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Carbon Metabolism in Spinach Leaves as Affected by Leaf Age and Phosphorus and Sulfur Nutrition[1]

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ABSTRACT

Spinach (*Spinacea oleracea*) plants were grown either continuously on complete nutrient solutions or for 2 weeks on media deficient in phosphate or sulfate. To characterize leaf carbohydrate metabolism, levels of phosphorylated intermediates, activities of enzymes involved in photosynthetic carbon metabolism, contents of soluble and acid hydrolyzable sugars were measured in leaves differing in age and mineral status and related to leaf rates of photosynthesis and assimilate partitioning. Concentrations of metabolites—particularly those which are preferentially compartmented in the cytosol—decreased from young to old leaves and were lowest in old phosphate starved leaves. Nutrient deficiency showed comparable effects on stromal and cytosolic intermediates. Whole leaf ATP to ADP ratios were dependent on the growth regime, but did not much change with leaf age. The assimilatory force increased in all leaves suffering from mineral deficiency; the assimilatory force was low when photosynthesis was high and vice versa. Sugars accumulated although enzyme activities were decreased under deficiency. The results show that growth of P- and S-starved plants is not limited by photosynthetic reactions.

During ontogenesis, leaves are first sinks for assimilates and other nutrients. Later, they assume a source function exporting assimilates to growing parts of the plants (11). Although the biochemistry and regulation of carbohydrate metabolism has been studied extensively in various herbaceous plants (*cf.* 10, 24), little is known about the effect of leaf age on carbohydrate metabolism. As will be shown in this communication, nutrient deprivation during growth also influences leaf development, source-sink relationships and carbohydrate metabolism.

Suboptimal phosphate availability during plant growth results in a depression of photosynthesis, changes in metabolite concentrations and carbon partitioning, and in an accumulation of starch (6, 17, 18, 25). However, little attention has been paid to the relationship between leaf age and plant nutrient status in terms of carbohydrate metabolism. Phosphorus starvation results in the mobilisation of P from old leaves which then suffer early senescence. As a consequence of the redistribution of P, young leaves do not show symptoms of P_i deficiency. This is in contrast to sulfur deficiency which retards the development of young leaves (4). It is evident from such observations that an analysis of all leaves is required for insight into the metabolic changes brought about by mineral deficiency. In this communication, such an analysis is presented for spinach plants whose phosphate or sulfate supply had been discontinued.

MATERIALS AND METHODS

Plant Growth and Metabolite Determinations

Spinach (*Spinacea oleracea*) was hydroponically grown in a greenhouse with supplementary light (250 μmol quanta m^{-2} s^{-1}). Four week old plants were transferred to phosphate- or sulfate-deficient medium or maintained on complete medium (for composition of the media see [4]). Two weeks after the transfer, the oldest primary (1 + 2 leaf), secondary (3 + 4 leaf), tertiary (5 + 6 leaf), quaternary leaf pair (7 + 8 leaf), and the remaining (9 + 10 + younger) leaves were harvested and analyzed separately. Six leaf discs of each sample were placed upside down into Petri dishes filled with water to guarantee efficient water supply. The discs were illuminated for 1 h at 250 μmol quanta m^{-2} s^{-1} at ambient CO_2 concentration (350 μL L^{-1}). They were rapidly frozen under continuous illumination. Metabolites were extracted and determined as described previously (2, 5). Chl and protein measurements were as described before (4).

Enzyme Activities

Leaf samples of 200 mg fresh weight were frozen in liquid N_2 and stored at −70 °C. The activities of GAPDH[2] (*cf.* 1), PGI (1), UDP glucose pyrophosphorylase (20) were measured spectrophotometrically. The samples were homogenized into the assay buffers (marked with * in the text) and the supernatant obtained by centrifugation was used for the determinations. The assays contained for PGI: 100 mmol L^{-1} tris-(hydroxymethyl)-aminomethane*-Cl (pH 8.0), 2 mmol L^{-1} EDTA*, 15 mmol L^{-1} $MgCl_2$*, 0.2 mmol L^{-1} $NADP^+$, 1.1 mmol L^{-1} Fru-6-P, 1.3 units mL^{-1} Glc-6-P dehydrogenase; for GAPDH: 100 mmol L^{-1} tris-(hydroxymethyl)-amino-

