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Mechanism of C_4 Photosynthesis

A Model Describing the Inorganic Carbon Pool in Bundle Sheath Cells

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ABSTRACT

A theoretical model of the composition of the inorganic carbon pool generated in C_4 leaves during steady-state photosynthesis was derived. This model gives the concentrations of CO_2 and O_2 in the bundle sheath cells for any given net photosynthesis rate and inorganic carbon pool size. The model predicts a bundle sheath CO_2 concentration of 70 micromolar during steady state photosynthesis in a typical C_4 plant, and that about 13% of the inorganic carbon generated in bundle sheath cells would leak back to the mesophyll cells, predominantly as CO_2. Under these circumstances the flux of carbon through the C_4 acid cycle would have to exceed the net rate of CO_2 assimilation by 15.5%. With the calculated O_2 concentration of 0.44 millimolar, the potential photorespiratory CO_2 loss in bundle sheath cells would be about 3% of CO_2 assimilation. Among the factors having a critical influence on the above values are the permeability of bundle sheath chloroplasts to HCO_3^-, the activity of carbonic anhydrase within these chloroplasts, the assumed stromal volume, and the permeability coefficients for CO_2 and O_2 diffusion across the interface between bundle sheath and mesophyll cells. The model suggests that as the net photosynthesis rate changes in C_4 plants, the level and distribution of the components of the inorganic carbon pool change in such a way that C_4 acid overcycling is maintained in an approximately constant ratio with respect to the net photosynthesis rate.

The generally accepted function of the C_4 pathway is to concentrate CO_2 in the bundle sheath cells where Rubisco and the PCR cycle are specifically located (4, 13). During photosynthesis in C_4 leaves, a pool of inorganic carbon develops to a substantially higher concentration than would be expected from equilibration with CO_2 in air (8, 12). This pool is presumed to represent the inorganic carbon concentrated in bundle sheath cells, but its composition is uncertain. At physiological pH, the pool would exist largely as bicarbonate if thermodynamic equilibrium is reached. However, effective suppression of the RuBP oxygenase activity of Rubisco, and associated photorespiration, would require that the CO_2 to HCO_3^- ratio be above the equilibrium value (8). It has not proved possible to experimentally determine the level of CO_2, as such, in leaves. For this reason it has been necessary to resort to modeling in an attempt to predict the status of the inorganic carbon pool. Recently, a model was proposed, based on the observed net fixation rate and pool size, which indicated the likelihood of a large CO_2 component (8). However, in this simple model the concentrations of CO_2 and HCO_3^-, and the fluxes of carbon were determined empirically and it was not possible to take detailed account of the intracellular distribution of inorganic carbon. The size and composition of such inorganic carbon pools would be affected by the pH of the various subcompartments and their permeabilities to bicarbonate. Uncertainty about the level and location of carbonic anhydrase is another complicating factor (2).

To operate effectively, the bundle sheath cells must restrict the diffusion of inorganic carbon back to the mesophyll, while still allowing the influx of C_4 acids and efflux of photosynthetically produced O_2 (13). The extent to which inorganic carbon leaks back to the mesophyll will depend not only on the permeability of the interface between bundle sheath and mesophyll cells to CO_2 and HCO_3^-, but also on the cytosolic concentrations of these components in bundle sheath cells during photosynthesis. Similarly, the flux of O_2 will be determined by the concentration in bundle sheath cells and its permeability. The factors that affect these steady-state concentrations of CO_2, HCO_3^-, and O_2 in bundle sheath cells, and the associated leakage of inorganic carbon from these cells, are critical to our understanding of the C_4 mechanism of photosynthesis. In this paper we describe a model which defines the major components of the inorganic carbon pool in bundle sheath cells. With certain assumptions, a theoretical solution has been derived which allows calculation of the CO_2, HCO_3^-, and O_2 concentrations for any given net photosynthesis rate and total inorganic carbon pool. Rates of inorganic carbon leakage from the bundle sheath to the mesophyll cells, and the consequent C_4 acid overcycling may then be estimated.

THEORY

Determination of Bundle Sheath CO_2, O_2, and HCO_3^- Concentrations and Leak Rates

The scheme in Figure 1 shows the major inorganic carbon pools in bundle sheath cells and the likely interchange between these pools. The scheme is based on the following assumptions: (a) that CO_2, not HCO_3^-, is the initial product of the different C_4 acid decarboxylation enzymes in bundle sheath cells (17), (b) that following decarboxylation of C_4 acids (which occurs in the bundle sheath cytosol, chloroplasts or mitochondria, depending on the C_4 subgroup; see refs. 4 and 13) at a

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rate v_1, released CO_2 rapidly equilibrates throughout all cell compartments to a uniform concentration, (c) that in the acidic vacuole there is negligible HCO_3^- formed, but that in the other cell compartments, which are more alkaline, conversion of CO_2 to HCO_3^- may occur, (d) that mitochondria are likely to be impermeable to HCO_3^- (22) so that CO_2 and HCO_3^- will be at thermodynamic equilibrium, (e) that in the chloroplast and cytosol the steady-state HCO_3^- concentrations will be dependent on the respective rates of HCO_3^- formation and removal; HCO_3^- will not necessarily be in thermodynamic equilibrium with the CO_2, and (f) that removal of inorganic carbon from the bundle sheath pool is by net fixation via the PCR cycle (rate v_2) and by diffusive efflux (leakage) of CO_2 (v_3) and HCO_3^- (v_4) back to the mesophyll cells.

The rates of leakage of CO_2 and HCO_3^- are a function of the permeability coefficients and the concentration gradients between the bundle sheath and mesophyll cells:

$$v_3 = P_{CO_2}([CO_2]^{BS} - [CO_2]^{Meso})$$

$$v_4 = P_{HCO_3}([HCO_3^-]^{BSCyt} - [HCO_3^-]^{Meso})$$

where P_{CO_2}, P_{HCO_3} are the permeability coefficients for diffusion of CO_2 and HCO_3^-, respectively, across the interface between bundle sheath and mesophyll cells, and the concentrations of CO_2 and HCO_3^- in the bundle sheath cytosol and mesophyll cells are indicated by the superscripts. The permeability coefficient parameter was previously termed the 'diffusion constant' in earlier, related studies (13, 33). This coefficient has been defined by Nobel (23) and in the current context it reflects the diffusive properties of a particular cell surface, or an interface between cells, to various molecules and ions in terms of flux per unit concentration gradient for a specified amount of tissue (usually defined by Chl content in this paper).

