthesis, while phosphorylase serves as a degradative enzyme.[7,21] It is possible that the increase in starch synthesizing ability in the mesophyll of maize leaves is due to an increase in activity of ADPG starch synthetase,

[18] HAWKER, J. S. and HATCH, M. D. (1965) *Physiol. Plant* **18**, 444.
[19] DE FEKETE, M. A. R. and VIEWEG, G. H. (1961) *Ber. Dtsch. Bot. Ges.* **84**, 475.
[20] VIEWEG, G. H. and DE FEKETE, M. A. R. (1972) *Planta* **104**, 257.
[21] AKAZAWA, T. (1965) in *Plant Biochemistry* (BONNER, J. and VARNER, J. E., eds.), p. 258, Academic Press, New York.

while an increase in phosphorylase is necessary for the clearing of starch from mesophyll cells during darkness. In this regard it is interesting that amylase did not change in distribution in the two light regimes.

The activities of starch synthetase and phosphorylase found in mesophyll cells of glasshouse-grown leaves, although low compared to the bundle sheath are, nonetheless, significant. The low starch level present in mesophyll cells, therefore, possibly reflects a localized deficiency of substrate for starch synthesis, since maize leaves given exogenous sucrose accumulate starch in the mesophyll.[22] The increased activity of starch synthetase and phosphorylase seen in the continuous light treatment is probably a response to elevated concentrations of soluble carbohydrate from prolonged photosynthesis.

EXPERIMENTAL

Growth of plants. Seedlings of maize (*Zea mays* L., cv. NES 1002) were grown in soil for 2–3 weeks in a naturally lit glasshouse during the period mid-August to mid-October (Southern Hemisphere). Average conditions were 26 ± 2° day/17 ± 3° night, 6·6 hr sunshine and total incident radiation of 420 cal/cm². In some experiments plants were removed from the glasshouse and placed in a growth cabinet under constant illumination of 0·16 cal/cm²/min (400–700 nm) for 2·5 days prior to extraction. The plants were also exposed to a thermoperiod of 18 hr at 21° and 6 hr at 17° throughout the continuous light treatment.

Plant extracts. Mesophyll and bundle sheath fractions from chilled maize leaves were prepared by the method of Woo *et al.*[23] Bundle sheath preparations, which consisted of bundle sheath cells attached to lengths of vascular bundles, were monitored by light microscopy to ensure that mesophyll contamination was minimal. All tissues were extracted in 50 mM Tris-acetate buffer pH 8·5, 20 mM EDTA, 11 mM DIECA, 15 mM cysteine–HCl and 6% Carbowax 4000 (polyethylene glycol approx MW 4000).[7] Extracts were filtered through Miracloth prior to assay. For amylase and invertase assays, extracts were dialyzed overnight against changes of 10 mM Tris-acetate buffer, pH 8·5 containing 1 mM EDTA. For the assay of sucrose synthetase and sucrose phosphate synthetase, 20 ml of filtrate through Miracloth was treated with 13 g of Carbowax 4000 followed by centrifugation at 20 000 *g* for 15 min. The ppt. was suspended in 1 ml of 5 mM Tris-HCl buffer, pH 7·0. All operations were performed at 0–4°.

Other analyses. Chlorophyll was determined according to Bruinsma.[24] For protein the method of Lowry *et al.*[25] was used, with bovine serum albumin as standard. Starch was determined as described by Ozbun *et al.*[26]

Enzyme assay. The activities listed are averages of means from 2 or 3 separate experiments. ADPG and UDPG starch synthetase, phosphorylase, ADPG and UDPG pyrophosphorylase, amylase, malic enzyme, sucrose synthetase, sucrose phosphate synthetase, sucrose phosphatase and malate dehydrogenase were assayed as described previously.[6,7,27,28] For invertase assay reaction mixtures contained 60 μmol NaOAc buffer pH 5·0, 60 μmol sucrose and enzyme in a final vol. of 1 ml. At intervals, 0·1 or 0·2 ml samples were withdrawn heated at 100° for 1 min and the reducing sugar content measured.[29]

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[23] WOO, K. C., ANDERSON, J. M., BOARDMAN, N. K., DOWNTON, W. J. S., OSMOND, C. B. and THORNE, S.W. (1970) *Proc. Nat. Acad. Sci. US* **67**, 18.
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[27] DOWNTON, W. J. S. (1970) *Can. J. Botany* **48**, 1795.
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