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Enzyme Sets of Glycolysis, Gluconeogenesis, and Oxidative Pentose Phosphate Pathway Are Not Complete in Nongreen Highly Purified Amyloplasts of Sycamore (*Acer pseudoplatanus* L.) Cell Suspension Cultures[1]

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ABSTRACT

Differential centrifugation and Percoll-gradient centrifugation of protoplast lysates of suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.) yielded pure amyloplasts. Contamination of the final amyloplast preparation by foreign compartments was assessed by measuring marker enzyme activities. The activity of alkaline pyrophosphatase was taken as a 100% plastid marker; relative to this marker, mitochondria (cytochrome c oxidase) averaged 0.34%, microbodies (catalase) 0.61%, and cytosol (alcohol dehydrogenase) 0.09%. Enzymatic activities of the glycolytic, gluconeogenic, pentose phosphate and the starch degradation pathways were found to be present in these amyloplast extracts in appreciable amounts. But the pyrophosphate-dependent phosphofructokinase and phosphoglyceromutase were judged to be essentially absent from amyloplasts because the activities of these enzymes were not enriched above the level of contaminating enzymatic activities in the amyloplast fractions. Additionally, the *in vitro* activities of starch phosphorylase, ATP dependent phosphofructokinase, NAD dependent glyceraldehyde-3 phosphate dehydrogenase, and glucose-6 phosphate dehydrogenase did not seem to support carbon fluxes from starch to triose phosphates as calculated from the rate of starch disappearance during carbon starvation of the cells. These results provide additional, indirect evidence for the recently emerged view that, in addition to the well known phosphate-triosephosphate translocator, another hexose phosphate and possibly also an ATP/ADP translocating system play major roles in nongreen plastids.

Function of Nongreen Plastids

Nongreen plastids, the counterparts of chloroplasts, obviously lack the main photosynthetic reactions which yield metabolic energy in the form of phosphorylation and reduction equivalents. Such nongreen plastids are therefore unable to drive the Calvin-Benson cycle. Nevertheless, these organelles are metabolically active. Tasks such as starch metabolism and the reactions involved in the biosynthesis of amino acids (22), nucleotides (5), lipids (25), and nitrate assimilation (4 and references therein) were found to be plastid bound. To achieve these and probably additional plastidic functions, metabolic energy is required in this compartment. Energy in such organelles is believed to be generated in the form of ATP and NAD(P)H equivalents by glycolysis and the oxidative pentose-P pathway (4). Although for such nongreen organelles no direct and detailed data are available about the permeability of the membranes with respect to small molecules, it is often assumed that roughly the same molecular species are transported across the membranes of nongreen plastids as across those of chloroplasts (12). To verify the former and obtain indicative data about the latter, we tried to examine the relation of starch metabolism, glycolysis, and the pentose-P pathway in plastids and in the cytosol of a white cell line of suspension-cultured sycamore cells. Extracts of pure amyloplasts were qualitatively and quantitatively compared with protoplast extracts with regard to the enzymatic activities of glycolysis, pentose-P pathway, and related enzymes. Analysis of the resulting absolute activities showed likely restrictions of carbon flux in the plastidic glycolysis, pentose-P pathway, and the phosphorolytic starch degradation. Incomplete or restricted sets of glycolytic activities in plastids of nongreen tissue have been reported (or can be estimated from data given in the reference after correcting for contaminating activities) in cauliflower buds (15), soybean suspension cultures (20), germinating castor bean endosperm (24), and developing endosperm (28) as well. Convincingly complete sets are reported for wheat endosperm amyloplasts (7). In this paper it is therefore argued that, in contrast to chloroplast membranes, hexose-P and adenylates are able to cross the amyloplast membranes, depending on tissue and/or plant species, at significant rates. This would explain the mechanism of starch degradation and carbon export from the amyloplast into the cytosol in the case of starving sycamore suspension-cultured cells (26).

