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Development and Intracellular Distribution of Enzymes of the Oxidative Pentose Phosphate Cycle in Radish Cotyledons[1]

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ABSTRACT

Developmental and compartmentation studies were used to evaluate the relative roles of the oxidative pentose phosphate cycle, the Calvin cycle, and the glycolysis in cotyledons of radish (*Raphanus sativus* L.).

Glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase, glucose-6-P isomerase, and the NAD-dependent glyceraldehyde-3-P dehydrogenase were present in high activity in ungerminated seeds, increased about 2-fold during germination in the dark, and were slightly enhanced by light. In contrast, NADP-dependent glyceraldehyde-3-P dehydrogenase was developed to only a small degree in the dark, but increased severalfold in continuous white or far red light. The activity of phosphofructokinase was low throughout germination.

The separation of cell compartment-specific isoenzymes showed that, except in ungerminated seeds, the plastid enzyme accounted for 40 to 45% of the total activity of 6-P-gluconate dehydrogenase and for 15 to 20% of glucose-6-P isomerase. The remaining activity was due to the cytosolic isoenzymes. The presence of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase in plastids was also established by their presence in the isolated organelle. The NAD-dependent glyceraldehyde-3-P dehydrogenase was mostly due to the cytosolic isoenzyme, whereas the NAD-dependent activity associated with the NADP-dependent glyceraldehyde-3-P dehydrogenase was very small.

The data indicate that the enzymes of the oxidative pentose phosphate cycle are present in the cytosol throughout germination. In the plastids these enzymes already became fully developed during early germination in the dark, whereas enzymes of the Calvin cycle increased only in the light. Glycolysis seemed to be of minor importance.

Enzymes involved in the sugar phosphate metabolism of plants have been found in two subcellular compartments, the chloroplasts and the cytosol (15, 25). Only enzymes specific for the Calvin cycle were restricted to the chloroplasts (12, 16, 27). When enzyme activities are present in both cell compartments, they are due to isoenzymes, one located in the chloroplasts and the other in the cytosol (1, 2, 4). This distribution also applies to the two key enzymes of the oxidative pentose phosphate cycle, glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase (11, 25, 26).

At present only little evidence is available about the intracellular distribution of enzymes in etiolated plant tissues, since most work has been performed using green leaves. In general, one can assume the same intracellular distribution of enzymes in etiolated tissues as in green leaves. In addition to changing levels of total enzyme activities in etiolated tissues (10, 28) it is possible that the quantitative distribution may be different in the two cellular compartments. Previous studies have measured only total enzyme activities from crude extracts, so that the extent of compartmentation of enzyme activities in the plastids and in the cytosol was not evaluated.

We chose cotyledons of germinating radish seedlings to investigate the intracellular distribution of enzymes of the oxidative pentose phosphate cycle in an etiolated tissue. The cotyledons can be obtained in an etiolated form when grown in the dark and are transformed to a green, photosynthetic form when exposed to light. Advantage has been taken of quantitative methods which we have recently developed (22, 23, 25) to separate two isoenzymes each of 6-P-gluconate dehydrogenase, glucose-6-P isomerase, and glyceraldehyde-3-P dehydrogenase, one isoenzyme specific for the activity in the plastids and the other for the activity in the cytosol. This was performed at different stages of development and, consequently, the relative activities in the plastids and in the cytosol could be calculated. In addition, we have established the presence of the key enzyme activities of the oxidative pentose phosphate cycle in the plastids isolated in sucrose density gradients by isopycnic centrifugation.

MATERIALS AND METHODS

Material and Growth Conditions. About 170 radish seeds (*Raphanus sativus* L.) were germinated on four layers of chromatography paper, well moistened with deionized H_2O in transparent plastic boxes (250 × 250 × 50 mm). The seedlings were incubated at 25 C continuously in the dark or transferred to 3000 lux of white light or to 1.3 w m^{-2} of far red light after 2 days of germination in the dark. It was necessary to add additional water after 2 days of germination to avoid water deficiency.

