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Redox Transfer across the Inner Chloroplast Envelope Membrane[1]

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ABSTRACT

In leaves of spinach plants (*Spinacia oleracea* L.) grown in ambient CO_2 the subcellular contents of adenylates, pyridine nucleotides, 3-phosphoglycerate, dihydroxyacetone phosphate, malate, glutamate, 2-oxoglutarate, and aspartate were assayed in the light and in the dark by nonaqueous fractionation technique. From the concentrations of NADP and NADPH determined in the chloroplast fraction of illuminated leaves the stromal NADPH to NADP ratio is calculated to be 0.5. For the cytosol a NADH to NAD ratio of 10^{-3} is calculated from the assay of the concentrations of NAD, malate, glutamate, aspartate, and 2-oxoglutarate on the assumption that the reactions catalyzed by the cytosolic glutamate oxaloacetate transaminase and malate dehydrogenase are not far away from equilibrium. For the transfer of redox equivalents from the chloroplastic NADPH to the cytosolic NAD two metabolite shuttles are operating across the inner envelope membrane: the triosephosphate-3-phosphoglycerate shuttle and the malate-oxaloacetate shuttle. Although both shuttles would have the capacity to level the redox state of the stromal and cytosolic compartment, this apparently does not occur. To gain an insight into the regulatory processes we calculated the free energy of the enzymic reactions and of the translocation steps involved. From the results it is concluded that the triosephosphate-3-phosphoglycerate shuttle is mainly controlled by the chloroplastic reaction of 3-phosphoglycerate reduction and of the cytosolic reaction of triosephosphate oxidation. The malate-oxaloacetate shuttle is found to be regulated by the chloroplastic NADP-malate dehydrogenase and also by the translocating step across the envelope membrane.

The metabolism of a leaf cell is distributed between various compartments, *e.g.* the cytosol, chloroplast stroma, and mitochondrial matrix. Each of the metabolic compartments has its specific function and hence also its special milieu. Specific translocators catalyze the transfer of metabolites between these compartments (16). For an understanding of cellular metabolism one needs to know how the metabolic processes in these various compartments are coordinated. The present report deals with the aspect of how redox processes occurring in the chloroplast stroma and the cytosol are interlinked. It has been shown in the past that redox equivalents can be transferred from the chloroplast stroma to the cytosol by two different metabolite shuttles, the triosephosphate-3-phosphoglycerate shuttle (14) catalyzed by the P_i-trioseP-3-PGA[3] translocator (6) and the malate-OAA shuttle (1) facilitated by specific transport of malate and OAA (12). As both metabolite shuttles would have the capacity to level the redox state of the stromal and cytosolic compartment, a regulation of these processes is required to maintain the specific redox states of the two metabolic compartments.

To gain an insight into such regulatory processes, we attempted in the present publication to analyze the redox state of pyridine nucleotides by the measurement of their concentrations and the concentrations of substrates of pyridine nucleotide-linked reactions in subcellular compartments of spinach leaves. These measurements were carried out mainly by nonaqueous fractionation of frozen leaves carried out by Heber (13) earlier and later refined in our laboratory (8). It will be shown that there exists a large difference in redox potentials between the NADPH/NADP in the stroma and the NADH/NAD in the cytosol and the processes being responsible for the maintenance of this redox gradient will be identified.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea*, U.S. hybrid 424, Ferry Morse Seed Co., Mountain View, Ca) was grown hydroponically in a climatized chamber with a 9 h light/15 h dark cycle, with a light intensity of 400 μE m^{-2} s^{-1}. The temperature in the light period was 22°C and in the dark 16°C. From these plants leaves were harvested by placing them immediately into liquid nitrogen, during which illumination or darkening was continued. The frozen leaves were then freeze dried and subcellular fractionated by a nonaqueous technique described in detail by Gerhardt and Heldt (8) with one modification. The samples for the metabolite analysis were taken up in 1.5 mL $CHCl_3$, 3.5 mL CH_3OH, and 0.6 mL buffer containing 20 mM Hepes (pH 8.0), 5 mM EGTA, and 5 mM NaF instead of

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[3] Abbreviations: trioseP, triosephosphate; 3-PGA, glycerate-3-phosphate; 1,3-BPGA, glycerate-1,3-bisphosphate; DHAP, dihydroxyacetonephosphate; GAP, glyceraldehyde-3-phosphate; GAPDH, glycerinaldehydephosphate dehydrogenase; GOT, glutamate oxaloacetate transaminase; MDH, malate dehydrogenase; OAA, oxaloacetate; 2-OG, 2-oxoglutarate; PGK, 3-phosphoglycerate kinase; TIM, triosephosphate isomerase.