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MATERIAL AND METHODS

Plant Material

The suspension culture of sycamore (*Acer pseudoplatanus* L.) used was the same as in Lescure (18) and a similar culturing procedure was employed. Suspension cultures were maintained weekly by aseptic transfer of 80 mL of suspension culture into 200 mL of new sterile culture medium [ingredients per liter of solution: Suc 20 g, $Ca(NO_3)_2 \cdot 4\ H_2O$ 290 mg, KCl 65 mg, KNO_3 1960 mg, $MgSO_4 \cdot 7\ H_2O$ 360 mg, KH_2PO_4 500 mg, Na_2HPO_4 88 mg, H_3BO_3 1.5 mg, KI 0.75 mg, $MnSO_4 \cdot H_2O$ 3.4 mg, $ZnSO_4 \cdot 2H_2O$ 1.5 mg, MoO_3 0.015 mg, $CrK(SO_4)_2\ 12H_2O_8$ 0.048 mg, $NiSO_4\ 6H_2O$ 0.05 mg, $Na_2WO_4\ 2H_2O$ 0.018 mg, $CoCl_2\ 6H_2O$ 0.059 mg, $CuSO_4\ 5H_2O$ 0.1 mg, $FeSO_4\ 7H_2O$ 27.9 mg, Na_2EDTA 3.785 mg, thiamine (HCl) 1 mg, 2,4-D 1.1 mg, pH about 5.8 before autoclaving 20 min at 121°C] in a 500 mL flask.

Chemicals

The coenzymes NAD(P)(H), ADP, and ATP used in the enzyme assays were from Oriental Yeast Co., Ltd, Japan; other phosphorylated substrates and the lyophylized coupling enzyme G6PDH[3] from *Leuconostoc* (G-5885) were from Sigma Chemical Co.; other coupling enzymes were from Boehringer. Soluble starch was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. The silica sol Percoll and the size exclusion columns (NAP—10 prepacked with Sephadex G-25) used to desalt enzyme solutions were from Pharmacia, Uppsala, Sweden.

Amyloplast Preparation

Amyloplasts were prepared according to Macherel *et al.* (21), and the following are modifications only. Cells were transferred into culture medium without Suc on the third day after subculture and culture was continued for 15 h (C-starvation). The cell-wall digesting medium was applied at a ratio of about 200 mL per 100 g of cells fresh weight, digestion was at 25°C; thereafter, all the handling was done on ice. The final protoplast pellet (5 min at 175 *g*) was resuspended in MEM buffer (21), without additives, at a ratio of 1 to 1.5 mL buffer per g of original fresh weight of the cells. The broken protoplast preparation (21) was centrifuged for 5 min at 1000 *g*, the pellet was gently resuspended using a soft paintbrush and at the same time adding MEM buffer slowly (final ratio about 10 mL/100 g initial fresh weight of the cells). The amyloplast pellet from the Percoll gradient was resuspended in a small volume of MEM buffer without rinsing (about 1 to 2 mL/100 g initial fresh weight of the cells). At all steps starting with the purified protoplasts, the volumes of all the fractions were carefully measured before samples were withheld and frozen at −80°C until assay of enzyme activities. This procedure allowed us to calculate recoveries for the centrifugation steps (recovery was calculated as the sum of the activities of an enzyme in all the fractions of a gradient as a percentage of the originally loaded amount).

[3] Abbreviations: G6PDH, glucose-6-P dehydrogenase; 6PGDH, gluconate-6-P dehydrogenase; ADH, alcohol dehydrogenase; PPase, alkaline pyrophosphatase; FBPase, fructose-1,6-bisP phosphohydrolase; PFK(ATP), fructose-6-P:ATP 1 phosphotransferase; PFK(PPi), fructose-6-P:pyrophosphate 1 phosphotransferase; FBP aldolase, fructose-1,6-bisP aldolase; TPI, triose-P isomerase; G3PDH(NAD), glyceraldehyde 3-P dehydrogenase; 3PG kinase, 3-P-glycerate kinase; PG mutase, P-glyceromutase; PGI, glucose-6-P isomerase; PGM, P-glucomutase; kat, one katal = 1 mol/s (unit of enzymic reaction rate).

