ABSTRACT

Spinach leaf chloroplasts isolated in isotonic media (330 millimolar sorbitol, -1.0 megapascals osmotic potential) had optimum rates of photosynthesis when assayed at -1.0 megapascals. When chloroplasts were isolated in hypertonic media (720 millimolar sorbitol, -2.0 megapascals osmotic potential) the optimum osmotic potential for photosynthesis was shifted to -1.8 megapascals and the chloroplasts had higher rates of CO₂-dependent O₂ evolution than chloroplasts isolated in 330 millimolar sorbitol when both were assayed at high solute concentrations.

Transfer of chloroplasts isolated in 330 millimolar sorbitol to 720 millimolar sorbitol resulted in decreased chloroplast volume but this shrinkage was only transient and the chloroplasts subsequently swelled so that within 2 to 3 minutes at 20°C the chloroplast volume had returned to near the original value. Thus, actual steady state chloroplast volume was not decreased in hypertonic media. In isotonic media, there was a slow but significant uptake of sorbitol by chloroplasts (10 to 20 micromoles per milligram chlorophyll per hour at 20°C). Transfer of chloroplasts from 330 millimolar sorbitol to 720 millimolar sorbitol resulted in rapid uptake of sorbitol (up to 280 micromoles per milligram chlorophyll per hour at 20°C) and after 5 minutes the concentration of sorbitol inside the chloroplasts exceeded 500 millimolar. This uptake of sorbitol resulted in a significant underestimation of chloroplast volume unless [¹⁴C]sorbitol was added just prior to centrifuging the chloroplasts through silicone oil. Sudden exposure to osmotic stress apparently induced a transient change in the permeability of the chloroplast envelope since addition of [¹⁴C]sorbitol 3 minutes after transfer to hypertonic media (when chloroplast volume had returned to normal) did not result in rapid uptake of labeled sorbitol.

It is concluded that chloroplasts can osmotically adjust in vitro by uptake of solutes which do not normally penetrate the chloroplast enve- loppe, resulting in a restoration of normal chloroplast volume and partially preventing the inhibition of photosynthesis by high solute concentrations. The results indicate the importance of matching the osmotic potential of isolation media to that of the tissue, particularly in studies of stress physiology.

In response to drought or salt-stress many plants lower their osmotic potential in order to maintain turgor. This osmotic adjustment may involve uptake and accumulation of inorganic ions or the synthesis of organic solutes (4, 8). Many metabolic processes are inhibited by high ionic strength and it has been suggested that as a part of osmotic adjustment, solutes which are not inhibitory to metabolism are accumulated in the cytoplasmic compartment of cells while inorganic ions are stored in the vacuole. Since much of the metabolism in leaf cells occurs in subcellular organelles, it is apparent that the osmotic potential inside these organelles must also decrease to prevent water loss and shrinkage of the organelles. Accumulation of compatible solutes inside these organelles would facilitate such osmo-regulation without impairing their metabolism.

There are already some experimental evidence for osmotic adjustment by chloroplasts in response to stress. Spinach plants exposed to salt-stress have decreased osmotic potentials, yet photosynthetic capacity is not decreased (12, 14), suggesting that osmotic adjustment in the chloroplasts somehow prevents the inhibition which is observed when chloroplasts are exposed to decreased osmotic potentials in vitro (2, 3, 5, 11). The concentration of inorganic ions increased in the leaf in response to salt stress but not in the chloroplast (12, 14) suggesting accumulation of organic solutes in the chloroplasts. Indeed, chloroplasts isolated from the salt-stressed plants had increased levels of sugars and amino acids (12) although these were not sufficient to account fully for the decrease observed in cell sap osmotic potential. Chloroplasts isolated from Mesembryanthemum grown under high salt had optimal photosynthetic rates at higher sorbitol concentrations in vitro than did chloroplasts grown in low salt (5). These observations are consistent with the accumulation of compatible solutes in chloroplasts to provide osmotic adjustment in response to salt stress.

