Photosynthetic Activity of Chloroplasts Isolated from Bermudagrass
(Cynodon dactylon L.), a Species With a High Photosynthetic Capacity

T. M. Chen, R. H. Brown, and C. C. Black, Jr.
Department of Agronomy and Department of Biochemistry, University of Georgia,
Athens, Georgia 30601

Received December 23, 1968.

Abstract. Chloroplasts have been isolated from bermudagrass (Cynodon dactylon L.) leaves and assayed for photophosphorylation and electron transport activity. These chloroplasts actively synthesize adenosine triphosphate during cyclic electron flow with phenazine methosulfate and noncyclic electron flow concurrent with the reduction of such Hill oxidants as neotetramide adenosine diphosphate, cytochrome c, and ferricyanide. Apparent Km values for the cofactors of photophosphorylation have been determined to be 5 × 10^{-5} M for phosphate and 2.5 × 10^{-5} M for adenosine diphosphate. The influence of light intensity on photophosphorylation has been studied and the molar ratio of cyclic to noncyclic phosphorylation calculated. It is concluded that the high photosynthetic capacity of bermudagrass leaves probably could be supported by the photophosphorylation capacities indicated in these chloroplast studies and the anomalous lack of data in chloroplast studies on the production of sufficient reductant for CO₂ assimilation at high light intensities has been noted.

Adequate evidence has been presented indicating that some species of plants photo-assimilate atmospheric carbon dioxide at higher rates than others (10, 38, 39). Species with a high photosynthetic capacity include Cynodon dactylon L., Amaranthus edulis, Saccharum officinarum, Zea mays, and Paspalum notatum (10, 15, 20, 25, 38). Leaves of these species have CO₂ uptake rates in the range of 50 to 70 mg dm⁻² hr⁻¹. Other plants, particularly from temperate climates are about one-half as efficient under comparable physiological conditions (10, 25, 38, 39). This difference is illustrated by the general photosynthesis response of these 2 groups of plants to increasing light intensity in Fig. 1. Studies from numerous laboratories have converged in comparing these 2 groups of plants. From these studies the following general observations appear to be valid for plants with a high photosynthetic capacity: a low (0.0005 % or less) CO₂ compensation concentration (12, 17); a lack of photorespiration (12, 17); no enhancement of photosynthesis by lowering the external O₂ concentration (11); high optimum temperature for photosynthesis in the range of 25 to 40° (15, 33, 38, 39); and possibly a major pathway for photosynthetic CO₂ fixation (21, 22, 23, 24, 28) other than the reductive pentose phosphate cycle (7). In contrast, the species with a low photosynthetic capacity, which include many plants such as tobacco, soybeans, and temperate climate grasses, have the following gen-

1 This research was supported in part by the National Science Foundation Grant GB 7772. University of Georgia College of Agriculture Experiment Stations Journal Series Paper No. 444, College Station, Athens.

Fig. 1. Photosynthesis by bermudagrass and orchardgrass leaves versus light intensities.
CO₂ fixation appears to be via the reductive pentose phosphate cycle (7). In addition to these dissimilarities between groups of species with varied photosynthetic capacity other workers have reported differences in anatomy of the vascular bundle sheath cells (12, 30), arrangement of mesophyll cells (12, 30), and chloroplast structure (40).

Since photosynthetic CO₂ fixation requires ATP and a reducing substance such as NADPH or reduced ferredoxin and research on the synthesis of these substances by chloroplasts has been confined almost exclusively to plants of low photosynthetic capacity, we initiated these studies on the activity of isolated chloroplasts from species with high photosynthetic capacity. To our knowledge only a very limited number of papers are available which describe the activity of chloroplasts isolated from species with high photosynthetic capacities. Miflin and Hageman (31, 32) reported the isolation of chloroplasts from maize which were active in cyclic photophosphorylation with PMS² and non-cyclic photophosphorylation concurrent with the reduction of ferricyanide. Moreland and Hill (34) also reported the reduction of ferricyanide with corn chloroplasts. In addition, other workers have examined a few enzymes involved in photosynthetic CO₂ fixation and sucrose synthesis in chloroplasts of corn, sorghum, and sugarcane (19, 41).