Enzyme Assays

Coupling enzymes supplied as ammonium sulfate suspensions were desalted before use by passage through NAP—10 columns; in all the enzyme assays described using the NADP-dependent G6PDH (*e.g.* from yeast), the *Leuconostoc* NAD(P)-dependent G6PDH was used and NADP substituted for NAD. This eliminated overestimations due to the presence of plant 6GPDH in some of the assayed samples. Measured rates of enzyme activities were corrected for the rates measured before adding substrates; assays were checked for linear response versus amount of extract added. We did not attempt to saturate the measured enzyme activities in the assays. Methods used to assay for enzyme activities and other analysis are given as follows: Name of the enzyme assayed, EC number, any modifications, and the corresponding reference are separated by semicolons from the next assay method. Cyt *c* NADPH reductase, EC 1.6.2.4 (13); Cyt *c* oxidase, EC 1.9.3.1 (13); catalase, EC 1.11.1.6, UV method in 1 mL of total volume (1); ADH, EC 1.1.1.1 (32); PPase, EC 3.6.1.1, up to 10 μL extract per 200 μL enzyme assay, the phosphate assay was reduced to a 1 mL final volume (8); starch, to 50 μL of extract 1 mL 80% ethanol was added, boiled for 10 min, and the procedure continued as for maize—autoclaving was in 250 μL water per starch sample, Glc was estimated using a commercial Glc oxidase based test kit (11); G6PDH, EC 1.1.1.49 (28); 6PGDH, EC 1.1.1.44 (28); transketolase, EC 2.2.1.1, xylulose 5-P and Rib-5-P were used at final concentrations of 1 mM each in method B and the assay volume was adjusted to 1 mL (17); transaldolase, EC 2.2.1.2 (30); FBPase, EC 3.1.3.11, cytosolic and plastidic assays exactly as described and Fru-1,6-bisP was pretreated as suggested to remove any traces of Fru-2,6-bisP (7); hexokinase as glucokinase, EC 2.7.1.2 (28); PFK(ATP), EC 2.7.1.11 (28); PFK(PPi), EC 2.7.1.90 (2); FBP aldolase, EC 4.1.2.13, activity calculated as the rate of triose-P formation (20); TPI, EC 5.3.1.1. (20); G3PDH(NAD), EC 1.2.1.13 (33); 3PG kinase, EC 2.7.2.3 (33); PG mutase, EC 2.7.5.3 (3); enolase, EC 4.2.1.11 (3); pyruvate kinase, EC 2.7.1.40 (28); PGI, EC 5.3.1.9 (28); PGM, EC 2.7.5.1 (28); phosphorylase, EC 2.4.1.1, phosphorolytic assay at pH 6.8 with 5 mg/mL of soluble starch in a final volume of 1 mL (27); protein (19). The unit katal (1 kat = 1 mol/s) is used to express enzymatic reaction rates.

RESULTS

Quality of Amyloplasts

To illustrate that our amyloplast preparations were consistently similar, we include the minimum and maximum values for the sediments and recoveries of the differential centrifugation and the Percoll gradient steps calculated from the

Table I. *Purity, Yield, and Content of Some Enzyme Activities of Amyloplasts of Sycamore Suspension Cells*

Protoplast lysate was subjected to differential centrifugation. The resulting pellet was separated on a Percoll density gradient. Activity for the protoplast lysate is expressed as the total activity of the lysate fraction divided by the fresh weight of the original cells without correction for protoplast yield. Data of up to three independent experiments were combined. If three values were available, the middle value of the three is given instead of their average. Ranges spanning from the smallest to the largest values determined are given to indicate the experimental variation of the centrifugation steps.

Enzyme	Protoplast Lysate Activity per Gram Fresh wt Equivalent	Differential Centrifugation			Stepped Percoll Density Gradient		
		Sediment as % of lysate (range)		Recovery range	Sediment as % lysate [amyloplast] (range)		Recovery range
	pkat	%			%		
Contamination markers							
Cyt *c* NADPH reductase	64	49	(30–67)	167[a]	1.7	(1.0–2.5)	88–173
Cyt *c* oxidase	817	21	(19–40)	80–111	0.04	(0.003–0.2)	68–165
Catalase	762	29	(23–44)	89–92	0.07	(nd[b,c]–0.07)	76–268
ADH	9990	14	(11–16)	76–92	0.01	(0.01–0.07)	64–85
Amyloplast markers							
PPase	2220	43	(36–64)	86–89	11.5	(8–11.5)	32–72
Starch (μmol)	1.0	58	(49–68)	100–107	25	(19–30)	97–99
Other							
PGI	3220	35	(33–46)	85–105	5.5	(4–7)	60–113
PGM	6070	11	(9–17)	74–95	0.8	(0.5–1.4)	47–110
Phosphorylase	75	41	(26–55)	76–94	2.7	(1–5)	NC[d]
Protein[e] (mg)	0.45	36		112	0.7		201