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[2] Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DHAP, dihydroxyacetone phosphate; Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-6-P, fructose-6-phosphate; Glc-6-P, glucose-6-phosphate; 3-PGA, 3-phosphoglyceric acid; P_0, organic phosphate; PGI, phosphoglucose isomerase; Ru-1,5-P_2, ribulose-1,5-bisphosphate; FA, assimilatory force.

methane*-Cl (pH 7.6*), 1 mmol L^{-1} EDTA*, 1.8 mmol L^{-1} $MgSO_4$*, 5 mmol L^{-1} dithiothreitol, 5.2 mmol L^{-1} 3-PGA, 1.4 mmol L^{-1} ATP, 0.4 mmol L^{-1} NADPH, 19 units mL^{-1} phosphoglycerate kinase; for UDPglucose pyrophosphorylase: tissue was homogenized in 50 mmol L^{-1} tris-(hydroxymethyl)-aminomethane*-Cl (pH 7.5), 10 mmol L^{-1} GSH*, 1 mmol L^{-1} EDTA*, and 5.3 mmol L^{-1} $Na_2S_2O_5$*. After centrifugation (5 min, 10,000*g*), 475 mg $(NH_4)_2SO_4$ per mL were added to the supernatant (pH 7.5). The pellet after centrifugation was dissolved and applied to a Sephadex HD 10 column. The high mol wt fractions were pooled and used for the determination. The spectrophotometric assay contained 80 mmol L^{-1} glycylglycine (pH 8), 5 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} NaF, 20 mmol L^{-1} cysteine, 1 mmol L^{-1} UDPglucose, 20 μmol L^{-1} glucose-1,6-bisphosphate, 2 mmol L^{-1} 3-PGA, 2.5 mmol L^{-1} pyrophosphate, 0.6 mmol L^{-1} $NADP^+$, 1 unit mL^{-1} phosphoglucomutase and 0.75 units mL^{-1} Glc-6-P dehydrogenase.

Incorporation of ^{14}C-CO_2 into Starch and Soluble Sugars

Leaves were illuminated with 250 μmol quanta m^{-2} s^{-1} in air (350 μL L^{-1} CO_2) for at least 30 min. Water was supplied to the leaves via the petioles. The cuvette (6 L) was then closed and ^{14}C-CO_2 (0.5 μCi g^{-1} fresh weight; specific activity: 5 μCi $mmol^{-1}$ $KHCO_3$) was liberated by acidifying the carbonate solution. After 10 min, the leaves were illuminated for further 10 min with opened cuvette. They were then frozen in liquid N_2 and ground to a fine powder which was suspended in H_2O (10 mL g^{-1} fresh weight). Trichloroacetic acid (final concentration 0.5% w/v) was added to an aliquot ('soluble sugars'). After centrifugation (10 min, 5000*g*), the supernatant was counted in a scintillation counter. Another aliqout ('acid-hydrolyzable sugars') was acidified with HCl, sugars were hydrolyzed at 100 °C for 1 h and determined as for 'soluble sugars.' A control experiment where starch was hydrolyzed enzymatically with amyloglucosidase gave comparable results.

RESULTS

General Characterization of the Leaves Differing in Age and Nutrient Availability

At harvest time, fertilized 6 week old spinach had 10 to 12 leaves with an area larger than 1 cm^2. The number of leaves of this size decreased to 5 to 10 when the supply with P or S was discontinued. The leaves were characterized in terms of measurements of leaf area, protein and Chl contents, F_A, and rates of photosynthesis (Table I). Reduced areas particularly of young leaves indicate retardation of growth under phosphate and sulfate deficiency. Protein and Chl contents and rates of photosynthesis were high in control leaves irrespective of leaf age. Photosynthesis was maximal in leaves 5 and 6. In old leaves of phosphate starved plants, reduced protein- and Chl-contents and low rates of photosynthesis indicated deficiency-induced senescence, whereas young phosphate starved leaves were comparable to the controls. As expected, sulfate deficiency had the largest effect on young leaves. The data show that the three treatments cause specific changes in plant development.

Leaf Concentrations of Phosphorylated Metabolites

Figure 1 shows concentrations of phosphorylated metabolites which are involved in photosynthesis, carbohydrate, or glycolytic metabolism. Prior to the metabolite determinations, the leaves were photosynthesizing under conditions similar to the growth conditions.

