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Raven, John A.; Beardall, John

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COMMENTARY

The ins and outs of CO₂

John A. Raven1,* and John Beardall2

1 Division of Plant Sciences, University of Dundee at the James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK†, and School of Plant Biology, University of Western Australia, M084, 35 Stirling Highway, Crawley, WA 6009, Australia

2 School of Biological Sciences, Monash University, VIC 3800, Australia

* To whom correspondence should be addressed. E-mail j.a.raven@dundee.ac.uk
† Permanent address of John Raven.

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Abstract

It is difficult to distinguish influx and efflux of inorganic C in photosynthesizing tissues; this article examines what is known and where there are gaps in knowledge. Irreversible decarboxylases produce CO₂, and CO₂ is the substrate/product of enzymes that act as carboxylases and decarboxylases. Some irreversible carboxylases use CO₂; others use HCO₃⁻. The relative role of permeation through the lipid bilayer versus movement through CO₂-selective membrane proteins in the downhill, non-energized, movement of CO₂ is not clear. Passive permeation explains most CO₂ entry, including terrestrial and aquatic organisms with C₃ physiology and biochemistry, terrestrial C₄ plants and all crassulacean acid metabolism (CAM) plants, as well as being part of some mechanisms of HCO₃⁻ use in CO₂ concentrating mechanism (CCM) function, although further work is needed to test the mechanism in some cases. However, there is some evidence of active CO₂ influx at the plasmalemma of algae. HCO₃⁻ active influx at the plasmalemma underlies all cyanobacterial and some algal CCMs. HCO₃⁻ can also enter some algal chloroplasts, probably as part of a CCM. The high intracellular CO₂ and HCO₃⁻ pools consequent upon CCMs result in leakage involving CO₂, and occasionally HCO₃⁻. Leakage from cyanobacterial and microalgal CCMs involves up to half, but sometimes more, of the gross inorganic C entering in the CCM; leakage from terrestrial C₄ plants is lower in most environments. Little is known of leakage from other organisms with CCMs, though given the leakage better-examined organisms, leakage occurs and increases the energetic cost of net carbon assimilation.

Key words: Aquaporins, bicarbonate, carbon concentrating mechanisms, C₄, carbon dioxide, crassulacean acid metabolism, leakage, lipid bilayer, permeability.

Introduction

The textbook equations for oxygenic photosynthesis and for dark respiration have CO₂ as, respectively, the inorganic C substrate and the inorganic C product. This is the case for the core autotrophic carboxylase of oxygenic photosynthetic organisms, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), with CO₂ as its inorganic carbon substrate, and for the decarboxylases of dark respiration with CO₂ as their inorganic C product (Raven, 1972a; Table 5.2 of Raven, 1984; Table 3 of Raven, 1997a). Add to this the Overton prediction over a century ago that CO₂ has a high permeability in lipid bilayers (see Endeward et al., 2014) and it appears at first sight that the textbook equations describe not just the inorganic
CO2 permeability of lipid bilayers and the role of CO2-conducting aquaporins and analogous protein pores

There is still significant uncertainty as to the mechanism of CO2 permeation of biological membranes (Boron et al., 2011; Itel et al., 2012; Endeward et al., 2014; Kai and Kaldendorf, 2014).
A particular problem is the role of proteinaceous CO₂ channels if the intrinsic CO₂ permeability of the lipid bilayer is very high, although there is evidence of increased photosynthesis and growth in terrestrial C₃ plants expressing CO₂-transporting aquaporins (Uehlein et al., 2003, 2008; Heckwolf et al., 2011) The most convincing evidence for the role of aquaporins in terrestrial C₃ plants comes from Hanba et al. (2004) and Tsuchihira et al. (2010). Table 1 shows the permeability coefficient for CO₂ of planar lipid bilayers of various compositions, and for the plasmalemma vesicles derived from high and low CO₂-grown Chlamydomonas reinhardtii. In all three cases attempts were made to eliminate the influence of diffusion boundary layers on each side of the membrane on the measured permeability.

**CO₂ entry in organisms lacking a biophysical CCM**

The classic example of these is the C₃ vascular land plants. It is now clear that CO₂ is the species of inorganic carbon entering the cells from the cell wall (Colman and Espie, 1985; Espie and Colman, 1986; Espie et al., 1986; Evans et al., 2009; Maberly, 2014). The assumption is that terrestrial C₄ and CAM vascular plants also rely on CO₂ entry from the cell wall to the cytosol where carbonic anhydrase equilibrates CO₂ with HCO₃⁻, the inorganic C substrate for PEPc (Colman and Espie, 1985; Nelson et al., 2005).

For C₃ plants the transport of CO₂ from the outside of the cell wall to Rubisco involves diffusion of CO₂ across the plasmalemma and across the outer and inner chloroplast membranes and, in the aqueous phase, through the cell wall, the cytosol and the stroma (Colman and Espie, 1985). It is implicitly assumed that there is no carbonic anhydrase in the cell wall (Raven and Glidewell, 1981; Colman and Espie, 1985) of C₃ plants, though there seems to be no experimental evidence demonstrating this. Carbonic anhydrases could equilibrate CO₂ and HCO₃⁻ in the cytosol and stroma and so enlist the predominant (at the pH of the cytosol and stroma) inorganic species, HCO₃⁻, in CO₂ transport across these aqueous phases, with the required H⁺ flux carried inwards by protonated buffers with a return flux outwards of the deprotonated buffers (Raven and Glidewell, 1981; Colman and Espie, 1985;)

### Table 1. Permeability coefficient for CO₂ in planar lipid bilayers and plasmalemma vesicles, corrected as far as possible for limitation by aqueous diffusion boundary layers

Also shown are the modelled ‘optimum’ or ‘maximum’ (for functioning in the CCM) CO₂ permeability of the wall of cyanobacterial carboxysomes and/or the estimated CO₂ permeability of the wall of cyanobacterial carboxysomes.

