Comparative Studies on the Activity of Carboxylases and Other Enzymes in Relation to the New Pathway of Photosynthetic Carbon Dioxide Fixation in Tropical Grasses

By C. R. Slack and M. D. Hatch*

David North Plant Research Centre, The Colonial Sugar Refining Co. Ltd., Indooroopilly, Queensland, Australia

(Received 11 November 1966)

1. The activity per unit of chlorophyll of certain carboxylases, and of other enzymes involved in photosynthesis, was determined in leaf extracts of the tropical grasses, sugar-cane, maize and sorghum, and compared with the activities for wheat, oat and silver-beet. Maximum rates of photosynthetic carbon dioxide uptake were also measured for comparison with enzyme activities. 2. Phosphopyruvate carboxylase activity was about 60 times greater in the tropical grasses than in wheat, oat and silver-beet and was severalfold higher than the rates of photosynthetic carbon dioxide uptake. Most of the enzyme was located in the chloroplast fraction of cell extracts. 3. Phosphopyruvate carboxylase was apparently the major photosynthetic carbon dioxide-fixing enzyme in the tropical grasses, although malic enzyme may contribute to a lesser extent. 4. Tropical grasses contained less than one-tenth of the ribulose diphosphate carboxylase activity present in wheat, oat and silver-beet. For the tropical grasses this activity, determined with a saturating concentration of bicarbonate, was approx. 10% of the rate of photosynthesis. 5. The fraction-1 protein content of leaf extracts paralleled the ribulose diphosphate carboxylase activity. 6. In contrast, the activity of several other enzymes of the Calvin cycle was similar in the different species examined.

Studies with sugar-cane leaves exposed to $^{14}$CO$_2$ have indicated that the path by which carbon dioxide is incorporated into sugar differs from the pathway generally accepted as operating in higher plants (Calvin & Bassham, 1962), at least in the nature of reactions leading to the formation of 3-phosphoglycerate (Kortschak, Hartt & Burr, 1965; Hatch & Slack, 1966). Our studies show that $^{14}$CO$_2$ is incorporated first into the C-4 of a C$_4$ dicarboxylic acid, either oxaloacetate or malate, and is subsequently transferred to the C-1 of 3-phosphoglycerate and then to sugars. The primary carbon dioxide acceptor was apparently a C$_3$ acid. Ribulose diphosphate carboxylase appeared to play, at the most, only a minor role in the overall process of carbon dioxide fixation. A survey of the early photosynthetic products (by short-time exposure to $^{14}$CO$_2$) in species from several families provided evidence that the pathway of photosynthetic carbon dioxide fixation in sugar-cane was also operative in a number of other grasses of tropical origin and a species of Cyperaceae (Hatch, Slack & Johnson, 1967). The labelling pattern obtained with other species was consistent with the operation of the Calvin cycle.

The aim of the present studies was to determine whether the different labelling patterns obtained with tropical grasses and other species could be related to differing activities of individual enzymes. We compared the activities of ribulose diphosphate carboxylase, other enzymes specifically associated with the Calvin cycle, and carboxylases that form malate and oxaloacetate, in leaves of three tropical grasses and three species in which the Calvin cycle is operative. The intracellular location of these enzymes was also examined.

MATERIALS

Phosphopyruvic acid, glyceraldehyde 3-phosphate, ribulose 1,5-diphosphate, glucose 6-phosphate, fructose 1,6-diphosphate, NADP, ATP, glucose 6-phosphate dehydrogenase and hexokinase were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). NaH$^{14}$CO$_3$ was prepared from Ba$^{14}$CO$_3$ supplied by The Radiochemical Centre (Amersham, Bucks.). Sodium mercaptothiazole was purchased from Hopkin and Williams Ltd. (London).

Ribose 5-phosphate isomerase free of phosphoribulo-
kinase was prepared from leaves of silver-beet as described by Hurwitz, Weissbach, Horecker & Smyrniotis (1956).

Ribulose diphosphate carboxylase was partially purified from leaves of silver-beet by fractionation with (NH₄)₂SO₄ and heat treatment as described by Racker (1962).

