

A Theory and Model for the Kinetics of the Two-Substrate Ordered Reaction of Rubisco with Rate-Determining Steps, and the Effects of RuBP Regeneration on the Hierarchy of Limitations.*

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Abstract: The two-substrate co-limiting model of Rubisco reaction by Farazdaghi & Edwards (Plant Cell & Environment 1988, 11, 789-798; 799-809) is extended to reflect recent experimental observations. The modifications include: 1) Consideration of two steps for the reaction, one for each substrate. 2) Consideration of the initial transitional reaction as a single step, and the steady state as a two-step reaction. 3) Application of single step limitation for low substrate levels, when free enzyme is available in the medium, enzyme limitation at saturation of both substrates, and co-limitation of the enzyme-substrate complexes of the two steps in between. 4) A biochemical model is developed in which the rate of reaction swings between the two substrates as they

become limiting. 5) Limitation of RuBP regeneration is considered to be due to either: a) Limitations of radiation (energy-limited) in the first step, or: b) Limitation of Calvin cycle (Calvin cycle capacity limited) in the second step of reaction. The model responded well to the data and can provide a sound biochemical basis for mechanistic extension of models for applications in genetic manipulation of plants, crop production in relation to environment, and changes in the global carbon cycle.

Introduction:

Carbon flux into the biosphere is mainly controlled by the global activity of Rubisco (ribulose-1,5-bisphosphate carboxylase/oxygenase) (Falkowski et al 2000, Cox et al 2000). The large majority of global CO₂ assimilation occurs in C3 plants in which Rubisco operates at relatively low carboxylation efficiency, in addition to oxygen inhibition and deficiency of CO₂ as substrate (Spreitzer and Salvucci 2002). Plant biologists have for decades attempted to model photosynthetic processes, in order to better understand the controlling mechanisms, and to predict the effects of greenhouse gas emissions, global warming, crop yield and biosphere productivity. More recently, modeling interest has focused on biome responses to global change (Baldocchi & Wilson 2001) and genetic manipulation for designing a better plant (Spreitzer and Salvucci 2002).

In the 1970's two major schools of thought arose regarding modelling of photosynthesis at the biochemical level. Earlier models (van Bavel 1975, Ku & Edwards 1977, Hall 1979) argued that CO₂ assimilation in vivo had to reflect the simultaneous availability of the RuBP and CO₂ substrates, O₂ in its oxygenation role, and the kinetics of the Rubisco enzyme. Effects of the environment, such as light availability, sink strength, temperature, humidity and nutrient supply could be understood and modelled via the effects of combined CO₂ and RuBP substrate supply and Rubisco kinetics. Proponents of this, (the single enzyme school) argued that this approach was robust, because it followed biochemical theory and focussed the prediction of CO₂ assimilation upon the Rubisco reaction, with its inputs and outputs, rather than distributing the control of reaction over processes removed from carboxylation to varying degrees.

Later models emphasised the dichotomy between the capacity of **Rubisco to consume RuBP**, and the capacity of light harvesting, electron transport, and Calvin cycle **to regenerate RuBP** (Farquhar et al 1980, Sharkey 1985). In contrast to these **dual system** models, the **single-enzyme** model was hampered by greater complexity, and substantial uncertainty at the time.

Farquhar and co-workers based their two-system model on the assumptions that carboxylation reaction was assumed to reflect a) the minimum of either the Rubisco capacity to consume RuBP, (the steep part of the CO₂ response curve), or b), the regeneration rate of RuBP for its replacement, (the plateau of the CO₂ response curve), as determined by electron transport rate, Calvin cycle capacity, or triose-phosphate regeneration capacity (Sharkey 1985). The two-system model received initial experimental support shortly after development (von Caemmerer & Farquhar 1981, Sharkey 1985). It has since been used in a wide variety of applications, ranging from anti-sense manipulation at single enzyme levels (von Caemmerer et al 1994, Whitney et al 1999) to modelling global scale changes in carbon flux (Kaduk & Heimann 1997, Baldocchi & Wilson 2001) and global changes in ozone*, photosynthesis and biochemical cycle (Woodward et al 1994, 1995).

Despite its success, limitations persist in the predictive power of the two-system model. The model is considered to be Blackman (1905) type in nature, leading it to an abrupt change that requires empirical correction coefficients such as the convexity factor to produce a smooth transition that reflects a shift of control from Rubisco to RuBP regeneration pathway, limited by an empirical J_{max} (Collatz et al 1990, de Pury & Farquhar 1997, von Caemmerer 2000).

The inability of the two-system model to cover different contingencies has led to attempts to improve the models (eg. Harley & Sharkey 1991), to reconsider the single enzyme approach (Farazdaghi & Edwards 1988a,b;1992), and other models (Laisk 1993). The single enzyme models, such as the model of Farazdaghi & Edwards (1989a, b) have been based on the controlling effect of substrates when their concentration is low, co-limitation of enzyme and substrates as the concentration of substrate increases, and enzyme limitation at saturated substrate levels. This is consistent with the widely accepted view that Rubisco is the “ultimate rate-limiting step” in carboxylation (Jensen 2000). The differences between the two schools and the scope of their effects can best be reflected by the following two quotations regarding the control over photosynthesis at ambient CO₂ (300μbar).

- i- Eckardt et al (1997) write: “Rubisco activity is one of the principal factors limiting photosynthesis at saturating light and atmospheric CO₂ concentrations.”.
- ii- Whitney et al (1999) write: “Since photosynthesis in control plants becomes limited by ribulose-P2 regeneration above 300 μbar CO₂ (Fig 6A), these plants are not suitable for measurement of wild-type Rubisco’s kinetic properties in vivo”.

The conclusion of Whitney et al (1999) is based on an important and basic assumption of the two-system theory of Farquhar et al (1980), that any deviation of the response of carboxylation to CO₂ from Michaelis-Menten curve should be due to a limitation in RuBP regeneration from Calvin cycle. This inevitable conclusion and its follow up recommendation can have significant impact on the

direction of research on photosynthesis and genetic manipulation of C3 plants, and if incorrect, may have serious consequences.

The objective of this communication is to re-examine the two-substrate ordered reaction of Rubisco, and determine the hierarchy of the factors that control the rate-determining step (RDS). Also, to formulate the corresponding kinetic models for the single-enzyme two-substrate reaction of Rubisco that can be verified by current experimental observations.

The Single-Enzyme Two-Substrate Ordered Reaction Model:

Following Farazdaghi & Edwards (1988b), we consider that any change in the rate of RuBP regeneration in the Calvin cycle would alter the RuBP concentration that reacts with enzyme in a sequentially ordered two-substrate reaction. The inputs, outputs and feedbacks of Rubisco reaction can be experimentally evaluated, and the rate of reaction can be independently modelled through the enzyme and its substrates. The activity of Rubisco can be adjusted by the regulatory and feedback mechanisms (eg, Mg, CO₂, Rubisco Activase, temperature, pH, and so on), while the capacity of the system is controlled by the activated state and turnover rate of Rubisco that is influenced. In this way carboxylation rate can be fully explained by the kinetics of Rubisco enzyme.