<table>
<thead>
<tr>
<th>Experimental system</th>
<th>CO₂ permeability m s⁻¹</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Planar lipid bilayer composed of 1:1 egg lecithin:cholesterol. 22–24 °C</td>
<td>3.5 ± 0.4.10⁻³ (standard error)</td>
<td>Gutknecht et al. (1977)</td>
</tr>
<tr>
<td>Plasmalemma vesicles of C. reinhardtii grown photolithotrophically in media with high low (350 μmol mol⁻¹ total gas) and high (50 mmol mol⁻¹ total gas) CO₂ for growth.? °C</td>
<td>0.76 ± 0.03–1.49 ± 0.2.10⁻⁵ (± standard error; low CO₂-grown cells) 1.21 ± 0.01–1.8 ± 1.7.10⁻⁵ (± standard error; high CO₂-grown cells)</td>
<td>Sültemeyer and Rinast (1996)</td>
</tr>
<tr>
<td>Planar lipid bilayer composed of (l) pure diphytanoyl-phosphatidyl choline (l) 3:2:1 cholesterol: diphytanoyl-phosphatidyl choline: egg sphingomyelin, and (l) mixture of lipids mimicking the red cell plasmalemma,.? °C</td>
<td>≥3.2 ± 1.6.10⁻⁵ (not clear what ± refers to; ≥3.2 refers to all three membrane compositions)</td>
<td>Missner et al. (2008)</td>
</tr>
<tr>
<td>Estimate of upper limit on CO₂ permeability of cyanobacterial carboxysome wall consistent with CCM function.</td>
<td>10⁻¹–2.5.10⁻⁶</td>
<td>Reinhold et al. (1987, 1991)</td>
</tr>
<tr>
<td>Estimate of CO₂ permeability of the carboxysome wall of Synechococcus assuming all of the limitation of CO₂ efflux from carboxysomes is in the carboxysome wall. 30 °C</td>
<td>2.2.10⁻⁷ (no estimates of error given)</td>
<td>Salon et al. (1996a,b), Salon and Carvin (1997)</td>
</tr>
<tr>
<td>Estimate of CO₂ permeability of the carboxysome wall in Anabaena variabilis assuming all of the limitation to CO₂ efflux from carboxysomes is in the carboxysome wall. 30 °C</td>
<td>2.8 ± 0.8.10⁻⁷ (standard error, n=9)</td>
<td>McGinn et al. (1997)</td>
</tr>
<tr>
<td>Estimate of ‘optimal’ CO₂ permeability of cyanobacterial carboxysome wall from CCM model.</td>
<td>10⁻⁶</td>
<td>Mangan and Brenner (2014)</td>
</tr>
<tr>
<td>CO₂ permeability of carboxysome wall in Prochlorococcus estimated from a model of CCM function.</td>
<td>10⁻⁷</td>
<td>Hopkinson et al. (2014)</td>
</tr>
<tr>
<td>Estimate of CO₂ permeability of the carboxysome wall in Prochlorococcus assuming all of the limitation to CO₂ efflux from carboxysomes is in the carboxysome wall.</td>
<td>10⁻⁶</td>
<td>Hopkinson et al. (2014)</td>
</tr>
</tbody>
</table>

Method for all three data sets involves measurement of inorganic carbon fluxes, expressed as CO₂, under a known CO₂ concentration difference across the membrane across planar membrane bilayers (Gutknecht et al., 1997; Missner et al., 2008) or plasmalemma vesicles of Chlamydomonas (Sültemeyer and Rinast, 1996). Carbonic anhydrase was added to both sides of the membrane to minimize the gradient of CO₂ across the aqueous diffusion boundary layers on each side of the membrane.
Evans et al., 2009; Niinemets et al., 2009; Tazoe et al., 2009, 2011). There is very significant interspecific variation in the magnitude of the mesophyll conductance (= permeability) of C3 seed plants (Evans et al., 2009; Niinemets et al., 2009; Tazoe et al., 2009; see Table 2).

Turning to submerged aquatic organisms, a number have CO2 entry followed by diffusive flux to Rubisco, resembling C3 land plants, although aquatic vascular plants lack stomata. These organisms include a number of freshwater and marine algae, aquatic bryophytes, and freshwater vascular plants (Raven, 1970; MacFarlane and Raven, 1985, 1989, 1990; Kübler et al., 1999; Sherlock and Raven, 2001; Maberly and Madsen, 2002; Raven et al., 2005; Maberly et al., 2009, Maberly, 2014). While these organisms share some of the physiological characteristics found in organisms with CCMs, e.g. the absence of a competitive interaction between CO2 and O2 in photosynthetic gas exchange (Kübler et al., 1999; Sherlock and Raven, 2001; Maberly et al., 2009), the overall influence of environmental factors points to diffusive CO2 entry.