Similarly, the HCO_3^- flux rate from bundle sheath chloroplasts to cytosol (v_9) is given by:

$$v_9 = P_{HCO_3}^{Chl}([HCO_3^-]^{BSChl} - [HCO_3^-]^{BSCyt})$$

where $P_{HCO_3}^{Chl}$ is the permeability coefficient for diffusion of HCO_3^- through the chloroplast membrane (usually assumed to be zero; see "Results and Discussion").

Assuming no carbonic anhydrase, the rates of CO_2 to HCO_3^- conversion in the bundle sheath cytosol (v_5) and chloroplast (v_7) are given by:

$$v_5 = K_c[CO_2]^{BS}$$

$$v_7 = K_c^{Chl}[CO_2]^{BS}$$

where K_c, K_c^{Chl} are constants comprising the nonenzymic rate constants (k_c, see below) at the pH of the bundle sheath cytosol and chloroplast, respectively, multiplied by the compartment volumes (to bring the rates to the correct units). Similarly, the HCO_3^- to CO_2 conversion rates are:

$$v_6 = K_b[HCO_3^-]^{BSCyt}$$

$$v_8 = K_b^{Chl}[HCO_3^-]^{BSChl}$$

where K_b, K_b^{Chl} are constants incorporating the nonenzymic rate constants (k_b, see below) at the appropriate pH values and the compartment volumes as above. These conversion rates may be increased over the nonenzymic rates by a specified factor to take into account various levels of carbonic anhydrase activity.

During steady-state photosynthesis,

$$v_9 = v_7 - v_8$$

which, substituting from above equations, can be written as

$$P_{HCO_3}^{Chl}([HCO_3^-]^{BSChl} - [HCO_3^-]^{BSCyt}) = K_c^{Chl}[CO_2]^{BS} - K_b^{Chl}[HCO_3^-]^{BSChl} \quad (1)$$

Also

$$v_4 = v_5 - v_6 + v_9$$

Therefore, from above, $v_4 = v_5 - v_6 + v_7 - v_8$ which, on substitution, becomes

$$P_{HCO_3}([HCO_3^-]^{BSCyt} - [HCO_3^-]^{Meso}) = K_c[CO_2]^{BS} - K_b[HCO_3^-]^{BSCyt} + K_c^{Chl}[CO_2]^{BS} - K_b^{Chl}[HCO_3^-]^{BSChl} \quad (2)$$

The total amount of inorganic carbon in bundle sheath cells (nmol[mg leaf Chl]$^{-1}$) is the sum of the amounts of CO_2 and HCO_3^- in all compartments:

$$T = CO_2^{BSCyt} + HCO_3^{-BSCyt} + CO_2^{BSChl} + HCO_3^{-BSChl} + CO_2^{BSmit} + HCO_3^{-BSmit} + CO_2^{BSvac} \quad (3)$$

where the pools of CO_2 and HCO_3^- in each compartment are specified by the superscripts, and T is the total inorganic carbon pool of the bundle sheath. Since the CO_2 concentration is taken to be the same throughout the cell, the amounts of CO_2 in the organelles relative to that in the cytosol is proportional to their volumes:

$$CO_2^{BSChl} = v_c/v_s\ CO_2^{BSCyt}$$

$$CO_2^{BSmit} = v_t/v_s\ CO_2^{BSCyt}$$

$$CO_2^{BSvac} = v_v/v_s\ CO_2^{BSCyt}$$

where v_s, v_c, v_t, v_v are the volumes (μL [mg leaf Chl]$^{-1}$) of the bundle sheath cytosol, chloroplast, mitochondria and vacuole, respectively. The amount of HCO_3^- in the mitochondria is a function of the amount of CO_2, and the equilibrium ratio $[HCO_3^-]$:$[CO_2]$, (r), at the mitochondrial pH:

$$HCO_3^{-BSmit} = r\ CO_2^{BSmit}$$

From above, $HCO_3^{-BSmit} = r\ v_t/v_s\ CO_2^{BScyt}$.

From Equation 3, and substituting:

$$T = (1 + v_c/v_s + v_t/v_s + r\ v_t/v_s + v_v/v_s)CO_2^{BScyt} + HCO_3^{-BScyt} + HCO_3^{-BSChl} \quad (4)$$

Taking into account the compartment volumes, Equations 1, 2, and 4 can be rearranged to give three simultaneous linear equations in the three unknowns CO_2^{BScyt}, HCO_3^{-BScyt}, and HCO_3^{-BSChl}. With given values for permeability coefficients, CO_2 to HCO_3^- interconversion rate constants, the total bundle sheath inorganic carbon pool, organelle volumes, and pH,

these equations may be solved by any convenient means (a matrix method is used here) to give values for $[CO_2]^{BSCyt}$, $[HCO_3^-]^{BSCyt}$, and $[HCO_3^-]^{BSChl}$. Knowing these concentrations, the rates of efflux of CO_2 (v_3) and HCO_3^- (v_4) from bundle sheath to mesophyll cells may then be calculated from above equations. The rate of C_4 acid decarboxylation (equivalent to the rate of the C_4 acid cycle) is then calculated from

$$v_1 = v_2 + v_3 + v_4$$

The overcycling of the C_4 pathway (C_4 acid overcycling), *i.e.* the difference between the rates of C_4 acid decarboxylation and net photosynthesis, is then calculated as a percentage of the net photosynthesis rate. This value is thus slightly larger than inorganic carbon 'leakage' which is usually given as a percentage of the C_4 acid decarboxylation rate (8).

Bundle sheath oxygen concentration, $[O_2]^{BS}$, is calculated from the equation:

$$v_{10} = P_{O_2}([O_2]^{BS} - [O_2]^{Meso})$$

where P_{O_2} is the permeability coefficient for O_2 diffusion across the bundle sheath-mesophyll cell interface (see below).

In practice, the above calculations were incorporated into a program and run on a microcomputer, which allows easy alteration of assumed parameters.

Values of Parameters used in the Model

Unless indicated otherwise, the following values were assumed in the application of the model: (a) total bundle sheath inorganic carbon pool is 55 nmol (mg leaf Chl)$^{-1}$, and the net photosynthesis rate is 6.4 μmol min^{-1} (mg Chl)$^{-1}$ (averages measured for six C_4 species, ref. 8); (b) C_4 leaves contain 2 mg Chl (g fresh weight)$^{-1}$ and 90% water, and the bundle sheath cell volume is 19% of total leaf volume (8); (c) as a percentage of bundle sheath cell volume the compartment volumes are vacuole, 51%; chloroplasts, 32%; cytosol, 15%; mitochondria, 2% (average values determined from electron micrographs of six C_4 species [14] and additional unpublished data kindly provided by S Craig); (d) the stroma occupies 55% of the chloroplast volume (the average we determined for five C_4 species by drawing random transects through electron micrographs of bundle sheath chloroplasts and measuring the overall lengths of each transect occupied by stroma or lamellae), (e) the pH of the mesophyll cell cytosol is 7.4, and for bundle sheath cell compartments, cytosol, 7.4 (20, 28), chloroplast stroma, 7.8 (16, 26, 35), mitochondria, 7.6 (22). When the pH of the bundle sheath cytosol was varied in the model (Fig. 5) the bundle sheath mitochondrial pH was also varied such that it remained greater by 0.2 pH units (22), and the chloroplast pH was varied such that it was 0.6 pH units greater at pH 7.0, but the same pH at a cytosolic pH of 8.5 (based on the observations of Heldt *et al.* [16] and Werdan *et al.* [35] for spinach chloroplasts).