Farazdaghi & Edwards (1988b) replaced RuBP with the equivalent radiation energy that was required for its regeneration in the Calvin cycle. They based derivation of their model on the initial slope or maximum efficiency of the carboxylation reaction (Ψ), which was more stable than Michaelis constant for Rubisco, and provided a simple linear function for CO₂ compensation concentration (Γ^*) in relation to oxygen concentration as:

$$\Gamma^* = 0.5(V_{\text{omax}}/K_o) \cdot O / (V_{\text{cmx}}/K_c) = 0.5\Psi_o \cdot O / \Psi_c \quad (1)$$

where, O is the partial pressure of oxygen at the site of reaction, Ψ_c and Ψ_o are the maximum carboxylation and oxygenation efficiencies respectively, and their ratio is consistent with the specificity constant of Jordan & Ogren (1984). The K_m values for carboxylation (K_c) and oxygenation (K_o) are specific to the rectangular hyperbola and can be written as:

$$K_m = V_{\text{max}}/\Psi \quad (2)$$

Therefore, the effects of competition between CO_2 and O_2 on the carboxylation and quantum efficiencies were described respectively as:

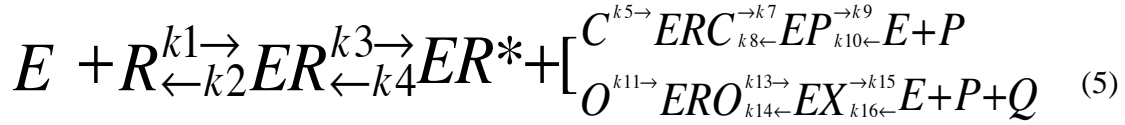
$$\Psi_a = \Psi_c / (1 + O/K_o) \quad (3)$$

$$\Phi = \Phi_m \frac{V_c}{V_c + V_o} = \Phi_m \frac{\Psi_c C}{\Psi_c C + \Psi_o O} = \Phi_m \frac{C}{C + 2\Gamma^*} \quad (4)$$

Where Ψ_a is the maximum apparent carboxylation efficiency, V_c and V_o are the velocities of carboxylation and oxygenation, and Φ and Φ_m , the actual and the maximum quantum efficiencies for RuBP regeneration respectively. At CO_2 compensation point, where $C = \Gamma^*$ in absence “dark” or “day-respiration (R_d), the amount of energy used in oxygenation is twice that used in carboxylation.

The Theory: 1-Rubisco Reaction in a Sequential Order:

The following equation is an extension of Farazdaghi & Edwards (1988b, 1992) and describes both carboxylation and oxygenation via fully activated Rubisco for the two-substrate ordered reaction:



The k values represent the rate constants for the forward and reverse reactions. E is free enzyme, R is RuBP, C is CO₂, O is O₂; ER, ERC, and ERO are enzyme-substrate complexes with RuBP, RuBP plus CO₂ and RuBP plus O₂ respectively. ER* is the enediol intermediate resulting from the reversible reaction of ER ↔ ER* (first step of the reaction). The reaction of ER* with CO₂, forming ERC, 3-keto-2-Carboxyarabinitol 2-phosphate, is irreversible (Andrews & Lorimer 1987 ; Chen & Spreitzer 1992). ERC undergoes a transformation, ERC ↔ EP, leading to carbon-carbon cleavage and releases PGA in the second step of the reaction (Pierce et al 1986). EX is the enzyme-product complex for oxygenation, and P & Q are the oxygenation products. Because of the irreversibility of carboxylation and oxygenation of enol-RuBP, k₆ and k₁₂ should be zero. Equation 6 provides a general form for the conservation of enzyme mass.

$$E_t = E + ER + ER^* + ERC + ERO + EP + EX \quad (6)$$

The components of equation 6 can be calculated in terms of EP for steady state conditions (Cleland 1991) as follows:

$$E_{t/EP} = (K_r/R) + (K_c/C) \cdot (1 + O/K'o) + 1 + \alpha_1 + \alpha_2 + K_r(k_2k_4/k_5 + k_{11} \cdot O/k_5) / RC \quad (7)$$

$$\text{Where, } K_r = (k_2 + k_3)k_9/k_1k_3; \quad (8)$$

$$K_c = (k_3 + k_4)k_9/k_3k_5; \quad (9)$$

$$K'o = k_{13} \cdot k_{15} \cdot (k_3 + k_4) / [k_3k_{11}(k_{13} + k_{14} + k_{15})]. \quad (10)$$

$$\alpha_1 = k_9/k_3, \quad (11)$$

$$\alpha_2 = K_p = (k_9 + k_8)/k_7. \quad (12)$$

The enzyme and substrate components of equation 7 can be separated as:

$$[Et - (1 + \alpha_1 + \alpha_2)EP]/EP = 1/Sf \quad (13)$$

$$\text{where, } 1/Sf = (K_r/R) + (K_c/C) \cdot (1 + O/K'_{o}) + K_r(k_2k_4/k_5 + k_{11} \cdot O/k_5)/RC \quad (14)$$

With standard Michaelis-Menten procedures, in which both the transitional and steady state maximum velocities are the same, equation 13 also leads to a similar single step co-limiting model as that of Farazdaghi & Edwards (1988b). In such models the rates of steady state reactions for different substrate levels follow a rectangular hyperbolic function with an inhibition of the maximum velocity (uncompetitive inhibition) by the intermediate enzyme-substrate complexes. In such derivations, the kinetic equations for two-substrate reactions, of either random or sequentially ordered, are basically the same and the order of the reaction cannot be enforced (Johnson 1992). Experimentally, this is also inconsistent with the results reported for two-substrate ordered reactions in general (Johnson 1992, Moulis et al 1991, Jamin et al 1991), and for Rubisco specifically (Laisk 1985, Ruuska et al 1998, and Laisk and Oja 1998). Ruuska et al (1998) demonstrated that, the transitional rate of Rubisco carboxylation for wild-type tobacco (WT) followed a rectangular hyperbola with a maximum of $V_{cmax} = 72.5 \mu\text{molm}^{-2} \text{s}^{-1}$. However, the steady state rate deviated significantly from the rectangular hyperbola, with a maximum of $V_{max} = 27 \mu\text{molm}^{-2} \text{s}^{-1}$ that was less than 50% of V_{cmax} .