[a] Recovery of differential centrifugation from one experiment only. [b] Not detectable. [c] In one experiment the catalase activity was below the detection limit of about 100 pkat/mL sample of this extract corresponding to a relative value of about 0.08%. [d] Not calculable, as the activity in this supernatant fraction was below the detection limit of this assay. [e] Determined in only one experiment.

analysis in up to three independent experiments (Tables I to III). The crude pellet of the differential sedimentation of the lysed protoplasts contains appreciable amounts of all of the determined activities (Tables I to III). This pellet could not be classified as enriched in amyloplasts because of its pattern of markers for the different subcellular compartments (Table I).

Judging by the plastid marker PPase (8, 9) about 12% of the amyloplasts originally present in the sycamore protoplast preparations were obtained as the sediment of the Percoll gradient (hereafter referred to as the amyloplast fraction) (Table I). Similar yields of amyloplasts using similar methods were reported for *Triticum* endosperm (20%; 8), soybean cell suspension cultures (20%, 20) and maize kernels (about 15%, 6). the starch yield of the sycamore amyloplast preparation was higher than for the PPase (25% for starch, 11.5% for PPase: Table I). This difference can be explained as follows. The amyloplast fraction consists of a pellet containing intact amyloplasts, including their respective enzymes, and starch granules derived from broken protoplasts. Both (large) starch granules and intact amyloplasts are heavy structures which sediment similarly through a density gradient. Therefore they

Table II. *Enzyme Activities of Glycolysis in Amyloplasts of Sycamore Suspension Cells*

Fractionation and calculation of data are the same as for Table I.

Enzyme	Protoplast Lysate Activity per Gram Fresh wt Equivalent	Differential Centrifugation			Stepped Percoll Density Gradient		
		Sediment as % of lysate (range)		Recovery range	Sediment as % lysate [amyloplast] (range)		Recovery range
	pkat	%			%		
Hexokinase	789	21	(21–35)	65–76	1.6	(1.6–3.7)	79–114
PFK (ATP)	668	12	(8–15)	77–97	1.0	(0.7–1.2)	79–124
PFK (PPi)	3010	19	(6–19)	86–133	0.01	(0.01–0.03)	83–103
FBP aldolase	6360	17	(9–25)	75[a]	2.5	(1–4)	109–169
TPI	15700	25	(22–29)	91–116	2.6	(2.3–8)	90–132
G3PDH (NAD)	3460	12	(8–19)	88–98	0.3	(0.1–0.7)	33–207
3PG kinase	2810	18	(18–43)	99–138	0.9	(0.8–1.4)	173–434
PG mutase	4570	11	(9–12)	86–100	0.01	(0.01–0.13)	93–145
Enolase	4750	18	(17–19)	88–92	1.2	(1.0–2.0)	53–84
Pyruvate kinase	1500	16	(8–22)	74–116	0.7	(0.4–0.7)	101–140

[a] Recovery of the differential centrifugation available for one experiment only.

Table III. *Enzyme Activities of the Pentose-P Pathway in Amyloplasts of Sycamore Suspension Cells*
Fractionation and calculation of data are the same as for Table I.

Enzyme	Protoplast Lysate Activity per Gram Fresh wt Equivalent	Differential Centrifugation			Stepped Percoll Density Gradient		
		Sediment as % of lysate (range)		Recovery range	Sediment as % lysate [amyloplast] (range)		Recovery range
	pkat	%			%		
G6PDH	690	10	(6–12)	80–92	0.35	(0.31–0.55)	80–93
6PGDH	1920	26	(24–45)	84–106	3.9	(3–7)	68–109
Transketolase	1360	45	(43–46)	83–85	8.8	(8–10)	62–133
Transaldolase	1000	20	(20–20)	61–61	10.7	(11–11)	145–169

will occur in the same pellet. As a result, the yield of complete amyloplasts is overestimated by the starch yield; values derived from other plastid components not closely associated with starch granules (*e.g.* PPase) will give better estimates of the complete plastid yield.

