Isolation of Intact and Functional Chloroplasts from Mesophyll and Bundle Sheath Protoplasts of the C₄ Plant Panicum miliaceum

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ABSTRACT

A procedure is described for isolating and purifying mesophyll protoplasts and bundle sheath protoplasts of the C₄ plant Panicum miliaceum. Following enzymic digestion of leaf tissue, mesophyll protoplasts and bundle sheath protoplasts are released and purified by density centrifugation. The lower density of mesophyll protoplasts allowed rapid separation of the two protoplast types. Evidence for separation of mesophyll protoplasts and bundle sheath protoplasts (up to 95% purity) is provided from light microscopy (based on size difference in both chloroplasts and protoplasts), levels of marker enzymes in the preparations (i.e. pyruvate, Pi dikinase and phosphoenolpyruvate carboxylase for mesophyll and ribulose-1,5-bisphosphate carboxylase for bundle sheath), and differences in substrate-dependent O₂ evolution by chloroplasts isolated from protoplasts. Chloroplasts were isolated from protoplasts by several passages of the protoplasts through a 20-micrometer nylon mesh. Mesophyll chloroplasts were judged approximately 90 to 95% intact and bundle sheath chloroplasts 80 to 90% intact based on retention of chloroplast marker enzymes and the ferricyanide test for intactness. It was necessary to include 10 millimolar MgCl₂ in media for osmotically shocking the chloroplasts in order to obtain maximum and linear rates of ferricyanide-dependent O₂ evolution.

Chloroplasts isolated from mesophyll protoplast preparations had low rates of light-dependent O₂ evolution in the presence of 10 millimolar NaHCO₃ (0.13 micromoles per milligram chlorophyll per minute) in comparison to bundle sheath chloroplasts (1 to 2.5 micromoles per milligram chlorophyll per minute). The mesophyll chloroplasts catalyze high rates of 3-phosphoglycerate-dependent O₂ evolution (2 to 4 micromoles per milligram chlorophyll per minute). Orthophosphate but not phosphoenolpyruvate inhibited the 3-phosphoglycerate-dependent O₂ evolution by the mesophyll chloroplasts. Rates of O₂ evolution were much higher with mesophyll than with bundle sheath chloroplasts in the presence of pyruvate plus oxaloacetate. The results are discussed in relation to the proposed function of these chloroplasts during C₄ photosynthesis.

Previous studies have established that there is differential compartmentation of photosynthetic functions between mesophyll and bundle sheath cells of C₄ plants (see 12 for review). While C₄ photosynthesis provides a mechanism for high rates of carbon assimilation, rapid inter- and intracellular fluxes of certain metabolites are required. Comparative studies on the regulation of photosynthetic metabolism and the mechanism of metabolite transport of mesophyll and bundle sheath chloroplasts are dependent on obtaining the respective organelles in a pure, intact, and functional state.

As effective means of isolating intact mesophyll chloroplasts of both C₃ and C₄ plants is by gentle lysis of MP² (4). Previously, enzymic digestion of leaf tissue of C₄ plants resulted in release of MP and strands of bundle sheath cells attached to vascular strands. The bundle sheath cell wall was much more resistant to enzymic digestion due either to its greater thickness or differing composition. Compared with bundle sheath strands, BSP would be a more convenient source of intact organelles and plasma membrane. In addition, contamination by membranes, enzymes, and organelles derived from vascular tissue would be eliminated.

In the present study, procedures for isolating mesophyll and bundle sheath protoplasts of Panicum miliaceum were developed and the quality of chloroplasts isolated from protoplasts was evaluated.

MATERIALS AND METHODS

Plant Material and Protoplast Isolation. P. miliaceum, variety Sirocket-5, was grown in soil in a greenhouse within a day-night temperature range of 30 to 20 °C. For isolation of MP and BSP, leaf tissue was taken 15 to 25 days after planting (leaves approximately 70–130 mm in length). In older tissue the bundle sheath strands became resistant to enzymic digestion which resulted in isolation of MP and bundle sheath strands. Eight to 10 leaves at a time were sampled, stacked, and cut by hand into small segments about 0.7 mm in width. Approximately 8 g of leaf tissue were prepared and subjected to enzymic digestion as previously described (5, 18). The enzyme medium contained 0.5 M sorbitol, 1 mM CaCl₂, 0.1% BSA, 2% cellulase (Onozuka 3S), and 0.3% pectinase (Macerozyme R-10) (pH 5.0). The tissue was incubated in a crystallizing dish in a 30 °C water bath under low light (500 ft·c or about 15 nE cm⁻² s⁻¹ between 400 to 700 nm from a Phillips 250-w lamp) for 4.5 h (similar yields are obtained