2- Kinetics of Limitations and Rate Determining Steps:

As the rate of each step in the reaction is proportional to the concentration of enzyme-substrate complex of that step, thus it can be limited either by the concentration of its substrate, or by the availability of enzyme for that step. The maximum velocity of the second step at RuBP saturation is dependent on the total capacity of Rubisco minus the

concentration of enzyme that is held in the second step. Therefore, the maximal velocity of the reaction varies, and its lower limit is reached when both substrates are saturating. Thus, in substrate response curves for the two-step reaction of Rubisco, the following phases can be observed:

i- **Transitional phase:** When RuBP saturated enzyme reacts with CO₂, it will result a single-step transitional phase, for which EP reaches its maximum value that is equal to Et at CO₂ saturation, resulting an initial or transitional maximum velocity for the reaction (V_{cmax}).

ii- **Substrate limitation phase:** The initial single-step transitional phase would be followed by a two-step steady state, in which the rate of carboxylation will be limited by the enzyme-substrate complex of the slower step (the step with the limiting substrate). This will be considered as the Rate-Determining Step (RDS). Therefore, when RuBP is limiting, equation (14) will be reduced to:

$$1/S_f = K_r/R \tag{15}$$

and when CO₂ is limiting:

$$1/S_f = (K_c/C).(1+O/K' o) \tag{16}$$

This is based on the assumption that when free enzyme is available in the medium the reaction of the faster step will be completed sooner and cannot limit the overall rate of reaction.

iii- **Co-Limitation Phase:** When both substrates are available at relatively high concentrations, but below their respective saturation levels, the rate of carboxylation is proportional to the enzyme-substrate concentration of the limiting substrate (slower step),

and its maximum is determined by the actual rate of the faster step. But, as free enzyme is reduced at higher substrate levels, the rate of the faster step becomes partly dependent on the release of enzyme that is controlled by the slower step. Thus as the substrate concentration of the slower step increases, the availability of enzyme for the faster step decreases and the two steps become co-limiting.

iv- **Enzyme Limitation Phase:** When the reaction has reached dual substrate saturation, the rate of reaction reaches its steady state maximum limit (V_{max}). This is because no free enzyme is left in the medium, and the enzyme-substrate complexes of the two steps have cumulatively reached the limit of total enzyme. Therefore, the maximum concentration of EP, (EP_{max}), which produces the maximum steady state rate $V_{max} = k_9 EP_{max}$, is smaller than the concentration of total enzyme that produces the transitional maximum, $V_{cmax} = k_9 E_t$, so that from equation (13) we get:

$$E_t = (1 + \alpha_1 + \alpha_2) EP_{max}. \quad (17)$$

Or: $V_{cmax}/V_{max} = 1 + \alpha_1 + \alpha_2 \quad (18)$

3- Limitations of RuBP regeneration:

The continuity of the reaction for in-vivo steady state is dependent on the rate of RuBP regeneration. RuBP regeneration can be limited either by the supply of radiation energy, or other factors that are required in its regeneration pathway. In **energy-limited** RuBP regeneration, carboxylation responds positively to changes in energy supply. When carboxylation does not respond to any increase in radiation (**energy-sufficient**), any factor from a large group of parameters may be limiting the reaction. These include a limitation in CO_2 supply (limitation of PGA), limited Rubisco capacity, or a limitation

in the capacity of Calvin cycle enzymes and metabolites that influence the utilisation of energy for RuBP regeneration (eg. inorganic phosphate). When RuBP is **Calvin cycle capacity limited**, PGA is accumulated and causes product or feedback inhibition (Sun et al 1997). When both substrates are saturating, the total **enzyme capacity**, that is distributed in enzyme-substrate complexes of the first [α_1 .EP] and the second step [$(1+\alpha_2)$ EP] would be co-limiting the reaction rate (**co-limitation of the two steps**). The magnitude of V_{max} is found to be between 30% to 50% of V_{cmax} (Laisk 1985, Ruuska et al 1998 and Laisk & Oja 1998).

At sub-saturation, the substrate that is limiting determines the rate of reaction (RDS), and the enzyme component that is engaged with the limiting substrate (E.RDS), makes the co-limiting component of the **enzyme for enzyme-substrate co-limitation**. Then (E-E.RDS) can be available in the form of enzyme-substrate complex of the faster step, and determines its potential rate which is the maximum rate of the slower step (often referred to as K_{cat}). Thus the apparent values of K_{cat} and K_m vary for different reaction rates, within the limits of V_{cmax} and V_{max} (Moulis et al. 1991, Jamin et al. 1991 Johnson 1992).

4- Effects of Limitations on Kinetic Equations:

When **RuBP is limiting due to the limitation of energy**, the limiting component of enzyme is α_1 EP, that is engaged with RuBP in $ER \leftrightarrow ER^*$. α_1 is the ratio of the rate of product release in the second step to the rate of production of enol-RuBP in the first step. The slower the rate of enol-RuBP production (smaller k_3), the larger is the value of α_1 and its limitation or inhibition effect. This means that the maximum available enzyme

from product release = $E_t - E \cdot RDS = E_t - \alpha_1 EP$, and the fraction of total enzyme that is potentially available for maximum rate of the first step (E_{m1}) is:

$$E_{m1}/E_t = (E_t - \alpha_1 EP)/E_t \quad (19)$$

When CO₂ is limiting the limitation is on the second step, and the maximum available enzyme for this step is given by the fraction of total enzyme (E_{m2}/E_t) that is available for producing ER^* , i.e. total enzyme minus the component that is engaged in the slower step:

$$E_{m2}/E_t = [E_t - (1 + \alpha_2)EP]/E_t \quad (20)$$

Equations 19 and 20 are particularly significant because of the separation of the two steps by the irreversible link found by Andrews and Lorimer (1987).

When RuBP is limiting due to a deficiency in the Calvin cycle, it causes an accumulation of PGA which has inhibitory effect on enzyme (product inhibition) and produces a limitation in the second step. This case can also be represented by equation (20) through an equivalent increase in the value of α_2 . Deficiencies in inorganic phosphate or its recycling rate observed by Sharkey (1985) or Zhang & Nobel (1996) can also be described by equation (20) in the same manner.

If the limitation in the second step is due to a limitation in recycling of inorganic phosphate, then it may also reduce the activity of Rubisco activase (Portis 1992, 1995) and may lead to an inhibition by RuBP substrate (Portis et al 1995) or CA1P (Hammond et al 1998). Thus, with regards to RuBP, if there is a shift from the limitation in energy supply to a limitation in the Calvin cycle, it should be evident through a shift in their responses from equation 19 to equation 20. But, when limitation is in the second step no such shift can be observed.