The P_{CO_2} value used was taken as 15 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} (average of values determined by Furbank *et al.* [9] and Jenkins *et al.* [18]), and the P_{HCO_3} value 4.8 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} (based on the average permeability coefficient determined for low mol wt organic acids into isolated bundle sheath cells [33] adjusted for the relative diffusivities of C_4 acids and HCO_3^- in water [19, 32]). The P_{O_2} was derived from the P_{CO_2} according to the following relationship:

$$P_{O_2} = [0.567 \times D_{O_2}^{H_2O}/D_{CO_2}^{H_2O} + 0.433 \times D_{O_2}^{L}/D_{CO_2}^{L}] \times P_{CO_2}$$

where $D_{O_2}^{H_2O}/D_{CO_2}^{H_2O}$ is the ratio of diffusivities of O_2 and CO_2 in water (1.72, ref. 30), and $D_{O_2}^{L}/D_{CO_2}^{L}$ is the ratio of permeabilities of O_2 and CO_2 through a hydrophobic, polymeric medium. A value of 0.2 was used for the latter which is the average ratio of permeabilities of O_2 and CO_2 through a wide range of synthetic plastics (see ref. 29). This expression for P_{O_2} includes components describing the flux of O_2 via an aqueous (plasmodesmatal) pathway and via an apoplastic path limited by a lipid polymer barrier. In the latter case the major resistance is assumed to be the lipid polymer of the suberized lamellae which occurs in the wall separating the bundle sheath and mesophyll cells of many C_4 species (13). The ratio of CO_2 diffusion through these alternative pathways is taken as 0.567:0.433 based on a P_{CO_2} through plasmodesmata of 8.5 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} (calculated from the permeability coefficient for C_4 acids into bundle sheath cells [33] and taking into account the relative diffusion coefficients of CO_2 and C_4 acids in water) and a total P_{CO_2} of 15 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} (see above). This treatment gives a value for P_{O_2} which is $1.06 \times P_{CO_2}$. The concentration of CO_2 in the mesophyll is assumed to be 4 μM, based on an average intercellular CO_2 partial pressure of 140 μbar at 350 μbar ambient pCO_2 (36), and that of O_2 was 0.24 mM based on its solubility in water at 28°C in equilibrium with air.

The rate constants for nonenzymic interconversion of CO_2 and HCO_3^- at the assumed pH values of cytosol and chloroplast were derived from:

$$k_c = 6.22 \times 10^{-11}/[H^+] + 3.8 \times 10^{-2}$$

$$k_b = 2 \times 10^{-4} + 4.96 \times 10^4\ [H^+]$$

where k_c is the sum of the rate constants for conversion of CO_2 to HCO_3^- and k_b is the sum of the rate constants for the reverse reactions. These equations are similar to those used by Farquhar (6) but have been revised to incorporate rate constants derived for ionic strength 0.1 on the assumption that this better approximates the physiological situation (for reactions and rate constants see refs. 10, 24, 25, 31; the value for $k_{H_2CO_3}$ of 4.96×10^4 was derived from the k_{CO_2} value of 3.8×10^{-2} and the apparent first ionization constant for H_2CO_3 at 0.1 M NaCl given in ref. 11). When the rate constants were increased to account for carbonic anhydrase activity, the rates of hydration and dehydration of CO_2 were multiplied by the same factor. The ratio of HCO_3^- to CO_2 concentrations in the mitochondria, and the HCO_3^- concentration in the mesophyll, were determined at the appropriate pH from the Henderson-Hasselbalch equation using a pK value of 6.116 (11) assuming an ionic strength of 0.1.

Potential photorespiratory CO_2 production was assessed from the calculated ratio of the rates of carboxylation and oxygenation, which would be catalysed by Rubisco,[2] at the particular $[CO_2]/[O_2]$ ratio according to the following Equa-

[2] Abbreviations: Rubisco, ribulose 1,5-bisphosphate carboxylase/oxygenase; PCR, photosynthetic carbon reduction.

tion (1), and assuming that one molecule of CO_2 would be released in the bundle sheath cells for every two oxygenations:

$$v_c/v_o = S_{REL} \times [CO_2]/[O_2]$$

In this equation, v_c and v_o are the respective rates of carboxylation and oxygenation, and S_{REL} is the specificity constant for higher plant Rubisco (taken as 100, ref. 1). The calculation gives a value for the potential for 'photorespiratory' CO_2 loss (relative to gross CO_2 assimilation) which exists in bundle sheath cells. Actual photorespiratory CO_2 production for intact leaves may be lower if some CO_2 is refixed in the mesophyll cells.

RESULTS AND DISCUSSION

Assumptions and Predictions about the Inorganic Carbon Pools

The inorganic carbon flux rates and concentrations shown in Figure 1 are calculated on the basis of the parameters and assumptions outlined in 'Theory.' This scheme aims to describe the inorganic carbon status in a typical C_4 plant during steady-state photosynthesis. Although C_4 acid decarboxylation is shown as occurring in the cytosol (as would occur in a PCK-type species), since CO_2 is assumed to rapidly equilibrate between all cell compartments the decarboxylation process could be in the chloroplast (NADP-ME type) or in the mitochondria (NAD-ME type) without altering the values calculated. It is assumed, for convenience, that half of the total photosynthetically produced O_2 is generated in bundle sheath cells of NAD-ME-type or PCK-type species (13). For NADP-ME types, where O_2 evolution in bundle sheath cells is low or negligible (3), the bundle sheath cell O_2 concentration would presumably be lower, but inorganic carbon fluxes and concentrations would not be affected.

