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The Biochemistry of C_4 Photosynthesis

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I. Introduction

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 C_4 pathway are located separately in these morphologically distinct cell types. In C_4 leaves, atmospheric 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 C_4 dicarboxylic acids are transported to BSC where they are decarboxylated, and the released 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 C_3 MC in carbon assimilation except for the presence of enzymes concerned with decarboxylation of C_4 acids.

The physiological significance of separate but coordinate function of the two cell types in C_4 photosynthesis is the specialization of MC for generation of a high concentration of 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 C_3 plants (*cf.*, Osmond and Grace, 1995), it is clear that C_4 plants have the capacity to perform effective photosynthesis under conditions in which RuBP oxygenase activity is restricted. C_4 photosynthesis can be visualized as a mechanism to provide Rubisco with near saturating CO_2 when C_4 plants can afford a high stomatal conductance, or to provide sufficient CO_2 for survival and growth when stomatal conductance is low.

During the evolution of C_4 photosynthesis from C_3 plants, the MC developed a high level of carbonic anhydrase (CA) and PEP carboxylase for initial CO_2 fixation in the cytoplasm, and pyruvate, orthophosphate (P_i) dikinase in the chloroplasts for provision of PEP, the HCO_3^- acceptor. It is equally important that the synthesis of some key photosynthetic enzymes in carbon metabolism of C_3 photosynthesis is repressed in MC of C_4 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 C_4 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 CO_2 concentration in BSC and the activity of Rubisco, which is exclusively localized in these cells, are discussed. In the third section, the energetics of C_4 photosynthesis is dealt with, including the theoretic maximum efficiency and *in vivo* energy requirements of C_4 plants. Although there is evidence for cooperation between the two cell types in C_4 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 C_4 photosynthesis see Edwards and Walker (1983), Hatch (1987), and Leegood and Osmond (1990).

A. The Three C_4 Subgroups

C_4 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 C_4 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 C_4 subgroups are summarized in Figs. 1, 2, and 3. Common to all C_4 plants is the initial fixation of HCO_3^- by PEP carboxylase to form oxaloacetate in the MC cytoplasm. As atmospheric CO_2 enters the MC via stomata, carbonic anhydrase in the MC cytoplasm helps to equilibrate the CO_2 to HCO_3^-. Malate and aspartate are formed from oxaloacetate in MC. As determined by $^{14}CO_2$-fixation experiments, the main initial product is malate in C_4 species of the NADP-ME type, whereas aspartate is the major product in the NAD-ME and PEP-CK types.

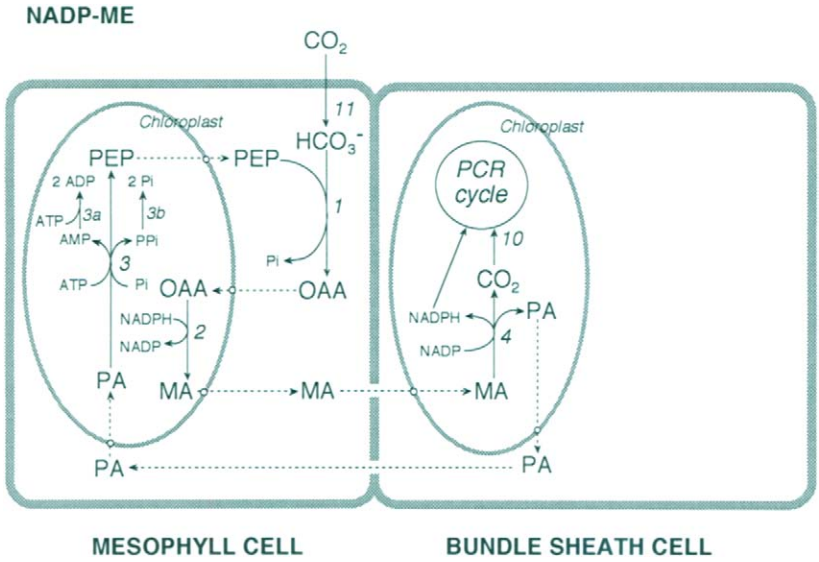


Figure 1 Subgroup of C_4 pathway: NADP-ME (malic enzyme) type. Compound abbreviations for Figs. 1–3: Ala, alanine; Asp, aspartate; MA, malate; OAA, oxaloacetate; PA, pyruvate; PEP, phosphoenolpyruvate; Pi, orthophosphate; PPi, pyrophosphate. Enzyme abbreviations: 1, PEP carboxylase; 2, NADP-malate dehydrogenase; 3, pyruvate phosphate dikinase; 3*a*, adenylate kinase; 3*b*, 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 C_4 species, bundle sheath chloroplasts of C_4 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 C_4 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 CO_2 and reduced NADP to the PCR cycle. The other product of decarboxylation, pyruvate, is returned to MC chloroplasts, where it is phosphorylated by pyruvate,P_i 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-

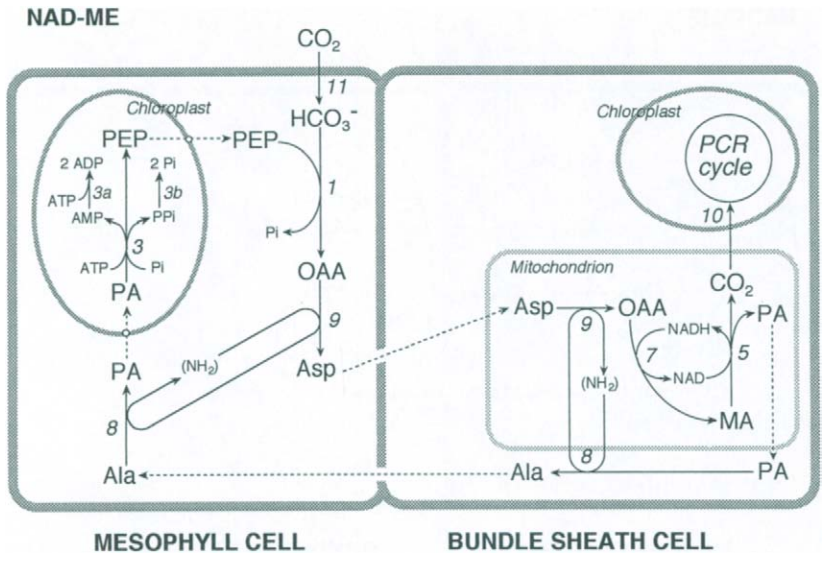


Figure 2 Subgroup of C_4 pathway: NAD-ME (malic enzyme) type. (See Fig. 1 legend for compounds and enzymes.)