Equation 13 was developed for full co-limitation of enzyme and substrates, without the consideration of RDS. In order to include RDS in this equation, equation 13 should be written for the limiting substrate with an adjustment in K_{cat} (or potential rate = V_m) for the fraction of total enzyme that can be available for that step. This is achieved by combining equation 13 for limitation of each substrate, with the fraction of enzyme used for its potential rate (equations 19 or 20) when the other substrate is not limiting, i.e.:

$$\{Et - EP(1 + \alpha_1 + \alpha_2)\}Et/\{EP(Et - \alpha_1 EP)\} = K'r/R \quad (21)$$

$$\{Et - EP(1 + \alpha_1 + \alpha_2)\}Et/\{EP(Et - (1+\alpha_2)EP)\} = (1+K'o/O)K'c/C \quad (22)$$

$$\{Et - EP(1 + \alpha_1 + \alpha_2)\}Et/\{EP(Et - (1 + \alpha_2)EP)\} = K'r/R \quad (23)$$

Considering that the velocity of steady state reaction is determined from its last step, then for RuBP limited carboxylation, $V_r = k_9 EP$, and for CO_2 supply limited $V_c = k_9 EP$. As $V_{cmax} = k_9 Et$, then, with simple substitution of V_r into equations (21) and (23), and V_c into equation (22), and rearrangements we get:

$$\alpha_1 V_r^2 - V_r [(1+\alpha_1+\alpha_2).R.V_{cmax}/K_r + V_{cmax}] + V_{cmax}^2 .R/K_r = 0 \quad (24)$$

$$(1+\alpha_2) V_c^2 - V_c [(1+\alpha_1+\alpha_2).C.V_{cmax}/K_c + V_{cmax}] + V_{cmax}^2 .C/K_c = 0 \quad (25)$$

$$(1+\alpha_2).V_r^2 - V_r [(1+\alpha_1+\alpha_2).R.V_{cmax}/K_r + V_{cmax}] + V_{cmax}^2 .R/K_r = 0 \quad (26)$$

Equations 24 to 26, can be written with respect to the maximum efficiency of the reaction according to equation (2) as follows:

$$\alpha_1 V_r^2 - V_r [(1+\alpha_1+\alpha_2).\Psi_r R + V_{cmax}] + V_{cmax}.\Psi_r.R = 0 \quad (27)$$

$$(1+\alpha_2) V_c^2 - V_c [(1+\alpha_1+\alpha_2).\Psi_c.C. + V_{cmax}] + V_{cmax}.\Psi_c.C = 0 \quad (28)$$

$$(1+\alpha_2).V_r^2 - V_r [(1+\alpha_1+\alpha_2).\Psi_r R + V_{cmax}] + V_{cmax}.\Psi_r.R = 0 \quad (29)$$

There is no basic difference between the two sets of equations (equations 24 to 26 and 27 to 29), the choice is rather based on clarity and ease of use, and the fact that K_c can not be measured accurately under steady state conditions (Laisk 1985, Ruuska et al 1998). In equations 27 to 29, the values of α_1 and $1+\alpha_2$ are related to the two separate steps of such reactions that can be applied to carboxylation. The findings of Andrews and Lorimer (1987) and Chen and Sprietzer (1992), of the irreversibility of the bindings of CO_2 with enolated RuBP provide experimental evidence for justification of such separation for Rubisco. However, there are additional complexities related to RuBP regeneration and oxygenation, but equations 27 to 29 provide logical bases for incorporation of such complexities based on the available information.

5-RuBP Regeneration and Radiation:

The amount of RuBP that is used in the reaction should be regenerated from the product of the reaction, PGA, in the triose- RuBP chain of the Calvin cycle, using the energy that is provided by NADPH and ATP. At CO_2 levels above compensation concentration (Γ), the PGA that is produced is not a limiting factor for the production of the consumed RuBP, thus, mole energy per mole RuBP can be substituted for RuBP with an appropriate change in the efficiency according to equation 4, therefore:

$$\Psi_{rR} = \Phi_{\max} \cdot E_i \cdot I = \Phi_m \cdot I \quad (30)$$

where, Φ_{\max} , is the maximum quantum efficiency for RuBP regeneration, the details of which are given by Farazdaghi & Edwards (1988b). E_i is the proportion of incident energy (I) that is absorbed, and Φ_m is the overall maximum quantum efficiency with respect to carboxylation, which is found to be constant among different C3 plants (Singsaas et al 2001). When CO_2 is not saturating, as the quantum efficiency for

carboxylation is dependent on the strength of its competitor, i.e. oxygenation, therefore, the effect of competition for RuBP is reflected in the net quantum efficiency of carboxylase described by equation 4. When CO₂ is saturating, the response of carboxylation to radiation can set the V_{max} of the CO₂ response at different light levels. By substituting equations (30) and (4) into equations (27) and (29) we get:

$$\alpha_1.V_i^2 - V_i[(1+\alpha_1+\alpha_2)\Phi.I + V_{cmax}] + \Phi.I.V_{cmax} = 0 \quad (31)$$

$$(1+\alpha_2).V_i^2 - V_i[(1+\alpha_1+\alpha_2)\Phi.I + V_{cmax}] + \Phi.I.V_{cmax} = 0 \quad (32)$$

where V_i is the rate of carboxylation relative to I for a given CO₂ level.

6-Net Assimilation Rate: The rates of net CO₂ assimilation, with respect to light (A_i) or CO₂, (A_c) respectively are:

$$A_i = V_i - 0.5V_o - R_d \quad (33)$$

$$A_c = V_c - 0.5V_o - R_d \quad (34)$$

However, although the first step of oxygenation is the same as that of carboxylation, the remainder of the oxygenation pathway is more complex and its specific modelling is beyond the scope of this communication. But as light intensity does not change the competitive ratios of V_o/V_i, therefore models of net assimilation rates with respect to radiation are provided in equations 35 and 36.

$$\alpha_1.A_i^2 - A_i[(1+\alpha_1+\alpha_2)\Phi.I + V_{cmax} - 2\alpha_1.R] + \Phi.(I - I_c).[V_{cmax} - R(1+\alpha_1+\alpha_2)] = 0 \quad (35)$$

$$(1+\alpha_2)A_i^2 - A_i[(1+\alpha_1+\alpha_2)\Phi.I + V_{cmax} - 2(1+\alpha_2)R] + \Phi.[I - I_c][V_{cmax} - R(1+\alpha_1+\alpha_2)] = 0 \quad (36)$$

The compensation point for light, I_c, is approximated by:

$$I_c = I^* + I_d \cong 0.5V_o / \Phi_o + R_d / \Phi_c \quad (37)$$

Where I^* and I_d represent close approximations for the components of light energy used by oxygenation and day respiration respectively at light compensation point.