10% $HClO_4$. The water soluble compounds were separated from the $CHCl_3$ phase by centrifugation, evaporated, and resolved in 1.5 mL H_2O. The metabolites were measured spectrophotometrically (3).

The samples for the analysis of the adenylates, DHAP, and 3-PGA were quenched in 10% $HClO_4$ (v/v), neutralized by 5 M KOH and 1 M triethanolamine, and the metabolite content was measured spectrophotometrically (21). For the determination of the pyridine nucleotides the fractions of the gradient were separated in two portions. The oxidized nucleotides were extracted by adding 10% $HClO_4$ (v/v), centrifuged for 10 min and neutralized by adding 5 M KOH and 1 M K_2HPO_4. To the samples of the reduced forms 1 M KOH in ethanol was added, then neutralized by 0.5 M triethanolamine, 0.5 M KH_2PO_4, 30 mM NH_4Cl, 5 mM 2-oxoglutarate to pH 7.8, centrifuged by 21,000*g*. NAD(P)H were subsequently oxidized by adding 1 U/mL glutamate dehydrogenase. After 15 min the oxidized nucleotides were extracted as described above and measured in a spectrophotometric test (19).

The distribution of metabolites between two subcellular compartments was evaluated according to Gerhardt and Heldt (8). For the evaluation of the metabolite distribution between three subcellular compartments a novel method (B Riens, unpublished results) has been used. The activity of marker enzymes of the three compartments (chloroplast, NADP-GAPDH; cytosol, phospho*enol*pyruvate carboxylase; vacuole, α-mannosidase), and the contents of metabolites were assayed in six fractions. A computer program was developed which calculates each possible metabolite distribution between the three compartments in steps of one percent according to the distribution of the marker enzymes. The calculated values were compared with the measured metabolite contents in the six fractions, and the distribution with the best fit was selected.

For the calculation of the combined equilibrium constant of the reactions of NADP-GAPDH, PGK, and TIM, intact chloroplasts from spinach leaves were isolated according to Heldt and Sauer (17). They were disrupted osmotically in 1 mL 50 mM Hepes buffer pH 8.0 (final Chl concentration 0.1 mg/mL) and centrifugated. To the supernatant 6 mM $MgCl_2$, 4 mM 3-PGA, 3 mM P_i, 1 mM ATP, 0.5 mM ADP, and 0.2 mM DHAP were added. This mixture was transferred to a cuvette and the extinction was determined spectrophotometrically at a wavelength of 334 nm. Then 0.5 mM NADPH and 0.3 mM NADP were added, and the extinction change was monitored at a temperature of 25°C. From the measured ΔE the NADPH and NADP in the steady state was calculated. $HClO_4$ (final concentration 10%) was added to denaturate the proteins and subsequently neutralized with 5 M KOH/1 M triethanolamine and the equilibrium concentrations of 3-PGA, P_i, ATP, ADP, and DHAP were determined (3, 21). The same experiment was carried out with NAD-GAPDH with one exception: the chloroplast extract was replaced by 10 U/mL NAD-GAPDH, PGK, and TIM from yeast (Boehringer, Mannheim, FRG) in 1 mL 50 mM Hepes buffer (pH 7.2 and 7.6). Chl was assayed according to Arnon (2).

RESULTS AND DISCUSSION

Subcellular Levels of Adenine Nucleotides, DHAP and 3-Phosphoglycerate

In the experiment shown in Table I ATP and ADP were determined in the chloroplastic and extrachloroplastic fraction of spinach leaves by a refined method of nonaqueous fractionation (8). Compared with the original method used for assay of ATP and ADP by Santarius and Heber (23) with our method the distribution of metabolites is calculated from the distribution of marker enzymes, ensuring a reliable correction for cross contamination of the subcellular compartments involved. This method enabled the assay of ATP and ADP in the chloroplastic and in the extrachloroplastic compartment. For our experiments the spinach plants were kept in hydroponic culture in a climatized growth chamber at a 9 h light/15 h dark cycle and exposed either to a 8.5 h light period or a 2 h dark period. To quench metabolism these leaves were rapidly detached and while exposed to the same light intensity immediately placed into liquid nitrogen. In Table II the resulting ATP to ADP ratios are shown. Both in the light and darkness the ATP to ADP ratio in the extrachloroplastic fraction is found to be higher than in the chloroplast compartment, which has been also found by Keys and Whittingham (18) in tobacco leaves and by Sellami (26) in wheat leaves. In illuminated leaves the chloroplast ATP to ADP ratio is found to be much higher than in the darkened one [see also Dietz and Heber (5)], the extrachloroplastic ATP to ADP ratio being less affected by illumination. The chloroplastic ATP to ADP values observed in our experiments are almost identical with those found by Dietz and Heber (5).