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² Abbreviations: MP: mesophyll protoplasts; BSP: Bundle sheath protoplasts; PGA: 3-phosphoglycerate; PEP: phosphoenolpyruvate; RuBP: ribulose 1,5-bisphosphate.
by digestion for 16 h at 18 C, although the shorter incubation period was used in the present study). Following incubation, the enzyme medium was gently discarded and the leaf segments washed several times with 0.5 mM sorbitol, 1 mM CaCl2, and 5 mM Hepes-KOH (pH 7.0) (hereafter referred to as the sorbitol medium). After each washing, the extract was filtered through a coarse mesh nylon kitchen strainer (1-mm apertures) and a layer of cheese cloth (apertures approximately 200 μm). The filtrate was centrifuged for 2 min at 300g and the supernatant discarded.

Protoplast Purification. I. The protoplast types were easy to recognize by light microscopy (see under “Results”) which aided in developing procedures for their separation. The BSP are generally larger and have a higher density than MP. Initially, the crude protoplast pellet was resuspended in four tubes (15 × 100 mm) each containing 4 ml of 0.5 mM sucrose, 1 mM CaCl2, 5 mM Hepes-KOH (pH 7.0). This mixture was overlaid with 2 ml of the sorbitol medium. After centrifugation for 5 min at 300g, the BSP partitioned at the interface between the layer of sucrose and sorbitol. The BSP sedimented to the bottom of the tubes and these were resuspended in medium containing 0.5 mM sucrose, 1 mM CaCl2, 5 mM Hepes-KOH (pH 7.0), and 9.1% dextran T40 (1 g dextran T40/10 ml). This mixture was overlaid with 2 ml of the sorbitol medium and, after centrifugation at 300g for 5 min, the BSP collected at the interface between the layer of sucrose-dextran T40 and sorbitol. The MP were washed once and resuspended in the sorbitol medium. The BSP fraction contained some very small, dense MP (as assays of PEP carboxylase verified that these were MP). These MP were partially removed by suspending the BSP preparation in 0.5 mM sucrose, 1 mM CaCl2, 5 mM Hepes-KOH (pH 7.0), and centrifuging at 200g for 5 min. The pellet was then resuspended in the sorbitol medium. This procedure for purification resulted in MP preparations which were about 95% pure and BSP preparations about 80% pure based on cross-contamination as indicated by photosynthetic marker enzymes of the protoplast types.

Protoplast Purification. II. A modification of the first purification procedure in the latter part of the study resulted in an increased purity of the BSP preparation up to approximately 95%.

In this method the crude protoplast pellet was resuspended in 0.5 mM sucrose, 5 mM Hepes-KOH (pH 7.0), and 1 mM CaCl2 with 13% dextran T40 (1.5 g/10 ml). Four ml of this suspension were added to each of four tubes. Two ml layers of 0.5 mM sucrose, 5 mM Hepes-KOH (pH 7.0), and 10.7% dextran T40 (1.2 g dextran T40/10 ml) and then 2 ml of the sorbitol medium were added on top of these solutions. After centrifugation at 300g for 5 min, a mixture of MP and BSP appeared at the upper interface (between the sorbitol layer and sucrose-dextran layer). The bands of protoplasts were collected with a Pasteur pipette and brought to a volume of 8 ml with the sorbitol medium. Four ml of this protoplast suspension were layered into two tubes over 10 ml of 0.4 mM sucrose, 0.1 mM sorbitol, 5 mM Hepes-KOH (pH 7.0), and 1 mM CaCl2 and centrifuged for 2 min at 300g. The supernatant contained MP and the pellet was an enriched preparation of BSP. The MP from the supernatant fraction were diluted with an equal volume of the sorbitol medium and centrifuged at 300g for 2 min. The pellet was resuspended in the sorbitol medium and taken as the MP purification.

The BSP pellet was resuspended in 4 ml of sorbitol medium and the purification repeated by layering on top of 10 ml of the medium containing 0.4 mM sucrose and 0.1 mM sorbitol (described above) and centrifuging at 300g for 1 min. The pellet was then taken as the BSP preparation. This purification procedure takes advantage of both size and density differences between the protoplast types and tends to eliminate any smaller and relatively dense MP from contaminating the BSP fraction. In either purification procedure from about 10 g of leaf tissue, the yield of MP on a Chl basis was 600 to 1,000 μg and that of BSP about 200 to 400 μg. Since there is about 1.8 mg Chl/g leaf tissue, the total yield of protoplasts from the leaf is roughly 5%. The remaining Chl is in undigested bundle sheath strands and broken protoplasts. After isolation, the protoplasts were stored at 0 C.

