Inhibition of Photosynthetic Carbon Dioxide Fixation in Isolated Spinach Chloroplasts Exposed to Reduced Osmotic Potentials

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ABSTRACT

Reduced osmotic potentials inhibited the rate of CO₂ fixation by isolated intact spinach (Spinacia oleracea) chloroplasts. This inhibition was observed immediately after transfer of chloroplasts from a solution containing 0.33 M sorbitol to higher sorbitol concentrations, and the depressed rate remained constant. The inhibited CO₂ fixation could not be attributed to a decreased rate of photosynthetic electron transport, since NADP reduction was unaffected by subjecting the chloroplasts to low potentials. It could also not result from restricted permeability to CO₂, as CO₂ concentrations had no effect on the relative inhibition induced by the lowered potential.

A procedure was developed for the determination of several enzymes of the photosynthetic carbon reduction cycle in the intact chloroplast without their being extracted. The activities of the combined three enzymes: ribose-5-phosphate isomerase, ribulose-5-phosphate kinase, and ribulose-1,5-diphosphate carboxylase and of ribulose-1,5-diphosphate carboxylase alone were found to be inhibited at low osmotic potentials. Analysis of the photosynthetic products showed that the formation of glycerate-3-phosphate was inhibited to a greater extent than the formation of all other products.

CO₂ fixation was partly resumed when chloroplasts were returned from a 0.67 M sorbitol to a 0.33 M sorbitol solution, regardless whether the transfer occurred in the light or in the dark.

The hydration level of the cytoplasmatic ultrastructure in which photosynthesis takes place may play an important role in photosynthesis reduction under decreased leaf water potential (8, 13, 14). If this reduced photosynthetic rate is controlled by chloroplasts, an inhibition in the photosynthetic activity of isolated chloroplasts should occur under reduced leaf water potentials. Sanatarius and Ernst (11), however, could not show depression of the Hill reaction with chloroplasts dehydrated by means of concentrated sugar solutions corresponding to a 90% water loss. Similarly, the reduction of NADP was hardly inhibited as long as water loss from leaves was less than 80% of that of saturation (12). Recently, Fry (4) demonstrated that water potentials of cotton leaves below −28 bars reduced about 50% the Hill activity of chloroplasts isolated from those leaves. An inhibition of O₂ evolution by chloroplasts of sunflower or pea leaves which were more moderately desiccated was shown by Boyer and Bowen (3). Even if water deficits have only a slight effect on photochemical activity in isolated chloroplasts, the restricted photosynthesis of intact plants under water stress may be due to inhibited CO₂ fixation. The purpose of the present work was to investigate the response of CO₂ fixation by isolated chloroplasts to reduced osmotic potentials. As the method of Jensen and Bassham (6)—which is based on the use of a buffered sorbitol solution at a concentration of 0.33 M—was used for chloroplast isolation, increased concentrations of sorbitol in the media thus may be used for the study of CO₂ fixation under reduced potentials.

MATERIALS AND METHODS

Spinach (Spinacia oleracea) plants were grown in an aerated, half-strength Hoagland solution at 24 C and 50 to 60% relative humidity under a 10-hr photoperiod. Light intensity was approximately 1000 ft-c at the level of the upper leaves. Expanded leaves were picked and floated on tap water at the same light intensity for about 1 hr before use. Chloroplast isolation was based on the method of Jensen and Bassham (6) with slight modification (10).

CO₂ fixation was initiated by adding aliquots of chloroplast suspensions containing 50 to 100 μg of chlorophyll to a 2-ml reaction mixture containing 5 μmoles of NaH¹⁴CO₃ unless otherwise indicated. Fixation was carried out in glass tubes, at 20 C, illuminated from opposite sides at an intensity of 2000 ft-c. Reaction mixtures were gently bubbled with N₂ or air before and during the experiments. Different osmotic potentials were obtained by varying the sorbitol concentration between 0.33 M and 1.00 M (equivalent to 8 and 24 bars, respectively, as measured cryoscopically) either in the isolation and suspension media or in the final reaction mixture. CO₂ fixation rates and products formation were determined as described in an earlier paper (10).