In this paper, I have investigated the possibility of inducing osmotic adjustment in chloroplasts in vitro in an attempt to determine the influence of such osmoregulation on photosynthesis. The results suggest that in vitro osmotic adjustment can occur by uptake of external solutes which only enter chloroplasts slowly under normal conditions.

MATERIALS AND METHODS

Plant Material. Spinach seeds (Spinacia oleracea L. cv Hybrid 102) were germinated in moist vermiculite and after 10 d the seedlings were transferred to hydroponic culture. Four seedlings were placed in each 6-L container with the following nutrient solution: 6 mm KNO₃, 2 mm MgSO₄, 1 mm KH₂PO₄, 4 mm Ca(NO₃)₂, 50 μM Fe Na (EDTA)₂, 50 μM H₂BO₃, 10 μM MnCl₂, 1 μM ZnSO₄, 0.5 μM Cu SO₄, and 0.1 μM Na₂ MoO₄. Plants were grown for 3 to 4 weeks in a glasshouse but the daylength was restricted to 12 h by a mechanical shutter and supplementary lighting was provided by fluorescent and incandescent lights to ensure a minimum light intensity of 100 μE·m⁻²·s⁻¹ (PAR). The pots were topped up with deionized water as required and were aerated continuously.

Chloroplast Isolation. Intact chloroplasts were isolated from spinach leaves and purified on a two-step Percoll gradient (15). All procedures were carried out at 0°C. Leaves (30–40 g) were ground for 3 s in a Polytron blender with 200 ml of 330 mm

Osmotic Adjustment by Intact Isolated Chloroplasts in Response to Osmotic Stress and Its Effect on Photosynthesis and Chloroplast Volume

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sorbitol, 5 mM MgCl₂, 10 mM NaH₂PO₄, 2 mM isocitrate, and 0.1% BSA (pH 6.5). The brei was squeezed through two layers of Miracloth containing a layer of cotton wool and the filtrate was centrifuged at 1700g for 1 min. The pellets were resuspended in 6 ml of 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 50 mM Heps-KOH, and 0.2% BSA (pH 7.6) and placed into two centrifuge tubes. Each was underlayered with 4 ml of the same medium plus 40% (v/v) Percoll, then the tubes were again centrifuged at 1700g for 1 min. Broken chloroplasts formed a band at the top of the Percoll layer, whereas intact chloroplasts were pelleted by this procedure. The supernatants were discarded and the pellets of intact chloroplasts were resuspended in the above medium. The total isolation procedure took 15 to 20 min and O₂ evolution by the chloroplasts could be measured within 30 min of harvesting the leaves. The chloroplasts were greater than 95% intact based on penetration of ferricyanide (13) and exhibited rates of CO₂-dependent O₂ evolution of 130 to 230 μmol·mg⁻¹·Chl·h⁻¹. The media described above had an osmotic potential of −1.0 MPa. For isolation of chloroplasts in lower osmotic potential, the sorbitol concentration was increased to 720 mM and such resulted in an osmotic potential of −2.0 MPa.

O₂ Evolution. CO₂-dependent O₂ evolution was measured at 20°C using Hansatech O₂ electrodes. Unless stated otherwise, the assay medium contained 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl₂, 1 mM MnCl₂, 50 mM Heps-KOH (pH 7.6), 4 mM NaHCO₃, 0.2 mM Pi, 1000 units·ml⁻¹ catalase. The suspension was illuminated with white light (1500 μE·m⁻²·s⁻¹ PAR). Chl was measured by the method of Arnon (1).

Osmotic Potentials. The osmotic potential of the media was determined by freezing point depression using a Knauer micro-osmometer.