Relationships similar to equation 37 can also be written with respect to CO_2 as follows:

$$\Gamma = \Gamma^* + \Gamma_d \cong (R_p + R_d) / \psi_c \cong 0.5V_o / \psi_c + R_d / \psi_c \quad (38)$$

where $\Gamma^* + \Gamma_d$ represent the components of CO_2 concentration related to photorespiration and day respiration respectively at CO_2 compensation point. The equation for net assimilation rate (39), can be written by considering equations 28 and 38, and an absence of photorespiration at CO_2 saturation.

$$(1+\alpha_2)A_c^2 - A_c[(1+\alpha_1+\alpha_2)\Psi_a C + V_{cmax} - 2(1+\alpha_2)R] + \Psi \cdot [C - \Gamma][V_{cmax} - R_d(1+\alpha_1+\alpha_2)] = 0 \quad (39)$$

Equation 35 represents limitation in the first step of reaction, while equations 36 and 39 represent those in the second step. If both sides of the equations 35, 36 and 39 are divided by $(1+\alpha_1+\alpha_2)$, assuming $\alpha_1/(1+\alpha_1+\alpha_2) = \alpha$, $(1+\alpha_2)/(1+\alpha_1+\alpha_2) = \beta$, and $V_{max} - R = A_{max}$, then the following equations can respectively be derived:

$$\alpha \cdot A_i^2 - A_i[\Phi \cdot I + V_{max} - 2\alpha R] + \Phi \cdot (I - I_c) \cdot A_{max} = 0 \quad (40)$$

$$\beta A_i^2 - A_i[\Phi \cdot I + V_{max} - 2\beta R] + A_{max} \cdot \Phi \cdot (I - I_c) = 0 \quad (41)$$

$$\beta A_c^2 - A_c[\Psi_a C + V_{max} - 2\beta R] + A_{max} \cdot \Psi_a (C - \Gamma) = 0 \quad (42)$$

Equations 40 to 42 have some similarity with the Blackman function with a convexity factor used by the two-system model. At the same time, they demonstrate how the convexity factor can interact with other components of the equations.

When $\alpha_1 \approx 1$ and $\alpha_2 \approx 0$ in equation 35, $\alpha \approx 0.5$ in equation 40, and we find:

$$0.5A_i^2 - A_i[\Phi I + A_{max}] + \Phi(I - I_c)A_{max} = 0 \quad (43)$$

where A_{max} is the observed saturation velocity of net assimilation. Similarly, when $\alpha_2 \approx 1$ and $\alpha_1 \approx 0$ in equation 41, an exact form of Blackman equation will be produced by employing equation 37, as follows:

$$A_i^2 - A_i[\Phi(I - I_c) + A_{max}] + \Phi(I - I_c)A_{max} = 0 \quad (44)$$

In general, three levels of limitation can be considered for the two-step reaction of Rubisco. The upper limit is set by the rate of transitional state, which obeys Michaelis Menten kinetics. The second limit is set by the first step of the reaction, which is related to energy limited supply of RuBP regeneration, and may also interact with the Rubisco activation level, which is energy dependent through Rubisco Activase. The third limitation is set by the second step for which a number of factors, including CO_2 or Calvin cycle may be responsible as discussed before.

7- Model Evaluation:

This section is primarily intended for experimental evaluation, with the objectives to verify that: a) the relationship envisaged by the theory, between the transitional and steady state carboxylation rates, can be supported by the experimental data. b) to verify the validity of the two steps, and the interaction with RuBP substrate in changing steps, that resulted from the concept of separation of energy from the Calvin cycle limitations on RuBP regeneration. In the following sections the responses to CO_2 , to light, and to variations of both CO_2 and light will be examined.

a-Response of Carboxylation to CO_2 :

The data of the carboxylation of wheat at 30 °C, for the ambient and 1% oxygen (Ku & Edwards 1977) are used for this examination. The results with a Michaelis-Menten equation for the transitional rate:

$$A + Rd = V_{cmax} \cdot \Psi_a(C - \Gamma^*) / (V_{cmax} + \Psi_a \cdot C) \quad (45)$$

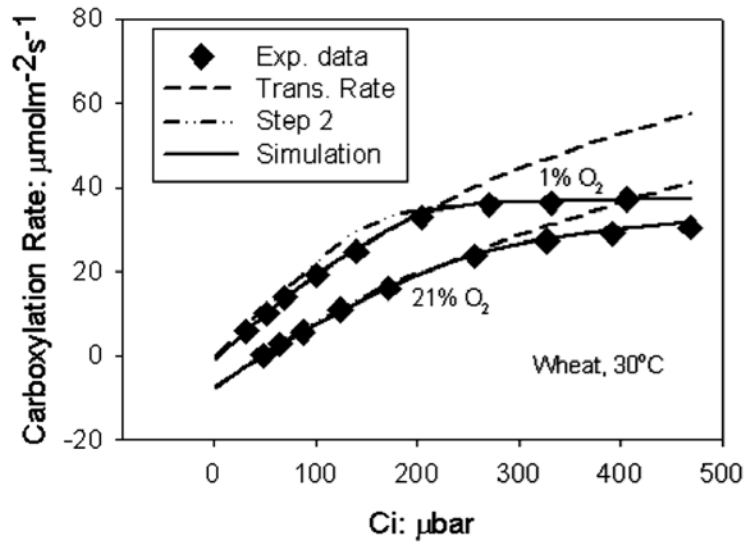


Fig. 1: Response of carboxylation to CO₂ for wheat (*Triticum aestivum*) at 1% and 21% O₂, upper and lower solid lines respectively, selected from the minimum of Michaelis-Menten equation (-.- lines), for the transitional rates and equation 39 (-- lines) for step 2 limitation. The parameters used are: $V_{cmax} = 120$ and $V_{max} = 38 \mu\text{mol m}^{-2} \text{s}^{-1}$; $Rd = 1$, $\alpha_1 + \alpha_2 = 2.158$, other parameters for 1% and 21% O₂ respectively are: $\alpha_2 = 2.058$ and 1.478 , $\Psi_a = 0.245$ and $0.16 \mu\text{mol m}^{-2} \text{s}^{-1} \mu\text{bar}^{-1}$, $\Gamma = 6.03$ and $48 \mu\text{bar}$, $\Gamma_d = Rd/\Psi_a$; experimental data from Ku & Edwards (1977).

and equation 39 for the limitation of the second step are presented in Fig. 1, with their relevant parameters in the legend. Equation 18 was used for determination of $\alpha_1 + \alpha_2$.

The results are very satisfactory for both ambient and low oxygen levels. Fig. 1 further confirms that, the steep part of the response curve is limited by substrate concentration, and the plateau of the curve by the enzyme activity. The ratio of steady state V_{max} to transitional V_{cmax} was 34% that is consistent with the range of values reported by Ruuska et al (1998) and Laisk & Oja (1998). Additional tests of the model with the data of Ku & Edwards (1977) for 25 and 35 °C, and also with the data of Ruuska et al (1998), Price et al (1995), Caemmerer and Farquhar (1981), Caemmerer et al (1994), for different C3 plants (not reported here) provided similar results. The close fit of the model to the data for different plants under diverse conditions added to its reliability and confirmed the validity of the model.

b- Response of Carboxylation to Irradiance:

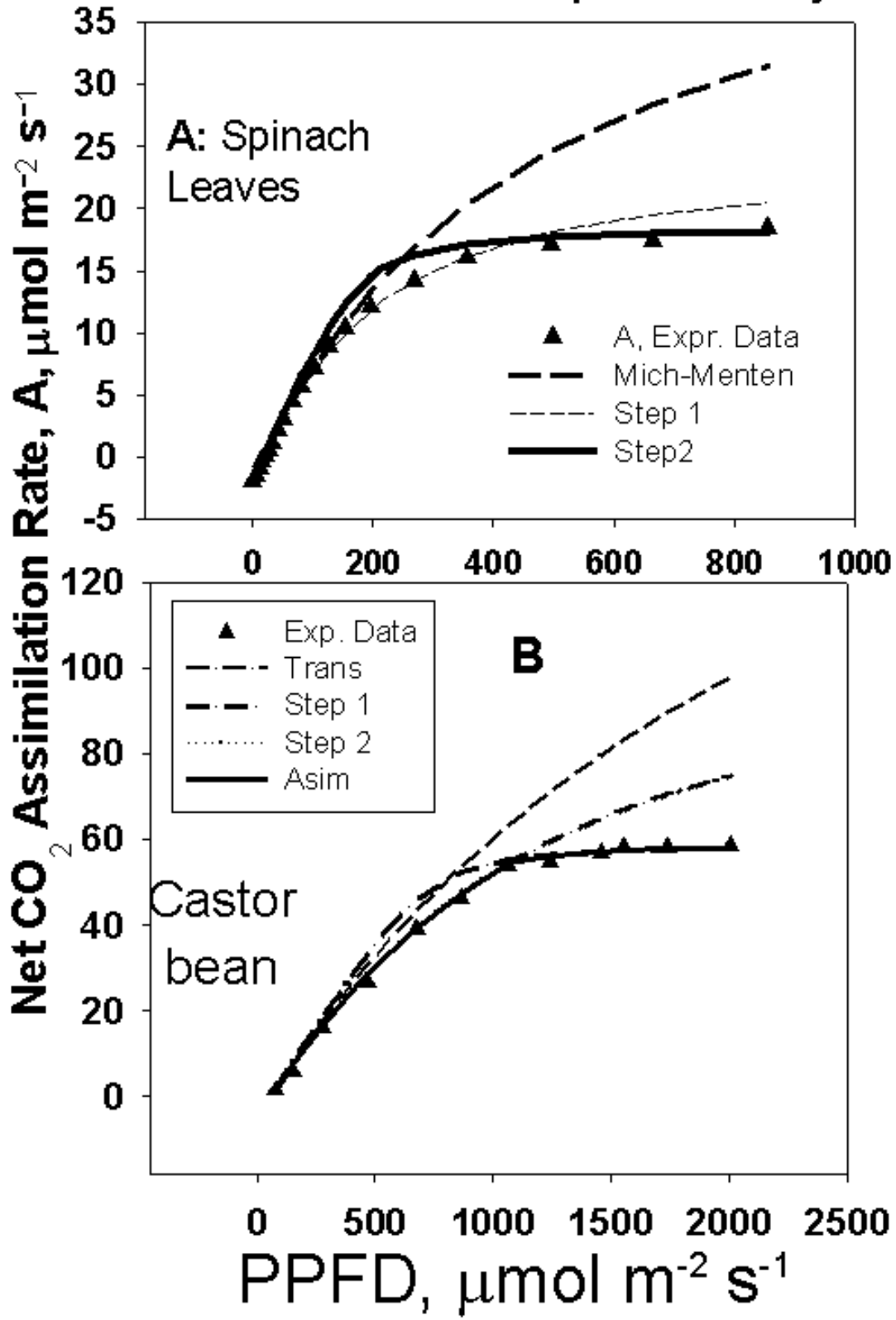
In this section, the hierarchy of limitations is examined, starting with: i) Rectangular hyperbola (equation 46) for the CO₂ saturated transitional state:

$$A = (V_{cmax} - R_d) \frac{\Phi(I - I_c)}{\Phi \cdot I + V_{cmax}} \quad (46)$$

ii) limitation of the first step when energy is limiting (equation 35); iii) limitation of the second step when other factors such as enzyme capacity or Calvin cycle capacity may be limiting (equation 36). The data for the response of spinach leaf disks to light at CO₂ saturation (Walker 1989) is used for the evaluation of equations 35 and 36. The results, in Fig. 2a, demonstrate that Rectangular hyperbola covers the initial part of the response curve, closely followed by equation 35 that fits the data up to the plateau of the curve. A rather abrupt change is observed which is defined by the equation of the second step (36).

It is of interest to note that transition from any step to another is very smooth and no external smoothing factor is required.