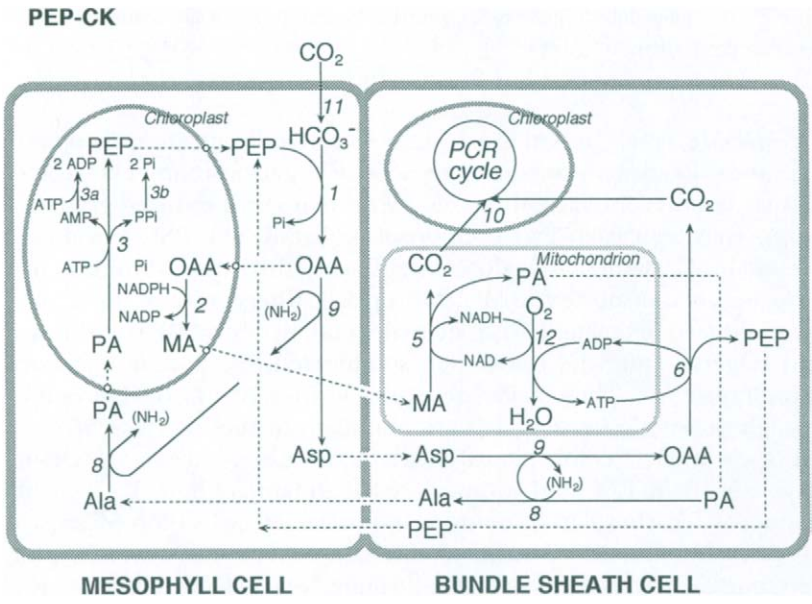


Figure 3 Subgroup of C_4 pathway: PEP-CK (carboxykinase) type. (See Fig. 1 legend for compounds and enzymes.)

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 C_4 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 $^{14}CO_2$-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 CO_2 to bundle sheath chloroplasts. Thus, bundle sheath mitochondria play a decisive role in this C_4 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 C_4 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 $^{14}CO_2$-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 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_i dikinase in PEP-CK C_4 plants compared with the other C_4 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 C_4 Pathway: Reaction and Properties

After proposing the C_4-dicarboxylic acid pathway of photosynthesis in 1966, Hatch and Slack identified many of the enzymes of the C_4 pathway and showed their activities were sufficient to account for *in vivo* photosyn-

Table I Summary of Enzyme Activities of the C_4 Pathway and Location in the Leaf

	Intercellular and intracellular enzyme location[a]	NADP-ME	NAD-ME	PEP-CK
		Enzyme activity in whole leaf extract (μmol min^{-1} mg^{-1} chlorophyll)		
PEP carboxylase[b,c]	M cyt	13~24	12~25	17~27
Pyruvate,Pi dikinase[b]	M chlt	4~8	4~9	2~4
Adenylate kinase[d]	M chlt >B	41~87	36~70	
Pyrophosphatase[d]	M chlt >B	37~57	52~74	
NADP-malate dehydrogenase[b]	M chlt >B	10~17	1~2	2~5
NADP-malic enzyme[b]	B chlt	10~16	<1	<1
NAD-malic enzyme[b]	B mit	<1	5~18	1~3
PEP carboxykinase[b]	B cyt	<1	<1	6~17
Aspartate aminotransferase[b]	M chlt >B	5~9		
	M cyt >B mit, cyt		27~46	44~60
Alanine aminotransferase[b]	M cyt =B cyt	3~8	30~63	38~45
RuBP carboxylase[c,e,f]	B chlt	1~4	1~3	1~4
Carbonic anhydrase[a]	M cyt	35~68	79~89	28

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.

[b] Hatch, 1987.

[c] Gutierrez *et al.*, 1974a.

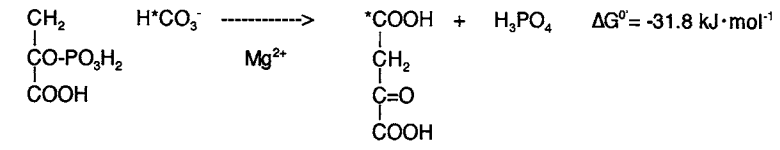
[d] Hatch and Burnell, 1990.

[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.

thetic rates (*ca.* 3–5 μmol CO_2 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 C_4 plants and their compartmentation are summarized in Table I.

1. PEP Carboxylase PEP carboxylase (EC 4.1.1.31) from C_4 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:



phosphoenol-pyruvate (PEP)

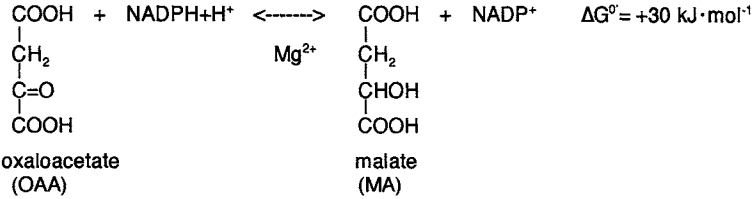
oxaloacetate (OAA)

(Reaction I)

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 m*M* and 1–2 m*M*, 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).