Coastal bermudagrass, a common forage in the southern United States, was chosen as a source of chloroplasts since it is easily available and has been shown to have a high photosynthetic rate (10, 13, 38), a low CO₂ compensation concentration (12) and an optimum temperature for photosynthesis near 35° (33, 38).

Materials and Methods

Isolation of Chloroplasts. Ten g of field or greenhouse grown Cynodon dactylon L. (Coastal bermudagrass) leaves were cut into short (3-4 mm) sections and ground in 40 ml of 0.02 M tris-HCl buffer containing 0.35 M NaCl at pH 8.0, with a prechilled mortar and pestle for 1 to 1.5 min. The slurry was squeezed through 4 layers of cheesecloth and centrifuged at 200g for 1 min. The supernatant was then centrifuged at 2000g for 5 min. The pellet was suspended in 10 ml of isolation medium and centrifuged again. The sedimented chloroplasts were resuspended in one-tenth strength of isolation medium to give a chlorophyll concentration around 0.5 mg/ml.

All isolation was made at 0 to 2°. The same isolation medium and procedures were employed to isolate chloroplasts from spinach and pokeweed (Phytolacca americana L.). Chlorophyll concentration was determined by the method of Arnon (1).

Photophosphorylation and Electron Transport. The reaction mixture for testing photophosphorylation with spinach and pokeweed chloroplasts contained the following (in μmoles/ml): Tricine pH 8.0, 50; MgCl₂, 2; ADP, 1; P₇ + 32P, 1; PMS, 0.02 (cyclic); or ferricyanide, 0.2 (non-cyclic) and from 10 to 30 μg of chlorophyll as chloroplasts. This reaction mixture was used to test the optimal level of each reagent for bermudagrass. As the optimal level of each reagent was established, the subsequent tests for other reagents switched to that optimal level.

The reaction mixtures were placed in vials (1.5 cm diameter) and illuminated laterally at room temperature (21–23°) with a 300 watt incandescent bulb which was immersed in a liter beaker, containing a 2% (w/v) CuSO₄ solution. The light intensity at the front surface of the reaction mixture was 3000 ft-c. For testing the effect of light intensities on photophosphorylation the reaction vials were placed at different distances from a 650 watt GE Mardi Gras Movie Light. A flat bottle, 8 cm thick, containing 2% (w/v) CuSO₄ solution was used as a filter. Light intensities were measured with a Weston Model 756 illumination meter with cosine correction and quartz filters. Reactions were terminated by turning off the light and adding 0.1 ml of 20% (w/v) trichloroacetic acid. After centrifugation, the supernatant was assayed for its ATP content according to Avron (3). Hill reaction measurement procedures have been described (3, 4, 16, 42).

Results

Since little research has been published using chloroplasts from species with high photosynthetic capacity and no results were available on bermudagrass we first tried to isolate chloroplasts which were active in the Hill reaction and photophosphorylation. The method we finally decided was adequate is given in Materials and Methods. We found little influence of adding such substances as ascorbate, bovine serum albumin, polyvinyl pyrrolidone of numerous other materials which have proven useful in isolating chloroplasts from other species.

Fig. 2 demonstrates that isolated bermudagrass chloroplasts are active catalysts of photophosphorylation in that the rate of ATP synthesis is proportional to chlorophyll concentration (Fig. 2A) and initially linear with time of illumination (Fig. 2B). Fig. 2C illustrates the stability of photophosphorylation in bermudagrass chloroplasts. In subsequent photophosphorylation experiments we used chlorophyll concentrations under 20 μg/ml, illuminated for less

² The following abbreviations are used: PMS, phenazine methosulfate; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; salicylanilide, 5-chloro, 3-p-chlorophenyl), 2', 4',5'-trichlorosalicylanilide; Tricine, tris (hydroxymethyl) methylglycine.
Final concentration 10^{-6}

Cytochrome c, 10^{-4} M

Ferricyanide, 2 \times 10^{-4} M

FMN, 10^{-4} M

PMS, 10^{-5} M

than 2 min, and utilized our chloroplasts within 40 min after preparation.