Preparation of Crude Extracts. Cotyledons of 50 seedlings were homogenized vigorously with mortar and pestle in 3 ml of 0.2 M potassium phosphate pH 8.6 and 10 mM dithioerythritol in the presence of 1.5 g of sea sand. The homogenate was transferred into centrifuge tubes using an additional 7 ml of buffer for rinsing. After centrifuging for 40 min at 48,000*g* the aqueous interface was decanted and used either for the determination of total enzyme activities or for the separation of isoenzymes. This and all other procedures were carried out at 4 C.

Isolation of Cell Organelles. About 10 g of radish cotyledons were gently homogenized in 30 ml of grinding buffer, squeezed through two layers of Miracloth, and layered onto a gradient of 25 to 60% sucrose. After centrifuging for 3 hr at 109,000*g* in an SW 25.2 rotor of a Beckman L3-50 ultracentrifuge at 4 C the gradients were fractionated from the bottom. All details have been published in a previous paper (24).

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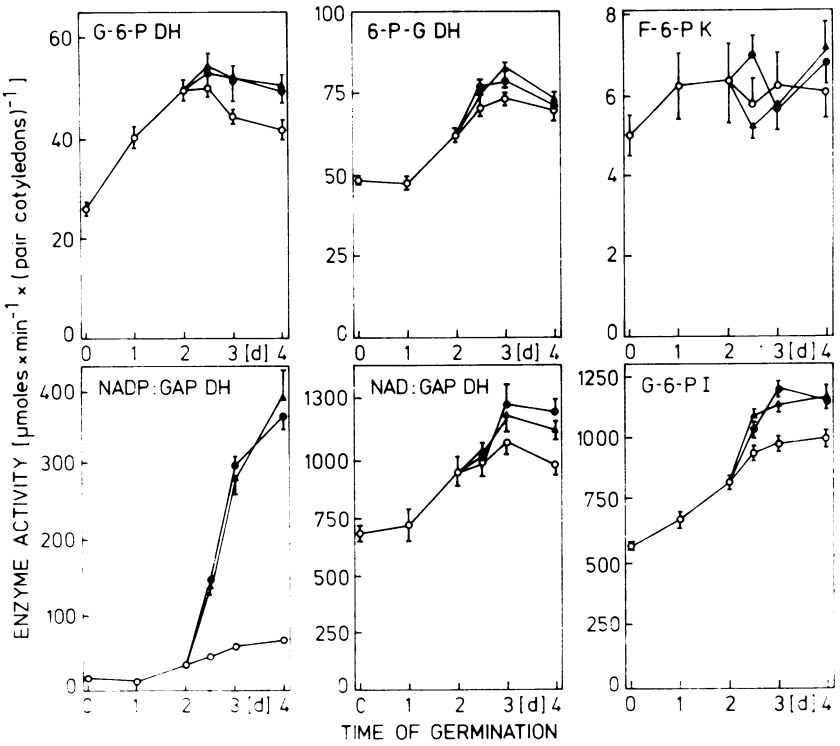

FIG. 1. Time course of some enzyme activities in radish cotyledons during the germination in continuous dark (○) and when transferred to white (●) or far red (▲) light after 2 days of germination in the dark. G-6-P DH: Glucose-6-P dehydrogenase; 6-P-G DH: 6-P-gluconate dehydrogenase; PFK: Phosphofructokinase; NADP:GAP DH and NAD:GAP DH: NADP-dependent and NAD-dependent glyceraldehyde-3-P dehydrogenase; G-6-P I: glucose-6-P isomerase.

$(NH_4)_2SO_4$ Gradient Solubilization. After the addition of 3 g of Hyflo Super Cel, proteins of a crude extract were precipitated by adding solid $(NH_4)_2SO_4$ to 80% saturation. The slurry was packed into a glass column 2 cm in diameter. The proteins were solubilized and eluted by a gradient of 200 ml 80 to 0% $(NH_4)_2SO_4$ in 0.2 M potassium phosphate, pH 7.5. The flow rate was 15 ml/hr^{-1} and fractions of 3 to 4 ml were collected.

