The Biochemistry of
\( \text{C}_4 \) Photosynthesis

Ryuzi Kanai and Gerald E. Edwards

I. Introduction

\( \text{C}_4 \) photosynthesis consists of the coordinated function of two cell types in the leaves, usually designated mesophyll cells (MC) and bundle sheath cells (BSC), because enzymes of the \( \text{C}_4 \) pathway are located separately in these morphologically distinct cell types. In \( \text{C}_4 \) leaves, atmospheric \( \text{CO}_2 \) enters through stomata and is first accessible to MC, where it is fixed by phosphoenolpyruvate (PEP) carboxylase to form oxaloacetate, and then malate and aspartate. These \( \text{C}_4 \) dicarboxylic acids are transported to BSC where they are decarboxylated, and the released \( \text{CO}_2 \) refixed by ribulose-1,5-bisphosphate (RuBP) carboxylase (Rubisco) and assimilated through the enzymes of the photosynthetic carbon reduction (PCR) cycle to form sucrose and starch. Although anatomic differentiation is apparent in BSC, they are functionally similar to \( \text{C}_3 \) MC in carbon assimilation except for the presence of enzymes concerned with decarboxylation of \( \text{C}_4 \) acids.

The physiological significance of separate but coordinate function of the two cell types in \( \text{C}_4 \) photosynthesis is the specialization of MC for generation of a high concentration of \( \text{CO}_2 \) in BSC in order to reduce the oxygenase activity of Rubisco and consequential reduction of photorespiration. Without consideration of a possible positive function of photorespiration in \( \text{C}_3 \) plants (cf., Osmond and Grace, 1995), it is clear that \( \text{C}_4 \) plants have the capacity to perform effective photosynthesis under conditions in which RuBP oxygenase activity is restricted. \( \text{C}_4 \) photosynthesis can be visualized as a mechanism to provide Rubisco with near saturating \( \text{CO}_2 \) when \( \text{C}_4 \) plants can afford a high stomatal conductance, or to provide sufficient \( \text{CO}_2 \) for survival and growth when stomatal conductance is low.
During the evolution of C₄ photosynthesis from C₃ plants, the MC developed a high level of carbonic anhydrase (CA) and PEP carboxylase for initial CO₂ fixation in the cytoplasm, and pyruvate, orthophosphate (P₄) dikinase in the chloroplasts for provision of PEP, the HCO₃⁻ acceptor. It is equally important that the synthesis of some key photosynthetic enzymes in carbon metabolism of C₃ photosynthesis is repressed in MC of C₄ plants. This includes Rubisco and phosphoribulokinase of the PCR cycle in MC chloroplasts, and enzymes of glycine decarboxylation in the photosynthetic carbon oxidation pathway (PCO cycle) in MC mitochondria. Differences in the C₄ pathway in three subgroups are illustrated in the first section of this chapter through highlighting differentiation in photosynthetic functions of MC and BSC. This is followed by concise information on the enzyme reactions and properties of the respective enzymes. In the second section, the CO₂ concentration in BSC and the activity of Rubisco, which is exclusively localized in these cells, are discussed. In the third section, the energetics of C₄ photosynthesis is dealt with, including the theoretic maximum efficiency and in vivo energy requirements of C₄ plants. Although there is evidence for cooperation between the two cell types in C₄ leaves from in vivo studies, cell separation techniques have allowed studies with isolated cell types as well as with intact organelles. These have been critical in understanding the division of labor and coordination of the two cell types in the intercellular and intracellular transport of metabolites. These are discussed in Sections IV and V. For previous reviews on the biochemistry of C₄ photosynthesis see Edwards and Walker (1983), Hatch (1987), and Leegood and Osmond (1990).

A. The Three C₄ Subgroups

C₄ plants have been separated into three subgroups based on differences in the enzymes of the decarboxylation step in BSC. These are the NADP-malic enzyme (NADP-ME), NAD-malic enzyme (NAD-ME), and PEP carboxykinase (PEP-CK) types. Each C₄ type shows not only morphologic differentiation in their arrangement of bundle sheath chloroplasts and ultrastructure, but also further biochemical differences between MC and BSC, and in the method of transport of metabolites between the cells (Gutierrez et al., 1974b; Hatch et al., 1975).

Biochemical pathways of the three C₄ subgroups are summarized in Figs. 1, 2, and 3. Common to all C₄ plants is the initial fixation of HCO₃⁻ by PEP carboxylase to form oxaloacetate in the MC cytoplasm. As atmospheric CO₂ enters the MC via stomata, carbonic anhydrase in the MC cytoplasm helps to equilibrate the CO₂ to HCO₃⁻. Malate and aspartate are formed from oxaloacetate in MC. As determined by ¹⁴CO₂ fixation experiments, the main initial product is malate in C₄ species of the NADP-ME type, whereas aspartate is the major product in the NAD-ME and PEP-CK types.
3. The Biochemistry of C₄ Photosynthesis

**Figure 1** Subgroup of C₄ pathway: NADP-ME (malic enzyme) type. Compound abbreviations for Figs. 1–3: Ala, alanine; Asp, aspartate; MA, malate; OAA, oxaloacetate; PA, pyruvate; PEP, phosphoenolpyruvate; Pi, orthophosphate; PPI, pyrophosphate. Enzyme abbreviations: 1, PEP carboxylase; 2, NADP-malate dehydrogenase; 3, pyruvate phosphate dikinase; 3a, adenylate kinase; 3b, pyrophosphatase; 4, NADP-malic enzyme; 5, NAD-malic enzyme; 6, PEP carboxykinase; 7, NAD-malate dehydrogenase; 8, alanine aminotransferase; 9, aspartate aminotransferase; 10, RuBP carboxylase; 11, carbonic anhydrase; 12, respiratory electron transport system.

1. **NADP-ME Type** In NADP-ME C₄ species, bundle sheath chloroplasts of C₄ grasses are usually arranged in a centrifugal position relative to the vascular bundle, and have thylakoid membranes with reduced grana stacking. As is evident from Fig. 1, chloroplasts in MC and BSC play a critical role in the C₄ pathway. Oxaloacetate, formed by PEP carboxylase in the cytoplasm, is transported to MC chloroplasts, where most of the oxaloacetate is reduced to malate (% is species dependent) by NADP-specific malate dehydrogenase and the remainder is converted to aspartate by aspartate aminotransferase. These acids are exported from MC to BSC, presumably through plasmodesmata, which are abundant at the interface of the two cell types. In BSC chloroplasts, malate is decarboxylated by NADP-malic enzyme to feed CO₂ and reduced NADP to the PCR cycle. The other product of decarboxylation, pyruvate, is returned to MC chloroplasts, where it is phosphorylated by pyruvate-Pi dikinase to form PEP, the acceptor of inorganic carbon. Decarboxylation through NADP-malic enzyme may also occur via aspartate being metabolized to malate in BSC through aspartate aminotransferase and malate dehydrogenase; alternatively in some NADP-
Figure 2  Subgroup of C₄ pathway: NAD-ME (malic enzyme) type. (See Fig. 1 legend for compounds and enzymes.)

Figure 3  Subgroup of C₄ pathway: PEP-CK (carboxykinase) type. (See Fig. 1 legend for compounds and enzymes.)
ME species, PEP carboxykinase may serve as a secondary decarboxylase (Gutierrez et al., 1974b; Walker et al., 1997).

2. **NAD-ME Type** Bundle sheath chloroplasts of NAD-ME C₄ species have thylakoid membranes with developed grana stackings. Both chloroplasts and mitochondria are located together in a centripetal position relative to the vascular bundle, except in some grass species of *Panicum* and *Eragrostis* (Ohsugi et al., 1982; Prendergast et al., 1986). The main initial product of \(^{14}\text{CO}_2\)-fixation is aspartate via aspartate aminotransferase in the MC cytoplasm. The aspartate is transported to BSC mitochondria, where it is deaminated by aspartate aminotransferase. The product oxaloacetate is reduced to malate by NAD-malate dehydrogenase and then the malate is decarboxylated by NAD-ME to feed \(\text{CO}_2\) to bundle sheath chloroplasts. Thus, bundle sheath mitochondria play a decisive role in this C₄ subtype, as illustrated in Fig. 2. The decarboxylation product, pyruvate, is converted to alanine, which is shuttled to the MC where it is used for resynthesis of PEP; alanine aminotransferases in the cytoplasm of MC and BSC have a key role in this process.

3. **PEP-CK Type** Bundle sheath chloroplasts of PEP-CK types have well-developed grana stacks. The chloroplasts are arranged evenly or in a centrifugal position in BSC of this C₄ subgroup. PEP carboxykinase in the bundle sheath cytoplasm is the main decarboxylation enzyme, but BSC mitochondria also possess appreciable activity of NAD-malic enzyme. Although aspartate is the main initial product of \(^{14}\text{CO}_2\)-fixation through the high aspartate aminotransferase activity in the MC cytoplasm, some malate is formed in MC chloroplasts. As shown in Fig. 3, aspartate transported from MC cytoplasm to BSC is deaminated and decarboxylated by PEP-CK, whereas malate transported to BSC mitochondria is decarboxylated by NAD-ME resulting in both decarboxylases feeding \(\text{CO}_2\) to BSC chloroplasts. The NADH formed by NAD-ME is oxidized through the mitochondrial electron transport chain to produce ATP by oxidative phosphorylation. The ATP is exported to the cytoplasm, where it is used for the PEP-CK reaction. Of the two decarboxylation products, pyruvate may return to MC chloroplasts through alanine, as noted in the NAD-ME type species. PEP is suggested to return directly to the MC cytoplasm, because only low activity of pyruvate kinase is detectable in BSC. Relatively low activity of pyruvate-P, dikinase in PEP-CK C₄ plants compared with the other C₄ types (cf., Table I) may also reflect the return of PEP. Mechanisms to balance distribution of nitrogen and phosphate between MC and BSC remain to be explored.