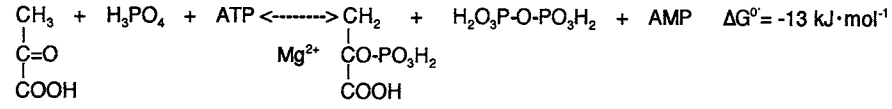
*2. **NADP-Malate Dehydrogenase*** Malate dehydrogenase specific to NADP (EC 1.1.1.82), found by Hatch and Slack (1969a) in the chloroplasts of



(Reaction II)

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 μM and 32 m*M*, respectively (Kagawa and Bruno, 1988). Light activation is mediated by the ferredoxin–thioredoxin *m* system, which reduces a disulphide 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,P_i Dikinase*** The enzyme activity first reported as "PEP synthetase" by Hatch and Slack in 1967 was identified as pyruvate, orthophosphate

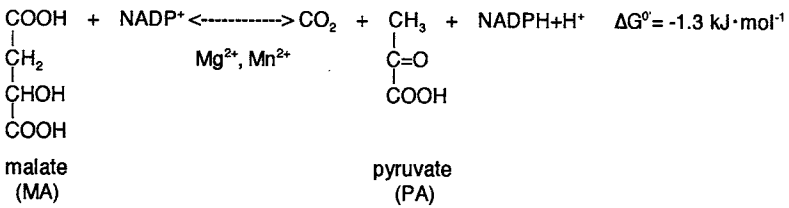


(Reaction III)

dikinase (EC 2.7.9.1), a new key enzyme in the C_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°C; K_m values for pyruvate, P_i, ATP, PEP, pyrophosphate and AMP are 250, 1,500, 15, 140, 40, and <10 μM, respectively (Sugiyama, 1973). Although the reaction itself is reversible, it proceeds to form PEP *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_i to form pyrophosphate and His residues at the active site of the enzyme with γ- and β-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 β-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 *et al.*, 1985).

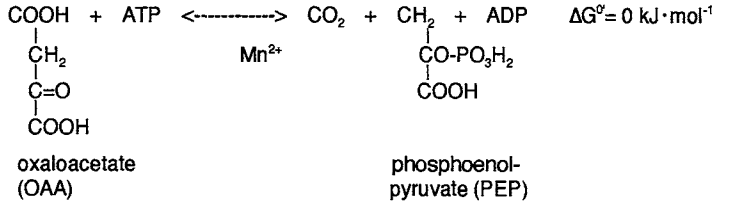
4. Enzymes of C_4 Acid Decarboxylation Although C_4 decarboxylating enzymes had already been discovered in plant tissues, their role in C_4 photosynthesis was revealed because of high activities in the BSC of each C_4 subgroup [by Slack and Hatch (1967) for NADP-malic enzyme (EC 1.1.1.40, Reaction IV); by Edwards *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 CO_2, and not HCO_3^-, after some debates; HCO_3^- is rather inhibitory to the decarboxylation reactions (Jenkins *et al.*, 1987).

NADP-malic enzyme in C_4 plants is located in BSC chloroplasts, whereas



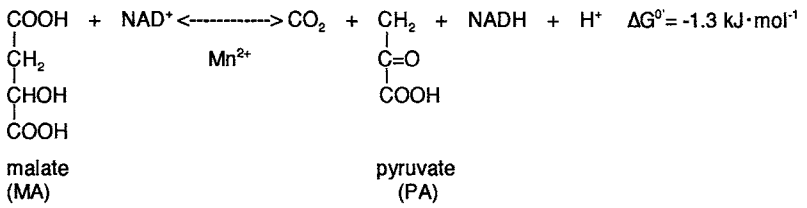
(Reaction IV)

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 μM, 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).



(Reaction V)

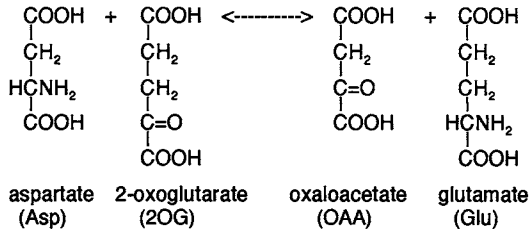
The localization of PEP carboxykinase in BSC cytoplasm was confirmed after some misinterpretation (Watanabe *et al.*, 1984). The purified enzyme from three C_4 species was reported as a hexamer with 68 kDa subunits having a specific activity of 36–51 U (molecular activity *ca.* 14,700–20,800) at pH 7.6 and 25°C, an absolute requirement for Mn^{2+}; and K_m values for oxaloacetate, ATP, and CO_2 of 12–25 μM, 16–25 μM, 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.



(Reaction VI)

NAD-malic enzyme located in the BSC mitochondria is the C_4 acid decarboxylase functioning in C_4 photosynthesis in NAD-ME type species; it also has a role in PEP-CK type C_4 plants (see Figs. 2 and 3). Different isoforms are widely reported in various tissues of plant species, even among the C_4 species (Hatch *et al.*, 1974; Artus and Edwards, 1985). The purified enzyme from *Eleusine coracana* is a homooctamer, having 63 kDa subunits with a molecular activity of 60,500 at pH 7.2 and 31°C (Murata *et al.*, 1989). Mn^{2+} is absolutely required for activity and acetyl CoA, CoA, and fructose 1,6-bisphosphate (FuBP) are potent activators. K_m values for malate and NAD^+ in the presence of FuBP are 2.2 and 0.63 m*M*, respectively. Antiserum raised against the enzyme from *E. coracana*, a monocot, does not inhibit the enzyme activity of a C_4 dicot, *Amaranthus edulis*. The purified enzyme from *Amaranthus hypocondriacus* 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).