Simulation of Different Steps of Carboxylation



The Hierarchy of Limitations

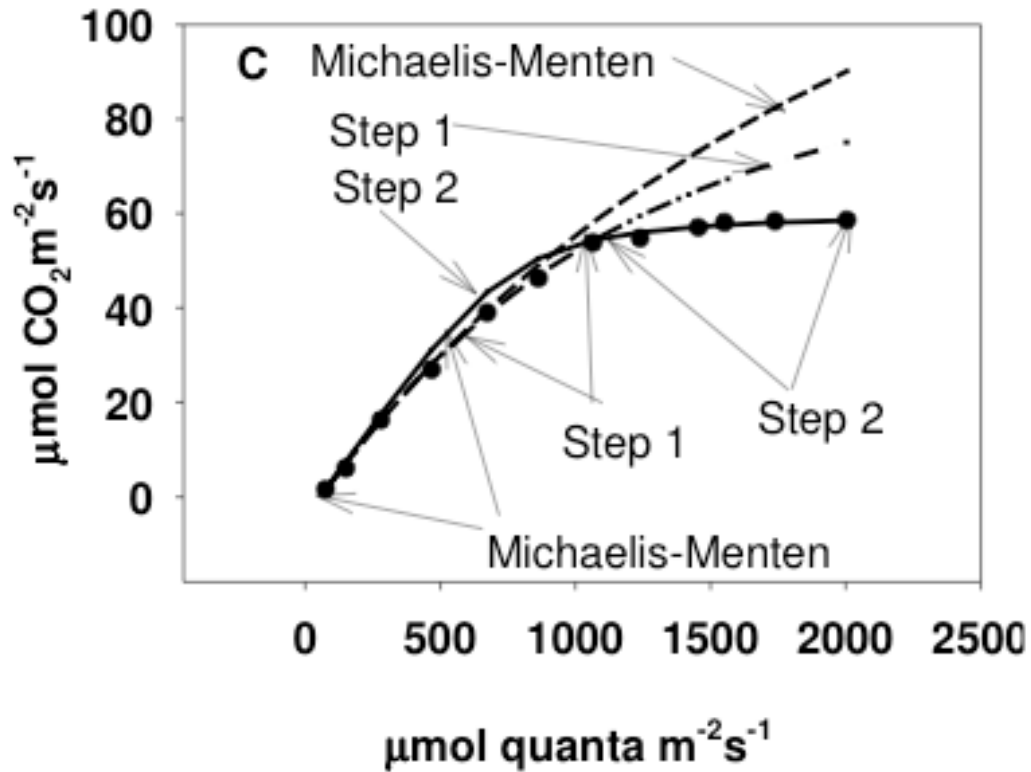


Fig.2. Response of carboxylation to light: A) A test of the validity of the two steps in the carboxylation reaction, using equation 46 (Rectangular hyperbola), and equations 35 and 36 for the two steps, with $V_{cmax} = 52.18$, $\Phi = 0.11$, $I_c = 19$, and $R_d = 2.09$ as common values, and step 1 with $V_{max} = 26.09$, $\alpha_1=1$, and $\alpha_2=0$; and Step 2 with $V_{max} = 20.59$, $\alpha_1=0.184$, and $\alpha_2=1.35$. Symbols denote experimental data of *Spinacea oleracea* L. (spinach leaf) at 20°C (Walker 1989). B) Photosynthesis of castor bean leaves (*Ricinus communis* L.) at 2% oxygen and 700 μbar CO_2 , data of Dai et al 1992. The simulations are: Transitional rate (broken line), $V_{cmax} = 240 \mu\text{mol m}^{-2}\text{s}^{-1}$, $\Phi = 0.09 \text{ mol/mol}$, $I_d = 56 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$, $R_d=5.04 \mu\text{molm}^{-2}\text{s}^{-1}$, and step 1 data: $V_{max}=120 \mu\text{mol m}^{-2} \text{ s}^{-1}$, $\alpha_1 = 1$, $\alpha_2 = 0$, and step2 data: $V_{max} = 64.5$, $\alpha_1 = 0$ and $\alpha_2 = 2.721$.

A change of limiting step can be a powerful tool for researchers interested in the effects of different factors, such as metabolites or acclimation of plants to environment, on Rubisco reaction. This is further evaluated with the data on the response of carboxylation of castor bean leaves to light, (Dai et al 1992), under non-photorespiring (2% O₂) and high intercellular CO₂ (700 μbar) in Fig. 2b and Fig. 2c.

Again, the hierarchy of limitations begins with equation 46 with $V_{cmax} = 220$ and $R_d = 4.85 \mu\text{molm}^{-2}\text{s}^{-1}$. Equation 35 is used next, with the same kinetic data and values of $\alpha_1 = 1$ and $\alpha_2 = 0$ for limitation of the first step. This equation covered only the steep part of the curve up to its plateau. Next, equation 36 was fitted to the plateau of the response curve with the same parameters and $A_{max} = 60 \mu\text{molm}^{-2}\text{s}^{-1}$. The values of $\alpha_1 + \alpha_2 = 2.412$ and $\alpha_1 = 0.07$ for limitation of the second step were found from this analysis using equation 18. A shift is observed in response from equation 35 for the first step, to equation 36 for the second step that covers the plateau of the response curve. This clearly confirms the validity of the observations made in Fig 2a, and the differentiation between the limitation of energy and other factors in response to light. The reverse order of the hierarchy is observed upward from the plateau of the curve (Fig. 2c).

c-Effects of Variations of both Radiation and CO₂ Concentration:

In this section an integrated simulation is intended with a relatively larger data set that contains the responses of carboxylation to both CO₂ and radiation. The data of Ogren (1993) on the photosynthesis of salix leaves grown under two different light levels, and the data of Ogren & Evans (1993) on photosynthesis of Eucalyptus leaves were successfully modelled, that yielded similar results. The integrated simulation has been

based on the consideration that, equation 35 is for energy-limited RuBP regeneration, in which the radiation component represents RuBP substrate. Thus, in the ordered reaction of Rubisco, the maximum of light saturation responses from equation 35 should be related together via equation 36 for the responses to CO₂ (double saturation curve). The simulation data of high light grown salix is presented here in Fig. 3a. The simulation is initiated for the data with the highest CO₂ level, with a rectangular hyperbola (equation 46) for RuBP-limited transitional state that covers the initial part of the curve (Fig 3a, dash-dot line), and with equation 35 that covers the rest of the curve. A V_{cmax} = 116 μmol m⁻² s⁻¹ for the Rubisco activated level and Φ_m = 0.085 mol/mol quanta is found from the rectangular hyperbola. Equation 39 is used with Ψ_a = 0.88 μmol m⁻² s⁻¹Pa⁻¹, with the same V_{cmax} for the light saturated response to CO₂. The light saturated steady state A_{max} for each CO₂ level is calculated from equation 39. The values of Φ, for each CO₂ level are found from equation 4. The values of α for each curve are calculated using equation 18. Thus all the parameters needed for equation 35 are known. Simulations for different curves are presented in Fig. 3a. The close proximity of the simulated responses with the experimental data in Fig 3a gives weight to the validity of the single enzyme theory and the equations that represent it.

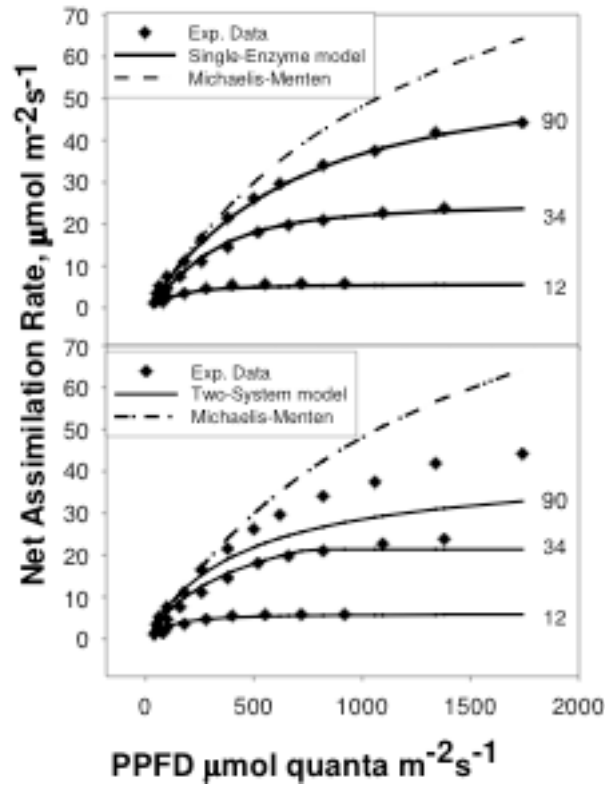


Fig. 3. Simulation of the carboxylation response of salix leaves grown under light intensity of $1400 \mu\text{mol quanta m}^{-2}\text{s}^{-1}$ for three different internal partial pressures of CO_2 [12. (\bullet), 34 (\circ) and 90 (\blacktriangledown) Pa]. Data of Ogren (1993). A) simulation with the single-enzyme model, starting with 1) Rectangular hyperbola (dash-dot lines) for transitional rate with $\Phi_m=0.085 \text{ mol mol}^{-1}$, $V_{c\text{max}}=116 \mu\text{mol m}^{-2} \text{ s}^{-1}$, $\Psi_a=.88 \mu\text{mol m}^{-2} \text{ s}^{-1} \text{ Pa}^{-1}$, and $\Gamma =4 \text{ Pa}$. $V_{c\text{max}}$ is found from equation 35 for highest C. Calculated Φ values from Φ_m were 0.038, 0.063, and 0.0758 from low to high C respectively. V_{max} values for different C levels were calculated from equation 36 with $V_{c\text{max}} = 116$, and $\alpha_1=0$, $\alpha_2 =1$. Values of α_1 and α_2 for light response curves from low to high C were: 9.508 & 5; 3.12 & 0.2, and 0.7 & 0.3 respectively. See text for details. B) Simulation based on the two-system model with $J_{\text{max}} = 210$, & $V_{c\text{max}} = 116 \mu\text{mol m}^{-2} \text{ s}^{-1}$, $K_{ca} = \text{apparent } K_c =$

$V_{cmax}/\Psi_a = 131.8 \text{ Pa}$, $\Gamma = 4 \text{ Pa}$, and θ values of 1, 1, and 0.7 from the highest to the lowest C respectively.