The highest metabolite concentrations were observed in young control leaves for Glc-6-P. Its concentration exceeded 500 μmol L^{-1} and decreased with leaf age. 3-PGA (380 μmol L^{-1}), Fru-6-P (190 μmol L^{-1}), and Ru-1,5-P_2 (>150 μmol L^{-1}) were also present in high concentrations. Levels of Fru-1,6-P_2 and DHAP were lower (<100 μmol L^{-1}). Metabolite concentrations changed with leaf age (see "*Discussion*").

Under phosphate deficiency and particularly in old leaves, concentrations of all phosphorylated metabolites were reduced. This coincides with low levels of extractable P_i (4) and protein and with low rates of photosynthesis (Table I). The metabolite status of young phosphate-deficient leaves (7 + 8) was similar to that of old control leaves (3 + 4). Table II shows ratios of metabolite concentrations of young to old leaves.

The metabolite pattern was different under conditions of sulfate starvation. With the exception of 3-PGA and Ru-1,5-P_2 which showed reduced concentrations, metabolite concentrations were similar in old leaves of sulfate starved and control plants. In young leaves of sulfur-starved plants, levels of most metabolites were low.

Status of the Adenylate System and Assimilatory Force

The energy status of the leaves was characterized by measuring ADP and ATP levels (Fig. 2). ATP and ADP concentrations decreased by a factor of two from young to old leaves under all growth regimes. The ATP to ADP ratio was comparable in control and sulfate-starved leaves and somewhat decreased under phosphate starvation, but not much affected by leaf age. A decreased ATP/ADP ratio does not necessarily indicate a decreased phosphorylation potential [ATP]/([ADP][P_i]). It may simply be a consequence of a decreased level of P_i. The ratio of DHAP to PGA can be used to calculate the assimilatory force F_A which drives photosynthesis (14). F_A is the product of phosphorylation potential and redox ratio [NADPH]/[$NADP^+$]. It reflects the balance between provision of ATP and NADPH by the chloroplast thylakoid system and consumption of ATP and NADPH in assimilation. F_A was increased under all deficiency regimes. However, there was an age-dependent gradient: old leaves of phosphate deficient plants and young leaves of sulfate-starved plants revealed the largest values of F_A indicating restricted use of ATP and NADPH by Calvin cycle reactions.

Changes in Organic Phosphate

The analysis of Figures 1 and 2 includes the dominant phosphorylated metabolites of spinach leaves. Summation over the phosphate content of these metabolites allows us to calculate P_O (Fig. 3). P_O was higher in control leaves than in corresponding deficient leaves; it was dramatically reduced in old leaves of phosphate deficient plants. In control plants, P_O increased by a factor of 1.8 from old (1 + 2) to young (7 + 8)

Table I. *Characterization of Spinach Leaves in Dependence of Age and Nutrient Status*

Six-week-old spinach grown on complete nutrient solutions is compared to spinach whose phosphate or sulfate supply in the medium had been discontinued for 2 weeks. The leaves emerging after the cotyledons are designated leaf 1 + 2; they are fully expanded old leaves. Leaves 9 + 10 are expanding young leaves.