Even our refined technique of nonaqueous fractionation of spinach leaves did not achieve a separation of the extrachloroplastic fraction into a cytosolic and mitochondrial portion. Hampp *et al.* (11) attempted to assay adenylate levels in the chloroplastic, cytosolic and mitochondrial fraction of oat leaf protoplasts using silicone layer filtering centrifugation. Since the time elapsed between disruption of the protoplasts and the quench of mitochondrial metabolism was 1 min, the validity of these measurements has been a matter of debate. In an alternative approach using a membrane filtering system with a quenching time in the order of 100 ms, Stitt *et al.* (27)

Table I. *Subcellular Content of ATP, ADP, 3-PGA, and DHAP in Spinach Leaves*

Spinach plants were grown in a climatized chamber with a 9 h light/15 h dark cycle and leaves were stopped in liquid N_2 after 8.5 h light or 2 h dark. The leaves were subcellular fractionated (8). The results are mean values ± standard deviation of six measurements from three preparations. The concentrations are calculated under an assumed chloroplast volume of 25 μL/mg Chl and a cytosolic volume of 20 μL/mg Chl (8).

Assay	Stroma	Cytosol + Mitochondria
	mM	
A. Light 8.5 h		
ATP	1.80 ± 0.28	2.55 ± 0.40
ADP	0.76 ± 0.12	0.65 ± 0.25
DHAP	0.39 ± 0.20	0.90 ± 0.25
3-PGA	4.00 ± 1.3	2.55 ± 0.40
B. Dark 2 h		
ATP	0.64 ± 0.12	1.45 ± 0.25
ADP	1.08 ± 0.28	0.80 ± 0.20
DHAP	<1	<1
3-PGA	4.8 ± 1.4	3.6 ± 0.8

Table II. *ATP/ADP Ratios*

Assay	ATP/ADP			
	Chloroplast	Cytosol + mitochondria	Cytosol	Mitochondria
		mM		
Light				
Spinach leaves[a]	2.4	3.9	(4.8)[b]	(1.9)
Wheat leaf protoplasts[c]	2.2	4.6	6.4	2.6
Dark				
Spinach leaves[a]	0.6	1.8	(3.0)	(0.2)
Wheat leaf protoplasts[c]	1.1	3.7	9.2	0.6

[a] Data from Table I. [b] The data in parentheses have been estimated from analogy with the subcellular distribution of adenylates in wheat leaf protoplasts on the assumption that 82% of the extrachloroplastic ADP + ATP are located in the cytosol and the rest in the mitochondria and that the factor between the cytosolic and mitochondrial ATP to ADP ratios is 2.5 in the light and 15 in the dark (27). [c] Data from Stitt *et al.* (27).

determined ATP to ADP ratios in the chloroplastic, cytosolic, and mitochondrial compartment of wheat leaf protoplasts, and these results were essentially confirmed by Gardeström (7) adapting the same method. With protoplasts the ATP to ADP ratio in the mitochondria was found to be lower and that in the cytosol higher than the average ATP to ADP ratio assayed for the extrachloroplastic fraction (Table II). As the ATP to ADP ratios assayed in spinach leaves are very similar to those found in wheat leaf protoplasts, for the leaves the cytosolic and the mitochondrial ATP to ADP ratios have been estimated on the assumption that the distribution of adenylates in spinach leaves follows the same pattern as in protoplasts (see values in brackets in Table II).

To correlate the adenylate levels in the chloroplastic and cytosolic compartments with the corresponding levels of DHAP and 3-PGA, the subcellular contents of the latter two compounds were analyzed in the same experiments (Table I). The values obtained in these measurements are similar to those of earlier measurements from our laboratory (9) and the stromal data by Dietz and Heber (5). Because DHAP and 3-PGA are not metabolized in the mitochondrial matrix, the contents of these substances in the extrachloroplast fraction can be regarded as cytosolic levels.