**CO2 entry in organisms expressing a biophysical CCM**

A biophysical CCM that involves diffusive CO2 entry was first proposed by Walker et al. (1980; see Briggs, 1959) for eckolicate giant internodal cells of freshwater green algal macrophytes of the Characeae growing in relatively alkaline waters. The localized active efflux of H+ across the plasmalemma causes a localized decrease in pH in the cell wall and diffusion boundary layer, to approximately 2 pH units below that in the medium. As HCO3- diffuses into the acid zone, the equilibrium CO2:HCO3- increases 100-fold, as does the rate of HCO3- conversion to CO2 in the absence of carbonic anhydrase (Walker et al., 1980). Subsequently, expression of carbonic anhydrase in the acid zones was demonstrated, further increasing the rate of HCO3- to CO2 conversion (Price et al., 1985; Price and Badger, 1985). Intracellular acid-base regulation requires alkaline zones between the acid zones. This mechanism also occurs in some freshwater flowering plants where the acid zone is on the abaxial leaf surface and the alkaline zone is on the adaxial leaf surface (Maberly and Madsen, 2002). The high CO2 concentration generated in the acid zones can, after crossing the plasmalemma by diffusion, give an internal CO2 concentration rather less than that in the acid zone but still sufficient to constitute a CCM with the CO2 concentration inside the cell higher than that in the bulk medium (Walker et al., 1980; Price et al., 1985, Price and Badger, 1985). As well as CO2 leakage from the acid zones to the bulk medium, CO2 could also leak from the cytosol back to the bulk medium through the alkaline zones.

A similar mechanism is thought to occur in many marine macrophytes as a mechanism of using external HCO3- (Raven and Hurd, 2012). However, the evidence for this is (a) inhibition of external HCO3- use by pH buffers that, ex hypothesis, eliminate the acid zones, (b) inhibition of external carbonic anhydrase using a membrane-impermeant inhibitor as well as, in some cases, (c) showing that photosynthesis is not decreased by inhibitors of one group of plasmalemma HCO3- transporters (Raven and Hurd, 2012). There have been no direct demonstrations of the acid zones in macroalge because, although they can be visualized when they occur in the freshwater Characeae and vascular macrophytes, they must (if they exist!) occupy smaller areas in marine macroalgae and in seagrasses (Raven and Hurd, 2012).

An analogous mechanism involves external HCO3- entry at the plasmalemma and across the chloroplast envelope membranes, not necessarily giving higher internal than external concentration, with HCO3- entry to the thylakoid lumen via (ex hypothesis) HCO3- transporting channels (Raven, 1997b; Jungnick et al., 2014; Raven et al., 2014). The low pH of the thylakoid lumen, with the presence (at least in

<table>
<thead>
<tr>
<th>Category of plant: flowering plant, hornwort, or liverwort</th>
<th>Mesophyll permeability m s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Herbaceous dicotyledonous flowering plant</td>
<td>2.16 ± 0.32.10⁻⁴ (standard error, n not clear)</td>
</tr>
<tr>
<td>Herbaceous monocotyledonous flowering plant</td>
<td>2.24 ± 0.29.10⁻⁴ (standard error, n not clear)</td>
</tr>
<tr>
<td>Woody deciduous dicotyledonous flowering plant</td>
<td>1.05 ± 0.12.10⁻⁴ (standard error, n not clear)</td>
</tr>
<tr>
<td>Woody evergreen dicotyledonous flowering plant</td>
<td>0.85 ± 0.08.10⁻⁴ (standard error, n not clear)</td>
</tr>
<tr>
<td>Hornwort</td>
<td>1.75.10⁻⁴ (no statistics provided by Meyer et al., 2008)</td>
</tr>
<tr>
<td>Unventilated liverwort</td>
<td>1.90 ± 0.15.10⁻⁴ (standard deviation, n=3)</td>
</tr>
<tr>
<td>Ventilated liverwort</td>
<td>0.80 ± 0.04.10⁻⁴ (standard deviation, n=3)</td>
</tr>
</tbody>
</table>

Conversion of photosynthetic rates for the plants on a projected leaf area basis (from Table 1 of Warren, 2008) to the area of mesophyll cells exposed to the intercellular gas space uses a ratio of 25 m² m⁻² mesophyll cells exposed to the intercellular gas space projected leaf area (from pp. 380-381 of Nobel, 2005). Conversion of the difference in CO2 concentration between the outside of the cell wall to the chloroplast stroma expressed in terms of atmospheric mol fraction (μmol CO2 mol⁻¹ total atmospheric gas) from Table 1 of Warren (2008) to mmol CO2 dissolved in each m² of leaf water uses a conversion factor of 1 mmol CO2 m⁻³ dissolved in leaf water for each 20.4 μmol CO2 mol⁻¹ total atmospheric gas (from pp. 377 and 384 of Nobel, 2005). For a ventilated thalloid liverwort the ratio of 9 m² mesophyll cells exposed to the intercellular gas space per m² projected thallus area (Green and Snelgar, 1982), and for a hornwort or and unventilated liverwort thallus the ratio is 1 (Green and Snelgar, 1982), with other data from Meyer et al. (2008).
Chlamydomonas) of a carbonic anhydrase, gives a rate of CO₂ production, and an equilibrium CO₂ concentration, similar to that in the extracellular acid zones of some freshwater macrophytes (Raven, 1997b; Moroney and Ynalvez, 2007; Jungnick et al., 2014; Raven et al., 2014). The final step in the CCM is diffusion of the CO₂ from the lumen to the stroma, and especially to the pyrenoid where most of the Rubisco occurs in Chlamydomonas (Raven, 1997b; Raven et al., 2014). CO₂ leakage could occur from the pyrenoid back to the bulk medium.