Chloroplast Volume. Chloroplast volume was determined by the silicone oil filtering centrifugation technique (7, 15). In this method, chloroplasts are incubated in a medium containing ³²H₂O and [¹⁴C]sorbitol then the chloroplasts are separated by rapidly centrifuging them through a layer of silicone oil into HClO₄ in the bottom of a microcentrifuge tube. The total volume pelleted, measured by ³²H₂O, represents the chloroplast volume plus a certain amount of the external medium which is carried through the silicone oil with the chloroplasts. Since it is considered that sorbitol does not penetrate the inner of the two chloroplast membranes, the amount of [¹⁴C]sorbitol pelleted with the chloroplasts is a measure of this volume of external medium which is carried through the silicone oil with the chloroplasts. Chloroplast volume is thus obtained by subtracting the [¹⁴C]sorbitol volume from the ³²H₂O volume and is the volume inside the inner envelope membrane, including the volume inside the thylakoids.

Chloroplasts were incubated at 20°C, without illumination, in the same medium as for measurement of O₂ evolution except that the Chl concentration was 50 μg·ml⁻¹. The chloroplasts were incubated for 3.25 min before adding ³²H₂O and [¹⁴C]sorbitol. Four replicate samples of 200 μl were immediately removed and added to microcentrifuge tubes containing silicone oil and HClO₄ and these were centrifuged for 15 s in a Beckman microfuge. Total incubation time was 4 min. The microfuge tubes contained 20 μl of 1 N HClO₄ and 75 μl silicone oil type AR200 (density 1.04 g·ml⁻¹, viscosity 200 centistokes) or 20 μl of 2 N HClO₄ and 75 μl silicone oil type AP150 (density 1.08 g·ml⁻¹, viscosity 150 centistokes) as indicated in the Figure legends. Since the [¹⁴C]sorbitol was added just prior to separating the chloroplasts by centrifugation, the actual chloroplast volume was measured by this procedure. When ³²H₂O plus [¹⁴C]sorbitol were added before the chloroplasts, any [¹⁴C]sorbitol entering the chloroplast during the incubation would result in an increased sorbitol volume and therefore an apparent decrease in chloroplast volume. Values obtained by incubating chloroplasts in ³²H₂O plus [¹⁴C]sorbitol from the outset are therefore referred to as "apparent chloroplast volume" and the difference between this and "actual chloroplast volume" is a measure of sorbitol uptake by the chloroplasts ("Results"). No correction was made for intrathylakoid volume, which comprises approximately 12% of the total chloroplast volume (7).

Chemicals. Silicone oil was a kind gift from Wacker, Australia. Radioisotopes were obtained from Amersham, United Kingdom, and all biochemicals from Sigma.

RESULTS

Chloroplast Integrity and Photosynthesis. The osmotic potential of spinach leaf sap was between −0.9 and −1.1 MPa and the normal media used for isolating chloroplasts (330 mM sorbitol, osmotic potential −1.0 MPa) was isotonic with the leaf tissue. The effect of varying the osmotic potential of the assay medium on chloroplast intactness and on the rate of CO₂-dependent O₂ evolution is shown in Figure 1. In this instance, glycinebetaine was used as the osmoticum but similar results were obtained