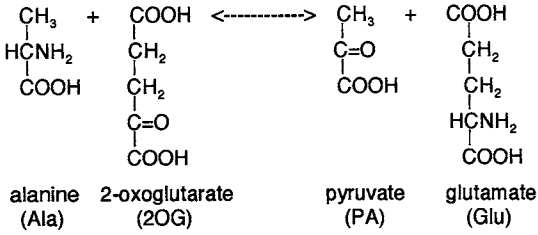
5. Aminotransferases By the work of Hatch and associates in the 1970s, aspartate aminotransferase (EC 2.6.1.1, Reaction VII) and alanine aminotransferase (EC 2.6.1.2, Reaction VIII) were proved to be essential in the C_4 pathway of NAD-ME and PEP-CK types (Hatch, 1987). These enzymes exist in both MC and BSC mainly as cytoplasmic and/or mitochondrial isoenzymes.



(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 C_4 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 C_3 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: K_m values for aspartate, 2-oxoglutarate, glutamate, and oxaloacetate are 1.3–3.0, 0.07–0.20, 8–32, 0.023–0.085 m*M*, respectively.

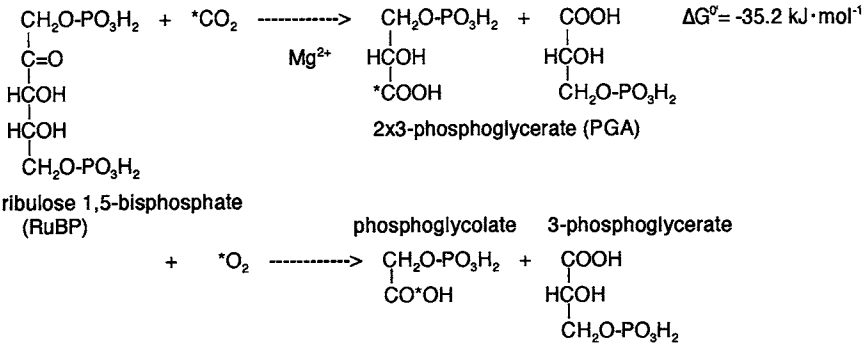
Three isoforms of alanine aminotransferase (AlaAT) exist in *P. miliaceum*



(Reaction VIII)

(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 C_4 photosynthesis, as the protein increases in parallel with other C_4 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 C_4 plants, like that of C_3 plants, consists



(Reaction IX)

of eight large subunits of 53.5 kDa and eight small subunits of 13 kDa (L_8S_8). Kinetic properties show some difference between C_3 and C_4 plants (Yeoh *et al.*, 1980; 1981). The range of $K_m(CO_2)$ values for the enzyme among C_3 species (12–25 μM) is lower than those in C_4 (28–34 μM), although variation of K_m(RuBP) values (15–82 μM in C_4) is not related to the photosynthetic pathway of higher plants. Among the C_4 grasses, the $K_m(CO_2)$ values of Rubisco in PEP-CK type (28–41, mean 35 μM) are significantly lower than those in NAD- and NADP-ME types (41–63, mean 53 μM). Molecular activities of some C_4 Rubiscos (2280–4020, mean of 3240 mol CO_2 mol enzyme^{-1} min^{-1}) are about twofold higher than those from C_3 higher plant species (Seemann *et al.*, 1984). These higher V_{max} values of Rubisco in C_4 plants accompanied by higher $K_m(CO_2)$ 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

$$CO_2 + H_2O \text{ <-----> } H_2CO_3 \text{ <-----> } H^+ + HCO_3^-$$

(Reaction X)

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 μM CO_2, were 28–89 μmol 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 C_4 photosynthesis, the level of CO_2 in BSC is influenced by a variety of factors, particularly (1) the intercellular CO_2 partial pressure, (2) the solubility of CO_2 as influenced by temperature, (3) the capacity for transport of C_4 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 CO_2 (and HCO_3^-) 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 CO_2 pool in BSC during C_4 photosynthesis (see He and Edwards, 1996; Chapter 6 by von Caemmerer and Furbank, this volume). Estimates indicate the C_4 cycle can raise the levels of CO_2 in the BSC to 30–75 μM (*ca.* 3 to 8 times higher than in C_3 plants), resulting in PCO cycle activity as low as 3% of net CO_2 uptake (Jenkins *et al.*, 1989; Dai *et al.*, 1993; He and Edwards, 1996).

The capacity of a C_4 plant to increase the supply of CO_2 to Rubisco is illustrated when comparing the response of photosynthesis in maize (C_4) to that of wheat (C_3) with varying intercellular levels of CO_2 (Fig. 4). The higher initial slope and lower CO_2 compensation point (intercept on the

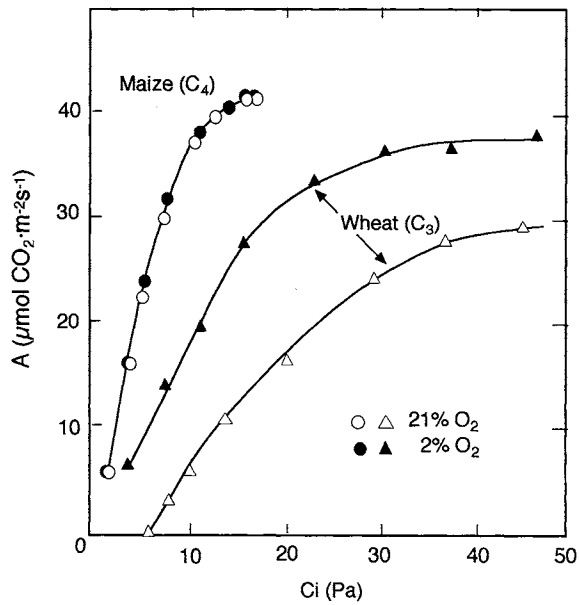


Figure 4 Response of the rate of CO_2-fixation in maize and wheat to varying intercellular levels of CO_2. At atmospheric level of CO_2 (33 Pa), the corresponding intercellular level of CO_2 (Ci) was approximately 16 Pa in maize and 21 Pa in wheat. (From Dai, Edwards, Ku, unpublished).