**B. Enzymes of the C₄ Pathway: Reaction and Properties**

After proposing the C₄-dicarboxylic acid pathway of photosynthesis in 1966, Hatch and Slack identified many of the enzymes of the C₄ pathway and showed their activities were sufficient to account for *in vivo* photosyn-
Ryuzi Kanai and Gerald E. Edwards

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>NADP-ME</th>
<th>Enzyme activity in whole leaf extract (µmol min⁻¹ mg⁻¹ chlorophyll)</th>
<th>NAD-ME</th>
<th>PEP-CK</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEP carboxylaseᵇ,e</td>
<td>M cyt</td>
<td>13~24</td>
<td>12~25</td>
<td>17~27</td>
</tr>
<tr>
<td>Pyruvate-Pi dikinaseᵇ</td>
<td>M chlt</td>
<td>4~8</td>
<td>4~9</td>
<td>2~4</td>
</tr>
<tr>
<td>Adenylate kinaseᵈ</td>
<td>M chlt &gt;B</td>
<td>41~87</td>
<td>36~70</td>
<td></td>
</tr>
<tr>
<td>Pyrophosphataseᵈ</td>
<td>M chlt &gt;B</td>
<td>37~57</td>
<td>52~74</td>
<td></td>
</tr>
<tr>
<td>NADP-malate dehydrogenaseᵇ</td>
<td>M chlt &gt;B</td>
<td>10~17</td>
<td>1~2</td>
<td>2~5</td>
</tr>
<tr>
<td>NADP-malic enzymeᶜ</td>
<td>B chlt</td>
<td>10~16</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>NAD-malic enzymeᵇ</td>
<td>B mit</td>
<td>&lt;1</td>
<td>5~18</td>
<td>1~3</td>
</tr>
<tr>
<td>PEP carboxykinaseᵇ</td>
<td>B cyt</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>6~17</td>
</tr>
<tr>
<td>Aspartate aminotransferaseᵈ</td>
<td>M chlt &gt;B</td>
<td>5~9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine aminotransferaseᵇ</td>
<td>M chlt &gt;B mit, cyt</td>
<td>27~46</td>
<td>44~60</td>
<td></td>
</tr>
<tr>
<td>RuBP carboxylaseᶜ,e,f</td>
<td>M chlt =B cyt</td>
<td>3~8</td>
<td>30~63</td>
<td>38~45</td>
</tr>
<tr>
<td>Carbonic anhydraseᶜ</td>
<td>M chlt</td>
<td>1~4</td>
<td>1~3</td>
<td>1~4</td>
</tr>
</tbody>
</table>

For values listed as < 1, the activity was less than 1 or not detectable.

ᵃ M, B, main localization in mesophyll or bundle sheath cell, respectively; M > B, more in mesophyll cell; M < B, more in bundle sheath cell; M = B, equally distributed; chlt, chloroplasts; cyt, cytoplasm; mit, mitochondria.
ᶜ Guderrez et al., 1974a.
ᵈ Hatch and Burnell, 1990.
e Hatch and Osmond, 1976.
f The lower activities in the range given are likely underestimates due to inactivation or loss of enzyme during extraction procedures because they are below rates of leaf photosynthesis.

Sthetic rates (ca. 3~5 µmol CO₂ min⁻¹ [mg Chl]⁻¹; see Hatch and Osmond, 1976). Subsequent studies established the distribution between MC and BSC and their intracellular localization. The enzyme activities on a chlorophyll basis in whole leaf extract of C₄ plants and their compartmentation are summarized in Table I.

1. **PEP Carboxylase** PEP carboxylase (EC 4.1.1.31) from C₄ leaves consists of a homotetramer with 110 kDa subunits. The purified enzyme from maize leaves has molecular activity (molar activity per minute per mol of enzyme: 

\[
\begin{align*}
\text{CH}_2 \quad \text{H}^+ \text{CO}_3^- & \quad \text{-------} \quad \text{°COOH} + \text{H}_2\text{PO}_4^- \\
\text{CO-P-O}_\text{H}_2 \quad \text{Mg}^{2+} \quad \text{CH}_2 \quad \text{C}=\text{O} \quad \text{COOH} \\
\text{COOH} \\
\text{phosphoenol-pyruvate (PEP)} & \quad \text{oxaloacetate (OAA)}
\end{align*}
\]

\(\Delta G^\circ = -31.8 \text{ kJ} \cdot \text{mol}^{-1}\)
mol min\(^{-1}\) [mol enzyme\(^{-1}\)] of 9920 at pH 7.0 and 22°C and \(K_m\) values for HCO\(_3^-\) and PEP are 0.02 mM and 1–2 mM, respectively (Uedan and Sugiyama, 1976). The catalytic mechanism starts with binding of metal\(^{2+}\), PEP and HCO\(_3^-\) in this order to the active site having Lys, His, and Arg residues. The chemical steps are summarized as follows: (1) phosphate transfer from PEP to form carboxyphosphate and enolate of pyruvate, (2) carboxyphosphate decomposes to form enzyme-bound CO\(_2\) and phosphate, (3) CO\(_2\) combines with the metal-stabilized enolate, and then (4) the products oxaloacetate and phosphate are released from the enzyme (for details, cf., Chollet et al., 1996).

Regulation of enzyme activity by metabolic control has been a subject of study especially at nearly physiological assay conditions, including positive and negative effectors such as glucose 6-phosphate and malate, respectively. In addition, light activation and diurnal changes in kinetic properties were shown to be via phosphorylation–dephosphorylation at a Ser residue near the C terminal of the protein by a specific protein kinase–phosphatase system. Interestingly, the phosphorylated enzyme, which occurs in the light in C\(_4\) plants (and the dark phase of CAM plants), is the active form that is more sensitive to positive effectors and less sensitive to negative effectors (Carter et al., 1996; Chollet et al., 1996).

2. NADP-Malate Dehydrogenase Malate dehydrogenase specific to NADP (EC 1.1.1.82), found by Hatch and Slack (1969a) in the chloroplasts of some C\(_4\) and C\(_3\) plants, was shown to be activated in the light (Johnson and Hatch, 1970). The enzyme purified from maize leaves is a homodimer with 43 kDa subunits with molecular activity of 60,500 at pH 8.5 and 25°C. The \(K_m\) values for NADPH, oxaloacetate, NADP\(^+\), and malate are 24, 56, and 73 \(\mu\)M and 32 mM, respectively (Kagawa and Bruno, 1988). Light activation is mediated by the ferredoxin–thioredoxin \(m\) system, which reduces a disulfide group on the enzyme (Edwards et al., 1985; Droux et al., 1987). Further modulation of the activation state occurs through a high NADPH–NADP\(^+\) ratio in MC chloroplasts in the light (Rebeille and Hatch, 1986).

3. Pyruvate, PEP Dikinase The enzyme activity first reported as "PEP synthetase" by Hatch and Slack in 1967 was identified as pyruvate, orthophosphate
Ryuzi Kanai and Gerald E. Edwards

\[
\text{CH}_3 + \text{H}_2\text{PO}_4 + \text{ATP} \rightleftharpoons \text{CH}_2 + \text{H}_2\text{O}_2\text{P-O-PO}_3\text{H}_2 + \text{AMP} \quad \Delta G^\circ = -13 \text{ kJ} \cdot \text{mol}^{-1}
\]

\[
\begin{array}{c}
\text{Pyruvate (PA)} \\
\text{CH}_2
\end{array} \rightleftharpoons
\begin{array}{c}
\text{Phosphoenolpyruvate (PEP)} \\
\text{H}_2\text{O}_3\text{P-O-PO}_3\text{H}_2 + \text{AMP}
\end{array} \quad \text{pyruvate (PEP) (PPi)}
\]

(Reaction III)

dikinase (EC 2.7.9.1), a new key enzyme in the C\textsubscript{4} pathway, which is also activated by illumination (Hatch and Slack, 1969b). The purified enzyme from maize leaves is a homotetramer with 94 kDa subunits, having a molecular activity of 2,600 at pH 7.5 and 22\textdegree C; \( K_\text{m} \) values for pyruvate, P\textsubscript{i}, ATP, PEP, pyrophosphate and AMP are 250, 1,500, 15, 140, 40, and <10 \( \mu \text{M} \), respectively (Sugiyama, 1973). Although the reaction itself is reversible, it proceeds to form PEP \textit{in vivo} because high activity of pyrophosphatase and adenylate kinase are present in the same compartment, the MC chloroplasts (cf., Figs. 1–3). The reaction mechanism includes phosphorylation of P\textsubscript{i} to form pyrophosphate and His residues at the active site of the enzyme with \( \gamma- \) and \( \beta- \) P of ATP, respectively, and then pyruvate reacts with the His-P residue to form PEP. As for light–dark regulation, the active enzyme having His-P is inactivated by phosphorylation of a specific Thr residue with \( \beta- \) P of ADP, and it is reactivated by phosphorolysis of Thr-P to form pyrophosphate. Interestingly, a single regulatory protein mediates both phosphorylation and dephosphorylation of the Thr residue (for details, cf. Edwards \textit{et al.}, 1985).

4. Enzymes of \textit{C}_4 Acid Decarboxylation

Although \textit{C}_4 decarboxylating enzymes had already been discovered in plant tissues, their role in \textit{C}_4 photosynthesis was revealed because of high activities in the BSC of each \textit{C}_4 subgroup [by Slack and Hatch (1967) for NADP-malic enzyme (EC 1.1.1.40, Reaction IV); by Edwards \textit{et al.} (1971) for PEP carboxykinase (EC 4.1.1.49, Reaction V); and by Hatch and Kagawa (1974) for NAD-malic enzyme (EC 1.1.1.39, Reaction VI)]. The inorganic carbon product of these three decarboxylation enzymes was proved to be \( \text{CO}_2 \), and not \( \text{HCO}_3^- \), after some debates; \( \text{HCO}_3^- \) is rather inhibitory to the decarboxylation reactions (Jenkins \textit{et al.}, 1987).