Subcellular Levels of the Pyridine Nucleotides

Table III shows the results of the subcellular content of pyridine nucleotides in leaves. The chloroplastic NADP system is partially reduced, more so in the light than in the dark. The NADPH to NADP ratio of 0.5 found in the light is identical to the value observed in chloroplasts isolated nonaqueously from spinach leaves by Heber and Santarius (15), but it is lower than assayed by Dietz and Heber (5). Hampp *et al.* (10) evaluated from fractionation of oat protoplasts a stromal NADPH to NADP ratio of 0.375 in the light and 0.89 in the dark. The NADP content evaluated for the cytosolic compartment is less than 10% of the total. Although due to this extreme distribution the accuracy of the determination is not very high, our results clearly show that the cytosolic NADP system is highly reduced concurring with earlier results by Heber and Santarius (15). The NAD system in the cytosol as well as in the stroma was found to be highly oxidized. We could not determine any NADH. These results also concur with those from Heber and Santarius (15), but are in contrast with data of Hampp *et al.* (10) who reported comparatively high NADH to NAD ratios in the chloroplastic and cytosolic fraction of oat protoplasts. It may be noted that in darkened chloroplasts the sum of NADPH and NADP was found to be lower, and the amount of NAD higher than the corresponding values assayed in the light. The reason for this is not known. It may be the result of the activity of the chloroplastic NAD kinase which is known to be a light activated enzyme (15). In the cytosol, such changes were not apparent.

Subcellular Levels of the Metabolites of the GOT and MDH Reactions

Using identical conditions as in the measurements of Tables I and III, the subcellular contents of glutamate, aspartate, 2-OG, and malate were assayed. The evaluation was done in a novel way (B Riens, manuscript in preparation; see "Materials and Methods"), allowing the determination of substrate levels in the vacuolar, chloroplastic and extrachloroplastic compartment. As shown in Table IV, in the chloroplastic and extrachloroplastic compartment very high concentrations of glutamate and aspartate are found. Compared to these the corresponding concentrations in the vacuole are low. Malate, on the other hand, is mainly found in the vacuolar compartment. Assuming a glutamate oxaloacetate transaminase equilibrium, from the concentrations of glutamate, aspartate, and 2-OG the concentrations of oxaloacetate have been evaluated.

Estimation of the Cytosolic NADH to NAD Ratio from Metabolite Equilibria

Because of the very low reduction state of NAD even a refined sensitive direct measurement of NADH contents would have been meaningless, because at a very low NADH

Table III. *Pyridine Nucleotide Content of Spinach Leaves*

Spinach plants were grown in a climatized chamber with a 9 h light/15 h dark cycle and leaves were stopped in liquid N_2 after 8.5 h light or 2 h dark. The leaves were subcellular fractionated as (8). The results are mean values ± standard deviation of six measurements from three preparations. The concentrations are calculated on the assumptions given in Table I.

Assay	Stroma	Cytosol + Mitochondria
	mM	
A. Light 8.5 h		
NADPH	0.29 ± 0.03	0.15 ± 0.06
$NADP^+$	0.59 ± 0.13	0.045 ± 0.033
NAD^+	0.19 ± 0.02	0.60 ± 0.13
$\frac{NADPH}{NADP}$	0.5	3.3
B. Dark 2 h		
NADPH	0.12 ± 0.02	0.15 ± 0.05
$NADP^+$	0.51 ± 0.12	0.038 ± 0.043
NAD^+	0.92 ± 0.36	0.73 ± 0.4
$\frac{NADPH}{NADP}$	0.23	4.0

Table IV. *Subcellular Content of Aspartate, Glutamate, 2-OG, and Malate*

Spinach plants were grown in a climatized chamber with a 9 h light/15 h dark cycle and leaves were stopped in liquid N_2 after 8.5 h light or 2 h dark. The leaves were subcellular fractionated as (8). The results are mean values from two independent experiments. The concentrations are calculated on the assumptions that the stromal, cytosolic and vacuolar volumes are 25, 20, and 150 μL/mg Chl, respectively.