Activity of several enzymes of the photosynthetic carbon reduction cycle was determined either in a crude chloroplast extract or directly in the intact organelle. Extracts were prepared after exposure of chloroplasts to light for 6 min in a reaction mixture containing different sorbitol concentrations and unlabeled NaHCO₃. After centrifugation at 2000g for 40 sec, the supernatant fluid was discarded and the chloroplasts were ruptured in a solution containing 10 μmoles of tris-HCl, pH 7.8. The suspension was centrifuged again at 12,000g and the supernatant liquid was retained for the determination of enzyme activity. The assays of ribulose-1,5-diP carboxylase and ribulose-5-P kinase were based on determination of ¹⁴CO₂ fixation rates by the extracts (5). For the assay of ribulose-1,5-diP carboxylase, the following were added in a volume of 0.6 ml: 200 μmoles of tris-HCl buffer, pH 7.8; 10 μmoles of MgCl₂; 50 μmoles of NaH¹⁴CO₃ (specific radioactivity 0.5

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Table I. Activity of the Combination of Ribose-5-P Isomerase, Ribulose-5-P Kinase and Ribulose-1,5-diP Carboxylase and of Ribulose-1,5-diP Carboxylase alone Determined at Different Conditions without Extraction from Chloroplasts

Chloroplasts were prepared and transferred to 1-ml reaction mixtures as described in Figure 1. NaHCO$_3$ was excluded from the mixtures and the sorbitol concentration was 0.25 M. Chloroplasts were preincubated in these mixtures under different conditions for 4 min at 25°C. The reaction was then initiated by the addition of 1 ml of 0.25 M sorbitol solution containing 5 μmoles of NaH$_2$CO$_3$ and was carried out in darkness. Chlorophyll concentrations were 36 μg/ml in experiment 1 and 39 μg/ml in experiment 2.

<table>
<thead>
<tr>
<th>Experiment No</th>
<th>Condition during Preincubation</th>
<th>Additions to Preincubation Medium</th>
<th>μC Incorporated during Initial 3 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Light</td>
<td>2 μmoles of ribose-5-P</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>8 μmoles of ATP</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>2 μmoles of ribose-5-P + 8 μmoles of ATP</td>
<td>2070</td>
</tr>
<tr>
<td>2</td>
<td>Light</td>
<td>2 μmoles of ribose-5-P + 8 μmoles of ATP</td>
<td>1660</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>2 μmoles of ribose-5-P + 8 μmoles of ATP</td>
<td>620</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>2 μmoles of ribulose-1,5-diP</td>
<td>9986</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>2 μmoles of ribulose-1,5-diP</td>
<td>9710</td>
</tr>
</tbody>
</table>

μC/μmole; 2 μmoles of GSH; 0.1 ml of extract; and 1 μmole of ribulose-1,5-diP (added last). Ribulose-5-P kinase, which was usually determined in combination with ribose-5-P isomerase and ribulose-1,5-diP carboxylase, was assayed in a similar reaction mixture but containing 2 μmoles of ATP and 2 μmoles of ribose-5-P instead of ribulose-1,5-diP. Reactions were carried out at 30°C and were terminated by the addition of concentrated formic acid (6% [v/v] final concentration) to samples taken at 2-min intervals. To assess the 14C fixed, 0.1 ml of the clear supernatant fluid was counted by means of a liquid scintillation counter. In the method developed for determination of enzyme activities in the intact organelle, chloroplasts were prepared in the usual way and added to 1 ml of the CO$_2$ fixation reaction mixture from which NaHCO$_3$ was excluded. They were preincubated in the light at 25°C for 4 min in a 0.25 M sorbitol solution containing either 2 μmoles of ribose-5-P and 8 μmoles of ATP, or 2 μmoles of ribulose-1,5-diP. The mixtures were then transferred to the dark and 5 μmoles of NaH$^{14}$CO$_3$ (specific radioactivity 2.0–3.0 μC/μmole) were added with 1 ml of sorbitol solution bringing the sorbitol to different final concentrations. Activity was determined by measuring the rate of 14C incorporation.

