Planta (1981) 153:430–435

Planta

Photosynthesis under osmotic stress

Differential recovery of photosynthetic activities of stroma enzymes, intact chloroplasts, protoplasts, and leaf slices after exposure to high solute concentrations

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Abstract. The reversibility of the inhibition of photosynthetic reactions by water stress was examined with four systems of increasing complexity – stromal enzymes, intact chloroplasts, mesophyll protoplasts, and leaf slices. The inhibition of soluble chloroplast enzymes by high solute concentrations was instantly relieved when solutes were properly diluted. In contrast, photosynthesis was not restored but actually more inhibited when isolated chloroplasts exposed to hypertonic stress were transferred to conditions optimal for photosynthesis of unstressed chloroplasts. Upon transfer, chloroplast volumes increased beyond the volumes of unstressed chloroplasts, and partial envelope rupture occurred. In protoplasts and leaf slices, considerable and rapid, but incomplete restoration of photosynthesis was observed during transfer from hypertonic to isotonic conditions. Chloroplast envelopes did not rupture in situ during water uptake. It is concluded that inhibition of photosynthesis by severe water stress is at the biochemical level brought about in part by reversible inhibition of chloroplast enzymes and in part by membrane damage which requires repair mechanisms for reversibility. Both soluble enzymes and membranes appear to be affected by the increased concentration of internal solutes, which is caused by dehydration.

Key words: Chloroplast – Photosynthesis (stress recovery) – Protoplast – *Spinacia* – Water stress.

Introduction

Water stress caused by increased concentrations of non-penetrating solutes, such as sorbitol, inhibits photosynthesis of intact chloroplasts, protoplasts, or leaf slices from spinach (Kaiser et al. 1981 b). A main reason for inhibition is the increased concentration of internal solutes to levels that cause inhibition of photosynthetic enzymes (Kaiser and Heber 1981). After mild or moderate stress, rapid partial recovery of photosynthesis has been reported for isolated chloroplasts (Plaut 1971). However, when plants are rewatered after periods of drought, recovery of CO_2-assimilation often requires hours or even days (Ashton 1956; Troughton 1969; Boyer 1971). Long periods of recovery have been attributed to high stomatal resistance which persists, owing to metabolic changes of stomata (Ashton 1956; Troughton 1969; Fischer et al. 1970), to damage at the mesophyll level (Santarius 1967) and to increased resistance to water transport in roots and shoots after periods of severe stress (Kramer 1950; Boyer 1971).

The diversity of factors thought to be responsible for the slow recovery of photosynthesis prompted us to reinvestigate the problem of reversibility of photosynthesis inhibition by water stress at the level of chloroplast enzymes, intact chloroplasts, mesophyll protoplasts, and leaf slices. These systems which differ in complexity are usually not limited in their activity by diffusional resistances, and their water potential can be easily controlled.

Materials and methods

Intact chloroplasts were isolated from freshly harvested spinach leaves (*Spinacia oleracea* cv. "Monatol"), according to Cockburn et al. (1968), with minor modifications described earlier (Kaiser et al. 1981 b). To measure CO_2-dependent O_2-evolution, 4 mmol l^{-1} $KHCO_3$, 0.2 mmol l^{-1} KH_2PO_4, and 1,000 U ml^{-1} catalase were added to the standard reaction medium. The percentage of intact chloroplasts was routinely determined by the ferricyanide method (Heber and Santarius 1970). It varied between 70 to 95%. Chlorophyll was measured according to Arnon's method (1949).

Spinach mesophyll protoplasts were obtained using the technique of Edwards et al. (1978), with some modifications described in a preceding paper (Kaiser et al. 1981 b).

Methods for preparing leaf slices and crude stroma extracts as well as conditions for $^{14}CO_2$-fixation in all these systems have been described earlier (Kaiser et al. 1981 b). Further details are given in the legends of the figures and tables.

0032-0935/81/0153/0430/$01.20

Chloroplast volumes were measured by coulter counter distribution with a coulter counter model Z_bI (with a 50 µm orifice), connected to a channelizer Model C-1000 and a x-y recorder.