DEAE-Cellulose Ion Exchange Chromatography. When this method was used, crude extracts were prepared in 0.1 instead of 0.2 M potassium phosphate. DE-32-cellulose (Whatman) was packed into a column 2 cm in diameter and 15 cm in length, and was equilibrated with a buffer consisting of 10 mM potassium phosphate pH 8.6 and 10 mM dithioerythritol. The crude extract was then diluted with deionized H_2O containing 2 mM dithioerythritol so that its conductivity was equivalent to a 20 mM KCl solution. After loading to the resin the column was washed with 50 ml of equilibrating buffer and the proteins were eluted with a gradient of 400 ml of 0 to 0.4 M KCl in the same buffer collecting 5-ml fractions.

Assays. Enzyme assays were performed at 25 C and recorded on a Zeiss PM 4 spectrophotometer with a 6-cuvette automation. All enzyme activities from crude extracts were determined within 9 hr after homogenization.

Glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were assayed following the reduction of NADP at 340 nm (25). For the determination of these activities in crude extracts it was necessary to adjust the assay buffer to pH 8 since at higher pH values, some precipitating material interfered with the assay. Glucose-6-P isomerase was measured with fructose-6-P as substrate and coupled to glucose-6-P dehydrogenase (23). Phosphofructokinase was coupled to fructose-diP aldolase, triose-3-P isomerase, and glycerol-P dehydrogenase (18). The assay mixture consisted of 20 mM tris/HCl, pH 8, 2 mM ATP, 15 mM $MgCl_2$, 5 mM dithioerythritol, 0.125 mM NADH, 1 unit per ml of each coupling enzyme, and 1.5 mM fructose-diP. Glyceraldehyde-3-P dehydrogenase was tested with both NADH and NADPH, generating glycerate-1,3-diP with phosphoglycerate kinase (12). Malate dehydrogenase and catalase were tested as previously described (24). Protein was determined according to Lowry (19), Chl according to Arnon (6), KCl concentrations conductometrically, and $(NH_4)_2SO_4$ concentrations with a refractometer.

RESULTS

Total Activity of Some Enzymes during Course of Germination. To evaluate the developmental regulation of sugar phosphate pathways in radish cotyledons the activity of some enzymes has been followed through germination (Fig. 1). The plants were grown continuously in the dark. After 2 days of germination some plants were transferred either to white light in order to induce all components of the photosynthetic apparatus, or to far red light to investigate only the morphogenic influence of phytochrome without the interference of photosynthesis.

The NADP-dependent glyceraldehyde-3-P dehydrogenase was present in very low activity in ungerminated seeds. This enzyme activity increased 5-fold during the germination in the dark. Irradiation on the 3rd and 4th day with either white or far red light enhanced this enzyme activity 7-fold above the dark control.

In contrast, glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were already high in the ungerminated seeds. They increased about 1.5- to 2-fold during the germination in the dark. Irradiation with white or far red light had a slightly stimulating effect.

The activities of glucose-6-P isomerase and NAD-dependent glyceraldehyde-3-P dehydrogenase showed a time course of development which was similar to that observed for the two