Light Microscopy. Protoplasts were fixed in 3% glutaraldehyde with 25 mM Hepes buffer (pH 7.0), and 0.3 mM sorbitol followed by 2% OsO4 in 25 mM phosphate buffer. Samples were then washed four times with the phosphate buffer and photographed, in buffer, with the light microscope using Kodak Panatomic X film.

Isolation of Chloroplasts from Protoplasts. Aliquots from the protoplast preparations were centrifuged and resuspended in an appropriate medium (see under “Results”) for chloroplast isolation. Protoplasts equivalent to 100 to 200 μg Chl in 0.4 to 0.8 ml of isolation media were broken by several passages through a 20-μm nylon mesh as previously described (5, 22). With less concentrated suspensions, it becomes difficult to break all of the protoplasts. Chloroplasts were then isolated by centrifugation at 300g for 90 s. The resuspended pellet, which contained essentially all of the Chl of the protoplasts extract, was used for determining percentage intactness of chloroplasts according to the ferricyanide test and for measuring substrate-dependent O2 evolution.

Enzyme Assays. Cross-contamination in the preparation of mesophyll and bundle sheath protoplasts was routinely checked by osmotically shocking samples of the protoplasts and assaying marker enzymes (PEP carboxylase, pyruvate, Pi dikinase for mesophyll and RubP carboxylase for bundle sheath) in the total protoplast extract. Pyruvate, Pi dikinase (for mesophyll chloroplasts) and RubP carboxylase (for bundle sheath chloroplasts) were also used to evaluate the degree of purity and intactness of chloroplasts isolated from protoplasts. The activity of a bundle sheath marker enzyme in MP preparations divided by the activity of the enzyme in pure bundle sheath cells times 100 would give the percentage contamination. Likewise, measurement of a marker enzyme for mesophyll cells would provide an estimate of MP contamination of BSP. With activity expressed per unit Chl, this will then give the percentage contamination on a Chl basis. Since the activity of marker enzymes in completely pure cells was unknown, the degree of purity was estimated as follows:

\[
\text{% Contamination of mesophyll protoplasts} = \left( \frac{A}{B} \right) \times 100
\]

where A and B equal the activity of the bundle sheath marker enzyme in the mesophyll and bundle sheath protoplast preparation, respectively.

\[
\text{% Contamination of bundle sheath protoplast preparation} = \left( \frac{C}{D} \right) \times 100
\]

where C and D equal the activity of the marker enzyme for mesophyll cells in the BSP and MP preparation, respectively. This is a satisfactory method when the preparations are relatively pure. The error is negligible when the respective cross-contaminations are 10% or less and only slightly overestimated with a cross-contamination up to 20%.

In the experiment reported (Table I), both the levels of enzymes in the protoplast preparations and the percentage intactness of the chloroplast based on retention of marker enzymes were determined. Aliquots of the mesophyll and bundle sheath protoplasts were pelleted and resuspended in the isolation medium containing 5 mM Tricine, 3 mM DTT, 3 mM MgCl2, and 0.3 mM sorbitol (pH 8.3). Enzymes were assayed in protoplast extracts, chloroplasts, and supernatant fractions isolated as described above. For assay of RubP carboxylase, a 0.1-ml aliquot was diluted to 0.3 ml with 5 mM Tricine, 3 mM DTT, and 3 mM MgCl2 (pH 8.3). Then 10 μl of 5% digitonin was added to each to ensure complete breakage of chloroplasts and the mixture incubated for 5 min at 30 C prior to assay. For PEP carboxylase, an aliquot of the samples was added to ½ volume of 0.5 mM Hepes-KOH (pH 7.0) for activation (11). For pyruvate, Pi dikinase, 50-μl aliquots were added to 10 μl 25

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mm Tricine (pH 8.3), 50 mM DTT, and 50 mM MgCl₂ plus 2.5 μl of 50 mM K₂HPO₄. To activate the enzyme, samples were gassed with N₂ and incubated for approximately 2 h at 25°C before assay.