Energetically downhill entry of CO₂ as part of a CCM occurs in cyanobacteria, although without localized surface acidification (see data of Maeda et al., 2002, and models of Mangan and Brenner, 2014; Eichner et al., 2015) (Fig. 1). Three further essential components are, first, active HCO₃⁻ influx at the plasmalemma, and the unidirectional conversion of CO₂ to HCO₃⁻ energized by the NDH₁ component of cyclic electron flow round photosystem I at the outer surface of the thylakoid membrane. Second, the carboxysomes, containing Rubisco and carbonic anhydrase, whose protein subunit walls probably have a limited permeability to CO₂ (see estimates in Table 1). The cytosolic HCO₃⁻ from these two sources enters carboxysomes through pores also allowing permeation of anions (Raven, 2006) and H⁺ (Menon et al., 2010) or, perhaps, OH⁻. Finally, HCO₃⁻ in the carboxysome lumen is acted on by carbonic anhydrase, producing CO₂ that is (mainly) consumed by Rubisco in the carboxysome, though some CO₂ could leak to the cytosol. The extent of CO₂ leakage through the carboxysomal wall is likely to be significant, even with a low CO₂ permeability coefficient across the carboxysomal wall with its positively charged pores, because of the large CO₂ accumulation factor (two to three orders of magnitude) in the carboxysome lumen relative to the cytosol during photosynthesis (Eichner et al., 2015). Modelling by Mangan and Brenner (2014) finds that the optimal carboxysome wall permeability coefficient for CO₂ for maximal CO₂ accumulation in the carboxysome lumen is around 10⁻⁵ m s⁻¹.

Hopkinson et al. (2011) and Hopkinson (2014) propose a general similar mechanism for diatoms, with active HCO₃⁻ influx at the plasmalemma (Nakajima et al., 2013) and parallel non-energized CO₂ influx (Fig. 2). These models involve cytosolic carbonic anhydrase to convert the CO₂ to the equilibrium concentration of HCO₃⁻, with active HCO₃⁻ uptake by chloroplasts. This latter step has not yet been identified in diatoms.

Is there a role for active transport of CO₂? The occurrence of a CO₂-stimulated ATPase from the ‘microsomal’ fraction (= plasmalemma?) of the freshwater green (chlorophycean) alga Eremosphaera viridis, the predominance of CO₂ uptake in photosynthesis in this alga, and the electroneutrality of CO₂ uptake (ruling out cation symport), is consistent with CO₂ uptake by primary active transport (Rotatore et al., 1992; Deveau et al., 1998; Huertas et al., 2000a; Deveau et al., 2001). No other CO₂ transporters that could reasonably function in active CO₂ transport are known. Accordingly, the possibility that other eukaryotes depend on a mechanism of the kind suggested by Beardall (1981) and Hopkinson et al. (2011), involving passive CO₂ entry at the plasmalemma and active HCO₃⁻ transport into the chloroplasts, cannot be ruled out for algae with a CCM and dominant CO₂ uptake, e.g. acidophilic eukaryotic algae, unless it has been shown that there is no HCO₃⁻ transporter at the chloroplast envelope. Rotatore and Colman (1990) showed that isolated chloroplasts of Chlorella ellipsoidea could take up HCO₃⁻ by active transport systems are shown in grey and high affinity systems are shown in black, and are found at the plasmalemma and/or thylakoid membrane. Transporters whose characteristics are unknown are shown in white. Redrawn after Fig. 1 of Price et al. (2002), Badger and Price (2003), and Giordano et al. (2005), (Price et al. 2002. Modes of active inorganic carbon uptake in the cyanobacterium Synechocystis sp. PCC7942. Functional Plant Biology 29, 131–149. CSIRO PUBLISHING (http://www.publish.csiro.au/nid/102/paper/PP01229.htm). (Badger and Price 2003. CO₂ concentrating mechanism in cyanobacteria: molecular components, their diversity and evolution. Journal of Experimental Botany 54, 609–622). (Giordano et al. 2005. CO₂ concentrating mechanisms in algae: mechanisms, environmental modulation, and evolution. Annual Review of Plant Biology 6, 99–131).
transport, but had no active CO₂ uptake; however, the HCO₃⁻ influx uptake by isolated chloroplasts is less than that at the plasmalemma on a per cell basis (Rotatore and Colman, 1991a,b). Rotatore and Colman (1991c) suggest that there is active uptake of CO₂ at the plasmalemma of *Chlorella saccharophila* and *C. ellipsoidea*, although the possibility suggested by Beardall (1981) and Hopkinson *et al.* (2011) of passive CO₂ followed by active entry of inorganic C into chloroplasts cannot be ruled out.