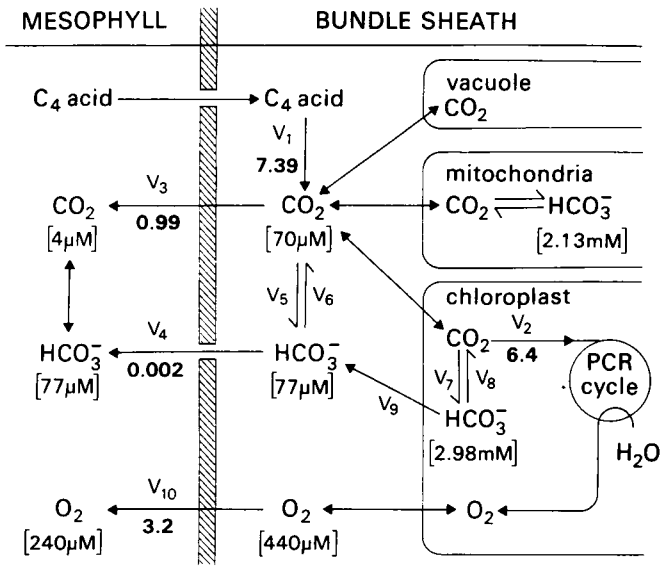

Figure 1. Schematic description of inorganic carbon fluxes and concentrations in C_4 leaves during steady-state photosynthesis. Double headed arrows indicate assumed equilibration of CO_2 and O_2 between compartments. The concentrations and flux rates were computed as described in "Theory" on the basis of assumed or derived values for the various parameters. The key values are: a net photosynthesis rate of 6.4 μmol min^{-1} (mg Chl)$^{-1}$, a total bundle sheath inorganic carbon pool of 55 nmol (mg Chl)$^{-1}$; and a bundle sheath cell volume 19% of the leaf volume. Intracellular compartment volumes (as percentage of cell volume) and pH values for these compartments were: cytosol, 15% (pH 7.4); chloroplasts, 17% (pH 7.8); mitochondria, 2% (pH 7.6); and vacuole, 51% (pH <5.5). The basis for using these values and other details are described in "Theory."

Using the same net photosynthesis rate and inorganic carbon pool size as assumed in an earlier study (8), the present model predicts a much lower bundle sheath cell CO_2 concentration (70 μM compared to 560 μM). This difference is mainly attributable to a large pool of HCO_3^- partitioned into the stroma of bundle sheath chloroplasts. Assuming no HCO_3^- flux through the limiting membranes of chloroplasts and mitochondria (see later discussion), the proportion of total bundle sheath cell inorganic carbon appearing as HCO_3^- in these organelles would be 81.8% and 6.6%, respectively, compared to 10.9% as CO_2 throughout the cell. The high level of HCO_3^- in chloroplasts and mitochondria means that both the cytosolic HCO_3^- concentration during steady-state photosynthesis, and the amount of carbon in this pool, are low (only 1.8% of the inorganic carbon pool).

Leakage of Inorganic Carbon and C_4 Acid Overcycling

The model developed in this study incorporates revised estimates for permeability coefficients for CO_2 (now directly measured, see refs. 9, 18) and HCO_3^- (see "Theory") diffusion from bundle sheath to mesophyll cells. These values are considerably greater than those assumed in previous calculations (8) so should give higher rates of diffusive efflux (leakage) of inorganic carbon from bundle sheath to mesophyll cells. However, in the current more elaborate model this trend is largely counteracted by the lower concentrations of CO_2 and HCO_3^- in the bundle sheath cytosol (discussed above). From Figure 1 it can be calculated that 13.4% of the inorganic carbon released from C_4 acid decarboxylation in bundle sheath cells will be lost to the mesophyll by leakage. Thus, despite the greater permeability coefficients, the leakage here remains similar to that estimated from the previous model (about 11% leakage; ref. 8). Theoretical estimations of inorganic carbon leak rates based on ^{13}C-isotope discrimination models gave values of about 20 to 40% (5, 6). In the present model, by far the largest gradient is for CO_2 and this is the predominant inorganic carbon component leaking back to the mesophyll cells (Fig. 1).

The sum of the leakage rates and net photosynthesis rate gives the steady-state rate of C_4 acid decarboxylation (and hence C_4 acid pathway cycling). With the values shown, this rate is 15.5% greater than the net photosynthesis rate (*i.e.* C_4 acid overcycling is 15.5%). The extent of overcycling will affect the quantum requirement for C_4 photosynthesis. The relationship between quantum requirements and various levels of overcycling are being considered currently, along with the possible involvement of a Q-cycle in C_4 photosynthesis, and will be published elsewhere.

$[CO_2]/[O_2]$ Ratio and Photorespiration

From consideration of the kinetics of Rubisco and the stoichiometry of photorespiration, a $[CO_2]/[O_2]$ ratio of about 0.15 or more would be necessary to get effective suppression of photorespiration in C_4 plants (photorespiratory CO_2 production 3.3% or less of CO_2 assimilation; see ref. 2). The set of values used for the scheme in Fig. 1 gave a $[CO_2]/[O_2]$ ratio of 0.159. By the same method as used earlier, this gives a low potential photorespiratory carbon loss in bundle sheath cells of 3.1% (Table I) as would be expected for C_4 plants (4, 7). Thus, despite a lower bundle sheath CO_2 concentration than was estimated earlier (8), the photorespiratory carbon loss corresponding to the set of assumptions used in Figure 1 remains within acceptable limits. The earlier model (8) predicted a CO_2 concentration in bundle sheath cells well above that required to effectively eliminate photorespiration. For C_4 species of the NADP-ME-type, the bundle sheath O_2 concentration would presumably be close to 0.24 mM (the O_2 concentration in solution in equilibrium with air) giving a $[CO_2]/[O_2]$ ratio of 0.292 and an even lower photorespiratory CO_2 loss of about 1.7% of assimilation.

Sensitivity of the Model to Varying Assumptions and Parameters

Table I records the effect of varying some of the assumptions and parameters used to derive the values shown in Figure 1 on the calculated CO_2 concentration, $[CO_2]/[O_2]$ ratio and consequent potential photorespiration, and C_4 acid overcycling. In each case, the only adjustment made to the calculation was of the single parameter shown. When the assumed net photosynthesis rate is reduced to half the original value, the CO_2 concentration remains the same but the O_2 concentration decreases, thus lowering the potential photorespiration. However, as a result of the lower photosynthesis rate, overcycling is increased to about double the original value. To avoid this result *in vivo*, it would appear likely that changes in net photosynthesis rates would probably be accompanied by proportional changes in the level and distribution of carbon in the bundle sheath cells. In fact, it has been shown that reductions in the rates of photosynthesis, induced by lowering either the light intensity or the ambient CO_2 concentration, are accompanied by lowering of the leaf inorganic carbon pool (8). Further consideration of these results is given below.