Phosphorylase activity cannot be taken as an amyloplast marker as it reflects the sum of different isozymes located in different compartments of the cell (27). In our case about 2.7% of this activity from the protoplast fraction was recovered in the amyloplast fraction (Table I). Only about 0.7% of the protoplast protein was recovered with the amyloplasts (Table I). Contaminating subcellular compartments in the amyloplast preparation were monitored by their respective marker enzymes and were found to be generally low. This was certainly true for mitochondria (0.04% of total Cyt *c* oxidase, Table I), microbodies (0.07% of total catalase, Table I) and cytosol (0.01% of total ADH, Table I), except for endomembranes and nuclei, which altogether added up to 1.7% of the content of the protoplast lysate in the amyloplast fraction as estimated by the marker Cyt *c* NADP reductase (Table I). This could be due to a close morphological association of endomembranes and amyloplasts or to nuclei which could well copurify with the amyloplasts owing to their huge size and high density. But these possibilities were not further investigated because a low contamination of such endomembranes was considered not to contribute significantly to the enzyme activities used in this study. Similar amyloplast preparations in our laboratory regularly proved to be more than 70% intact as estimated by latency tests. Latency was tested by comparing the 6PGDH activity measured in fresh amyloplast preparations (in osmotically stabilized assays—value for the activity not enclosed in intact membranes) with a parallel assay including a detergent to disrupt membranes (value for the total activity and results not shown; procedure essentially as in Entwistle and apRees [7]).

ENZYMATIC EQUIPMENT

Glycolysis and Gluconeogenesis

The set of enzymatic activities involved in glycolysis was not found to be complete (Table II). Our amyloplast preparation contained appreciable amounts of hexokinase (1.6% of total, Table II). In maize kernel amyloplasts hexokinase was not detected at all (6). The ATP and the PPi-dependent P-fructokinases were differentially localized in sycamore protoplasts. PFK(ATP) (1% of total, Table II) was partially associated with the amyloplast preparation and, in contrast, PFK(PPi) was detectable only as a contamination of the amyloplast fraction (0.01%, Table II; *cf.* to ADH, 0.01%, Table I).

A similar distribution of these two P-fructokinases was found in *Triticum* endosperm amyloplasts (7). Sycamore amyloplast preparations contained most of the glycolytic activities from FBP aldolase through pyruvate kinase (Table II). Exceptions to this distribution were G3PDH(NAD) activity (with a marginal abundance in the amyloplast preparations—0.3% of total, Table II) and PG mutase activity, which was not recovered in excess of cytosolic markers (ADH, 0.01% in amyloplast fractions, Table I). Similarly, low relative activities of PG mutase and G3PDH(NAD) were found in amyloplast preparations of *Brassica* flower buds (15) and of developing *Ricinus* endosperm (28). Also in *Cucurbita* amyloplast preparations, the relative abundance of PG mutase and enolase were reported to be negligible (3). In contrast, *Triticum* endosperm amyloplast preparations were fully competent for the complete set of glycolytic activities (7).

Pentose-P Pathway

Some of the enzyme activities peculiar to the oxidative pentose-P pathway have been determined in the fractions derived from sycamore protoplasts (Table III). The activities of 6GPDH, transketolase, transaldolase, and PGI were present in the amyloplast fractions in substantially higher proportions than contaminations (3.9%, 8.8%, 10.7% [Table III], 5.5% [Table I], respectively). In contrast, the activity of G6PDH, the entry point of the pentose-P pathway, was almost absent from the amyloplast fractions (0.35%, Table III). Such results have been obtained earlier with different nongreen plastid preparations (6, 24, 28). This kind of uneven distribution of some activities of the pentose-P pathway into two separate compartments, namely, plastid and cytosol, is at first surprising. If this pathway is to function *in vivo*, then a well developed shuttle for transporting substrate across the separating membrane systems has to operate. This is especially true at high fluxes or intensive cycling, when a small proportion of an enzyme activity in a given compartment cannot fully account for the activity needed therein (*e.g.* G6PDH in the amyloplast fractions, Table III and discussion below).

