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Compartmentation of labeled fixation products in intact mesophyll protoplasts from *Avena sativa* L. after in-situ inhibition of the chloroplast phosphate translocator

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Abstract. Leaf mesophyll protoplasts of oat (*Avena sativa* L.) were allowed to fix ^{14}C-labeled bicarbonate in the absence or presence of pyridoxal phosphate (PLP), a specific inhibitor of the phosphate translocator of the inner envelope membrane of chloroplasts. The incubation was terminated by a method of rapid integrated protoplast homogenization and fractionation, and compartmented levels of label contained in sugars, phosphate esters, amino acids and organic acids were determined. The results show that the addition of PLP to a suspension of intact protoplasts causes an accumulation of phosphate esters in the chloroplast stroma for up to 2.5 min of incubation, with a corresponding decrease in the cytosol. Prolonged treatment of protoplasts with PLP in the light resulted in a decrease of starch-associated label, combined with higher levels of labeled sugars in the cytosol, indicating a switch from phosphorolytic to hydrolytic starch degradation. Together with the determination of pool sizes of triose phosphates and of inorganic phosphate, the results demonstrate that the method employed is an important tool in investigating processes of intracellular regulation. They are discussed with respect to the permeability and possible side reactions of PLP, as well as in the light of reports on PLP action on isolated chloroplasts.

Key words: *Avena* (photosynthesis) – CO_2 fixation – Compartmentation – Phosphate translocator (chloroplast) – Pyridoxal 5′-phosphate.

Abbreviations: P_i = orthophosphate; PLP = pyridoxal 5′-phosphate; TP = triosephosphate

Introduction

The phosphate translocator of the inner membrane of the chloroplast envelope controls in exchange with inorganic phosphate the export of phosphate esters from the chloroplast stroma (Heldt 1976). This major protein of the inner envelope membrane (Flügge and Heldt 1976) catalyses a specific anion counter-exchange. As a result of its action, during photosynthetic carbon fixation, the net uptake of inorganic phosphate into chloroplasts is counterbalanced by the release of triosephosphates (TP) and 3-phosphoglyceric acid. It has been shown that high external inorganic phosphate enhances the export of 3-phosphoglyceric acid and TP, thereby increasing the lag in light-dependent oxygen evolution (Walker 1976), whereas inhibition of the exchange mechanism reduces orthophosphate (P_i) inhibition of CO_2 fixation. Pyridoxal 5′-phosphate (PLP), which is known to react with lysine residues, is an inhibitor of the P_i transport in isolated spinach chloroplasts (Fliege et al. 1978). While incubation of chloroplasts with PLP resulted in a build up of carboxylation products in the stroma, their release to the surrounding medium was largely decreased (Flügge et al. 1980). From the observation that the pattern of stromal metabolites observed in the presence of PLP was similar to that observed when there was a P_i deficiency in the medium, the authors concluded that PLP does not penetrate the chloroplast envelope and thus has no marked effect on stromal reactions (see Herndon et al. 1982). In all respective experiments isolated chloroplasts from spinach were used to elucidate this translocation mechanism.

In order to gain an insight into the action of the phosphate translocator within the intact cell, we tried to assess the effect of PLP on the compartmented distribution of labeled fixation products in photosynthesizing intact protoplasts from oat mesophyll. The results demonstrate that the incubation of intact protoplasts with PLP results in specific effects on the distribution of labeled fixation products between chloroplast; stroma and cytosol.

Material and methods

Plant material and culture conditions. Seedlings of *Avena sativa* L. (cv. Arnold) were grown in hydroponic culture for 7 d. Illumination (about 9 W m^{-2}: Osram HQLS, 400 W + two Osram concentra PAR spots, 75 W each) was started 4 d after germination in the dark at 26° C and 80% rel. humidity.

Isolation of protoplasts. Enzymatic isolation of protoplasts from 0.5- to 1-mm-wide leaf segments, as well as their purification, were carried out as reported earlier (Hampp and Ziegler 1980; Goller et al. 1982). Protoplast numbers were counted using a Neubauer double haemocytometer; chlorophyll was determined according to Arnon (1949).