NADP-malic enzyme in \textit{C}_4 plants is located in BSC chloroplasts, whereas

\[
\begin{array}{c}
\text{COOH} + \text{NADP}^+ \rightleftharpoons \text{CO}_2 + \text{CH}_3 + \text{NADPH} + \text{H}^+ \quad \Delta G^\circ = -1.3 \text{ kJ} \cdot \text{mol}^{-1}
\end{array}
\]

\[
\begin{array}{c}
\text{Malate (MA)} \\
\text{CH}_2
\end{array} \rightleftharpoons
\begin{array}{c}
\text{Pyruvate (PA)} \\
\text{CH}_2
\end{array} \quad \text{malate (MA) pyruvate (PA)}
\]

(Reaction IV)
that in CAM plants is located in the MC cytoplasm (cf., Edwards and Andreo, 1992). The enzyme purified from sugarcane leaves has a molecular activity of 17,750 at pH 8.0 and 30°C; $K_m$ values for malate and NADP$^+$ are 120 and 5 $\mu$M, respectively. The major form of the enzyme is a homotetramer with 62 kDa subunits at pH 8.0, but a homodimer at pH 7.0 (Iglesias and Andreo, 1990). The former is considered the active form in the light as it has higher $V_{max}$ and lower $K_m$ (NADP$^+$) and higher affinity for Mg$^{2+}$ compared with the latter. A possible light–dark regulation by the ferredoxin–thioredoxin system was also suggested from the thiol–disulfide interchange of the maize enzyme (Drincovich and Andreo, 1994). Although the enzyme activity is reversible ($K_m$ for CO$_2$, 1.1 mM), the ratio of the decarboxylation–carboxylation reaction was about 10/1 at pH 8.1 and 0.6 mM CO$_2$ (Jenkins et al., 1987).

\[
\text{COOH} + \text{ATP} \rightleftharpoons \text{CO}_2 + \text{CH}_2 + \text{ADP} \quad \Delta G^\circ = 0 \text{kJ} \cdot \text{mol}^{-1}
\]

The localization of PEP carboxykinase in BSC cytoplasm was confirmed after some misinterpretation (Watanabe et al., 1984). The purified enzyme from three C$_4$ species was reported as a hexamer with 68 kDa subunits having a specific activity of 36–51 U (molecular activity ca. 14,700–20,800) at pH 7.6 and 25°C, an absolute requirement for Mn$^{2+}$; and $K_m$ values for oxaloacetate, ATP, and CO$_2$ of 12–25 $\mu$M, 16–25 $\mu$M, and 2.5 mM, respectively (Burnell, 1986). Later work, however, showed that these purifications yielded a truncated form of the subunit at the N-terminal end due to rapid proteolysis. The native form, isolated from five C$_4$ species, has 67–71 kDa subunits (Walker and Leegood, 1996). The enzyme having the larger subunit, for example, from Panicum maximum, is phosphorylated–dephosphorylated by dark–light treatment of the leaves in vivo, but its role in regulation of the enzyme activity remains to be studied.

\[
\text{COOH} + \text{NAD}^+ \rightleftharpoons \text{CO}_2 + \text{CH}_2 + \text{NADH} + \text{H}^+ \quad \Delta G^\circ = -1.3 \text{kJ} \cdot \text{mol}^{-1}
\]
NAD-malic enzyme located in the BSC mitochondria is the C₄ acid decarboxylase functioning in C₄ photosynthesis in NAD-ME type species; it also has a role in PEP-CK type C₄ plants (see Figs. 2 and 3). Different isoforms are widely reported in various tissues of plant species, even among the C₄ species (Hatch et al., 1974; Artus and Edwards, 1985). The purified enzyme from Eleusine coracana is a homoctamer, having 63 kDa subunits with a molecular activity of 60,500 at pH 7.2 and 31°C (Murata et al., 1989). Mn²⁺ is absolutely required for activity and acetyl CoA, CoA, and fructose 1,6-bisphosphate (FuBP) are potent activators. Kₘ values for malate and NAD⁺ in the presence of FuBP are 2.2 and 0.63 mM, respectively. Antiserum raised against the enzyme from E. coracana, a monocot, does not inhibit the enzyme activity of a C₄ dicot, Amaranthus edulis. The purified enzyme from Amaranthus hypochondriacus is a heterotetramer composed of two 65 kD α-subunits, which are responsible for catalytic activity, and two 60 kD β-subunits (Long et al., 1994).

5. Aminotransferases

By the work of Hatch and associates in the 1970s, aspartate aminotransferase (EC 2.6.1.1, Reaction VII) and alanine aminotransferase (EC 2.6.1.2, Reaction VIII) were proved to be essential in the C₄ pathway of NAD-ME and PEP-CK types (Hatch, 1987). These enzymes exist in both MC and BSC mainly as cytoplasmic and/or mitochondrial isoenzymes.

\[
\begin{align*}
\text{COOH} + \text{COOH} & \leftrightarrow \text{COOH} + \text{COOH} \\
\text{CH}_2 & \text{CH}_2 \\
\text{HCNH}_2 & \text{C-O} \\
\text{COOH} & \text{COOH} \\
\text{aspartate (Asp)} & \text{2-oxoglutarate (2OG)} & \text{oxaloacetate (OAA)} & \text{glutamate (Glu)} \\
\end{align*}
\]

(Reaction VII)

A main aspartate aminotransferase (AspAT) isoform located in the MC cytoplasm was first purified from P. maximum (PEP-CK type). It is a homodimer with 42 kDa subunits having molecular activity of 18,200 at pH 8.0 and 25°C (Numazawa et al., 1989). Of the three AspAT isoforms in Panicum miliaceum and E. coracana (NAD-ME type), two major isoforms are specific to C₄ plants and are localized in the MC cytoplasm (cAspAT) and BSC mitochondria (mAspAT). The third minor component is a plastidic isoform (pAspAT) that is similar to that of C₃ plants (Taniguchi and Sugiyama, 1990; Taniguchi et al., 1996). All isoforms have been purified and consist of a homodimer with approximately 40 kDa subunits. Although the isoelectric points of the proteins and cross-reactivity against antisera are different, kinetic properties are similar: Kₘ values for aspartate, 2-oxoglutarate, glutamate, and oxaloacetate are 1.3–3.0, 0.07–0.20, 8–32, 0.023–0.085 mM, respectively.

Three isoforms of alanine aminotransferase (AlaAT) exist in P. miliaceum
3. The Biochemistry of C₄ Photosynthesis

\[
\begin{align*}
\text{alanine (Ala)} & \quad \text{2-oxoglutarate (2OG)} \quad \text{pyruvate (PA)} \quad \text{glutamate (Glu)} \\
\text{(Reaction VIII)}
\end{align*}
\]

(NAD-ME type) leaves; the major forms occurring in the MC and BSC cytoplasm have been purified (cAlaAT). The enzyme is a homodimer with 50 kDa subunits having a molecular activity of 39,500 at pH 7.5 and 25°C. \( K_m \) values for alanine, 2-oxoglutarate, pyruvate and glutamate are 6.7, 0.15, 0.33 and 5.0 mM, respectively. The cAlaAT seems to be functional in C₄ photosynthesis, as the protein increases in parallel with other C₄ enzymes during greening of seedlings (Son et al., 1991).

6. RuBP Carboxylase–Oxygenase

Ribulose-1,5-bisphosphate carboxylase—oxygenase (Rubisco, EC 4.1.1.39) of C₄ plants, like that of C₃ plants, consists of eight large subunits of 53.5 kDa and eight small subunits of 13 kDa (L₈S₈). Kinetic properties show some difference between C₃ and C₄ plants (Yeoh et al., 1980; 1981). The range of \( K_m(\text{CO}_2) \) values for the enzyme among C₃ species (12–25 \( \mu \text{M} \)) is lower than those in C₄ (28–34 \( \mu \text{M} \)), although variation of \( K_m(\text{RuBP}) \) values (15–82 \( \mu \text{M} \) in C₄) is not related to the photosynthetic pathway of higher plants. Among the C₄ grasses, the \( K_m(\text{CO}_2) \) values of Rubisco in PEP-CK type (28–41, mean 35 \( \mu \text{M} \)) are significantly lower than those in NAD- and NADP-ME types (41–63, mean 53 \( \mu \text{M} \)). Molecular activities of some C₄ Rubiscos (2280–4020, mean of 3240 mol CO₂ mol enzyme⁻¹ min⁻¹) are about twofold higher than those from C₃ higher plant species (Seemann et al., 1984). These higher \( V_{\text{max}} \) values of Rubisco in C₄ plants accompanied by higher \( K_m(\text{CO}_2) \) may be an
evolutionary change that allows a high activity per unit Rubisco protein under high levels of CO$_2$ in BSC in the light. However, there are no clear differences between the C$_4$ and C$_3$ Rubisco in their specificity factor ($S_{rel}$), that is, the relative specificity to react with CO$_2$ versus O$_2$, which is based on $V_{max}$ values and Michaelis constants for the two gases. An improved measurement of the specificity factor of Rubisco resulted in a value of 79 mol/mol for maize leaves, which is marginally smaller than those of five C$_3$ higher plant species (82–90 mol/mol) (Kane et al., 1994).