The model in Figure 1 was also sensitive to changes in total inorganic carbon pool size (Table I). When this parameter alone was varied, a linear relationship between pool size and C_4 acid overcycling was observed (not shown; only two points have been selected for inclusion in Table I). As expected, the CO_2 concentration and $[CO_2]/[O_2]$ ratio increased or decreased *pro rata* with changes in the total inorganic carbon pool size. Of course, if the pool size changes correspond *pro rata* with varying photosynthesis rate (see above), then there may be little or no effect on either photorespiratory CO_2 loss or C_4 acid overcycling. With regard to variation in inorganic carbon pool size between species, an earlier study showed differences in pool sizes of between 15 to 97 nmol (mg Chl)$^{-1}$ for a range of C_4 plants. These differences seemed to be correlated more with C_4 photosynthetic type than with photosynthetic rate (8). Thus, the range indicated in Table I represents a realistic range for C_4 plants.

Bundle sheath cells contain little or no carbonic anhydrase, certainly less than 1% of total leaf activity (2). From this evidence and consideration of the earlier model (8), it was proposed that low bundle sheath cell carbonic anhydrase activity may be essential for effective operation of C_4 pathway photosynthesis (2). The basis for this conclusion was the necessity for maintaining a high $[CO_2]$:$[HCO_3^-]$ ratio, well above that at thermodynamic equilibrium. In contrast, with the current more elaborate inorganic carbon model, the concentration of CO_2 and the overcycling of the C_4 acid pathway are much less sensitive to carbonic anhydrase activity in the bundle sheath cytosol (Table I). It is necessary to increase this activity more than 100-fold over the nonenzymic rate to have a major effect on overcycling, and the bundle sheath CO_2

Table I. *Effect of Changes in Values of Assumed Parameters on the Calculated Concentrations of CO_2 and O_2 in Bundle Sheath Cells, the Resulting Percentage Photorespiratory CO_2 Loss, and on C_4 Acid Overcycling*

Unless stated otherwise, all parameters were as for Figure 1.

Assumptions	$[CO_2]$	$[CO_2]/[O_2]$	Photorespiratory CO_2 Loss[a]	C_4 Acid Overcycling[b]
	μM		%	%
As in Fig. 1.	70	0.159	3.1	15.5
0.5 × Net photosynthesis rate	70	0.206	2.4	31.0
0.5 × Pool size	34	0.078	6.4	7.1
1.5 × Pool size	106	0.241	2.1	23.9
CA 10^2 × nonenzymic[c]	69	0.158	3.2	18.3
CA 10^3 × nonenzymic[c]	65	0.149	3.4	35.9
$P_{O_2} = 1.72 \times P_{CO_2}$[d]	70	0.193	2.6	15.5
$P_{O_2} = 0.2 \times P_{CO_2}$[d]	70	0.054	9.3	15.5

[a] Percentage of gross photosynthetic CO_2 fixation. [b] Percentage by which rate of C_4 acid cycle exceeds the rate of CO_2 assimilation in bundle sheath cells. [c] Carbonic anhydrase activity in the bundle sheath cytosol. [d] The factors 0.2 and 1.72 represent the ratios of diffusivities of CO_2 and O_2 through a lipid-polymer medium or water, respectively, as described in "Theory."

concentration is only slightly changed even with a 1000-fold increase.

If a very low carbonic anhydrase activity in the bundle sheath cytosol is not an essential requisite for effective functioning of the C_4 pathway, as the model suggests, it is interesting to consider why the activity should be so low. One possibility is simply that it may not be needed. In C_3 mesophyll cells, where the CO_2 concentration would be low during photosynthesis (about 7 μM, ref. 4), carbonic anhydrase has been proposed to function to assist CO_2 diffusion within the chloroplasts (see ref. 27). In C_4 bundle sheath cells, where there is a relatively high CO_2 concentration during steady-state photosynthesis (about 70 μM from the present study), carbonic anhydrase may not be required for this purpose.

In deriving the model described in Figure 1, an attempt was made to assess diffusion of O_2 from bundle sheath to mesophyll cells via two pathways (see "Theory" section). These comprise an aqueous, plasmodesmatal path and an apoplastic path in which diffusion limitation is likely to be due to the suberin lamellae, a lipid-polymer component in the cell wall (13). Assumptions about these paths are especially significant to the bundle sheath O_2 concentration and consequent $[CO_2]/[O_2]$ ratio since the diffusivity of O_2 in water is substantially greater than for CO_2, but in hydrophobic polymers (similar to the composition of suberin) the reverse holds true (see "Theory"). In Table I, values were calculated assuming O_2 diffusion via a totally aqueous ($P_{O_2} = 1.72\ P_{CO_2}$) or a totally lipid-polymer path ($P_{O_2} = 0.2\ P_{CO_2}$). In the former case the $[CO_2]/[O_2]$ ratio is increased and potential photorespiratory CO_2 loss is slightly reduced (Table I). In contrast, in the latter case the $[CO_2]/[O_2]$ ratio is lowered and photorespiration is increased by three-fold. The potential for photorespiratory carbon loss is therefore rather sensitive to P_{O_2}, but in either case it is less than 10% of net assimilation.

Effect of Varying Stromal Volume

In the present model a large proportion of inorganic carbon is allocated to the HCO_3^- pool in bundle sheath chloroplasts, mainly due to the higher pH and low HCO_3^- permeability of this compartment. Since this component was important in determining the amount of inorganic carbon in other compartments, we examined the effect of varying the percentage volume of the chloroplast that was assumed to be stromal. The results (Fig. 2) show that this is a critical parameter in determining the bundle sheath CO_2 concentration and also the percentage of C_4 acid overcycling. With the average volumes of cell compartments used to derive the model, a stromal volume less than about 40% of the bundle sheath chloroplast volume gives a marked increase in bundle sheath cell CO_2 concentration and consequent overcycling.