-[14C]Oxaloacetate was prepared from phosphopyruvate and NaH¹⁴CO₃ by using a partially purified preparation of maize-leaf phosphopyruvate carboxylase. When the reaction was complete HCl was added to a final concentration of 0.1N. The mixture was treated with Dowex 50 to remove Mg²⁺ and tris and stored at −15° in 0.1N-HCl.

Plant material. The young fully-expanded leaves were obtained from plants of sugar-cane (Saccharum officinarum, var. Badilla), maize (Zea mays, var. DS 606A), hybrid sorghum (Sorghum, var. Rio), oat (Avena sativa, var. Benton), wheat (Triticum sativum, var. Gala) and silver-beet (Beta vulgaris) grown in nutrient culture in full sunlight.

METHODS

Measurement of the rate of photosynthetic carbon dioxide uptake. Leaves were detached by cutting under water and placed in a Perspex chamber (29 cm. x 22 cm. x 8cm.) with their bases submerged in water. Initially, leaves were illuminated at 9000 ft.-candles, by a Philips 400w HPL lamp shielded by a 1 in.-thick water filter. Air, saturated with water vapour, was passed through the chamber at a rate sufficient to maintain the CO₂ concentration at approx. 0.0032% (v/v). At 10 min. intervals the chamber was sealed and the rate of decline of the CO₂ concentration from 0.0029% to 0.0032% (v/v) was measured with a Beckman infrared gas analyzer (model 15A). Rates were determined at several light-intensities in the range 2000-9000 ft.-candles.

Preparation of leaf extracts. Leaf lamina (2g.) was macerated in a Serval Omnimix at maximum speed for 2 min. with 15 ml. of 0.1M-tris–HCl buffer, pH 7.8, containing 10 mM-2-mercaptoethanol. The blending was performed under N₂ at 0°, the N₂ and mercaptoethanol being added to prevent phenol oxidation. The liquid obtained by expressing the macerated material through muslin was sampled for chlorophyll determinations and the remainder centrifuged at 20000g for 10 min. A 1 ml. sample of the supernatant was applied to a Sephadex G-25 column (5ml.), previously equilibrated with 50 mM-tris–HCl buffer, pH 7.4, and the protein component was collected in 1 ml. All steps were conducted at 5° and most enzymes were assayed within 10 min. of preparing the extracts. Spectrophotometric assays were conducted at 22° and the remaining assays at 30°.

For the preparation of extracts used for ultracentrifuge analysis the following modified procedure was employed. Leaf laminae (8g.) were macerated in 16 ml. of 0.1M-tris–HCl buffer, pH 8.2, containing 50 mM-sodium mercapto- benzothiazole and 1 mM-EDTA at 0° under N₂. The juice, obtained by expressing the macerated tissue through muslin, was rapidly frozen, thawed and centrifuged at 25000g for 10 min. (Singer, Eggman, Campbell & Wildman, 1952). After treatment on a Sephadex G-25 column, equilibrated with 0.1M-tris–HCl buffer, pH 7.4, samples of the preparation were either assayed for ribulose diphosphate carboxylase or centrifuged at 20° in a Spinco model E ultracentrifuge for protein analysis. The extracts for the ultracentrifuge studies contained 3-4.5 mg. of protein/ml. as determined by the method of Warburg & Christian (1941). Non-aqueous isolation of chloroplasts from maize leaf. Half-fully-expanded maize leaves were freeze-dried as described by Stocking (1959) then stored in vacuo over H₂SO₄ and solid NaOH at −25° until required. The tissue was macerated in a hexane–carbon tetrachloride mixture (density either 1.35 or 1.37) with a glass Ten-Broek homogenizer, and separated into fractions of different density as described by Smillie (1963).