After activation pretreatments described above RuBP carboxylase (21), PEP carboxylase (11), and pyruvate, Pi dikinase (1) were assayed as previously described except that the pyruvate, Pi dikinase assay mixture included 5.5 mM (NH₄)₂SO₄. The temperatures of the assays were 30°C for RuBP carboxylase and 22°C for all other enzymes.

**RESULTS AND DISCUSSION**

**LIGHT MICROSCOPY**

*P. miliaceum* is an NAD-malic enzyme type C₄ species characterized anatomically by a centripetal location of the bundle sheath chloroplasts. Like many species of this C₄ subgroup, the bundle sheath chloroplasts are considerably larger than the mesophyll chloroplasts (7, 10).

Figure 1 shows preparations of MP and BSP of *P. miliaceum*. In general, both the bundle sheath protoplasts and their chloroplasts are larger than those of the mesophyll preparations. Most of the freshly prepared and fixed BSP showed an asymmetric orientation of chloroplasts characteristic of their position in vivo. This feature is clearly evident in only a small proportion of these protoplasts when they are randomly orientated and photographed in narrow plane of focus. Both size and density differences in the two types of protoplasts allowed their separation (see under "Materials and Methods"). The reason for the greater density of BSP is uncertain although it might be due to a relatively lower volume of vacuole per unit volume of cytoplasm than in the mesophyll cells. The tissue was in a rapid state of growth and was sampled early in the morning so that little starch accumulation in either mesophyll or bundle sheath chloroplasts was expected.

Previously, enzymic digestion of C₄ species resulted in the isolation of MP and bundle sheath strands (e.g. ref. 14). *P. miliaceum* is the first C₄ species from which BSP have been isolated. Recently, mesophyll and BSP were readily isolated from mature leaves of *Amaranthus graezius* using a similar procedure (Ku and Edwards, unpublished). Differences in the composition of bundle sheath cell walls among C₄ species and in the commercial sources of cellulase and pectinase will likely be important factors in further efforts to isolate BSP from other C₄ species.
PURITY OF PREPARATIONS

Besides light microscopy, the purity of the preparations was tested by assaying marker enzymes for MP and BSP. In the experiment of Table 1, MP were about 95% pure based on cross-contamination by RuBP carboxylase

\[
\begin{align*}
0.185 \mu\text{mol mg}^{-1} \text{Chl min}^{-1} \text{ in mesophyll preparation} \\
3.7 \mu\text{mol mg}^{-1} \text{Chl min}^{-1} \text{ in bundle sheath preparation} \\
(100) = 5%
\end{align*}
\]

(also see under "Materials and Methods"). Likewise, BSP was about 93% pure based on cross-contamination by pyruvate, Pi dikinase and PEP carboxylase. If corrections are applied for a pyruvate, Pi dikinase assay blank and PEP carboxylase activity associated with bundle sheath cells (see Table 1 footnotes), BSP purity was about 97%.

INTACTNESS OF CHLOROPLASTS ISOLATED FROM PROTOPLASTS

Retention of Chloroplast Marker Enzymes. Pyruvate, Pi dikinase is localized in the stroma of C3 mesophyll chloroplasts (8, 9), and its retention by chloroplasts prepared from MP indicates that the chloroplasts are about 90% intact. Likewise, bundle sheath chloroplasts were considered about 90% intact based on the retention of RuBP carboxylase in the 200g chloroplast pellet (Table 1). PEP carboxylase, previously concluded to be a cytoplasmic enzyme (8, 9), was associated with the supernatant fraction from mesophyll protoplast extracts confirming its extrachloroplastic location (Table 1).

Ferricyanide Test. The impermeability of ferricyanide to intact chloroplasts can be used as a method of determining the intactness of a chloroplast preparation (19). Ferricyanide-dependent O2 evolution is determined before and after osmotic shock. According to this method, chloroplasts prepared from protoplasts of *P. miliaceum* (protoplast preparation as in Table I) were 98% and 82% intact for mesophyll and bundle sheath chloroplasts, respectively. Both retention of chloroplast marker enzymes and the ferricyanide test indicate that high proportions of intact chloroplasts can be obtained from both protoplast types. Slight discrepancies between the two methods probably occur due to the use of separate isolations. Reducing conditions (inclusion of DTT) were considered essential for extraction for the enzyme assays (Table I) while DTT was eliminated from the preparations for ferricyanide measurements in order to avoid its interference with the assay of ferricyanide-dependent O2 evolution.
SUBSTRATE-DEPENDENT O₂ EVOLUTION

Chloroplasts isolated from MP had very low rates of O₂ evolution in the light with or without HCO₃⁻ whereas bundle sheath chloroplasts had relatively high rates of O₂ evolution with HCO₃⁻ as a substrate (Table II). These results are consistent with previous evidence that bundle sheath but not mesophyll cells contain the Calvin pathway enzymes and capacity for net carbon assimilation (4, 12).