Incubation of protoplasts. Purified protoplasts were resuspended in 0.5 M sorbitol, 7.5 mM $CaCl_2$, 25 mM 2-(N-morpholino) ethanesulfonic acid (Mes), pH 6.0 (about 0,12 mg Chlorophyll ml^{-1}) and incubated in a Clark-type oxygen electrode at 20° C (Delieu and Walker 1972). The $CaCl_2$ was added in order to inhibit photosynthesis of chloroplasts, set free from broken protoplasts (Wirtz et al. 1980). Illumination was provided by two opposing 250-W slide projectors, yielding about 600 W m^{-2} at the surface of the electrode vessel. Pyridoxal 5′-phosphate was dissolved in 0.5 M sorbitol, and added to the protoplast suspension either at the beginning of a 2-min dark interval, followed by a second light treatment, or after 8 min of continuous illumination. Controls contained an appropriate amount of 0.5 M sorbitol. Immediately before switching on the light, labeled bicarbonate was added (final concentration 1.6 mM, specific activity 2.14 MBq μmol^{-1}).

Fractionation of protoplasts. The incubation of protoplasts was terminated by centrifugal filtration under the same conditions as during incubation (illumination or darkness). Fifty-µl aliquots of the protoplast suspension were pipetted into 400-µl centrifuge tubes containing a 20-µm nylon mesh and several hydrophobic and hydrophilic layers. For preparation and handling of these microgradient systems see Hampp (1980) and Goller et al. (1982). This integrated system of protoplast homogenisation and fractionation delivers three fractions (chloroplasts, mitochondria and the remainder of the cell) which are largely metabolically quenched within seconds. Procedures for the correction for cross contamination, as well as a critical assessment of this technique in general, have been given in detail (Hampp et al. 1982; Goller et al. 1982).

Separation of labeled fixation products. After centrifugal fractionation of the protoplasts, the microtubes were cut in the middle of the oil layers and below the net and the fractions of 4 parallels were extracted together in 1.0 ml of methanol: chloroform: water: formic acid (12:5:2:1, by vol.). The extraction was repeated twice. The remainder was resuspended and centrifuged in methanol: chloroform (12:5; v/v) and starch was determined from the radioactivity in the residue. The combined aqueous methanol phases were reduced in volume to about 200 µl under a stream of N_2 and the pH was adjusted to a value of 6 to 7. Aliquots of 50 µl were used to separate sugars, phosphate esters, amino acids and organic acids by microscale ion-exchange chromatography on Sephadex-QAE-A-25-formate and Sephadex-SP-C-25-H^+ (both Pharmacia, Freiburg, FRG; Redgwell 1980). The fractions, washed from the columns, were made up to 2.0 ml with distilled water and radioactivity was countered by liquid scintillation. Of the added radioactivity and labeled markers 100 ± 2% were recovered.

Determination of inorganic phosphate and of triose phosphates. Perchloric-acid extracts were neutralized as described before (Hampp et al. 1982) and aliquots (2 and 10 µl, respectively) used to assay P_i and TP by enzymatic cycling (Lowry and Passonneau 1972).

Results

Effect of pyridoxal phosphate on light-dependent oxygen evolution of intact protoplasts. In Fig. 1 a and b, oxygen-electrode traces for protoplast suspensions are given. In the absence of PLP, protoplasts only show a short lag in light-dependent oxygen evolution (Fig. 1 a). When PLP is added 2 min prior to illumination, there is a concentration-dependent second induction phase in parallel to a decrease in the rate of oxygen evolution when a new steady state is reached. The rather steep increase in O_2 consumption upon illumination is in part the result of some oxidation of PLP in the light (less than 0.1% of the total PLP present). In addition, there is a kind of Mehler reaction (Mehler 1951) by the chloroplasts as a consequence of a reduced NADPH oxidation inPLP-treated systems (Hampp, unpublished). It is not a consequence of increased respiration, as protoplasts in the dark did not respond to PLP at all concentrations tested (0.1 to 5 mM; data not shown). When PLP was added under continued illumination (Fig. 1 b), it caused a reduced rate of O_2 evolution after reaching again a steady state in the light (Fig. 2). Under these experimental conditions, the inhibitor-induced lag phase was shorter than when the PLP preincubation was carried out in the dark.

The lag caused by a given PLP concentration was to some extent dependent on changes in the sensitivity of the protoplast preparation over the experimental period. Therefore, before each experiment, a PLP concentration was selected which caused the protoplasts to take about 3–4 min in order to come to a positive O_2 balance.