Assay of ribulose diphosphate carboxylase (EC 4.1.1.39). The formation of radioactive 3-phosphoglycerate was measured in reactions provided with H⁺¹⁴CO₃⁻ and ribulose 1,5-diphosphate. Reaction mixtures contained 10 μmoles of tris–HCl buffer, pH 8.3, 1 μmole of 2-mercaptoethanol, 1 μmole of MgCl₂, 7.5 μmoles of NaH¹⁴CO₃ (containing 6.2 x 10⁶ disintegrations/min.), 0.2 μmole of ribulose 1,5-diphosphate and the leaf extract in a total volume of 0.13 ml. The volume of leaf extract was adjusted to give a linear reaction rate for 10 min. at 30°. Reaction mixtures were preincubated at 0° for 10 min. without ribulose 1,5-diphosphate to prevent any initial lag in the reaction rate (Calvin & Pon, 1959). Samples (0.04 ml.) of the reaction mixture were removed after 4 and 8 min. and transferred to 0.04 ml. of 10% trichloroacetic acid. Portions of the acidified reaction mixtures were spotted within a 1 in.-diameter circle on Whatman no. 1 filter paper, dried in an air stream, and counted with a 2 in.-diameter Geiger–Müller tube. The counting efficiency was 5%.

Assay of glyceraldehyde phosphate dehydrogenase (EC 1.2.1.13). The assay procedure described by Gibbs (1955) was employed.

Assay of hexose diphosphatase (EC 3.1.3.11). Activity was measured by the release of Pi from fructose 1,6-diphosphate as described by Racker & Schroeder (1958). Control reactions established that fructose 6-phosphate was not hydrolysed under the conditions employed.

Assay of glucose 6-phosphate dehydrogenase (EC 1.1.1.49). The enzyme was assayed as described by Kornberg & Horecker (1955).

Assay of phosphoribulokinase (EC 2.7.1.19). The formation of ribulose 1,5-diphosphate from ribulose 5-phosphate was determined in the presence of an excess of ribose 5-phosphate isomerase. Reaction mixtures contained 7-8 μmoles of tris–HCl buffer, pH 8.4, 0.37 μmole of ribose 5-phosphate, 0.02 μmole of ATP, 1 μmole of MgCl₂, 1 μmole of 2-mercaptoethanol, 1 μmole of KH¹⁴CO₃ (containing 2.9 x 10⁶ disintegrations/min.), ribose 5-phosphate isomerase (2 units, 30°, pH 8.4) and the leaf extract in a total volume 0.15 ml. In control reaction mixtures 0.08 μmole of ribulose 1,5-diphosphate replaced ribose 5-phosphate. Reaction mixtures were incubated at 30° for 5 and 15 min., transferred to a boiling-water bath for 90 sec., then cooled. The remaining ATP in the reaction mixtures was removed by incubating solutions with hexokinase and an excess of glucose. Ribulose diphosphate carboxylase (0.5 unit, 30°, pH 8.4) was added and after the solutions were incubated at 30° for 10 min. they were acidified and samples counted by the procedure already described. Control reactions to which ribulose 1,5-diphosphate was added showed that the method afforded a quantitative measure of ribulose 1,5-diphosphate formation.

Assay of phosphopyruvate carboxylase (EC 4.1.1.31). Activity was determined in uncentrifuged leaf extracts by
measuring the incorporation of $\text{H}^4\text{CO}_2^-$ in the presence of phosphopyruvate. Reaction mixtures contained 5$\mu$moles of tris–HCl buffer, pH 8-3, 1$\mu$moles of 2-mercaptoethanol, 0-5$\mu$moles of MgCl$_2$, 0-2$\mu$moles of sodium phosphopyruvate, 0-5$\mu$moles of NaH$_4$CO$_3$ (containing $1-25 \times 10^4$ disintegrations/min.), 0-5$\mu$moles of sodium glutamate and the leaf extract in a total volume 0-08ml. The amount of leaf extract used was adjusted so that the reaction rate was linear for at least 5 min. at 30°. An equal volume of 10% trichloroacetic acid was added to reaction mixtures after 2 and 4 min. and samples were counted as already described.