The osmotic potential of the various solutions was calculated from the freezing point depression as determined with a Knauer Semi-micro Osmometer Type M 21.20.

Results and discussion

Enzymes. Under water stress, cellular solutes are concentrated and photosynthesis is inhibited. Enzymes in crude stromal extracts of chloroplasts were found to be sensitive to high concentrations of various solutes, such as sorbitol, glycerol, glycinebetaine, or KCl and NaCl (Kaiser and Heber 1981). Enzyme inhibition was thought to be caused by solute effects on the proteins.

After transfer from solutions containing inhibitory concentrations of sorbitol or KCl to a sorbitol – or KCl-free medium, the activity of enzymes involved in CO_2-fixation with ribose-5-phosphate and ATP as the substrates was restored (Table 1). Recovery was so fast that its kinetics could not be resolved (not shown). If inhibition of photosynthesis of chloroplasts or protoplasts under hypertonic stress were exclusively caused by solute effects on enzymes, recovery in vivo should be expected to be as fast and complete as with isolated enzymes.

Chloroplasts. Isolated intact chloroplasts were inhibited by osmotic stress to about the same extent or even more than protoplasts or leaf slices (Kaiser et al. 1981 b). Contrary to the reports of Plaut (1971), after back-transfer of intact chloroplasts from hypertonic media to isotonic solutions, photosynthesis did not recover. Rather, additional inhibition was observed (Fig. 1). Increased inhibition was already significant after back-transfer from solutions of moderate osmotic potentials (Fig. 1 A). At 24 bar, inhibition was usually relatively small (e.g., 28% in Fig. 1, A). After back-transfer to the normal reaction medium, photosynthesis was completely inhibited.

Intact chloroplasts have been shown to respond to an increased osmotic potential of the suspending medium by decreasing their volumes (Kaiser et al. 1981). Chloroplast shrinkage follows the Boyle-Mariotte relation

$$\pi \times V = \text{const.}$$

Table 1. $^{14}CO_2$-fixation by a crude, membrane-free stromal extract, with 1 mmol l^{-1} ribose-5-phosphate and 2 mmol l^{-1} ATP as substrates, at different osmotic potentials. Intact chloroplasts were lysed in 1 ml sorbitol-free medium (see Materials and methods). 200 µl aliquots were added to 200 µl of 3 different solutions: a) lysing medium, b) lysing medium containing 4 mol l^{-1} sorbitol, c) lysing medium containing 1.2 mol l^{-1} KCl, giving osmotic potentials of 2.0, 58.0 and 40.0 bar, resp. These enzyme solutions were left on ice for 20 min. The reaction was started by adding 5 µl of each solution to the various reaction media (total volume 500 µl). After 1–2–4 min, 150 µl aliquots were sampled and injected into 400 µl methanol. The samples were acidified with glacial acetic acid and flushed with air until dry. Radioactivity was estimated by liquid scintillation counting. Rates are based on the chlorophyll content of the chloroplasts from which the enzymes were obtained

osmotic potential of preincubation medium (bar)	osmotic potential of reaction medium (bar)	$^{14}CO_2$-fixation (µmol mg^{-1} chl. h^{-1})	relative rate (%)
2	2	169	100
58 (sorbitol)	58 (sorbitol)	16	9
58 (sorbitol)	2.6 (sorbitol)	138	82
40 (KCl)	40 (KCl)	21	12
40 (KCl)	2.4 (KCl)	174	103