Table I shows that the incorporation of 14C was due to enzymatic activity, since limited radioactivity was detected when either ribose 5-P and ATP or when ribulose-1,5-diP were absent. It is most likely that the added ATP supported the reaction, but mainly when added in the light, suggesting that light stimulated the penetration of ATP, and that there was little leaching of enzymes from chloroplasts. Ribulose-1,5-diP carboxylase alone was active when chloroplasts were preincubated in the dark.

NADP reduction rates were determined after transfer of the intact chloroplast preparation to a reaction mixture which contained no sorbitol, causing immediate rupture of the chloroplasts. This mixture consisted of: tris-HCl buffer, pH 7.6, 30 μmoles; MgCl$_2$, 2 μmoles; KH$_2$PO$_4$, 1 μmole; ADP, 2 μmoles; NADP, 1 μmole; ferredoxin, 2.6 units; and chloroplast fragments containing 20 to 50 μg of chlorophyll. The total volume was 2.5 ml. After illumination under the same conditions as described for CO$_2$ fixation, NADP was assayed as described by Turner et al. (15). Chlorophyll was determined by the method of Arnon (1).

**RESULTS**

When chloroplasts were prepared at the standard concentration of 0.33 M sorbitol and then transferred for CO$_2$ fixation to reaction mixtures of higher concentrations, a decrease in the CO$_2$ fixation rate was observed (Fig. 1). A rise in concentration from 0.33 M to 0.50 M (equivalent to –24 bars) reduced the fixation rate about 30%. At further concentration the increase of inhibition was relatively less marked. Chloroplasts continued to fix CO$_2$ even at a sorbitol concentration of 1.00 M (equivalent to –24 bars), suggesting that in spite of the inhibited rate, structural integrity was maintained. However, when chloroplasts were transferred to a reaction mixture lacking sorbitol hardly any fixation was found, implying destruction of the membranes and loss of enzymes (6). The decrease in fixation rate at elevated solute concentrations seems to be unrelated to factors responsible for the initial lag (2), since the reduction in fixation rates at those concentrations was observed during the lag phase.

The possibility that the restricted CO$_2$ fixation under decreased osmotic potential is reversible, was examined in two ways. (a) Chloroplasts were prepared in the usual manner, but transferred to suspension media (solution B according to Jensen and Bassham [6]) at two sorbitol concentrations for a 6 min

![Fig. 1: Effect of osmotic potentials of the CO$_2$ fixation media on CO$_2$ fixation by isolated chloroplasts. Chloroplasts were isolated and suspended according to Plaut and Gibbs (10) and transferred to 2-ml reaction mixtures of different osmotic potentials at the start of the illumination period. Potentials of 0, 8, 12, 16, and 24 atm were obtained by adding sorbitol to a final concentration of 0, 0.33, 0.50, 0.67, and 1.00 M, respectively. The reaction mixture also contained 100 μmoles of tricine adjusted with NaOH to pH 8.1; 4 μmoles of NaNO$_3$; 4 μmoles of EDTA (di-potassium salt); 2 μmoles of MnCl$_2$; 2 μmoles of MgCl$_2$, 1 μmole of K$_2$HPO$_4$; 5 μmoles of Na$_3$PO$_4$, 5 μmoles of NaH$_2$CO$_3$, and 90 μg of chlorophyll.](image-url)
preincubation period in the dark. They were then transferred to the CO₂ fixation media at identical or different concentrations as lighting was resumed (Fig. 2a). (b) The preincubation at different sorbitol concentrations was carried out in the CO₂ fixation media in light. The transfer of chloroplasts to fresh reaction mixtures resulted in dilution of the labeled products which were formed during the initial preincubation of 6 min, accounting for the drop in graphs (Fig. 2b).