x axis) in the C_4 plant is a consequence of the CO_2 concentrating mechanism. In C_4 plants, the large increase in efficiency of Rubisco to function in CO_2 fixation through the C_4 system allows for a lower investment in Rubisco protein than in C_3 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 C_4 Photosynthesis

A. Minimum Energy Requirements in the Three C_4 Subgroups

The minimum energy requirements for CO_2 fixation to the level of triose phosphate in C_3 plants are 3 ATP and 2 NADPH per CO_2 in the absence of photorespiration. The same is true for the refixation of CO_2 generated by C_4 acid decarboxylation in BSC of C_4 plants by Rubisco and subsequent PCR cycle enzymes, because the oxygenase activity of Rubisco is negligible due to high CO_2 concentration in BSC in the light. In C_4 leaves, however, extra energy is required for regeneration of PEP, the acceptor for the initial CO_2 fixation, from pyruvate through pyruvate,P_i dikinase and adenylate kinase in mesophyll chloroplasts; it costs 2 ATP per CO_2 fixed by PEP carboxylase. Differentiation of the energy requirements among C_4 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 CO_2 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 CO_2 fixed) and 75% converted to aspartate. In this case, the ATP requirement to form PEP in mesophyll chloroplasts is only 0.5 ATP per CO_2

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 CO_2 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 C_4 Plants

For the estimation of energy requirements of C_4 photosynthesis *in vivo* it is necessary to evaluate several factors, such as the leakage of CO_2 from BSC, costs of CO_2 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 ^{14}C labeling (Hatch *et al.*, 1995), carbon isotope discrimination (Evans *et al.*,1986; Henderson *et al.*, 1992), O_2 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 C_4 cycle ranging from 8% to 50%. Using a leakage value of 20% as a reasonable estimate, for every 5 CO_2 delivered to the BSC by the C_4 cycle, 1 CO_2 would leak, resulting in a C_4 cycle expense of 2.5 ATP per net CO_2 fixed by PCR cycle. Besides the C_4 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 CO_2 fixed in mature leaves (Furbank *et al.*, 1990; Edwards and Baker, 1993).

Although photorespiration via the PCO cycle is strongly suppressed during C_4 photosynthesis, it is not completely eliminated. The extent of photorespiration may vary with leaf age, environmental conditions that affect intercellular CO_2 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 $^{18}O_2$ into glycolate under normal atmospheric levels of CO_2 and O_2 indicated that the velocity of oxygenase (v_o) was about 3% of the net rate of CO_2 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 C_3 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 CO_2 and O_2 is shown in Table II. This analysis shows a total of 5.7 ATP and 2 NADPH per CO_2 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 O_2 evolution (J_{O_2}) to net CO_2 uptake (A) in this case is 1.1. This value is remarkably consistent with the J_{O_2}/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 J_{O_2} and A in maize under varying temperature, light intensity, and CO_2 concentrations fits well the theoretical energy requirements for C_4 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 J_{O_2}/A ratio in C_4 plants compared to C_3 plants suggests that respiratory processes during C_4 photosynthesis are low (Badger 1985; Edwards and Baker, 1993). From measurements of J_{O_2} by O_2 isotope analysis on three monocot species of each C_4 subgroup at ambient external CO_2 levels, the average rate of O_2 uptake for NADP-ME was only 4% of J_{O_2}, for NAD-ME 9% of J_{O_2}, and for PEP-CK 22% of J_{O_2} (Furbank and Badger, 1982). Photorespiration and the associated O_2 consumption during C_4 photosynthesis could occur by PCO cycle activity, pseudocyclic electron flow (linear

Table II Estimate of Energy Required in NADP-malic Enzyme Type C_4 Species (e.g., Maize) per CO_2 Fixed Considering Bundle Sheath Leakage of CO_2, Carbohydrate Synthesis, a Low Level of PCO Cycle Activity, and Nitrate Assimilation

Function	ATP	NADPH
1. C_3 pathway per net CO_2 fixed	3	2
2. C_4 pathway, allowing for 25% overcycling	2.5	0
3. ATP per C in triose-P converted to carbohydrate (sucrose)	0.08	0
4. Rubisco oxygenase[a] where v_o is 3% of the net rate of CO_2 fixation (A)	0.14	0.09
5. Nitrate assimilation[b]	0.02	0.11
Total	5.7	2.2

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

[a] The activity of Rubisco oxygenase used is that from measurements on the rates of incorporation of $^{18}O_2$ into glycolate and CO_2 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 O_2 evolution with respect to Rubisco (J_{O_2}) = $v_c + v_o$, that the net rate of CO_2 fixation with respect to Rubisco (A) = $v_c - 0.5\ v_o$, and that for each O_2 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).

[b] 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_{O_2}) 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_{O_2}, which, along with RuBP oxygenase activity, may account for this subgroup having the highest rates of O_2 uptake (22% of J_{O_2}).