The mesophyll chloroplasts show a high capacity for PGA-dependent O₂ evolution supporting previous conclusions that C₄ mesophyll chloroplasts have the capacity to reduce PGA to triose-P (12). Light-dependent O₂ evolution in the presence of oxaloacetate was severalfold higher in mesophyll than in bundle sheath chloroplasts. This was stimulated by pyruvate only in mesophyll preparations. Maximum rates of noncyclic electron flow depend on both ATP and NADPH being utilized. In mesophyll chloroplasts, limitation of NADPH-dependent oxaloacetate reduction via NADP malate dehydrogenase would be relieved by pyruvate-mediated ATP utilization via pyruvate, Pi dkinase (4, 17). Thus, the levels of substrate-dependent O₂ evolution by the preparation with bicarbonate, PGA, and oxaloacetate + pyruvate further substantiate the differing photosynthetic functions of these chloroplast types.

Photosynthesis by the isolated bundle sheath chloroplasts was found to be strongly dependent on Pi (Fig. 2). Very low levels of Pi (0.1 mM) were found to be sufficient to provide maximum activity. High levels of Pi inhibited photosynthesis and this could be overcome by the addition of PGA (Fig. 3). Similar results with C₃ chloroplasts such as spinach and wheat have been interpreted as a requirement for transport on the phosphate translocator in order to obtain maximum rates of photosynthesis (5, 23).

One might expect that with isolated chloroplasts, carbon assimilation to starch and its associated O₂ evolution would not require exogenous Pi. Since bundle sheath chloroplasts of C₄ species have been noted for their capacity to accumulate large quantities of starch, the strong dependence of photosynthesis of these chloroplasts of P. milieaceum on Pi (Fig. 2) was unexpected.

The likely transport processes involving three-carbon phosphorylated compounds into or from C₄ mesophyll chloroplasts differ from those operating in C₃ chloroplasts. For instance, 3-PGA derived from bundle sheath cells is probably taken up and reduced (12, 17). This could occur in exchange for triose-P produced as the result of 3-PGA reduction. The C₄ chloroplasts possess an additional translocator which catalyzes Pi uptake in exchange for PEP during the regeneration of the acceptor for the C₄ cycle (16). In contrast, in isolated spinach (C₃) chloroplasts, Pi, triose-P, and PGA are transported on the phosphate translocator. In vivo, these chloroplasts are thought to take up Pi in exchange for triose-P (13, 23).

The extent to which interactions occur in the exchange of Pi, PEP, PGA, and triose-P across C₄ mesophyll chloroplasts is uncertain. In the present study, the apparent Kₘ for PGA was 0.3 mM for PGA-dependent O₂ evolution with the mesophyll chloroplasts (Fig. 4). The theoretical Vₘₙₐₓ was higher than the experimental Vₘₙₐₓ (Fig. 3) which could be due to a photochemical limitation at the higher PGA concentration (analogous to that occurring with HCO₃⁻ dependent O₂ evolution with isolated spinach chloroplasts (20)). Pi was found to inhibit PGA-dependent O₂ evolution which could occur if Pi is competing for PGA for uptake by the chloroplasts (Fig. 5). The results indicate that the cytoplasmic

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**Table II.** Summary of some data on substrate-dependent O₂ evolution by mesophyll and bundle sheath chloroplasts of Panicum milieaceum.

Chloroplasts were isolated, resuspended, and assayed in 0.3 M sorbitol, 25 mM Tricine-KOH, pH 8.0, and 10 mM EDTA. The assay medium also contained 200 units of catalase in 1.8 ml of reaction mixture.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Mesophyll Chloroplasts</th>
<th>Bundle Sheath Chloroplasts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
<td>2</td>
</tr>
<tr>
<td>none</td>
<td>--</td>
<td>0.13</td>
</tr>
<tr>
<td>10 mM bicarbonate, 0.2 mM Pi</td>
<td>0.18</td>
<td>0.13</td>
</tr>
<tr>
<td>4 mM PGA, 0.2 mM Pi</td>
<td>2.67</td>
<td>--</td>
</tr>
<tr>
<td>4 mM PGA</td>
<td>--</td>
<td>3.95</td>
</tr>
<tr>
<td>4 mM PGA, 10 mM bicarbonate, 0.2 mM Pi</td>
<td>3.13</td>
<td>3.47</td>
</tr>
<tr>
<td>0.5 mM Pi</td>
<td>--</td>
<td>0.06</td>
</tr>
<tr>
<td>0.5 mM Pi, 1.5 mM OAA</td>
<td>--</td>
<td>0.82</td>
</tr>
<tr>
<td>0.5 mM Pi, 1.5 mM OAA, 5 mM pyruvate</td>
<td>--</td>
<td>1.65</td>
</tr>
</tbody>
</table>