Table IV. *Comparison of Enzymatic Capacities of Sycamore Amyloplasts with the Starch Degradation Rate during Carbon Source Deprivation*

Name of Activity	Activity in the Amyloplast Preparation	
	As hexose equivalents[a]	Calculated rate of starch degradation
	pkat/g fresh wt equivalent	%
Calculated rate of starch degradation	10[b]	100
Starch degradation (starch → Glc-6-P)		
Phosphorylase	2	20
PGM	49	490
Hexokinase	13	130
Glycolysis (Glc-6-P → triose-P)		
PGI	180	1800
PFK (ATP)	6.7	67
FBP aldolase	80	800
TPI	410	4100
G3PDH (NAD)	5	50
3PG kinase	13	130
Pentose-P pathway (Glc-6-P → triose-P)		
G6PDH	0.8	8
6PGDH	25	250
Transketolase	60	600
Transaldolase	110	1100
PGI	90	900

[a] Absolute enzymatic activities for the amyloplast fractions were calculated using data from Tables I through III. These activities are converted to reflect the activity of conversion of hexoses toward triose-P in mol-hexose-equivalents, *e.g.* while 1 mol of Glc-6-P is converted to triose-P by the pentose-P pathway, the G6PDH has to process a total of 3 mol of Glc-6-P. Therefore, the *in vitro* G6PDH activity is reduced to one-third. [b] The starch degradation rate at 15 h of carbon starvation was estimated from data of Rébeillé *et al.* (26) and reduced to the overall yield of amyloplasts from these cells.

DISCUSSION

The method presently employed yielded approximately 11% of the amyloplasts from the starting sycamore protoplasts (PPase, Table I), this value being in the same range of those obtained from other species (6, 7, 20). The yield of protoplasts from the suspension cells was about 13%, as judged from the yield of 6PGDH activity (results not shown). Therefore, the combined overall yield of amyloplasts from the starting cell material was calculated to be about 1.4%. This value is higher than yields of nongreen plastids obtained directly from tissue by mechanical means (*e.g.* about 0.4% in *Brassica*, calculated from Table I in ref. 15). The purity of the sycamore amyloplast preparation compares favorably with mechanically obtained plastids. For example, the ratio of mitochondria to amyloplasts in the present preparations was calculated to be about 0.3% (0.04% Cyt *c* oxidase divided by 11.5% PPase, Table I), whereas for *Brassica* this ratio was calculated to be about 5% (using the marker enzymes in Table I in ref. 15). The yield of mechanically obtained plastids in the case of pea is reported to be 25% from the starting root tissue, but the ratio of contamination by mitochondria was calculated to be as high as 5% (from Table I in ref. 4). Other authors mentioned in the present paper did not report overall yields for their amyloplast preparations from the starting tissue or any suitable values from which to calculate overall yield.

We assume for our preparations that (a) the relatively long overall preparation time of about 5 h (from the collection of the cells to the final amyloplast suspension) barely modified the bulk activities of the enzymes in question; we particularly assume for the discussion that (b) the preparation procedure did not distort their distribution pattern among the organelles. These assumptions are supported by the fact that the same cells lost only about 50% of their original glycolytic activities over a 50-h period of culture without Suc (14). In our case, the 15 h starvation period of the cells prior to digestion of the cell walls changed their metabolic status to mobilizing reserves (14), and we cannot exclude slight changes of some enzyme activities.

When cells are growing heterotrophically in the absence of an external carbon source, it is assumed that carbohydrates are mainly respired for cell life maintenance. In such a case, the enzymes involved in starch degradation and at least in the first part of glycolysis would be expected to sustain the experimentally observed carbohydrate fluxes. In a simple approach, we assume for our experimental system that the glycolytic activities determined *in vitro* can be taken as approximations for catalytic activities *in vivo*. We are aware that the enzymatic activities determined *in vitro* are not necessarily maximum catalytic activities; and even if they were, they may not reflect the maximal possible *in vivo* rates of substrate conversion. Keeping this in mind, and for the sake of comparison, we used the measured activities as reasonable approximations for *in vivo* enzymatic capacities. We compared the glycolytic rates

calculated from starch degradation with the measured enzyme activities in amyloplasts. A starch degradation rate of approximately 0.72 nmol Glc equivalents per s per g fresh weight of sycamore cells between 10 and 16 h of starvation was calculated from Rébeillé *et al.* (26). Applying the present overall yield factor for amyloplasts obtained from cells (about 1.4%, see above) to the observed starch degradation rate resulted in a rate of 10 pmol Glc equivalents per s per amyloplast fraction from 1 g fresh weight of cells (Table IV). This value was used directly for comparison with the absolute enzymatic activities of isolated amyloplast fractions.