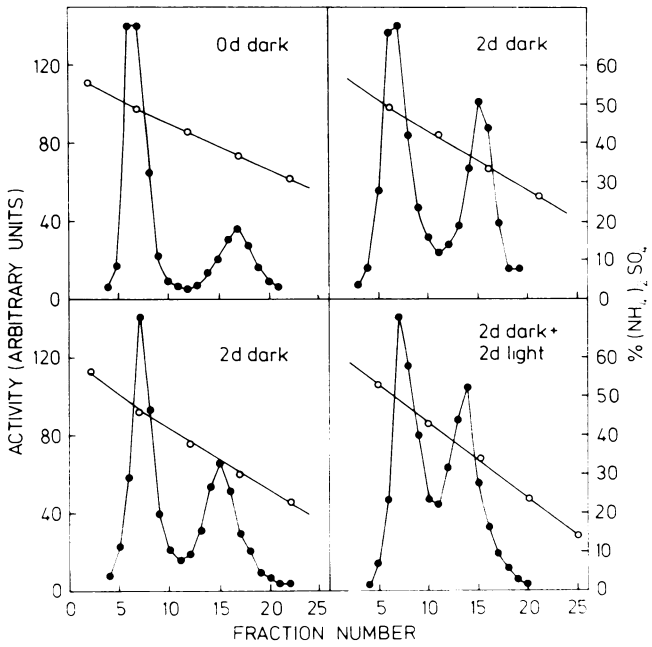

FIG. 2. Separation of 6-P-gluconate dehydrogenases (●) by $(NH_4)_2SO_4$ gradient solubilization at various times of germination. The left peak represents the cytosolic isoenzyme and the right peak the plastid isoenzyme.

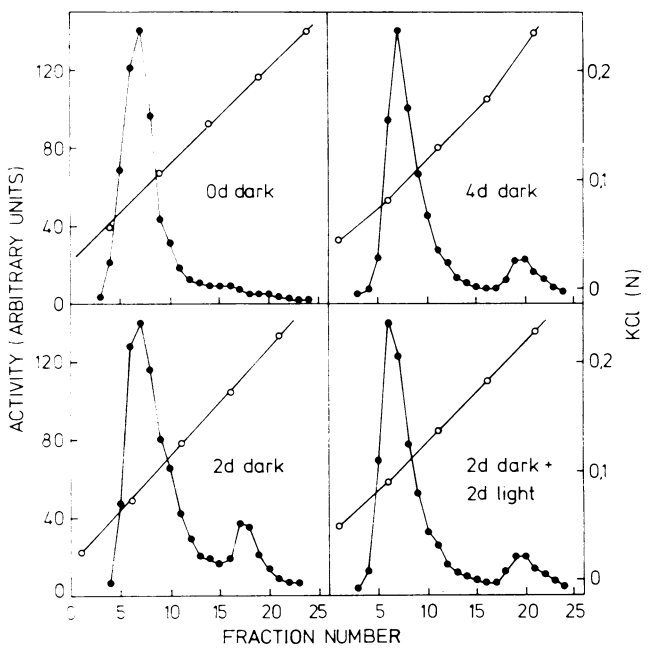

FIG. 3. Separation of glucose-6-P isomerases (●) by DEAE-cellulose ion exchange chromatography at various times of germination. The left peak represents the cytosolic isoenzyme and the right peak the plastid isoenzyme.

enzymes of the oxidative pentose phosphate cycle. Phosphofructokinase was present in ungerminated seeds in very low activity and this did not change greatly during the germination in the dark or in the light.

If crude extracts were desalted on Sephadex G-25, only 40 to 70% of the activity was recovered for glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase, phosphofructokinase, and NADP-dependent glyceraldehyde-3-P dehydrogenase, but virtually 100% for glucose-6-P isomerase and NAD-dependent glyceraldehyde-3-P dehydrogenase. The lower values of some enzyme activities were disregarded as artificial due to inactivation.

The substitution of phosphate buffer by tris-HCl buffer had little effect on enzyme activities, when measured directly after homogenization. Phosphate buffer was far more favorable for the stability of most enzyme activities. As a typical example, the activity of glucose-6-P dehydrogenase disappeared to more than 90% within 20 hr after homogenization in tris/HCl with and without dithioerythritol (50 mM), to more than 80% in phosphate buffer without dithioerythritol, and to only 60% in phosphate buffer including dithioerythritol. The presence or absence of 50 mM dithioerythritol or dithiothreitol in the grinding medium had no effect on the activity of phosphofructokinase, but the presence of either of these compounds reduced the activity of glucose-6-P dehydrogenase by 20% when the seedlings were grown for more than 2 days either in the dark or in the light.