Bermudagrass chloroplasts exhibit an apparent \( K_m \) of 5 \times 10^{-5} M for phosphate (Fig. 3A) although complete saturation was not reached until 2 \times 10^{-3} M, which was the concentration routinely employed. The nucleotide specificity data for photophosphorylation is given in Table I and the apparent \( K_m \) values calculated from Fig. 3B are ADP 2.5 \times 10^{-5} M, GDP 5 \times 10^{-5} M, and IDP 12 \times 10^{-5} M. Routinely 0.25 \mu mole/ml of ADP was used since inhibition occurred at higher concentrations, Fig. 3B.

The pH optimum was about 8.0 for ferricyanide supported photophosphorylation and the PMS system exhibited a similar optimum with a slight shift toward pH 7.5, Fig. 3D. In subsequent experiments pH 8.0 was employed. The concentrations of PMS, 2 \times 10^{-5} M, and ferricyanide, 2 \times 10^{-4} M employed in the experiments in this paper are both within optimal concentrations ranges, Fig. 3C.

We also examined the metal requirements for photophosphorylation and found Mg^{2+} at 2 \times 10^{-3} M to be the most effective metal.

The ability of bermudagrass chloroplasts to synthesize ATP concurrent with the reduction of typical Hill oxidants is given in Table II. The ATP/2e\(^{-}\) ratios are slightly lower than routinely observed with spinach but certainly tend to approach unity. The uncoupler salicylanilide (8) and the electron transport inhibitor DCMU give results similar to those commonly obtained with spinach.

Table I. Nucleotide Specificity of Photophosphorylation With Coastal Bermudagrass Chloroplasts

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>Relative rates of photophosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Noncyclic</td>
</tr>
<tr>
<td>ADP</td>
<td>100 (^1)</td>
</tr>
<tr>
<td>CDP</td>
<td>0.5</td>
</tr>
<tr>
<td>GDP</td>
<td>53</td>
</tr>
<tr>
<td>IDP</td>
<td>53</td>
</tr>
<tr>
<td>UDP</td>
<td>18</td>
</tr>
</tbody>
</table>

\(^1\) In noncyclic photophosphorylation with ferricyanide the ADP catalyzed rate was 108 \mu moles of ATP mg chlorophyll\(^{-1}\) hr\(^{-1}\) and 481 for cyclic.

Table II. Electron Transport Activity and Concurrent Photophosphorylation With Coastal Bermudagrass Chloroplasts

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>ATP/2e(^{-})</th>
<th>Photophosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DCMU(^2)</td>
</tr>
<tr>
<td>NADPH, 2 \times 10^{-4} M(^4)</td>
<td>0.6 to 0.7</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome c, 1.0 \mu g/ml</td>
<td>0.0 to 0.7</td>
<td>0</td>
</tr>
<tr>
<td>Ferricyanide, 2 \times 10^{-4} M</td>
<td>0.7 to 0.8</td>
<td>0</td>
</tr>
<tr>
<td>FMN, 10^{-4} M</td>
<td>...</td>
<td>0</td>
</tr>
<tr>
<td>PMS, 10^{-5} M</td>
<td>...</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^1\) Final concentration in reaction mixtures.

\(^2\) Final concentration 2 \times 10^{-6} M, chosen to give only partial inhibition of cyclic photophosphorylation.

\(^3\) Final concentration 10^{-6} M.
The relationship between the species, however, will remain essentially the same. The experiment conditions employed in Fig. 1 and described by Brown et al. (10) are an approximation of physiological conditions. In bermudagrass leaves, photosynthesis at 50 mg of CO₂ fixed dm⁻²hr⁻¹ is equivalent to approximately 470 μmoles of CO₂ fixed mg chlorophyll⁻²hr⁻¹.