A simulation is also performed for the data of salix leaves with the model of Farquhar et al (1980). The simulation is based on instructions of Caemmerer & Farquhar (1981, equations A13 and A14), and the results are shown in Fig. 3b. Similar results were obtained with the data of Ogren & Evans(1993) from experiments on Eucalyptus leaves. It is evident that the performance of Two-System model deteriorates as the number of data sets in an integrated model increases

8-Discussion and Conclusions:

The single enzyme theory of Rubisco carboxylation considers that, the activated enzyme reacts with RuBP first, producing enol-enzyme complex that in turn reacts irreversibly with the second substrate, CO_2 or O_2 . The second reaction also leads to the formation of other enzyme-substrate intermediate complexes (3-keto-carboxy-arabinitol bisphosphate that transforms to gem diol) and leads to carbon-carbon cleavage and the release of product (PGA). The general model of two-substrate (R and C) ordered reaction can be given by:

$$\left(\frac{V}{V_{c \max}}\right)^2 \left(\frac{\alpha 1 K_r}{R} + \frac{(1 + \alpha 2) K_{ca}}{C}\right) - \left(\frac{V}{V_{c \max}}\right) \left[1 + \alpha 1 + \alpha 2 + \frac{K_r}{R} + \frac{K_{ca}}{C}\right] + 1 = 0 \quad 47$$

Where, the apparent $K_{ca} = K_c(1 + O/K_o)$. In equation 47, the velocity of reaction is controlled either by R, or C, depending on which one of the two substrates is limiting the reaction rate (substrate-controlled Rate-Determining Step). In Rubisco reaction, when RuBP is saturating (physical infinity), the first step is not limiting, therefore its effect is equal to zero ($K_r/R = 0$). Equation 28 can be derived by replacing $1/\Psi_c \cdot C$ for

$K_c/V_{cmax}.C$ in equation 47. Similar operations can be performed for RuBP limitation in the first step (limitation of energy) or in the second step (limitation of Calvin cycle capacity) to derive the equation for each special case. When both substrates are saturating (no free enzyme in the medium), neither of the two would be limiting, but the reaction will be co-limited by the enzyme-substrate complexes of both substrates (including inhibitions), that constitute the total concentration of enzyme. The maximum rate of steady state reaction will be given by:

$$V_{\substack{R \rightarrow \infty, \\ C \rightarrow \infty}} = V_{max} = V_{cmax}/(1 + \alpha_1 + \alpha_2) \quad (48)$$

Equation 48 is the same as equation (18), and shows the magnitude of the inhibitory effects of intermediary products. When there is a difference between the transitional and steady state rates, the values of both K_c and V_{cmax} should be determined from the transitional rates.

The biochemical equation 5 can also be viewed as partly quick equilibrium between PGA and RuBP. As the level of energy increases both the activated level of enzyme (through Rubisco Activase), and the concentration of RuBP (through consumption of PGA in Calvin Cycle) are increased, resulting in a raise in the level of activity. This can also be observed from the structure of equation 47 which is composed of two parts: a) V^2 and its coefficient, and b) the rest of the equation. Part “b” represents the equation for quick equilibrium without the product of the substrates ($K_c K_i / R.C$), which is due to the irreversibility of the carboxylation reaction, and partly reflects the rate-determining function. The sum of values, $(1 + \alpha_1 + \alpha_2)$, denotes co-limitation of the two steps. Part “a”, reduces the effect of limitation to only one of the steps, (either α_1 or $1 + \alpha_2$), before saturation of both substrates, when the enzyme for the sum of the two steps becomes the limiting factor.

This consideration further suggests that, α_1 and α_2 are equilibrium constants, and if so, concentration dependent.

The transitional rate of reaction (single turnover) however, is only a single step reaction as the total enzyme reacts sequentially with each substrate, thus it follows Michaelis-Menten equation. Moulis et al (1991) had experiments with 11 different two-substrate ordered reactions. They recorded that, the initial K_{cat} and K_m of the reactions decreased in subsequent turnovers, until they stabilized at the steady state rate. Observations with Rubisco reaction have shown to be rather different. Experimental evidence (Kieller & Walker 1990, Walker et al 1983) shows that the transitional rate of the reaction undergoes a sinusoidal oscillation before it settles into steady state conditions. One interpretation for this is that, the reduction in K_{cat} that was observed by Moulis et al (1991) after consecutive turnovers, can present itself as oscillation due to an irreversible link between the two steps of reaction. If correct, it suggests that the periodic time of the first oscillation should be equal to the single turnover time of the enzyme.

Validation of the model is performed exclusively with well-established published data sets, so that the reader can concentrate on the model itself, free from complexities of the experimental methods. The limitations in CO_2 supply, with and without a competition of oxygen were tested in Fig. 1. In response to light, limitations of RuBP regeneration by energy or by the Calvin cycle capacity were tested in Fig.2. The model was also validated for changes of carboxylation rate, under different CO_2 and light intensities (Fig 3a). It was shown that the same V_{cmax} that governs the effect of CO_2 , does also govern the effect of light as a representative of RuBP supply. Also, the responses to light were tested for the hierarchy of limitations. In addition to the data reported here, the model was

tested and positively validated with a larger number of data sets, the results of which are not included in this report (eg; Price et al 1995, Caemmerer & Farquhar 1981, Whitney et al 1999). It appears that the main cause of the wide acceptance of the two-system model by plant physiologists has been its single factor limitation and the convexity factor that made it flexible enough to provide good response curves to the data of photosynthesis. However, present biochemical model (equation 47), which is derived to swing between the two substrates as they become limiting, considers single substrate limitation at low substrate levels, co-limitation of enzyme-substrate complexes of the two steps at saturation of both substrates and co-limitation of the enzyme and substrate concentrations of the limiting step in between. The biochemical nature of the model provides it with the capabilities to reflect or interpret the influence of the factors that affect photosynthesis through inputs, or outputs (feedbacks) by the established biochemical methods. Thus, it can help improve the predictability and accuracy of the models on genetic manipulation of plants, microclimatic management of crop production, and prediction of the climatic changes through the global carbon cycle.

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Correction of previous draft: Fig 2b was misplaced for Fig 2a; corrected Aug. 30th 2004

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