7. Carbonic Anhydrase  Carbonic anhydrase (CA: carbonate hydratase, EC 4.2.1.1), which catalyses the first step in C$_4$ photosynthesis, is located in the mesophyll cell cytoplasm (Gutierrez et al., 1974a; Ku and Edwards, 1975), where it equilibrates the aerial CO$_2$ to HCO$_3^-$. Some activity may also be associated with the MC plasmamembrane (Utsunomiya and Muto, 1993). HCO$_3^-$ is the form of inorganic carbon used as a substrate in the PEP carboxylase reaction. In the absence of CA it is presumed that the rate of nonenzymatic equilibration of atmospheric CO$_2$ to HCO$_3^-$ would be rate limiting for C$_4$ photosynthesis, although this has not been proved. Its virtual absence in BSC is understandable because the inorganic carbon substrate used by Rubisco is CO$_2$, which is also the direct product of the three decarboxylation enzymes (Hatch and Burnell, 1990). Maize CA has been purified, but its molecular and kinetic properties were not reported (Burnell, 1990). Antibodies from maize CA cross-reacted with a 24–25 kDa subunit in a crude extract of C$_4$ plants by Western blot analysis. Studies with several C$_4$ leaf CA showed a $K_m$(CO$_2$) of 0.8–2.8 mM at 0°C and maximum activity at 35 mM CO$_2$, which was far higher than the maximum photosynthetic rates of C$_4$ plants. However, their activities measured under conditions considered to exist in vivo, pH 7.2 and 4 $\mu$M CO$_2$, were 28–89 $\mu$mol CO$_2$ hydration min$^{-1}$ (mg Chl)$^{-1}$, just sufficient for C$_4$ photosynthesis (Hatch and Burnell, 1990).

II. CO$_2$ Concentration and Rubisco Activity in Bundle Sheath Cells

As mentioned in the introduction, the C$_4$ pathway of photosynthesis evolved to concentrate CO$_2$ around Rubisco in BSC, which allows much more efficient CO$_2$ assimilation than in C$_3$ plants under limiting CO$_2$ in the atmosphere. In C$_3$ plants, PCO cycle activity is a consequence of the kinetic properties of Rubisco; O$_2$ competes with CO$_2$ to produce phosphoglycolate, which is metabolized in the glycolate pathway to glycerate.
In C₄ photosynthesis, the level of CO₂ in BSC is influenced by a variety of factors, particularly (1) the intercellular CO₂ partial pressure, (2) the solubility of CO₂ as influenced by temperature, (3) the capacity for transport of C₄ acids from MC through plasmodesmata (cf., Osmond and Smith, 1976), (4) the reactions of decarboxylation followed by (5) refixation via Rubisco in the BSC, and (6) leakage of CO₂ (and HCO₃⁻) from BSC to MC by passive diffusion, which depends on diffusive resistance of the BSC cell wall (cf., Section IIIB).

Various models have been used to estimate the size of the CO₂ pool in BSC during C₄ photosynthesis (see He and Edwards, 1996; Chapter 6 by von Caemmerer and Furbank, this volume). Estimates indicate the C₄ cycle can raise the levels of CO₂ in the BSC to 30–75 μM (ca. 3 to 8 times higher than in C₃ plants), resulting in PCO cycle activity as low as 3% of net CO₂ uptake (Jenkins et al., 1989; Dai et al., 1993; He and Edwards, 1996).

The capacity of a C₄ plant to increase the supply of CO₂ to Rubisco is illustrated when comparing the response of photosynthesis in maize (C₄) to that of wheat (C₃) with varying intercellular levels of CO₂ (Fig. 4). The higher initial slope and lower CO₂ compensation point (intercept on the

![Figure 4](image)  
**Figure 4** Response of the rate of CO₂-fixation in maize and wheat to varying intercellular levels of CO₂. At atmospheric level of CO₂ (33 Pa), the corresponding intercellular level of CO₂ (Ci) was approximately 16 Pa in maize and 21 Pa in wheat. (From Dai, Edwards, Ku, unpublished).
x axis) in the C₄ plant is a consequence of the CO₂ concentrating mechanism. In C₄ plants, the large increase in efficiency of Rubisco to function in CO₂ fixation through the C₄ system allows for a lower investment in Rubisco protein than in C₃ plants (Ku et al., 1979; Sage et al., 1987). For example, maize has about half the Rubisco content of wheat (Edwards, 1986).

III. Energetics of C₄ Photosynthesis

A. Minimum Energy Requirements in the Three C₄ Subgroups

The minimum energy requirements for CO₂ fixation to the level of triose phosphate in C₃ plants are 3 ATP and 2 NADPH per CO₂ in the absence of photorespiration. The same is true for the refixation of CO₂ generated by C₄ acid decarboxylation in BSC of C₄ plants by Rubisco and subsequent PCR cycle enzymes, because the oxygenase activity of Rubisco is negligible due to high CO₂ concentration in BSC in the light. In C₄ leaves, however, extra energy is required for regeneration of PEP, the acceptor for the initial CO₂ fixation, from pyruvate through pyruvate, P₁ dikinase and adenylate kinase in mesophyll chloroplasts; it costs 2 ATP per CO₂ fixed by PEP carboxylase. Differentiation of the energy requirements among C₄ subgroups takes place by the difference in decarboxylation enzymes and transport metabolites between MC and BSC.

In the NADP-ME type (cf., Fig. 1), primary shuttle metabolites are malate/pyruvate, and the NADPH used for the reduction of oxaloacetate to malate in MC chloroplasts is regenerated in BSC chloroplasts by NADP-ME for the PCR cycle; thus, 5 ATP and 2 NADPH are required per CO₂ assimilated. The NAD-ME type (cf., Fig. 2) retains the same energy requirement; the primary shuttle metabolites are aspartate/alanine, no additional energy is required for transamination reactions in MC and BSC, and NADH produced by NAD-malic enzyme is recycled in BSC mitochondria.

The situation is complicated in the PEP-CK type (cf., Fig. 3) because PEP carboxykinase and NAD-malic enzyme function together in the decarboxylation step and the relative stochiometries are uncertain. NADH is generated by malate decarboxylation through NAD-malic enzyme. Assuming a maximum of 3 ATP are generated per NADH oxidized through mitochondrial phosphorylation, NAD malic enzyme would have to operate at a minimum of one-third of the rate of PEP carboxykinase (see Carnal et al., 1993, in which interregulation of the two decarboxylases is discussed). With this stochiometry, 25% of the oxaloacetate formed in MC cytoplasm would be reduced to malate in MC chloroplasts (requirement of 0.25 NADPH per CO₂ fixed) and 75% converted to aspartate. In this case, the ATP requirement to form PEP in mesophyll chloroplasts is only 0.5 ATP per CO₂
fixed because of direct return of PEP (three-fourths of the decarboxylation products via PEP-CK) from BSC. With this stoichiometry, the minimum energy requirements of the PEP-CK type are estimated to be 3.5 ATP and 2.25 NADPH per CO₂ assimilated. However, adopting the recent estimate of mitochondrial ATP generation of 2.5 ATP per NADH oxidized (Hinkle et al., 1991), the energy requirements in the PEP-CK type will be increased to 3.57 ATP and 2.29 NADPH, and NAD-malic enzyme would need to operate at 40% of the rate of PEP carboxykinase. Another possibility for ATP supply to PEP carboxykinase is through the oxidation of triose phosphate to 3-phosphoglycerate in the cytoplasm. In fact, isolated BSC of PEP-CK species decarboxylate oxaloacetate when supplied with triose phosphate, NAD⁺ and ADP (Burnell and Hatch, 1988a).

B. In Vivo Energy Requirements in C₄ Plants

For the estimation of energy requirements of C₄ photosynthesis in vivo it is necessary to evaluate several factors, such as the leakage of CO₂ from BSC, costs of CO₂ assimilation to form carbohydrates and nitrate assimilation, and level of photorespiration, if any. The magnitude of leakiness of BSC has been estimated using techniques such as ¹⁴C labeling (Hatch et al., 1995), carbon isotope discrimination (Evans et al., 1986; Henderson et al., 1992), O₂ sensitivity of photosynthesis (He and Edwards, 1996) and application of mathematical models (cf., He and Edwards, 1996; Chapter 6 by von Caemmerer and Furbank, this volume), with values as a percentage of the rate of C₄ cycle ranging from 8% to 50%. Using a leakage value of 20% as a reasonable estimate, for every 5 CO₂ delivered to the BSC by the C₄ cycle, 1 CO₂ would leak, resulting in a C₄ cycle expense of 2.5 ATP per net CO₂ fixed by PCR cycle. Besides the C₄ and PCR cycles, other demands for assimilatory power are for the synthesis of carbohydrate and assimilation of nitrate, namely the cost of conversion from triose phosphate to carbohydrates (sucrose and starch) and from nitrate to glutamate, respectively. It is apparent that these do not add much to the total assimilatory power requirements per CO₂ fixed in mature leaves (Furbank et al., 1990; Edwards and Baker, 1993).

Although photorespiration via the PCO cycle is strongly suppressed during C₄ photosynthesis, it is not completely eliminated. The extent of photorespiration may vary with leaf age, environmental conditions that affect intercellular CO₂ levels, and species (see Marek and Stewart, 1983; de Veau and Burris, 1989; Dai et al., 1993, 1995, 1996). In maize, PCO cycle activity measured by incorporation of ¹⁸O₂ into glycolate under normal atmospheric levels of CO₂ and O₂ indicated that the velocity of oxygenase (νₙ) was about 3% of the net rate of CO₂ assimilation (A) in mature leaves of 3-month-old plants, and 11% in leaves of 9-day-old seedlings (as opposed to 54% in wheat, a C₃ plant; de Veau and Burris, 1989).
Considering the previously mentioned factors, an estimate of the energy requirements for mature leaves of maize, an NADP-ME species, under normal levels of CO₂ and O₂ is shown in Table II. This analysis shows a total of 5.7 ATP and 2 NADPH per CO₂ fixed. About 90% of the reductive power is accounted for by the reduction of 3-phosphoglycerate to triose phosphate. The ratio of the true rate of O₂ evolution (Jo₂) to net CO₂ uptake (A) in this case is 1.1. This value is remarkably consistent with the Jo₂/A values determined by oxygen isotope exchange (Badger, 1985) and by chlorophyll fluorescence analysis (Genty et al., 1989; Krall and Edwards, 1992; Oberhuber and Edwards, 1993; Oberhuber et al., 1993). The relationship between Jo₂ and A in maize under varying temperature, light intensity, and CO₂ concentrations fits well the theoretical energy requirements for C₄ photosynthesis (Edwards and Baker, 1993). However, under low temperature stress in the field there is evidence that additional electron sinks may be induced (Baker et al., 1995).