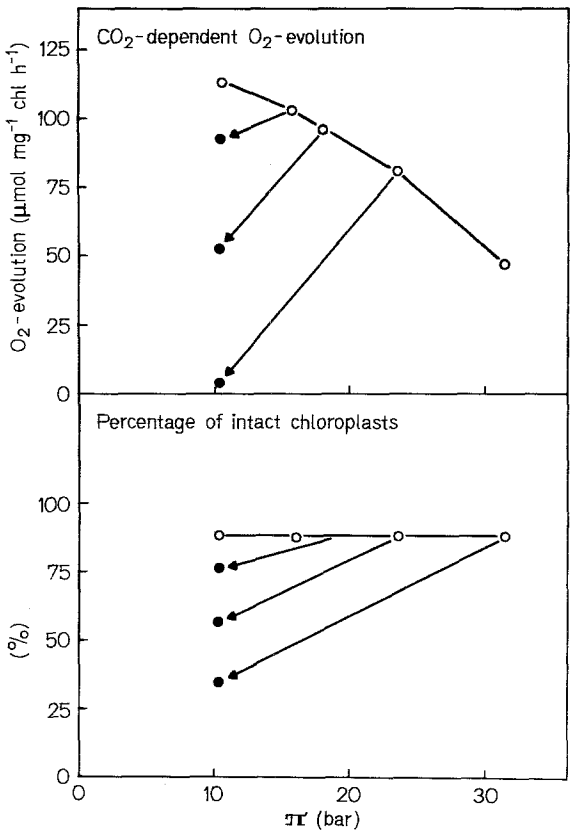

Fig. 1. CO_2-dependent O_2-evolution and chloroplast integrity during and after hypertonic stress. 50 µl of intact chloroplasts (about 50 µg chlorophyll) were added to 2.5 ml of media with various sorbitol concentrations, and CO_2-dependent O_2-evolution and intactness were measured as described in materials and methods. For following after-stress response (arrows), 50 µl of chloroplasts were supended in 0.5 ml of solutions of different hypertonicity, and after 3 min in the dark, they were brought back to the standard osmotic potential (10 bar) by slowly adding sorbitol-free medium; subsequently, O_2-evolution and integrity were measured as above

Table 2. Chloroplast volume during and after hypertonic stress (sorbitol as osmoticum). Intact chloroplasts were suspended in media with various sorbitol concentrations for 2 min at room temperature (20° C). After that, aliquots (20 µl) were injected into 20 ml of media with the same or with different sorbitol concentrations, and chloroplast volume was immediately determined by coulter counter distribution

osmotic potential during preincubation (bar)	osmotic potential during measurement (bar)	chloroplast volume (μm^3/chloroplast)	percentage of intact chloroplasts (%)
10	10	38	85
21	21	24	85
21	10	45	55
32	32	20	85
32	10	55	30
10	2	68	0

rather closely. π is the osmotic potential of the suspending medium and V the chloroplast volume. When osmotic stress is relieved and chloroplasts are transferred from a hypertonic to an isotonic medium, chloroplasts should be expected to swell in accordance with the above relation. Swelling is indeed observed, but Table 2 shows that it is much more extensive than expected. Strongly swollen chloroplasts reduced ferricyanide which does not penetrate the envelopes of intact chloroplasts (Fig. 1, B). In contrast to hypertonic treatment alone, which leaves chloroplast envelopes intact, hypotonic swelling following hypertonic shrinkage thus caused damage to chloroplast envelopes. This may explain, at least in part, why photosynthesis does not recover and actually exhibits increased inhibition, when chloroplasts are returned to isotonic conditions after exposure to hypertonic stress (Fig. 1, A).

Usually, water stress was relieved by mixing a hypertonic chloroplast suspension with a hypotonic medium of proper strength. Dilution was performed under vigorous stirring either rapidly or slowly to vary the rate of water uptake by the chloroplasts. Results were very similar, indicating that osmotic shock by rapid volume expansion of water-stressed chloroplasts was not responsible for chloroplast damage. When chloroplasts were transfered from an isotonic to a hypotonic medium (i.e., from an osmotic potential of 10 bar to 5 bar) and subsequently returned to isotonicity, their envelopes remained intact, and photosynthesis, which had been inhibited under hypotonic stress, was restored (Kaiser et al. 1981 a). In contrast, transfer from isotonic to hypertonic solutions and then back considerably decreased chloroplast intactness as measured by the ferricyanide method and increased the photosynthesis inhibition which was initially produced by hypertonic stress (Fig. 1). The conclusion is inevitable, that hypertonic chloroplast shrinkage, while not causing envelope rupture, produced envelope alterations which led to rupture when the chloroplasts expanded.