With all PLP concentrations tested, protoplasts regained their ability to evolve oxygen in the light, although this was at reduced rates and was dependent on the concentration of PLP. In a wide range of experiments it has been observed that any dam-

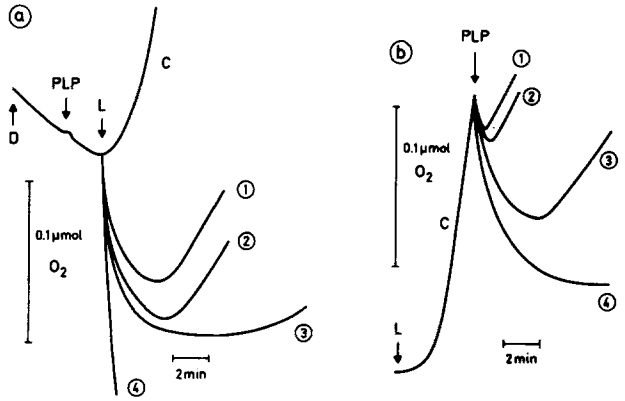

Fig. 1a, b. Oxygen-electrode tracing of oxygen consumption and evolution by suspensions of intact oat mesophyll protoplasts. The 1-ml reaction medium contained protoplasts corresponding to about 90 µg of chlorophyll. The rate of light-dependent oxygen evolution of the control (*c*) was about 65 µmol mg chlorophyll^{-1} h^{-1}. *D*, dark; *L*, light; *1* to *4*, different concentrations of pyridoxal phosphate (PLP, mM); *1*, 2.0; *2*, 2.2; *3*, 2.6; *4*, 5.2. Changes in treatment are indicated by arrows

age to the plasma membrane of oat protoplasts, causing some leakage of solutes, results in a loss of their ability to evolve oxygen in the light. They do not recover from this (data not shown). It thus appears conceivable that PLP (or pyridoxal, which could be formed during the incubation by enzymes leaking from broken protoplasts) is able to penetrate the plasma membrane, and that the effects of this are caused by an action upon the chloroplasts contained within intact protoplasts, rather than by their action on protoplast integrity. This assumption is further supported by the determination of labeled compounds after $^{14}CO_2$ fixation in the supernatant of protoplast suspensions, filtered through silicone oil according to Hampp et al. (1982). This determination is routinely used to correct for metabolites originating from broken protoplasts (Hampp et al. 1982; Goller et al. 1982). The percentage of label retained by filtered protoplasts as determined in this way was similar for suspensions incubated in the absence or presence of up to 2.5 mM PLP.

Time course of light-dependent ^{14}C incorporation by intact protoplasts. In order to prelabel the metabolite pools, protoplasts were allowed to fix labeled bicarbonate for 8 min before the injection of PLP. Addition of PLP under continued illumination caused a reduction of ^{14}C incorporation with a most pronounced effect between 2 and 4 min after injection (Fig. 2). When PLP was introduced at the beginning of a 2 min dark interval, the reduction in $^{14}CO_2$ fixation during the following illumination was less pronounced (Fig. 2).

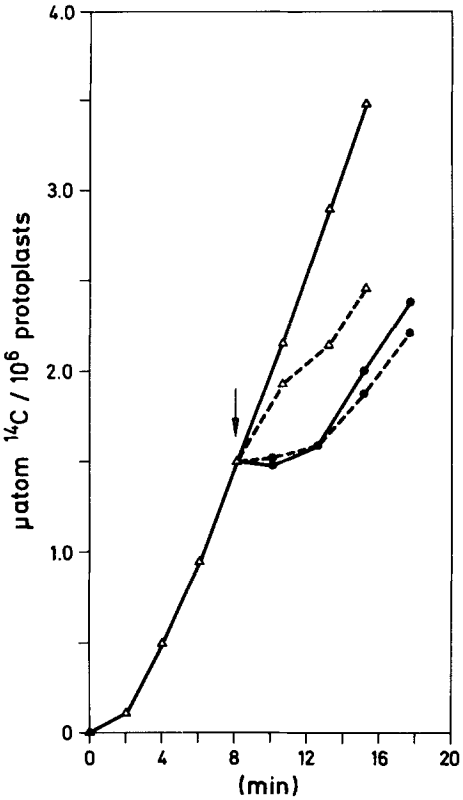

Fig. 2. Time course of incorporation of labeled bicarbonate into suspensions of intact oat protoplasts. *Solid line:* control, *dashed line:* + pyridoxal phosphate (PLP, final concentration 2.4 mM). Pyridoxal phosphate (*arrow*) was added 8 min after starting the illumination, either under continued light treatment (△) or together with the start of a 2-min dark interval (●; 8–10 min), followed by a second period of illumination