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 $H^+/\bar{e}$) 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 CO_2 fixed would require 8.4 quanta be used for whole chain transfer of 4.2 $\bar{e}$, 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

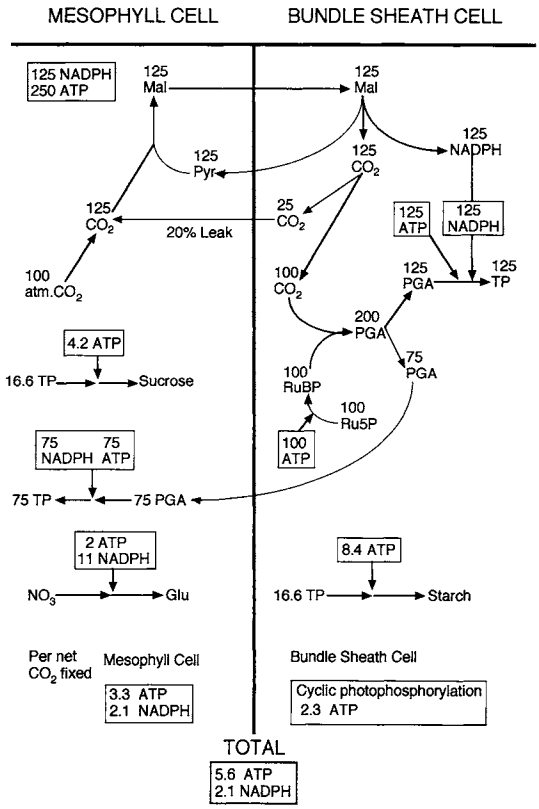


Figure 5 Illustration of energy requirements of mesophyll and bundle sheath cells of NADP-ME C_4 species. Note that 125 TP generated in BSC + 75 TP generated in MC equals a total of 200 TP; 166.6 TP are needed for regeneration of RuBP and the remainder used for synthesis of sucrose and starch. Abbreviations: 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 $J_{O_2(\text{Rubisco})} = v_c + v_o$, and $A = v_c - 0.5\ v_o$, the increase in quantum requirement (QR) due to RuBP oxygenase activity per net CO_2 fixed can be calculated.

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

$$= \frac{[v_c\ (8\ \text{quanta}/CO_2) + v_o\ (8\ \text{quanta}/O_2)]}{(v_c - 0.5\ v_o)} - 8$$

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 C_4 photosynthesis has the additional energy requirements for the C_4 cycle. However, under extremely high CO_2 levels (10%), the maximum quantum yields per O_2 evolved in the C_4 plants maize (NADP-ME) and *P. miliaceum* (NAD-ME) are as high as 0.105, which is the same as in C_3 plants under high CO_2 (Lal and Edwards, 1996). This suggests that in C_4 plants under very high CO_2 there is direct fixation of atmospheric CO_2 in the BSC through the PCR cycle, that the C_4 pathway is inhibited, and that all the assimilatory power is used to support reactions of the C_3 pathway as in C_3 plants.

IV. Coordination of the Two Cell Types in C_4 Photosynthesis

A. Separation of the Two Cell Types and Enzyme Distribution

Following the discovery of C_4 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 C_4 pathway as well as ^{14}C-intermediates of the pathway (Slack, 1969; Slack *et al.*, 1969). Using aqueous media, brief grindings repeated with C_4 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 C_4 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 C_4 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 C_4 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 C_4 pathway, PCR and PCO cycles, and other enzymes of primary carbon metabolism are listed in Table III, based on data from some representative C_4 species of the three C_4 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_i 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 C_3 plants where the PCR cycle is located. Nitrate reductase and nitrite reductase are localized in MC cytoplasm and chloroplasts, respectively, in most C_4 species (Rathnam and Edwards, 1976). Cytoplasmic and chloroplastic isoforms of glutamine synthetase are distributed evenly in the two cell types; but ferredoxin-dependent glutamate synthase (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 C_4 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 C_4 pathway of photosynthesis necessitates a rapid bidirectional movement of various metabolites between MC and BSC. The intercellular transport of metabolites includes C_3/C_4 acids, as illustrated in Figs. 1–3, C_3 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 C_4 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 III Intra- and Intercellular Localization of Enzymes in the Mesophyll Cell and Bundle Sheath Cell of C_4 Plants

	MC/BSC enzyme activity: μmol hour^{-1} (mg Chl)$^{-1}$		
	NADP-ME type *Zea mays*	PEP-CK type *Panicum maximum* (*Urochloa panicoides*)	NAD-ME type *Eleusine indica* (*Panicum miliaceum*)
C_4 pathway			
Carbonic anhydrase[a,b]	98/2	(98/2)[c]	(93/7)
PEP carboxylase[d,e]	864/14	2590/3	2400/10
Pyruvate,Pi dikinase[b]	188/15	(66/1)	82/1
Adenylate kinase[a,f]	56/10		
Pyrophosphatase[a,f]	49/16		
NADP-malate dehydrogenase[b,d]	805/<1	72/25	175/51
NADP-malic enzyme[b,d]	<1/1690	<1/85	7/28
PEP carboxykinase[d]	<1/<1	<1/542	<1/1
NAD-malic enzyme[d]	<1/126	62/263	11/554
Aspartate aminotransferase[d]	324/70	(1150/753)	1400/205
Alanine aminotransferase[d]	33/53	(612/335)	942/579
PCR cycle			
RuBP carboxylase[b,d]	<1/389	4/249	1/395
Phosphoribulokinase[b,d]	<1/2940	(75/1310)	24/2450
Phosphoriboisomerase[d]	75/1500		
PGA kinase[b]	1290/2450	(2110/701)	2350/1090
NADP-triose-P dehydrogenase[b,d]	705/1400	(240/457)	250/477
Triose-P isomerase[b]	526/545	(277/481)	209/813

Photorespiratory glycolate pathway			
Glycolate oxidase[b]	1.2/10	(1/26)	1.2/22
Hydroxypyruvate reductase[b]	20/184	(15/330)	40/526
Catalase ($\times 10^{-3}$)[b]	9/60	(11/46)	11/60
Glycerate kinase[g]	7.5/<1		(22/2)
Others (sucrose synthesis, glycolysis, oxidative pentose-P pathway, respiration)			
UDPG pyrophosphorylase[g]	797/285		(392/443)
Sucrose-P synthase[g]	56/3.2		
Phosphoglyceromutase[b]	160/23	95/35	150/115
Enolase[b]	203/18	181/100	174/95
Glucose-6-P dehydrogenase[b]	20/9	(37/18)	(24/34)
6-Phosphogluconate dehydrogenase[b]	10/8	(28/18)	11/38
Cytochrome oxidase[b]	33/146	(78/149)	46/117

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

[a] Relative value.