Assay	Stroma	Cytosol + Mitochondria	Vacuole
		mM	
A. Light 8.5 h			
Glutamate	25.2	39.3	0.82
Aspartate	25.5	43.7	2.06
2-OG	0.16	0.58	0.025
Malate	3.0	1.0	26.7
OAA[a]	0.025	0.098	
B. Dark			
Glutamate	49.6	48.3	3.68
Aspartate	35.8	30.5	3.53
2-OG	0.39	0.70	0.065
Malate	1.0	1.0	26.7
OAA[a]	0.043	0.067	

[a] Calculated from GOT equilibrium ($K = 6.61$).

content most of it will be bound to enzymes and for these reasons the NADH content could not be used for the evaluation of NADH concentrations (4). Alternatively, NADH to NAD ratios can be estimated from the concentration of metabolites supposed to be in near equilibrium with the NADH to NAD couple. Because of the high activity of MDH and GOT in the cytosol, the reaction sequence catalyzed by these two enzymes appears to be suitable for an estimation of the cytosolic NADH to NAD ratio. For these estimations a pH value has to be introduced. Since the cytosolic pH value of illuminated leaf cells is not exactly known, we based all pH dependent calculations alternatively on pH 7.2 and 7.6.

According to the equation

$$\frac{\text{NADH}}{\text{NAD}} = \frac{(\text{Glu})\cdot(\text{malate})\cdot K_1\cdot K_2}{(\text{2-OG})\cdot(\text{Asp})\cdot(\text{H}^+)}$$

$K_1 = 6.61$ (GOT) (29) and $K_2 = 2.78\cdot10^{-12}$ (MDH) (29). From the data of Table IV and assuming a pH of 7.2, the cytosolic NADH to NAD ratio in the light is calculated as $0.5\cdot10^{-3}$ which in the presence of 0.6 mM NAD corresponds to an NADH concentration of 0.3 μM. At an assumed cytosolic pH of 7.6, a NADH to NAD ratio of $1.1\cdot10^{-3}$ and a NADH concentration of 0.7 μM is evaluated. For leaves kept in darkness very similar values are found, the NADH to NAD ratio amounting to $0.7\cdot10^{-3}$ (pH 7.2) and $1.7\cdot10^{-3}$ (pH 7.6) corresponding to a NADH concentration of 0.5 and 1.2 μM, respectively. It may be noted that the determination of the extrachloroplastic malate concentration may involve some error. A minor error could be also introduced by the simplification in attributing the extrachloroplastic metabolites to the cytosolic compartment only. Nevertheless, even if there were an error of a factor of 5 in either direction, the cytosolic NADH concentration would be in a range below 5 μM. Since the malate-OAA shuttles from the chloroplast stroma and the mitochondrial matrix into the cytosol appear to be operating in the direction of the cytosol, the cytosolic malate dehydrogenase will catalyze the flux from malate to OAA. If this reaction were not in equilibrium, the actual NADH to NAD ratio in the cytosol would be even lower than evaluated by our calculation above. An error in the assumption of the cytosolic volume given in Table I will not influence the result of the calculation, because it would change numerator and denominator by the same factor. In summarizing, it can be concluded from our data that in a leaf in the steady state of photosynthesis the NADH to NAD ratio in the cytosol is at the order of 10^{-3}.

This value may seem very low at first, but it is very similar to the steady-state concentration ratio of NADH to NAD found in the cytosol of animal tissues, such as liver (4). The validity of the cytosolic NADH concentrations in spinach leaves determined in the preceeding is supported by our recent studies of spinach nitrate reductase, a major NADH consuming enzyme of the cytosolic compartment. Applying a novel coupled enzyme activity assay the apparent K_m of this enzyme for NADH, as measured in the presence of 0.5 mM NAD, was determined as 1.4 μM (22). Since the K_m of an enzyme is usually above the substrate level occurring under physiological conditions, these results indicate that nitrate reductase normally operates at an NADH to NAD ratio in the order of 10^{-3}, which is of the same order as the cytosolic NADH to NAD ratio determined above.

Direct measurements of NADPH and NADP show that in illuminated leaves the stromal NADPH to NADP ratios are about 0.5 (see above), which is three orders of magnitude higher than the NADH to NAD ratio found for the cytosol. The question arises which processes are responsible that such a gradient is maintained.