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FIG. 1. Chloroplast intactness and CO₂-dependent O₂ evolution as a function of osmotic potential of the assay medium for chloroplasts isolated in normal media (−1.0 MPa), or in high sorbitol media (−2.0 MPa). ○. The assay media contained varying concentrations of glycinebetaine as indicated. Maximum rates of CO₂-dependent O₂ evolution were 152 μmol·mg⁻¹·Chl·h⁻¹ for chloroplasts prepared in −1.0 MPa and 165 μmol·mg⁻¹·Chl·h⁻¹ for chloroplasts prepared in −2.0 MPa.
with other solutes, as discussed below. For chloroplasts prepared in normal media (–1.0 MPa), the percentage of intact chloroplasts decreased if the glycinebetaine concentration was below 300 mM. The rate of O₂ evolution also decreased at less than 300 mM glycinebetaine but the inhibition was greater than would be expected to result from the decrease in percentage of intact chloroplasts. This has been observed previously (10) and probably results from swelling of those chloroplasts which remained intact. At glycinebetaine concentrations above 300 mM, the rate of photosynthesis was also decreased although the percentage of intact chloroplasts was not decreased (Fig. 1). When chloroplasts were isolated in media with 720 mM sorbitol (osmotic potential –2.0 MPa), optimum osmotic potential in the assay medium for O₂ evolution was shifted to –1.8 MPa (Fig. 1). Chloroplasts isolated in 720 mM sorbitol were more readily ruptured at low solute concentrations, the percentage of intact chloroplasts declining when the assay medium had less than 630 mM glycinebetaine (–2.0 MPa). The decrease in O₂ evolution at osmotic potentials above and below the optimum paralleled that in the normal chloroplast preparation; the main effect of isolating in 720 mM sorbitol was to shift the optimum osmotic potential to more negative values. In the experiment shown in Figure 1, the maximum rates of CO₂-dependent O₂ evolution by the two chloroplast preparations were similar but it was generally observed that chloroplasts isolated in high sorbitol had lower maximum rates than those isolated in normal media. For seven experiments in which the two chloroplast preparations were made from the same batch of leaves, the maximum rate of O₂ evolution of chloroplasts isolated in 720 mM sorbitol ranged from 61 to 109% of normally prepared chloroplasts with a mean value of 81%. By comparison, normally prepared chloroplasts assayed in 720 mM sorbitol had, on average, 60% of the rate of O₂ evolution observed in 330 mM sorbitol. When assayed at the optimum osmotic potential, the pH optimum and optimal Pi concentration for O₂ evolution by the chloroplasts were the same for chloroplasts isolated in 720 mM sorbitol and in 330 mM sorbitol.

It was not necessary to maintain high sorbitol throughout the isolation procedure to observe osmotic adjustment. Similar shifts in the optimum osmotic potential for O₂ evolution were observed when normally prepared chloroplasts were diluted with 720 mM sorbitol and washed once (data not shown). The shift in optimum osmotic potential for photosynthesis was also observed when chloroplasts were isolated using other solutes as osmoticia (Table I). For both glycinebetaine and sorbitol, chloroplasts isolated in –2.0 MPa more readily ruptured on transfer to –0.5 MPa than did chloroplasts isolated in isotonic media. Chloroplasts isolated in KCl showed a decrease in percent intact in –0.5 MPa media whether they were isolated in –1.0 MPa or –2.0 MPa media. For all three solutes, isolation of chloroplasts in media of –2.0 MPa shifted the optimum osmotic potential for O₂ evolution to more negative values. Chloroplasts isolated in media with KCl as the osmoticum consistently exhibited higher rates of O₂ evolution than chloroplasts isolated in sorbitol or glycinebetaine (Table I).

**Chloroplast Volume.** The results in Figure 1 suggest that chloroplasts isolated in 720 mM sorbitol osmotically adjust by taking up solutes from the medium. If this is true, the chloroplasts would have the same volume at –1.8 MPa as normally prepared chloroplasts at –1.0 MPa and both preparations would swell on transfer to lower solute concentrations. In measuring chloroplast volume by the silicone oil technique using [³H₂O] and [¹⁴C]sorbitol it was noticed that the incubation procedure influenced the values obtained. The method assumes that [³H₂O] penetrates the chloroplast and is indicative of the total aqueous volume pelletted whereas sorbitol does not penetrate the inner chloroplast envelope membrane and measures the volume external to the chloroplast. The chloroplast volume is then obtained by subtracting the [¹⁴C]sorbitol volume from the [³H₂O] volume (6, 7). When normally prepared chloroplasts were incubated in an assay medium of varying osmotic potential and containing [¹⁴C]sorbitol and [³H₂O], chloroplast volume decreased as the osmotic potential was reduced from –0.5 to –2.0 MPa but was constant at lower osmotic potentials (Fig. 2, apparent volume). However, if the [¹⁴C]sorbitol and [³H₂O] were added after the chloroplasts had been incubated in the same media for 3 min the chloroplast volume did not decrease for osmotic potentials between –1.0 and –3.0 MPa (Fig. 2, actual volume). The difference between apparent and actual chloroplast volume was significant at –1.0 MPa and increased between –1.0 MPa and –3.0 MPa. The results suggest that sorbitol does penetrate the chloroplast envelope under these conditions and that the increase in [¹⁴C]sorbitol pelletted reflects uptake of sorbitol by the chloroplasts rather than shrinkage of the chloroplasts. This is discussed further in the following section but in subsequent measurements the actual chloroplast volume was measured by incubating chloroplasts in media without labeled sorbitol, which was added just prior to separating the chloroplasts by centrifugation.