**Assay of malic enzyme** [L-malate–NADP oxidoreductase (decarboxylating), EC 1.1.1.40]. The formation of radioactive malate was measured in reaction mixtures containing the leaf extract, 5$\mu$moles of sodium pyruvate, 0-1$\mu$moles of NADP, 1$\mu$moles of MgCl$_2$, 1-5$\mu$moles of glucose 6-phosphate, 0-5 unit of glucose 6-phosphate dehydrogenase, 15$\mu$moles of tris–HCl buffer, pH 7-4, and 12-5$\mu$moles of NaH$_4$CO$_3$ (13 $\times$ $10^4$ disintegrations/min.) in a total volume 0-3ml. Reactions were incubated at 30° in small stoppered tubes in which the air volume was only 0-2ml. At intervals 0-05ml. samples were withdrawn through the stopper with a syringe, and acidified and counted as previously described. The reaction rate was linear for at least 30 min.

**Assay of malate dehydrogenase** (EC 1.1.1.37). The procedure involving the measurement of NADH$_2$ formation in potassium glycinate buffer, pH 9-4, was employed (Wolfe & Neilands, 1956).

**Assay of aspartate aminotransferase** (EC 2.6.1.1). The formation of radioactive aspartate was measured in reaction mixtures containing [4-14C]oxaloacetate and glutamate. Reaction mixtures contained 10$\mu$moles of tris–HCl buffer, pH 8-0, 0-5$\mu$moles of [4-14C]oxaloacetate (containing $1-4 \times 10^4$ disintegrations/min.), 4$\mu$moles of potassium glutamate, 2$\mu$g. of pyridoxal phosphate and the leaf extract in a final volume 0-2ml. At intervals 0-04ml. samples were transferred to 0-01ml. of 0-5M-CuSO$_4$ in 0-33M-KH$_2$PO$_4$ and heated at 100° for 90 sec. to convert the remaining oxaloacetate into unlabelled pyruvate and $^{14}$CO$_2$ (Vennesland, 1962). The radioactivity remaining as aspartate was counted as described above.

**Assay of acid phosphatase.** Acid phosphatase was assayed at pH 5-3 with p-nitrophenol phosphate as substrate, as described by Lowry (1957).

---

**Table 1. Rates of photosynthetic carbon dioxide uptake of detached leaves and activities of Calvin-cycle enzymes in leaf extracts**

<table>
<thead>
<tr>
<th>Species</th>
<th>Photosynthesis (mole of CO$_2$ fixed/mg. of chlorophyll/min.)</th>
<th>Ribulose diphosphate carboxylase</th>
<th>Phosphoribulokinase</th>
<th>Hexose diphosphatase</th>
<th>Glyceraldehyde phosphate dehydrogenase (NADP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar-cane</td>
<td>2-9</td>
<td>0-30</td>
<td>8-1</td>
<td>0-76</td>
<td>5-1</td>
</tr>
<tr>
<td>Maize</td>
<td>3-5</td>
<td>0-62</td>
<td>11-1</td>
<td>0-80</td>
<td>4-5</td>
</tr>
<tr>
<td>Sorghum</td>
<td>3-1</td>
<td>0-35</td>
<td>14-8</td>
<td>0-96</td>
<td>3-3</td>
</tr>
<tr>
<td>Wheat</td>
<td>1-7</td>
<td>4-7</td>
<td>28-0</td>
<td>1-21</td>
<td>4-8</td>
</tr>
<tr>
<td>Oats</td>
<td>1-6</td>
<td>4-5</td>
<td>25-0</td>
<td>1-15</td>
<td>9-7</td>
</tr>
<tr>
<td>Silver-beet</td>
<td>1-7</td>
<td>4-2</td>
<td>21-0</td>
<td>0-76</td>
<td>2-6</td>
</tr>
</tbody>
</table>

**Assay of invertase.** The procedure described by Hatch, Sacher & Glaziov (1963) was employed.

**Chlorophyll determinations.** Chlorophyll was determined as described by Arnon (1949).

---

**RESULTS**

**Rates of photosynthetic carbon dioxide uptake and activity of Calvin-cycle enzymes.** The rates of photosynthetic carbon dioxide uptake shown in Table 1 were determined at saturating light-intensities; approx. 8000 ft.-candles for the tropical grasses, sugar-cane, maize and sorghum, and 5000 ft.-candles for wheat, oats and silver-beet. The rates of photosynthetic carbon dioxide uptake/mg. of chlorophyll for all three tropical grasses were approximately twice the rates for the other species examined.