	Control Plants			
	1 + 2	5 + 6	9 + 10	
Photosynthesis on a unit area basis	11.0 ± 2.4	15.4 ± 2.3	10.5[a]	μmol CO_2 m^{-2} s^{-1}
Photosynthesis on a unit fresh weight basis	31 ± 7	62 ± 9	54	nmol CO_2 g^{-1} s^{-1}
Chl	0.82 ± 0.05	1.80 ± 0.23	1.63 ± 0.23	mg g^{-1} fresh wt
Protein	14.8 ± 0.9	23.0 ± 1.4	25.0 ± 1.7	mg g^{-1} fresh wt
F_A	152	132	192	L mol^{-1}
Leaf area	12 ± 3	73 ± 16	12 ± 11	cm^2
GAPDH	54 ± 12	80 ± 8	51 ± 13	nkat g^{-1} fresh wt
PGI	17 ± 4	36 ± 9	45 ± 12	nkat g^{-1} fresh wt
UDPglc PP_iase	74 ± 19	224 ± 34	147 ± 26	nkat g^{-1} fresh wt
Soluble sugars	1.6 ± 0.7	3.4 ± 1.6	2.2 ± 0.4	mg g^{-1} fresh wt
Hydrolyzable sugars	1.8 ± 0.6	3.0 ± 0.6	4.0 ± 1.4	mg g^{-1} fresh wt
Sum of sugars	3.4	6.4	6.2	mg g^{-1} fresh wt
% sugars as starch	53	47	65	%
% ^{14}C incorporation into starch	22	11	4	%
	Phosphate-Starved Plants			
	1 + 2	5 + 6	9 + 10	
Photosynthesis on a unit area basis	4.8 ± 2.8	11.0 ± 4.8	10.7 ± 1.3[b]	μmol CO_2 m^{-2} s^{-1}
Photosynthesis on a unit fresh weight basis	15 ± 8	46 ± 20	56 ± 7	nmol CO_2 g^{-1} s^{-1}
Chl	0.61 ± 0.24	1.77 ± 0.42	1.75 ± 0.16	mg g^{-1} fresh wt
Protein	12.3 ± 0.9	18.8 ± 3.1	23.3 ± 0.4	mg g^{-1} fresh wt
F_A	299	242	195[b]	L mol^{-1}
Leaf area	9 ± 1	22 ± 5	0.7 ± 0.2	cm^2
GAPDH	36 ± 15	45 ± 13	70 ± 19[b]	nkat g^{-1} fresh wt
PGI	16 ± 6	29 ± 12	29 ± 12[b]	nkat g^{-1} fresh wt
UDPglc PP_iase	34 ± 6	90 ± 21	74 ± 21[b]	nkat g^{-1} fresh wt
Soluble sugars	3.4 ± 0.5	3.9 ± 0.8	4.8 ± 0.6	mg g^{-1} fresh wt
Hydrolyzable sugars	2.8 ± 0.5	4.8 ± 1.2	6.0 ± 0.5	mg g^{-1} fresh wt
Sum of sugars	6.2	8.7	10.8	mg g^{-1} fresh wt
% sugars as starch	45	55	56	%
% ^{14}C incorporation into starch	32	17	4	%
	Sulfate-Starved Plants			
	1 + 2	5 + 6	9 + 10	
Photosynthesis on a unit area basis	6.1 ± 3.9	4.4 ± 1.2	1.8[a]	μmol CO_2 m^{-2} s^{-1}
Photosynthesis on a unit fresh weight basis	17 ± 11	18 ± 5	7	nmol CO_2 g^{-1} s^{-1}
Chl	0.62 ± 0.18	0.53 ± 0.10	0.38 ± 0.02[b]	mg g^{-1} fresh wt
Protein	11.4 ± 0.9	12.2 ± 1.5	10.8 ± 1.3	mg g^{-1} fresh wt
F_A	311	302	560[b]	L mol^{-1}
Leaf area	12 ± 2	13 ± 6	2 ± 1	cm^2
GAPDH	17 ± 3	19 ± 3	5[a]	nkat g^{-1} fresh wt
PGI	14 ± 4	11 ± 8	15 ± 5	nkat g^{-1} fresh wt
UDPglc PP_iase	36 ± 8	52 ± 11	29 ± 11	nkat g^{-1} fresh wt
Soluble sugars	2.6 ± 0.5	3.8 ± 0.8	5.1 ± 1.1	mg g^{-1} fresh wt
Hydrolyzable sugars	3.3 ± 0.3	4.8 ± 0.8	4.9 ± 1.9	mg g^{-1} fresh wt
Sum of sugars	5.9	8.6	10.0	mg g^{-1} fresh wt
% sugars as starch	56	56	49	%
% ^{14}C incorporation into starch	30	24	21 21	%

[a] Result of one experiment. [b] Result for leaves 7 + 8 instead of leaves 9 + 10.

leaves. Under conditions of phosphate deficiency, P_0 increased from old to young leaves by a factor of 2.7. This compares to an increase by a factor of 1.45 under conditions of sulfate starvation. The ratio of P_0 to inorganic phosphate (4) increased from old to young leaves and was, as expected, largest under conditions of phosphate starvation (data not shown).