On the Maintenance of a Redox Gradient in a Malate-OAA Shuttle

A calculation of the hypothetic equilibrium NADPH to NADP ratio in the stroma of illuminated leaves from the concentrations of glutamate, malate, 2-OG and aspartate in Table IV, assuming a stromal pH of 8.0 yields a value of $3.4\cdot10^{-2}$, which is about 15 fold lower than the measured stromal NADPH to NADP ratio (Table III). For the darkened leaves the calculated NADPH to NADP ratio at an assumed pH of 7.6 is $2.6\cdot10^{-3}$, which is 88-fold less than the measured value. Apparently, in the stroma the reaction sequence catalyzed by the GOT and MDH reaction is far from equilibrium yielding an erroneous calculation for the value of the NADPH to NADP ratio. On the reasonable assumption that the deviation from equilibrium is not due to the transaminase reaction, it appears that the stromal OAA concentrations are much higher than those concentrations which would be in equilibrium with the stromal NADPH to NADP. In the case of darkness the reason for this is obvious, since the stromal NADP malate dehydrogenase is light activated by the thioredoxin system (24) and for this reason not expected to be active in the dark. But also in the state of illumination the NADP-malate dehydrogenase is strongly controlled in its activity by the stromal NADP concentration in such a way that the enzyme is active

at high NADPH to NADP ratios only (24). The function of the stromal malate dehydrogenase has been therefore compared with a valve allowing the release of excessive redox equivalents from the stroma (24). The results of our stromal metabolite measurement strongly support this notion. The considerable deviation from equilibrium observed above suggests that the stromal NADP malate dehydrogenase is an important factor in maintaining a redox gradient between the NADPH to NADP system in the stroma and the NADH to NAD system in the cytosol.

In addition to this, metabolite transport across the inner envelope membrane also contributes to the maintenance of the redox gradient. The concentration ratio (Glu)·(malate)/(2-OG)·(Asp) in the stroma appears to be about 12-fold higher than in the cytosol (Table V). In darkened leaves, where a malate-OAA shuttle should not operate (see above), this gradient is almost abolished. It cannot be decided at present how this gradient observed during illumination is maintained.

Redox Transfer by TrioseP-3-PGA Shuttle

A trioseP-3-PGA shuttle represents another possibility for a redox transfer from the chloroplast stroma into the cytosol (14). The operation of this shuttle would require steps in the reaction sequence causing altogether a redox gradient of $5 \cdot 10^2$. The question is which are these steps. One possible step could have been the phosphate translocator catalyzing the counterexchange of trioseP and 3-PGA across the chloroplast envelope. This, however, is not the case. Measurements of subcellular DHAP and 3-PGA levels clearly show that the ratio of DHAP to 3-PGA is even higher in the cytosol than in the stroma (Table V). This effect, due to the well established stimulation of trioseP *versus* 3-PGA export by light (6) makes it seem impossible that in a trioseP-3-PGA shuttle from the stroma to the cytosol the translocator step plays any major role in the maintenance of a redox gradient.

To identify limiting steps responsible for a redox gradient, the concentration ratios of NADPH to NADP (stroma) and NADH to NAD (cytosol) in equilibrium with the corresponding subcellular metabolite concentrations (Tables I and II) were calculated according to the following equation:

$$\frac{\text{NAD(P)H}}{\text{NAD(P)}} = \frac{(\text{DHAP})\cdot(\text{P}_i)\cdot(\text{ADP})}{(\text{3-PGA})\cdot(\text{H}^+)\cdot(\text{ATP})\cdot K}.$$

K is the product of the equilibrium constants of (a) phosphoglycerate kinase (3):

$$K_1 = \frac{(\text{1,3-BPGA})\cdot(\text{ADP})}{(\text{3-PGA})\cdot(\text{ATP})} = 2.9\cdot 10^{-4}$$

(b) GAPDH (3):

$$K_2 = \frac{(\text{GAP})\cdot(\text{P}_i)\cdot(\text{NAD(P)})}{(\text{1,3-BPGA})\cdot(\text{NAD(P)H})\cdot(\text{H}^+)} = 2.0\cdot 10^{7}.$$

(c) triose isomerase (3):

$$K_3 = \frac{(\text{DHAP})}{(\text{GAP})} = 22.$$

From these literature data the resulting value for $K = K_1 \cdot K_2 \cdot K_3$ is $1.3 \cdot 10^5$.