There were only small species differences in the activities of phosphoribulokinase, hexoseid phosphate and glyceraldehyde phosphate dehydrogenase (NADP) in extracts from the leaves of tropical grasses and wheat, oats and silver-beet (Table 1). However, the activity of ribulose diphosphate carboxylase was much lower in extracts from the tropical grasses. Similar ribulose diphosphate carboxylase activities were obtained with uncentrifuged leaf extracts or when sodium mercaptoethanol was used as a phenol oxidase inhibitor in the extraction medium. We could find no evidence for the inactivation of the enzyme during the preparation of leaf extracts of tropical grasses or for the presence of an inhibitor.

**Fraction-1 protein content of leaf extracts.** There is considerable support for the belief that fraction-1 protein (Singer et al. 1952) and ribulose diphosphate carboxylase are the same protein entity (Weissbach, Horecker & Hurwitz, 1956; Lyttleton & Ts'o, 1958; Park & Pon, 1961). A direct relationship...
between ribulose diphosphate carboxylase activity and the fraction-1 protein content in the leaves of the different species we examined would therefore provide additional evidence that enzyme activity was indicative of enzyme concentration.

In the analytical ultracentrifuge, extracts of wheat, oats, silver-beet and the three tropical grasses revealed a component that moved as a sharp symmetrical peak with a sedimentation coefficient of between 16 and 17s, properties typical of fraction-1 protein. However, with extracts of the tropical grasses only 4-10% of the total soluble protein was located in this peak, compared with approx. 50% for the other species. The remaining protein was located in a slower-moving peak that diffused rapidly with time. The proportion of the total protein in each peak was determined by measuring the area of the peaks on enlarged ultracentrifuge diagrams. Ultracentrifugation patterns for extracts of sorghum and silver-beet leaves are shown in Fig. 1. The ratio of ribulose diphosphate carboxylase activity (μmoles/mg. of chlorophyll/min.) to the amount of fraction-1 protein (mg. of protein/mg. of chlorophyll) was similar for extracts of all species: wheat (0-90), oats (0-78), silver-beet (0-87), sugar-cane (0-97), maize (1-15) and sorghum (0-78).

Activity of enzymes involved in the synthesis and interconversion of C₄ dicarboxylic acids. Phosphopyruvate carboxylase and malic enzyme were identified in leaf extracts of all the species examined (Table 2). However, the activities of both enzymes were 40- to 60-fold greater in leaf extracts of the tropical grasses than in extracts from wheat, oats and silver-beet. In all extracts phosphopyruvate carboxylase was considerably more active than malic enzyme and its activity in the tropical grasses was severalfold greater than the rates of photosynthetic carbon dioxide uptake recorded in Table 1. Phosphopyruvate carboxylase, previously described in leaves of a number of species (Mazulis & Vennesland, 1957), was not detectable with the H¹⁴CO₃⁻-oxaloacetate exchange assay procedure (Vennesland, Evans & Altman, 1947) in extracts of any of the species we investigated.

Since malate, aspartate and oxaloacetate contain almost all the fixed label in leaves of tropical grasses after a brief exposure to ¹⁴CO₂ (Hatch et al., 1967), we determined the activities of enzymes catalysing the interconversion of these compounds. Aspartate aminotransferase activity was somewhat greater, and malate dehydrogenase activity similar in extracts from the tropical grasses compared with the other species (Table 2). In separate studies no evidence was obtained for a NADP-specific malate dehydrogenase in leaves of sugar-cane, maize or sorghum.