Actual chloroplast volume as a function of osmotic potential of the assay medium is shown in Figure 3 for chloroplasts prepared in –1.0 MPa or –2.0 MPa media. For the normal preparation, chloroplast volume increased when the solute concentration was below that of the isolation media but was relatively constant at osmotic potentials below –1.0 MPa. For cho-

<table>
<thead>
<tr>
<th>Isolation Media</th>
<th>Chloroplasts Intact Measured in -0.5 MPa</th>
<th>Maximum Rate O₂ Evolution μmol·mg⁻¹ Chl·h⁻¹</th>
<th>Osmotic Potential MPa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbitol 330 mM (–1.0 MPa)</td>
<td>83</td>
<td>97</td>
<td>152</td>
</tr>
<tr>
<td>Sorbitol 720 mM (–2.0 MPa)</td>
<td>56</td>
<td>95</td>
<td>165</td>
</tr>
<tr>
<td>Glycinebetaine 300 mM (–1.0 MPa)</td>
<td>86</td>
<td>97</td>
<td>150</td>
</tr>
<tr>
<td>Glycinebetaine 630 mM (–2.0 MPa)</td>
<td>9</td>
<td>95</td>
<td>131</td>
</tr>
<tr>
<td>KCl 200 mM (–1.0 MPa)</td>
<td>47</td>
<td>91</td>
<td>322</td>
</tr>
<tr>
<td>KCl 440 mM (–2.0 MPa)</td>
<td>38</td>
<td>92</td>
<td>224</td>
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glycinebetaine plus medium. The assay medium contained varying concentrations of glycinebetaine plus 5 mM sorbitol. \(^3\)H\(_2\)O and \(^{14}\)C\(_{\text{sorbitol}}\) were added initially to measure apparent volume or 0.75 min prior to centrifugation to measure actual volume. Total incubation was for 4 min. The silicone oil was type AR200. The volume was corrected for the percentage of intact chloroplasts in each case.

Chloroplasts isolated in \(-2.0\) MPa media, chloroplast volume also increased at solute concentrations less than the isolation medium but was constant at higher solute concentrations. Between \(-0.25\) and \(-1.5\) MPa, the chloroplasts isolated in \(-2.0\) MPa media had larger volumes than normally prepared chloroplasts, as predicted, but the volumes were similar for the two preparations between \(-1.5\) MPa and \(-3.0\) MPa.

When the rate of \(\text{CO}_2\)-dependent \(\text{O}_2\) evolution was plotted against actual chloroplast volume, the curves for the two different chloroplast preparations were quite similar in shape and in the optimum chloroplast volume for photosynthesis. As the glycinebetaine concentration was increased, chloroplast volume decreased and \(\text{O}_2\) evolution increased until a maximum was reached at 29.1 \(\mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\) for the normal chloroplasts and at 28.4 \(\mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\) for the chloroplasts isolated in \(-2.0\) MPa media (Fig. 4). These optimum volumes were attained at different assay medium osmotic potentials for the two preparations: at \(-1.0\) MPa for the chloroplasts isolated in \(-1.0\) MPa media and at \(-1.8\) MPa for the chloroplasts isolated in \(-2.0\) MPa media. For both preparations, further increases in glycinebetaine concentration resulted in only a small decrease in actual chloroplast volume but a dramatic loss of \(\text{O}_2\) evolution activity (Fig. 4).