In Table IV the starch degrading, glycolytic, and the pentose-P cycle activities are tabulated as determined and converted to reflect the capacity of conversion of hexose units. This now allows a direct comparison of the activities confined to amyloplasts with the starch degradation rate during starvation (26). Major restrictions are obvious for the carbon flux from starch to triose-P in the starch degrading phosphorylase (20% of the starch degradation rate, Table IV), PFK(ATP) (67% of the starch degradation rate, Table IV), G3PDH(NAD) (50% of the starch degradation rate, Table IV) and a total block at PG mutase (Table II). Based on these observations, starch degradation seems to proceed mainly by a hydrolytic pathway, which includes activation of the resulting Glc by hexokinase. The latter activity appears to be present in adequate amounts in amyloplasts (30%, Table IV).

Glycolysis as a whole or at least as far as formation of the commonly exportable triose-P cannot function in the amyloplasts for the following reasons: (a) There does not appear to be a way around the restriction at PFK(ATP), the entry point of glycolysis. PFK(PPi) is totally absent from the amyloplast compartment (Table II); (b) at PG mutase the glycolysis is largely or even completely blocked and G3PDH(NAD) is severely reduced (Table IV). Alternatively, one could think of the pentose-P cycle as yielding triose-P from Glc-P inside the amyloplast. However, the pentose-P cycle's entry point, G6PDH, is also severely reduced in amyloplasts (Table IV). Its activity toward triose-P formation is reduced because it has to cycle three hexose units to effectively absorb one hexose unit and thereby yield only one exportable triose-P unit. These considerations support the view that in some cases of nongreen plastids, carbon is mainly transported across the amyloplast membranes as hexose units, most likely as hexose-P (4, 7, 16, 20, 28, 31). Therefore, either a hexose-P translocator or a triose-P translocator with a substrate range including at least one species of the hexose-P family must allow for carbon exchange with the cytosol. During normal growth the sycamore cells contain starch and consequently will synthesize it in their amyloplasts. If carbohydrate used for starch synthesis had to cross the inner plastid membrane as triose-P, then, in order for the reversal of glycolysis to work, appreciable activities of FBPase should be detectable in such amyloplasts. We attempted to obtain data concerning the FBPase activity in plastids and its distribution within the cells. The photometric assays are well established and are designed to distinguish the plastidic from the cytosolic FBPase (29). But due to the very low FBPase activity in the sycamore extracts tested (our unpublished data) and the presence of PFK(PPi) activity in all but the amyloplast fractions (Table II), it was not possible to unequivocally assign the observed increases of absorbance to distinct activities (see also 7). No FBPase-like activity could be demonstrated in amyloplast fractions, using either the assay for the plastidic or that for the cytosolic FBPase (Entwistle and apRees [7] modified from Stitt *et al.* [29]). This negative result implies that gluconeogenesis in our plastids from triose-P will not occur or will occur only at insignificant rates. This absence of FBPase in plastids is additional indirect evidence for a transport mechanism capable of importing and exporting one or several kinds of hexose-P into and out of the plastid (10).

Incomplete glycolysis in the amyloplast not only renders the interconversion of carbon skeletons difficult but also impairs the generation of ATP in the compartment itself. Based on the above results we therefore propose that an ATP/ADP translocating system connects both the plastidic and the cytosolic adenylate pools. Recently, immunochemical evidence for the presence of an ATP/ADP translocator-like polypeptide residing in the inner membrane fraction of sycamore amyloplasts has been provided (23). In conclusion, our study of enzymatic activities shows an incomplete glycolytic pathway in amyloplasts. This, in turn, suggests a hexose-P and an ATP/ADP translocator which play major roles in connecting the plastidic metabolism to the cytosolic one. Further investigations are being undertaken to present more direct functional and molecular evidence of such translocators.

Note Added in Proof

The presence of ATP/ADP translocator in the envelope membranes of amyloplasts has been demonstrated by employing the "double" silicone oil layer filtering centrifugation technique (Presented at XV The International Carbohydrate Symposium, Yokohama. August 12–17, 1990).

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