Carbohydrate Content and Incorporation of Newly Fixed CO_2 into Starch

Sugar content decreased from more than 6 mg g^{-1} fresh weight in young leaves to 3.4 mg g^{-1} fresh weight in old leaves of control plants (Table I). Both concentrations of soluble and acid hydrolyzable sugars increased under P- or S-deficiency. However, the acid hydrolyzable sugars (*i.e.* starch) as fraction of the total sugar pool changed only within narrow limits. In contrast to increasing levels of carbohydrates from old to young leaves, incorporation of newly fixed $^{14}CO_2$ into starch decreased. However, in all leaves from nutrient-deficient plants, the percentage of newly fixed carbon which is partitioned into starch synthesis was increased as compared with the corresponding controls (*cf.* 25).

Activities of GAPDH, PGI, and UDPglucose Pyrophosphorylase

GAPDH, PGI, and UDPglucose pyrophosphorylase were chosen as examples for the enzymatic characterization of the Calvin cycle, starch, and sucrose synthesis. The activity profile of GAPDH changed in parallel to the photosynthetic activity and was maximal in leaf 5 to 8 of control plants. Highest PGI activities were observed in young control leaves. They reflect the changes in hexosemonophosphate concentrations. UDPglc pyrophosphorylase showed high activities in leaves with high rates of photosynthesis. With the exception of GAPDH in young phosphate-deficient leaves, all enzyme activities were decreased under mineral deficiency.

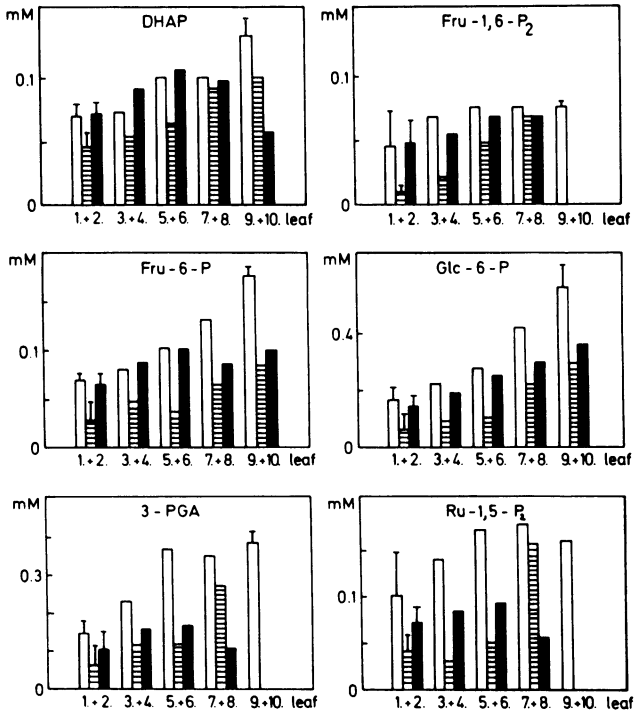

Figure 1. Metabolite concentrations in leaves of different age from control (open columns), phosphate-deficient (hatched columns), or sulfate deficient plants (black columns). Metabolite levels were related to the water content of the leaves. Leaves 1 + 2 correspond to the oldest leaves which emerged first after the cotyledons. The leaves are numbered in consecutive order, *i.e.* leaves 1 + 2 are old and leaves 9 + 10 young expanding leaves (*cf.* Table I).

Table II. *Metabolite Ratios of Young to Old Leaves in Control (leaf 9 + 10 ↔ leaf 1 + 2) and starved plants (7 + 8 ↔ 1 + 2)*

For the sulfur-deprived plants, also the metabolite ratio of leaves 5 + 6 to leaves 1 + 2 is given (in brackets). Data were taken from Figure 1.

	Increase in Metabolite Content from Old to Young Leaves $[metabolite]_{young\ leaves}/[metabolite]_{old\ leaves}$		
	Control plants	Starved plants	
		Phosphate	Sulfate
Fru-1,6-P_2	1.65	7.50	1.43 (1.49)
3-PGA	1.69	3.72	1.06 (1.43)
Ru-1,5-P_2	1.75	3.67	0.76 (1.64)
DHAP	1.89	2.00	1.36 (1.27)
Fru-6-P	2.54	2.66	1.30 (1.73)
Glc-6-P	3.35	3.50	2.05 (1.53)