Because of the complexity of the reaction we did not want to rely on these literature values and therefore determined K by adding substrates to a stromal lysate obtained by osmotic shock of spinach chloroplasts and assaying the metabolite concentrations after equilibrium has been reached (see "Materials and Methods"). A similar experiment was also done with a mixture of commercially available phosphoglycerate kinase, NAD glyceraldehyde phosphate dehydrogenase and triose isomerase. In this way the following equilibrium constants were experimentally obtained:

1. For the chloroplastic reaction sequence, determined at pH 8.0:

$$K = \frac{\text{DHAP}\cdot\text{P}_i\cdot\text{ADP}\cdot\text{NADP}}{\text{3-PGA}\cdot\text{H}^+\cdot\text{ATP}\cdot\text{NADPH}} = 3.4\cdot 10^5.$$

2. For the cytosolic reaction sequence, determined at pH 7.2:

$$K = \frac{\text{DHAP}\cdot\text{P}_i\cdot\text{ADP}\cdot\text{NAD}}{\text{3-PGA}\cdot\text{H}^+\cdot\text{ATP}\cdot\text{NADH}} = 4.4\cdot 10^4.$$

At pH 7.6, for the above reaction an apparent equilibrium constant of $1.1 \cdot 10^5$ was determined. These constants are similar to the products of the equilibrium constants reported from literature shown above. The small deviations are probably the result of pH dependent changes in the charges of the components involved, which are included in this "apparent" equilibrium constants.

Introducing the experimentally obtained values of K, the stromal concentrations of DHAP, ADP, 3-PGA and ATP listed in the Tables I and II, assuming a P_i concentration of 5 mM (20) and a stromal pH of 8.0 into the above equation, the NADPH to NADP ratio in equilibrium with these substrates is calculated as:

$$\frac{\text{NADPH}}{\text{NADP}} = 0.06.$$

The value thus obtained is by a factor of 8 lower than the NADPH to NADP ratio measured in the stroma, clearly indicating that the reaction sequence catalyzed by PGK, NADP-GAPDH, and TIM in the stroma shows a considerable deviation from a equilibrium.

The analogous calculation of the NADH to NAD in the cytosol assuming P_i = 5 mM and pH = 7.2 or 7.6 and introducing the corresponding K values (see above) yields in

Table V. *Metabolite Ratios: Values from Tables I and IV*

Assay	$\frac{(\text{Glu})\cdot(\text{Mal})}{(\text{2-OG})\cdot(\text{Asp})}$	$\frac{(\text{DHAP})}{(\text{PGA})}$
A. Light 8.5 h		
Stroma	18.5	0.098
Cytosol	1.6	0.35
B. Dark 2 h		
Stroma	3.6	
Cytosol	2.3	

both cases:

$$\frac{\text{NADH}}{\text{NAD}} = 0.13.$$

This value is nearly similar to the stromal ratio, but by a factor of 260 (pH 7.2) and 120 (pH 7.6) higher than the cytosolic NADH to NAD determined from malate dehydrogenase equilibrium (see above). It appears therefore, that also in the cytosol the reaction sequence catalyzed by PGK, NAD-GAPDH, and TIM does not result in an equilibrium.

CONCLUSION

We have shown that in a spinach leaf cell there is a redox gradient between the NADPH to NADP in the stroma and the NADH to NAD in the cytosol in order of 10^3. Assuming a stromal pH of 8.0 and cytosolic pH of either 7.2 or 7.6 the observed redox gradient reflects a change in the free energy of $\Delta G = -13$ kJ/mol. A redox transfer between these NAD(P)H/NAD(P) pools by Mal/OAA or DHAP/3-PGA shuttle has to account for the fact that this driving force is maintained. In Figure 1, from the results obtained in the preceeding the changes in free energy for the individual steps of both shuttles have been evaluated. For the NAD-GAPDH, PGK reaction where the evaluated ΔG depends on the assumed pH in the cytosol, the value calculated for pH 7.6 is given in brackets. Our results show that an uncertainty of the cytosolic pH does not markedly affect the result. Also the assumptions about the volumes of the subcellular compartments required to convert the results of our subcellular metabolite analyses into concentrations have no effect, since in the equations used for our calculations, these volumes represent equal factors in the numerator and the denominator. Due to uncertainties of parameters, *e.g.* pH values and P_i concentrations, and the possibility that substrates are bound, the results of the calculations, as summarized in Figure 1, can be regarded as rough estimations only. Nevertheless these results summarize in which way in both shuttles the redox gradient is maintained. It appears that in the case of the Mal/OAA shuttle the loss of free energy occurs mainly at the site of NADP-malate dehydrogenase and the transfer of metabolites across the envelope, whereas in the case of trioseP-3-PGA shuttle the transport across the envelope is even an uphill process, and that the change of free energy occurs to some extent at the PGK-GAPDH sequence in the stroma and mainly at the PGK-GAPDH sequence in the cytosol. The experiments in the preceeding have been carried out at ambient CO_2 and the light intensity of our normal growing conditions. In experiments by Dietz and Heber (5) analyzing nonaqueously isolated chloroplasts from spinach leaves the determined concentrations ratios of