**HCO₃⁻ entry in organisms expressing a biophysical CCM**

Use of HCO₃⁻ is indicated by more rapid photosynthesis than can be accounted for by the uncatalysed rate of HCO₃⁻ to CO₂ conversion (Briggs, 1959). The ‘direct’ use of HCO₃⁻ involves influx of the anion at the plasmalemma, as compared with the ‘indirect’ use by external conversion to CO₂ as described in the previous section (see Briggs, 1959). The physiological methods of demonstrating direct use of HCO₃⁻ involve the known absence, or inhibition, of external carbonic anhydrase(s). In cyanobacteria (Eichner *et al.*, 2015; Hopkinson *et al.*, 2014) and eukaryotic algae such as diatoms (Nakajima *et al.*, 2013), HCO₃⁻ entry has been shown by physiological methods, and also by molecular genetic techniques, including ectopic expression and tests of functionality of the HCO₃⁻ transporter gene. The processes in cyanobacteria (Mangan and Brenner, 2014; Eichner *et al.*, 2015) (Fig. 1) and diatoms (Hopkinson *et al.*, 2011; Hopkinson, 2014) (see Fig. 2) have been recently modelled.

In other algae HCO₃⁻ entry has been shown by physiological methods, including the absence of inhibition of photosynthesis by pH buffers or by inhibition of external carbonic anhydrase, and inhibition by inhibitors of anion exchange proteins (Raven and Hurd, 2012). In some cases, e.g. the eustigmatophycean marine microalga *Nannochloropsis gaditana*, all of the inorganic carbon entering in the CCM involves direct entry of HCO₃⁻ (e.g. Munoz and Merrett, 1989; Huertas and Lubián, 1997; Huertas *et al.*, 2000b, 2002).

In most cases, there is entry of both CO₂ and HCO₃⁻ in CCMs (Korb *et al.*, 1997; Tortell *et al.*, 1997; Burkhardt *et al.*, 2001; Giordano *et al.*, 2005; Rost *et al.*, 2006a,b, 2007; Tortell *et al.*, 2008) and, in a few cases (see above) only CO₂ enters in algae with CCMs.

Isolated, metabolically active chloroplasts of some green algae with CCMs show CO₂ and HCO₃⁻ uptake into the chloroplasts as well as into whole cells (Amoroso *et al.*, 1998, van Hunnik *et al.*, 2002, Giordano *et al.*, 2005, and references therein). Yamano *et al.* (2015) show cooperative expression of the plasmalemma HCO₃⁻ HLA3 ABC transporter and the chloroplast envelope LCIA formate/nitrite transporter homologue (Wang and Spalding, 2014) in *C. reinhardtii*. LCIA is probably a HCO₃⁻ channel (Wang and Spalding, 2014; Yamano *et al.*, 2015); Wang and Spalding (2014) point out that such a channel could not act to accumulate HCO₃⁻ in the stroma relative to the cytosol since the electrical potential difference across the chloroplast envelope is stroma negative relative to the cytosol.

**Leakage from the intracellular inorganic C pool of CCMs**

Significant attention has been paid to leakage of CO₂ from terrestrial C₄ plants; this has been thoroughly reviewed by Kromdijk *et al.* 2014 (Table 3 and Supplementary Table S1, available at *JXB* online). For typical C₄ anatomy with mesophyll cells with intercellular gas spaces and a single bundle sheath layer with limited exposure to intercellular gas spaces...
and, in some cases, a suberin (mestome) sheath that could further limit CO₂ leakage. Kromdijk et al. (2014) give an excellent critique of the methods used to determine the leakiness to CO₂ (CO₂ efflux as a fraction of gross CO₂ influx) and list their outcomes. These are ¹⁴CO₂ labelling to determine the size of the bundle sheath inorganic carbon pool (and hence CO₂ efflux) or to directly estimate CO₂ efflux, the deviation size of the quantum yield of CO₂ assimilation from the value predicted from biochemistry assuming no CO₂ leak, and the natural abundance of stable carbon isotopes in the organic matter of the plant (determined by destructive sampling, or from online measurements of CO₂ before and after gas flow over photosynthesizing plants) relative to that of source CO₂.

All of these methods have problems (Kromdijk et al., 2014; von Caemmerer et al., 2014). The values for leakiness vary between −0.03−0.70. For the much less common case of terrestrial single-cell C₄ photosynthesis, the leakiness is similar to that for typical C₄ anatomy determined by similar methods (King et al., 2012). The permeability of the bundle sheath cells for CO₂ (1.6−4.5.10⁻⁶ m s⁻¹; Table 4), derived from the CO₂ efflux from the pool accumulated by the CCM and the driving force of the difference in CO₂ concentration between the CCM pool and the medium, is at least an order of magnitude higher than the permeabilities for cyanobacteria (Table 4). However, the bundle sheath permeability is two

**Table 3. Leakage of inorganic C from CCMs as a fraction of the inorganic C pumped into the intracellular pool for terrestrial C₄ flowering plants, hornworts, eukaryotic algae, and cyanobacteria**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Range of CO₂ leakage estimates as a fraction of gross CO₂ entry, from Supplementary Table S1</th>
<th>Mean leakage from estimates in Supplementary Table S1 or (C₄ terrestrial flowering plants) the more detailed data in Table 1 of Kromdijk et al. (2014)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₄ terrestrial flowering plants</td>
<td>–0.03−0.70</td>
<td>0.260±0.108 (standard deviation, n=20)</td>
</tr>
<tr>
<td>Hornworts with CCMs</td>
<td>0.170, 0.304, 0.31</td>
<td>0.263±0.066 (standard deviation, n=3)</td>
</tr>
<tr>
<td>Eukaryotic algae</td>
<td>0.01−0.80</td>
<td>0.36±0.16 (standard deviation, n=14); using results from MIMS only.</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.09−0.78</td>
<td>0.407±0.214 (standard deviation, n=5); using results from MIMS only.</td>
</tr>
</tbody>
</table>