When the photosynthetic response of bermudagrass leaves to increasing light intensity (Fig. 1) is considered in relation to the electron transport and ATP synthesis systems which have been studied in isolated chloroplasts of such plants as spinach it is clear that only cyclic photophosphorylation with PMS (2,4, Fig. 4A, B, and C) continues to increase with intensity of illumination. In spinach chloroplasts the rate of NADP⁺ (42), trichloroindophenol (4), and vitamin K₃ (2), reduction reach a plateau at 1000 to 2000 ft-c as well as the synthesis of ATP concurrent with the reduction of Hill oxidants such as NADP⁺ and spinach ferredoxin (16, 42). CO₂ fixation by isolated chloroplasts also reaches a plateau at comparable intensities (26, 42). Bermudagrass phosphorylation (Fig. 4C) responds in similar fashion and the reduction of Hill oxidants (unpublished data) is similar. Thus the synthesis of ATP during cyclic electron flow observed in isolated chloroplasts could theoretically support the CO₂ fixation of intact leaves of plants with high photosynthetic capacity at higher light intensities, but the production of sufficient amounts of a reductant for CO₂ fixation cannot be explained from any available data. The authors recognize that the absolute rates of reduction of most Hill oxidants with isolated chloroplasts often are as high as CO₂ fixation in intact leaves. But these Hill reaction assays usually are supplemented with saturating concentrations of all components and invariably saturate at low light intensities. 1 to 2000 ft-c, which contrasts sharply with the light intensity response curve for bermudagrass leaves, Fig. 1. If one can legitimately add photosynthesis to photorespiration in plants with a low photosynthetic capacity, it also is difficult to explain the production of sufficient CO₂ reductant in these plants at high light intensities. Of course there also is some doubt that the in vitro chloroplast studies on cyclic photophosphorylation in the presence of the nonphysiological substance PMS can be extrapolated to intact leaves.

The affinity of bermudagrass chloroplasts for P₅ seems to be higher than other plant chloroplasts. An apparent P₅ Km of 5 × 10⁻⁵ m for bermudagrass is at least 10-fold lower than the range of 4 × 10⁻⁴ to 2 × 10⁻³ m reported for spinach and swiss chard (3, 4, 5). In addition, the rate of bermudagrass phosphorylation was saturated at about 2 × 10⁻⁵ m whereas spinach and swiss chard chloroplasts are difficult to saturate with P₅ (3, 4). Bermudagrass chloroplasts also have a high affinity for ADP as indicated by an apparent Km of 2.5 × 10⁻³ m which is lower than the apparent Km of 5 to 6 × 10⁻⁴ m.

Discussion

In those species with a high photosynthetic capacity the maximum rate of photosynthesis occurs at higher light intensities than in those species with a low photosynthetic capacity. In Fig. 1, bermudagrass shows the general characteristics of plants with high photosynthetic capacity and orchardgrass (Dactylis glomerata L.) illustrates the response of a species with low photosynthetic capacity to increasing illumination intensity. Note that dark respiration (2-4 mg CO₂ dm⁻²hr⁻¹) has been added to all values. Of course, these responses are general in that variation of factors such as CO₂ concentration and temperature can change light response curves.

From these experiments there did not appear to be anything strikingly unusual about the activity of chloroplasts from bermudagrass when compared to other plant chloroplasts such as spinach. We then decided to examine the effect of light intensity on photophosphorylation in comparison with spinach and pokeweed (Fig. 4A, B, and C). The most striking response of bermudagrass chloroplasts occurred at low light intensities, 1200 ft-c or less. At all intensities studied cyclic photophosphorylation activity was higher than noncyclic in bermudagrass chloroplasts. Indeed the sigmoid shaped curve of photophosphorylation with spinach (42) and pokeweed (Fig. 2A, B) was not as evident with bermudagrass, Fig. 4C. The molar ratio of cyclic to noncyclic ATP in Fig. 4A, B and C is plotted together with data at higher intensities in Fig. 4D; again indicating that cyclic photophosphorylation in bermudagrass is quite active.

![Image of Figure 4. Comparison of cyclic (PMS) and noncyclic (ferricyanide) photophosphorylation in isolated chloroplasts of spinach (A), pokeweed (B) and bermudagrass (C), and the molar ratio of cyclic to noncyclic photophosphorylation (D) versus light intensity.](image-url)

Note that dark respiration (2-4 mg CO₂ dm⁻²hr⁻¹) has been added to all values.
for spinach \((6,37)\). An apparent \(K_m\) of 5 to 7 \(\times 10^{-6}\) m for ADP with swiss-chard chloroplasts has been reported \((3)\). These data on \(P_i\) and ADP suggest that bermudagrass chloroplasts should be quite active as catalysts of photophosphorylation. The effectiveness of diphosphate nucleotides other than ADP as phosphate acceptor was similar to spinach and swiss chard \((3,29)\). It is likely that nucleoside diphosphokinase is present in the bermudagrass chloroplasts although we have not examined this enzyme.