**Sorbitol Uptake.** The chloroplast envelope is generally considered to be impermeable to sorbitol (6, 7, 16, 17) so the above results prompted a study of sorbitol uptake by chloroplasts. This was measured by incubating chloroplasts in \(^{14}\)C\(_{\text{sorbitol}}\) for varying times and separating the chloroplasts from the medium by centrifugation through silicone oil. Actual chloroplast volume and volume external to the chloroplast inner envelope were measured by incubating parallel samples in unlabeled sorbitol and adding \(^3\)H\(_2\)O and \(^{14}\)C\(_{\text{sorbitol}}\) just prior to centrifugation to minimize any uptake of labeled sorbitol. The difference between these samples and those incubated in \(^{14}\)C\(_{\text{sorbitol}}\) from the outset was a measure of sorbitol uptake by the chloroplasts. For normally prepared chloroplasts suspended in 330 mM sorbitol, there was a slow but significant uptake of sorbitol which continued linearly with time (Fig. 5). Calculated rates of sorbitol uptake were 10 to 20 \(\mu\text{mol}\cdot\text{mg}^{-1}\text{Chl}\cdot\text{h}^{-1}\) at 20\(^\circ\)C, supporting the previous findings that the chloroplast envelope is relatively impermeable to sorbitol. Nevertheless, incubation of chloroplasts in \(^{14}\)C\(_{\text{sorbitol}}\) for more than 2 to 3 min would allow sufficient sorbitol uptake to introduce significant errors into measurements of chloroplast volume (Figs. 2, 5). After 5 min in 330 mM sorbitol the concentration of sorbitol inside the chloroplast was calculated to be 60 to 100 mM.

Upon transfer of chloroplasts from 330 mM sorbitol (\(-1.0\) MPa) to 720 mM sorbitol (\(-2.0\) MPa) there was a 5-fold increase in the amount of \(^{14}\)C\(_{\text{sorbitol}}\) pelleted with the chloroplasts. This could result from shrinkage of the plastids, which would increase the extrachloroplast volume pelleted, or from uptake of sorbitol by the chloroplasts. For chloroplasts incubated in \(^{14}\)C\(_{\text{sorbitol}}\), the amount pelleted was maintained at this increased level but for chloroplasts in unlabeled sorbitol the amount of \(^{14}\)C\(_{\text{sorbitol}}\) pelleted declined after the first 1 min of transfer to 720 mM sorbitol (Fig. 6). After 4 to 5 min, a constant value was attained which was 1.5 to 3 times that of chloroplasts in 330 mM sorbitol. Chloroplast volume initially decreased on transfer to...
720 mM sorbitol but subsequently increased until a steady state was achieved after 5 to 6 min (Fig. 6). The final volume was similar to the original chloroplast volume in 330 mM sorbitol. The results suggest that on transfer to hypertonic media the chloroplasts initially shrink in response to the decreased osmotic potential and this increases the amount of ["C]sorbitol pelleted because of the increase in extrachloroplast volume. The chloroplasts subsequently take up sorbitol and gradually swell back to their original volume. The ["C]sorbitol pelleted is increased because of this solute uptake and results in a low apparent chloroplast volume (Figs. 2, 6). By incubating chloroplasts in unlabeled sorbitol, solute uptake does not contribute significantly to the amount of ["C]sorbitol pelleted and actual chloroplast volume is measured.