Effect of PLP on the subcellular compartmentation of labeled compounds under continuous illumination. In all fractionation studies, label associated with the mitochondrial fraction was comparatively low, especially for shorter periods of photosynthesis. For this reason, results are only given for chloroplast and extrachloroplast spaces, the latter, however, are corrected for mitochondria.

In Fig. 3a, a comparison of total incorporation of label into chloroplasts, cytosol and non-fractionated protoplasts is given. After about 2 min of PLP treatment the increase in extrachloroplast label is already greatly reduced, while that of the chloroplast compartment is even slightly increased and only shows a reduction after prolonged PLP incubation. An analysis of the distribution of label among the four major groups of fixation products is given in Fig. 3b for chloroplasts. The effect of PLP addition is not uniform and can be divided into two phases. Within the first 2.5 min of PLP treatment, there is much more label in phosphate esters than in the untreated control, which is also

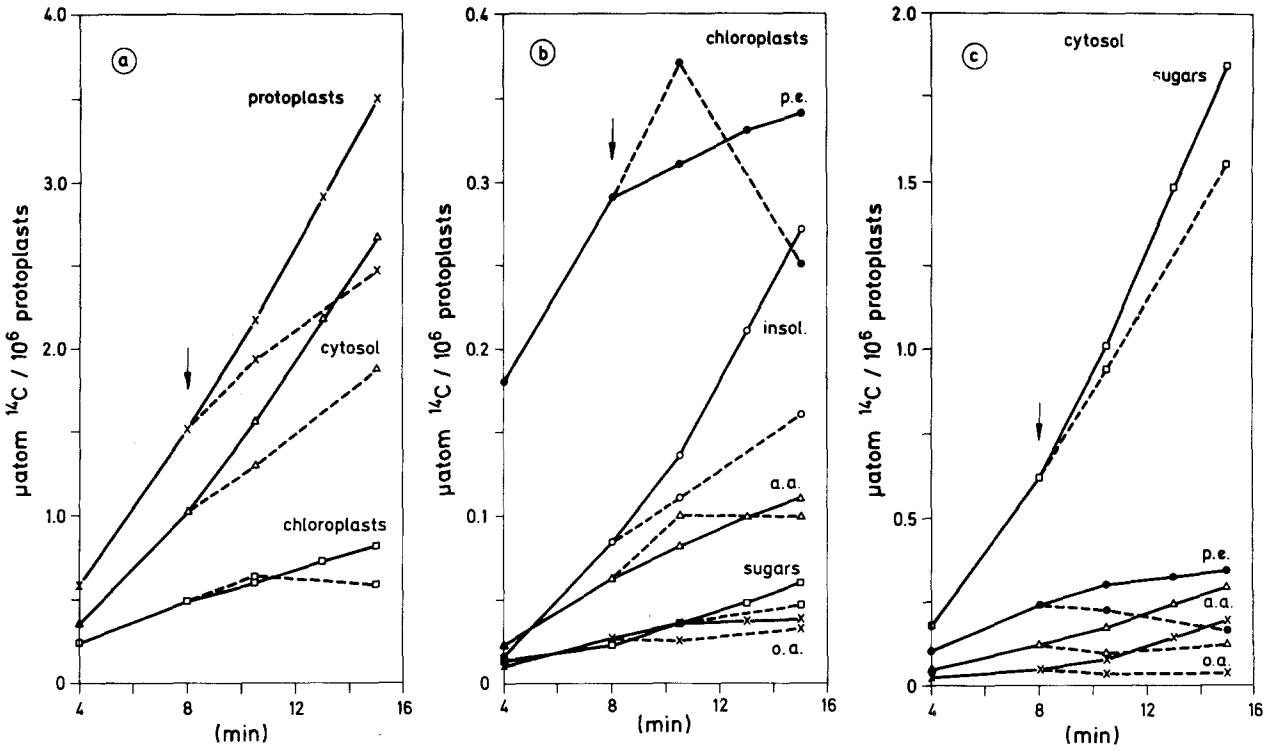

Fig. 3a–c. Time course of incorporation of labeled bicarbonate into different subcellular and metabolite fractions under continuous illumination. *Solid lines*, controls; *dashed lines*, addition of pyridoxal phosphate (final concentration 2.4 mM) after 8 min of light treatment (*arrows*). **a** Total label associated with intact protoplasts (×), chloroplasts (□), and the extrachloroplast space, corrected for mitochondria (△). **b** Fractionation of chloroplast-associated label into phosphate esters (p.e.; ●), insolubles (○), sugars (□), amino acids (a.a.; △), and organic acids (o.a.; ×). **c** Fractionation of extrachloroplast label; symbols as in **b**. Values are means of three independent experiments

to some extent true for amino acids. In contrast, label is reduced in the organic-acid fraction and in the insoluble compounds. Sugars do not show any changes during this period. Incubation times longer than 2.5 min lead to a sharp reduction in phosphate-ester label, no further increase in amino acids, a smaller increase in sugars and a slightly enhanced level of organic acids. The formation of starch, however, is still linear although with a much smaller rate than in the control.