A rupture of part of the chloroplasts appeared not to be the only reason for decreased rates of photosynthesis after transfering chloroplasts back from a hypertonic to an isotonic medium. It has previously been reported that hypertonic stress caused a transient loss of metabolites from intact chloroplasts (Kaiser and Heber 1981). After having lost part of their metabolites and cofactors, those chloroplasts which escaped rupture during osmotic expansion suffered from dilution of external solutes. Hypotonic treatment of chloroplasts has shown such dilution to bring about photosynthesis inhibition (Kaiser et al. 1981 a).

Protoplasts. In contrast to chloroplast photosynthesis, carbon assimilation of mesophyll protoplasts was

Table 3. Recovery of protoplast photosynthesis, integrity of protoplast plasmalemma, and intactness of chloroplasts obtained from protoplasts during and after osmotic stress. CO_2-dependent O_2-evolution was measured as described in materials and methods. To measure the distribution of labelled compounds between protoplasts and medium, protoplasts were illuminated in the standard reaction medium (18 bar) for 10 min (600 W m^{-2}, 20° C, 73 µg chlorophyll, $KHCO_3$ 4 mmol l^{-1}, spec. activity $6.1 \cdot 10^4$ Bq µmol^{-1}, total volume 1.64 ml). A 25 µl aliquot was used to determine $^{14}CO_2$-fixation which showed similar rates as O_2-evolution. 3×450 µl were added to 450 µl of media containing either 0.5 mol l^{-1} sorbitol (a), or 2.8 mol l^{-1} sorbitol (b, c). After 5 min in the dark, 2 ml of media containing 0.5 mol l^{-1} sorbitol (a), 1.65 mol l^{-1} sorbitol (b) or no sorbitol (c) were added to bring the final osmotic potential to 18 bar (a), 44 bar (b), and back to 18 bar (c). After 5 min more in the dark, 500 µl aliquots from these suspensions were centrifuged (5 min at 100 *g*), and an aliquot (200 µl) of the clear supernatant was acidified with glacial acetic acid and treated as usually. Chloroplasts were obtained from protoplasts according to Edwards et al. (1978) by passing the protoplasts through a 20 µm nylon net. Intactness of these chloroplasts was measured as usually (see Materials and methods)

osmotic potential of preincubation medium (bar)	osmotic potential of reaction medium (bar)	O_2-evolution (µmol mg^{-1} chl. h^{-1})	labelled compounds in protoplast supernatant (% of total fixation)	percentage of intact chloroplasts (%)
a) 18	18	67	13	99
b) 44	44	15	7	99
c) 44	18	40	29	94

largely restored upon transfer of protoplasts from hypertonic to isotonic conditions. Still, restoration was not complete (Table 3). In order to find out whether some protoplasts are damaged during transfer, a protoplast suspension was permitted to photosynthesize in the presence of $^{14}CO_2$. In a subsequent dark period, the protoplasts were exposed first to hypertonic stress, and then stress was relieved. If a significant percentage of protoplasts had been damaged during transfer (i.e., if the plasmalemma had been broken), labeled metabolites should have leaked out. As shown in Table 3, 13% of the total fixed carbon was found in the supernatant of a slightly hypertonic protoplast suspension after a period of photosynthesis. This is attributed in part to slow loss of some metabolites from intact protoplasts and in part to protoplast damage during the experimental treatment. Even under severe hypertonic stress, metabolite loss was not increased, indicating that osmotic stress had left the plasmalemma intact and did not even cause a transient loss of metabolites such as was observed in intact chloroplasts (Kaiser and Heber 1981). After transfer of protoplasts from a hypertonic to an isotonic medium, the percentage of labeled metabolites in the medium increased from 7 to 29%. Apparently, some of the protoplasts ruptured during back-transfer. This was also confirmed by phase-contrast microscopy (not shown). Thus, damage of the protoplast plasmalemma contributes to the incomplete recovery of photosynthesis which was observed after osmotic stress was relieved, but is insufficient to explain it completely.