Effect of HCO_3^- Permeability and Carbonic Anhydrase Activity of Bundle Sheath Chloroplasts

If, as the model predicts, a high level of HCO_3^- develops in bundle sheath chloroplasts, then there will be a large HCO_3^- gradient between the chloroplast and the cytosol (Fig. 1). Available evidence suggests that chloroplasts are largely impermeable to HCO_3^- (15, 34) but, clearly, even with a low

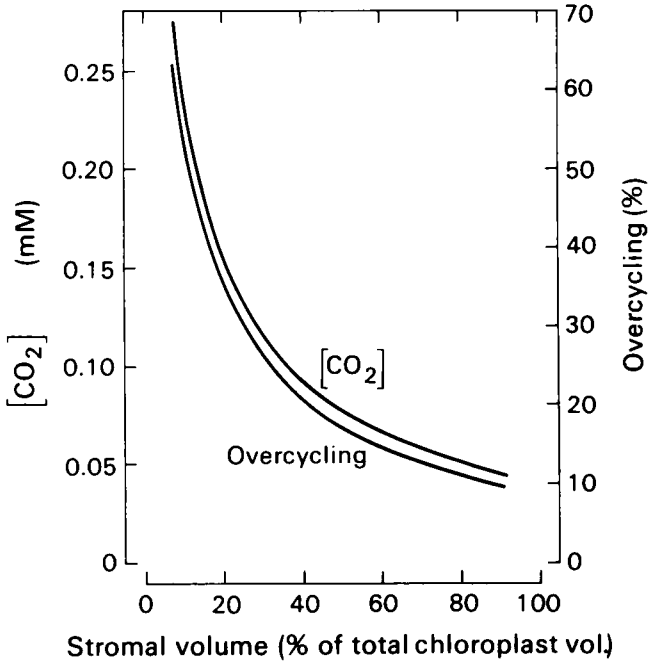

Figure 2. Effect of varying the volume assumed for stroma on the calculated bundle sheath CO_2 concentration and percentage C_4 acid overcycling. Stromal volume is expressed as a percentage of the total volume of bundle sheath chloroplasts. The values for other parameters were as used for Figure 1.

HCO_3^- permeability (relative to CO_2 permeability) significant flux of HCO_3^- may occur with such a large gradient. To examine the effect of allowing a small flux of HCO_3^- out of the chloroplast, the effect of varying $P^{Chl}_{HCO_3}$ values was examined, while maintaining all other parameters constant (Fig. 3A). The results indicate that as $P^{Chl}_{HCO_3}$ increases there is a dramatic shift in the concentrations of the bundle sheath inorganic carbon components, with the HCO_3^- concentration in the chloroplast decreasing and the cell CO_2 concentration increasing. The HCO_3^- concentration in the cytosol remains fairly constant during these changes (results not shown) but there is a large increase in the overcycling of the C_4 acid pathway (to compensate for the greater CO_2 efflux from the cells) to maintain the net photosynthesis rate. To emphasize how little flux of HCO_3^- through the chloroplast membrane is required to give these large changes, $P^{Chl}_{HCO_3}$ values of 0.01 and 0.05 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} would give flux rates equivalent to only 0.16 and 0.22%, respectively, of the net photosynthesis rate. It should be noted that a $P^{Chl}_{HCO_3}$ value of 0.05 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} for HCO_3^- permeability into chloroplasts is still more than four orders of magnitude lower than the permeability coefficient for CO_2 entry (probably greater than 10^3 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} based on C_3 mesophyll cell CO_2 permeabilities, see refs. 9, 18).

These results on the effects of varying $P^{Chl}_{HCO_3}$ suggest several possibilities for the *in vivo* situation. First, C_4 bundle sheath chloroplasts may be virtually impermeable to HCO_3^- (with a maximum flux rate for HCO_3^- at least 10^3-fold lower than the photosynthesis rate). A second possibility is that under conditions where significant HCO_3^- flux occurs, the inorganic

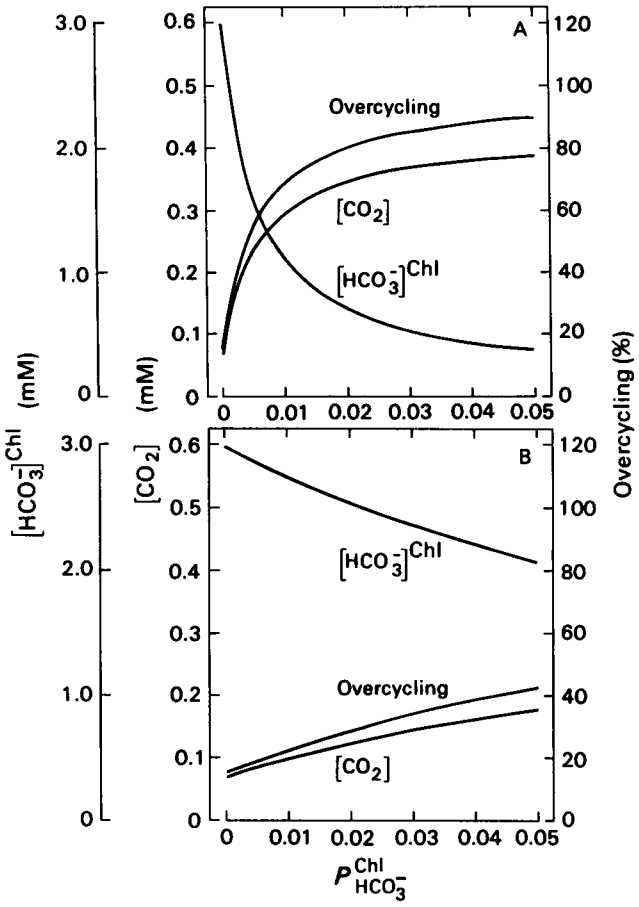

Figure 3. Effect of varying the permeability coefficient for HCO_3^- diffusion across the bundle sheath chloroplast envelope, $P^{Chl}_{HCO_3}$, on the calculated bundle sheath CO_2 concentration, chloroplast HCO_3^- concentration, and percentage C_4 acid overcycling. A, Other assumed values were as for Figure 1; B, A bundle sheath chloroplast carbonic anhydrase activity giving 20 times the nonenzymic rate was assumed.

carbon components in the bundle sheath chloroplast are brought closer to thermodynamic equilibrium by carbonic anhydrase activity. For example, Figure 3B shows the effect of varying $P^{Chl}_{HCO_3}$ over the same range but where a very low level of carbonic anhydrase activity (20 × the nonenzymic rate) is assumed within the bundle sheath chloroplast. Under these conditions, the chloroplast HCO_3^- remains the predominant component of the inorganic carbon pool, and overcycling remains closer to energetically acceptable levels. In relation to this, Figure 4 shows the effect of varying bundle sheath chloroplast carbonic anhydrase activities on bundle sheath cell CO_2 concentration and C_4 acid overcycling for two different permeabilities of the chloroplast to HCO_3^-. Even with the higher permeability coefficient value, a carbonic anhydrase activity in bundle sheath chloroplasts of as little as 30 times the nonenzymic rate would decrease the overcycling to below 40%.