The transient increase of label in stromal phosphate esters runs parallel to an immediate reduction in the cytosolic phosphate-ester label – for about the same absolute amount (Fig. 3c). Quantitatively, a comparable pattern can be observed for amino acids. The label in organic acids, on the other hand, exhibits a reduction in both compartments. Sugars show only a rather limited effect with respect to the cytosolic labeling, the absolute levels being about four times of those within the stroma. Although there was a rather small effect of PLP on total labeling, when applied at the beginning of a 2-min dark interval (Fig. 2), changes in the distribution of radioactivity between stroma and cytosol, as well as between the four groups of metabolites in question, are quite pronounced. Figure 4a gives a comparison of the total fixed label contained in intact protoplasts, chloroplasts and in the extrachloroplast space (corrected for mitochondria) under these experimental conditions. During the 2-min dark period, there is a retention of chloroplast-associated label under PLP treatment compared with a slight decrease within the extra-chloroplast space. This is in contrast to the control; here the dark interphase is characterized by an export of label from the chloroplast stroma toward the cytosol. This difference is mainly caused by the labeling of phosphate esters (Fig. 4b, c). In the absence of PLP, phosphate-ester-associated label largely decreases upon switching off the light, accompanied by an increase of label in the other compounds. With PLP present, however, label in stromal phosphate esters stays at a high level, whereas that in the extrachloroplast space is even lower than in the control. In addition, there is an increase in starch labeling in the dark. Stromal radioactivity, identified in sugars, organic acids and amino acids, shows no