Intracellular location of phosphopyruvate carboxylase and malic enzyme in maize leaves. Chloroplasts isolated from maize leaves in aqueous media containing 0·35M-sodium chloride retained little of the total tissue complement of either phospho-

---

**Table 2. Activity of phosphopyruvate carboxylase, malic enzyme, aspartate aminotransferase and malate dehydrogenase in leaf extracts**

Methods for the preparation of leaf extracts and the assay of enzymes are described in the Methods section. The rates of photosynthetic CO₂ uptake for these plants, expressed in the same units, are provided in Table 1.

<table>
<thead>
<tr>
<th>Species</th>
<th>Phosphopyruvate carboxylase</th>
<th>Malic enzyme</th>
<th>Aspartate aminotransferase</th>
<th>Malate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sugar-cane</td>
<td>18-5</td>
<td>0-48</td>
<td>5-6</td>
<td>8-1</td>
</tr>
<tr>
<td>Maize</td>
<td>17-5</td>
<td>0-45</td>
<td>3-6</td>
<td>6-5</td>
</tr>
<tr>
<td>Sorghum</td>
<td>15-8</td>
<td>0-45</td>
<td>4-7</td>
<td>8-9</td>
</tr>
<tr>
<td>Wheat</td>
<td>0-39</td>
<td>0-01</td>
<td>2-1</td>
<td>8-1</td>
</tr>
<tr>
<td>Oats</td>
<td>0-33</td>
<td>0-01</td>
<td>1-9</td>
<td>7-9</td>
</tr>
<tr>
<td>Silver-beet</td>
<td>0-35</td>
<td>0-02</td>
<td>2-3</td>
<td>10-4</td>
</tr>
</tbody>
</table>
pyruvate carboxylase or malic enzyme. Several enzymes of the Calvin cycle are lost from chloroplasts isolated in aqueous media but remain associated with the chloroplasts isolated in non-aqueous media (Stocking, 1959; Smillie & Fuller, 1959; Smillie, 1960). This method is based on the assumption that enzymes originally located in the chloroplasts, but not other enzymes, will remain associated with chlorophyll during fractionation. We applied this procedure to maize leaves and compared the distribution of phosphopyruvate carboxylase and malic enzyme with that of several other enzymes including two, ribulose diphosphate carboxylase and glyceraldehyde phosphate dehydrogenase (NADP), previously shown to be located entirely in the chloroplasts (Smillie, 1963). The results of two experiments are shown in Table 3. The distribution of phosphopyruvate carboxylase and malic enzyme was similar to that for ribulose diphosphate carboxylase and glyceraldehyde phosphate dehydrogenase (NADP) but markedly different from the distribution of invertase and acid phosphatase. Malate dehydrogenase and glucose 6-phosphate dehydrogenase showed an intermediate pattern of distribution. In Expt. 2 the fractions of increasing density contained respectively 41%, 33% and 26% of the total malate dehydrogenase and 37%, 32% and 31% of the glucose 6-phosphate dehydrogenase.

**DISCUSSION**

The present studies reveal that the unique labelling pattern previously observed when illuminated leaves of tropical grasses were exposed to $^{14}$CO$_2$ (Hatch & Slack, 1966; Hatch et al. 1967) is correlated with marked differences in the activity of certain enzymes and in rates of photosynthetic carbon dioxide uptake. The observation that the maximum rates of photosynthetic carbon dioxide uptake per mg. of chlorophyll for the tropical grasses are higher than those for other species is in agreement with the results of Hesketh & Moss (1963). Compared with wheat, oats and silver-beet, the ribulose diphosphate carboxylase activity, measured at an optimum concentration of bicarbonate, was much lower in the tropical grasses and was only about one-tenth of the maximum carbon dioxide-fixation rates. These findings support our earlier conclusion that 3-phosphoglycerate is not the product of the major photosynthetic carboxylation reaction in the tropical grasses (Hatch et al. 1967). Owing to its high $K_m$ for bicarbonate of between 11mM and 30mM (Weissbach et al. 1956; Peterkofsky & Racker, 1961), the potential activity of ribulose diphosphate carboxylase in vivo may be considerably less than estimates based on the assay of the isolated enzyme. The problems of relating the activity of this enzyme in extracts to its activity in vivo have been considered by Racker (1957) and Peterkofsky & Racker (1961).