Given a substantial leakage of HCO_3^- from bundle sheath chloroplasts, further modeling suggested that another option available to plants for resolving the consequent problem of

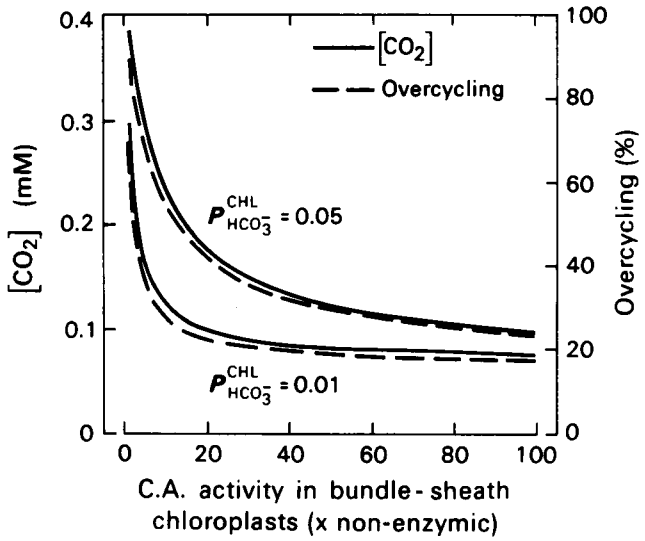

Figure 4. Effect of varying the bundle sheath chloroplast carbonic anhydrase activity on calculated bundle sheath cell CO_2 concentration and percentage C_4 acid overcycling. The data is presented for two values of the chloroplast HCO_3^- permeability coefficient. Other parameters were as for Figure 1.

high overcycling would be, paradoxically, to reduce the overall size of the inorganic carbon pool (results not shown). Thus, the net effect of bundle sheath chloroplasts either being impermeable to HCO_3^-, or having significant permeability in combination with either a low level of carbonic anhydrase or a reduced total inorganic carbon pool size, is the same with respect to overcycling. Hence we retained the original assumption that $P^{Chl}_{HCO_3}$ is 0 (*i.e.* the chloroplasts are impermeable to HCO_3^-) for the remainder of the modeling described here.

Effect of Varying pH

The pH of the cytosol of C_4 bundle sheath cells has not been determined but we assumed a value of 7.4 (20, 28). Since both the concentrations of CO_2 and HCO_3^- at thermodynamic equilibrium, and the rate constants for the interconversion of these species, are pH dependent, assumptions about bundle sheath cytosolic pH would clearly be important. The effect of varying this parameter was examined and the mitochondrial and chloroplast pH was correspondingly varied as described in "Theory." The CO_2 concentration, $[CO_2]/[O_2]$ ratio, and C_4 acid overcycling decreased as cytosolic pH was increased (Fig. 5). However, within the range usually assumed for cytosolic pH, the derived values remained within acceptable limits. For example, a $[CO_2]/[O_2]$ ratio of 0.1 corresponds to a potential photorespiration rate of 5% of net photosynthesis. Figure 5 shows that a threshold value for a $[CO_2]/[O_2]$ ratio giving greater than 5% photorespiration therefore occurs at a cytosolic pH of 7.8. C_4 acid overcycling remains below 20% throughout the pH range down to about pH 7.2.

Varying stromal pH alone (rather than in combination with cytosolic pH) had a qualitatively similar effect (results not shown). For instance, reducing the stromal pH over the range from 8.0 to 7.4 increased overcycling from 11 to 31% while decreasing potential photorespiration from 4.4 to 1.6%.

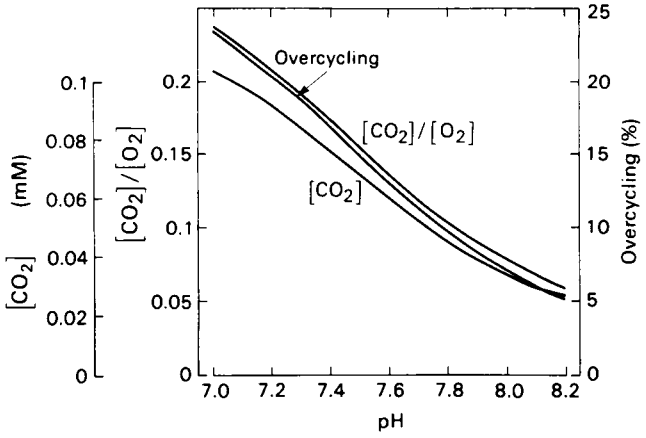

Figure 5. Effect of varying the bundle sheath cell cytosolic pH on the calculated bundle sheath cell CO_2 concentration, $[CO_2]/[O_2]$ ratio, and percentage C_4 acid overcycling. Other assumed values were as for Figure 1. With varying cytosolic pH, the bundle sheath chloroplast and mitochondrial pH were correspondingly varied as described in "Theory."

Effect of Bundle Sheath Cell Permeability to CO_2

Another critical parameter in the model is the P_{CO_2} value. As outlined in "Theory" we used a value of 15 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} which represents an average for a range of C_4 species from determinations using either isolated bundle sheath cells or intact leaves (values ranged from 6–30 μmol min^{-1} [mg Chl]$^{-1}$ mM^{-1} from refs. 9 and 18). The effect of varying this parameter is shown in Figure 6. The major effect of increasing P_{CO_2} is that overcycling is increased in a linear fashion. This is a consequence of the model (Fig. 1) in that, as P_{CO_2} increases, the distribution of total inorganic carbon during steady state photosynthesis remains the same but the CO_2 leakage increases. This necessitates a greater rate of CO_2 supply by C_4 acid decarboxylation and hence overcycling.

While the CO_2 concentration remains constant, the concentration of O_2 gradually decreases (Fig. 6) since, for the purposes of the model, P_{O_2} is related to the P_{CO_2} (see "Theory"). At P_{CO_2} values in the range above 20 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} the $[CO_2]/[O_2]$ ratio increases only slightly, while in the range below this value the ratio decreases rapidly. However, based on calculations of potential photorespiratory CO_2 loss relative to CO_2 assimilation, P_{CO_2} would have to drop below 6.5 μmol min^{-1} (mg Chl)$^{-1}$ mM^{-1} (all other parameters remaining constant) before the rate of photorespiration increases to above 5% of photosynthesis rate. This value is close to the lower limit of values experimentally determined for P_{CO_2}, as mentioned above.