DISCUSSION

Metabolite Compartmentation in Relation to Leaf Age and Mineral Deficiency

Whole leaf measurements integrate over all compartments of a leaf. Stitt *et al.* (21) and Gerhardt *et al.* (12) performed a detailed analysis on metabolite distribution between cytosol and stroma in protoplasts and whole leaves. Their work demonstrated that, depending on the length of the light period to which the leaves had been exposed prior to harvest, 20 to 40% of the cellular Glc-6-P, 40 to 56% of the Fru-6-P, 47 to 73% of the DHAP, 70 to 80% of the 3-PGA, and 90 to 93% of the Fru-1,6-P_2 are localized in the stroma, the remainder being compartmentalized in the cytosol. Interestingly, the relative decrease in metabolite concentration from young to old leaves (Fig. 1) showed a remarkable correlation to the reported distribution of the metabolites between cytosol and stroma. The decrease from young to old leaves was large for metabolites which are predominantly localized in the cytosol. For example in control leaves, a 3.4-fold decrease of metabolite content was measured for Glc-6-P. The factor was 2.5 in the case of Fru-6-P, 1.9 for DHAP, 1.7 for 3-PGA, and 1.7 for Fru-1,6-P_2 (Table II). Furthermore, levels of predominantly or exclusively stromal metabolites did not change significantly between leaves 5 + 6 and leaves 9 + 10, whereas concentrations of Glc-6-P and Fru-6-P changed by a factor of two.

In contrast to control plants, not only the concentrations of presumably stromal but also of cytosolic metabolites were drastically reduced in old as compared to young leaves of phosphate starved plants. The data show that the leaves reduce Calvin cycle activities maintaining catabolic pathways.

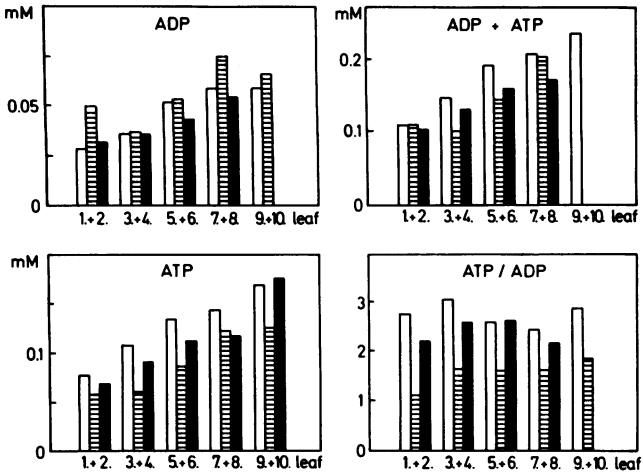

Figure 2. Adenylate concentrations in leaves differing in age and mineral status: control plants, open column; phosphate deficient plants, hatched column; sulfate deficient plants, black column (see also legend to Fig. 1).

Under sulfur starvation, concentrations of the 'stromal metabolites' Fru-1,6-P_2, 3-PGA, and Ru-1,5-P_2 decreased from leaves 5 + 6 to leaves 1 + 2 to an extent comparable to the controls, whereas 'cytosolic' metabolites decreased less.

Relationship between Carbohydrate Metabolism and Volume of the Cytoplasm

The interpretation of leaf metabolite levels could be extended, provided they were related to the volumes of the chloroplastic and cytosolic compartments. However, such data are difficult to obtain *in vivo*. A simplified approach was chosen to estimate the volumes of the compartments from measured protein and Chl contents (Table III). Some assumptions were made: (a) Spinach chloroplasts occupy a volume of 30 μL mg^{-1} Chl. (b) In mesophyll cells, chloroplasts comprise 50% of the cytoplasmic volume ('cytoplasm' includes all plasmatic compartments [19]). Thus, in leaves, where the cytosol to chloroplast volume is increased due to non-green cells, we assumed that the chloroplasts accounts for 40% of the total volume of the cytoplasm. (c) By far the largest portion of the leaf protein is compartmentalized in the cytoplasm (16). The cytoplasmic protein concentration was assumed to be constant. (d) The Chl to chloroplast volume was assumed not to change with leaf age or growth condition. This assumption is critical. It is only valid if the development of the thylakoid system is strictly coordinated with that of the stroma. Some evidence for this assumption may be derived from the changes in activities of stromal enzymes and from the changes in concentrations of Calvin cycle intermediates (Table 1; Fig. 1; [8]) and from the reduced number of chloroplasts under sulfate-deficiency (5). All these parameters indicate a coordinated reduction of chloroplast activities. Table III shows the result of the calculation. The volume of the cytosol decreased from 100 μL g^{-1} fresh weight in young leaves to 60 μL g^{-1} fresh weight in old leaves. Under nutrient deficiency, the volumes of the cytosol was decreased. It increased from old to young phosphate-deficient leaves and was constant in leaves of different age under conditions of sulfur deprivation.