In the Calvin cycle, 3 moles of ATP and 2 moles of NADPH apparently participate in the fixation of 1 mole of \(CO_2\) \((7)\). For each \(CO_2\) fixed there may be 2 ATP’s and 2 NADPH’s from noncyclic photophosphorylation and 1 ATP presumably comes from cyclic photophosphorylation. There are enzyme data indicating that reactions for the formation of hexoses and other products of the Calvin cycle are operating in plants with high photosynthetic capacity \((21,41)\). However, there are also data indicating that plants with high photosynthetic capacity have another pathway of \(CO_2\) fixation involving the carboxylation of phosphopyruvate forming oxaloacetate which equilibrates with malic and aspartic acid \(\text{C}_4\) \((21,22,23,24,27,28,41)\). In short time \((\text{under 10 sec})\) \(^{14}\text{CO}_2\) fixation studies these compounds are labeled and only later does \(^{14}\text{C}\) appear in PGA and hexoses. We have obtained data in unpublished experiments in which bermudagrass leaves fixed \(^{14}\text{CO}_2\) into compounds similar to those in corn and sugarcane leaves. Thus, we tentatively consider that bermudagrass has the \(\text{C}_4\) cycle of \(CO_2\) assimilation.

In the \(\text{C}_4\) cycle the formation of phosphopyruvate catalyzed by phosphopyruvate synthetase \((\text{pyruvate, } \text{Pi} \text{ dikinase})\) is shown in equation I \((22,23)\).

\[
\text{Pyruvate} + \text{ATP} + \text{Pi} \rightarrow \text{phosphopyruvate} + \text{AMP} + \text{PP}
\] (I)

In order to operate the \(\text{C}_4\) cycle, this reaction would require 2 moles of ATP: 1 ATP as given in equation I; and then probably 1 ATP would be needed to regenerate ADP from the AMP in equation I, possibly through the action of adenyl kinase. It has been postulated that this \(\text{C}_4\) cycle might operate in conjunction with the Calvin cycle in plants such as sugarcane by supplying a carboxyl carbon of the \(\text{C}_4\) acids to the Calvin cycle and generating pyruvate \((21)\). Two theoretical stoichiometries for photosynthetic \(CO_2\) fixation could be calculated as follow: A) Plants containing only the Calvin cycle \((7)\): 1 \(CO_2\): 3 ATP: 2 NADPH; B) Plants containing the Calvin cycle operating in conjunction with the \(\text{C}_4\) cycle: 1 \(CO_2\): 5 ATP: 2 NADPH. The 2 extra molecules of ATP would be needed to continuously generate phosphopyruvate \((\text{equation I})\). Hence it appears that plants containing the \(\text{C}_4\) cycle may require a high rate of photophosphorylation, presumably cyclic, to furnish the extra ATP required for the simultaneous operation of the \(\text{C}_4\) cycle and the Calvin cycle.

High photosynthetic capacity usually is accompanied by the presence of the \(\text{C}_4\) cycle \((9,12)\,\) but it is not clear how the \(\text{C}_4\) cycle could contribute to a higher photosynthetic capacity. Possibly the phosphopyruvate carboxylase which has a low \(K_m\) for \(\text{CO}_2\) \((43)\) with respect to ribulose-1, 5-diP carboxylase would enable these plants to use low concentrations of \(\text{CO}_2\) and recycle \(\text{CO}_2\) evolved from respiration \((14)\). This could account for the low \(\text{CO}_2\) compensation concentration and the apparent lack of photorespiration. However, it is pertinent to this point to note that Goldsworthy found the same \(K_m\) for \(\text{CO}_2\) during photosynthesis with maize, sugarcane, and tobacco \((18)\). There are reports that plants with high photosynthetic capacity lack glycolate oxidase \((35)\), hence glycolate oxidation and the associated \(\text{CO}_2\) production may not occur.

Considering the theoretical stoichiometries given above for \(\text{CO}_2\) fixation, indicating the high use of ATP in plants with high photosynthetic capacity, our data on the affinity of bermudagrass chloroplasts for \(P_i\) and ADP and the molar ratio of cyclic to noncyclic ATP, support the possibility that these plants may be capable of producing more ATP than low photosynthetic capacity plants.

Literature Cited