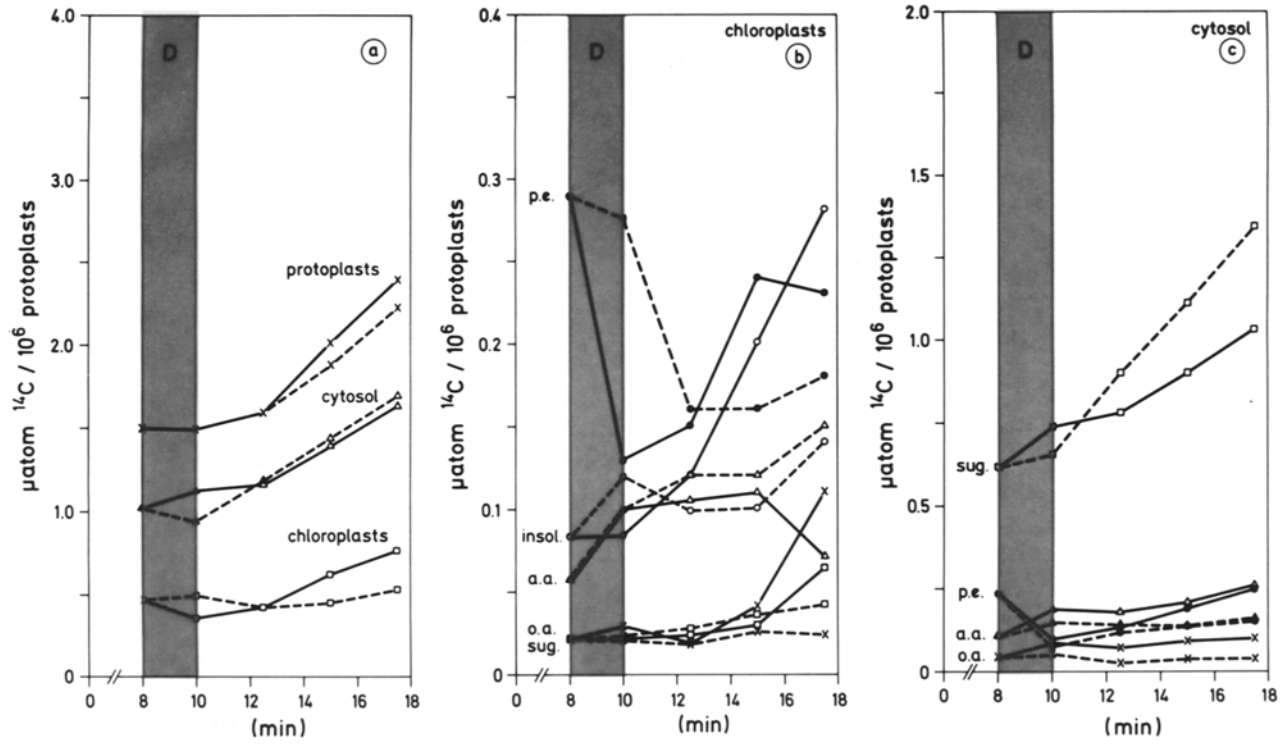

Fig. 4a–c. Time course of incorporation of labeled bicarbonate into different subcellular and metabolite fractions. Protoplast suspensions were incubated for 8 min in continuous light, followed by a 2-min dark interval (*D*) and a second illumination period. *Solid lines*, control; *dashed lines*, +pyridoxal phosphate (final concentration 2.4 mM; added at the beginning of the dark treatment). **a** Total label associated with intact protoplasts (×), chloroplasts (□) and the extrachloroplast space, corrected for mitochondria (△). **b** Fractionation of chloroplast-associated label into phosphate esters (p.e.; ●), insolubles (○), sugars (□), amino acids (a.a.; △), and organic acids (o.a.; ×). **c** Fractionation of extrachloroplast label; symbols as in **b**. Values are means of three independent experiments

changes in this period, whereas that of the cytosol is lower compared to the control.

In the following second light treatment, incorporation of label in the control starts after a short lag, with similar characteristics in the stroma and the extra-chloroplast space (Fig. 4a). Addition of PLP decreases chloroplast label in total (Fig. 4b) while that of the extra-chloroplast space shows a corresponding increase (Fig. 4c). This observation can be explained mainly by comparing radioactivity associated with phosphate esters and sugars. Upon illumination in the presence of PLP, phosphate esters are strongly reduced in the chloroplast stroma, while the label in cytosolic sugars increases.

Discussion

As shown by Fliege et al. (1978), transport of inorganic phosphate is inhibited by PLP by forming a Schiff base with lysine residues of the translocator protein. Because of its anionic charge and from biochemical evidence (Flügge et al. 1980), the authors suggested that this compound does not readily permeate the inner envelope membrane and therefore reacts preferentially with the carrier proteins exposed to the outer surface of the inner envelope membrane. In this respect, this membrane differs from the plasma membrane, which obviously possesses some permeability toward PLP, although it can not be excluded that part of the added PLP is hydrolyzed to pyridoxal by enzymes originating from broken protoplasts. Pyridoxal causes comparable effects (not shown). There are, however, reports which demonstrate that, e.g. the inner membrane of rat liver mitochondria can be permeated by PLP via facilitated passive diffusion (Lui et al. 1982). In any case, incubation of intact mesophyll protoplasts of oat in the presence of PLP causes a characteristic alteration of their pattern of photosynthetic ^{14}C labeling. While the overall fixation rate is stepwise reduced with increasing PLP concentration, there are specific effects on the compartmented levels of label in different groups of metabolites.