The relatively low Jo₂/A ratio in C₄ plants compared to C₃ plants suggests that respiratory processes during C₄ photosynthesis are low (Badger 1985; Edwards and Baker, 1993). From measurements of Jo₂ by O₂ isotope analysis on three monocot species of each C₄ subgroup at ambient external CO₂ levels, the average rate of O₂ uptake for NADP-ME was only 4% of Jo₂, for NAD-ME 9% of Jo₂, and for PEP-CK 22% of Jo₂ (Furbank and Badger, 1982). Photorespiration and the associated O₂ consumption during C₄ photosynthesis could occur by PCO cycle activity, pseudocyclic electron flow (linear

Table II  Estimate of Energy Required in NADP-malic Enzyme Type C₄ Species (e.g., Maize) per CO₂ Fixed Considering Bundle Sheath Leakage of CO₂, Carbohydrate Synthesis, a Low Level of PCO Cycle Activity, and Nitrate Assimilation

<table>
<thead>
<tr>
<th>Function</th>
<th>ATP</th>
<th>NADPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. C₃ pathway per net CO₂ fixed</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2. C₄ pathway, allowing for 25% overcycling</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>3. ATP per C in triose-P converted to carbohydrate (sucrose)</td>
<td>0.08</td>
<td>0</td>
</tr>
<tr>
<td>4. Rubisco oxygenase* where vi is 3% of the net rate of CO₂ fixation (A)</td>
<td>0.14</td>
<td>0.09</td>
</tr>
<tr>
<td>5. Nitrate assimilation*</td>
<td>0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>Total</td>
<td>5.7</td>
<td>2.2</td>
</tr>
</tbody>
</table>

This analysis of energy requirements assumes no dark-type mitochondrial respiration is occurring in the light.

* The activity of Rubisco oxygenase used is that from measurements on the rates of incorporation of ^18O₂ into glycolate and CO₂ fixation in leaves of 3-month-old maize plants (de Veau and Burris, 1989). The ATP and NADPH requirement was calculated considering that the true rate of O₂ evolution with respect to Rubisco (Jo₂) = v₁ + u₁, that the net rate of CO₂ fixation with respect to Rubisco (A) = v₁ - 0.5 u₁, and that for each O₂ reaction with RuBP approximately 3 ATP and 2 NADPH are consumed in the PCO cycle and conversion of the products to RuBP (Krall and Edwards, 1992).

* Based on a C/N ratio in maize of 35/1, assuming all nitrate is assimilated in leaves in the day, and that carbon loss by respiration in the dark is 20% of carbon gain in the light (see Edwards and Baker, 1993).
flow with $O_2$ as electron acceptor), or normal mitochondrial respiration. First, all C$_4$ plants examined have lower rates of $O_2$ uptake than C$_3$ plants due to the low RuBP oxygenase activity. The differences between the three C$_4$ subgroups in $O_2$ uptake may be explained by differences in the mechanism of generation of the additional ATP required in C$_4$ photosynthesis above what is produced from linear electron flow to NADP. The BSC chloroplasts of NADP-ME species are specialized for production of ATP by cyclic photophosphorylation which contributes additional ATP without contributing to $O_2$ uptake (see Edwards and Baker, 1993). The very low rate of $O_2$ uptake (4% of $J_{o2}$) in NADP-ME species may be accounted for by low PCO cycle activity (Table II). The lack of production of $O_2$ in BSC by PSII activity may contribute to low PCO cycle activity in this subgroup. Neither NAD-ME species nor PEP-CK species have chloroplasts that are specialized in cyclic photophosphorylation. The higher rate of $O_2$ uptake in NAD-ME species compared to NADP-ME species may be due to production of additional ATP for the C$_4$ cycle via pseudocyclic electron flow and/or a higher RuBP oxygenase activity in BSC. In PEP-CK species, additional ATP for the C$_4$ cycle is provided via BSC mitochondrial respiration, which contributes to $O_2$ uptake. According to the minimum energy requirements for PEP-CK species (cf., Section III.A), the rate of $O_2$ uptake associated with respiratory production of ATP (using NADH produced via NAD-malic enzyme) would be about 12.5% of $J_{o2}$, which, along with RuBP oxygenase activity, may account for this subgroup having the highest rates of $O_2$ uptake (22% of $J_{o2}$).

C. Energy Requirements of C$_4$ Photosynthesis Compared to Maximum Quantum Yield

Based on the biochemistry of C$_4$ photosynthesis, the requirements for assimilatory power have been defined (cf., Section III.A). Knowing the energy requirements per CO$_2$ fixed, and considering the photochemical efficiency for production of ATP and NADPH using absorbed quanta, the theoretical quantum yield for CO$_2$ fixation (or $O_2$ evolved) can be calculated. The maximum experimental quantum yield can be determined under limiting light and compared to the theoretical values (Ehleringer and Björkman, 1977; Monson et al., 1982; Ehleringer and Pearcy, 1983; Furbank et al., 1990; Edwards and Baker, 1993; Lal and Edwards, 1995). Theoretical considerations include the degree of engagement of the Q-cycle, the H$^+$/ATP ratio for ATP synthase, the degree of overcycling of the C$_4$ cycle, and the extent to which cyclic versus pseudocyclic electron flow contributes additional ATP.

Calculation of the theoretical maximum quantum yield versus the experimental quantum yield can be illustrated for maize, an NADP-ME species.
The energy requirements for MC and BSC are shown in Fig. 5, in which NADPH production is assumed to be produced photochemically only in the MC chloroplasts.

If the Q-cycle is fully engaged (3 H⁺/ē) and 4 H⁺ are required per ATP synthesized (Rumberg and Berry, 1995), then 3 ATP would be synthesized per 2 NADPH generated by whole chain electron flow. In this case, production of the 2.1 NADPH in MC chloroplasts per CO₂ fixed would require 8.4 quanta be used for whole chain transfer of 4.2 ē, which would generate 3.15 ATP according to the previously mentioned stoichiometry. If the additional 0.15 ATP needed in MC were generated by pseudocyclic electron

![Diagram of energy requirements for mesophyll and bundle sheath cells of NADP-ME C₄ species.](image)

**Figure 5** Illustration of energy requirements of mesophyll and bundle sheath cells of NADP-ME C₄ species. Note that 125 TP generated in BSC + 75 TP generated in MC equals a total of 200 TP; 166.6 TP are needed for regeneration of RuBP and the remainder used for synthesis of sucrose and starch. Abbreviations: TP, triose-P; Mal, malate; Pyr, pyruvate; PGA, 3-phosphoglycerate; Glu, glutamate. (See Table II for overall summary of ATP and NADPH requirements.)
transport with the same efficiencies, this would require an additional 0.4 quanta. Production of 2.3 ATP by cyclic electron flow in BSC chloroplasts per CO$_2$ fixed would require an additional 4.6 quanta (assuming 2 H$^+$ generated by cyclic electron flow per quanta and 4 H$^+$ used per ATP produced). Overall, a minimum of 13.4 quanta are required in this example. This ratio will increase due to PCO cycle activity and the expense can be calculated as follows, assuming each O$_2$ reacting with RuBP consumes approximately 2 NADPH (cf., Krall and Edwards, 1992). Considering $J_{O_2}(Rubisco) = v_t + v_o$, and $A = v_t - 0.5 v_o$, the increase in quantum requirement (QR) due to RuBP oxygenase activity per net CO$_2$ fixed can be calculated.

$$\text{Increase in QR due to PCO cycle activity} = \frac{[J_{O_2}(Rubisco)]}{A} - 8$$

$$= \frac{(v_t (8 \text{ quanta/CO}_2) + v_o (8 \text{ quanta/O}_2))}{(v_t - 0.5 v_o)} - 8$$

For example, in mature leaves of maize, $v_o$ was found to be 3% of $A$ (de Veau and Burris 1989), which would require an additional 0.35 quanta per CO$_2$ fixed. In young leaves of maize, de Veau and Burris (1989) found $v_o$ was 11% of $A$, which would require an additional 1.32 quanta. In summary, the minimum quantum requirement would be 13.4 without PCO cycle activity, and 13.8 and 14.7 per CO$_2$ fixed, with $v_o$ equal to 3% of $A$, and with $v_o$ equal to 11% of $A$, respectively. The maximum efficiency of transfer of excitons from light harvesting chlorophyll to the reaction centers under limiting light must also be considered; assuming an efficiency of 85% (Krause and Weis, 1991), then the quantum requirement on the basis of absorbed quanta in these examples would be 15.8, 16.2, and 17.3, respectively (i.e., maximum quantum yields of 0.057–0.063). For example, in maize (NADP-ME), the measured minimum quantum requirement is approximately 17 quanta/CO$_2$ fixed—that is, a maximum quantum yield of 0.059 (Monson et al., 1982; Ehleringer and Pearcy, 1983; Dai et al., 1993). In general, experimental values of the maximum quantum yields in C$_4$ plants under limiting light are about half of the theoretical maximum of 0.125 quanta per CO$_2$ for generation of 2 NADPH per CO$_2$ fixed.