Isolated protoplasts yield chloroplasts of excellent intactness (Edwards et al. 1978), when the protoplasts are mechanically ruptured by passing them through a nylon net of suitable mesh width (20 µm). This method permitted us to determine whether chloroplast integrity decreased not only in vitro but also in vivo when hypertonic stress was decreased. Chloroplasts were released from protoplasts at various stages of osmotic stress and chloroplast intactness was measured by the ferricyanide method. The results shown in Table 3 indicate that chloroplasts remain intact inside the protoplasts during a stress cycle. Thus, protoplasts offer very effective protection to chloroplasts during changes of osmotic conditions.

Leaf slices. Photosynthesis of spinach leaf slices was inhibited by osmotic stress to a similar extent as photosynthesis of protoplasts, but often less than photosynthesis of chloroplasts (Kaiser et al. 1981 b). Since diffusional distances in thin leaf slices are short (500 µm), it was possible to infiltrate leaf slices with hypertonic solutions and to release stress by transfer of the slices into a fresh, hypotonic medium for 10 to 30 min. This permitted the use of solutions of low osmotic potentials or even water. In the experiments shown in Fig. 2, leaf slices were first infiltrated with media of different osmotic potential (from 1.2 to 47 bar, sorbitol as osmoticum), and after 15 min in the dark some samples were transferred back to hypotonic solutions (1.2 bar). From all states of inhibition, significant but incomplete recovery of photosynthesis was observed. After exposure of leaf slices to osmotic potentials, causing 50% inhibition of photosynthesis (about 30 to 40 bar), the rate of photosynthesis after back-transfer to hypotonic solution usually reached 70 to 80% of the control rate in a hypotonic solution (Fig. 2). Within 10 min to 3 h after release of stress, the extent of recovery did not change. Experiments with leaf slices prelabeled under hypotonic conditions with $^{14}CO_2$ showed that release of metabolites from slices during a stress cycle (1.2 bar – 44 bar – 1.2 bar) was less than 10% of the total labeled soluble material

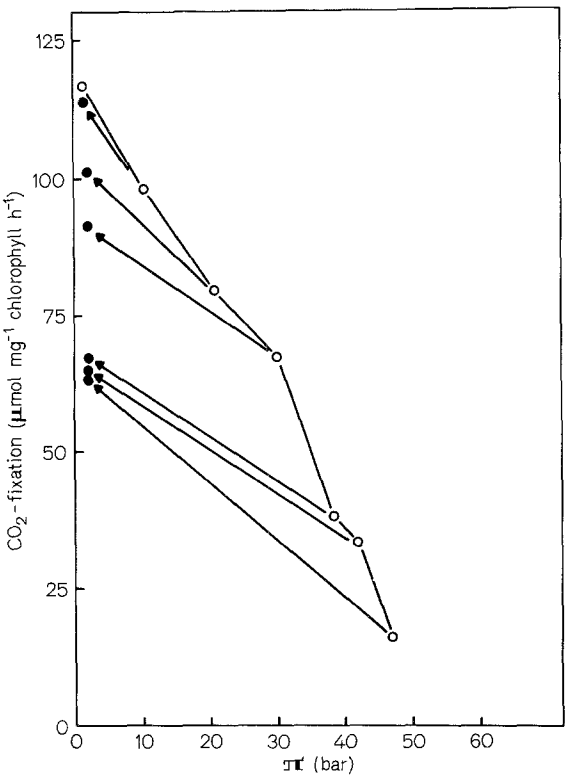

Fig. 2. Inhibition of $^{14}CO_2$-fixation of spinach leaf slices during osmotic stress (sorbitol as osmoticum), and partial recovery after back-transfer to hypotonic solution. Leaf slices were vacuum infiltrated and preincubated for 15 min in solutions containing various sorbitol concentrations. A proportion of the slices was transferred back into hypotonic solutions, and the rest remained in the various media for 15 min more in the dark. Subsequently, $NaH^{14}CO_3$ (specific activity $1.47 \cdot 10^3$ Bq µmol^{-1}) was added, to give a final concentration of 10 mmol l^{-1} HCO_3^-, and the slices were illuminated for 15 min at 20° C (165 W m^{-2}). for further details see Materials and methods