[b] Ku and Edwards, 1975.

[c] R. Kanai, unpublished results.

[d] Kanai and Edwards, 1973a.

[e] Gutierrez *et al.*, 1974a.

[f] Slack *et al.*, 1969.

[g] Usuda and Edwards, 1980a.

feeding of $^{14}CO_2$) and pulse-chase data ($^{14}CO_2$ pulse and $^{12}CO_2$ 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 $^{14}CO_2$-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 $^{14}CO_2$-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) $^{14}CO_2$-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 $^{14}CO_2$-fixation, and then reillumination in CO_2-free air. Preillumination of leaves significantly enhanced the subsequent dark $^{14}CO_2$-fixation into malate and aspartate, but no label was transferred to 3-phosphoglycerate and sugar phosphates. The transfer of ^{14}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

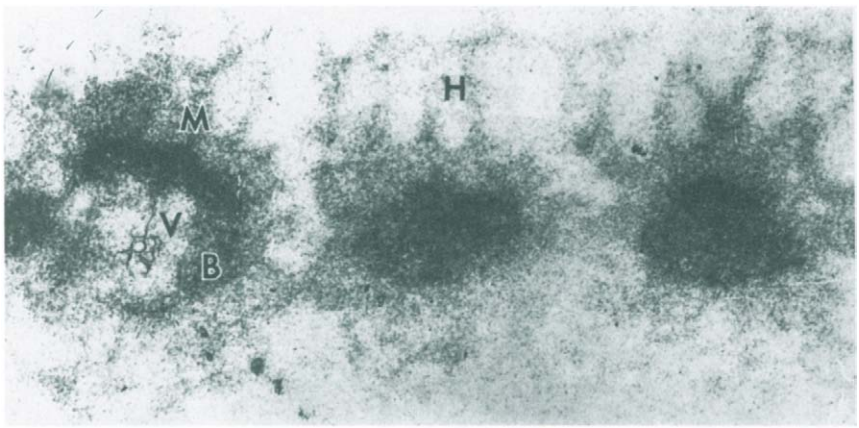


Figure 6 Microautoradiograph of a transverse section of *Atriplex spongiosa* leaf tissue after 2 second $^{14}CO_2$-photosynthesis. M, mesophyll cells; B, bundle sheath cells; V, vascular bundle; H, hypodermis. [Reprinted with permission from C. B. Osmond (1971). Metabolite transport in C_4 photosynthesis. *Aust. J. Biol. Sci.* **24,** 159–163, with permission.]

for use of the C_4 acids as donors of CO_2 to the PCR cycle in BSC. Interestingly, when a ^{14}C-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 CO_2 directly supplied to BSC by the vascular tissue is fixed by the PCR cycle, whereas atmospheric CO_2 is fixed into C_4 acids by the C_4 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 C_4 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 m*M* 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 C_4 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 m*M*, respectively, whereas that of 3-phosphoglycerate from BSC to MC was 9 m*M* (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 C_4 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 transport 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 differential centrifugation in sucrose density gradients, chloroplasts, mitochondria 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 quantities 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 μM) 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 m*M*, 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 chloroplast 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

(Huber and Edwards, 1977a,b). Although pyruvate uptake was mediated by a new translocator in the envelope of C_4 mesophyll chloroplasts, PEP was transported by a phosphate translocator having the extra capacity to carry PEP in addition to P_i, 3-phosphoglycerate and triose phosphate, which are tranported by the phosphate/triose–phosphate translocator in C_3 chloroplasts. In *P. miliaceum,* both MC and BSC chloroplasts are able to transport PEP (Ohnishi *et al.,* 1989). The P_i translocator of maize MC chloroplasts possesses a higher affinity for all transported substrates compared to that of C_3 chloroplasts: 50 times higher for PEP and 2-phosphoglycerate (compounds with the phosphate group at the C-2 position), and 6 times higher for P_i, 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 C_3 and C_4 species, those from *Flaveria trinervia* (C_4) and *F. pringlei* (C_3) 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 C_4 phosphate translocator (Fischer *et al.,* 1994).

3. Active Pyruvate Transport After the study of pyruvate tranport 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 m*M* 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, C_4 plants can be divided into two groups: a H^+ type and a Na^+ type. NADP-ME type C_4 species of Arundineleae and Andropogoneae in the Gramineae belong to the former group, whereas most of the other C_4 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 PEP^{3-} 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

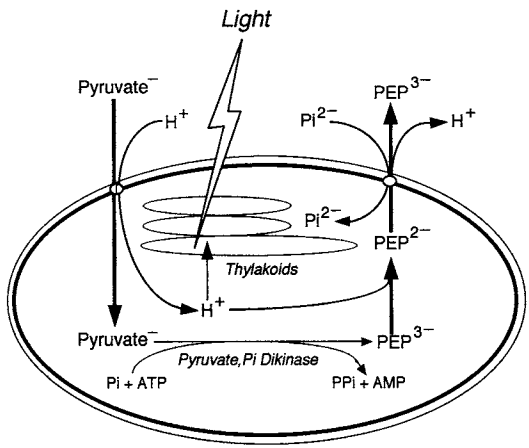


Figure 7 Scheme of H^+ mobilization accompanied with pyruvate import and phosphoenolpyruvate export in the light in mesophyll chloroplasts of C_4 species having H^+ type pyruvate transport. [Reprinted with permission from N. Aoki and R. Kanai (1995). The role of phosphoenolpyruvate in proton/pyruvate cotransport into mesophyll chloroplasts of maize. *Plant Cell Physiol.* **36,** 187–189, with permission.]