At the beginning of photosynthetic CO_2 fixation, phosphate-ester-associated label is mainly contained in triose phosphates and phosphogly-

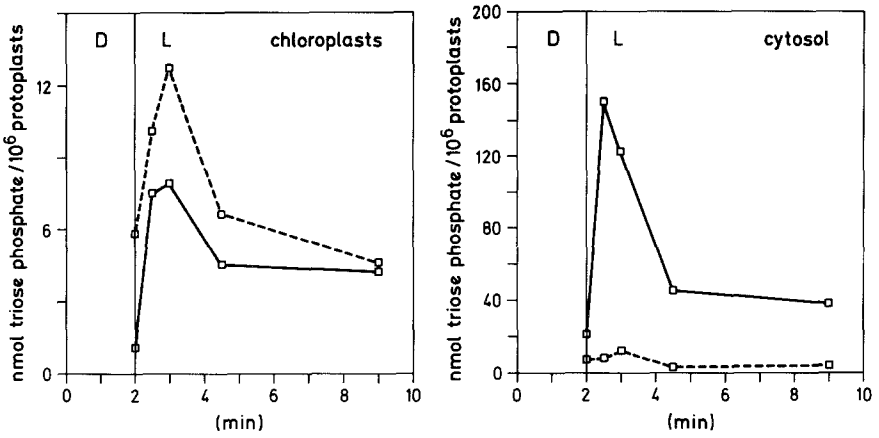

Fig. 5. Total pool sizes of acid-extractable triose phosphates within chloroplasts and cytosol of oat mesophyll protoplasts. Before illumination (*L*) protoplasts were incubated for 2 min in the dark (*D*). *Solid lines*, control; *dashed lines*, +pyridoxal phosphate (final concentration 4.8 mM; added at the beginning of the dark treatment. Fifty nmol are equivalent to a stroma concentration of about 20 mM

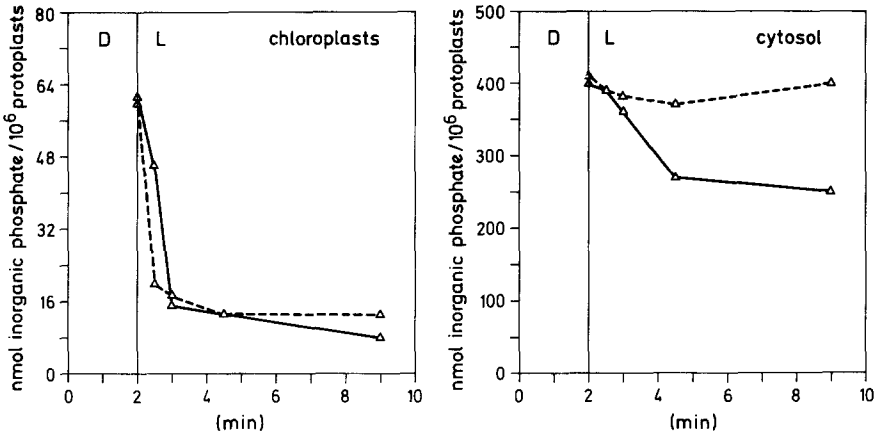

Fig. 6. Total pool sizes of inorganic phosphate within chloroplasts and the extrachloroplast space of oat mesophyll protoplasts. *Solid lines*, control; *dashed lines*, +pyridoxal phosphate. Conditions as in Fig. 5. The dark level of 400 nmol is equivalent to an overall concentration (cytosol+vacuole) of about 20 mM

ceric acid and then only increases in pentose and hexose monophosphates (Giersch et al. 1980). Experiments with isolated spinach chloroplasts showed that during induction of photosynthetic CO_2 fixation, stromal metabolites (TP, PGA) are built up and subsequently are transported to the surrounding medium (Lilley et al. 1977). This is also the case in intact protoplasts, in the form of an export from the chloroplast stroma to the cytosol (Figs. 3, 4; controls). Independently of the manner of PLP application, either under continued illumination (Figs. 2, 3) or at the beginning of a 2-min dark interval (Fig. 4), it is primarily the level of phosphate esters that is affected. They tend to be sequestered within the chloroplast stroma, with a concomittant decrease in the extrachloroplast space.