The maximum quantum yields measured in C$_3$ and C$_4$ plants are similar under current atmospheric CO$_2$ at approximately 25°C, with the C$_3$ plants having an additional investment in PCO cycle activity and the C$_4$ plants having an additional investment in the C$_4$ pathway. Higher temperatures at atmospheric levels of CO$_2$, or subatmospheric levels of CO$_2$ at moderate temperatures, decrease the maximum yield in C$_3$ plants to values below that of C$_4$ plants due to increased RuBP oxygenase activity (see Leegood and Edwards, 1996; Chapter 7, this volume).

It is well known that the maximum quantum yield in C$_3$ plants is higher than that of C$_4$ plants with sufficient CO$_2$ enrichment to minimize PCO
cycle activity (ca. 3 times atmospheric levels) because C₄ photosynthesis has the additional energy requirements for the C₄ cycle. However, under extremely high CO₂ levels (10%), the maximum quantum yields per O₂ evolved in the C₄ plants maize (NADP-ME) and P. miliaceum (NAD-ME) are as high as 0.105, which is the same as in C₃ plants under high CO₂ (Lal and Edwards, 1996). This suggests that in C₄ plants under very high CO₂ there is direct fixation of atmospheric CO₂ in the BSC through the PCR cycle, that the C₄ pathway is inhibited, and that all the assimilatory power is used to support reactions of the C₃ pathway as in C₃ plants.

IV. Coordination of the Two Cell Types in C₄ Photosynthesis

A. Separation of the Two Cell Types and Enzyme Distribution

Following the discovery of C₄ photosynthesis and its association with two photosynthetic cell types, there were questions not only about the mechanism, but controversy also developed over the localization of key enzymes. Roger Slack was the first to separate chloroplasts of the two cell types with nonaqueous mixtures of hexane and carbon tetrachloride, and he demonstrated differential location of some enzymes in the C₄ pathway as well as ¹⁴C-intermediates of the pathway (Slack, 1969; Slack et al., 1969). Using aqueous media, brief grindings repeated with C₄ leaf segments were later devised for the separation of MC and BSC contents in the form of partial leaf extracts. The “differential” grinding methods in the early 1970s brought about controversy over the localization of some enzymes, such as PEP carboxylase and Rubisco in the two cell types (see Chapter 2 by Hatch, this volume). However, careful handling of the procedure was subsequently effective to obtain intact MC and BSC chloroplasts and bundle sheath strands (e.g., Jenkins and Russ, 1984; Jenkins and Boag, 1985; and Sheen and Bogorad, 1985, respectively).

In a limited number of C₄ species such as Digitaria sanguinalis and other species of the genus, intact MC and bundle sheath strands were separated by simple maceration of the leaf segments with a mortar and pestle and subsequent filtration with nylon nets (Edwards and Black, 1971). Treatment of C₄ leaf segments with digestive enzymes such as pectinase and cellulase proved to be a very effective method for separating the two cell types from various species in a pure and intact state, namely mesophyll protoplasts and bundle sheath strands (Kanai and Edwards, 1973a,b). Improvements in commercial enzymes and testing protocols and the use of young seedlings has allowed the enzymatic method to be applicable to a variety of C₄ plants, especially for isolating intact organelles from protoplasts (Edwards et al., 1979; Moore et al., 1984; Watanabe et al., 1984). A disadvantage of the
method is the limited quantity of organelles in spite of the high degree of intactness.

Distribution of enzyme activities related to the C₄ pathway, PCR and PCO cycles, and other enzymes of primary carbon metabolism are listed in Table III, based on data from some representative C₄ species of the three C₄ subgroups. Very low activity in a specific cell type may indicate either a low degree of contamination by the other cell type or actual presence of the enzyme—for example, PEP carboxylase, pyruvate, P dikinase, and NADP-malate dehydrogenase in BSC, or Rubisco, phosphoribulokinase, and malic enzymes in MC. As for the photorespiratory glycolate pathway, BSCs possess most of the enzymes except for the absence of glycerate kinase, whereas MCs lack Rubisco in the chloroplasts and enzymes of glycine decarboxylation in the mitochondria. Carbonic anhydrase is localized in MC; little activity occurs in BSC chloroplasts in contrast to high activities in chloroplasts of C₃ plants where the PCR cycle is located. Nitrate reductase and nitrite reductase are localized in MC cytoplasm and chloroplasts, respectively, in most C₄ species (Rathnam and Edwards, 1976). Cytoplasmic and chloroplastic isoforms of glutamine synthetase are distributed evenly in the two cell types; but ferredoxin-dependent glutamate synthetase (Fd-GOGAT) is mostly located in BSC chloroplasts in maize leaf (Harel et al., 1977; Becker et al., 1993). Thus, nitrate assimilation is restricted to MC, whereas recycling of ammonia produced by the photorespiratory pathway may occur in BSC. In situ immunolocalization studies have also been a valuable tool in demonstrating inter- and intracellular location of certain key enzymes in C₄ photosynthesis (e.g., Hattersley et al., 1977; Wang et al., 1992, 1993).

B. Intercellular Transport of Metabolites

Because of differential location of the enzymes, operation of the C₄ pathway of photosynthesis necessitates a rapid bidirectional movement of various metabolites between MC and BSC. The intercellular transport of metabolites includes C₃/C₄ acids, as illustrated in Figs. 1–3, C₃ compounds in the reductive phase of the PCR cycle, and glycerate of the photorespiratory PCO cycle. As the enzymes of the reductive phase of the PCR cycle are distributed in both cell types, MC chloroplasts are able to share this function, especially in many C₄ species of the NADP-ME type, where the BSC chloroplasts have reduced grana and are deficient in Photosystem II activity. The glycerate formed by the glycolate pathway in the BSC is apparently transported and converted to 3-phosphoglycerate in MC due to exclusive localization of glycerate kinase in MC chloroplasts (Usuda and Edwards, 1980a,b).

C. Evidence for Coordination of Two Cell Types

In addition to the enzyme distribution evidence, there is a large body of deductive evidence based on a combination of time-course (during
<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>PEP-CK Type</th>
<th>C₄ Pathway</th>
<th>PCR Cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zea mays</td>
<td>Panicum maximum</td>
<td>Eleusine indica</td>
</tr>
<tr>
<td></td>
<td>(Urochloa panicoides)</td>
<td>(Panicum miliaceum)</td>
<td></td>
</tr>
<tr>
<td>NADP-ME type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₄ pathway</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbonic anhydrase&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>98/2</td>
<td>(98/2)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>(93/7)&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>PEP carboxylase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>864/14</td>
<td>2590/3</td>
<td>2400/10</td>
</tr>
<tr>
<td>Pyruvate, Pi dikinase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>188/15</td>
<td>(66/1)</td>
<td>82/1</td>
</tr>
<tr>
<td>Adenylate kinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56/10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyrophosphatase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>49/16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP-malate dehydrogenase&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>805/&lt;1</td>
<td>72/25</td>
<td>175/51</td>
</tr>
<tr>
<td>NADP-malic enzyme&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;1/1690</td>
<td>&lt;1/85</td>
<td>7/28</td>
</tr>
<tr>
<td>PEP carboxykinase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;1/&lt;1</td>
<td>&lt;1/542</td>
<td>&lt;1/1</td>
</tr>
<tr>
<td>NAD-malic enzyme&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;1/126</td>
<td>62/263</td>
<td>11/554</td>
</tr>
<tr>
<td>Aspartate aminotransferase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>324/70</td>
<td>(1150/753)</td>
<td>1400/205</td>
</tr>
<tr>
<td>Alanine aminotransferase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>33/53</td>
<td>(612/335)</td>
<td>942/579</td>
</tr>
<tr>
<td>PCR cycle</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RuBP carboxylase&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>&lt;1/389</td>
<td>4/249</td>
<td>1/395</td>
</tr>
<tr>
<td>Phosphoribulokinase&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>&lt;1/2940</td>
<td>(75/1310)</td>
<td>24/2450</td>
</tr>
<tr>
<td>Phosphoribooisomerase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>75/1500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PGA kinase&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1290/2450</td>
<td>(2110/701)</td>
<td>2550/1090</td>
</tr>
<tr>
<td>NADP-triose-P dehydrogenase&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>705/1400</td>
<td>(240/457)</td>
<td>250/477</td>
</tr>
<tr>
<td>Triose-P isomerase&lt;sup&gt;d&lt;/sup&gt;</td>
<td>526/545</td>
<td>(277/481)</td>
<td>209/813</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Activity</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>---------------------------------------</td>
<td>------------</td>
<td>------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Glycolate oxidase</td>
<td>1.2/10</td>
<td>Ku and Edwards, 1975</td>
<td></td>
</tr>
<tr>
<td>Hydroxypyruvate reductase</td>
<td>20/184</td>
<td>Ku and Edwards, 1975</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>9/60</td>
<td>Kanai and Edwards, 1973a</td>
<td></td>
</tr>
<tr>
<td>Glycerate kinase</td>
<td>7.5/&lt;1</td>
<td>R. Kanai, unpublished results</td>
<td></td>
</tr>
<tr>
<td>Others (succrose synthesis, glycolysis, oxidative pentose-P pathway, respiration)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UDPG pyrophosphorylase</td>
<td>797/285</td>
<td>Gutierrez et al., 1974a</td>
<td></td>
</tr>
<tr>
<td>Sucrose-P synthase</td>
<td>56/3.2</td>
<td>Slack et al., 1969</td>
<td></td>
</tr>
<tr>
<td>Phosphoglyceromutase</td>
<td>160/23</td>
<td>Kanai and Edwards, 1973a</td>
<td></td>
</tr>
<tr>
<td>Enolase</td>
<td>203/18</td>
<td>Kanai and Edwards, 1973a</td>
<td></td>
</tr>
<tr>
<td>Glucose-6-P dehydrogenase</td>
<td>20/9</td>
<td>Gutierrez et al., 1974a</td>
<td></td>
</tr>
<tr>
<td>6-Phosphogluconate dehydrogenase</td>
<td>10/8</td>
<td>Gutierrez et al., 1974a</td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>33/146</td>
<td>Usuda and Edwards, 1980a</td>
<td></td>
</tr>
</tbody>
</table>

For values listed as <1, the activity was less than 1 or not detectable.