Phosphopyruvate carboxylase is apparently mainly responsible for the rapid fixation of carbon dioxide into C$_4$ dicarboxylic acids by illuminated leaves of tropical grasses. Its activity in leaves of tropical grasses was well in excess of the maximum rates of photosynthesis and was about 60-fold the activity in leaves of wheat, oats and silver-beet. In

### Table 3. Enzyme activities and their relation to the chlorophyll content of maize-leaf fractions isolated in non-aqueous media

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Chlorophyll content (mg./fraction)</th>
<th>Glyceraldehyde phosphate dehydrogenase (NADP)</th>
<th>Phosphopyruvate carboxylase</th>
<th>Malic enzyme</th>
<th>Invertase</th>
<th>Acid phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg./fraction)</td>
<td>(%) of total</td>
<td>(%) of total</td>
<td>(%) of total</td>
<td>(%) of total</td>
<td>(%) of total</td>
</tr>
<tr>
<td>1</td>
<td>&lt;1:35</td>
<td>0-029</td>
<td>48</td>
<td>55</td>
<td>53</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>1:35-1:37</td>
<td>0-010</td>
<td>17</td>
<td>23</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>1:37-1:40</td>
<td>0-013</td>
<td>16</td>
<td>14</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>&gt;1:40</td>
<td>0-008</td>
<td>13</td>
<td>4</td>
<td>7</td>
<td>8</td>
</tr>
<tr>
<td>2</td>
<td>&lt;1:37</td>
<td>0-044</td>
<td>58</td>
<td>60</td>
<td>55</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>1:37-1:40</td>
<td>0-020</td>
<td>27</td>
<td>31</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>&gt;1:40</td>
<td>0-011</td>
<td>15</td>
<td>9</td>
<td>14</td>
<td>18</td>
</tr>
</tbody>
</table>
addition, evidence provided in Table 3 suggests that most of the phosphopyruvate carboxylase complement of maize leaves is associated with the chloroplasts. Malic enzyme may have an auxiliary role in photosynthetic carbon dioxide fixation in tropical grasses. This enzyme was also associated with the chloroplast fraction of maize leaves and was much more active in the tropical grasses than in wheat, oats or silver-beet. However, its activity in leaves of tropical grasses was considerably less than the maximum rates of photosynthesis.

The present studies provide information which supports and supplements a scheme for photosynthetic carbon dioxide fixation originally proposed for sugar-cane (Hatch & Slack, 1966) and recently extended to a number of other tropical grasses (Hatch et al. 1967). The additional evidence for the non-participation of ribulose diphosphate carboxylase and the implication of phosphopyruvate carboxylase as the major photosynthetic carboxylase raises the question of the origin of phosphopyruvate. If the transfer of the C-4 of the dicarboxylic acid pool is accompanied by pyruvate production, as previously proposed, then the efficient conversion of pyruvate into phosphopyruvate becomes a prerequisite of the scheme. Cooper & Kornberg (1965) recently described an enzyme from Escherichia coli that catalyses the conversion of pyruvate and ATP into phosphopyruvate, AMP and P₄. Our unpublished studies indicate that a similar enzyme is present in the leaves of maize.

In formulating the scheme for photosynthesis in sugar-cane (Hatch & Slack, 1966) the possibility of similarities with the Calvin cycle, including a role for ribulose 1,5-diphosphate, was not excluded. Since the different species examined during the present study contained similar amounts of glyceraldehyde phosphate dehydrogenase (NADP) and hexose diphosphatase, the pathway for synthesis of hexoses from 3-phosphoglycerate in the tropical grasses is probably the same as for the Calvin cycle. The high activity of phosphoribulokinase in leaves of tropical grasses is indicative of a role for ribulose 1,5-diphosphate. Whether it functions as an acceptor for the transcarboxylation reaction proposed in the scheme remains to be determined.

We wish to thank Mr C. J. Leeder of the Biochemistry Department, University of Queensland, for performing the ultracentrifugal analyses.

REFERENCES