Table 4. Photosynthesis and intactness of chloroplasts isolated from leaf slices infiltrated with media of various osmotic potentials. Spinach leaves were cut into 1 mm broad slices and vacuum infiltrated with blending medium A (see materials and methods) containing 0.33 mol l^{-1} sorbitol (a) or 1.33 mol l^{-1} sorbitol (b, c). After 15 min in the dark, a portion of the slices infiltrated at 38 bar was transferred back to the standard medium (10 bar), and left there for 15 min more (c). Subsequently, the different fractions of leaf slices were homogenized in a blender and chloroplasts were isolated from each fraction by the standard procedures. Oxygen evolution and intactness were measured as described in Materials and methods

osmotic potential of infiltration media (bar)	osmotic potential of isolation media (bar)	O_2-evolution (μmol $mg^{-1}chl \cdot h^{-1}$)		percentage of intact chloroplasts (%)	
		at 10 bar	at 38 bar	at 10 bar	at 38 bar
a) 10	10	78	35	70	70
b) 38	38	0	24	15	79
c) 38 → 10	10	66	35	76	76

(data not shown). Thus, incomplete recovery of photosynthesis could not be due to rupture of mesophyll cells. In fact, the osmotic response of leaf slices could be visually observed. During stress, leaf slices curled up, but within 5 to 10 min after transfer to a sorbitol-free solution, they stretched out and became fully turgid again. Since cell rupture is not a sufficient cause for incomplete recovery of photosynthesis of protoplasts and leaf slices after stress, other factors must be considered. On transfer to hypertonic conditions, isolated chloroplasts became transiently leaky (Kaiser et al. 1981b). In vivo, this effect would decrease recovery of photosynthesis in protoplasts and leaf slices. Also, a transient unspecific increase in membrane permeability might occur not only in the chloroplast envelope, but possibly in other intracellular membranes as well. Leakiness of the tonoplast would result in release of vacuolar acids and decrease of the pH of the cytoplasm. This in turn would affect photosynthesis which is known to be pH-controlled (Heldt 1979).

To examine effects of osmotic stress on chloroplast integrity in vivo, leaf slices were infiltrated with isolation media containing different sorbitol concentrations. After 15 min incubation in the dark, a portion of the slices was transferred back to an isotonic medium and incubated for another 15 min in order to allow equilibration of the osmotic values between medium and tissue. Chloroplasts isolated from these slices were then assayed for CO_2-dependent O_2-evolution and intactness at 10 bar and 38 bar. For comparison, chloroplasts were also isolated in hypertonic media from hypertonically infiltrated slices (38 bar), and in isotonic media from isotonically infiltrated slices (10 bar). Table 4 shows that chloroplasts from leaf slices which had been exposed to stress were as intact as those from unstressed controls after back-transfer to isotonic conditions, but showed somewhat lower rates of photosynthesis. Thus, contrary to the situation in vitro, stress and release of stress in vivo did not seem to affect chloroplast integrity. Once again, decreased rates of photosynthesis (with intactness being unchanged) might be explained by a transient efflux of metabolites during the stress cycle. It should be noted, however, that in the experiment from Table 4 leaf slices were mechanically ruptured and only a small percentage of the released chloroplasts was selected by differential centrifugation. Still, the results agree with data from chloroplasts which had been released under comparable conditions from protoplasts. In this case, all released chloroplasts were assayed (Table 3).

This work was supported in part by the Deutsche Forschungsgemeinschaft. The skilled technical assistance of Mrs. Annette Weber is gratefully acknowledged.

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Received 4 April; accepted 10 August 1981