A recovery in CO₂ fixation can be seen when chloroplasts were transferred from 0.67 M to 0.33 M sorbitol (Fig. 2a and b). The slopes obtained for those chloroplasts became steeper about 6 min after transfer compared with those obtained for chloroplasts which remained at the higher concentrations. While this partial recovery was only detected at least after 6 min, the inhibition in fixation when chloroplasts were transferred to higher concentrations took place immediately.

The delay in partial recovery was utilized to study whether the observed reduction in CO₂ fixation may be related to photoinhibitory electron transport in chloroplasts under reduced osmotic potentials. Since whole chloroplasts have to be fragmented in order to measure NADP reduction, their preincubation at different osmotic potentials was carried out prior to NADP reduction and CO₂ fixation measurements. Chloroplasts were isolated, resuspended, and subsequently preincubated for 4 min in the dark at different sorbitol concentrations. They were then transferred simultaneously to appropriate reaction mixtures for CO₂ fixation (containing 0.33 M sorbitol) or NADP reduction (containing no sorbitol) at identical concentrations (Table II). CO₂ fixation and NADP reduction rates were then determined before any recovery of CO₂ fixation took place. While the inhibition of CO₂ fixation was about 50%, and 90% when chloroplasts were preincubated in 0.67 M and 1.00 M sorbitol, respectively, with 0.33 M, NADP reduction was not affected.

Table III shows that the decrease in fixation rate at lowered osmotic potential cannot be attributed to a repressed ¹⁴CO₂ penetration into chloroplasts. The relative decrease in fixation rate at 0.67 M compared with 0.33 M sorbitol is unaffected by NaHCO₃ concentration either much below saturation level (experiment 1) or above this level (experiment 2). Apparent Kₘ (HCO₃⁻) values for carbon fixation at both 0.33 M and 0.67 M sorbitol were found to be in the range of 0.6 to 0.8 mM for the initial 6 min following the lag period. The similar Kₘ values at different concentrations, which are also in good agreement with those reported earlier (6), thus cannot be a factor responsible for the different fixation rates.

The distribution of photosynthetic products formed by chloroplasts which were fixing ¹⁴CO₂ at two different osmotic potentials is shown in Figure 3. Formation of glycylate-3-P, the most distinctive product in the control, was reduced by 70% at the higher sorbitol concentration. On the other hand, the formation of triose-P (consisting primarily of dihydroxyacetone-P) and sugar mono and di-phosphates was inhibited to a much smaller extent. The inhibition of glycylate-3-P formation was found in experiments carried out under N₂ when the counts found in glycylate were only 5% of the total (Fig. 3b), as well as under air, when its formation was about 23% of all the products (Fig. 3a). Synthesis of end products such...
The results show that CO₂ fixation by intact chloroplasts was reduced when the concentration of the incubation medium was increased up to 1 M sorbitol. This could be due either to a dehydration of chloroplasts or to direct inhibition by the penetration of sorbitol. While dehydration of chloroplasts may be a rapid process at hypertonic concentrations, the penetration of sorbitol would continue for a much longer time due to its larger molecules. Since the reduced fixation was detected immediately on transferring the chloroplasts to a higher sorbitol concentration, and the reduced rate was constant with time, it may be concluded that chloroplast dehydration was the predominant process. Furthermore, sorbitol concentrations below 0.25 M decreased CO₂ fixation, possibly because of partial rupture of the chloroplasts. If sorbitol had a direct inhibitory effect on assimilation of CO₂, this decrease would be hard to interpret.