The experiment of Figure 6 was performed at 2°C to allow resolution of the kinetics of this rapid process but qualitatively similar results were observed at 20°C. The calculated rates of sorbitol uptake following transfer to 720 mM sorbitol were 140 to 180 µmol-mg−1 Chl·h−1 at 2°C and greater than 280 µmol-mg−1 Chl·h−1 at 20°C. The concentration of sorbitol in the chloroplast reached 500 to 550 mM after 5 min in 720 mM sorbitol. The shrinkage and subsequent swelling observed in Figure 6 with sorbitol was also observed with other solutes. After 5 min incubation in media of −2.0 MPa, actual chloroplast volume was similar to that in media of −1.0 MPa whether sorbitol, glycinebetaine, or proline was used as the osmoticum (data not shown) implying uptake of each of these solutes for osmotic adjustment by the chloroplasts in response to hypertonic conditions.

**DISCUSSION**

The results demonstrate that osmotic adjustment can occur in chloroplasts in vitro as a result of uptake and retention of external solutes when chloroplasts are transferred to hypertonic media. This shifted the optimum osmotic potential for O2 evolution to more negative values (Fig. 1) irrespective of the solute used (Table I). As a result of this osmotic adjustment, the chloroplasts prepared in high sorbitol had higher rates of O2 evolution than normal chloroplasts when both were assayed at high sorbitol concentrations. The reverse was true when chloroplasts were assayed at normal sorbitol concentrations since the chloroplasts prepared in high sorbitol were more swollen and a lower percentage remained intact upon return to normal media (Figs. 1 and 3).

CO2-dependent O2 evolution by chloroplasts was very sensitive to changes in chloroplast volume and the main consequence of osmotic adjustment was to shift the osmotic potential at which critical chloroplast volume was attained to more negative values (Figs. 3 and 4). When the solute concentration was reduced below this value the chloroplasts swelled and the rate of O2 evolution declined. This occurred at higher solute concentrations for the chloroplasts isolated in 720 mM sorbitol (Fig. 1). At solute concentrations above the optimum there was only a minor decrease in steady state chloroplast volume but O2 evolution was greatly inhibited in both normal chloroplasts and those isolated...
in 720 mM sorbitol (Fig. 4).

It has been suggested that the inhibition of photosynthesis observed in hypertonic media results from shrinkage of the chloroplasts and concentration of stromal solutes (2, 3, 11). The present results suggest that on transfer to media of more negative osmotic potential (higher solute concentrations), chloroplasts do initially shrink but then subsequently swell again as uptake of external solutes occurs, until the chloroplast volume is restored (Fig. 6). At 20°C, chloroplast volume returned to normal within 2 to 3 min of transfer to hypertonic media and the actual steady state chloroplast volume was not decreased when the solute concentration was increased above that in which the plastids were isolated (Fig. 2). This is borne out by the results since inhibition of O₂ evolution occurred without any significant decrease in actual chloroplast volume (Fig. 4). The inhibition of photosynthesis in hypertonic media may have resulted from the accumulation of external solutes inside the chloroplasts since the sorbitol concentration was above 500 mM in the chloroplasts when they were suspended in 720 mM sorbitol. This seems unlikely, however, since similar inhibition of O₂ evolution was observed with osmotically equivalent concentrations of glycinebetaine or proline, which are considered to be physiological solutes (data not shown). The inhibition of photosynthesis is more likely a consequence of the more negative osmotic potential inside the chloroplast as a result of accumulation of external solutes. Kaiser and Heber (9) have shown that a number of stromal enzymes are inhibited by increased solute concentration.