¹Calculated from sum of means of ranges in Table 1 of Kromdijk et al. (2014), using data from all methods of estimation. Where values are given for more than one irradiance the value from the highest irradiance was used. The theoretically impossible value of −0.03 of leakage obtained by the quantum yield methods was retained rather than being rounded to zero; this made no difference to the outcome.

²Estimates from C isotope method, acknowledging that the pyrenoid-based CCM in hornworts may be subject to over-estimation as a result of internal recycling discussed for eukaryotic algae (see Wang and Spalding, 2014).

³Estimates from the C isotope method for leakage from a cyanobacterium in excess of 1.0 are theoretically impossible; these and other very high values obtained by this method for the cyanobacteria, are not given here. Possible reasons for these very high values are discussed by Eichner et al. (2015). For eukaryotic algae an analogous over-estimate of leakage using the C isotope method to that suggested for cyanobacteria could also occur, at least in Chlamydomonas (Wang and Spalding, 2014), but in the case of the eukaryotic algae none of the leakage estimates from using the C isotope method in Supplementary Table S1 are higher than the highest estimates from the MIMS method.

**Table 4. Permeability coefficients, on a cell surface area basis, for CO₂ and HCO₃⁻ determined for efflux of inorganic carbon from the intracellular pool accumulated by CCMs in cyanobacteria and for the bundle sheath of C₄ plants**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Inorganic carbon species</th>
<th>Permeability coefficient m s⁻¹</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synechococcus (Cyanobacterium)</td>
<td>CO₂</td>
<td>10⁻⁷ m s⁻¹ (no estimates of errors given)</td>
<td>Badger et al. (1985)</td>
</tr>
<tr>
<td>Synechococcus</td>
<td>CO₂</td>
<td>2.49±0.13.10⁻⁶ m s⁻¹ (standard error, n=4) −3.36±0.14.10⁻⁶ m s⁻¹ (standard error, n=18)</td>
<td>Salon et al. (1996a,b)</td>
</tr>
<tr>
<td>Synechococcus¹</td>
<td>HCO₃⁻</td>
<td>1.47±0.23.10⁻⁹ m s⁻¹ (standard error, n=7) −1.84±0.17.10⁻⁹ m s⁻¹ (standard error, n=7)</td>
<td>Salon et al. (1996a,b); Salon and Canvin (1997)</td>
</tr>
<tr>
<td>Anabaena variabilis (Cyanobacterium)</td>
<td>CO₂</td>
<td>9.8±1.5.10⁻⁶ m s⁻¹ (standard error, n=10)</td>
<td>McGinn et al. (1997)</td>
</tr>
<tr>
<td>Anabaena variabilis¹</td>
<td>HCO₃⁻</td>
<td>7.6±0.9.10⁻⁹ m s⁻¹ (standard error, n=7)</td>
<td>McGinn et al. (1997)</td>
</tr>
<tr>
<td>C₄ terrestrial flowering plants (5 species)</td>
<td>CO₂</td>
<td>1.6−4.5.10⁻⁶ m s⁻¹ (no estimates of errors given)</td>
<td>Furbank et al. (1999)</td>
</tr>
</tbody>
</table>

¹The quantification of the efflux of HCO₃⁻ is less direct than that of CO₂ efflux. As mentioned by Salon et al. (1996b), the permeability coefficient for HCO₃⁻ is a minimal value since the inside-negative electrical potential difference across the plasmalemma is not accounted for in the calculations.
orders of magnitude less than the mesophyll permeability in C3 plants (Table 2).

Leakage of an increased fraction of the CO2 released into the bundle sheath by the biochemical CO2 pump at low light is thought to be a reason for the rarity of shade-adapted C4 plants (see Bellasio and Griffiths, 2014). Bellasio and Griffiths (2014) point out that there is an ontogenetic shading of older leaves in high light-adapted C4 plants, and that up to 50% of C4 crop photosynthesis occurs in shaded leaves, and investigated CO2 leakage in shade-acclimated leaves of the sun-adapted Zea mays. They found that CO2 leakage as a fraction of PEPc activity (= biochemical CO2 pump) stayed constant with decreasing light, thus differing from expectation of a relative increase in leakage. The basis for this constancy is a decreased PEPc activity relative to that of Rubisco, and fixation of an increased fraction of the CO2 generated from respiration in bundle sheath cells.

Less attention has been paid to leakage of CO2 from terrestrial CAM plants (Cockburn et al., 1979; Winter and Smith, 1996; Nelson et al., 2005; Nelson and Sage, 2008; Winter et al., 2015).