Levels of most metabolites and consequently the size of the P_0 pool decreased from young to old leaves. This is partly explained by a decreased volume of the cytoplasma. The vacuole of young expanding leaves has not yet reached its final size. When P_0 is related to the calculated cytoplasmic volumes (Table III), a rather constant value of about 12 is obtained for all leaves which show efficient photosynthesis. The ratio increased in young sulfur-starved leaves and decreased in old phosphate-starved plants. Under phosphate starvation, concentrations of P_0 of leaves 1 to 6 were about half that of control plants. These data are similar to the results of Rao *et al.* (18) which were obtained for P_i-deficient sugar beet leaves. However, in our case, very young leaves revealed metabolite levels close to control leaves. Metabolite levels were increased under sulfur deficiency when related to cytoplasmic volumes. This is particularly interesting as the activities of all measured enzymes were drastically reduced. In fact, the reduction in GAPDH and PGI activity was correlated to the cytoplasmic volumes. Reduced enzyme activities coincide with high substrate concentrations.

Regulation of Carbohydrate Metabolism

Photosynthesis per unit leaf area is decreased under mineral deficiency. However, on a Chl basis, photosynthesis was comparable irrespective of age and mineral status (4). This observation poses the question whether growth of the deficient plants is limited by photosynthetic reactions. Although the ATP to ADP ratio was lower under phosphate starvation than in the controls, F_A was as high or even higher (leaves 1–6) than in the controls. Efficient photosynthesis may either be driven by a high phosphorylation potential or a high redox ratio of NADPH to $NADP^+$ (13). Thus, the results make it unlikely that plant growth under conditions of P- or S-starvation is limited by the energy status of the leaves.

In all plants, increasing leaf age decreased the concentra-

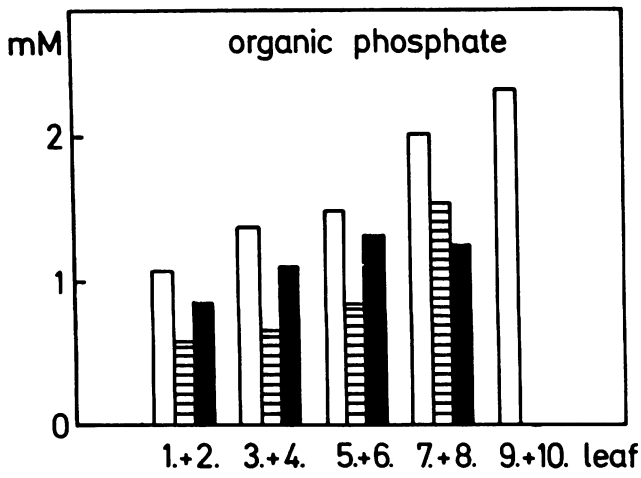

Figure 3. Organic phosphate concentrations in leaves differing in age and mineral status. Open columns show the result of control plants, hatched columns that of phosphate-deprived plants, and black columns that of sulfate-deprived plants (see also legend of Fig. 1).

Table III. *Estimation of Chloroplastic and Cytosolic Volumes in Spinach Leaves*

Chl and protein contents were measured (*cf.* Table I and ref. 4). Volumes were calculated under the following assumptions: in leaves 5 + 6 of control plants, the chloroplast volume accounts for 40% of the total leaf cytoplasm. One mg Chl corresponds to 30 μL chloroplast volume. Chl content C gives an approximation of chloroplast volume, *i.e.* 1.8 mg Chl g^{-1} fresh weight → 54 μL chloroplast g^{-1} fresh weight. Protein content P allows to approximate cytoplasmic volume. It should be mentioned that leaves contain Chl-free cells which naturally contribute to the total volume of the cytosol. Therefore, the volume of the cytosol appears to be large as compared to the volume of the chloroplast. Using these relationships, the cytosolic volume V_{cyt} was calculated:

$$V_{cyt}[\mu L\ g^{-1}\ \text{fresh weight}] = (5.9\ P - 30\ C).$$

Growth Condition	Leaf	Chloroplast Volume	Cytosol Volume	Cytoplasm Volume V_{cpl}	P_o/V_{cpl}
			μL g^{-1} fresh wt		*rel. unit*
Control	1 + 2	25	63	88	12.6
	3 + 4	44	72	116	12.1
	5 + 6	54	81	135	11.2
	7 + 8	55	108	163	12.5
	9 + 10	49	98	147	15.9
Phosphate-starved	1 + 2	18	54	72	8.0
	3 + 4	29	51	80	8.4
	5 + 6	53	56	109	7.8
	7 + 8	56	70	126	12.5
Sulfate-starved	1 + 2	19	49	68	12.9
	3 + 4	18	51	69	16.4
	5 + 6	16	55	71	19.0
	7 + 8	11	51	62	20.5

tions of sugar phosphates which have a central function in leaf metabolism. PGI catalyzes the isomerization of Glc-6-P to Fru-6-P. At thermodynamic equilibrium, the ratio of Glc-6-P to Frc-6-P is between 4 and 3 (depending on temperature). When starch synthesis proceeds at high rates, ratio of Glc-6-P/Fru-6-P decreases in spinach chloroplasts to values as low as 1. This shows that the plastidic isomerase becomes partially rate controlling in starch synthesis (3). The cytosolic reaction is usually close to equilibrium (26). In young leaves of all plants, the ratio of Glc-6-P to Fru-6-P was about 3 and decreased in old leaves. There, the rate of incorporation of newly fixed ^{14}C into starch was increased as compared to young leaves (Table I).

Fructosebisphosphatase catalyzes hydrolysis of Fru-1,6-P_2 to Fru-6-P and P_i. At thermodynamic equilibrium, hydrolysis is essentially complete. However, a rather constant ratio of Fru-1,6-P_2 to Fru-6-P of 0.3 to 0.5 demonstrates careful control (2). It is mediated by a chloroplastic and a cytosolic enzyme. The stromal FBPase is of prime importance in regulating the diversion of carbon between regeneration of Ru-1,5-P_2 and starch synthesis on one side and export of triosephosphates on the other side. The cytosolic FBPase controls photosynthetic sucrose synthesis. It is regulated by Fru-2,6-P_2 (22). Spinach leaves pretreated so as to alter their sucrose content exhibited a correlation between sucrose contents and leaf levels of Fru-2,6-P_2 (23). Increased sucrose levels resulted in increased levels of Fru-2,6-P_2 and phosphorylated metabolites (23). Our results show that this relationship does not hold under all conditions. High levels of phosphorylated intermediates were detected in young control leaves in the absence of high concentrations of soluble sugars. In young phosphate deficient leaves, the sugar phosphates comprised only a small fraction of P_o as compared to the controls although leaf contents of soluble sugars were very high. The results show that the regulatory state of carbohydrate metabolism changes during leaf ontogenesis and that results from leaves of different age and growth condition may not directly be comparable.

Metabolite Levels and Carbohydrate Status of the Leaves

In control plants, high concentrations of soluble sugars were present in leaves with efficient photosynthesis. Sugar concentrations decreased with decreasing rates of photosynthesis. As all leaves were harvested in the middle of the light period, soluble sugars are likely to represent mainly assimilates stored in the vacuole. They may be translocated or metabolized in the dark (12, 15). The balance between synthesis, storage, and transport of soluble sugars is disturbed under conditions of P- or S-deficiency. Sugar and starch levels are increased. Carbohydrates were abundant in deficient plants as compared to well-fertilized plants, although leaf levels of phosphorylated metabolites decreased. The results demonstrate that the inhibition of growth is not a consequence of the capacity of the leaves to synthesize carbohydrates in the light. Growth control is achieved at another level of regulation. Decreased levels of total nucleic acids in all leaves under P- or S-deficiency point to a sensor of the deficiency which is

more sensitive than photosynthesis and carbohydrate metabolism (4, 9). Little is known about the regulation of gene expression in response to mineral deficiency and nothing about the nature of the sensor.

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