Since chloroplasts only shrink transiently on exposure to hypertonic conditions it is not clear why isolation of chloroplasts in media of −2.0 MPa should confer an advantage over transfer of normally prepared chloroplasts to −2.0 MPa at the assay stage. At assay medium osmotic potentials below −1.5 MPa, the steady state volume of chloroplasts isolated in −2.0 MPa was similar to normal chloroplasts (Fig. 3) yet their rate of O₂ evolution was higher (Fig. 1). It may be that by isolating chloroplasts in hypertonic media (at 0°C) the shrinkage/swelling cycle occurred more slowly than when chloroplasts were transferred to hypertonic assay medium (at 20°C). Certainly, sorbitol uptake was slower at 2°C than at 20°C. Kaiser and Heber (9) reported that transfer of chloroplasts to hypertonic media induced a transient alteration in the envelope membrane permeability and resulted in loss of metabolites from the chloroplasts. Exposure of chloroplasts to osmotic stress at 0°C rather than at 20°C may reduce this permeability change and restrict the loss of chloroplast metabolites. Thus, chloroplasts isolated in 330 mM sorbitol but assayed in 720 mM sorbitol would also osmotically adjust to maintain chloroplast volume but in doing so may lose chloroplast metabolites, and hence reduce their photosynthetic capacity. This implies that much of the inhibition of photosynthesis by osmotic stress in vitro results from loss of metabolites from the chloroplast due to the sudden change in osmotic potential and not from the reduced osmotic potential per se.

The chloroplast envelope is considered to be impermeable to sorbitol and it is assumed that chloroplasts shrink and swell in response to changes in sorbitol concentration (3, 6, 7, 10, 16, 17). In isotonic media, uptake of sorbitol by chloroplasts was slow (Fig. 5) and chloroplasts did swell when transferred to media of lower solute concentration (Fig. 3). However, the shrinkage of chloroplasts in hypertonic media was only transient and was followed by swelling of chloroplasts as external solutes were taken up (Fig. 6). Kaiser and Heber (9) also found that chloroplasts swell subsequent to shrinkage in hypertonic media and suggested that this might indicate solute uptake. Previous reports of chloroplast shrinkage have measured chloroplast volume in the initial period of shrinkage or have been in error because of the failure to take account of uptake of solutes from the medium. Thus, in preparation of chloroplasts for EM, the chloroplasts were added to hypertonic solutions containing fixative so that the chloroplasts would not have subsequently swollen in response to solute uptake (3, 6). Measurements of chloroplast volume by Coulter counter distribution (10) were made as soon as the chloroplasts were added to the medium and before subsequent swelling occurred. Determination of chloroplast volume by silicone oil centrifugation (2, 6) included [14C]sorbitol from the outset and would have measured uptake of sorbitol, thus understimating chloroplast volume (Fig. 2).

It is clear that the permeability of the chloroplast envelope does change transiently when the chloroplasts are subjected to osmotic stress, increasing both the influx of solutes from the medium (Fig. 6) and the efflux of metabolites from the chloroplast (9). Under normal conditions it is unlikely that chloroplasts would be subjected to such rapid changes in osmotic potential in vivo hence such permeability changes would not be a part of normal osmoregulation by chloroplasts. The slow uptake of even relatively impermeable solutes (Fig. 5) would allow osmotic adjustment to the gradual changes in osmotic potential likely to occur in vivo. Nevertheless, effective osmoregulation must occur in response to changes in cell osmotic potential in order to

Fig. 6. Uptake of [14C]sorbitol by chloroplasts following transfer to high sorbitol. Chloroplasts were isolated in normal media (−1.0 MPa) but transferred to media with 720 mM sorbitol (−2.0 MPa) at zero time. Incubation was at 2°C. Actual chloroplast volume was determined by adding [3H₂O and [14C]sorbitol 0.75 min prior to centrifuging. For determination of [14C]sorbitol pelletted, the chloroplasts were incubated in [14C]sorbitol from the outset (top curve) or it was added 0.75 min prior to centrifuging (lower curve). Silicone oil was type AP150.
maintain chloroplast volume constant. It is clear from the results (Fig. 4) that photosynthesis is extremely sensitive to changes in actual chloroplast volume. To prevent changes in chloroplast volume during isolation and assay, the osmotic potential of the media should be chosen so as to be isotonic with the leaf sap. This is particularly important in studies of stress physiology where osmotic adjustment may result in considerable changes in leaf osmotic potential. Failure to match the osmotic potential of the media to that of the tissue could result in both loss of solutes from the chloroplast and uptake of external solutes as well as loss of photosynthetic activity.

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