Cockburn et al. (1985) examined the shootless orchid Chiloschista usneoides where CAM occurs (in the absence of other photosynthetic structures) in the astomatous velameniferous root. The absence of stomata means that the usual terrestrial CAM method of diurnal closure of stomata decreasing CO2 leakage during deacidification and CO2 refixation by Rubisco is unavailable. Cockburn et al. (1985) showed that the intercellular CO2 concentration during deacidification is not significantly different from that of the surrounding atmosphere, while the intercellular CO2 concentration during dark acidification is lower than that of the surrounding atmosphere. While lower intercellular CO2 concentration in the deacidification phase than is the case of stomata-bearing CAM structures decreases the leakage of CO2 from intercellular gas spaces in the stomata-less roots, it also means that carboxylase activity of Rubisco is likely to be substantially below saturation, and the Rubisco oxygenase activity is likely to be significant.

For aquatic vascular plants with CAM there is also no possibility of stomatal limitation of leakage of CO2 produced during deacidification in the light. For isoetids there is very little loss from the possible leakage of CO2 from the astomatous, cuticularized, photosynthetic part of the leaf, and even loss from any lower, less cuticularized, part of the leaf might be limited or abolished by the high CO2 concentration in the surrounding sediment that contains mineralizing particulate organic matter derived by sedimentation from the plankton. The non-isoetid submerged aquatic CAM flowering plant Crassula helmsii also lacks the leakage-limiting stomatal closure mechanism of terrestrial Crassula spp. C. helmsii can show net CAM fixation from external CO2 in the dark and also net photosynthetic C3 CO2 assimilation from external CO2 in parallel with refixation of internal CO2 generated in deacidification from malic acid, with, presumably, implications for CO2 leakage (Newman and Raven, 1995; Maberly and Madsen, 2002; Klavsen and Maberly, 2010).

Of course, the great majority of aquatic primary producers carrying out almost all of the aquatic primary productivity involving CCMs do not express CAM. Essentially all of the work on leakage of CO2 from intracellular pools of the CCM in aquatic organisms comes from cyanobacteria and eukaryotic microalgae; very little is known of leakage of CO2 from algal macrophytes or submerged aquatic vascular macrophytes that concentrate CO2 by C3 metabolism or a biophysical CCM. Perhaps the clearest example of CO2 leakage comes from the work of Tchernov et al. (1997, 1998, 2003) using membrane inlet mass spectrometry (MIMS). This method gives estimates of changes in CO2 and O2 in solution, with the difference between the two (if the photosynthetic quotient is assumed to be 1) representing the HCO3− flux, typically (see above) HCO3− influx. Tchernov et al. (1997, 1998, 2003) found an increase in external O2 and also CO2, with the computed HCO3− influx exceeding the organic carbon production rate computed from O2 production. Especially at high light, the HCO3− influx can significantly exceed the rate of photosynthesis, with the ‘excess’ inorganic carbon lost as CO2 in (for example) the cyanobacterium Synechococcus and the eustigmatophycean eukaryotic alga Nannochloropsis (Tchernov et al., 1997, 1998, 2003). There are also cases of CO2 influx exceeding the organic C production, implying net HCO3− efflux.

However, the general case with MIMS measurements is that of CO2 decrease, or at least no increase, in the light. Here the MIMS method can be used to estimate CO2 efflux in the light from the CO2 influx immediately after the cessation of illumination (Badger et al., 1994; Salon et al., 1996a,b; Eichner et al., 2015; Table S1 and Supplementary Table S1, available at JXB online). Badger et al. (1994) found a leakage of not more than 0.1 of net photosynthesis in low inorganic carbon-grown cells of Synechococcus, while for low CO2 grown Chlamydomonas the corresponding leakage is 0.5 at low inorganic C and 0.1 at high inorganic C. Again using Synechococcus, Salon et al. (1996a,b) and Salon and Canvin (1997) were able to distinguish CO2 efflux from HCO3− efflux immediately after darkening; the total inorganic C efflux in the presence of carbonic anhydrase was measured, as was the CO2 efflux under non-equilibrium conditions, and the difference is the HCO3− efflux. The CO2 efflux was only 0.08 of the maximum CO2 influx, while the HCO3− efflux was 0.45 of the maximum HCO3− influx. The CO2 permeability coefficient determined from the measurements and expressed in terms of the plasmamem area was 3.10−8 m s−1, while it was 1.6–2.5 m s−1 in terms of the carboxysome area (Tables 1, 14). The HCO3− permeability coefficient expressed in terms of the plasmamem area is at most 1.4–1.7 10−9 m s−1 (Table 4); the value is an upper limit because the inside-negative electrical potential component was not used in the calculation (Salon et al. 1996b; see also Ritchie et al. 1996).

In the case of Trichodesmium the leakage (CO2 efflux: gross inorganic carbon uptake) calculated using MIMS is 0.3–0.7 for two CO2 levels and with or without NO3− (Eichner et al.,
2015), as compared with values of 0.5–0.9 in previous work on this organism (see Kranz et al., 2009, 2010) (Table 3).

The other main method for estimating leakage of CO₂ from aquatic organisms expressing a CCM is from natural abundance ¹³C/¹²C of particulate organic matter gained by photo-

...ction, and that algae relying on diffusive CO₂ entry from the medium to Rubisco would be more common in low-irradiance habitats (review by Raven et al. 2000). The limited data available agree with these predictions (Raven et al., 2000, 2002; Burkhhardt et al., 2001; de Araujo et al., 2011; Cornwall et al., 2015; see Table 3). Turning to temperature, Raven and Beardall (2014) show that algal CCMs occur at lower temperatures than does terrestrial C₄ photosynthesis. Kranz et al. (2015) showed that the energy cost of algal CCMs decreased at low temperatures; it is not known if this is the case for terrestrial C₄ photosynthesis.