Separation of 6-P-Gluconate Dehydrogenases. Isoenzymes of 6-P-gluconate dehydrogenase have been separated at various times of germination by $(NH_4)_2SO_4$ gradient solubilization (Fig. 2). There were always two peaks of activity present. The peak at high $(NH_4)_2SO_4$ concentration has previously been shown to belong to the cytosolic isoenzyme and the other to the chloroplast isoenzyme of spinach leaves (25). After the separation of these two isoenzymes it was possible to estimate the proportion of 6-P-gluconate dehydrogenase in the plastids and in the cytosol of radish cotyledons. At all stages of development the isoenzyme of the cytosol contributed most to the total activity of 6-P-gluconate dehydrogenase. The plastid isoenzyme accounted for 40 to 45% of the total activity after 2 days or 4 days of germination either in the dark or in the light. In ungerminated seeds the plastid isoenzyme represented only in the order of 30% of the total activity.

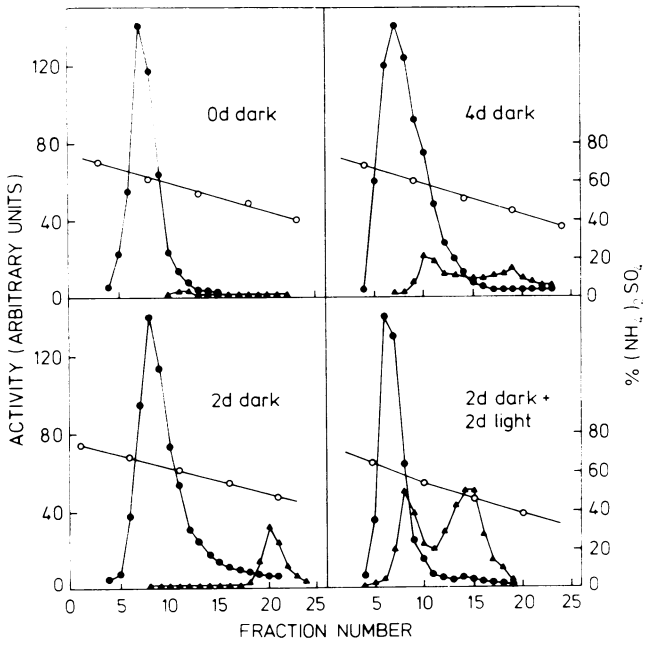

FIG. 4. Separation of NAD- (●) and NADP- (▲) dependent glyceraldehyde-3-P dehydrogenases by $(NH_4)_2SO_4$ gradient solubilization. The main NAD-dependent peak represents the cytosolic enzyme and the NADP-dependent activity is plastid specific. The activity shown on the figure represents the actual activity for the NAD-dependent activity and 10 times the actual activity for the NADP-dependent activity.