While the differences between controls and PLP-treated samples in regard to phosphate-ester labeling are reproducible but relatively small, the analysis of changes in total metabolite pools is much more convincing. Figures 5 and 6 show the pool sizes of TP and inorganic phosphate associated with the chloroplast stroma and the extrachloroplast space minus mitochondria. Illumination of controls causes, in both compartments, a transient increase in the level of TP, as the result of an accumulation in the stroma and a subsequent export to the cytosol which was followed by conversion into sucrose or 3-phosphoglyceric acid. In parallel, P_i levels in both compartments are reduced. Addition of PLP at the beginning of a 2-min preincubation in the dark causes considerable changes. Stroma levels of TP are higher by the end of the dark treatment and do not show any changes in the extrachloroplast space upon illumination. On the other hand, P_i exhibits an even faster decrease in illuminated chloroplasts without significant changes in the rest of the cell.

In isolated spinach chloroplasts, the addition of PLP similarly enhanced the formation of carboxylation products in the stroma, but strongly inhibited their release to the medium (Flügge et al. 1980). An analysis of the pattern of stromal metabolites observed in the presence of PLP (Heldt et al. 1978) showed similarities to a deficiency of inorganic phosphate in the medium.

All these findings indicate that it is the phos-

phate translocator of the inner chloroplast envelope membrane that has been selectively inhibited in our protoplast experiment. If side reactions of PLP should occur, or a reactive degradation product should be formed in the protoplast cytosol (due to an interference with lysine residues of, e.g. kinases), then these effects obviously do not interfere with the TP shuttle per se.

In intact protoplasts, optimal concentrations of stromal and cytosolic metabolites exist, enabling a maximal rate of CO_2 fixation. As the result of the counter-exchange catalyzed by the P_i translocator, the sum of P_i and phosphate esters tends to come to a more or less constant level. In the presence of PLP, this counter-exchange is severely affected (Figs. 3–6): phosphate esters transiently accumulate in the stroma and are reduced in the cytosol. When PLP is applied at the beginning of a dark interval, the retention of phosphate esters in the stroma gives rise to a continued formation of starch, whereas there is none in the control (Fig. 4b). This should be due to a stimulation of starch formation by a high 3-phosphoglyceric acid/P_i ratio (Heldt et al. 1977), which has been reported to activate ADP glucose phosphorylase, the enzyme controlling starch synthesis (Preiss and Levi 1977).

As consequence of the inhibited translocator activity, chloroplasts suffer from a P_i deficiency (Fig. 6), which results in a reduced rate of CO_2 fixation in the light. This also affects the regulation between starch formation and degradation. Starting a second period of illumination after a dark interval causes an immediate increase in starch labeling in the control, compared with a decrease in PLP-treated protoplasts. In parallel, there is an accumulation of sugars in the cytosol (Fig. 4b, c) which is probably caused by an enhanced amylolytic starch degradation under P_i deficiency in the chloroplast stroma in the light.

For isolated chloroplasts, Stitt and Heldt (1981) have shown that under illumination, starch formation and degradation occur simultaneously, mainly regulated by the extent of triose-phosphate exchange against external P_i. In this system, starch accumulation is enhanced when export of triose phosphate in exchange for P_i is restricted (Heldt et al. 1977). The depletion of the chloroplast stroma of P_i in the light followed by the addition of PLP leads to a reduction in the level of phosphate esters (Fig. 4b). As a consequence there should now be a switch from phosphorolytic to hydrolytic starch breakdown as reported for isolated spinach chloroplasts (Stitt and Heldt 1981). This will result in higher levels of neutral compounds (e.g., sugars). As shown in Fig. 4b, stroma-localized sugars increase slightly over control values at the beginning of the second light period. In addition, cytosolic sugar labeling is considerably increased (Fig. 4c). This can be taken as evidence for an enhanced hexose export from the chloroplast stroma, possibly by means of a glucose transporter as characterized by Schäfer et al. (1977).

The important role of the phosphate translocator in regulating the exchange of photosynthetic products between the chloroplast and the extra-chloroplast spaces (i.e. the regulation of starch formation in stroma and cytosolic sucrose synthesis), as well as its importance in exporting reducing equivalents, in combination with the ability of specific inhibitors to permeate the plasma membrane of intact protoplasts, opens interesting aspects for the investigations of intracellular regulation.

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