**Leakage from the photorespiratory carbon oxidation cycle(s)**

Tcherkez (2013) gives an excellent critique of the CO₂ fluxes associated with C₃ photosynthesis, photorespiration, and respiration. With a carboxylase:oxygenase ratio of Rubisco in vivo in a C₃ plant in the present atmosphere of 3:1, CO₂ production in the photorespiratory carbon oxidation cycle is 0.167 of gross CO₂ assimilation in photosynthesis (Raven, 1972a,b; Tcherkez, 2013). There is about 15% recycling of the photorespiratory CO₂ and ‘dark’ respiratory CO₂ production in photosynthesizing structures (Raven, 1972a,b; Tcherkez, 2013), so the CO₂ release into the environment as a fraction of gross photosynthesis is 0.167 × 0.85 or 0.14; it is likely that an upper limit is 0.20. This is at the low end of the range for leakage in C₄ plants and in algae CCMs (Table 3).

The various C₃–C₄ intermediate flowering plants have photosynthetic gas exchanges that show varying mixtures of C₃ and C₄ characteristics (Hylton et al., 1988; Rawsthorne et al., 1988a,b; von Caemmerer, 1989; Rawsthorne and Hylton, 1991; Morgan et al., 1993). This work shows the expression of most or all the glycine decarboxylase activity, and some of the Rubisco carboxylation–oxygenase, in bundle sheath cells. This location of the decarboxylase of the photorespiratory carbon oxidation cycle, with some Rubisco, in tightly packed bundle sheath cells increases recycling of CO₂ from glycine decarboxylase by the carboxylase activity of Rubisco relative to leakage of CO₂ back to the intercellular spaces.

CCMs increase the steady-state CO₂/O₂ ratios at the site of Rubisco activity; this decreases the ratio of Rubisco oxygenase activity to that of Rubisco carboxylase activity. The decreased rate of production of phosphoglycolate involves a decreased rate of the pathway(s) converting phosphoglycolate into phosphoglycerate and triose phosphate that can be used in core metabolism and/or complete oxidation to CO₂ (Eisenhut et al., 2008; Hagemann et al., 2010; Young et al., 2011; Raven et al., 2012). Even this low flux is essential, since deletion of all three of the pathways of phosphoglycolate metabolism (photorespiratory carbon oxidation cycle; tartronic semialdehyde pathway; complete oxidation via oxalate) is lethal (Eisenhut et al., 2008; Hagemann et al., 2010; Raven et al., 2012). Comparable work has not been yet been carried out in photosynthetic eukaryotes with CCMs where, at least in embryophytes, the pathway of phosphoglycolate metabolism is the photorespiratory carbon oxidation cycle. However, it is known that the C₄ and CAM CCMs decrease the rate of phosphoglycolate synthesis and flux through the photorespiratory carbon oxidation cycle relative to what occurs in otherwise comparable C₃ plants.

**Conclusions**

Quantifying the flux of CO₂ into and out of cells is difficult. All known irreversible decarboxylases produce CO₂; CO₂ is also the product/substrate of enzymes that can act as carboxylases and decarboxylases. Whether reversible or irreversible, decarboxylases produce CO₂, which can potentially leak out of cells. Some irreversible carboxylases also have CO₂ as their substrate; others use HCO₃⁻.

There is still controversy as to the relative role of permeation through the lipid bilayer and of movement through membrane proteins such as CO₂-selective aquaporins in the downhill, non-energized, movement of CO₂. Such movement is involved in CO₂ entry in terrestrial and aquatic organisms with C₃ physiology and biochemistry, as well as terrestrial C₄
plants and all CAM plants. Although there is also some evidence of active CO₂ transport at the plasmalemma of algae, downhill CO₂ transport is part of some mechanisms involved in the use of external HCO₃⁻ and CCM function. Further work is needed to test the validity of the mechanism based on localized surface acidification in marine macrophytes, and on HCO₃⁻ conversion to CO₂ in the thylakoid lumen.

HCO₃⁻ active influx at the plasmalemma underlies all cyanobacterial and some algal CCMs. HCO₃⁻ can also enter chloroplasts of some algae, possible as part of a CCM. Leakage from the intracellular CO₂ and HCO₃⁻ pool of CCMs sometimes occurs as HCO₃⁻, but typically occurs as CO₂. Leakage from cyanobacterial and microalgal CCMs, and terrestrial C₄ plants and hornworts with CCMs, usually involve half or less of the gross inorganic C entering in the CCM, but can be as high as 80%. CO₂ leakage to the environment from photospiration in C₃ plants is less than 20% of gross photosynthesis. Leakage from terrestrial CAM plants, algal macrophytes, and vascular aquatic macrophytes with CCMs (C₄, CAM, biophysical CCMs) has been less extensively examined. From what is known, CO₂ leakage can be appreciable in many photoautotrophs with CCMs and increases the energetic cost of net inorganic carbon fixation (see Raven et al., 2014).

Supplementary data

Supplementary data are available at JXB online.

Table S1. Leakage of inorganic C from CCMs as a fraction of the inorganic C pumped into the intracellular pool.

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