* Relative value.
* R. Kanai, unpublished results.
* Gutierrez et al., 1974a.
* Slack et al., 1969.
* Usuda and Edwards, 1980a.
feeding of $^{14}\text{CO}_2$ and pulse-chase data ($^{14}\text{CO}_2$ pulse and $^{12}\text{CO}_2$ chase) under steady state photosynthesis. All of this information supports cooperative interaction between cells as the only reasonable explanation for C$_4$ photosynthesis and allows precise deductions about how it occurs (cf., Hatch, 1987; see also Chapter 2 by Hatch, this volume).

One of the most direct visual proofs for metabolite transport from MC to BSC is an autoradiograph of a transverse leaf section of *Atriplex spongiosa*, an NAD-ME C$_4$ dicot, after 2 sec of $^{14}\text{CO}_2$-fixation in the light (Fig. 6). This shows most of the label in BSC and the remaining label in MC and hypodermal cells (Osmond, 1971). Within such a short time of $^{14}\text{CO}_2$-photosynthesis, about 90% of the label was found in C$_4$ acids; mostly aspartate, as would be expected for this subtype. The microautoradiography clearly indicates rapid transport of these C$_4$ acids from MC cytoplasm to BSC.

A unique approach to separate functions of the two cell types during C$_4$ photosynthesis *in vivo* was a light-enhanced dark (LED) $^{14}\text{CO}_2$-fixation experiment using intact leaves of maize (Samejima and Miyachi, 1971, 1978, or *Cynodon dactylon* (Black et al., 1973). Leaves were illuminated in CO$_2$-free air, followed by dark $^{14}\text{CO}_2$-fixation, and then reillumination in CO$_2$-free air. Preillumination of leaves significantly enhanced the subsequent dark $^{14}\text{CO}_2$-fixation into malate and aspartate, but no label was transferred to 3-phosphoglycerate and sugar phosphates. The transfer of $^{14}\text{C}$ to the PCR cycle intermediates and synthesis of sucrose occurred only when the leaves were reilluminated. This experiment apparently separates the function of MC and BSC in time, and demonstrates that light is required
for use of the C₄ acids as donors of CO₂ to the PCR cycle in BSC. Interestingly, when a ¹⁴C-bicarbonate solution was supplied directly to BSC by vacuum infiltration through vascular tissue of cut maize leaves, the main initial photosynthetic products in the light were 3-phosphoglycerate and sugar phosphates. This indicates that CO₂ directly supplied to BSC by the vascular tissue is fixed by the PCR cycle, whereas atmospheric CO₂ is fixed into C₄ acids by the C₄ pathway. These experiments provide further evidence for the photosynthetic function of MC and BSC and the cooperation required in light-mediated processes.

In general, intercellular transport of metabolites is mediated by apoplastic and/or symplastic processes. Although the former is via the cell wall and entails movement across the two plasma membranes, the latter occurs between the cytoplasm of two cells through the plasmodesmata. Osmond calculated the flux of C₄ acids through the plasmodesmata, which occupy approximately 3% of the cell wall interface between MC and BSC. He suggested that the rapid movement of metabolites between the two cell types may be accomplished by a diffusive process in the symplasm, with approximately 10 mM concentration gradient; thus it is not necessary to postulate a special mechanism for active transport (for detail, cf., Osmond and Smith, 1976). Electron micrographs of C₄ leaves show many plasmodesmata at the interface of MC and BSC and, in many species, a thick, suberized layer in the BSC cell wall (Laetsch, 1971; Evert et al., 1977).

Actual estimations of concentration gradients of the intermediates in leaves of maize and Amaranthus edulis were made by partial mechanical separation of MC and BSC followed by enzymatic assay of the metabolites (Leegood, 1985; Stitt and Heldt, 1985; Leegood and von Caemmerer, 1988). For example, in photosynthesizing maize leaves, gradients of malate and triose phosphates from MC to BSC were 18 and 10 mM, respectively, whereas that of 3-phosphoglycerate from BSC to MC was 9 mM (Stitt and Heldt, 1985). However, the estimated concentration of pyruvate between MC and BSC was similar. This unexpected result can be explained by active accumulation of pyruvate in MC chloroplasts in the light (cf., Section V.A.3), which allows a concentration gradient of pyruvate to be maintained from the cytosol of BSC to the cytosol of MC. Large concentration gradients guarantee rapid intercellular transport of each metabolite.

Isolated bundle sheath strands were obtained from several C₄ species and used for studying the permeability of compounds of different molecular sizes (Weiner et al., 1988; Valle et al., 1989). The BSC in these preparations retained their structural integrity and transport into BSC via severed plasmodesmata, which were originally connected to MC. The size exclusion limit into BSC was estimated to be approximately 900–1000 kDa by inhibition of alanine aminotransferase using inhibitor dyes having various molecular weights and by observations on plasmolysis of the bundle sheath strands.
with polyethyleneglycol (PEG) of various molecular sizes. The apparent
diffusion rates of the compounds with smaller molecular weight were in
the range of 2 to 5 mmol min\(^{-1}\) (mg Chl)\(^{-1}\) per mmolar gradient between
bundle sheath strands and the suspending medium. This suggests that
various metabolites of low molecular weight can readily diffuse between
MC and BSC through plasmodesmata.

V. Intracellular Transport of Metabolites

Compared with C\(_3\) plants, C\(_4\) species, by necessity, have a more extensive
and diverse intracellular transport of metabolites, namely the unique trans-
port of C\(_3\) and C\(_4\) acids associated with intercellular transport, PEP and
phosphorylated C\(_3\) compounds in the PCR cycle, and glycerate in the PCO
cycle. Differentiation in the transport properties of respective organelles
in MC and BSC is also expected because of compartmentation of key
enzymes and the unique feature of intercellular coordination. Using differ-
ential centrifugation in sucrose density gradients, chloroplasts, mitochon-
dria and peroxisomes have been separated from mesophyll and bundle
sheath protoplasts isolated from some C\(_4\) species (Gutierrez et al., 1975;
Moore et al., 1984; Watanabe et al., 1984; Ohnishi et al., 1985). However,
direct studies on carrier-mediated mechanisms of metabolite transport and
their kinetic properties have been performed mainly with MC chloroplasts.
This is largely because of technical limitations in isolating sufficient quanti-
ties of intact organelles from BSC.

A. Mesophyll Chloroplasts

1. Translocators of C\(_4\) Acids In NADP-ME (and to some extent PEP-CK) C\(_4\)
plants, the initial CO\(_2\)-fixation product in MC, oxaloacetate, is transported
from the cytoplasm to the chloroplasts by a specific translocator having a
high affinity for oxaloacetate \(K_m: ca. 45 \mu M\) but very low affinity for malate
(Hatch et al., 1984). Malate (and some aspartate in NADP-ME-type plants)
formed in mesophyll chloroplasts is exported to the cytoplasm by a C\(_4\)
dicarboxylic acid translocator \(K_m\) for malate: 0.5 mM, Day and Hatch,
1981), which is similar to that of C\(_3\) chloroplasts. Purification and molecular
cloning of these C\(_4\) acid translocators can be anticipated for C\(_4\) mesophyll
chloroplasts, because a malate/2-oxoglutarate translocator from the chloro-
plast envelope of spinach has already been cloned (Weber et al., 1995).

2. Translocator of P\(_i\)/Triose Phosphate and PEP Because pyruvate,P\(_i\) dikinase
is localized in the chloroplasts and PEP is used by PEP carboxylase in the
cytoplasm of MC, transport of pyruvate and PEP was logically discovered
using mesophyll chloroplasts of Digitaria sanguinalis, an NADP-ME C\(_4\) plant
3. The Biochemistry of C₄ Photosynthesis

(Huber and Edwards, 1977a,b). Although pyruvate uptake was mediated by a new translocator in the envelope of C₄ mesophyll chloroplasts, PEP was transported by a phosphate translocator having the extra capacity to carry PEP in addition to Pᵢ, 3-phosphoglycerate and triose phosphate, which are transported by the phosphate/triose-phosphate translocator in C₃ chloroplasts. In *P. miliaceum*, both MC and BSC chloroplasts are able to transport PEP (Ohnishi et al., 1989). The Pᵢ translocator of maize MC chloroplasts possesses a higher affinity for all transported substrates compared to that of C₃ chloroplasts: 50 times higher for PEP and 2-phosphoglycerate (compounds with the phosphate group at the C-2 position), and 6 times higher for Pᵢ, triose phosphates and 3-phosphoglycerate (compounds with phosphate group at C-3 position) (Gross et al., 1990). Among the phosphate translocator cDNAs cloned recently from several C₃ and C₄ species, those from *Flaveria trinervia* (C₄) and *F. pringlei* (C₃) showed 94% homology of amino acids in the mature protein. A computer-aided molecular modeling of the translocators suggests that minor changes in amino acids at the translocation pore might be sufficient to extend high substrate specificity to PEP in the C₄ phosphate translocator (Fischer et al., 1994).