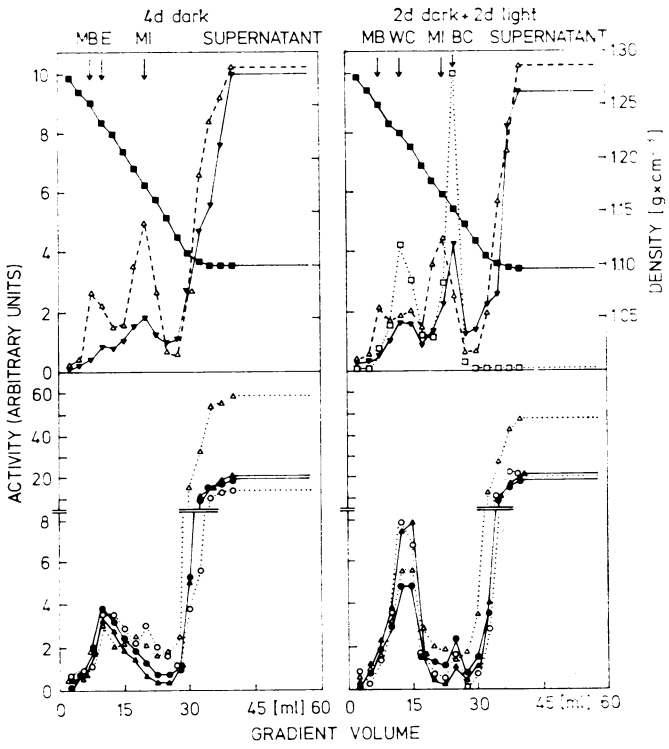

FIG. 5. Isolation of cell organelles from dark- and light-treated cotyledons in sucrose density gradients after isopycnic centrifugation. Markers used were the peak of malate dehydrogenase(△---△) at the highest sucrose density (■——■) for microbodies (MB), the main particulate peak of malate dehydrogenase for mitochondria (MI), the main peak of chlorophyll (□····□) for broken chloroplasts (BC), and the particulate activities of the NADP-dependent (○····○) and NAD-dependent (△····△) glyceraldehyde-3-P dehydrogenase for etioplasts (E) and whole chloroplasts (WC). Glucose-6-P dehydrogenase (●——●) and 6-P-gluconate dehydrogenase (▲——▲) are present in both isolated etioplasts and whole chloroplast protein (▼——▼).

Separation of Glucose-6-P Isomerases. Two isoenzymes of glucose-6-P isomerase could also be found in radish cotyledons (Fig. 3). The cytosolic isoenzyme eluting at low salt concentration contributed more than 80% to the total activity of glucose-6-P isomerase. The plastid isoenzyme accounted for about 15% after 2 days of germination and thereafter. In ungerminated seeds the activity of the plastid isoenzyme was hardly detectable.

Separation of Glyceraldehyde-3-P Dehydrogenases. Glyceraldehyde-3-P dehydrogenase activity from spinach leaves can be separated into one NAD- and two NADP-dependent activities by $(NH_4)_2SO_4$ gradient solubilization, the latter two having also a NAD-dependent activity (C. Schnarrenberger, unpublished). In radish cotyledons (Fig. 4) the main activity was due to the cytosolic NAD-dependent activity which was solubilized at high $(NH_4)_2SO_4$ concentration. The NAD-dependent activity was solubilized at lower $(NH_4)_2SO_4$ concentrations and resolved into two peaks which are two forms of the plastid enzyme. The NADP-dependent activity appeared only after 2 days of germination and only in the one form. During prolonged germination in the dark or in the light this activity was found in both forms. The NAD-dependent activity of the NADP-dependent glyceraldehyde-3-P dehydrogenase was very low and could hardly be detected on the gradients because the high activity of the cytosolic NAD-dependent glyceraldehyde-3-P dehydrogenase trailed too far into the region of the NADP-dependent enzyme.

Enzyme Activities in Cell Organelles Isolated in Sucrose Gradients. Further evidence for the intracellular location of enzymes of the oxidative pentose phosphate cycle was obtained from the distribution of enzyme activities in sucrose density gradients after isolation of cell organelles by isopycnic centrifugation (Fig. 5). Markers used for cell organelles were as follows: the peak of malate dehydrogenase at the highest sucrose density for microbodies, the main particulate peak of malate dehydrogenase for mitochondria, the major peak of Chl for broken chloroplasts, the particulate activity of NADP-dependent glyceraldehyde-3-P dehydrogenase for whole plastids, and the high enzyme activities on the top of the gradient for soluble or solubilized enzyme activities. The NADP- and NAD-dependent glyceraldehyde-3-P dehydrogenase, glucose-6-P dehydrogenase, and 6-P-gluconate dehydrogenase were all present in the particulate fraction of the plastids of both dark- and light-treated cotyledons. When compared with the activity of the NADP-dependent glyceraldehyde-3-P dehydrogenase, the high activity of NAD-dependent glyceraldehyde-3-P dehydrogenase, glucose-6-P dehydrogenase, and 6-P-gluconate dehydrogenase in the supernatant fraction are highly indicative of the presence of cytosolic isoenzymes in this fraction.