$$\frac{\text{(DHAP)}\cdot\text{(ADP)}\cdot\text{(NADP)}}{\text{(3-PGA)}\cdot\text{(ATP)}\cdot\text{(NADPH)}}$$

were very similar to our data and did not differ for a range of different CO_2 concentrations and resulting different rates of photosynthesis. It may be concluded from these results that the reaction sequence catalyzed by PGK and NAD(P)-GAPDH in the stroma and in the cytosol are regulated.

An understanding of these deviations from the equilibrium in the chloroplast and the cytosolic compartments requires knowledge of the regulatory properties of the enzymes involved. Turner and Turner (28) discussed that triosephosphate isomerase may not catalyze an equilibrium. This enzyme is competitively inhibited by several compounds of the Calvin cycle although to low extent. If the chloroplastic triosephosphate isomerase were the limiting step in the reaction of 3-PGA reduction due to the inhibitory effect of various metabolites, glyceraldehyde phosphate should accumulate and be exported into the cytosol, in order to be oxidized to 3-PGA. Therefore a limitation of the chloroplast triosephosphate isomerase would not affect the redox transfer across the envelope membrane. The NADP-glyceraldehyde dehydrogenase is known to be regulated in a complex manner. It has been reported to be regulated by thioredoxin, ATP and NADPH (30, 31), but little is known about the fine regulation of the fully activated enzyme. Inhibitory effects of sedoheptulose mono- and bisphosphate on the catalytic activity of NADP-GAPDH could be involved (25). The limitation of the cytosolic reaction sequence cannot be explained at present. A flux control by the cytosolic triosephosphate isomerase seems to be unlikely, since this limitation would also affect sucrose synthesis. A proposed physiological role of effects of heptulose phosphates on the cytosolic NAD-GAPDH cannot be ascribed as the cytosolic concentrations of heptulose phosphates are

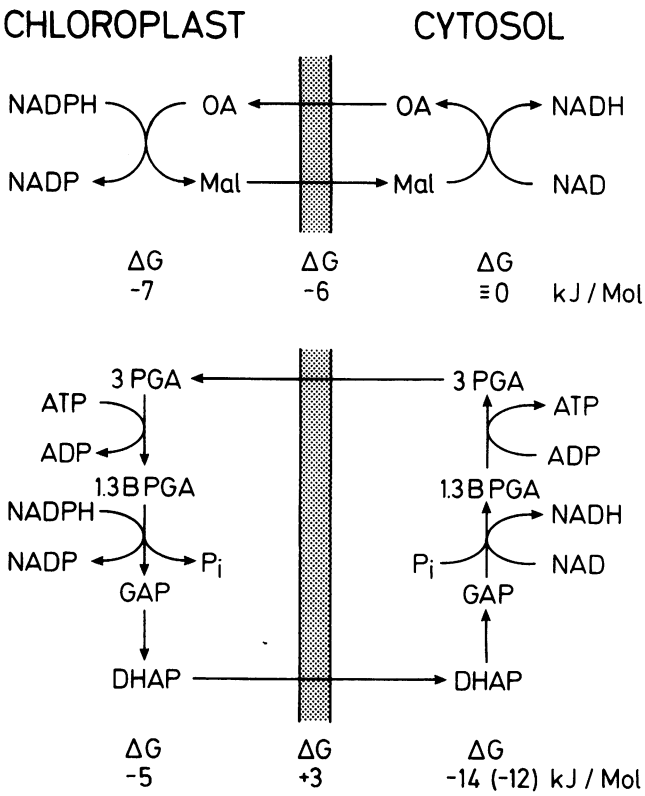

Figure 1. Transfer of redox equivalents across the envelope membrane. The values of free energy are calculated from the results in the light of Tables I, II, III, IV, and V, and from the equilibrium constants given in the text. The cytosolic values are calculated on the assumption of a cytosolic pH of 7.2. The value in parentheses is calculated at the assumption of pH 7.6.

unknown (25). The regulation of phosphoglycerate kinase seems to be caused only by the availability of both nucleotides ATP and ADP (15). It remains to be elucidated how a regulation of a DHAP/3-PGA shuttle is achieved.

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