The possibility that the reduced fixation in dehydrated chloroplasts may partly originate from increased internal concentration of ions due to H₂O loss is, however, not ruled out. Estimates of the extent of dehydration of fragmented chloroplasts when incubated in sugar, sorbitol, or saline solutions of different concentrations were presented by Santarius and Ernst (11) and are extremely high. Water withdrawal from cells can be considered more properly in terms of cell water potentials, matrix, and osmotic than in terms of water content. It is difficult to relate total water withdrawal from cells or organelles to dehydration of specific structures in the cell or to changes in water potential in the stroma.

The decrease in CO₂ fixation under lowered potentials was primarily associated with the inhibition of a combination of three enzymes: ribose-5-isomerase, ribulose-5-P kinase, and ribulose-1,5-diP carboxylase (Fig. 4), leading most likely to a limited generation of the CO₂ acceptor. The carboxylation site of the carbon cycle was certainly much more sensitive to decreased osmotic potential than the reduction site. This can be concluded from the remarkable response of glycerate-3-P formation compared with that of triose-P or sugar-P at lowered potentials (Fig. 3).

It was demonstrated, however, that the availability of CO₂ could not be responsible for the decrease in CO₂ fixation (Table III), and that the activity of ribulose-1,5-diP carboxylase alone was less sensitive to the lowered potential than the combination of the three enzymes. Since the activity of ribose-5-P isomerase was most likely not limited, the inhibition may be attributed mainly to ribulose-1,5-diP carboxylase and ribulose 5-P kinase. This kinase has also been found to be a control enzyme responsible for the Warburg effect in chloroplasts (7). The inhibition of the enzyme activity by O₂ was demonstrated in extracts of chloroplasts treated prior to extraction, which was not the case, however, in the present study. Enzyme extracted from chloroplasts subjected to different osmotic potentials exhibited identical activities. It may be suggested that the reduced potentials gave rise to conformational changes of the
enzyme or its association with a specific structure in the chloroplast. Nir et al. (9) have demonstrated distinctive changes in ultrastructure of root plastids when subjected to dehydration. If similar changes took place in chloroplasts, they may be responsible for the decreased activities of certain enzymes. Restoration of the organelles' structure was demonstrated after dehydration, which is in agreement with the recovery of CO₂ fixation found when potentials were reversed (Fig. 2).

The experiments indicate that photo reduction of NADP was unaffected by lowering osmotic potentials (Table II). This is in accordance with the findings of Santarius (12) that no depression of ferricyanide reduction by chloroplast fragments is obtained in sugar or sorbitol solutions even at much higher concentrations than those used in this study. On the other hand, when intact plants were subjected to water stress, a consistent reduction of the Hill reaction was found in whole chloroplasts which were isolated from those plants (4). Similarly, an inhibition of O₂ evolution by intact chloroplasts was demonstrated by Boyer and Bowen (3). In intact chloroplasts O₂ evolution is closely coupled to CO₂ fixation rather than being controlled strictly by photochemical processes. Walker et al. (16) have shown that in intact chloroplasts the photosynthetic quotient was unity when CO₂ assimilation and its associated O₂ evolution were measured simultaneously.

The possibility that lowered osmotic potentials inhibited ATP formation primarily by a decrease of cyclic phosphorylation cannot be ruled out (8). This may cause limited formation of ribulose-1,5-diP which, in turn, would bring about a decrease in CO₂ fixation associated with a steep reduction in glyceraldehyde-3-P formation.

The effect of low osmotic potential of CO₂ fixation was reversible in our experiments with isolated intact chloroplasts and also in the experiments of Fry (4) and with intact leaf tissue (12). On the other hand, Boyer and Bowen (3) have shown that when chloroplasts were dehydrated in the intact leaf, restoration of O₂ evolution after isolation of chloroplasts and their incubation at 0.33 m sorbitol was negligible. The possibility that a partial restoration did take place, however, cannot be ignored, since the degree of initial inhibition was not measured in the dehydrated leaf. Restoration also may be progressive with time and therefore was not detected.

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LITERATURE CITED