3. Active Pyruvate Transport

After the study of pyruvate transport in *D. sanguinalis* in MC in the dark (Huber and Edwards, 1977a), pyruvate uptake in maize and *P. miliaceum* MC chloroplasts was shown to be promoted by light (Flügge et al., 1985; Ohnishi and Kanai, 1987a, respectively). In illuminated chloroplasts of *P. miliaceum*, the initial rate of transport was 7–10 times higher and the amount of pyruvate accumulated was 10–30 times higher than in the dark. Good correlations between the light-dependent pyruvate uptake and stromal alkalization of illuminated chloroplasts suggested that active transport is primarily driven by the pH gradient, which is formed across the envelope (Ohnishi and Kanai, 1987b). In maize MC chloroplasts, in fact, generation of an artificial pH gradient by a pH shift from 7 to 6 in the suspension medium (a H⁺-jump) induced a 5- to 10-fold enhancement of pyruvate uptake in the dark (Ohnishi and Kanai, 1990). In *P. miliaceum*, however, addition of 10 mM Na⁺ into the medium (a Na⁺-jump) induced a similar enhancement of pyruvate uptake in the dark (Ohnishi and Kanai, 1987c). In terms of the pyruvate uptake induced by these cation jumps in the dark, C₄ plants can be divided into two groups: a H⁺ type and a Na⁺ type. NADP-ME type C₄ species of Arundinellae and Andropogoneae in the Gramineae belong to the former group, whereas most of the other C₄ monocots and all dicots thus far studied belong to the latter (Aoki et al., 1992).

In the H⁺ type, the sole source of driving force for the active pyruvate uptake in MC chloroplasts is the H⁺ gradient formed across the envelope and H⁺ and pyruvate are cotransported in a one-to-one ratio in the light
(Aoki et al., 1994). Moreover, the pH gradient is maintained by co-export of H⁺ and PEP³⁻ via the phosphate translocator as illustrated in Fig. 7 (Aoki and Kanai, 1995).

In the Na⁺ type, the driving force for the active pyruvate transport in MC chloroplasts was originally considered to be by a Na⁺ gradient generated by light dependent H⁺ uptake with Na⁺ efflux, because Na⁺ and pyruvate were apparently cotransported in the light and darkness (Ohnishi et al., 1990). In this case, light (or ATP) dependent Na⁺ efflux or Na⁺/H⁺ antiport across the envelope would be expected. However, in a Na⁺ jump experiment in the dark, there is no evidence for measurable H⁺ efflux, nor change in ATP content in the chloroplast. On the contrary, addition of K-pyruvate or Na-pyruvate decreases the stromal pH only in the light with the Na-pyruvate being most effective (Fig. 8).

Furthermore, illumination and Na⁺-jump treatments were cooperative in promoting the initial rate of pyruvate uptake in MC chloroplasts of P. miliaceum suspended in medium at pH 7.8. Thus, in the Na⁺ type, pyruvate uptake may occur by Na⁺ dependent acceleration of pyruvate-H⁺ cotransport (Aoki and Kanai, 1997).

**B. Bundle Sheath Chloroplasts**

The BSC chloroplasts have a phosphate transporter (Flugge and Heldt, 1991) and a glycolate transporter (Ohnishi and Kanai, 1988) that have

![Figure 7](image-url)  
*Figure 7*  Scheme of H⁺ mobilization accompanied with pyruvate import and phosphoenolpyruvate export in the light in mesophyll chloroplasts of C₄ species having H⁺ type pyruvate transport. [Reprinted with permission from N. Aoki and R. Kanai (1995). The role of phosphoenolpyruvate in proton/pyruvate cotransport into mesophyll chloroplasts of maize. *Plant Cell Physiol.* 36, 187–189, with permission.]
8. The Biochemistry of C₄ Photosynthesis

---

Figure 8 Changes in stromal pH of Panicum miliaceum mesophyll chloroplasts in the light by the addition of K- or Na-pyruvate. Intact chloroplasts isolated from mesophyll protoplasts of P. miliaceum were suspended in the medium of pH 7.8 and preincubated for 5 min in the light (500 μmol quanta m⁻² s⁻¹). Stromal pH was calculated from the uptake of [¹⁴C]-5,5-dimethyl oxazolidine-2,4-dione by silicone-oil filtering centrifugation. Addition at 0 time: (+) water, (●) 5 mM Na-gluconate, (○) 5 mM K-gluconate, (△) 5 mM K-pyruvate, (▲) 5 mM Na-pyruvate. There were no significant changes in stromal pH in darkness by these additives (cf. Aoki and Kanai, unpublished, 1997).

similar properties to these transporters in C₃ chloroplasts although the metabolic exchanges during C₄ photosynthesis are different due to the coordinated functions between MC and BSC chloroplasts. During CO₂ fixation by RuBP carboxylase, BSC chloroplasts export some of the product PGA in exchange for triose-P or P₃. This occurs by the exported PGA being converted to triose-P in MC chloroplasts and re-entering the BSC chloroplast, or the triose-P being metabolized to sucrose, releasing P₃, which is taken up by the BSC chloroplast. Whereas C₃ chloroplasts export glycolate in exchange for glycerate during photorespiration, in C₄ plants the glycolate synthesized in BSC during photorespiration is exported, metabolized to glycerate and the glycerate imported by MC chloroplasts, where it is converted to PGA (cf. Section IV). This transport is mediated by a glycolate/glycerate transporter in BS and MC chloroplasts (Ohnishi and Kanai, 1988).
BSC chloroplasts of NADP-ME species like maize also transport malate and pyruvate. Intact BSC chloroplasts isolated from maize leaves are capable of high rates of malate decarboxylation and $^{14}$CO$_2$-assimilation only in the presence of 3-phosphoglycerate and/or triose phosphate, with significant enhancement of the rates by the addition of aspartate (Boag and Jenkins, 1985; Taniguchi, 1986). The former compounds are required for the generation of intermediates of the PCR cycle as well as for recycling of NADPH/NADP$^+$ in the decarboxylation reaction. As aspartate itself is not metabolized, nor does it influence the decarboxylation reaction, the enhancement effect suggests a malate translocator, which differs from C$_4$ acid translocators in C$_3$ and C$_4$ MC chloroplasts.

These differences in the characteristics of malate transport in MC and BSC chloroplasts of maize are illustrated in Fig. 9. The initial rate and final level of $^{14}$C-malate uptake in maize MC chloroplasts (Fig. 9A) were the highest in the absence of aspartate (−Asp in the figure). The rate was reduced by adding aspartate together with malate (+Asp) and even more by preincubation with aspartate for 5 min before malate addition (pre+Asp). However, $^{14}$C-malate uptake into maize BSC chloroplasts (Fig. 9B) was enhanced by adding aspartate (+Asp), and even more so by preincubation with aspartate (pre+Asp). In contrast, $^{14}$C-aspartate uptake with/without malate was essentially the same in MC and BSC chloroplasts of maize; namely, the rate was slightly reduced by adding malate and further inhibited by preincubation with malate (data not shown). Interestingly, addition of pyruvate had an enhancement effect on $^{14}$C-malate uptake in maize BSC chloroplasts similar to that of aspartate (Fig. 9C). These results indicate that the envelope of BSC chloroplasts possesses a new malate transport system in addition to the C$_4$ acid and/or aspartate translocators that have previously been found in C$_3$ and C$_4$ MC chloroplasts (Day and Hatch, 1981; Werner-Washburne and Keegstra; 1985). Although there is evidence that BSC chloroplasts can transport pyruvate (Taniguchi, 1986; Ohnishi and Kanai, 1987a), the mechanism of this transport relative to that in MC chloroplasts remains to be fully characterized.

**VI. Summary**

It is clear that since the late 1960s the biochemistry of C$_4$ photosynthesis in three C$_4$ subgroups has become well defined and functional characteristics of many of the key translocators have been elucidated. However, much work remains to be done on isolation of translocators and description of their physical and molecular properties. The two photosynthetic cells, MC and BSC, must cooperate in production of ATP and NADPH to meet the energy requirements of the C$_4$ cycle and PCR cycle; the relative contribu-
Figure 9 $^{14}$C-Malate uptake by mesophyll and bundle sheath chloroplasts isolated from maize leaves: effects of aspartate and pyruvate. BSC chloroplasts from maize leaves were isolated according to Jensen and Boag (1985). Intactness of the BSC chloroplasts was more than 80%, whereas that of mesophyll chloroplasts was more than 90%; estimation of intactness was made with phase-contrast microscopy and an NADP-triose phosphate dehydrogenase activity test (instead of ferricyanide test) resulted in similar percentage intactness. Uptake of $^{14}$C-malate (0.5 mM, in the medium) was measured by silicone-oil filtering centrifugation at 5°C and pH 8.0 after preincubation in the light (300 μmol quanta m$^{-2}$ s$^{-1}$). A, B: Malate uptake by mesophyll and BSC chloroplasts, respectively, without (-Asp), with (+Asp), and after 5 min preincubation with (pre+Asp) 0.5 mM aspartate. The initial uptake rates (in mmol [mg Chl$^{-1}$ hour$^{-1}$] at 10 sec in mesophyll chloroplasts were 3.9, 2.8, and 1.5, respectively, whereas those in BSC chloroplasts were 1.1, 1.6, and 2.3, respectively. C: Effect of pyruvate on malate uptake without (-PA), with (+PA), and after preincubation with (pre+PA) 5 mM pyruvate; the initial uptake rates were 1.1, 1.4, and 3.2, respectively (Taniguchi and Kanai, unpublished).

...tions of the two chloroplast types and degree of flexibility in shared production of assimilatory power is not clear. Although metabolic control and balance within and between the C$_4$ and PCR cycle are required, they are poorly understood. One can presently only speculate on how the two cycles are coordinated under a range of environmental conditions to provide a sufficient level of CO$_2$ to the BSC to minimize RuBP oxygenase activity and at the same time avoid excessive overcycling and leakage of CO$_2$ from BSC. Molecular research supported by solid plant biochemistry (Hatch, 1986)
continues to be important in considering the origin of enzymes and translocators essential to C₄ photosynthesis. Finally, the relevance of the variations on the biochemistry of C₄ photosynthesis and Kranz type leaf anatomy to survival and performance in different habitats remains to be elucidated.

References


3. The Biochemistry of $C_4$ Photosynthesis


3. The Biochemistry of C₄ Photosynthesis