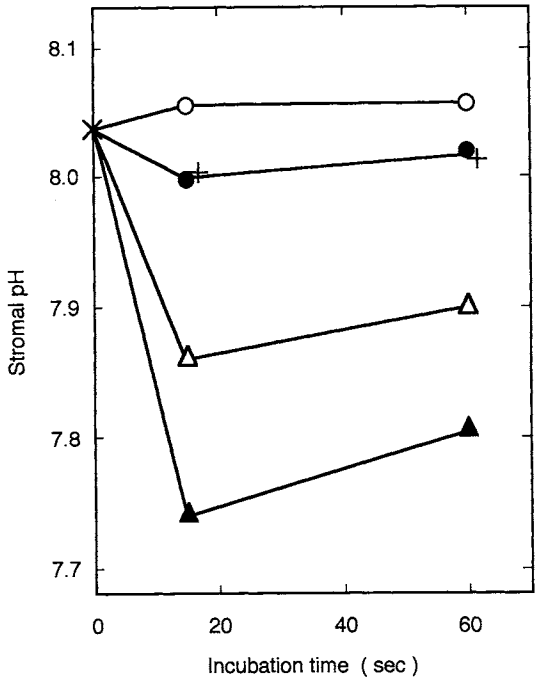


Figure 8 Changes in stromal pH of *Panicum miliaceum* mesophyll chloroplasts in the light by the addition of K- or Na-pyruvate. Intact chloroplasts isolated from mesophyll protoplasts of *P. miliaceum* were suspended in the medium of pH 7.8 and preincubated for 5 min in the light (500 μmol quanta m^{-2} s^{-1}). Stromal pH was calculated from the uptake of [^{14}C]-5,5-dimethyloxazolidine-2,4-dione by silicone-oil filtering centrifugation. Addition at 0 time: (+) water, (●) 5 m*M* Na-gluconate, (○) 5 m*M* K-gluconate, (Δ) 5 m*M* K-pyruvate, (▲) 5 m*M* 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 C_3 chloroplasts although the metabolic exchanges during C_4 photosynthesis are different due to the coordinated functions between MC and BSC chloroplasts. During CO_2 fixation by RuBP carboxylase, BSC chloroplasts export some of the product PGA in exchange for triose-P or P_i. 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 P_i, which is taken up by the BSC chloroplast. Whereas C_3 chloroplasts export glycolate in exchange for glycerate during photorespiration, in C_4 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-

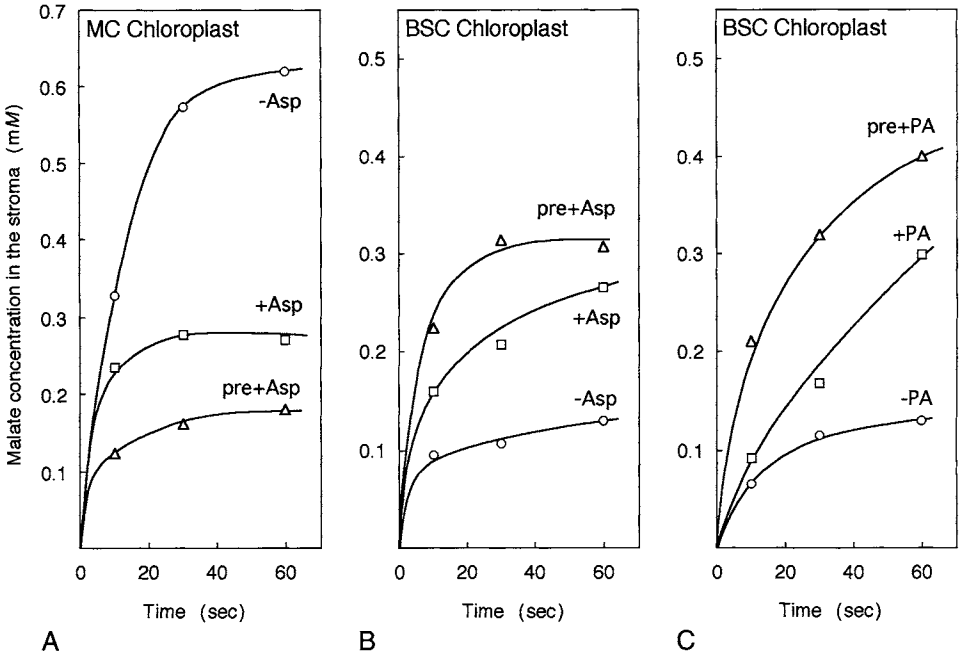


Figure 9 ^{14}C-Malate uptake by mesophyll and bundle sheath chloroplasts isolated from maize leaves: effects of aspartate and pyruvate. BSC chloroplasts from maize leaves were isolated according to Jensen and Boag (1985). Intactness of the BSC chloroplasts was more than 80%, whereas that of mesophyll 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 m*M*, 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 m*M* aspartate. The initial uptake rates (in mmol [mgChl]$^{-1}$ hour^{-1}) at 10 sec in mesophyll chloroplasts were 3.9, 2.8, and 1.5, respectively, whereas those in BSC chloroplasts were 1, 1.6, and 2.3, respectively. C: Effect of pyruvate on malate uptake without (-PA), with (+PA), and after preincubation with (pre+PA) 5 m*M* pyruvate; the initial uptake rates were 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 C_4 photosynthesis. Finally, the relevance of the variations on the biochemistry of C_4 photosynthesis and Kranz type leaf anatomy to survival and performance in different habitats remains to be elucidated.

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