Application of the Model to Experimental Data

The inorganic carbon pool in *Urochloa panicoides* has been measured at the different steady-state rates of photosynthesis induced by varying the CO_2 concentration (8). This data was used in the model to examine the effect of varying the photosynthesis rate on the bundle sheath cell CO_2 concentration and overcycling. The computed overcycling remains almost constant over the range of net photosynthesis rates

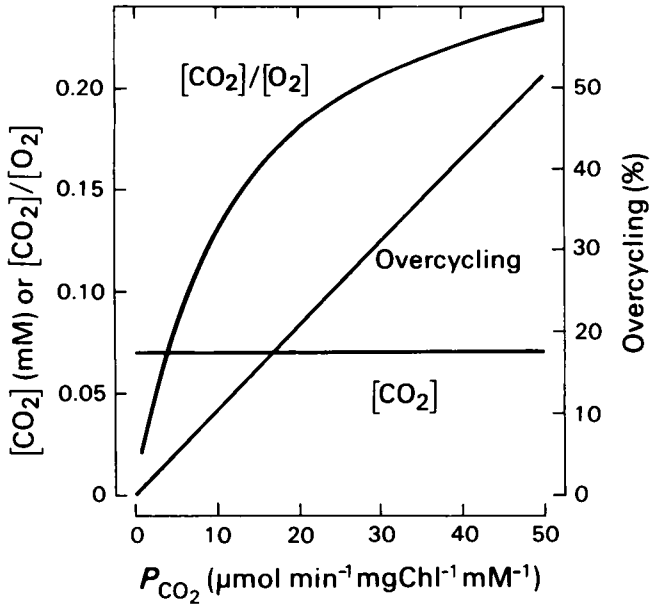

Figure 6. Effect of varying the permeability coefficient for CO_2 diffusion across the bundle sheath-mesophyll interface, P_{CO_2} on the calculated bundle sheath cell CO_2 concentration, $[CO_2]/[O_2]$ ratio, and percentage C_4 acid overcycling. Other assumed values were as for Figure 1.

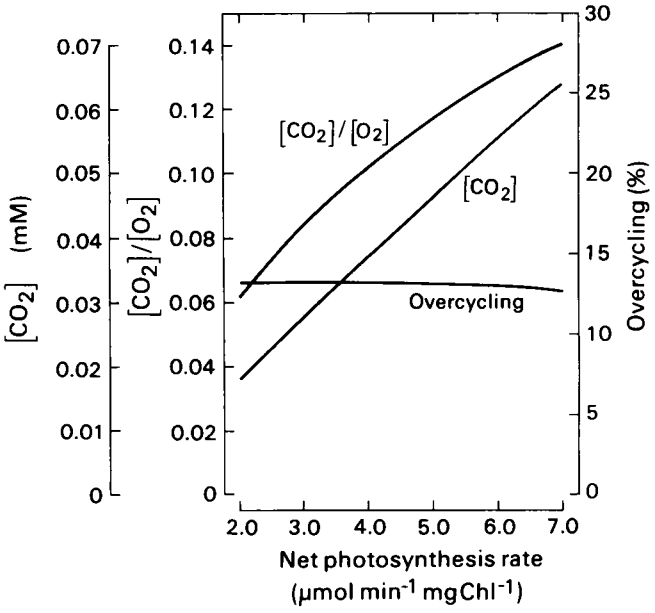

Figure 7. Effect of varying the net photosynthesis rate and inorganic pool size (by changing ambient CO_2 concentration) on the calculated bundle sheath cell CO_2 concentration, $[CO_2]/[O_2]$ ratio, and percentage C_4 pathway overcycling. For each level of CO_2 that was supplied to the leaf, the experimentally determined photosynthesis rate and pool size (from ref. 8) were used along with the mesophyll CO_2 concentration (calculated assuming appropriate ratios of intercellular to ambient CO_2 concentrations for leaves in humidified air derived from the data in ref. 21). These values were incorporated into the inorganic carbon pool model along with other assumed values as for Figure 1.

(Fig. 7). These results suggest that the net photosynthesis rate, bundle sheath cell CO_2 concentration, and inorganic carbon pool size may be adjusted in C_4 leaves in such a way that overcycling remains a constant percentage of photosynthesis rate.

The outcome described in Figure 7 may represent an efficiency optimisation aimed at keeping the energy cost associated with inorganic carbon leakage and overcycling to a minimum while trying to maintain a sufficient CO_2 concentration in bundle sheath cells to largely prevent photorespiration. For instance, from the predicted $[CO_2]/[O_2]$ ratios in Figure 7 photorespiration would increase from 3.6% to only 8.2% as the photosynthesis rate decreases from 7 to 2 μmol min^{-1} (mg Chl)$^{-1}$. This is broadly consistent with the observations of Furbank and Badger (7) that photorespiration was almost undetectable in many C_4 plants, even near the CO_2 compensation point.

CONCLUDING REMARKS

So far, it has not proved possible to directly determine the composition of the inorganic carbon pool that develops in leaves of C_4 plants during steady-state photosynthesis. Hence, the concentration of CO_2 in bundle sheath cells, the inorganic carbon species critical for suppressing photorespiration, remains unknown. The present paper describes a model of C_4 photosynthetic metabolism in bundle sheath cells which permits predictions about the concentration of the various components of the inorganic carbon pool and the fluxes between these. This model represents a major sophistication of the simpler empirical model presented earlier (8). With 'best estimate' assumptions, the present model predicts lower CO_2 concentrations during steady-state photosynthesis than previous estimates, combined with a slightly higher rate of C_4 acid overcycling. These differences are attributable to more detailed subcompartmentation of cellular inorganic carbon, and the use of a higher, directly determined permeability coefficient for CO_2 efflux from bundle sheath cells.

The model highlights the factors likely to be important determinants of the cellular CO_2 concentration, the associated $[CO_2]/[O_2]$ ratio, and C_4 acid overcycling. Critical to the bundle sheath cell CO_2 concentration is the large allocation of carbon to the HCO_3^- pool in the high pH chloroplast compartment. Other of these factors are not intuitively obvious. For instance, the model suggests that effective suppression of photorespiration, combined with energetically acceptable C_4 acid overcycling, only prevails when either chloroplasts are impermeable to HCO_3^-, or when a degree of HCO_3^- permeability is coupled with a low but significant level of chloroplast carbonic anhydrase activity. Thus, contrary to an earlier conclusion, a low, defined activity of carbonic anhydrase in bundle sheath chloroplasts may be essential if the latter situation exists *in vivo*. Cytosolic pH and cytosolic carbonic anhydrase activity, although having a marked influence, were less critical than might have been anticipated or previously supposed (2, 8).

The function of C_4 photosynthesis is to effectively eliminate photorespiration by generating a sufficiently high $[CO_2]/[O_2]$ ratio in bundle sheath cells. This must be achieved without energetically unacceptable overcycling of the 'CO_2 pump'—the C_4 acid cycle that transfers CO_2 to these cells. The bundle sheath cells must be sufficiently impermeable to CO_2 to prevent excessive loss of CO_2 generated in these cells; at the same time O_2 generated during photosynthesis must escape sufficiently fast to prevent the development of steady-state concentrations that adversely affect the maintenance of high $[CO_2]/[O_2]$ ratios. The model clearly demonstrates that this is an exercise in optimization.

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