*Assays were made 10 h after isolation of bundle sheath protoplasts which may account for lower activity in this preparation.*
Fig. 3. Light-dependent $O_2$ evolution by bundle sheath chloroplasts of $P. miliaceum$. Chloroplasts were isolated, resuspended, and assayed in 0.3 M sorbitol, 25 mM Tricine-KOH (pH 8.0), and 10 mM EDTA. In addition, the assay medium contained 10 mM NaHCO$_3$, 200 units of catalase/1.8 ml and (a) 0.05 mM Pi, (b) 4 mM Pi + addition of PGA as indicated. Numbers in parentheses indicate rate of $O_2$ evolution as $\mu$mol mg$^{-1}$ Chl min$^{-1}$. 

Variables:
- $O_2$ evolution
- $0.05$ mM Pi
- $4$ mM Pi
- PGA addition (4 mM)
- Time (Minutes)
Fig. 4. Influence of PGA concentration on rate of light-dependent $O_2$ evolution by mesophyll chloroplasts of *P. miliaceum*. See Table II for isolation and assay media. Inset shows a double reciprocal plot of rate of photosynthesis versus PGA concentration.

Fig. 5. Influence of Pi concentration on rate of PGA-dependent $O_2$ evolution of mesophyll chloroplasts of *P. miliaceum*. See Table II for description of isolation and assay medium. In the assay medium contained 1 mM PGA.

Table III. PGA-dependent $O_2$ evolution with chloroplasts isolated from mesophyll protoplasts of *Panicum miliaceum*. See Table III for components of the isolation and assay medium.

| Addition                | Rate  
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>umol mg$^{-1}$ Chl min$^{-1}$</td>
</tr>
<tr>
<td>(Preparation 1)</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.13</td>
</tr>
<tr>
<td>1 mM PGA</td>
<td>2.10</td>
</tr>
<tr>
<td>5 mM PEP</td>
<td>0.07</td>
</tr>
<tr>
<td>1 mM PGA, 5 mM PEP</td>
<td>2.30</td>
</tr>
<tr>
<td>(Preparation 2)</td>
<td></td>
</tr>
<tr>
<td>0.3 mM PGA</td>
<td>2.30</td>
</tr>
<tr>
<td>0.3 mM PGA, 3 mM PEP</td>
<td>2.45</td>
</tr>
<tr>
<td>0.5 mM PGA</td>
<td>3.22</td>
</tr>
<tr>
<td>0.5 mM PGA, 1 mM pyridoxal phosphate</td>
<td>0.08</td>
</tr>
<tr>
<td>0.5 mM PGA, 5 mM Pi</td>
<td>1.38</td>
</tr>
</tbody>
</table>

mic Pi concentration could influence PGA reduction by the mesophyll chloroplasts in vivo.

If PGA and PEP were transported on a common carrier in the envelope of $C_4$ chloroplasts, PEP should inhibit PGA-dependent $O_2$ evolution. However, as shown in Table III, up to 5 mM PEP, or concentrations of PEP 5- to 10-fold higher than that of PGA, had no effect on PGA-dependent $O_2$ evolution. This suggests that PEP and PGA transport occur on separate carriers. Pyridoxal phosphate has been found to be a strong inhibitor of the phosphate translocator in spinach chloroplasts (6). Likewise, pyridoxal phosphate was very inhibitory to PGA-dependent $O_2$ evolution with the $C_4$ mesophyll chloroplasts (Table III) which could be due to inhibition of carrier-mediated transport.

Some evidence exists for specific carrier-mediated transport of PEP, Pi, and pyruvate with $C_4$ mesophyll chloroplasts (15, 16). Further direct studies on exchange of a number of metabolites across envelopes of both $C_4$ mesophyll and bundle sheath chloroplasts will be required to understand the transport mechanisms and their regulation in $C_4$ photosynthesis.

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LITERATURE CITED