DISCUSSION

Information can be obtained about the regulatory processes in cotyledons of germinating seedlings by following the level of enzyme activities. For this purpose radish seedlings were grown in complete darkness to obtain them in an etiolated form. Some of them were transferred to white light to induce autotrophic growth or to far red light to investigate the morphogenic modulation by phytochrome without the interference of photosynthesis. By this type of experimental device the enzyme activities of the oxidative pentose phosphate cycle and of the Calvin cycle could easily be distinguished by their activity levels in the dark and in the light. Glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase, the two key enzymes of the oxidative pentose phosphate cycle, reached high levels of activity in the dark and were only slightly stimulated by light. The NADP-dependent glyceraldehyde-3-P dehydrogenase, a Calvin cycle enzyme, was developed to only low activity in the dark, but rose several-fold during irradiation with white or far red light. A special feature of the two enzymes of the oxidative pentose phosphate cycle seemed to be the very high activity already present in the ungerminated seeds. Similar observations for these two types of enzymes were already known from studies with other plant cells (8, 10, 15, 21).

In spinach (13, 17) and in pea plants (3, 5), glucose-6-P dehydrogenase has been reported to be inactivated by light and by dithiothreitol. The inactivation by light could not be observed by us in radish cotyledons, although we could confirm an inhibitory effect by dithiothreitol or dithioerythritol. This latter inhibition was observed with light-treated and dark-grown plants, if they were older than 2 days. Thus, our data with radish cotyledons do not support the suggestion that dithiothreitol may simulate the inhibition by light, as has been previously discussed by some authors (3, 5). Also, a reported inactivation (14) by dithiothreitol or light of phosphofructokinase could not be observed by us.

In order to determine quantitatively the intracellular compartmentation of enzymes of the oxidative pentose phosphate cycle in etiolated cotyledons, we separated the two isoenzymes of 6-P-gluconate dehydrogenase from the plastids and the cytosol. This showed that 40 to 45% of the total activity of 6-P-gluconate dehydrogenase is located in the plastids and the other part in the cytosol. Since the activity of the NADP-dependent glyceraldehyde-3-P dehydrogenase is low in etioplasts, the oxidative pentose phosphate cycle may have to be considered as the major sugar phosphate pathway in the etioplasts with respect to the

Calvin cycle. A reversal of this situation only occurs during irradiation with light.

Furthermore, the activities of glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were also found in isolated plastids from both light-treated and dark-grown plants. This also indicates that the oxidative pentose phosphate cycle is definitely already present in the etioplasts and confirms the conclusion drawn from the separation of isoenzymes of 6-P-gluconate dehydrogenase. The high activity in the supernatant of the sucrose gradients was indicative of cytosolic activities when compared to the activity profile of the NADP-dependent glyceraldehyde-3-P dehydrogenase. Particulate enzyme activities of the oxidative pentose phosphate cycle have been reported previously from etiolated tissues, also associated with other cell organelles such as mitochondria (7) or oxidative particles (9, 20). These activities may have been due also to etioplasts or proplastids.

In comparison to enzyme activities of the oxidative pentose phosphate cycle, the activity of phosphofructokinase, a marker enzyme of glycolysis, was low throughout germination. Since the enzymes of the oxidative pentose phosphate cycle increased during germination, the ratio of activity between the two pathways would change in favor of the oxidative pathway during germination.

In addition to the enzyme activities so far discussed, we also investigated the glyceraldehyde-3-P dehydrogenases in radish cotyledons. Here, the activity is mainly due to the cytosolic NAD-dependent isoenzyme, and the concomitant activity with NADP-dependent enzyme in the plastids is almost negligible.

Glucose-6-P isomerase shows a time course of activity during germination similar to the enzymes of the oxidative pentose phosphate cycle. The separation of compartment specific isoenzymes clearly demonstrated that the plastid enzyme was fully developed during the early germination, even though the cytosolic isoenzyme always accounted for more than 80% of the total activity. Thus, in addition to glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase, other enzyme activities also seem to be present in the etioplasts and add up to the enzyme complement of the oxidative pentose phosphate cycle in this cell organelle.

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